

Targeting Nrf2 in Inflammation and Cancer

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*This thesis is dedicated to my parents, Katherine Ashdown and Julian Cowan.
This is mostly because of their unwavering support of my science education,
but also because dedicating it to Spider-Man is apparently “immature”.*

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Abstract

The transcription factor Nrf2 protects against cellular stress by inducing cytoprotective proteins. Activation of Nrf2 protects against inflammation and oxidative damage in disease models *in vitro* and *in vivo*. Nrf2 activation may be a good therapeutic strategy in these diseases.

Some dietary components activate Nrf2, which may be partially responsible for their beneficial effects in preventing disease. In this study a novel organosulfur compound from garlic, diallyl pentasulfide (DAPS), was investigated. DAPS strongly activated the Nrf2 pathway. Furthermore, it was a much more powerful activator of heme oxygenase-1 than any diallyl sulfides reported to date.

Nrf2 is regulated by Keap1, which targets it for degradation. Disruption of the Nrf2/Keap1 interaction results in Nrf2 activation. In this study, a novel cell-penetrating peptide, based on the Keap1-binding site of Nrf2, disrupted the Nrf2/Keap1 interaction, and activated the Nrf2 pathway. Furthermore, it demonstrated anti-inflammatory activity, significantly inhibiting LPS-induced TNF expression in THP-1 monocytes, suggesting that the interaction is a valid therapeutic target in inflammation.

In cancer, Nrf2 plays a dual role. Activation of Nrf2 protects cells from carcinogens. However, once a tumour has developed, Nrf2 can be hijacked by cancer cells to induce chemoresistance. This study examined the role of Nrf2 in malignant melanoma cells. Nrf2 was found to be overexpressed in 11 human melanoma cell lines in comparison with melanocytes. Chemoresistance to dacarbazine, doxorubicin and cisplatin correlated with Nrf2 expression, and Nrf2 siRNA increased the susceptibility of M202 and SK-MEL-5 cells to cisplatin, suggesting that Nrf2 plays a role in chemoresistance in melanoma.

In conclusion, this study has identified novel activators of Nrf2, including a dietary compound and a cell penetrating peptide which inhibits inflammation *in vitro*. In addition, Nrf2 inhibition sensitises melanoma cells to chemotherapy. These results suggest that targeting Nrf2 is a viable strategy in both inflammation and cancer.

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Abbreviations

ARE	Antioxidant response element
Bach1/2	BTB and CNC homology 1/2
BCP	1-Bromo-3-chloropropane
BTB	Broad-Complex, tramtrack and bric à brac
bZip	Basic leucine zipper
CNC	Cap 'n' collar
CO	Carbon monoxide
CRE	cAMP Response Element
Cul3	Cullin 3
DADS	Diallyl disulfide
DAPS	Diallyl pentasulfide
DAS	Diallyl sulfide
DATS	Diallyl trisulfide
DATTS	Diallyl tetrasulfide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GCL	Glutamate-cysteine ligase
GCS	Glutathione synthetase
GFP	Green fluorescent protein
Gpx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSK-3 β	Glycogen synthase kinase 3 β
GST	Glutathione-s-transferase
H ₂ DCFDA	2, 7'-dichlorodihydrofluoresceindiacetate
H ₂ O ₂	Hydrogen peroxide
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
IFN	Interferon
IL-1 β	Interleukin 1 β
IL-6	Interleukin 6

IL-8	Interleukin 8
iNOS	Nitric oxide synthase
IVR	Intervening region
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MARE	Maf recognition element
MCP-1	Monocyte chemoattractant protein-1
MgCl ₂	Magnesium Chloride
MGST	Microsomal glutathione-s-transferase
mRNA	Messenger Ribonucleic acid
MRP	Multidrug-associated resistance protein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Neh1-7	Nrf2-ECH homology 1-7
NF-E2	Nuclear factor, erythroid 2
NF-κB	Nuclear factor-κB
NO	Nitric Oxide
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf1/2/3	Nuclear factor erythroid 2 related factor 1/2/3
OGG1	Oxoguanine glycosylase
PBS	Phosphate buffered saline
PKC	Protein kinase C
Prdx	Peroxiredoxin
PVDF	Polyvinylidene difluoride
Rbx1	RING-box protein 1
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide Dismutase
SQSM1	Sequestosome 1
Srx	Sulfiredoxin
tBHQ	<i>tert</i> -Butylhydroquinone
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRE	12-o-tetradecanoylphorbol 13-acetate Response Element
Trx	Thioredoxin
UGT1A6	UDP glucuronosyltransferase 1A6

Publications and Presentations

- Steel R., Cowan J., Payerne E., O'Connell M.A., Searcey M., 2012. Anti-inflammatory effect of a cell penetrating peptide targeting the Nrf2/Keap1 interaction, *ACS Medicinal Chemistry Letters* 3(5), pp407-410.

In Progress:

- Cowan, J., Arbach, M., Mallett, A., Gohil, S., Groom, M., Hamilton, C., O'Connell, M. A. (2014) Long chain polysulfides from garlic activate the Nrf2 pathway in human monocytic cells via p38 MAP kinase.

Poster Abstracts:

- Cowan, J., Akhtar, F., Sherwood, V., O'Connell, M.A. (2012) Analysing Expression of Nrf2 Signalling Pathway Members in Melanoma. *UEA School of Pharmacy Research Day, 2012.*
- Cowan, J., O'Connell, M. A. (2012) Nrf2: Co-ordinating the defence against cell damage. *Showcase of Postgraduate Research.*
- Cowan, J., Arbach, M., Mallett, A., Hamilton, C., O'Connell, M.A. (2013) Garlic polysulfides activate the Nrf2 pathway in THP-1 monocytic cells. *Nutrient mechanisms in inflammation, infection and immunity.*
- Cowan, J., Arbach, M., Mallett, A., Hamilton, C., O'Connell, M.A. (2013) Garlic polysulfides activate the Nrf2 pathway in monocytes, revealing a correlation between potency and chain length. *UEA School of Pharmacy Research Day 2013.*

Chapter 1: Introduction

1.1 The role of Nrf2 in protection against disease

Oxidative stress and inflammation are major causative factors in a wide range of diseases. They are central in the pathogenesis of chronic age-related diseases, such as cardiovascular diseases, neurodegenerative diseases, and cancer, but are also major factors in other non-age related diseases, such as asthma, or inflammatory bowel disease (Koskela et al., 2012; Elnakish et al., 2013; Paschos et al., 2013; Sanders and Greenamyre, 2013; Stoner et al., 2013; Read and Douglas, 2014; Zhang and Li, 2014).

Inflammation and oxidative stress involve a wide variety of processes that, if left unregulated, can cause cellular damage. This leads to either malfunctioning cells or cell death, which can cause a variety of problems (Gafer-Gvili et al., 2013; Hanus et al., 2013). In the most extreme cases these include the development of tumour cells or necrosis, which can cause organ failure and eventually death (Ma et al., 2013; Zhang et al., 2014).

There are many mechanisms by which cells protect themselves from damage, and reduction of oxidative stress and inflammation is an effect that many of them share. Drug metabolism is one such process, which can detoxify oxidants to prevent them from causing oxidative stress (Copple et al., 2010). Likewise, transcription of pro-inflammatory mediators can be inhibited, which prevents excessive damage from inflammation (Di Paola et al., 2013).

One molecule that plays a key role in regulating these processes is Nuclear factor erythroid 2 related factor 2 (Nrf2). Nrf2 is a transcription factor that induces expression of a wide range of proteins including heme oxygenase-1 (HO-1), NAD(P)H:Quinone oxidoreductase 1 (NQO1), Ferritin, glutathione synthesis enzymes, and multidrug-associated resistance proteins (Pietsch et al., 2003; Vollrath et al., 2006; Li et al., 2009; Liang et al., 2013). Many of the enzymes induced by Nrf2 are phase II and III detoxification enzymes, which

convert toxins into less harmful metabolites and/or facilitate their export from the cell (Cornejo et al., 2013). Others, such as HO-1, have both antioxidant and anti-inflammatory effects (Rushworth et al., 2008; Zhao et al., 2013a). The most striking element of Nrf2-regulated genes is that although they have different specific roles, almost all of those roles prevent cellular damage (Table 1.1).

Full Name	Abbreviation	Function	Reference
BTB and CNC homology 1	Bach1	Suppressor of ARE-dependent transcription	Jyrkkänen et al., 2011
Ferritin H	N/A	Regulation of iron homeostasis	Pietsch et al., 2003
Ferritin L	N/A	Regulation of iron homeostasis	Theil, 2013; Choi et al., 2014
Glutamate-Cysteine Ligase	GCL	GSH synthesis	Li et al., 2009
Glutathione Peroxidase	Gpx	Catalyses the detoxification of peroxides by GSH	Kurzatkowski et al., 2013
Glutathione Reductase	GR	Produces GSH from GSSG	Kurzatkowski et al., 2013
Glutathione-s-Transferase	GST	Conjugation of GSH to substrate	Lin et al., 2012
Glutathione Synthetase	GCS	Glutathione synthesis	Chan et al., 2001
Heme Oxygenase-1	HO-1	Catabolism of heme	Liang et al., 2013
Microsomal Glutathione-s-Transferase	MGST	Conjugation of GSH to substrate	Kelner et al., 2000; Choi et al., 2014
Multidrug Resistance-Associated Proteins	MRP	Cellular Export Of Various Molecules	Vollrath et al., 2006; Xu et al., 2010
NAD(P)H Quinone Oxidoreductase 1	NQO1	Detoxification of quinones	Liang et al., 2013
Oxoguanine glycosylase	OGG1	DNA repair	Singh et al., 2013
Peroxiredoxin	Prdx	Redox homeostasis	Kim et al., 2011
Sulfiredoxin	Srx	Redox homeostasis	Soriano et al., 2009
Superoxide Dismutase	SOD	Redox homeostasis	Zhang et al., 2012
Thioredoxin	Trx	Redox homeostasis	Im et al., 2012
UDP glucuronosyltransferase 1A6	UGT1A6	Glucuronidation reaction to aid molecule excretion	Mackenzie et al., 1997; Choi et al., 2014

Table 1.1 Examples of Nrf2 target genes. Nrf2 regulates transcription of many genes involved in cellular protection.

Nrf2, therefore, is considered a master regulator of cytoprotection, and this is backed up by disease models *in vivo* and *in vitro*. Although Nrf2 is not essential for development, Nrf2 knockout mice display increased sensitivity to a range of adverse conditions including respiratory disease, autoimmune diseases, and sepsis (Chan et al., 1996; Chan and Kan, 1999; Yoh et al., 2001; Iizuka et al., 2005; Kong et al., 2010) (Table 1.2). This is backed up by *in vivo* models showing that upregulation of Nrf2 activity protects against disease and tissue damage, including but not limited to brain injury, ischemia, and inflammatory diseases (Zhao et al., 2011; Kudoh et al., 2014; Lee et al., 2014a; Mo et al., 2014; Xu et al., 2014a). Nrf2 has also been shown to provide protection against carcinogenesis, most likely via inhibition of oxidative stress (Kavitha et al., 2013; Cheung et al., 2014; Tasaki et al., 2014).

Nrf2 is just one member of a family of transcription factors that bind to similar regulatory regions, and therefore share overlap in the genes they regulate and processes that they are involved in (Motohashi et al., 2002; Jaiswal, 2004; Biswas and Chan, 2010).

Disease Model	Abnormalities	Reference
None	Normal development and behaviour	Chan et al., 1996; Chan and Kan, 1999; Yoh et al., 2001; Lee et al., 2004; Pacchioni et al., 2007
	Anemia	
	Age-related lupus-like symptoms	
Injury	Decreased fracture healing	Wang et al., 2007; Liu et al., 2009; Lippross et al., 2014
	Increased injury in the brain and kidney	
Inflammatory and immune diseases	Increased susceptibility to:	Yoh et al., 2001; Cho et al., 2004; Iizuka et al., 2005; Khor et al., 2006; Osburn et al., 2007; Wang et al., 2007; Reddy et al., 2009; Kong et al., 2010
	<ul style="list-style-type: none"> • Bacterial infection • Sepsis • Lung inflammation • Brain inflammation • Colitis • Lupus-like symptoms 	
Respiratory diseases	Increased susceptibility to:	Chan and Kan, 1999; Cho et al., 2004; Iizuka et al., 2005; Rangasamy et al., 2005
	<ul style="list-style-type: none"> • Asthma • Emphysema • Pulmonary fibrosis • Airway inflammation • Acute respiratory stress syndrome 	
Liver disease	Increased susceptibility to steatohepatitis	Chowdhry et al., 2010
Diabetes	Increased susceptibility to diabetic nephropathy	Jiang et al., 2010
Cancer	Increased carcinogenesis:	Iida et al., 2004; Khor et al., 2008
	<ul style="list-style-type: none"> • Urinary bladder carcinoma • Colorectal cancer 	

Table 1.2 Nrf2 knockout mice studies. Nrf2 knockout mice are susceptible to numerous diseases.

1.2 Cap 'n' Collar Transcription Factors

Nrf2 is a member of the cap 'n' collar (CNC) family of transcription factors, which are defined by two overlapping motifs. The first is a basic leucine zipper (bZip), and the second is a CNC motif, homologous to a similar sequence in the drosophila protein of the same name. CNC family members do not necessarily have any homology outside of these motifs (Caterina et al., 1994; Itoh et al., 1995).

The bZip functions as both a protein binding and a DNA binding motif (Fig 1.1). Alone, it is incapable of inducing transcription, but as a dimer, it forms a full DNA binding domain, activating the transcription factor. bZip cofactors that dimerise with CNC proteins are typically small maf proteins, but can also include other bZip transcription factors, such as Fos or c-Jun (Newman and Keating, 2003). Different combinations of bZip transcription factors (which may or may not include a CNC transcription factor) bind different DNA sequences, but there is enough overlap that these sequences share homology. They include the antioxidant response element (ARE), the maf recognition element (MARE), the NF-E2 site, and the 12-O-tetradecanoylphorbol-13-acetate response element (TRE) (Rushmore et al., 1991; Kataoka et al., 1994; Nguyen et al., 1994; Xie et al., 1995; Koyanagi et al., 2011) (Table 1.3).

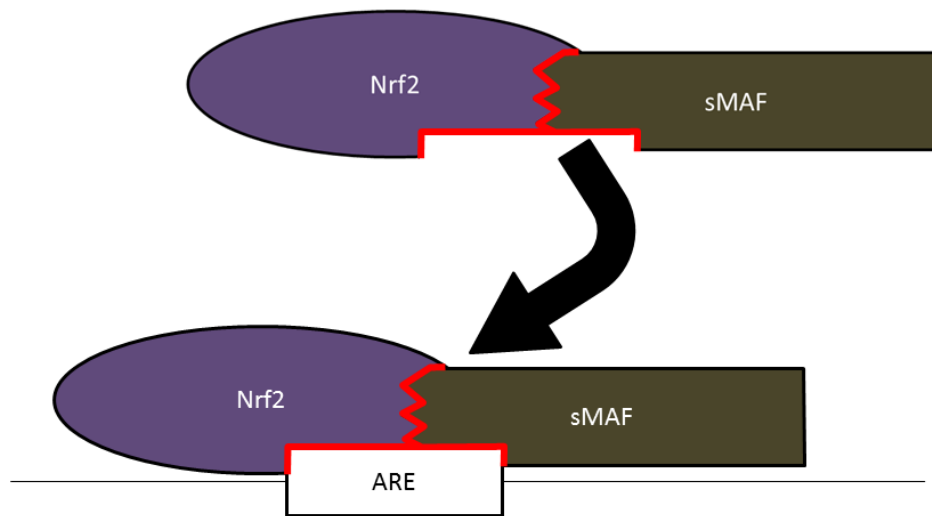


Fig 1.1 The bZip motif. Nrf2 binds small maf proteins and the ARE using the bZip motif, outlined in red. bZip dimerization is required to form the DNA binding site.

Several of the CNC transcription factors, including Nrf2, bind to the ARE, and are able to activate or suppress transcription of genes containing this sequence in their promoter. The ARE was discovered in 1990, and recognised as a *cis*-acting regulatory region of antioxidant gene expression a year later (Rushmore and Pickett, 1990; Rushmore et al., 1991). It is now known to regulate a variety of genes in addition to antioxidant enzymes, almost all of them protective (Chen and Kunsch, 2004; Reddy, 2008; Nguyen et al., 2009). The core ARE sequence is 5'-TGACnnnGC-3' although the importance of each base varies. For example, the 5' terminal thymine is essential for all ARE-dependant transcription, but the 3' terminal guanine is required only for inducible activity (Rushmore et al., 1991).

Response Element	Sequence	Reference
Antioxidant Response Element (ARE)	5'- TGAC <i>CnnnGC</i> -3'	Rushmore et al., 1991
Maf Recognition Element (T-MARE)	5'-TGCT TGA (G/C)TCAGCA-3'	Kataoka et al., 1994
Maf Recognition Element (C-MARE)	5'-TGCT TGA (GC/CG)TCAGCA-3'	Kataoka et al., 1994
cAMP Response Element (CRE)	5' TG (A/C)CGT(C/A)(G/A)-3'	Koyanagi et al., 2011
12-o-tetradecanoylphorbol 13-acetate Response Element (TRE)	5'- TGA (C/G)TCA-3'	Nguyen et al., 1994; Xie et al., 1995

Table 1.3 The antioxidant response element and its related sequences. ARE-like elements (shown in bold) overlap with other sequences, showing that transcription factors may be able to share the same promoter region, and raising the possibility of cross-pathway regulation.

Nrf2 is by far the best characterised member of the CNC family, but all members are related in structure and function. These include NF-E2, Nrf1, Nrf3, Bach1 and Bach2.

1.2.1 NF-E2

Nuclear factor, erythroid 2 (NF-E2) was originally reported to bind the porphobilinogen deaminase promoter in mouse erythroleukemia cells in 1989 (Mignotte et al., 1989). It contains a transactivation domain and a CNC bZip domain, and forms a functional heterodimer with small maf proteins (Blank et al., 1997). It binds a DNA sequence very similar to the TRE (Table 1.3), which is GCTGA(G/C)TCA (Ney et al., 1990).

It is expressed primarily in hematopoietic cells (Andrews et al., 1993; Ney et al., 1993). Little is known about its regulation, but it has been shown that NF-

E2 plays a non-vital role in transcription of globin genes, and is important for megakaryocyte development (Andrews et al., 1993; Blank et al., 1997; Andrews, 1998).

1.2.2 Nrf1

Nrf1 was the first of the NF-E2 related proteins to be discovered, and was originally considered responsible for constitutive ARE-dependent gene expression (Chan et al., 1993). Although it has been shown that this is not quite true, Nrf1 has much more stable regulation than Nrf2. It has a half life of 5 hours, and is regulated by switching between three isoforms. The largest, p120, contains a 30-residue N-terminal sequence which confines it to the endoplasmic reticulum (Wang and Chan, 2006). Cleavage of the N-terminal sequence is controlled by deglycosylation, and is required for transcriptional activation. The cleaved form, p95, is the active form of Nrf1, which induces ARE-dependent gene expression (Zhang et al., 2007). The final form of Nrf1, p65, is truncated. While it retains its ability to bind DNA, it lacks a transactivation domain and inhibits activation of ARE activity (Wang et al., 2007a).

Functionally, Nrf1 shares a lot of redundancy with Nrf2. However, unlike Nrf2, Nrf1 knockout mice are not viable suggesting that the two transcription factors have their own distinct functions (Chan et al., 1998). Although their differential regulation may account for part of this, Nrf1 has been shown to have its own transcriptional targets. Metallothionein-1, metallothionein-2 and proteasomal subunits are regulated by Nrf1, but not Nrf2 (Ohtsuji et al., 2008).

1.2.3 Nrf2

In 1994, Moi and coworkers detailed the discovery and characterisation of Nrf2. The Nrf2 gene was cloned in K562 cells, and was found to consist of 1807 base pairs, which predicts a 66.1kDa protein consisting of 589 amino acids (Moi et al., 1994). One of the main features of Nrf2 is that it is inducible (Table 1.4a and b). Under normal conditions it is targeted for degradation by a protein called Kelch-like ECH-associated protein 1 (Keap1) (Itoh et al., 1999; Itoh et al., 2003). When a protective response is required, Nrf2 degradation is halted by disrupting the interaction between Nrf2 and Keap1. This allows it to initiate transcription of genes containing the ARE sequence in their promoter (Jaiswal, 1994; Itoh et al., 2003).

Classification	Molecule	Reference
Isothiocyanate	Sulforaphane	Zhou et al., 2014
Carotene	Lycopene	Sahin et al., 2010b
Carotenoid	Apo-8'-lycopenol	Yang et al., 2012
Catechin	Epigallocatechin	Ogborne et al., 2008
Catechin	Epigallocatechin-3-gallate	Sahin et al., 2010a
Coumarin	Osthole	Yang et al., 2014b
Curcuminoid	Curcumin	Gao et al., 2013
Diallyl polysulfide	DAS	Chen et al., 2004
Diallyl polysulfide	DADS	Chen et al., 2004
Diallyl polysulfide	DATS	Chen et al., 2004
Diallyl polysulfide	DATTS	Saidu et al., 2013
Dicarboxylic acid	Dimerumic acid	Lee et al., 2013a
Dihydroxybenzoic acid	Protocatechuic acid	Vari et al., 2011
Fatty Acid	Nitro-oleic acid (OA-NO ₂)	Kansanen et al., 2011
Flavonoid	Isorhamnetin	Yang et al., 2014a
Flavonoid	Quercetin	Domitrović et al., 2012
Fumaric acid ester	Dimethyl fumarate	Linker et al., 2011
Glycoside	Rutin	Pan et al., 2014
hydroxyalkenal	4-HNE	Numuzawa et al., 2003
Isoflavone	Genistein	Wang et al., 2013a
Metal	Zinc	Zhang et al., 2014b
N/A	Arsenite	Wang et al., 2008a

Table 1.4a Activators of the Nrf2 pathway.

Classification	Molecule	Reference
N/A	dexamethasone methylate	Dinkova Kostova et al., 2002
N/A	Hexabromocyclodecanes	Zou et al., 2013
N/A	hypochlorous acid	Forquet et al., 2010
N/A	Monomethylarsonic acid	Wang et al., 2008a
N/A	NONOate	Forquet et al., 2010
N/A	Phorone	Numuzawa et al., 2003
N/A	s-nitrocysteine	Forquet et al., 2010
Organosulfur Compound	Allicin	Li et al., 2012c
Peroxide	Hydrogen Peroxide	Forquet et al., 2010
Phenethylamine	Deprenyl	Xiao et al., 2011
Phenol	tBHQ	Yamamoto et al., 2008
Polyamine	spermine	Forquet et al., 2010
Polyphenol	3,4,5-trihydroxycinnamic acid	Lee et al., 2014a
Polyphenol	Salvianolic Acid	Zhang et al., 2014a
Protein	Sequestosome-1	Jain et al., 2010
Protein	Ulinastatin	Song et al., 2014
Stilbene	α -veniferin	Dilshara et al., 2014
Stilbenoid	Resveratrol	Palsamy and Subramanian, 2011
Triterpenoid	Bardoxalone methyl	Wu et al., 2014
Triterpenoid	CDDO	Cleasby et al., 2014
Triterpenoid	Dihydro-CDDO-trifluorothyl amide	Li et al., 2014

Table 1.4b Activators of the Nrf2 pathway.

Nrf2 has been classically considered to contain 6 domains, named Neh1-6. Recently Neh7 has been identified as an additional domain with a unique function (Itoh et al., 1999; Wang et al., 2013a) (Table 1.5). Neh1 is the domain containing the bZip and CNC motifs, and is therefore the domain responsible for heterodimerisation with bZip transcription factors and DNA binding (Itoh et al., 1999). Neh2 and 6 are negative regulatory domains that target Nrf2 for degradation. Neh2 is the Keap1 binding domain, whereas Neh6 promotes Keap1-independent Nrf2 degradation (Itoh et al., 1999; Rada et al., 2011). Neh3, 4, and 5 are transactivation domains, which can be phosphorylated to interact with transcription cofactors and enhance transcription (Nioi et al., 2005; Apopa et al., 2008).

Domain	Amino Acids	Function
Neh1	434-561	DNA binding (Itoh et al., 1999) CNC bZip region for heterodimerization with small Maf proteins (Itoh et al., 1999)
Neh2	1-86	Keap1 binding (Itoh et al., 1999) Ubiquitinated by Keap1/E3 ubiquitin ligase complex (Huang et al., 2002; Zhang et al., 2004)
Neh3	561-604	Required for transcriptional activation (Nioi et al., 2005)
Neh4	112-134	Phosphorylated prior to transcriptional activation (Apopa et al., 2008) Binds nuclear cofactors (Katoh et al., 2005; Kim et al., 2013)
Neh5	182-200	Phosphorylated prior to transcriptional activation (Apopa et al., 2008) Binds nuclear cofactors (Katoh et al., 2005; Kim et al., 2013)
Neh6	336-386	Phosphorylated by GSK-3 β (Rada et al., 2011) Ubiquitinated by the SCF/TrCP E3 ubiquitin ligase complex (Rada et al., 2011)
Neh7	209-316	Directly interacts with the DNA binding region of retinoic X receptor alpha. This interaction inhibits Nrf2 transcriptional activity (Wang et al., 2013a)

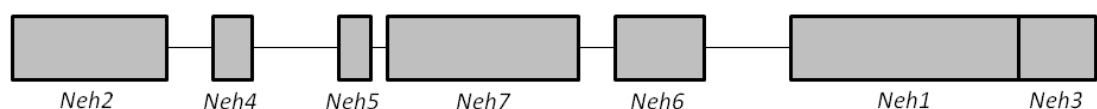


Table 1.5 A summary of domain functions in Nrf2. Each of the 7 domains in Nrf2 has a specific function. A graphical representation of the relative position of each domain within the protein is also provided.

1.2.4 Nrf3

Nrf3 (nuclear factor-erythroid 2 related factor 3) contains an N-terminal Neh3-like domain and a transactivation domain similar to that of Nrf1. Like Nrf1, it contains an N-terminal sequence that targets it to the ER. Not much is known about Nrf3, but it is highly expressed in the placenta, and not at all in the heart or liver. Nrf3 null mice show no obvious phenotype, and no differences were observed compared to the wild type after viral infection. Nrf1/Nrf2 double knock-outs show residual ARE expression, which may be due to Nrf3. Data showing a 4-5 fold increase in Nrf3 expression in skin cells in Nrf2^{-/-} mice seems to support this, but other data shows that double knock-outs of Nrf3/Nrf2 and Nrf3/p45 did not increase mortality over knock-outs of Nrf2 or p45 alone. Nrf3 is not well characterised, but it may play a role in protecting against inflammation and carcinogenesis (Derjuga et al., 2004; Zhang et al., 2009a; Chevillard and Blank, 2011).

1.2.5 Bach1

Bach1 (BTB and CNC homology 1) is a CNC bZip transcription factor that is highly involved in Nrf2 regulated gene expression. It is unlike the Nrf-transcription factors because it contains a Broad-Complex, tramtrack and bric à brac (BTB) domain, which is a protein-binding domain. In the case of Bach1 it is used to bind small maf proteins (Oyake et al., 1996; Igarashi et al., 1998). Bach1 binds to the ARE, and suppresses gene transcription in an antagonistic relationship with Nrf2 (Sun et al., 2002).

Bach1 has been shown to negatively regulate ARE dependent gene transcription in many cell types. For example, bach1^{-/-} mice expressed more HO-1 in the brain, increased HO-1 mediated suppression of atherosclerosis, and increased HO-1 expression after ischemia in cardiac cells, which led to

increased protection of heart tissue (Yano et al., 2006; Sakoda et al., 2008; Watari et al., 2008).

Although the absence of *bach1* has a beneficial effect when protecting the cell from damage, it is not desirable to have a constitutively active antioxidant response. Firstly, constitutive activation of Nrf2 leads to hyperkeratosis (Sato et al., 2009). Secondly, although cytoprotection is important when the cell is under threat from toxins and oxidative stress, these processes tend to inhibit apoptosis, which is an important mechanism in tumour prevention (Hayes and McMahon, 2001).

1.2.6 *Bach2*

Bach2 (BTB and CNC homology 2) is very similar to *bach1*, although its expression is constrained mainly to B cells and neuronal cells (Hoshino and Igarashi, 2002; Chen et al., 2013). Like *bach1*, it mainly acts as a transcription repressor. There are two isoforms of *Bach2*. A 110kDa form, and a 136kDa form. For most of its known functions, it forms heterodimers with MafK, and binds the MARE promoter sequence, although it can also form a homodimer to bind the TRE (Oyake et al., 1996).

Current research suggests that the role of *Bach2* is somewhat different from the other CNC transcription factors, and despite the sequence similarity between the MARE and the ARE there is no evidence that it plays a role in Nrf2-related cell signalling. *Bach2* is mainly involved in maturation and differentiation of B cells into plasma cells. Expression profiles show that *Bach2* is highly expressed in early stages of B cell development, but levels drop once the fate of the cell has been decided (Ochiai et al., 2008).

1.2.7 CNC transcription factors regulate the antioxidant response

The antioxidant response is induced by, and combats, oxidative stress (Nguyen et al., 2009). A common theme of CNC transcription factors is that they regulate antioxidant enzymes, often via binding the ARE (Jaiswal et al., 1994; Sankaranarayanan and Jaiswal, 2004; Dhakshinamoorthy et al., 2005; Zhang et al., 2007; Chowdhury et al., 2009; Chen et al., 2013b).

It is Nrf2, however, which is considered the primary regulator of the antioxidant response (Nguyen et al., 2009). This is because of the large array of enzymes that it regulates, ranging from phase II and III detoxifying enzymes to anti-inflammatory mediators and DNA repair enzymes (Cornejo et al., 2013; Singh et al., 2013a).

1.3 Nrf2 in Oxidative Stress

Although Nrf2 is credited with many beneficial biological effects, it is most commonly associated with the antioxidant response. This is unsurprising; oxidative stress is involved in many diseases, including inflammatory diseases, neurodegenerative diseases and cardiovascular diseases (Dumont and Beal, 2011; Berg et al., 2011; Rosenbaugh et al., 2013). Combating oxidative stress is a key mechanism (although often not the only mechanism) by which Nrf2 has been shown to protect against them (Howden, 2013; Ruiz et al., 2013; Gan and Johnson, 2014).

Oxidative stress is an imbalance in cellular redox homeostasis, and is characterised by an excessive amount of reactive oxygen species (ROS) which, unless detoxified, cause damage to cellular components. ROS are oxygen-containing molecules which are reactive. Many are free-radicals, and can react with many molecules around the cell, disrupting their function (Baker and Staecker, 2012; Sin et al., 2013; Zuo et al., 2013).

Oxidative stress has many causes, largely because ROS have useful functions in the body provided that they are properly regulated. For example, ROS are used by the immune system to combat pathogens (Zurita et al., 2013; Genestet et al., 2014). They are also used as second messenger molecules in cell signalling pathways (Valko et al., 2007; Ray et al., 2012). Oxidative stress tends to occur when this balance is upset. Major causes of this often come from outside sources, such as food, radiation, or pollution. UV light, for example, causes free-radical production (Jurkiewicz et al., 1994; Murapa et al., 2012; Menichini et al., 2013) and is thought to be a major cause of skin cancer. A high fat diet is also known to cause oxidative stress (Charradi et al., 2013).

Nrf2 is a key regulator of oxidative stress. Nrf2 knockout mice are more susceptible to oxidative stress-related diseases, are badly equipped to deal with injured tissue, and have an overactive inflammatory response (Kong et al., 2010; Wruck et al., 2011; Okada et al., 2013; Zhang et al., 2013a). Nrf2 knockdown *in vitro* renders cells susceptible to ROS accumulation and induced toxicity, whereas Nrf2 induction has been shown to reduce ROS and increase cell viability in their presence (Li et al., 2012a; Deng et al., 2013; Hao et al., 2013).

Both Nrf2 and its inhibitor, Keap1, can act as a ROS sensor. ROS can cause disruption of the Nrf2/Keap1 interaction and the activation of Nrf2 transcriptional activity (Wakabayashi et al., 2004). Therefore, Nrf2 acts as a negative feedback mechanism to reduce cellular ROS. A large number of Nrf2 target genes have antioxidant activity, but the specific functions that they perform are diverse.

1.3.1 HO-1, Ferritin and Iron Homeostasis

HO-1 is a well-studied Nrf2 target gene, which is responsible for the rate limiting step in the conversion of heme to bilirubin (Fig 1.2) in a reaction that also produces carbon monoxide and Fe^{2+} ions (Kirkby and Adin, 2006). Bilirubin is a powerful antioxidant that directly scavenges free radicals (Stocker et al., 1987). It also inhibits the production of superoxide by NADPH oxidase (Fujii et al., 2010). Although no single study has linked Nrf2, HO-1 and bilirubin to antioxidant activity, there are many smaller studies that strongly support this theory when the research is combined.

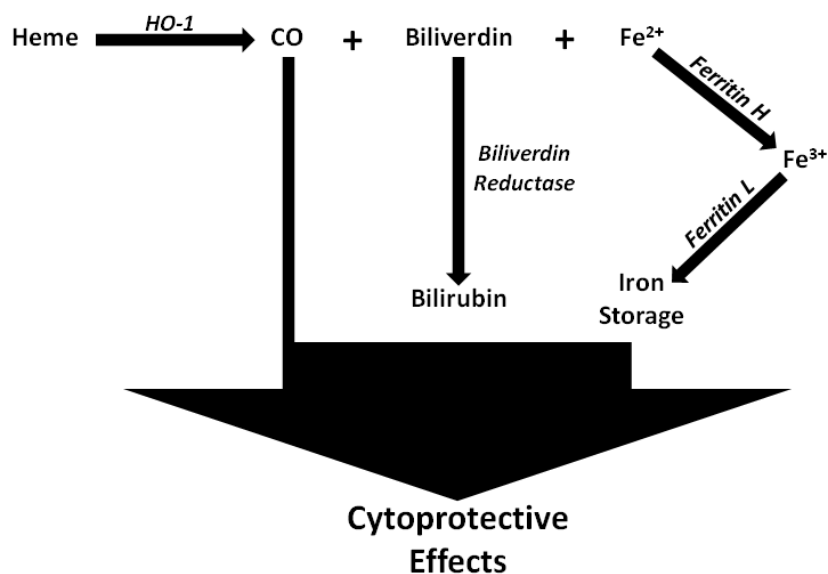


Fig 1.2. HO-1 and iron homeostasis. HO-1 catalysis the rate-limiting step in the catabolism of heme. The products of the breakdown of heme, CO and bilirubin, have been shown to play roles in defending against oxidants and inflammation. Ferritin, another Nrf2 driven enzyme, plays a key role in neutralising the harmful effects of Fe²⁺.

Increased HO-1 expression is often linked with protection against oxidative stress. Induced pluripotent stem cells lacking HO-1 have been shown to be more susceptible to oxidative damage than their HO-1 expressing counterparts (Lin et al., 2012a). Blood plasma was analysed in HO-1 knockout mice, which revealed elevated lipid peroxidation in the knockouts, suggesting increased oxidative stress (Ishikawa et al., 2012). Inducers of HO-1 such as sulforaphane, salvianolic acid A, and genistein have all been shown to protect against oxidative stress via the Nrf2 pathway, suggesting that Nrf2 is a key regulator of the protection provided by HO-1 (Kleszczyński et al., 2013; Wang et al., 2013a; Zhang et al., 2014a).

Another product of HO-1 activity is carbon monoxide (CO), which can also be protective at sub-cytotoxic levels. CO has been shown to inhibit ROS

production in RAW264.7 cells stimulated with LPS (Srisook et al., 2006). In a model of hyperoxia CO was able to inhibit apoptosis and caspase-8 activation, possibly by the inhibition of ROS (Wang et al., 2007)

However, not every product of HO-1 is an antioxidant. Fe^{2+} is produced from the degradation of heme. Fe^{2+} can become oxidised to Fe^{3+} by H_2O_2 in a reaction that produces a hydroxyl radical (Jomova et al., 2010). Fortunately, another Nrf2-mediated enzyme, Ferritin, is able to detoxify free Fe^{3+} , protecting the cell from any damage it may cause. Ferritin consists of 24 subunits, of which there are two types. The role of the H subunit is to catalyse an alternative reaction for the oxidation of Fe^{2+} which does not involve the production of hydroxyl radicals, whereas the L subunit binds and stores Fe^{3+} in a non-toxic state, controlling its bioavailability (Alkhateeb and Connor, 2013).

The antioxidant activity of ferritin has been documented in several *in vitro* studies. Ferritin correlates with improved cell viability after porcine aortic endothelial cells were incubated with H_2O_2 and hematin. Furthermore, preloading cells with ferritin confers protection against cytotoxicity caused by H_2O_2 /hemin, which an inactive ferritin H mutant does not (Balla et al., 1992). In addition, overexpression of both ferritin H and ferritin L has been shown to protect against doxycycline-induced ROS accumulation in HeLa cells (Orino et al., 2001). In a model of acute pancreatitis in Wistar rats and AR42J pancreatic cells, ferritin was subject to degradation, which correlated with increased ROS production (Sledzinski et al., 2013).

1.3.2 NQO1

Another Nrf2 target, NQO1, provides a more direct way of tackling ROS and oxidative stress. The primary role of NQO1, as the name suggests, is to reduce quinones to hydroquinones using NADPH as a reductant. This has the effect of preventing the unfavourable reduction of quinones by reactions which produce free radicals (Bianchet et al., 2004). Quinones are common molecules, and sources of quinone-induced oxidative stress can range from environmental toxins (Laskin et al., 1995; Prisby et al., 2008) to hormones (for example, metabolites of oestrogens (Singh et al., 2012)). NQO1 may also have a secondary role as a direct scavenger of superoxide (Siegel et al., 2004).

NQO1 has been shown to protect against oxidative stress caused by a large range of inducers. For example, both tectorigenin and glycitein are NQO1 inducers that protect rat astrocytes from H₂O₂-induced ROS and cell death. The NQO1 inhibitor dicoumarol prevented the protective effects of these compounds (Park et al., 2011a). Furthermore, NQO1 silencing exacerbated cisplatin-induced ROS in human renal tubular cells (Gang et al., 2013). NQO1 is also believed to play an integral role in the detoxification of the metabolites of benzene, many of which are quinones. A C609T mutation in NQO1 has been associated with an increased risk to the toxic effects of benzene, possibly by inhibiting the production of NQO1 (Moran et al., 1999).

In addition to detoxifying quinones from external sources, a potential role for NQO1 is arising in protecting against neurodegenerative diseases. Metabolites of dopamine are thought to be involved in the development of Parkinson's disease by blocking 20s proteasome activity, a process that NQO1 can prevent (Zafar et al., 2006).

1.3.3 Glutathione

Glutathione (GSH) is probably the most direct regulator of ROS, and is extremely prevalent in animal cells, available at concentrations in the mM range. GSH is a tripeptide of glutamic acid, cysteine and glycine, although the cysteine and glutamic acid are bound through the carboxyl group of the glutamate side chain, rather than its backbone. GSH exerts its activity through the thiol group of the cysteine residue, which can directly neutralise oxidants by acting as a reducing agent. Although the oxidised form of GSH is a reactive free radical, it doesn't exist in this form, and instead reacts with itself to form a stable disulfide, GSSG. This is normally catalysed by GSH peroxidase (Deponter, 2013).

Biologically, GSH has been well documented to regulate redox homeostasis, and Nrf2 has been shown to act upstream of GSH activity (Hayes and McLellan, 1999; Jozefczak et al., 2012). The role of Nrf2 in GSH biochemistry is to induce enzymes that are involved in the production of GSH. Nrf2 knockout mice are susceptible to oxidative stress because they have impaired GSH production, which has been attributed to an inhibition of glutamate cysteine ligase (GCL) and GSH synthetase expression (Chan et al., 2001). Similarly, isorhamnetin was able to induce Nrf2 and GCL in HepG2 cells. Depletion of Nrf2 inhibited GCL expression. t-BHP-induced GSH depletion, oxidative stress and cytotoxicity were all inhibited by isorhamnetin (Yang et al., 2014a).

1.4 Inflammation

In the last decade, Nrf2 has also been shown to have anti-inflammatory effects (Kong et al., 2010; Foresti et al., 2013). Inflammation is a key process that protects the body by creating localised areas of stress, which is important for activating the immune system and wound healing, and creating a hostile environment for pathogens (Shi and Pamer, 2011). However, left unchecked, this can rapidly become harmful to host tissue (Mu et al., 2013). Chronic inflammation plays a major role in a wide variety of diseases, ranging from those for which inflammation is a primary factor, such as inflammatory bowel disease or sepsis, to those where inflammation is an underlying component of the pathogenic process, such as neurodegenerative diseases and cardiovascular diseases (Lohner et al., 2013; Stoner et al., 2013; Amor et al., 2014; Zhang and Li, 2014). Even conditions that appear unrelated, such as depression, have been linked with inflammatory mediators (Vogelzangs et al., 2012). This wide range of effects makes inflammation an important process to target therapeutically, and over the last decade it has become increasingly apparent that activation of Nrf2 is a very promising avenue to pursue.

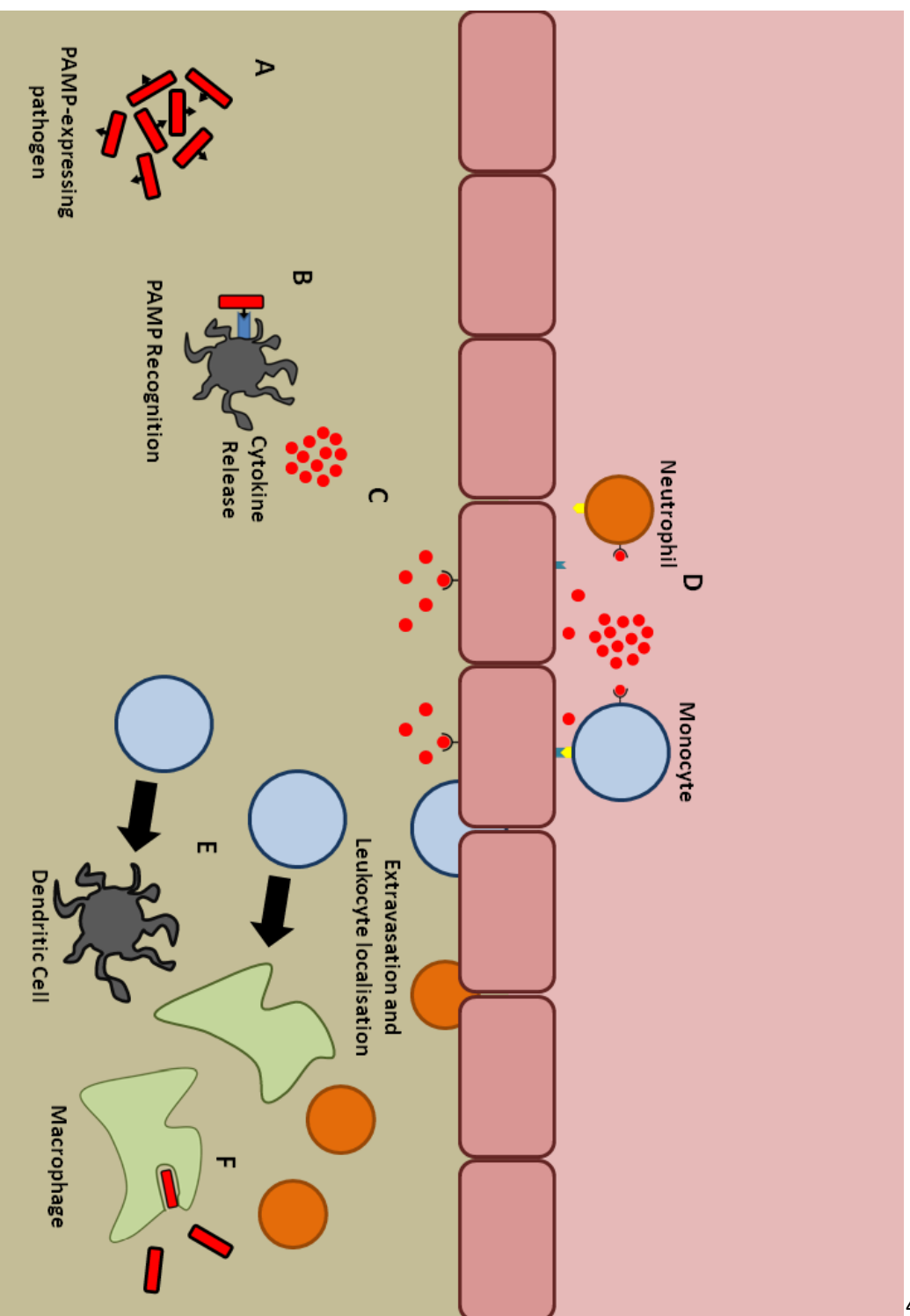
1.4.1 The inflammatory response

Inflammation has been characterised for approximately 2000 years by its cardinal signs of redness, heat, swelling and pain, originally noted by Cornelius Celsus. Today inflammation is better understood mechanistically as a response of the body to damage, which can refer to anything from a physical wound to an invasion of pathogens. The inflammatory response encompasses a wide array of processes ranging from recognition of the

threat, up to activation of the adaptive immune system (Alessandri et al., 2013) (Fig 1.3).

Fig 1.3. The inflammatory response.

(A) The inflammatory cascade is often started by infection of tissue by pathogens. **(B)** Pathogens are recognised by tissue-resident leukocytes, which activates a pro-inflammatory signalling cascade resulting in **(C)** cytokine release. Cytokines have many functions, including activating the inflammatory response in other leukocytes. One way of doing that is **(D)** initiation of leukocyte extravasation and localisation to the point of infection. Neutrophils arrive first and exacerbate the inflammatory response if necessary. **(E)** Monocytes differentiate into dendritic cells and macrophages. **(F)** Phagocytes destroy invading pathogens.



1.4.1.1 Pattern recognition receptors.

Pattern recognition receptors (PRRs) are expressed in a wide range of immune cells. They bind “pathogen-associated molecular patterns” (PAMPs), which are essentially common structures found in a wide range of pathogens. An example would be lipopolysaccharide (LPS), which is expressed in all gram negative bacteria. Although there are many types of LPS, they all share the same basic structure, which can be recognised by the PRR toll-like receptor 4 (TLR4). PRRs can be externally expressed on the cell membrane or internally expressed in the cytoplasm. Typically PRRs on the cell membrane recognise PAMPs which are externally expressed by pathogens. Cytoplasmic PRRs recognise PAMPs that become exposed to the cell’s cytoplasm, which can occur when a pathogen is internalised by the cell either by invasion or phagocytosis (Takeda and Akira, 2005). PRR activation activates signalling pathways that result in the transcription and translation of pro-inflammatory cytokines. The specific PAMP and PRR involved will often activate specific pathways, which control the cytokines that are produced (Teixeira-Coelho et al., 2014).

The TLR family are a versatile set of PRRs that can recognise molecules belonging to bacteria, fungi or viruses (Table 1.6). They are expressed by a wide range of leukocytes and epithelial cells (Brigotti et al., 2013; Krejsek et al., 2013; Ling et al., 2014; Ortega-González et al., 2014; Teixeira-Coelho et al., 2014). Lipopolysaccharide (LPS), a component of gram negative bacterial cell walls, is a powerful activator of monocytes and binds TLR4 (Hoshino et al., 1999). LPS activation of monocytes will be utilised as an *in vitro* model of inflammation in this study.

TLR	Pathogens	Ligands	Reference
TLR1/2	Bacteria	Triacyl Lipopeptides	Takeda et al., 2002
	Gram Positive Bacteria	Peptidoglycan, Lipotechoic Acid	Schwandner et al., 1999
TLR2	Bacteria	Lipoproteins	Aliprantis et al., 1999
	Yeast	Zymosan	Dillon et al., 2006
TLR3	Virus	Double Stranded RNA	Alexopoulou et al., 2001
TLR4	Gram Negative Bacteria	LPS	Hoshino et al., 1999
TLR5	Bacteria	Flagellin	Hayashi et al., 2001
TLR2/6	Mycoplasma	Diacyl Lipopeptides	Into et al., 2004
TLR7	RNA Virus	Single Stranded RNA	Heil et al., 2004
TLR8	RNA Virus	Single Stranded RNA	Heil et al., 2004
TLR9	Bacteria and Viruses	Unmethylated CpG DNA	Bauer et al., 2001

Table 1.6 Toll-like receptors. A non-exhaustive list of the PAMPs that are commonly associated with each TLR.

TLR4 uses adaptor proteins to bind LPS. The first of these is LPS binding protein (LBP), which facilitates the binding between LPS and a second adaptor protein, CD14. LBP may be able to extract LPS from bacteria directly (Jerala et al., 2007). CD14 has been implicated in activating differential signalling pathways depending on the structure of the LPS (Regen et al, 2011). The final adaptor protein required for LPS signalling via TLR4 is a member of the ML superfamily called MD-2. When MD-2 is bound to TLR4 its affinity for LPS increases (its affinity for TLR4 is constant whether LPS is bound or not) (Mitsuzawa et al, 2006). LPS, bound to CD14, will bind MD-2 monomers, of which there are two bound to a TLR4 homodimer. This is essential for LPS signalling, and no role for TLR4 has been identified without MD-2 (Shimazu et al, 1999).

1.4.1.2 TLR4 Signalling

TLR4 signals through two major pathways (Fig 1.4). These are known as the myeloid differentiation primary response gene (88) (MyD88) dependent pathway and the MyD88 independent pathway. Most TLRs signal through the MyD88 dependent pathway (Kumar et al, 2009). MyD88 is an adaptor protein that is recruited to the cytoplasmic domain of TLR4 upon its activation. A second adaptor protein, TIRAP (also known as MAL), acts to increase the affinity of the interaction between MyD88 and TLR4. This complex, and the downstream targets that it recruits, is called the myddosome (reviewed in: Gay et al., 2011).

MyD88 then recruits IL-1 receptor associated kinase-4 (IRAK-4) to the receptor complex. IRAK-4 is essential for MyD88 signalling but other IRAK proteins may not be. IRAK-4 interacts with IRAK-1, although IRAK-1 knockout mice only had a partially inhibited MyD88 dependent pathway. IRAK-2 may also play a role in signalling downstream of IRAK-4 (Wesche et al., 1997; Suzuki et al., 2002; Keating et al., 2007; Lu et al., 2008).

TNF receptor associated factor 6 (TRAF6) lies downstream of IRAK-4 and IRAK-1, and has been reported to be essential for MyD88 dependent signalling. This suggests that any variance in substrates of IRAK-4 converge again on TRAF6. TRAF6 forms a complex with Tab1 and Tab2 to activate TAK1 (Jiang et al., 2002). This results in activation of I κ B Kinases (IKKs), which phosphorylate inhibitor of κ B proteins (I κ Bs). I κ Bs are inhibitors of nuclear factor- κ B (NF- κ B), which are deactivated by phosphorylation (Lu et al., 2008; Avila et al., 2012).

NF- κ B is the most important transcription factor for the inflammatory response, but others are also activated by the MyD88 dependent pathway.

These include interferon regulatory transcription factor 5 (IRF5), activator protein-1 (AP-1), and mitogen activated protein kinases (MAPK) (Guha and Mackman, 2001; Moynagh, 2005; Lu et al., 2008).

TLR4 is unique among the TLRs in that knocking down MyD88 does not completely abolish NF- κ B activation. Instead, NF- κ B is activated by the MyD88 independent pathway (also known as the TRIF dependent pathway), which happens to be the same pathway through which TLR3 signals (Piao et al., 2013).

Upon TLR4 activation both TIR-domain-containing adaptor-inducing interferon β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited to the receptor complex. Both are essential for MyD88 independent signalling. TRIF recruits TRAF3. TRAF3 controls downstream signalling by activating a complex of TRAF family member-associated NF- κ B activator (TANK), TANK binding kinase 1 (TBK1) and IKKi. TBK1 and IKKi are involved in dimerization and nuclear translocation of the transcription factor IRF3, which results in production of type I interferons (IFN α/β) (Youn et al., 2005; Lu et al., 2008).

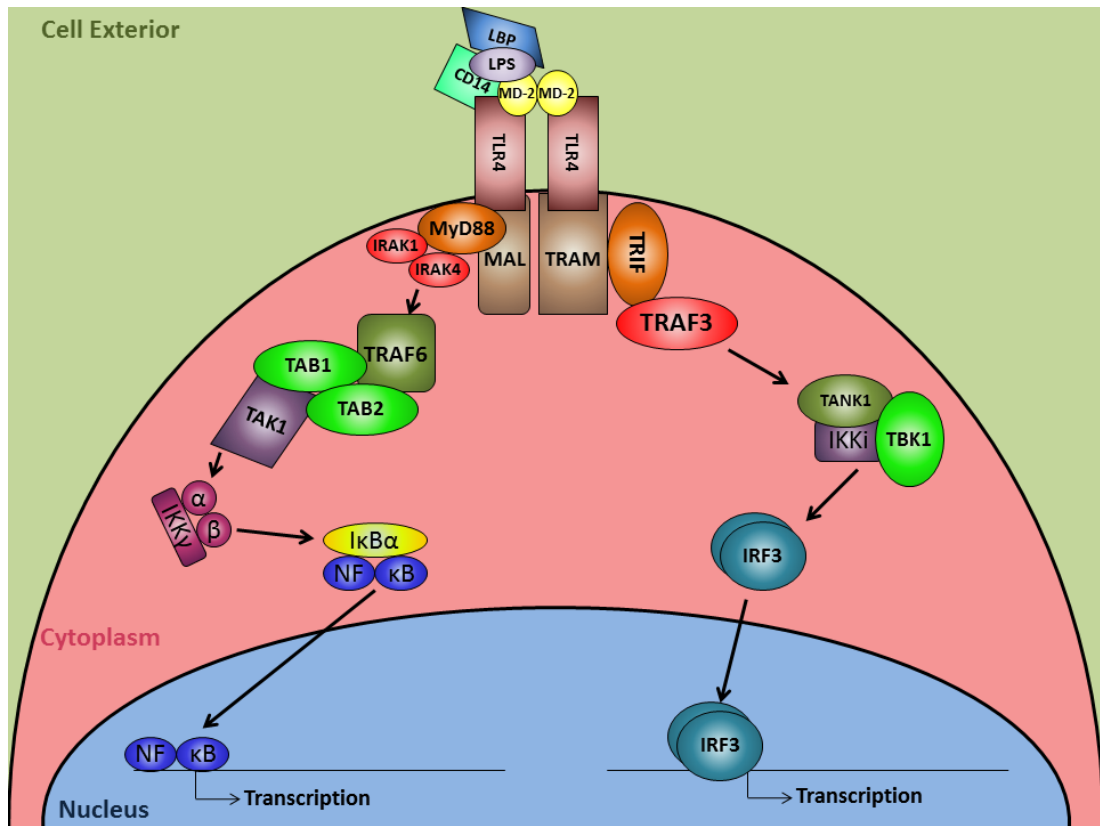


Fig 1.4. TLR4 Signalling. TLR4 signals via the MyD88 dependent and MyD88 independent pathway.

TRIF also activates the serine/threonine kinase RIP1. RIP1 is involved in the MyD88 independent activation of NF-κB and MAPK (Youn et al., 2005).

1.4.1.3 Cytokines, chemokines and NF-κB

NF-κB refers to a small family of transcription factors comprising v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA; also known as p65), RELB, C-Rel, p50 and p52, which all contain an N-terminal Rel homology domain. p50 and p52 contain no transactivation sites of their own, and frequently form heterodimers with RelA, RelB, or c-Rel (Hoesel and Schmid, 2013).

NF- κ B is an important mediator of inflammation. Constitutive activation of NF- κ B in mice caused death after 7-10 days. The mice had severe dermatitis, increased concentrations of monocytes and macrophages in the spleen, and overproduction of inflammatory mediators (Klement et al., 1996). Furthermore, anti-inflammatory mediators are often linked with inhibition of NF- κ B (Ivanenkov et al., 2011; Oh et al., 2013; Wang et al., 2014a).

The reason that NF- κ B is so important for inflammation is that its function is to initiate transcription of cytokines that regulate the innate and adaptive immune responses (Hoesel and Schmid, 2013; Gerondakis et al., 2014). Cytokines are messengers of the immune system, and are usually secreted by leukocytes to regulate processes of surrounding leukocytes. Common cytokines include the interleukin family, tumour necrosis factor (TNF), and the interferons (Table 1.7). Once these are produced at the site of damage or infection, they are secreted into the extracellular environment to find and activate other immune cells. IL-1 β does exactly this by stimulating the production of adhesion molecules in endothelial cells, and by stimulating further release of pro-inflammatory cytokines in other leukocytes (Tsang et al., 1997; Kasza et al., 2013). TNF plays a similar role, and also activates phagocytes. Some cytokines, called chemokines, regulate chemotaxis (Zelová and Hošek, 2013). These typically activate leukocyte extravasation, and subsequent migration to the site of infection. Neutrophils are the first to arrive. Neutrophils release defensins, which are proteases and lysozymes that are secreted to kill bacteria from range (Baines et al., 2011). In addition, neutrophils also release reactive oxygen species to kill pathogens. These mediators, and the debris caused by destroyed bacteria, can harm host cells and are the cause of the fourth cardinal sign of inflammation, pain (Dupré-Crochet et al., 2013).

Full Name	Abbreviation	Role
Type 1 Interferons	IFN α/β	Induction of antiviral activity (Arimori et al., 2013) Monocyte differentiation into dendritic cells (Farkas and Kemény, 2011)
Interleukin 1 β	IL-1 β	Upregulation of many pro-inflammatory cytokines (Kasza et al., 2013) Upregulation of tissue factor and coagulation (Yang et al., 2013) Induces selectins on endothelial cells (Tsang et al., 1997)
Interleukin 6	IL-6	Th17 cell differentiation (Zhang et al., 2013b) Key regulator of acute phase proteins (Bode et al., 2012)
Interleukin 8	IL-8	Induction of MCP-1 (Zhang et al., 2013c) Neutrophil chemotaxis (Harada et al., 1994)
Monocyte Chemoattractant Protein-1	MCP-1 (CCL2)	Monocyte chemotaxis (Chuang et al., 2011)
Tumour Necrosis Factor	TNF	Upregulation of pro-inflammatory cytokines (Kearney et al., 2013) Induction of chemotaxis adhesion molecules (Nallasamy et al., 2014; Thichanpiang et al., 2014)

Table 1.7 Important cytokines in inflammation. Many cytokines play an important role in inflammation, but these are important in the amplification of inflammatory signalling.

In addition to leukocyte recruitment, cytokines stimulate the release of acute phase proteins (APPs). These are proteins that are either upregulated (positive APPs) or downregulated (negative APPs) in inflammation. If cytokines are the messengers of the immune system, positive acute phase proteins can be considered those that carry out the functions.

Some of the first APPs to be activated in the inflammatory response are Opsonins. Opsonins are proteins that enhance phagocytosis. Phagocytosis is

the process by which leukocytes can engulf and break up pathogens (Sjöwall and Wetherö, 2007). Neutrophils, monocytes, dendritic cells and macrophages are capable of phagocytosis. Monocytes are large phagocytes, which are precursor cells to macrophages and dendritic cells. Upon arriving at the site of infection monocytes differentiate into one of these types of cell depending on the cytokines that are present in the extracellular matrix (Huber et al., 2014). Macrophages and dendritic cells are antigen presenting cells, meaning that they can activate the adaptive immune system via T-cell activation by presenting fragments of pathogens on their cell membrane (Dresch et al., 2012; Altaf and Revell, 2013).

Opsonins include C-reactive protein and components of the complement system. C-reactive protein is a PRR that recognises phosphocholine on apoptotic cell membranes, and is largely involved in initiating clearance of dead tissue, but is also involved in bacterial recognition. The complement system is a protein cascade that interacts directly with bacteria and fungi. It plays several roles in the immune system including marking bacterial cells for phagocytosis, and killing pathogens directly. One part of the complement system, the membrane attack complex, makes pores in the cell membrane of the pathogen, causing cell contents to leak and apoptosis to occur (Sjöwall and Wetherö, 2007; Tegla et al., 2011; Barratt-Due et al., 2012).

Other APPs include coagulants, which function to clot blood. In inflammation, the function of this is to isolate the infected area from the rest of the body (O'Brien, 2012; Woel-A-Jin et al., 2012).

Some APPs are negative regulators of inflammation and contribute to the resolution of inflammation. The resolution of inflammation is the process by which inflammation is ended when it is no longer needed. For example, serpins are anti-coagulants, and work to restore the vascular system. Another important process in the resolution of inflammation is induction of apoptosis

and subsequent clearance of accumulated leukocytes. If resolution of inflammation doesn't occur then inflammatory diseases can manifest.

1.4.1.4 The development of inflammatory diseases

Inflammation is an essential function of the body to defend against injury and infection, but if it is not properly regulated it can manifest into an inflammatory disease. Inflammatory diseases are characterised by conditions whereby leukocytes over-produce ROS, which causes extensive damage to host tissue, and over-stimulate lymphocytes, which can lead to autoimmunity.

Sepsis is a good example of the consequences of an unregulated inflammatory response. It is a systemic inflammatory disease, which is normally caused by an over-reaction to bacterial or fungal infection. In sepsis, an unregulated inflammatory response can lead to necrosis, multiple organ failure and death (Wiersinga et al., 2014). The first problem is overproduction of inflammatory mediators and ROS by leukocytes. ROS react with host tissue, causing necrosis. This causes more ROS production from processes such as lipid peroxidation (Andrades et al., 2011). The overproduction of inflammatory mediators further exacerbates the inflammatory response. Blood pressure can be drastically lowered due to increased vascular permeability, leukocytes can harm host tissues, but perhaps the most damaging aspect is the overproduction of coagulants. This leads to blood clotting and ischemia, eventually causing multiple organ failure and subsequent death (Takala et al., 2010; Semeraro et al., 2012; Wiersinga et al., 2014).

1.4.2 Nrf2 in inflammatory diseases

Nrf2 is known to protect against inflammation and down-regulate pro-inflammatory cytokines *in vivo*. Nrf2 knockout mice display characteristics of an unregulated inflammatory response, and lupus-like autoimmune symptoms (Ma et al., 2006). In a mouse model of elastin-induced emphysema Nrf2 knockout was accompanied with susceptibility to the disease, which was linked with a high level of inflammation. This was characterised by alveolar oedema, haemorrhage, and neutrophil infiltration. Furthermore, increased neutrophils and macrophages were found in bronchiolar fluid compared to WT mice (Ishii et al., 2005). In a model of ConA-induced liver inflammation in mice, hepatocyte-specific Keap1 knockout inhibited macrophage and neutrophil accumulation in the liver. Furthermore, hepatocytic pro-inflammatory cytokines, IFN- γ , TNF, Mip-2, and MCP-1 were inhibited compared to WT mice (Osburn et al., 2008). *In vitro*, Nrf2 plays a natural role in regulating inflammation. For example, our lab has previously shown that Nrf2 knockdown by siRNA leads to an exacerbation of LPS-induced TNF and IL-1 β in THP-1 monocytes (Rushworth et al., 2008). Several studies have shown that induction of Nrf2 with different classes of Nrf2 activators *in vitro* and *in vivo* provide protection against pro-inflammatory mediators. In RAW264.7 macrophages dihydro-CDDO-trifluoroethyl amide activated Nrf2 and inhibited LPS-induced MCP-1 and MIP-1 β . The suppression of pro-inflammatory cytokines was lost in the presence of Nrf2 siRNA (Li et al., 2014a). LPS is not the only activator of inflammation to be inhibited by Nrf2. S100b-induced TNF and IL-1 in THP monocytes is blocked by dimeric acid, except in the presence of Nrf2 siRNA (Lee et al., 2013a).

There has therefore been a lot of interest in finding activators of Nrf2 that can provide these protective effects. Nrf2 activation has been associated with anti-inflammatory effects caused by epigallocatechin-3-gallate (EGCG) in mice with lupus (Tsai et al., 2011) and in a rat model of bleomycin-induced pulmonary fibrosis (Sriram et al., 2009). Although no causal relationship with Nrf2 has been observed, EGCG-mediated inhibition of TNF-induced ICAM-1 and ROS in A549 cells was abrogated by HO-1 siRNA (Lee et al., 2013b). Although causal relationships are important to show, correlation of Nrf2 activation with anti-inflammatory activity is common. In May 2014 alone diallyl sulfide, 3,4,5-trihydroxycinnamic acid, zinc, α -viniferin, rutin, ulinastatin, and osthole have all been suggested to protect against inflammation via the Nrf2 pathway (Dilshara et al., 2014; Ho et al., 2014; Lee et al., 2014a; Pan et al., 2014; Song et al., 2014; Yang et al., 2014b; Zhang et al., 2014b).

The mechanisms behind Nrf2-mediated anti-inflammatory activity are complex and numerous, with many studies proposing different processes. However, they can be split into two main groups: those that act via Nrf2 target genes, and those that do not.

Nrf2 target genes are known to inhibit inflammatory mediators. We have previously shown that overexpression of NQO1 and/or HO-1 inhibits TNF and IL-1 β expression in monocytes in response to LPS, whereas knockdown of HO-1 and NQO1 has the inverse effect (Rushworth et al., 2008). HO-1 in particular has been linked with anti-inflammatory effects, potentially linked to CO production (Onyiah et al., 2013; Anyanwu et al., 2014). Peritoneal macrophages from HO-1 deficient mice produce increased levels of ROS and inflammatory mediators including IL-6 and MCP-1. This has been linked with an increased susceptibility to atherosclerosis (Orozco et al., 2007). HO-1 has

also been linked to protection from ischaemia (Richards et al., 2010; Park et al., 2013; Huang et al., 2014).

However, it has become apparent that Nrf2 target genes are not the only mechanism by which Nrf2 exerts anti-inflammatory effects, and crosstalk between the Nrf2 signalling pathway and the NF- κ B pathway in particular has been proposed by numerous lab groups. Furthermore, Keap1 may be a key regulator of this anti-inflammatory activity, rather than Nrf2 itself (Lee et al., 2009a; Kim et al., 2010).

The Nrf2 inhibitor, Keap1 is also a negative regulator of IKK β and interacts with it using the same core binding sites as it does when it binds Nrf2 (Kim et al., 2010). Although they use the same binding site, computational docking research has suggested that the binding of both Nrf2 and IKK β are slightly different (Jiang et al., 2013). IKK β is a positive regulator of NF- κ B. Keap1 siRNA has been shown to activate IL-6 via NF- κ B signalling and exacerbate TNF-induced NF- κ B activity in HEK293 and breast cancer cells (Lee et al., 2009a; Kim et al., 2010; Lv et al., 2013). However, Keap1 silencing has been used to cause Nrf2 overexpression, which has been shown to protect against inflammation in the vast majority of the literature, so the significance of this is debatable (Osburn et al., 2008; Blake et al., 2010; Lu et al., 2014a).

Not every study agrees that Nrf2 is protective against inflammation, however. Nrf2 may be involved in the NLRP3 inflammasome formation. Nrf2 knockout mice showed a decrease in inflammatory response, which was believed to signal through ROS production (Zhao et al., 2014). Furthermore, the involvement of Nrf2 in the NLRP3 inflammasome may also play a role in atherosclerosis (Freigang et al., 2011). Interestingly, some evidence that points to anti-inflammatory roles of Nrf2 may act by inhibiting the NLRP3 inflammasome (Tsai et al., 2011; Yang et al., 2013). More research is required to bring these opposing roles together, but it is possible that Nrf2 plays a

larger role in regulating the NLRP3 inflammasome that goes beyond simple inhibition of inflammatory activity.

It is difficult to reconcile the research that shows a pro-inflammatory role for Nrf2 with that which shows that Nrf2 is an important anti-inflammatory mediator, although evidence supporting the latter overwhelms the former. Understanding regulation of Nrf2 activity will be important if Nrf2 is to be seriously considered as a drug target for inflammatory diseases.

1.5 Nrf2 Regulation

Nrf2 expression is tightly regulated by several mechanisms, and typically Nrf2 only mediates gene transcription under conditions of cellular stress. Under normal conditions Nrf2 is found in the cytoplasm in a complex with its inhibitor Keap1, and only when this interaction is disrupted, Nrf2 accumulates in the nucleus (Itoh et al., 1999). Keap1 is the primary regulator of Nrf2, but it is not the only mechanism by which Nrf2 activity is controlled. Other regulators include microRNAs, Nrf2 phosphorylation, *de novo* synthesis of Nrf2 mRNA, and other CNC transcription factors (Huang et al., 2002; Rushworth et al., 2008; Kaspar and Jaiswal, 2010; Cheng et al., 2013).

1.5.1 Keap1

Keap1 was originally identified in 1998 as a protein consisting of 624 amino acids with five domains. Keap1 is classified as a member of the kelch repeat superfamily due to the presence of six double glycine repeat (also known as kelch repeat) motifs in the kelch domain (Table 1.8). Kelch domains form propeller-like structures called β -propellers. Each kelch repeat consists of 44-56 amino acids and forms an anti-parallel β -sheet, which consists of 4 β -strands. Repeats in a kelch domain will then form a ring—like structure with each individual repeat becoming a propeller blade of the larger structure (Adams et al., 2000). The kelch domain is a protein binding domain, and has been shown to anchor keap1 to the actin cytoskeleton and to bind directly to the Neh2 domain of Nrf2. Actin binding has been shown to be essential for keap1 activity (Itoh et al., 1999; Kang et al., 2004).

Domain	Function
N-terminal	Unknown
BTB	Cul3-dependent E3 ubiquitin ligase complex binding (Canning et al., 2013; Chauhan et al., 2013) Cysteine 151 regulates Nrf2 activity (Yamamoto et al., 2008)
IVR	Redox sensitive regulation of Nrf2 ubiquitination (Yamamoto et al., 2008; Kansanen et al., 2011)
Kelch	Nrf2 binding (Itoh et al., 1999) Other E3 ubiquitin substrate binding (IKK β , BCL-2) (Tian et al., 2012)
C-terminal	Unknown

Table 1.8 A summary of domain functions in Keap1. The functions of both terminal domain have not attracted much interest, but the BTB, IVR, and Kelch domains are integral to Keap1 function.

1.5.2 The E3 ubiquitin ligase complex

Keap1, although considered an inhibitor of Nrf2 activity, is actually an adaptor protein for the E3 ubiquitin ligase complex, which transfers ubiquitin from E2 ubiquitin ligase to a substrate protein (Zhang et al., 2004; Furukawa and Xiong, 2005). Polyubiquitination acts as a marker for protein degradation (Fig 1.5). An E3 ubiquitin ligase complex typically consists of a scaffold, an E2 binding RING protein, a bridge and an adaptor protein. The variability of the individual components of the complex allow for some degree of specificity within the system. The cullin family forms the scaffold of the ubiquitin ligase complex, and acts as adaptor protein binding sites. Cul3 is an interesting member of the cullin family, because it is thought that in Cul3 based ubiquitin ligase complexes the adaptor protein contains both the Cul3 and

substrate binding domains, eliminating the need for a bridge (Cullinan et al., 2004; Furukawa and Xiong, 2005).

In the case of Keap1, the Nrf2 binding Kelch domain acts as the adaptor, and the Cul3 binding BTB domain acts as the bridge (Pintard et al., 2004; Cullinan et al., 2004). Rbx1 is a RING protein that associates with Cul3 and Keap1, and its presence increases ubiquitination of Nrf2 (Small et al., 2010). Its function is to bind E2 ubiquitin ligase, which brings it into close proximity with Nrf2 (Furukawa and Xiong, 2005).

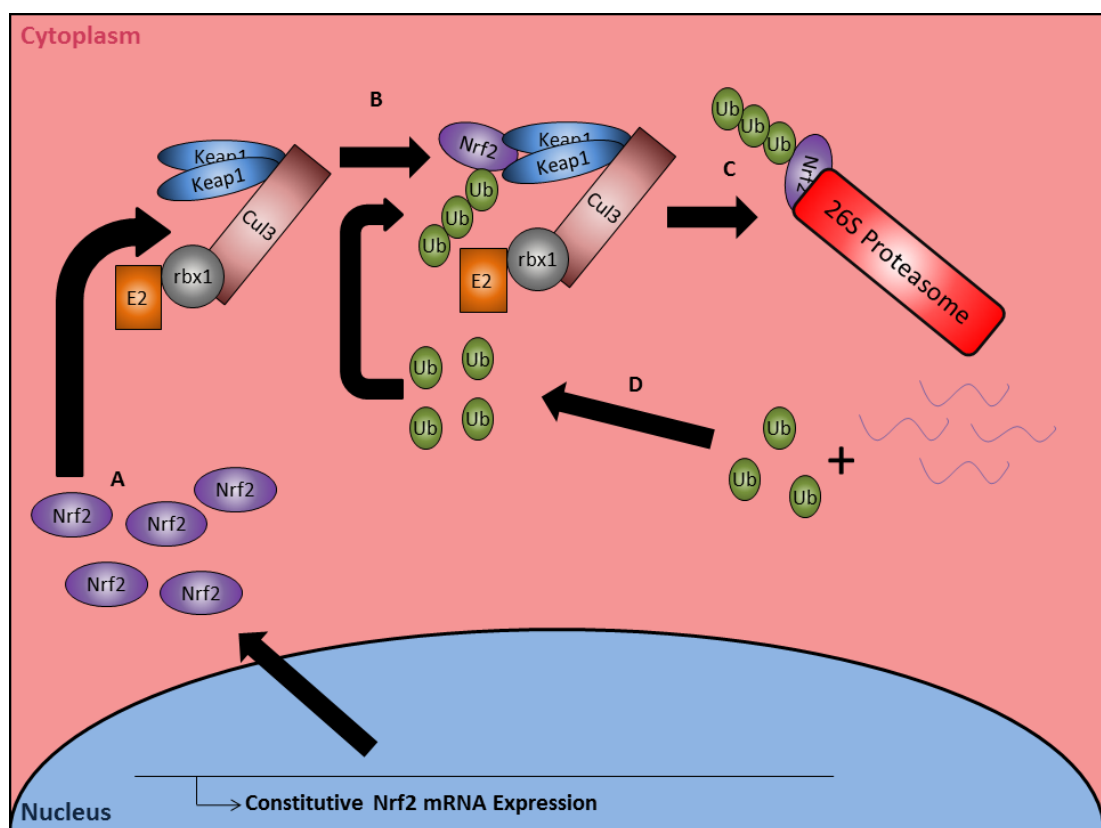


Fig 1.5 Nrf2 ubiquitination and degradation. (A) Nrf2 is constitutively expressed, and binds Keap1 homodimers in the cytoplasm. (B) Keap1 facilitates Nrf2 ubiquitination by the E3 ubiquitin ligase complex. (C) This targets Nrf2 for degradation by the 26S proteasome, and (D) allows ubiquitin to be recycled.

Ubiquitination of Nrf2 occurs on multiple lysine residues, supported by data showing that lysine to alanine substitutions at positions 19, 23, and 30 blocked degradation of Nrf2. This supports the same group's predicted structure of the Neh2 domain, in which the N-terminal sub domain is α -helical with these three lysine residues clustered on one side of the helix. A conserved region throughout CNC transcription factors is a DLG motif at residues 29-31. The leucine appears to be the most important residue because a mutation, L30A, inhibited the Nrf2-Keap1 interaction and subsequent Nrf2 ubiquitination (Katoh et al., 2005). Displacement of this motif may cause a conformational change in Nrf2, which prevents ubiquitination of the nearby lysine-rich region (Tong et al., 2006).

Keap1, therefore, is a chief negative regulator of Nrf2 activity. There are several proposed mechanisms for the regulation of the Nrf2/Keap1 complex. However, they all have the same underlying principles. In essence, disrupting Nrf2/Keap1 binding blocks Nrf2 ubiquitination (Fig 1.6). This prevents Nrf2 degradation. Newly synthesised Nrf2 is no longer sequestered in the cytoplasm by Keap1, and can enter the nucleus to promote transcription.

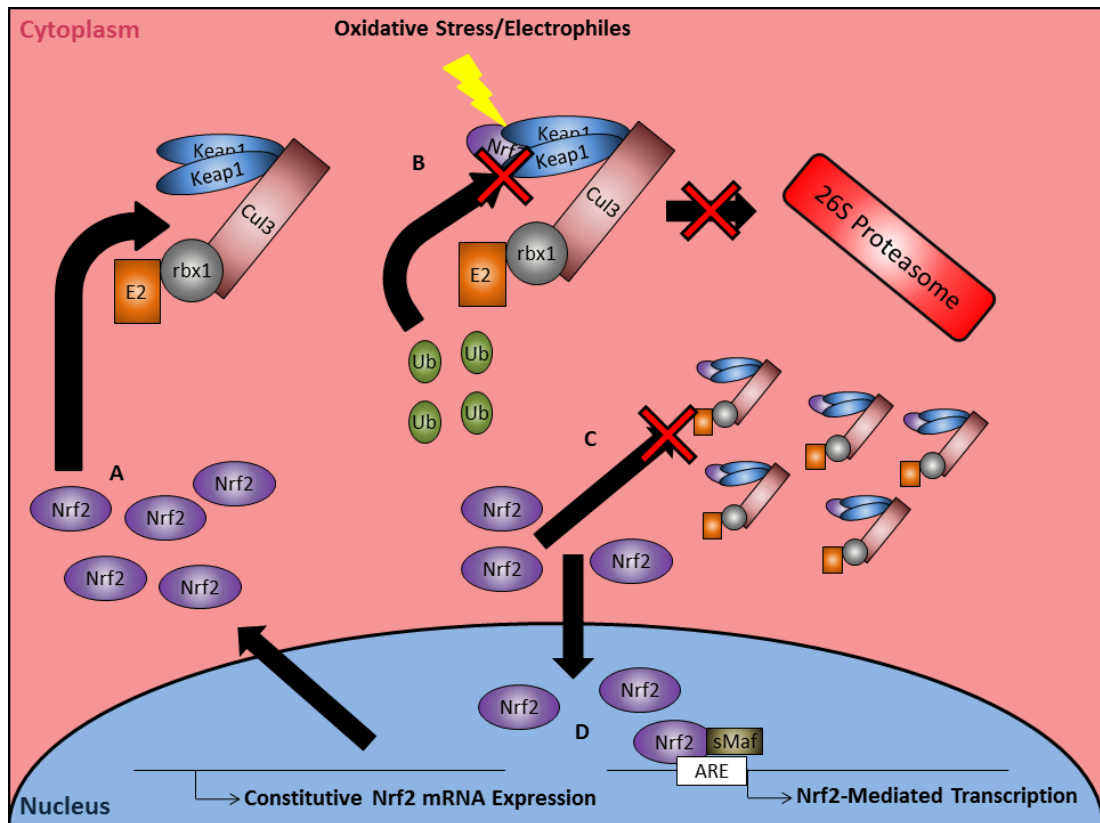


Fig 1.6 Nrf2 activation. (A) Nrf2 is constitutively expressed, and binds Keap1 homodimers in the cytoplasm. **(B)** Modification of Keap1 by inducers cause a conformational change in the Nrf2/Keap1 interaction, blocking ubiquitination and subsequent degradation. **(C)** This results in Keap1 saturation, causing accumulation of newly created Nrf2. **(D)** Nrf2 can now translocate to the nucleus to start transcription.

1.5.3 The Nrf2/Keap1 interaction

Since the discovery of Keap1 there have been several proposed models for the disruption of the Nrf2/Keap1 interaction (Tong et al., 2006a; Baird et al., 2013). The first of which is the sequester and release model, which originally proposed that activation of Nrf2 was caused by its complete dissociation of Keap1. This was more of an assumption than a supported model, and was largely based on observations showing that Keap1 anchors Nrf2 in the cytoplasm, and Nrf2 nuclear accumulation can be caused by conformational changes in Keap1 (Itoh et al., 1999; Zipper and Mulcahy, 2002; Itoh et al., 2003).

The problem with this model of Nrf2 regulation became apparent as it was shown that Nrf2 nuclear accumulation could be induced without altering its binding to Keap1 (Kobayashi et al., 2006). In addition, it was shown that the Neh2 domain of Nrf2 contains two Keap1 binding sites, each of which binds one Kelch domain in a Keap1 homodimer (Fig 1.7). The two sequences are ²⁹DLG³¹ and ⁷⁹ETGE⁸². The strong binding ETGE sequence anchors Nrf2 to Keap1, whereas the weak binding DLG sequence acts as a regulatory element for Nrf2 ubiquitination (Katoh et al., 2005; McMahon et al., 2006; Tong et al., 2006b; Tong et al., 2007).

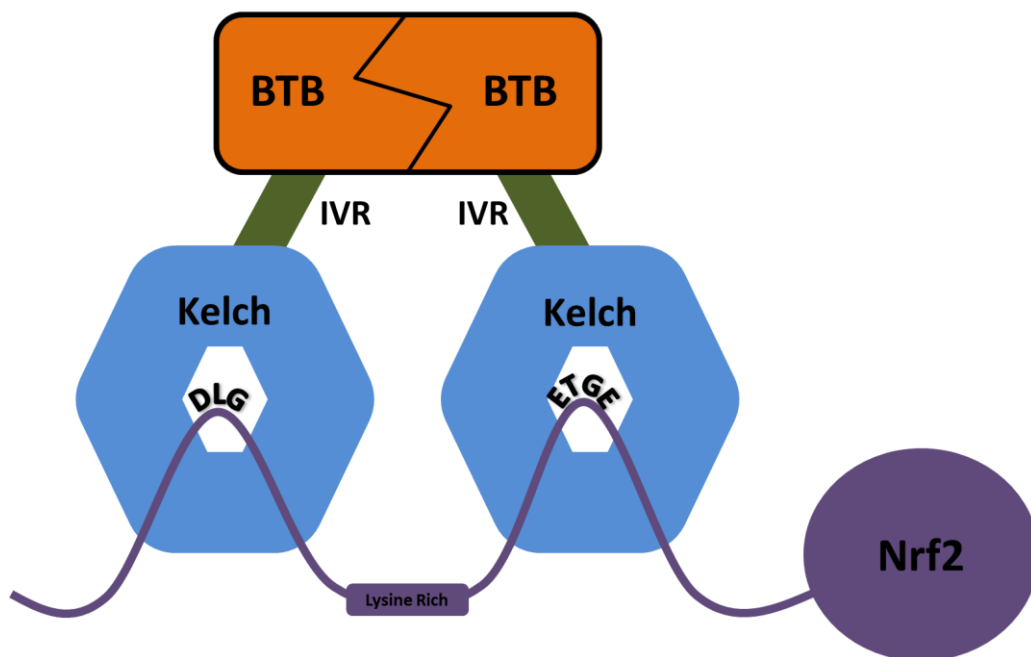


Fig 1.7 The Nrf2/Keap1 interaction. The N-terminal Neh2 domain of Nrf2 contains two Keap1 binding sites. The DLG motif binds one kelch domain of a Keap1 homodimer, and the ETGE motif binds the other.

This configuration of the Nrf2/Keap1 interaction led to a new explanation for how Nrf2 is regulated. This was called the hinge and latch mechanism, and proposed that activation of Nrf2 is caused by displacement of the weaker DLG motif while the ETGE motif remains bound to Keap1. The displacement of the DLG motif blocks Nrf2 ubiquitination, and Keap1 becomes saturated due to the lack of Nrf2 degradation. This allows newly synthesised Nrf2 to enter the nucleus. The key implication of this model is that a drug designed to induce Nrf2 activation would only need to inhibit the weaker DLG/Kelch interaction, which is a much easier task and may be possible using ETGE-like sequences (Tong et al., 2006a; Hancock et al., 2012). This hinge and latch model is also purely hypothetical with little experimental evidence backing it up, and recent evidence suggests that it is only half right.

The Nrf2/Keap1 interaction is slightly more dynamic than previously thought. Using a FRET-based technique the binding between Nrf2 and Keap1 was examined experimentally, and has led to the suggestion of a conformational cycling model. Under resting conditions Nrf2 binds Keap1 in two conformations, an open conformation and a closed conformation. Like the hinge and latch mechanism, the open and closed conformations revolve around the binding of the DLG motif. However, in contrast to the implications of the hinge and latch model, Nrf2 activators were shown to induce the closed conformation. Based on this evidence, the authors propose a mechanism whereby Nrf2 binds Keap1 via the stronger ETGE motif first (the open conformation), and then binds with the weaker DLG motif (the closed conformation). This means that at any one time there are two conformations of the Nrf2/Keap1 interaction. The closed conformation brings Nrf2 into proximity of the E3 ubiquitin ligase complex, promoting ubiquitination and degradation of Nrf2. In the presence of inducers, the closed conformation is still promoted, but it is proposed that these inducers cause conformational changes to Keap1 by reacting with cysteines around the Kelch and IVR

domains. These conformational changes are suggested to alter the positional relationship between Nrf2 and the E3 ubiquitin ligase complex, inhibiting its ubiquitination. The details concerning the E3 ubiquitin ligase complex have not been shown experimentally (Baird et al., 2013).

Current research suggests that the conformational cycling method is the best suggested mechanism of the Nrf2/Keap1 interaction, although it is difficult to see how this method would work with competitive inhibitors. Therefore, neither the hinge and latch or the sequester and release model can be completely discarded yet as a secondary mechanism of Nrf2 regulation.

Blockage of Nrf2 ubiquitination by the above mechanisms is proposed to come about by inducers causing conformational changes in Keap1 or Nrf2, or by competitive inhibition for the Keap1 binding site. The two broad mechanisms by which this occurs are by modification of Keap1 cysteine residues, and by Nrf2 phosphorylation.

1.5.4 Modification of Keap1 cysteine residues

Human Keap1 contains 27 cysteine residues (Eggler et al., 2005), including several in its kelch and IVR domains. These are thought to be modified by electrophiles and oxidants, which cause a conformational change in Keap1, blocking ubiquitination of Nrf2 and inducing its nuclear accumulation. The best studied cysteine residue in Keap1 is C151, which is located in the BTB domain. A C151S mutation in the mouse protein rendered Nrf2 signalling non-responsive to tBHQ in mouse embryonic fibroblasts, highlighting the importance of the residue (Yamamoto et al., 2008). However, the mutation did not affect Nrf2 levels under basal conditions, suggesting that it did not affect the Nrf2/Keap1 interaction or Keap1 binding to Cul3. Due to the position of C151 in the BTB domain of Keap1, it has been hypothesised that

the residue might work as a molecular switch. Modification of C151 by reactive oxygen species may cause a minor alteration in Keap1 structure to redirect degradation from Nrf2 to Keap1. This is backed up by evidence that shows C151S mutations block Keap1 dissociation from Cul3, and other evidence that shows mutations in the BTB domain increasing degradation of Keap1 and decreasing degradation of Nrf2 (Sekhar et al., 2010).

Caution should be exercised when interpreting Keap1 cysteine data, however. It has been noted that while C151 modification is well supported as a mechanism of Nrf2 activation, this appears to be inducer-dependent. It has been shown, for example, that C151 is not required for induction of Nrf2 by arsenite or monomethylarsonic acid (Wang et al., 2008a). In addition, experimental data can also be method-dependent (Eggler et al., 2007), suggesting that some research on the reactivity of Keap1 cysteine residues may be flawed.

Other cysteine residues have also been suggested to play a role in the regulation of Nrf2 activation. Most of these lie either in the IVR domain or the Kelch domain, and are generally believed to affect Nrf2 ubiquitination (Table 1.9).

Domain	Residue	Nrf2 inducers linked with residue
N-terminal	C13	-
N-terminal	C14	-
N-terminal	C23	-
N-terminal	C38	Sulforaphane (Hu et al., 2011), OA-NO ₂ (Kansanen et al., 2011)
BTB	C77	Sulforaphane (Hu et al., 2011)
BTB	C151	Sulforaphane (Hu et al., 2011), CDDO (Cleasby et al., 2014), tBHQ, Diethylmaleate, Dimethylfumarate (Takaya et al., 2012), H ₂ O ₂ , S-nitrocysteine, Spermine NONOate, Hydrochlorous acid (Fourquet et al., 2010)
BTB	C171	-
BTB	C196	-
IVR	C226	Sulforaphane (Hu et al., 2011), H ₂ O ₂ , S-nitrocysteine, Spermine NONOate, Hydrochlorous acid (Fourquet et al., 2010)
IVR	C241	-
IVR	C249	-
IVR	C257	Dexamethasone mesylate (Dinkova-Kostova et al., 2002), OA-NO ₂ (Kansanen et al., 2011)
IVR	C273	Dexamethasone mesylate (Dinkova-Kostova et al., 2002), OA-NO ₂ (Kansanen et al., 2011)
IVR	C288	Dexamethasone mesylate (Dinkova-Kostova et al., 2002), OA-NO ₂ (Kansanen et al., 2011), Diallyl trisulfide (Kim et al., 2014)
IVR	C297	Dexamethasone mesylate (Dinkova-Kostova et al., 2002)
IVR	C319	Sulforaphane (Hu et al., 2011)
Kelch	C368	Sulforaphane (Hu et al., 2011)
Kelch	C395	-
Kelch	C406	-
Kelch	C434	Sulforaphane (Hu et al., 2011)
Kelch	C489	Sulforaphane (Hu et al., 2011), OA-NO ₂ (Kansanen et al., 2011)
Kelch	C513	-
Kelch	C518	-
Kelch	C583	-
C-terminal	C613	H ₂ O ₂ , S-nitrocysteine, Spermine NONOate, Hydrochlorous acid (Fourquet et al., 2010)
C-terminal	C622	-
C-terminal	C624	-

Table 1.9 Keap1 cysteine residues. Keap1 contains 27 cysteine residues, which are believed to be modified prior to Nrf2 activation.

Although cysteine modification is suggested by many to be the primary mechanism of regulating the Nrf2/Keap1 interaction, it has been shown that in response to antioxidants C151 modification alone is not sufficient for Nrf2 release from Keap1. It seems that phosphorylation of Nrf2 is also required (Niture et al., 2009). Another interesting hypothesis put forward by Li and Kong (2009) is that a cysteine residue at position 183 in Nrf2 may also be a redox sensor. It certainly has been shown to be susceptible to modification, although its importance is unknown (Sekhar et al., 2010).

1.5.5 Nrf2 phosphorylation

Nrf2 activation is often blocked by inhibition of various kinases (Rushworth et al., 2005; Rushworth et al., 2006; Niture et al., 2009). Although there is much research linking upstream kinases to Nrf2 activation, the mechanisms involved are unclear. So far, the only phosphorylation site shown to be involved in disruption of the Nrf2/Keap1 complex is serine 40 (S40) within the Neh2 domain. This was originally shown *in vitro* using rat proteins by competitively inhibiting protein kinase C (PKC)-dependent phosphorylation of Nrf2 using small peptide sequences of amino acids 35-44 of Nrf2. It was then found that mutating the serine residue at position 40 prevented phosphorylation without affecting ARE binding (Huang et al., 2002). These results were verified in cell culture, and it was shown that although S40 was not essential for nuclear translocation of Nrf2, mutating the serine residue to alanine caused Nrf2 to have a much tighter association with Keap1 (Bloom and Jaiswal, 2003). Therefore, the evidence suggested that phosphorylation of S40 was important for preventing Nrf2 from being sequestered by Keap1 in the cytoplasm. Although PKC was the first, several kinases have been implicated in inducing Nrf2 activity, and these will be briefly discussed below.

1.5.5.1 PKC

PKC has been implicated in Nrf2 activation in response to many stimuli including epigallocatechin, phorone, curcumin, LPS, and tBHQ (Numazawa et al., 2003; Rushworth et al., 2005; Rushworth et al., 2006; Ogborne et al., 2008; Niture et al., 2009). There are 11 isoforms of PKC, all of which can be categorised into one of three subtypes. Classical PKCs are those that require diacylglycerol (DAG) and Ca^{2+} for activation, novel PKCs require DAG but not Ca^{2+} , and atypical PKCs require neither. Due to the differing requirements for activation, isoforms from the three subtypes can be activated by different mechanisms (Liu and Heckman, 1998). It is therefore noteworthy that at least one isoform from all three subtypes has been implicated in Nrf2 activation.

Although direct phosphorylation by PKC has never been shown in cell culture, *in vitro* kinase assays using purified proteins have revealed that PKC isoforms α , η , δ , and θ are all capable of phosphorylating Nrf2 (Niture et al., 2009). Although the only known PKC site is S40, three other possible sites exist at positions S387, S447, and S597 (Numazawa et al., 2003).

PKC- δ has been shown to be involved in Nrf2 activation in response to more stimuli than other isoforms. These stimuli include NO, curcumin and epigallocatechin (Rushworth et al., 2006; Ogborne et al., 2008; Zhang et al., 2009b). In response to NO, Nrf2 seems to be regulated as a negative feedback mechanism via PKC- δ to protect cells from NO-induced apoptosis. Curcumin, an anti-inflammatory compound found in turmeric, also upregulates ARE dependent gene expression via a PKC- δ mediated pathway. Rottlerin, a PKC- δ inhibitor, abolished HO-1 gene expression and ARE binding induced by curcumin, which indicates that at some stage downstream of PKC- δ , Nrf2 was activated (Rushworth et al., 2006). PKC- δ was also required for Nrf2 activation by tBHQ. Interestingly, this was shown to be insufficient

for Nrf2 activation, with covalent modification of C151 on Keap1 also required (Niture et al., 2009).

Previous work from our laboratory has shown that a pan PKC inhibitor, Ro-31-8220 completely inhibited LPS induced HO-1 mRNA expression in THP-1 cells, but rottlerin, a PKC- δ inhibitor, did not. Instead Go6976, a PKC α/β 1 inhibitor did inhibit HO-1 expression, but a PKC β inhibitor did not. This indicates that PKC- α is involved in Nrf2 activation (Rushworth et al., 2005).

PKC- ι has also been implicated in Nrf2 activation. Phorone and 4-HNE induced Nrf2 nuclear translocation and ARE reporter gene activity in WI-38 and COS-7 cells were abolished in cells expressing a null mutant of PKC- ι . Furthermore, the same group have cited unpublished data that shows a similar effect with another atypical isoform of PKC, PKC- ζ (Numazawa et al., 2003).

1.5.5.2 Other Kinases

There is evidence supporting a role for MAP kinases (Mitogen Activated Protein Kinases) in Nrf2 activation, although the exact mechanisms involved are unknown. p38 and ERK (extracellular signal-regulated kinase) inhibitors have been shown to block Nrf2 expression induced by diallyl sulfide and apo-8'-lycopenal (Ho et al., 2012; Yang et al., 2012). Inhibition of p38 also blocked Nrf2 expression caused by myocardial infarction in Wistar rats, whereas ERK has been shown to contribute to the upregulation of Nrf2 expression by deprenyl (Xiao et al., 2011; Mitra et al., 2014). A JNK (c-jun N-terminal kinase) inhibitor blocks protocatechuic acid-induced Nrf2 expression in J774A.1 macrophages (Vari et al., 2011).

PI3K (phosphatidylinositol-3 kinase) acts upstream of Nrf2 activation in response to deprenyl and hexabromocyclodecanes (Xiao et al., 2011; Zou et al., 2013). In addition, Nrf2 has been shown to play a role in the unfolded protein response, and is regulated by PERK (PKR-like endoplasmic reticulum kinase) (Cullinan and Diehl, 2006).

These studies all block Nrf2 accumulation by kinase inhibition, but they offer no insights into the phosphorylation sites involved, nor do they offer any evidence to suggest whether they interact directly with Nrf2. It is unknown, therefore, whether they act by disruption of the Nrf2/Keap1 interaction.

1.5.6 Sequestosome 1

A cellular regulator of autophagy, sequestosome 1 (p62 or SQSM1) is also believed to activate Nrf2 expression by competing directly for the Keap1 binding site, although the Keap1 binding sequence of SQSM1 does not have the same affinity as that of Nrf2 (Copple et al., 2010; Jain et al., 2010; Lau et al., 2010; Hancock et al., 2012). Overexpression of SQSM1 leads to an increase in Nrf2 expression and a decrease in Keap1 expression. Crucially, knockdown of SQSM1 does not affect Nrf2 activation by tBHQ or sulforaphane, showing that it is not part of the “canonical” mechanisms. Instead, SQSM1 binding to Keap1 targets Keap1 for degradation by autophagy (Jain et al., 2010).

1.5.7 Other mechanisms of Nrf2 regulation

It has been suggested that Keap1 may be able to enter the nucleus. Keap1 is not required for Nrf2 transcriptional activity (Kim et al., 2012a; Williamson et al., 2012), but there is some evidence suggesting that Keap1 nuclear import can play an inhibitory role. Nuclear import of Keap1 has been shown to inhibit Nrf2 transcriptional activity in two independent studies, one of which associates this with an increase in Nrf2 ubiquitination in nuclear extracts of HepG2 cells (Nguyen et al., 2005; Sun et al., 2011). Currently there is no evidence that the Keap1/Nrf2 complex enter the nucleus together.

There are other regulators of Nrf2 activity besides the Nrf2/Keap1 interaction. For example, our group has previously shown that LPS induces Nrf2 mRNA expression in THP-1 cells (Rushworth et al., 2008). MicroRNAs have been shown to regulate Nrf2 translation. So far Nrf2 translation has been shown to be inhibited by miR-153, miR-27a, miR-142-5p, MiR-144, MiR28, and miR-34a (Sangokoya et al., 2010; Li et al., 2011; Yang et al., 2011b; Narasimhan et al., 2012). In addition, the transcription repressors Bach1 and potentially Bach2 compete with Nrf2 for the binding of the ARE. Bach1 in particular has been shown to play a key role in the regulation of the Nrf2 target gene HO-1 (Miyazaki et al., 2010). Bach1 may bind to the ARE more strongly than Nrf2, and there is some evidence to suggest that its phosphorylation and subsequent nuclear export is required prior to the activation of Nrf2-dependent transcription (Kaspar and Jaiswal, 2010). Nrf2 also has phosphorylation sites in its Neh4 and 5 domains that are believed to alter its transcriptional ability, possibly by enhancing its ability to bind other transcription apparatus (Pi et al., 2007; Apopa et al., 2008).

The final mechanism by which Nrf2 is regulated is in its deactivation. Currently little has been reported on nuclear export of Nrf2. Two independent lab groups have uncovered a key role for GSK-3, although they

each assign a different mechanism to it. Jaiswal showed that GSK-3 β activation results in the activation of Fyn, a tyrosine kinase that phosphorylates nuclear Nrf2 on Y568 (Jain and Jaiswal, 2007). This causes Nrf2 to lose affinity for the ARE and associate with the nuclear export protein Crm1 instead (Jain and Jaiswal, 2006).

However, GSK-3 β has also been shown to phosphorylate Nrf2 directly on serine residues in its Neh6 domain. Nrf2 then binds SCF/ β -TrCP, an adaptor protein for a Cul1 based E3 ubiquitin ligase complex, and is subsequently degraded (Rada et al., 2012; Chowdhry et al., 2013). This research is consistent with previous work by the same group showing that the function of the Neh6 domain is to promote Keap1 independent degradation of Nrf2 (McMahon et al., 2004; Rada et al., 2011). Together, this evidence suggests a dual role for GSK-3 β in which it induces Nrf2 nuclear export via Fyn, and then later induces its degradation via the E3 ubiquitin ligase complex (Fig 1.8).

The strict regulation of Nrf2 combined with its protective effects, especially in oxidative stress and inflammation, makes it a promising target for protecting against disease. Currently a significant amount of research is being conducted on investigating the role of dietary components in activating Nrf2 and their potential as nutraceuticals in the prevention of chronic diseases.

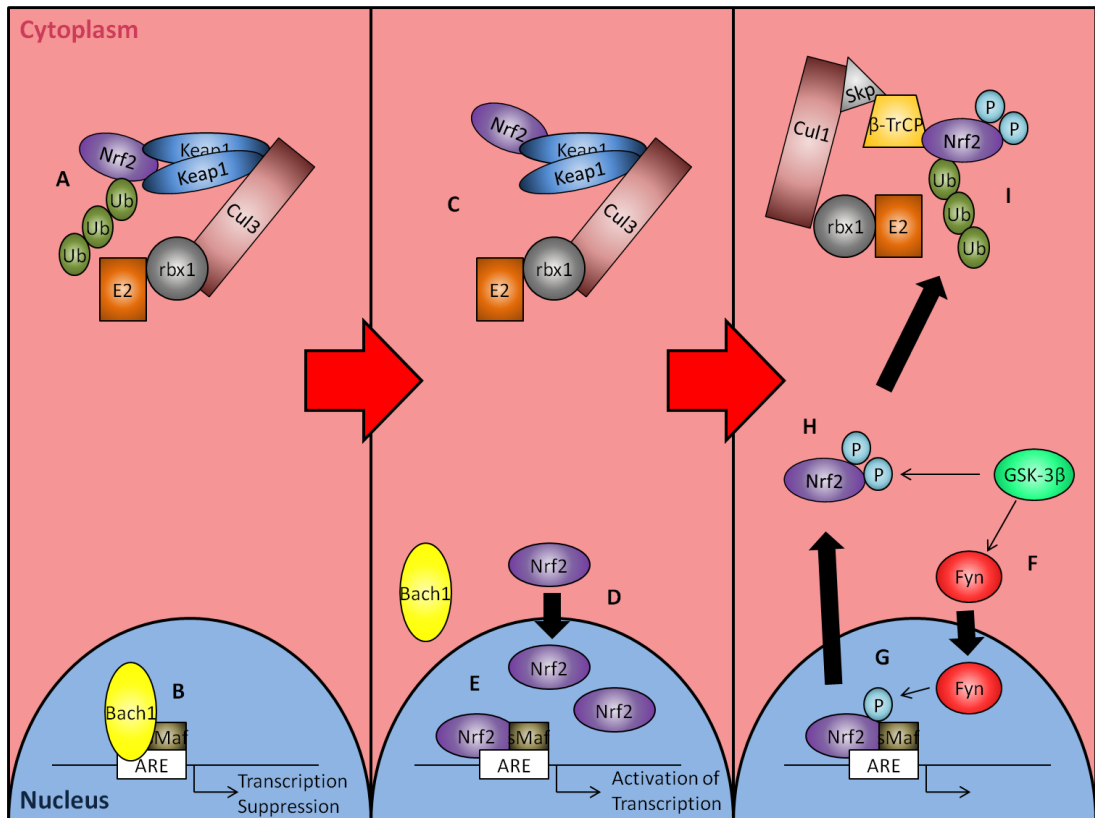


Fig 1.8 An overview of the Nrf2 signalling pathway. (A) Under basal conditions Nrf2 is bound to the Keap1 homodimer, and ubiquitinated by the E3 ubiquitin ligase complex. **(B)** The absence of Nrf2 results in the suppression of transcription of ARE containing genes. Sometimes Bach1 is bound to the ARE, which suppresses transcription further. **(C)** Inducers of Nrf2 result in the disruption of the Nrf2/Keap1 interaction. This prevents ubiquitination of Nrf2 and its subsequent degradation, leading to saturation of Keap1. **(D)** With no Keap1 to bind, Nrf2 translocates to the nucleus, where it forms heterodimers with small Maf proteins on the ARE to initiate transcription **(E)**. Bach1, on the other hand dissociates from the ARE, and is exported from the nucleus. **(F)** GSK-3 β activates Fyn, which then translocates into the nucleus to phosphorylate Nrf2 on Y-568 **(G)**. This initiates nuclear export of Nrf2, and suppression of transcription of ARE containing genes. **(H)** Once in the cytoplasm, Nrf2 is phosphorylated again in its Neh6 domain by GSK-3 β . This results in a high affinity for β -TrCP **(I)**. Once bound to β -TrCP, Nrf2 is ubiquitinated by a Cul1-based E3 ubiquitin ligase complex and subsequently degraded.

1.6 Activation of the Nrf2 pathway by dietary compounds

The diet has been identified as significant contributory factor to general health, and protection against disease. For example, a diet high in fat and simple sugars has been identified as a risk factor in cardiovascular disease and diabetes. In contrast a diet high in fruits, vegetables, and wholegrain foods, or a Mediterranean diet are associated with a decreased risk of these conditions (Salas-Salvado et al., 2011; Rees et al., 2013; Van Baak, 2013). These foods have been linked with molecules that carry out these effects. For example, flavonoids have been identified as bioactive components of fruits and vegetables, which may protect against cardiovascular disease (Toh et al., 2013).

Molecules such as these are sold as health supplements, used for health advice, and have been used either as drugs or drug precursors in medical research (Marik and Flemmer, 2012; NHS, 2012; Lenzi et al., 2014). Therefore, it is in the public interest to pursue dietary components for potential protective effects. Nrf2 can be activated by a wide range of dietary compounds, and many have been implicated in Nrf2-mediated cytoprotective effects *in vitro* and *in vivo* (Table 1.10).

Molecule	Food	Details	Dose	Reference
Allicin	Garlic	Increased Nrf2 expression in the hippocampus of cognitively impaired mice.	180 mg/kg for 56 days	Li et al., 2012c
Curcumin	Turmeric	Increased Nrf2 in arsenic challenged mouse liver	200 mg/kg twice a week for 6 weeks	Gao et al., 2013
Epigallocatechin-3-gallate	Green tea	Increased Nrf2 and HO-1, and inhibited NF-κB in cisplatin challenged rat kidney.	100 mg/kg for 2 days	Sahin et al., 2010a
Lycopene	Tomatoes	Increased Nrf2 and HO-1, and inhibited NF-κB in cisplatin challenged rat kidney.	6 mg/kg for 30 days	Sahin et al., 2010b
Quercetin	Capers, radish, dill, fennel, red onion	Induced Nrf2 and inhibited NF-κB in CCL4-induced liver injury.	50 mg/kg for 5 days	Domitrović et al., 2012
Resveratrol	Grapes	Normalises renal Nrf2 expression in diabetic rats.	5 mg/kg for 30 days	Palsamy and Subramanian, 2011
Sulforaphane	Cruciferous vegetables (broccoli, cabbage, brussels sprouts)	Induced Nrf2 in mouse liver.	50 mg/kg for 5 days	Zhou et al., 2014

Table 1.10. Dietary compounds that activate Nrf2 *in vivo*.

For example, sulforaphane is an isothiocyanate most commonly found in cruciferous vegetables, which include broccoli, cabbage and Brussels sprouts (Zhang et al., 1992). *In vitro*, sulforaphane activates the Nrf2 pathway, which has been linked to protection from ionising radiation-induced damage in human skin cells (Kleszczyński et al., 2013; Mathew et al., 2014). It also has anti-inflammatory effects via the Nrf2 pathway in smooth muscle cells and peritoneal macrophages (Lin et al., 2010; Zhao et al., 2013b). Curcumin, a compound found in turmeric, has shown similar effects via the Nrf2 pathway. TNF-induced adhesion between monocytes and keratinocytes was inhibited

by inhibiting the adhesion molecule, ICAM-1. Nrf2 siRNA prevented this effect (Youn et al., 2013). The flavonoid quercetin, commonly found in capers, radish, dill and red onion, inhibits ROS generation and NF- κ B activation by ochratoxin A in HepG2 cells. This was accompanied by Nrf2 activation (Bhagwat et al., 2011; Ramyaa et al., 2014). In addition, HaCaT keratinocytes were protected from UV-induced ROS production and cell death by quercetin in a Nrf2-dependent manner (Kimura et al., 2009).

In vitro studies are useful for finding new compounds and targets, and for investigating mechanisms. In support of the *in vitro* research, there are many *in vivo* models that support a protective role for Nrf2 activators in the diet.

Oral administration of sulforaphane in mice showed that it has high bioavailability, reaching many tissues including the liver, kidney, brain and colon (Clarke et al., 2011). Feeding mice with a diet containing 300ppm sulforaphane causes anti-inflammatory effects. Extracted aortic epithelium from mice fed with a sulforaphane rich diet did not bind monocyte-derived macrophages as strongly as mice on a normal diet. Furthermore, monocyte-derived macrophages from the mice demonstrated lower expression of ICAM-1 after TNF stimulation in mice fed with a sulforaphane rich diet (Nallasamy et al., 2014). Sulforaphane also has protective effects against mental and neurodegenerative conditions, adipogenesis, thrombosis and mutagenesis (Chen et al., 2012; Abel et al., 2013; Jayakumar et al., 2013; Morroni et al., 2013; Choi et al., 2014; Lee et al., 2014b). Activation of Nrf2 is thought to regulate many of sulforaphane's effects, although much of the *in vivo* evidence is circumstantial. In a mouse model of type 2 diabetes mellitus, 4 months sulforaphane treatment alleviated some of the effects of the disease, including fibrosis and an increase in arterial wall thickness. In addition, it was found that mice with type 2 diabetes had decreased aortic SOD1 and HO-1 expression, which accompanied increased apoptosis and

oxidative damage. Sulforaphane reversed these effects, and was found to increase levels of aortic Nrf2 (Wang et al., 2014b). In a mouse model of induced stroke, sulforaphane was shown to protect the blood brain barrier, and induce Nrf2 activity (Alfieri et al., 2013).

Like sulforaphane, quercetin has also been shown to have therapeutic effects *in vivo*. The vast majority of the effects of quercetin are anti-inflammatory. For example, examination of livers revealed that inflammation induced by feeding mice with a high fat diet is alleviated by quercetin supplementation. Nuclear NF- κ B, iNOS, TNF, IL-6 were among the inflammatory mediators that were inhibited, and GST, catalase, and SOD were among the antioxidant enzymes that were induced by quercetin. Encouragingly, leaf extract of *moringa oleifera*, a plant in which quercetin is believed to be a bioactive component, had the same effect (Das et al., 2013). In mouse livers, steatohepatitis-induced inflammatory mediators including TLR4, TNF, NF- κ B and MMP9 were also inhibited by quercetin (Marcolin et al., 2012). Inflammation may play a role in type 2 diabetes, and obese mice fed with quercetin were shown to regain some insulin sensitivity. In addition, GLUT4 receptor expression was increased in mice treated with quercetin, whereas inflammatory mediators TNF, iNOS and NF- κ B were inhibited (Anhê et al., 2012). Despite the evidence linking quercetin to Nrf2 *in vitro*, Nrf2 dependent effects have not been shown *in vivo*. However, Nrf2 has been shown to be induced by quercetin in rat liver, which correlated with inhibition of CCL4-induced NF- κ B, TNF and Cox2 (Domitrović et al., 2012).

Allicin and diallyl sulfide have been shown to protect mice from various conditions, possibly involving Nrf2 upregulation. Cognitive impairment in aged mice was significantly reduced by allicin, which was accompanied with a rise in Nrf2 expression in the hippocampus. Diallyl sulfide was shown to protect rats from inflammation induced by the antibiotic gentamicin in the

kidney, possibly by inhibiting NF- κ B signalling (Kalayarasan et al., 2009; Li et al., 2012c). Similarly, Epigallocatechin-3-galate ameliorates nephrotoxicity in rats by activating Nrf2 and inhibiting NF- κ B (Sahin et al., 2010).

Some Nrf2 activators have been used in epidemiological and clinical trials in humans to assess their use in disease prevention. The potential therapeutic effects that have been demonstrated by sulforaphane have led to human epidemiological trials involving broccoli to investigate whether sulforaphane may help to detoxify carcinogens. Subjects were fed a sulforaphane-rich broccoli derived beverage, and had their urine tested for mercapturic acids of known carcinogens that are commonly found in pollution. Urine after treatment with the beverage had higher levels of mercapturic acids than urine before treatment began, suggesting an activated detoxification system caused by the high-sulforaphane beverage (Kensler et al., 2012). The effect of sulforaphane on phase II detoxification enzymes has also been observed in humans. After consumption of a broccoli homogenate, subjects had higher expression of HO-1, NQO1, GSTM1 and GSTP1 in nasal cells (obtained by nasal lavage). Furthermore, this occurred in a dose dependent manner, and did not occur after consumption of control alfalfa sprouts, which do not contain sulforaphane (Riedl et al., 2009).

Quercetin has also been used in human clinical trials, with varying levels of success. 72 type 2 diabetic women received quercetin supplementation for 10 weeks. This lowered systolic blood pressure, but did not affect other markers of cardiovascular disease such as serum cholesterol levels. In addition, the inflammatory mediators IL-6 and TNF were not significantly affected compared to placebo (Zahedi et al., 2013). Systolic blood pressure was also lowered by quercetin after eating a lipid rich meal. Subjects treated with quercetin had lower systolic blood pressure and 11% lower triacylglycerol levels 4h after eating. These effects did not last. Interestingly,

like the findings of Zahedi and co-workers, inflammatory markers measured did not change with quercetin treatment (Pfeuffer et al., 2013).

Although dietary compounds such as quercetin and sulforaphane are exciting molecules, they may have off-target effects. For example, in LPS-induced inflammation sulforaphane has been suggested to inhibit TLR4 signalling by binding cysteine residues in the receptor itself, inhibiting oligomerization (Youn et al., 2010). Further complicating matters is the controversial role of Nrf2. Youn and co-workers (2010) claim that Nrf2 is not involved in the anti-inflammatory role of sulforaphane in mouse embryonic fibroblasts (MEFs), whereas in mouse peritoneal macrophages, Nrf2 knockout abrogated the effects of sulforaphane (Lin et al., 2010).

There is strong evidence that Nrf2 activators can help defend against diseases, especially inflammatory diseases and oxidative stress related conditions. Although the role of Nrf2 in *in vivo* studies is less conclusive, at minimum they show that Nrf2 is worthy of research interest, and is a very promising drug target.

1.7 Targeting the Nrf2/Keap1 interaction

1.7.1 The shortcomings of Nrf2 activators

Targeting the Nrf2 pathway to protect against disease shows a lot of promise both for therapeutic action in disease, and preventative measures in dietary health. However, a distinction must be made between drug targeting and dietary targeting. The aim of dietary research is to improve general health, and to introduce measures into peoples' lifestyle as a means of lowering the risk of disease. As such, while the diet can be altered to alter intake of specific substances, the amount that is eaten will typically be quite low. *In vitro* studies use much higher concentrations of compounds than would be consumed, which is practical for identifying effects and targets but is not an accurate model from which to formulate conclusions on health. Drugs, on the other hand, are needed to help fight a disease that has already occurred, and thus are required to have a potent, acute effect. Therefore, the body is exposed to much higher concentrations of the molecule, and is much more susceptible to undesirable side effects. Interest in Nrf2 is high in both these fields, but the approach and conclusions from each may not be compatible with each other. It is not a coincidence that some of the most successful Nrf2 activators are derived from natural products, but are not dietary compounds.

One of the recent success stories is dimethyl fumarate, which has been successfully used in clinical trials to treat multiple sclerosis, and has been approved for use by the U.S Food and Drug Administration (Ruggieri et al., 2014). The protective effects of dimethyl fumarate have been attributed to its ability to downregulate inflammatory mediators and oxidative stress. Both of these functions in the central nervous system are dependent on Nrf2 (Linker et al., 2011; Scannevin et al., 2012). Dimethyl fumarate has other protective effects. It is used in combination with other molecules to treat psoriasis, and has been linked with functions for which it has not yet been

medically approved. These include cardioprotection and prevention of vascular calcification (Ashrafian et al., 2012; Ha et al., 2014).

Although the broad range of activators of Nrf2 in protection looks promising, it is not necessarily as good as it first appears. Many of the molecules that activate Nrf2 are not protective, and even those that have promising results in the lab have failed at clinical trial. Recently bardoxolone methyl (also known as CDDO-Me), a synthetic triterpenoid, was used in clinical trials for stage 4 chronic kidney disease. Bardoxolone methyl is known to activate Nrf2 and, if it acts in a similar manner to CDDO, then it may do so by modifying C151 in the BTB domain of Keap1 (Cleasby et al., 2014; Wu et al., 2014). The clinical trial was stopped because of serious side effects, including heart failure (de Zeeuw et al., 2013).

The reason that a lot of these molecules fail is that Nrf2 is a stress sensor, and so its activation and subsequent protective effects are products of cell damage. This causes production of reactive molecules, including ROS and electrophiles, which cause conformational changes in the structure of Keap1 and Nrf2, resulting in the activation of protective genes. A lot of molecules that activate Nrf2 like this, and any protective effects caused by Nrf2 are a response to initial stress caused by the drug. It's not necessarily a bad approach, and there is evidence that it can work. Both sulforaphane and quercetin are widely considered to prevent oxidative stress, but have also been shown to induce Nrf2 expression by inducing ROS (Lapidot et al., 2002; Lee et al., 2012a). The problem with activating Nrf2 in this way is not just the production of reactive molecules, however. It can result in non-specific interactions and activation of other mediators. To use one of the most promising examples, sulforaphane directly binds to TLR4 (Koo et al., 2013) and Hsp90 (Li et al., 2012b), activates TGF β /Smad signalling (Kaminski et al., 2010), and has been shown to both protect against and induce apoptosis (Mi

et al., 2007; Hsu et al., 2013; Ziaei et al., 2013; Jiang et al., 2014b). Drugs derived from these molecules should be expected to have adverse effects in clinical trials, and should be treated with caution. Ideally drugs targeting the Nrf2 pathway would do so without the accompanying damage, and with higher specificity (Fig 1.9 and 1.10). Therefore, research is currently underway to inhibit the Nrf2/Keap1 interaction directly.

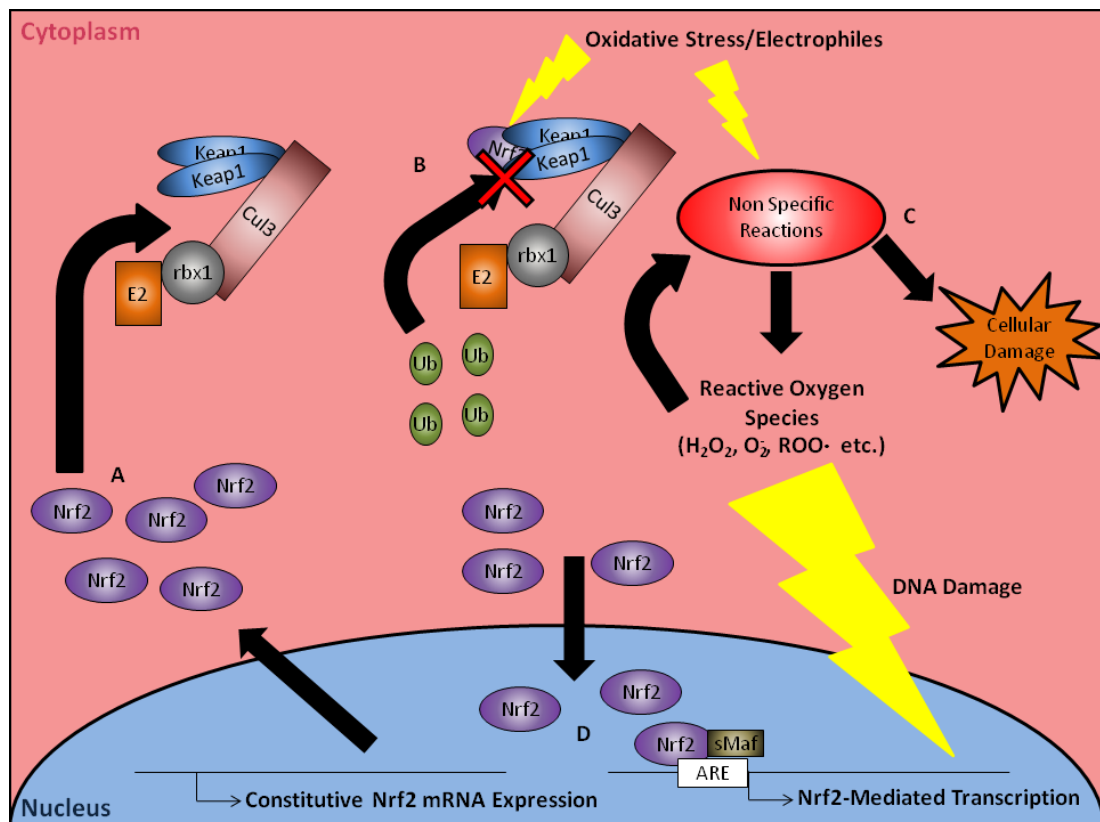


Fig 1.9 Targeting the Nrf2/Keap1 with ROS/electrophiles. (A) Nrf2 is produced and binds Keap1. **(B)** ROS/electrophiles modify the structure of Keap1, blocking Nrf2 degradation. **(C)** These molecules also react with other molecules in the cell, activating other signalling pathways and producing reactive compounds which have the potential to cause cell damage. **(D)** Nrf2 is activated and can initiate transcription. Under the right conditions the advantages of increased Nrf2 expression can outweigh the effects of the cell damage.

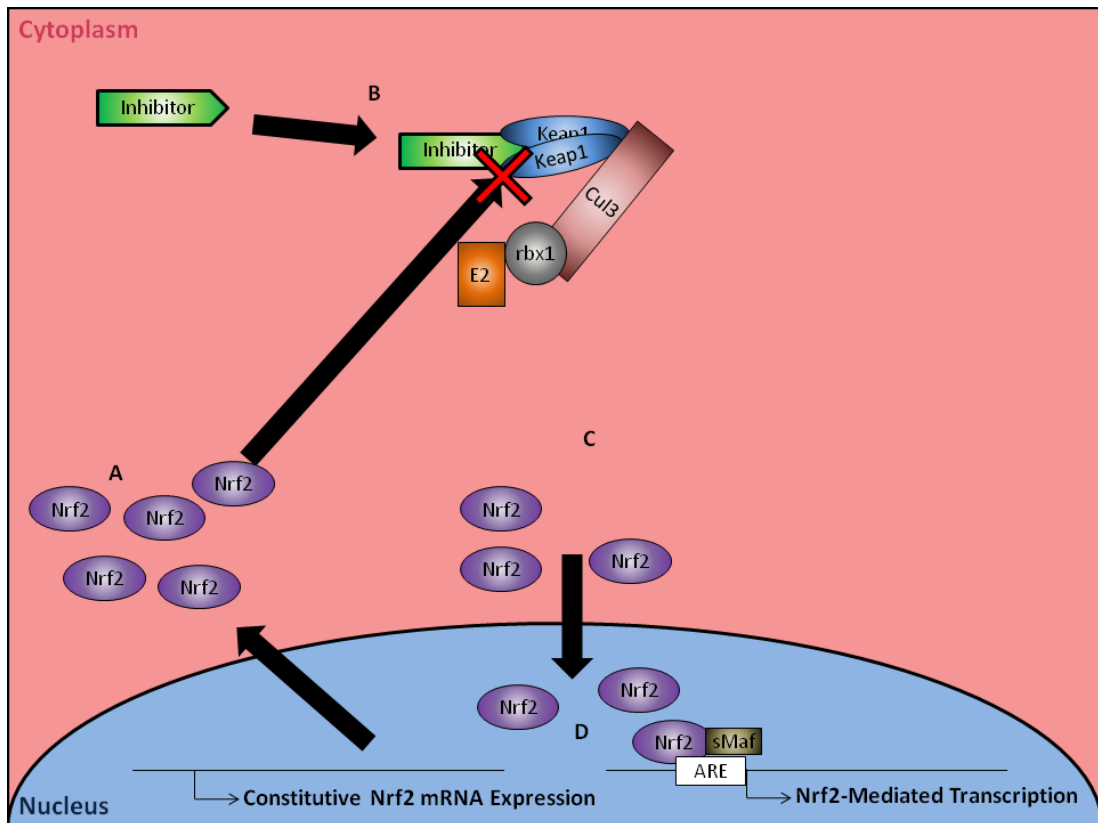


Fig 1.10 Targeting the Nrf2/Keap1 interaction with competitive inhibitors. (A) Nrf2 is produced and binds Keap1. (B) A direct inhibitor blocks the Keap1 binding site. (C) Nrf2 builds up and translocates to the nucleus to (D) initiate transcription.

1.7.2 Competitive inhibitors of Keap1

In 2006, Lo and co-workers found that small peptide sequences mimicking the ETGE-containing motif of the Neh2 domain are capable of binding Keap1 and inhibiting the interaction between Keap1 and Nrf2. This work showed that it is possible to displace Nrf2 from the Nrf2/Keap1 complex, and showed that competitive inhibition is a viable strategy in targeting Nrf2. Since then there have been several strategies to find suitable inhibitors, and to investigate the biological effects of targeting the Nrf2/Keap1 interaction in this way.

There have been two approaches used to find small inhibitors to target the Nrf2/Keap1 interaction. The first is to use the Neh2 mimetic peptides as a template, and modify the sequence and structure accordingly. Using this approach, Hancock and co-workers were able to improve upon the Keap1 binding site of Nrf2 by introducing elements from the binding site of sequestosome 1 (Hancock et al., 2012; Hancock et al., 2013). This method is the slower of the two methods, but is more likely to produce a specific drug, because it is basing the structure on existing binders of Keap1.

The second method that has been used to find small molecules to target the Nrf2/Keap1 interaction is screening large libraries of compounds. Wang and co-workers (2012) narrowed down a screen of over 300,000 compounds to two using a fluorescence polarization assay. One was discounted because it was believed to be a electrophilic, which means that there is a strong chance it would react with non-specific nucleophiles, causing off-target effects. The other was tested for biological activity. It was found to activate an ARE-driven beta-lactamase reporter assay in HepG2 cells. It was also non-toxic in both HepG2 cells and HEK293 cells. Surface plasmon resonance was used to calculate its binding affinity for Keap1, which was 1 μ M. Another molecule was found by narrowing down a >300,000 compound search by structure based virtual screening. The remaining 65 compounds were narrowed down by Fluorescence polarization. The most active compound was found to induce nuclear accumulation of Nrf2 in PC12 cells at a concentration of 4 μ M (Zhuang et al., 2014). By far the most promising compound, however, was found from a library of 250,000 compounds by fluorescence polarization. Of the 18 hits, one induced ARE-luciferase activity and NQO1 expression, which was inhibited by Nrf2 siRNA. Furthermore, the compound does not modify cysteine residues, which suggests that it would not have many non-specific effects (Marcotte et al., 2013). This compound was then modified by adding two CH₂COOH groups in order to reach additional binding pockets in the

Kelch domain. In addition to being the strongest small-molecule binder of Keap1 to date, it also induced ARE-luciferase activity in HepG2 cells and HO-1, NQO1, and GCLM in HCT116 cells (Jiang et al., 2014). The main drawback with this method is that target specificity is less assured. They are not derived from anything that binds Keap1 under normal conditions, and therefore off target effects are more likely despite measures to reduce reactivity in the compound structures.

Research into Nrf2 activators is making rapid progress, which is looking very promising for several diseases. However, this is not the case in cancer.

1.8 Cancer

1.8.1 The hallmarks of cancer

Cancer one of the major causes of death worldwide. In 2012 14.1 million adults were diagnosed with cancer, and it was the cause of 8.2 million deaths worldwide (CRUK, 2014a). In the UK, lung, breast, bowel and prostate cancers caused approximately 46% of all cancer deaths in 2011, with lung cancer causing 22% alone (CRUK, 2014b).

Cancer is defined by the World Health Organisation as “the uncontrolled growth and spread of cells”, which is a simplistic but accurate definition. Typically cancer cells lose their ability to control their own growth, replication and function which invades surrounding tissues, eventually obstructing essential bodily functions, causing death. More specifically, cancer is more difficult to define although the prevailing opinion is that there are eight hallmarks of cancer (Table 1.11), as proposed by Hanahan and Weinberg (2000; 2011).

Cancer is caused by mutations which affect the function of genes involved in regulating mitosis, and therefore promoting one or more of the eight hallmarks. Therefore, only mutations which promote the hallmarks of cancer will contribute to its onset. For example, a mutation that impairs the function of p53 will promote tumour-like characteristics (Wang et al., 2014c). On the other hand, a null mutation will have no physiological effect, and mutations that activate apoptosis will prevent tumour progression. Despite this, cancer is still extremely difficult to treat, because there is still a near infinite combination of mutations that can cause cancer. Therefore, each cancer has

Hallmark	Explanation	Example
Resisting Cell Death	Most mutations to DNA activate DNA repair pathways. If these fail, the cell is driven towards apoptosis. Cancer cells must evade apoptosis if tumour-promoting mutations are to prevail.	Nrf2 protects cells from apoptosis through the upregulation of DNA repair enzymes and detoxifying enzymes (Chen et al., 2013).
Sustaining Proliferative Signalling	Cancer cells have overactive growth signalling pathways. This can be due to increased expression of growth factors, alterations to growth factor receptors, or deregulation of cell cycle checkpoints.	Hepatoma-derived growth factor expression has been linked with progression of malignant melanoma (Tsai et al., 2013a)
Evading Growth Suppressors	The cell cycle has many ways to regulate cell division. Evading these allows the cell to bypass cell cycle checkpoint and ignore suppressors of cell growth.	p53 regulates cell growth. Mutations in p53 have been associated with various cancers and cell survival (Muller and Vousden, 2014).
Enabling Replicative Immortality	Cancer cells overcome senescence to improve long term tumour survival.	Telomeres are repeated segments on the end of chromosomes that get progressively shorter with each replication. Some cancer cells can maintain telomere length, leading to replicative immortality (Henson et al., 2002)
Inducing Angiogenesis	Tumours require nutrients to be delivered to cells in the centre. The way they achieve this is by making new blood vessels that deliver nutrients straight to the tumour.	VEGF is a growth factor that can stimulate the production of new blood vessels. It has been shown to be overexpressed in some cancers, resulting in angiogenesis (Wang et al., 2013b; Zhou et al., 2014).
Activating Invasion and Metastasis	Metastasis is the spreading of cancer from the original tumour to other sites in the body. This allows new tumour growth in these new sites.	RhoC is a G-protein that is involved in regulating cell migration through actin rearrangement. Its overexpression promotes metastasis in cancer cells (Rosenthal et al., 2012).
Deregulating Cellular Energetics	Cancer cells have been observed to prefer anaerobic respiratory pathways to aerobic respiration, relying on glycolysis for ATP production. The functional advantages to this are unknown, although it may be because of the lack of oxygen found at the centre of tumours, especially without angiogenesis.	Glucose transporters are upregulated in cancers, which increases glucose uptake and therefore ATP production by glycolysis (Cho et al., 2013).
Avoiding Immune Destruction	Lymphocytes, natural killer cells, and macrophages are known to target cancer cells. Evading this increases survivability of cancer cells.	It is thought that cancer cells can release regulators into their microenvironment that modulate the activity of leukocytes. For example, it has been suggested that tumours expressing soluble BAG6 inhibit the cytotoxicity of natural killer cells (Reiners et al., 2013).

Table 1.11 The hallmarks of cancer. Cancer cells acquire abilities to survive and propagate better than normal cells (Hanahan and Weinberg, 2011).

the potential to be unique. There are patterns, however. Over 60% of melanomas contain a mutation in the BRAF gene. The vast majority of these are a V600E substitution (Sclafani et al., 2013). Therefore, a targeted approach is viable for large portions of the population. Alternatively, cancer cells can be killed by non-specific chemotherapeutics, such as cisplatin or doxorubicin. These work by activating apoptosis mechanisms, usually by strongly damaging DNA to overpower aberrant survival mechanisms (Siddik, 2003). The problem with these drugs is that often they are not effective at concentrations that are safe for the patient. This is due to off-target effects. For example, cisplatin causes nephrotoxicity, which limits its usefulness in chemotherapy (Zirak et al., 2014).

Although it is impossible to prevent cancer completely, it is certainly possible to reduce the chances of it developing by minimizing exposure to carcinogens and by increasing antioxidant defences. The latter may be possible to achieve by activation of Nrf2.

1.8.2 Nrf2 promotes both cytoprotection and chemoresistance in cancer

The Nrf2-mediated antioxidant defence is known to detoxify carcinogens and prevent DNA damage. For example several studies have linked Nrf2 expression to protection against UV damage. Nrf2 knockout mice are more susceptible to sunburn and show higher formation of 8-hydroxydeoxyguanosine (8-OHdG) lesions in DNA (Kawachi et al., 2008). They are also susceptible to DMBA and TPA induced skin tumourigenesis (Xu et al., 2006). Nrf2 upregulation protects keratinocytes from UV radiation (Tian et al., 2011). 6-thioguanine is a drug used to treat inflammatory bowel disease, but is also thought to be a carcinogen. High Nrf2 expression reduces its incorporation into DNA (Kalra et al., 2011). Furthermore, Nrf2 can upregulate

DNA repair enzymes. Both BHA and Vitamin C restore oestrogen inhibited 8-oxoguanine glycosylase (OGG1) expression, which is dependent on Nrf2. OGG1 expression is accompanied by a decrease in 8-OHdG lesions (Singh et al., 2013a).

Crucially, several upregulators of Nrf2 found to protect against cancer are found in the diet, including sulforaphane and curcumin, suggesting that there may be protective measures against cancer that can be taken through the diet (Lee et al., 2013c).

Unfortunately, Nrf2 plays a dual role in some cancers. While it protects against tumour development, if a cell becomes cancerous Nrf2 promotes the survival of that cell. Several human cancers have been shown to overexpress Nrf2 (Table 1.12), and Nrf2 has been verified to protect cancer cells against chemotherapy *in vitro*. Inhibiting Nrf2 in lung cancer cells renders them more susceptible a range of cytotoxic therapies including mitoxantrone, topotecan (Singh et al., 2010a), cisplatin, doxorubicin and etoposide (Wang et al., 2008b). Cervical cancer cells were found to become more sensitive to treatment with doxorubicin, cisplatin, paclitaxel, and 5-fluorouracil. Nrf2 deficiency combined with cisplatin was also shown to inhibit tumour growth *in vivo* (Ma et al., 2012). Type II endometrial cancer cells derived from patients also showed similar patterns. Cell lines derived from patients with high Nrf2 expression were more resistant to chemotherapeutic drugs than those derived from patients with low Nrf2 expression. Overexpression of Keap1 in cells with high expression of Nrf2 inhibited Nrf2 expression, which rendered cells more susceptible to cisplatin and paclitaxel treatment (Jiang et al., 2010).

Cancer	Study Description	Reference
Colorectal Cancer	Nrf2 mRNA and protein were overexpressed in patient samples compared to normal cells.	Hu et al., 2013
Endometrial Cancer	Nrf2 expression was detected in 89% of endometrial serous carcinomas, 28% of endometrial endometrioid carcinomas, and 0% of benign uteri.	Jiang et al., 2010
Gallbladder Cancer	Keap1 mutations were found in 31% patient samples.	Shibata et al., 2008a
Gastric Cancer	Nrf2 was overexpressed in 44% of gastric cancer patient samples.	Yoo et al., 2010
Lung Cancer	19% of non-small-cell lung carcinoma patient samples had Keap1 mutations.	Singh et al., 2006
Ovarian Cancer	29% of patient samples were found to have a mutation in Keap1.	Konstantinopoulos et al., 2011

Table 1.12 Nrf2 in cancer patients. Nrf2 is often overexpressed in cancer, which is often linked with mutations in Keap1.

The function of Nrf2 is not different in cancer cells to normal cells, but the cytoprotective pathways can be hijacked by cancer cells to protect against various forms of therapy. The reason for this is that anti-cancer therapy is designed to drive the cancer cells towards apoptosis, a function that Nrf2 can inhibit by several mechanisms. Nrf2 upregulates detoxification enzymes and drug transporters, which reduces the bioavailability of any chemotherapeutics. Nrf2 expression and chemoresistance in A549 and H460 lung cancer cells has been linked to the expression of ABCG2, a drug transporter (Singh et al., 2010a). Nrf2 has also been shown to regulate expression of other ABC transporters, including MRP1, 2, 3, and 4 (Aleksunes et al., 2008; Ji et al., 2013a; Wang et al., 2014d). Radiotherapy and many drugs cause DNA damage by inducing ROS, which Nrf2 is capable of removing. Nrf2 has been shown to protect A549 and H460 cells from the effects of ionizing radiation (Singh et al., 2010b). Nrf2 has also been suggested to mediate chemoresistance by activating autophagy, the

inhibition of which was linked to decreased resistance to cisplatin in ovarian carcinoma cells (Bao et al., 2014).

Nrf2 can be overexpressed in cancer cells by several mechanisms. Keap1 mutations are associated with increased Nrf2 expression and have been found in 31% of gallbladder cancers (Shibata et al., 2008a), 19% of non-small-cell lung carcinomas (Singh et al., 2006), and 29% of clear-cell ovarian carcinomas (Konstantinopoulos et al., 2011). Keap1 mutations commonly occur in the IVR/Kelch domain, and research into some of these mutations has revealed that mutant Keap1 cannot sequester Nrf2 (Hast et al., 2014).

Keap1 can also be silenced in cancers. Breast cancer cells were shown to silence Keap1 through expression of a microRNA, miR-200a (Eades et al., 2011). Another mechanism of Keap1 silencing is through hypermethylation of its promoter, inhibiting its transcription, which has been shown in lung cancer and prostate cancer cells (Wang et al., 2008b; Zhang et al., 2010).

Keap1 is not the only member of the Nrf2 pathway to be affected in cancer. Out of 103 lung cancers and 12 head and neck cancers, 10% of lung cancer patients and 25% of head and neck cancer patients had mutations in Nrf2. 11 of the 14 mutations found were in either the DLG or ETGE binding sites (Shibata et al., 2008b).

These observations show that Nrf2 is upregulated in a significant number of cancer patients, and that this confers chemoresistance and apoptosis-evasion properties to the cancer cells. Chemotherapy supplemented with Nrf2 inhibition is more effective than chemotherapy alone, raising new possibilities for cancer treatment (Ren et al., 2011).

1.8.3 Inhibitors of the Nrf2 pathway.

The dual role of Nrf2 in cancer has provided a possibility for the use of Nrf2 inhibitors as supplemental drugs. Unfortunately, there has not been as much progress in the field of Nrf2 inhibition as there has been in targeting it for activation. A few small molecules have been found to inhibit Nrf2 nuclear accumulation. One of these is brusatol, which was shown to inhibit ARE-driven luciferase activity, Nrf2 expression and Nrf2 target gene expression. It was then shown to increase Nrf2 ubiquitination, while decreasing Keap1 ubiquitination. Its effectiveness on cancer cells was also investigated, and was found to sensitise A549 cells to cisplatin. In cells without Nrf2 there was no difference between the effectiveness of cisplatin with or without brusatol, but in cells expressing Nrf2, cisplatin and brusatol was superior to cisplatin alone (Ren et al., 2011). Another small molecule, trigonelline, has also been shown to inhibit the Nrf2 pathway in HT29 cells (Boettler et al., 2011).

The current standard of evidence clearly shows that Nrf2 is an important regulator in health and disease that is simultaneously a mediator that requires consideration in a wide range of diseases and a promising drug target. In a healthy individual, the overwhelming consensus is that it is a helpful mediator of protective pathways, and shows a lot of promise as a drug target in many diseases.

1.9 Summary

Nrf2 is a key regulator of important cytoprotective mechanisms that protect against diseases, which can include respiratory diseases, inflammatory diseases and bacterial infections (Iizuka et al., 2005; Reddy et al., 2009; Kong et al., 2010). Nrf2 activity is regulated by a cellular inhibitor, Keap1. Disruption of the Nrf2/Keap1 interaction is an inducible process that activates the Nrf2 pathway when protective mechanisms are required (Turpaev, 2013). It is therefore possible that Nrf2 can be activated through the diet to improve general health, or that the Nrf2/Keap1 interaction can be targeted specifically for medical intervention (Egglar et al., 2008; Hancock et al., 2012).

Cancer is an exception to the benefits of Nrf2-mediated protection. Although Nrf2 protects against the onset of cancer, once cancer has already developed the protective mechanisms that it promotes can be hijacked by cancer cells, which gain resistance to chemotherapy and radiotherapy (Fig 1.11). Nrf2 has been reported to be elevated in many cancers, including lung (Yang et al., 2011), breast (Hartikainen et al., 2012), endometrial (Li et al., 2013) and pancreatic cancer (Soini et al., 2014), and inhibiting Nrf2 expression has been shown to improve susceptibility of some cancer cells to therapy *in vivo* and *in vitro* (Singh et al., 2010a; Singh et al., 2010b; Ma et al., 2012; Arlt et al., 2013).

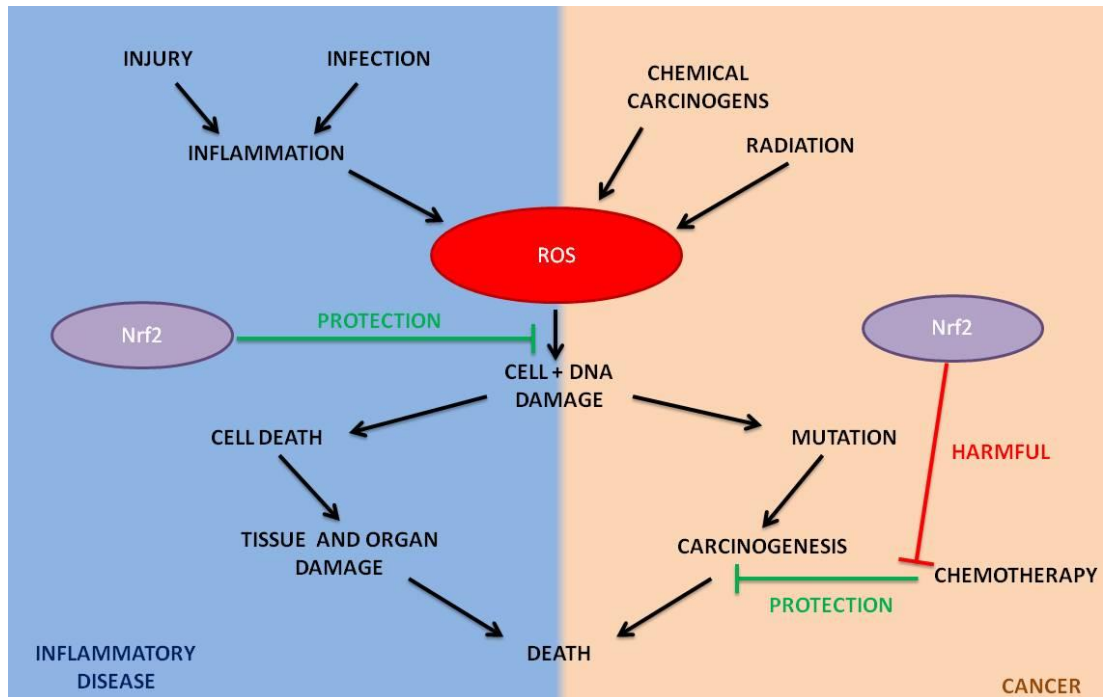


Fig 1.11 The dual roles of Nrf2. The main function of Nrf2 is to protect against reactive oxygen species (ROS). In Inflammation, ROS cause cell damage which harms the host. In cancer, these protective effects of Nrf2 prevent drugs from killing tumours.

Research on inhibiting the Nrf2 pathway is not as well developed as activating it. There are few known inhibitors of Nrf2 activity (Ren et al., 2011; Arlt et al., 2013), and evidence to support further research into it is not as well documented. Currently researchers are focusing on improving understanding of the potential roles an Nrf2 inhibitor could play in the clinic, with drug design considered a long-term prospect.

1.10 Aims of this study

1. Establish a reporter assay for Nrf2 for use in further laboratory studies.
2. Investigate the effects of garlic oils and a novel long chain diallyl polysulfide on Nrf2 activation and examine the mechanisms involved.
3. Examine the effects of novel cell-penetrating peptides targeted to Nrf2/Keap1 and validate this protein-protein interaction as a therapeutic target in inflammation.
4. Examine the expression of Nrf2 in malignant melanoma cells and investigate the role of Nrf2 in chemoresistance in these cells.

Chapter 2: Materials and methods

2.1 Consumables and chemicals

Contents of all buffers can be found in Appendix I.

2.1.1 General reagents and consumables

Dimethyl sulfoxide (DMSO), hydrogen peroxide, N-acetylcysteine (NAC), tert-Butylhydroquinone (tBHQ), cisplatin, doxorubicin, and dacarbazine were obtained from Sigma-Aldrich (Poole, UK). Dulbecco's PBS was obtained from PAA (Yeovil, UK). Lipopolysaccharide (Calbiochem[®] E.Coli 0111: B4) was purchased from Merck Chemicals Ltd (Nottingham, UK). The p38 MAP kinase inhibitor, SB203580, was obtained from Tocris (Bristol, UK).

1.5 mL eppendorf tubes were obtained from Starlab (Milton Keynes, UK). 15 mL and 50 mL falcon tubes, 24-well tissue culture plates, and 96-well tissue culture plates were obtained from Corning (Amsterdam, The Netherlands). Pipettes were purchased from Gilson (Luton, UK). All other reagents, equipment, plastics, and disposables (including those listed in Appendix I) were purchased from Fisher Scientific (Loughborough, UK) unless stated otherwise.

2.1.2 Keap1 targeting peptides

TAT-10, TAT-14, TAT-sc, 14mer and TAT-16 (Table 2.1) were synthesised and purified as previously described (Steel et al., 2012), by Richard Steel, a member of Professor Mark Searcey's lab group (School of Pharmacy, University of East Anglia, UK).

Peptide	Sequence
TAT-10	YGRKKRRQRRR-LDEETGEFLP
TAT-14	YGRKKRRQRRR-LQLDEETGEFLPIQ
TAT-16	YGRKKRRQRRR-AFFAQLQLDEETGEFL
14mer	LQLDEETGEFLPIQ
TAT-sc	YGRKKRRQRRR-EFGTDIQLIEPQLE

Table 2.1. Keap1 targeting peptides.

2.1.3 Garlic oils and diallyl polysulfides

Diallyl polysulfide compounds and a diallyl polysulfide mixture (PS Mix) were synthesised and purified by Miriam Arbach, a member of Dr Chris Hamilton's lab group (School of Pharmacy University of East Anglia, UK) (Wang et al., 2012b). The garlic oil Naturex was supplied by Naturex (Avignon, France), whereas the garlic oil Stringer was sourced from Stringer Flavours Ltd (Tring, UK).

2.2 Cell Culture

All cell lines were cultured at 37°C, 5% CO₂ under humidified conditions. Cell culture media was pre-warmed to 37°C before use. Foetal bovine serum (PAA, Yeovil, UK) was heat-inactivated at 56°C for 50 min and filter-sterilised using a 0.45 µm filter (Corning) before it was used for supplementation of media.

2.2.1 Cell Maintenance

THP-1 is a human monocytic cell line derived from a 1-year-old male leukemia patient, and was obtained from the European Collection of Cell cultures (Salisbury, UK) (Tsuchiya et al., 1980). THP-1 cells were cultured in RPMI 1640 with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (PAA). THP-1 cells were maintained in T75 tissue culture flasks (Corning) at concentrations between 2.5×10^5 and 8×10^5 cells/mL and passaged every 3.5 days. To passage, the cell density was calculated, and the cells were diluted to 2.5×10^5 cells per mL in fresh media. THP-1 cells were not used after passage 25.

MCF-7 is a human breast cancer cell line derived from a 69-year-old Caucasian woman, and was obtained from the European Collection of Cell cultures (Salisbury, UK) (Soule et al., 1973). MCF-7 cells were grown in DMEM (PAA) with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. They were passaged every 3.5 days. The MCF-7 cells were not used after passage 50.

HepG2 is a human liver carcinoma cell line derived from a 15-year-old Caucasian male, and was obtained from the European Collection of Cell cultures (Salisbury, UK) (Knowles et al., 1980). HepG2 cells were cultured in DMEM with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. They were passaged every 3.5 days. The HepG2 cells were not used after passage 20.

The human malignant melanoma cell lines A375, A2058, Colo 829, M202, M238, M263, M308, Mel 501, SK-MEL-5, SK-MEL-28, and UACC 1273 (Table 2.2) were a kind donation from Dr Toni Ribas (UCLA, USA) (Niehr et al., 2011). All of the melanoma cell lines were cultured in RPMI 1640 with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL

streptomycin. They were passaged every 3.5 days. The melanoma cells were not used after passage 20.

HepG2, MCF-7 and all melanoma cells were passaged by removing the media, washing the cells for 10 seconds with 10 mL pre-warmed PBS and then adding 2.5 mL trypsin/EDTA (PAA). After incubation at room temperature for 10 seconds, 2 mL trypsin was removed, and the cells were incubated at 37°C for 1-2min until they were detached from the flask. The cells were resuspended in 10 mL fresh media and diluted as required.

Cell Line	Age	Gender
A2058	43	Male
A375	54	Female
Colo 829	45	Male
M202	Unknown	Unknown
M238	Unknown	Unknown
M263	Unknown	Unknown
M308	Unknown	Unknown
Mel 501	Unknown	Unknown
SK-MEL-5	24	Female
SK-MEL-28	51	Male
UACC 1273	54	Male

Table 2.2. Melanoma cell lines.

HEMa-LP are human epidermal melanocytes from lightly pigmented adult skin, which were obtained from Life Technologies (Paisley, UK) (Bhawan et al., 1997). HEMa-LP cells were grown in medium 254 (Life Technologies) supplemented with 1% HMGS-2 (Life Technologies). Media was changed

every 2 days, and cells were passaged when they reached 90% confluency. To passage, media was removed from the cells and 4 mL of 1x trypsin/EDTA was added. The flask was briefly rocked to ensure all the cells were covered. 3 mL trypsin/EDTA was removed. The cells were incubated at room temperature until the cells appeared round with no processes. The flask was tapped gently to detach the cells. 6 mL trypsin neutralizer solution was added to the cells, and the entire contents of the flask were transferred to a 15 mL falcon tube. The cells were centrifuged at 180 x g for 7min. The supernatant was removed, and the cells were resuspended in 4 mL media. The cells were counted and reseeded at 1.25×10^5 cells per 25cm² flask. Cells were not used past passage 6.

2.2.2 Calculating cell density

Cells were counted using a Malassez haemocytometer (Fisher Scientific), which contains a gridded chamber. Each grid counted holds 0.1 µL, and therefore the number of cells within the confines of a grid is the number of cells in 0.1 µL of the sample being counted.

Before counting, the cells were diluted in trypan blue (Sigma-Aldrich) in a 1:1 ratio. 20µL cells were loaded onto the hemocytometer and viewed under a light microscope. The cells in four chambers were counted and recorded. Cells stained blue were ignored (the blue staining only affects dead cells). In the case of cells on the border of the grid, only cells on the top or the left hand side were counted. Once four grids were counted, the cell count was averaged and the cell density was calculated using the following equation.

$$\text{Cell Density (cells/mL)} = (\text{Average cell count} \times 10,000)/2$$

In the event that a variant of this equation was needed, it could be adjusted by altering the following parameters. 10,000 is the amount to multiply the chamber size (0.1 μ L) by to reach 1 mL. 2 is the trypan blue dilution factor.

2.2.3 Freezing Cells

For suspension cells, 5×10^6 cells were collected from a stock density of 3×10^5 cells/mL. For adherent cells, 1 flask of cells at approximately 70% confluency was trypsinised. Cells were centrifuged at 300 x g for 5min. The supernatant was removed and the cells were resuspended in 1 mL freezing media. The cells were then transferred to ice immediately. The cells were stored in a polystyrene box, wrapped in tissue paper at -80°C for 24h. The cells were permanently stored in liquid nitrogen.

2.2.4 Thawing Cells

The cells were removed from liquid nitrogen and immediately thawed in a 37°C waterbath. Once thawed, the cells were added to 9 mL media. The cells were then centrifuged at 300 x g for 5min. The media was removed, and the cell pellet was resuspended in 6 mL media. THP-1 cells were transferred to a T25 cell culture flask (Corning) and cultured at 37°C . Adherent cells were transferred to a T75 cell culture flask, and a further 6 mL media was added. The cells were then cultured at 37°C .

2.3 Cell Stimulation

The following methods were used unless a variant is stated elsewhere in this chapter.

2.3.1 THP-1 cells

THP-1 cells were counted and diluted to 5×10^5 cells per mL. 2 mL cells were added to each well of a 24 well plate. Any unused well was filled with 2 mL sterile H₂O. The plate was incubated at 37°C for 24h. Without disturbing the cells, 1 mL media was removed from the top of each sample. This was to reduce the total cell volume to 1 mL, in order to reduce the amount of any compounds needed for treatment. The cells were then treated in an experiment-specific manner (details can be found in the relevant figure legends). Common treatments are described in Table 2.3. Previous experiments in the laboratory have documented that none of the vehicle controls used have any effect on Nrf2 or its target genes compared to unstimulated cells.

Compound	Vehicle Control	Final Concentration
TAT-14	PBS	75 μ M
TAT-sc	PBS	75 μ M
Garlic Oil	DMSO	10 μ g/mL
DAPS	DMSO	20 μ M
tBHQ	DMSO	50 μ M
NAC	H ₂ O	1 mM
LPS	RPMI	10 μ g/mL

Table 2.3. Common cell treatments.

2.3.2 Adherent cells

Following trypsinisation, the cells were resuspended in 10 mL media and transferred to a 15 mL Falcon tube. The cells were counted and the cell density calculated. The required volume of cells required for the experiment

was centrifuged at 300 x g for 5min. The supernatant was removed and the cells were resuspended to the desired concentration (Table 2.4). Cells were plated out as stated and empty wells were filled with H₂O before the plate was incubated at 37°C for 24h.

Cell Line	Plate	Volume	Cell Density (Cells/mL)
A375	96-well	100 µL	5x 10 ⁴
HepG2	24-well	500 µL	4x 10 ⁵
M202	6-well	2 mL	5 x 10 ⁵
M202	96-well	100 µL	8x 10 ⁴
M238	96-well	100 µL	1x 10 ⁵
MCF-7	96-well	100 µL	8x 10 ⁴
SK-MEL-5	6-well	2 mL	5x 10 ⁵
SK-MEL-5	96-well	100 µL	1x 10 ⁵
THP-1	24-well	2 mL	5x 10 ⁵
UACC 1273	96-well	100 µL	8x 10 ⁴

Table 2.4. Common cell seeding densities.

2.4 RT-qPCR

2.4.1 Sample collection

Cells were stimulated as previously described. After cell treatment the media was removed either by pipetting (adherent cells) or by transferring them to a 1.5 mL eppendorf tube and centrifuging at 5000 rpm for 5min and pipetting off the supernatant. The cells were resuspended in 1 mL Tri-Reagent (Life Technologies) and incubated at room temperature for 5min to allow for cell lysis. The samples were then stored at -80°C.

For the expression data in Chapter 6 the cells were not seeded. Instead, cells were detached from the flask using trypsin/EDTA and counted. 1 x 10⁶ cells

were transferred to a separate tube and centrifuged at 300 x g for 5min. The supernatant was removed, and the cells were resuspended in 1 mL Tri-Reagent. The samples were incubated at room temperature for 5min before storage at -80°C.

2.4.2 RNA extraction

The samples were thawed before adding 100 µL 1-bromo-3-chloropropane (BCP) (Sigma-Aldrich). The samples were shaken vigorously for 10 seconds, and incubated at room temperature for 10min. The samples were centrifuged for 20min at 12, 000 x g at 4°C. The aqueous layer was transferred to a new tube; the rest was discarded. 500 µL 2-propanol (Sigma-Aldrich) was added to each sample followed by a harsh vortex for 10 seconds. The samples were incubated at room temperature for 15min before centrifuging at 12,000 x g at 4°C. The supernatant was discarded, and 1 mL 70% molecular biology grade ethanol (Sigma-Aldrich) was added to the pellet. The samples were gently vortexed with 6 short pulses before immediately centrifuging at 4°C for 10min. All ethanol was removed from the pellet by pipetting and evaporation. 20 µL nuclease free H₂O was added to each sample before storing at -80°C overnight.

2.4.3 Nanodrop

The RNA concentration for each sample was measured by nanodrop using a ND-1000 Spectrophotometer (Labtech, Ringmer, UK). 1 µL was loaded, and the absorbance spectrum from 200-300 nm was measured. From the absorbance at 260 nm the quantity of RNA was calculated using the Beer-Lambert law. RNA purity is calculated using absorbance at 230 nm to measure phenol contamination and 280 nm to measure protein

contamination. Dividing the absorbance at 260 nm by the absorbance at 230 nm or 280 nm should give a value of 1.8-2.0 in a purified RNA sample. All RNA samples were diluted to a concentration of 200 ng/ μ L. In cases where this was not possible, the samples were diluted to either 100 ng/ μ L, 66 ng/ μ L, or 50 ng/ μ L (which ever was largest).

2.4.4 cDNA

For each sample, 200 ng RNA was diluted with nuclease free H₂O (Fisher Scientific) to a total volume of 4.5 μ L. A mastermix was made up of 45% MgCl₂, 18% 10xRT buffer, 18% deoxyribonucleotide triphosphate, 4.5% Reverse Transcriptase, 4.5% RNase inhibitor, and 9% random hexamers (Applied Biosystems, Paisley, UK). 5.5 μ L master mix was added to each sample. The samples were then loaded into a PTC-100 thermal cycler (Labcare Service, Braintree, UK) and cycled through the following temperatures: 21°C for 10min, 42°C for 15min, 99°C for 5min, 4°C for 5min. Upon completion, the samples were diluted with 20 μ L nuclease free H₂O.

2.4.5 Real-time PCR

5 μ L of each sample was added to an empty 0.1 mL PCR tube (Qiagen, Crawley, UK) for each gene of interest. For each gene, a separate mastermix was made up, consisting of 66.7% SYBR green (Sigma-Aldrich), 26.7% H₂O and 6.7% primers (Life Technologies; table 2.5). 15 μ L of primer mix was added to each sample. The samples were probed for the expression of specific genes by real time PCR using a QIAGEN Rotor-Gene Q Series 5-Plex. The following temperature cycle was used: 2min at 95°C, 40 cycles of {15s at 95°C followed by 40s at 60°C}, finishing off with a melting assay ramping from 60°C -94°C.

The results were analysed using the standard curve method and Rotor-Gene Q Series Software 1.7 (Build 94).

Gene	Primer Sequence	
Nrf2	Forward	5'-AAC CAC CCT GAA AGC ACA GC-3'
	Reverse	5'-TGA AAT GCC GGA GTC AGA ATC-3'
HO-1	Forward	5'-ATG GCC TCC CTG TAC CAC ATC-3'
	Reverse	5'-TGT TGC GCT CAA TCT CCT CCT-3'
NQO1	Forward	5'-CGC AGA CCT TGT GAT ATT CCA G-3'
	Reverse	5'-TCC TAT GAA CAC TCG CTC AAA CC-3'
Ferritin H	Forward	5'-ACC AAC GAG GTG GCC GAA T-3'
	Reverse	5'-TTC AGC CCG CTC TCC CAG T-3'
TNF	Forward	5'-GCC CAG GCA GTC AGA TCA TC-3'
	Reverse	5'-CGG TTC AGC CAC TGG AGC T-3'
IL-1 β	Forward	5'-GGA CAA GCT GAG GAA GAT GC-3'
	Reverse	5'-TCG TTA TCC CAT GTG TCG AA-3'
GAPDH	Forward	5'-AAC AGC CTC AAG ATC ATC AGC A-3'
	Reverse	5'-TGC TAA GCA GTT GGT GGT GC-3'

Table 2.5. Primer sequences.

2.5 Western immunoblotting

2.5.1 Sample preparation

Cells were stimulated as previously described. 30min before the cells were due to be harvested, 2x NuPAGE SDS sample buffer (Life Technologies) and sterile PBS (PAA) were pre-warmed to 37°C. 1x SDS sample buffer was made by diluting 2x SDS sample buffer in PBS in a 1:1 ratio. After cell treatment the media was removed either by pipetting (adherent cells) or by transferring

cells to a 1.5 mL Eppendorf tube and centrifuging at 5000 rpm for 5 min. The cells were resuspended in 100 μ L 1x SDS sample buffer and boiled in a water bath for 5min. The samples were then either stored at -80°C or protein was quantified immediately.

For the expression data in Chapter 6 the cells were not seeded. Instead, cells were detached from the flask using trypsin and counted. 1×10^6 cells were transferred to a separate tube and centrifuged at $300 \times g$ for 5min. The supernatant was removed, and the cells were resuspended in 100 μ L 1x SDS sample buffer. The samples were boiled in a water bath for 5min before storage at -80°C .

2.5.2 Protein quantification

Protein concentration of each sample was measured by absorbance at 280 nm using a ND-1000 spectrophotometer. 2 μ L was loaded, and the absorbance spectrum from 200-300 nm was measured. From the absorbance at 280 nm the quantity of protein was calculated using the Beer-Lambert law. This information was used to calculate the amount of each sample to load onto the SDS PAGE gel to ensure equal protein loading of all samples. 50 μ g of protein was used for each sample except in circumstances of dilute samples where 50 μ g would not fit onto the gel. In these cases the amount of protein loaded was reduced for all samples of the experiment to the highest possible amount.

2.5.3 SDS PAGE Gel and transfer

The Novex Western blotting system was used for Figures 4.2, 4.11, 4.13, and Chapter 5

The Bio-Rad Western blotting system was used for Figures 4.4, 4.6, 4.10, 4.12, and Chapter 6

2.5.3.1 The Novex system

The *SureLock*[®] Mini-Cell gel rig (Life Technologies) was set up according to the manufacturer's instructions, using pre-made NuPAGE 4-12% Bis-Tris gels (Life Technologies). NuPAGE running buffer (Life Technologies) was added to the inner chamber so that it covered the top of the gel. 500 µL antioxidant solution (Life Technologies) was added to the running buffer in the inner chamber. The outer chamber was filled with running buffer to approximately 2/3 of the height of the gel. The wells of the gel were washed by pipetting running buffer into them.

If samples were frozen they were first boiled for 5min. The required volume of sample was added to a fresh 1.5 mL Eppendorf tube. NuPAGE Sample Reducing Agent (10X) (Life Technologies) was added to make up 10% of the total volume. The samples were boiled for a further 5min. Each sample was loaded into the wells of the gel, and 3 µL pre-stained broad range molecular weight marker (Bio-Rad, Hemel Hempstead, UK) was loaded into a separate well. The gel was run at 150 V for 5min to ensure even running. Then the gel was turned up to 200V until the leading band reached the foot of the gel (approximately 50min).

The polyvinylidene fluoride (PVDF) membrane (Bio-Rad) was activated by washing in methanol (Fisher Scientific) for 30 seconds. It was then rinsed in deionised H₂O and washed in transfer buffer for 20min. Blotting pads were soaked in NuPAGE transfer buffer (Life Technologies) and all air bubbles were removed. The gel was then removed from its case and placed adjacent to the PVDF membrane. Two pieces of Whatman blotting paper (Fisher Scientific) were soaked in transfer buffer before sandwiching the gel and membrane.

Two blotting pads were loaded into the deeper negative electrode of the XCell II™ blot module (Life Technologies). The gel “sandwich” was added on top with the membrane side facing away from the negative electrode. Four blotting pads were loaded on top, before adding the positive electrode. The XCell II™ blot module was then loaded into the *SureLock*® Mini-Cell cell and clamped shut. Transfer buffer was added to the inner chamber, submerging the gel, membrane and blotting pads. The transfer was run at 30 V for 1 hour.

2.5.3.2 The Bio-Rad system

Polyacrylamide gels were made using the components described in Appendix I, using the apparatus supplied with the Mini PROTEAN Tetra Cell (Bio-rad). The Mini Protein spacer plates were cleaned with deionised H₂O and ethanol, and clamped together in the gel casting equipment as specified in the manufacturer’s instructions. The resolving gel was made and added in between the glass plates using a pipette. A gap of about 1.5-2 cm was left at the top of the casting equipment. This gap was filled immediately with 2-propanol. The gel was left to set for 30min. The 2-propanol was removed. The stacking gel was mixed and added to the top of the resolving gel. The comb was added immediately, and the gel was allowed to set for 30min. The comb was removed, and the plates removed from the clamp. The gel, still inside the spacer plates, was wrapped in damp tissue paper and cling film. It was stored at 4°C for up to 5 days.

The Mini PROTEAN Tetra Cell rig (Bio-Rad) was set up according to manufacturer’s instructions. Running buffer (Appendix I) was added to the inner chamber, and the wells of the gel were washed out using a pipette.

Running buffer was added to the outer chamber up to a level specified by the chamber case.

The samples were boiled for 5min and the desired amount was transferred to fresh Eppendorf tubes. NuPAGE Sample Reducing Agent (10x) was added to make up 10% of the final volume of the tube. The samples were boiled again and loaded into the wells of the gel. 3 μ L pre-stained molecular weight marker was also added to a spare well. The gel was run at 120 V until the leading band was past the stacking gel. The voltage was increased to 200 V for approximately 1h, until the leading band reached the bottom of the gel.

The PVDF membrane was activated by washing in methanol for 30 seconds. It was then rinsed in deionised H₂O and washed in transfer buffer for 20min. Blotting pads were soaked in transfer buffer (Appendix I) and all air bubbles were removed. The gel was then removed from its case and placed adjacent to the PVDF membrane. The gel and membrane were then flanked by two pieces of Whatman paper and placed on a blotting pad. Another blotting pad was added on top, and the entire pile was clamped into a Bio-Rad Tetra Blotting Module (Bio-Rad). The membrane and gel were orientated so that the membrane was on the side of the positive electrode. The transfer chamber was filled with transfer buffer, and an ice pack was added. The transfer was run at 100 V for 1h.

2.5.4 Immunoblotting

The membrane was placed in 5% milk, made up from Marvel Original Dried Skimmed Milk Powder (Premier International Foods, Dublin, Ireland) and TBST, for 1 hour on a shaker at room temperature. The primary antibody (Table 2.6) was made up in 5 mL 5% milk in a Falcon tube. The membrane was then incubated in the antibody on rollers at room temperature for 1h.

This was followed by three 5min washes in 5% milk, and three 5min washes in TBST. The membrane was incubated in the relevant HRP-conjugated secondary antibody (Table 2.7) for 30min at room temperature. This was followed by another 3 x 5min washes in 5% milk, and another 3 in TBST. Following this, Amersham ECL Prime solutions A and B (GE Healthcare, Little Chalfont, UK) were mixed and added to the membrane for 5min. The ECL was blotted off and the membrane encased in acetate. The membrane was imaged using a Syngene G:Box (Cambridge, UK).

Antibody	Host species	Source	Dilution
Nrf2	Rabbit	Abcam	1/2000
HO-1	Goat	R&D	1/500
β -actin	Mouse	Sigma Aldrich	1/100 000

Table 2.6. Primary Antibodies.

Antibody	Host species	Source	Dilution
Anti-Goat	Donkey	Santa Cruz Biotechnology (Heidelberg, Germany)	1/1000
Anti-Mouse	Goat	Dako (Cambridge, UK)	1/1000
Anti-Rabbit	Goat	Santa Cruz Biotechnology	1/1000

Table 2.7. Secondary Antibodies.

2.5.5 Reprobing

All Westerns were re-probed for beta actin, which was used as a loading control. In order to do this stripping solution was made by diluting 2 mL 10x

Reblot plus (strong) (Millipore, Watford, UK) in 18 mL ddH₂O. The membrane was then incubated in the stripping solution for 30min on a shaker. Following this the membrane was washed twice for 5min in blocker. From here the membrane can be probed with a different primary antibody.

2.6 MTS assay

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is a tetrazolium dye that is used to measure cytotoxicity, which is a broad term that includes apoptosis, and inhibition of cell proliferation. It is reduced to a molecule that absorbs light at 490-500nm in a process that is dependent on NAD(P)H-dependant oxidoreductase enzymes. Therefore, the assay measures cytotoxicity by detecting the activity of metabolic processes that produce NAD(P)H.

100 µL cells were seeded in a 96-well plate (Table 2.8). Adherent cells were incubated at 37°C overnight, suspension cells treated immediately. Cells were treated in triplicate with various concentrations of compounds of interest (details in figure legends). 3 wells were reserved for media alone (blank), and another 3 wells contained cells alone (positive control). Cells were incubated at 37°C for the indicated time period before 10 µL MTS (Cell Titer 96 Aqueous One solution Cell Proliferation Assay, Promega, Southampton, UK) was added to every well. Cells were then incubated for 3h. Absorbance was measured at 492 nm using a POLARstar Optima microplate reader (BMG Labtech; Aylesbury, UK).

2.7 TNF ELISA

2.7.1 Cell stimulation and sample preparation

1 mL THP-1 cells were seeded at a density of 0.5×10^5 cells/mL and incubated at 37°C for 24h. The top 500 µL medium was removed. Cells were treated with 75 µM TAT-14 or TAT-sc for 4h prior to addition of 50 ng/mL LPS for a further 4h. Cells were transferred to Eppendorfs, and pelleted by centrifugation at 5000 rpm for 5min. Supernatants were collected, and frozen at -80°C until use.

2.7.2 ELISA

The BD Bioscience TNF Elisa Kit (BD Bioscience, Oxford, UK) was performed according to manufacturer's instructions. TNF capture antibody was diluted 1:250 in coating buffer. A Nunc F96 Maxi-sorb immune plate (Fisher Scientific) was coated with 100 µL anti-human TNF capture antibody solution. The plate was sealed and stored at 4°C overnight. The wells were aspirated and washed three times in wash buffer. The plates were then blocked in assay diluent and incubated at room temperature for 1h. The plate was washed three times in wash buffer. Standards were made up by diluting known concentrations of TNF in assay diluent. Samples were made up by diluting supernatants 1:2 in assay diluent. 100 µL of each standard and sample were added to the appropriate wells, and incubated for 2 hours at room temperature. The plate was washed five times in wash buffer. 100 µL detection antibody was added to each well for 1 hour at room temperature. The plate was washed seven times in wash buffer. 100 µL TMB substrate

solution (BD Bioscience) was added to each well for 30min in the dark at room temperature. 50 μ L stop solution (0.5 M sulfuric acid) was added to each well. Absorbance at 450 nm was measured on a POLARstar Optima microplate reader.

2.8 Preparation and purification of plasmids

Detailed methods can be found in Chapter 3.2

2.8.1 Preparation of LB Broth and Agar Plates

Lysogeny broth (LB Broth) was prepared by dissolving 20 g LB broth powder (Sigma-Aldrich) in 1 L ddH₂O. The LB broth was sterilised by autoclaving at 123°C for 15min before use. Agar was prepared by adding 15 g bacteriological agar powder (Sigma-Aldrich) to 1 L LB broth. The solution was sterilised by autoclaving at 123°C for 15min. 500 μ L Ampicillin (Sigma-Aldrich) from a 50 mg/mL stock was added to the agar to give a final concentration of 50 μ g/mL. Agar plates were poured in a sterile LAF hood and left to set for 45min.

2.8.2 Extracting plasmids from blotting paper

The 8xARE and 4xARE plasmids were a gift from Professor Roland Wolf (University of Dundee, UK), supplied on blotting paper. They were extracted from the blotting paper by resuspending in 50 μ L nuclease free H₂O and stored at 4°C overnight. The blotting paper was removed and the DNA concentration was measured by nanodrop. The pGL4.73 and pGL4.32

plasmids (Promega), and the GFP plasmid (Lonza, Slough, UK) were commercially sourced, and did not require extracting from blotting paper.

2.8.3 Amplification of plasmids

Silver efficiency α -select *E. coli* (Bioline, London, UK) were transformed with 200ng of one of the plasmids by heat-shock transformation. The bacteria were grown in 960 μ L SOC media (Sigma-Aldrich) at 37°C for 1h. 100 μ L were spread over an agar plate, and colonies were grown overnight at 37°C. A colony was picked for each plasmid, and grown up in LB broth over 48h. In the first 24h the colony was grown in a total volume of 7 mL at 37°C with agitation. For the second 24h 2 mL of the bacteria was grown in 120 mL LB broth under the same conditions.

2.8.4 Plasmid Purification

Plasmids were harvested from the bacteria using an EndoFree Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. DNA concentration was then recorded by nanodrop before sending non-commercially sourced plasmids for sequencing for sequence verification at The Genome Analysis Centre (TGAC; Norwich, UK). Sequences were analysed using ApE v2.0.44 (Downloaded from: www.biologylabs.utah.edu; Created by M.Wayne Davis).

2.8.5 Measuring reporter gene activity

THP-1, MCF-7, or HepG2 cells were seeded and transfected with the luciferase plasmids as described in Chapter 3.2 using Lipofectamine technology (Life Technologies). The cells were treated as described before

measuring reporter gene activity. The luciferase assay was carried out using the Promega Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. The cells were transferred to a solid white 96-well plate (Fisher Scientific) as described in chapter 3.2 and added to an equal volume of luciferase substrate reagent. The cells were incubated in the dark for 10min and firefly luminescence was recorded using a POLARstar Optima microplate reader. Stop and Glo solution was added to the samples. The cells were incubated in the dark for 10min and renilla luminescence was recorded.

For analysis, non-transfected cell readings were subtracted from all samples. Firefly luciferase luminescence was divided by renilla luciferase luminescence and expressed as fold induction over unstimulated cells.

2.9 siRNA transfections

Cells were seeded 48h before transfection and grown to between 3×10^5 and 4×10^5 cells/mL (THP-1 cells) or 50-60% confluency (melanoma cells). Cells were counted, and 2×10^6 cells were transferred to a 15 mL falcon tube for each transfection sample required. The cells were centrifuged at $90 \times g$ for 10min. The media was removed, and the cells were resuspended in 92 μ L un-supplemented RPMI 1640 media. 200 nM (THP-1 and M202 cells) or 250nM (SK-MEL-5 cells) Nrf2 or negative control siRNA (Ambion®, Life Technologies) was added to each sample. The samples were transferred to a cuvette (Lonza) and electroporated in an Amaxa Nucleofector II Device (Lonza) on setting V-001. The cells were diluted to the desired density (Table 2.4) in fully supplemented RPMI 1640. The cells were incubated at 37°C for 24h and stimulated as described in the relevant figure legends. Cytotoxicity assays used the MTS assay protocol in section 2.2.7.

2.10 Fluorescence polarization assay

The fluorescence polarization assay works on the principle that if a fluorophore absorbs polarized light, the light that is emitted will lose polarization if the fluorophore is able to move freely. A fluorophore attached to a larger molecule tumbles more slowly in solution, and therefore the light emitted will retain its polarization. This method can be used to measure whether a fluorescent peptide is bound to the Kelch domain of Keap1. If the fluorescent peptide is bound, it will emit highly polarized light compared to unbound fluorescent peptide.

Plasmid DNA encoding the Kelch domain (residues 321-609) of Keap1 was a kind gift from Mark Hannink (University of Missouri, USA). This Keap1 protein was expressed and purified by Richard Steel.

90 μ L of interaction solution (Appendix I) was added to 50 wells of a low-binding black 96-well microplate. The inhibitor solution was made up of either TAT-14 or 14mer diluted in sodium phosphate buffer to a concentration of 50 μ M or 10 μ M. For each starting concentration a total of seven ten-fold serial dilutions was produced in sodium phosphate buffer (Appendix I) for a total of 16 concentrations. 10 μ L of each inhibitor solution was added in triplicate to the wells of the microplate containing the interaction solution. 10 μ L sodium phosphate buffer was added to the final two control wells. Solutions were mixed by pipetting, and any bubbles were removed. Plates were incubated in the dark for 15min at room temperature. The fluorescence intensity of each well was read at 485/520 nm in order to ensure that the inhibitor did not interfere with the fluorescence of the reaction mix. The fluorescence polarization gain was adjusted to the control wells. Fluorescence polarization was read ten times for each well. These readings were averaged and the fluorescence polarization for each concentration of inhibitor was analysed in triplicate by non-linear regression.

2.11 ROS assay

100 μ L M202 cells were seeded at a density of 1×10^5 cells/mL in a 96 well black plate with a transparent bottom (Fisher Scientific). The cells were incubated at 37°C for 24h. The media was removed, and replaced with 50 μ M 2, 7'-dichlorodihydrofluoresceindiacetate (H₂DCFDA) (Sigma-Aldrich) for 40min. H₂DCFDA was removed and replaced with fresh media. The cells were incubated at 37°C for 20min. Basal fluorescence was measured at 485/520 nm on a POLARstar Optima. Cells were stimulated with 10 μ M, 20 μ M, 50 μ M, or 100 μ M cisplatin for 4h. Fluorescence was read at 485/520 nm. Basal fluorescence was subtracted from the fluorescence in treated cells to calculate the amount of fluorescence caused by cisplatin.

2.12 Statistical analysis and presentation of data

All experiments were carried out at least three times and data presented as mean + SD, unless otherwise stated. Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software Inc, (C) 1992-2007). Unless stated otherwise, data was subjected to a one-way ANOVA with a Tukey's multiple comparison test. Values of $P \leq 0.05$ were considered statistically significant.

Chapter 3: Development of luciferase reporter assays to measure NF- κ B and Nrf2 transcriptional activity

3.1 Introduction

In the last decade Nrf2 has emerged as a key regulator of protective mechanisms within the cell. These include drug detoxification and inhibition of inflammation. Nrf2 knockout mice are susceptible to inflammatory diseases such as sepsis, and are susceptible to oxidative damage. Nrf2 activators are frequently examined for their ability to alleviate the effects of toxic molecules and inflammatory mediators (Kong et al., 2010; Khodaghali et al., 2011; Zhao et al., 2013b; Cheung et al., 2014).

Nrf2 is a key regulator of these protective processes because it upregulates enzymes, such as HO-1, NQO1, and glutamine cysteine ligase, which all play roles in protecting against ROS-induced damage and inflammation (Rushworth et al., 2008; Noll et al., 2012; Huang et al., 2013a; Liang et al., 2013). Nrf2 regulates these genes by binding to an ARE sequence in their promoters (Itoh et al., 1997).

One key transcription factor involved in inflammatory processes is NF- κ B, which is involved in the up-regulation of pro-inflammatory cytokines. Inhibition of NF- κ B activation is often considered a marker of anti-inflammatory activity. NF- κ B is inhibited by Nrf2 activation, and this has been linked to Nrf2 target genes, including HO-1 and NQO1 (Rushworth et al., 2008; Wang et al., 2014a)

A large range of compounds activate Nrf2 including phenols (such as flavonoids), quinones (for example, tBHQ), and isothiocyanates (which include sulforaphane) (reviewed in Magesh et al., 2012). New activators are continually being found (Dassano et al., 2014; Joo Choi et al., 2014). Many of these molecules are protective. For example sulforaphane, quercetin, and tBHQ all inhibit oxidative stress and inflammation (Jin et al., 2010; Kleszczynski et al., 2013; Zhao et al., 2013b; Lu et al., 2014b; Ramyaa et al.,

2014). Finding new Nrf2 activators is an important part of research into targeting the Nrf2 pathway to resolve inflammation. A reliable methodology that can be used for this is the luciferase assay.

A Luciferase assay is an experiment that tests for the activation of a transcription factor by measuring transcription of a gene under control of a specific promoter sequence. The gene under control of this sequence is one that encodes for a luciferase protein. Luciferase proteins are found in a variety of organisms including fireflies and sea pansies. They oxidise compounds called luciferins to create oxyluciferin, in a reaction that produces light. Luciferases and luciferins are organism specific, which means that they are also specific to each other. This allows them to be used in bioluminescence assays in conjunction with each other. In the dual luciferase assay two plasmids are co-transfected into the cell line of interest. One contains firefly luciferase under control of the promoter of interest. The second contains renilla luciferase, which is constitutively expressed under control of the SV40 promoter. This allows the renilla luciferase to be used as a control for transfection efficiency and cell viability. The Promega Dual-Glo Luciferase Assay System is designed for exactly this purpose, and contains a substrate for firefly luciferase, and a substrate for renilla luciferase mixed with an inhibitor of firefly luciferase (Deluca and McElroy, 1974; Matthews et al., 1977).

In this study two luciferase assays were established, using the Dual Luciferase Assay System as a detection mechanism. The first was to measure activation of the NF- κ B response element (κ B-RE), and the second was to measure activation of the ARE.

3.2 Aims

1. To develop a luciferase assay for the laboratory that can be used to identify activators of ARE-dependent transcription.
2. To develop a luciferase assay to measure activation of the NF- κ B pathway in THP-1 cells.

3.3 Methods

3.3.1 Bacterial transformations and plasmid purification.

The renilla plasmid, pGL4.73, and the NF- κ B-RE plasmid, pGL4.32, were purchased from Promega (Southampton, UK). 4xARE and 8xARE plasmids were a gift from Professor Roland Wolf (University of Dundee, UK). 8xARE and 4xARE plasmids were extracted from blotting paper by resuspending in 50 μ L nuclease free water and stored at 4°C overnight. The blotting paper was removed and the DNA concentration was measured.

200 ng of each plasmid was added to 40 μ L α -select bacteria (Bioline; London, UK). The bacteria were then put on ice for 30min, incubated at 42°C for 40 seconds, followed by a further 2min on ice. 960 μ L SOC media was added to the bacteria before placing in a rotary incubator for 1 hour.

100 μ L of bacteria was added to an agar plate, and spread over the surface. Bacteria were then grown overnight at 4°C. A colony was then picked from one plate for each plasmid using a pipette tip and grown up overnight in 7 mL LB broth with 50 μ g/mL ampicillin. The following day 2 mL of bacteria was grown up in 120 mL media.

Plasmids were harvested from the bacteria using an EndoFree Plasmid Maxi Kit (Qiagen; Crawley, UK) according to the manufacturer's instructions. DNA concentration was then recorded by nanodrop before sending for sequencing at The Genome Analysis Centre (TGAC; Norwich, UK) for analysis.

3.3.2 Transfections

3.3.2.1 THP-1 cells

1 mL THP-1 cells were seeded at a density of 1×10^6 cells per mL in a 24-well plate and incubated for 1h in a tissue culture incubator at 37°C, 5% CO₂. For each sample 400 ng firefly plasmid DNA, 100 ng pGL4.73, and 0.5 µL PLUS™ reagent were added to 100 µL Opti-MEM medium (Life Technologies). The solution was incubated at room temperature for 15min before adding 2.25 µL lipofectamine per sample. The mixture was incubated at room temperature for 25min. 100 µL solution was added to each transfected well. 100 µL Opti-MEM was added to non-transfected controls. Cells were incubated at 37°C for 24h before stimulation.

3.3.2.2 MCF-7 cells

100 µL MCF-7 cells were seeded at a density of 8×10^4 cells per mL in a 96-well plate and incubated at 37°C for 24h. For each sample 80 ng firefly plasmid DNA, 20 ng pGL4.73, and 0.1 µL PLUS™ reagent was added to 20 µL Opti-MEM medium. The solution was incubated for 15min at room temperature. 0.45 µL lipofectamine per sample was added to the mixture and incubated at room temperature for 25min. 20 µL of the solution was added to each well of MCF-7 cells to be transfected. Non-transfected controls were treated with 20 µL Opti-MEM instead. Cells were incubated for 24h before adding the desired stimulus.

3.3.2.3 HepG2 cells

500 µL HepG2 cells were seeded at a density of 4×10^5 cells per mL in a 24-well plate and incubated at 37°C for 24h. For each sample 400 ng firefly plasmid DNA, 100 ng pGL4.73, and 0.5 µL PLUS™ reagent was added to 20 µL Opti-MEM medium. The solution was incubated for 15min at room temperature. 2.75 µL lipofectamine per sample was added to the mixture

and incubated at room temperature for 25min. 100 μ L of the solution was added to each well of MCF-7 cells to be transfected. Non-transfected controls were treated with 100 μ L Opti-MEM instead. Cells were incubated for 24h before adding the desired stimulus.

3.3.3 Dual-Glo Luciferase Assay

Luminescence was measured using the Dual-Glo Luciferase Assay System by Promega. This kit contains a luciferase substrate reagent and a Stop and Glo reagent. The first contains a lysis buffer and the substrate for firefly luciferase, and the second contains both an inhibitor of firefly luciferase activity and the substrate for renilla luciferase.

3.3.3.1 THP-1 cells

After transfected cells were incubated with the desired stimulus for the indicated period of time, they were centrifuged at 5000 rpm for 5min. Media was removed and cells were resuspended in 80 μ L RPMI media (Fisher). 75 μ L of each sample was transferred to a solid white 96-well plate, ensuring to keep a gap of at least one well between adjacent samples. 75 μ L luciferase substrate reagent was added to each sample and mixed by pipetting. The plate was incubated in the dark for 10min. Luminescence was read using a POLARstar Optima (BMG Labtech; Aylesbury, UK). The plate was read a second time after a further 10min to ensure signal stability.

3.3.3.2 MCF-7 Cells

After transfected cells were incubated with the desired stimulus for the indicated period of time, 100 μ L luciferase substrate solution was added to each sample. Cells were incubated in the dark at room temperature for 10min. Samples were transferred to a solid white 96-well plate, taking care

to avoid bubbles. Luminescence was recorded using a POLARstar Optima (BMG Labtech). 10min later luminescence was read again to ensure signal stability.

3.3.3.3 HepG2 cells

After transfected cells were incubated with the desired stimulus for the indicated period of time, 350 μ L media was removed from each sample. 150 μ L luciferase substrate solution was added to each sample and incubated in the dark for 10min. Each sample was thoroughly mixed and 75 μ L transferred to a solid white plate. Luminescence was read using a POLARstar Optima (BMG Labtech). Luminescence was read 10min later to ensure signal stability.

3.3.3.4 *Renilla Luciferase Activity*

75 μ L (THP-1 and HepG2 cells) or 100 μ L (MCF-7 cells) Stop and Glo solution was mixed into each sample by pipetting and incubated in the dark for 10min. Luminescence was measured. This is the renilla luminescence. The plate was read a second time 10min later to ensure signal stability. Results were analysed by subtracting luminescence of the non-transfected control away from all other luminescence readings. Then firefly luminescence was normalised to renilla luminescence and expressed as fold induction over transfected cells containing vehicle control.

3.4 Results

3.4.1 Optimising an NF- κ B-RE driven luciferase assay in THP-1 cells

Before setting up a luciferase assay, it was important to set up a reliable method of transfection. Lipofectamine was chosen because it was deemed appropriate for optimisation and large-scale studies. THP-1 cells were seeded in a 24-well plate at various concentrations before transfecting with 500ng GFP-plasmid, as advised by manufacturer's instructions. After 24h, cells were viewed using fluorescence microscopy. There was a modest increase in the number of transfected cells in samples with higher seeding densities (Fig 3.1). There was no observed effect of seeding density on cell morphology. Therefore, total cells transfected was preferred over transfection efficiency in order to maximise the signal strength for the luciferase assay. The results suggest that there is little correlation between cell density and the amount of cells transfected. A seeding density of 1×10^6 cells/ml was used for all subsequent THP-1 luciferase experiments.

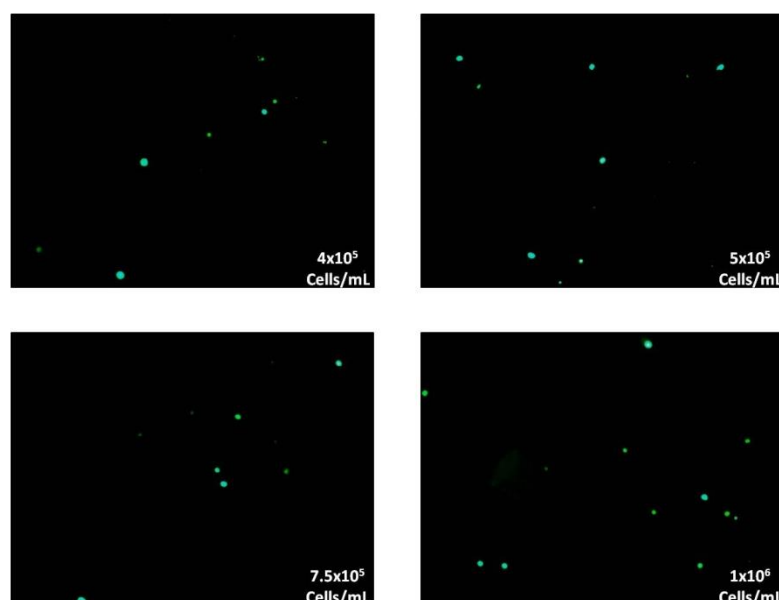


Fig 3.1. Transfection of GFP in THP-1 cells. The indicated number of THP-1 cells were transfected with 500ng GFP plasmid in 1 mL media using lipofectamine. Cells were pelleted and resuspended in PBS before observing fluorescence at 40x magnification.

LPS is an activator of monocytes and has been previously shown to activate NF- κ B in THP-1 cells (O'Connell et al, 1998; Essafi-Benkhadir et al., 2012; Wang et al., 2013c). Therefore the conditions for an LPS-induced NF- κ B-driven luciferase assay were optimised. pGL4.32 (Fig 3.2A) is a plasmid that contains a firefly luciferase gene driven by five repeating NF- κ B-RE sequences. pGL4.73 (Fig 3.2B) is a control plasmid which constitutively expresses Renilla luciferase under control of a SV40 promoter. THP-1 cells were transfected with pGL4.32 and pGL4.73 at a ratio of 4:1. A total of 500ng DNA was used per sample. 24h after transfection cells were stimulated with 10 μ g/mL LPS for up to 8h. Samples were pelleted and resuspended in 75 μ L RPMI media before transferring to a solid white 96-well microplate. Firefly and renilla luciferase activity were measured using the Dual Glo Luciferase Assay System. The results show that LPS-induced NF- κ B driven luciferase activity increased from 2-8h. Activity peaked at 7h with a 5.8 fold induction ($p < 0.001$), but there was little difference between any of the time points tested from 4h onwards (Fig 3.2C). 7h was used for the remainder of the experiments.

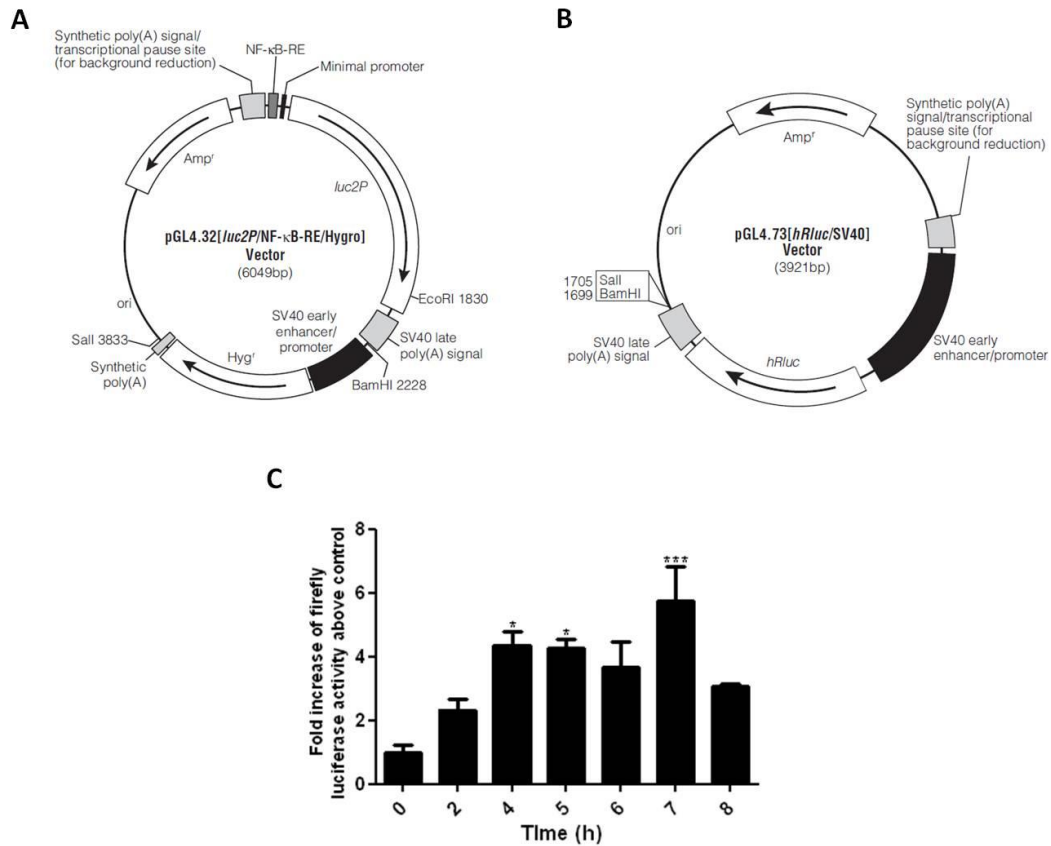
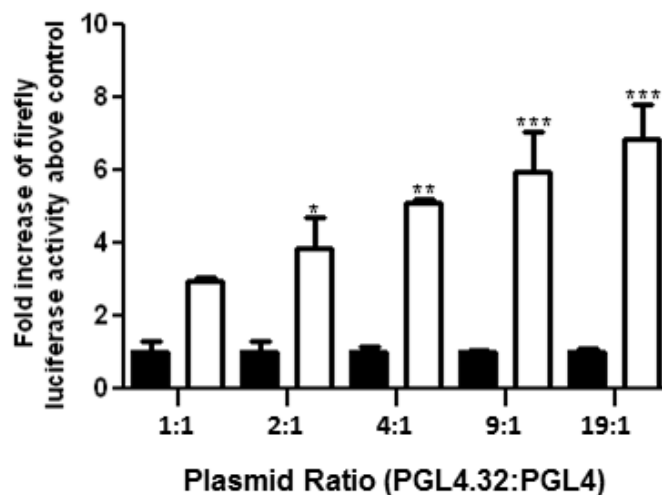


Fig 3.2. LPS activates the NF-κB-RE in THP-1 cells. Schematics of plasmids **(A)** pGL4.32 and **(B)** pGL4.73 (Copyright Promega, 2007). **(C)** THP-1 cells were transfected with 400ng pGL4.32 and 100ng pGL4.73. 24h after transfection cells were incubated with 10 μg/mL LPS for the indicated time period. Luminescence was read using the Promega Dual-Glo Luciferase Assay System, and firefly luminescence was normalised to renilla luminescence. N=3, mean±SEM; *p<0.05, ***p<0.001; One way ANOVA with Tukey's multiple comparison test.

To ensure maximal accuracy from luminescence readings it was important to optimise the luminescence created from the firefly luciferase plasmid. The easiest way to do this was to adjust the relative concentrations of the two plasmids. Therefore, THP-1 cells were transfected with various pGL4.32:pGL4.73 plasmid ratios in duplicate for 24h. One of each pair of samples was left untreated, and the other stimulated with LPS for 7h. The results show that LPS induces NF-κB-RE activity more effectively in samples with higher ratios of pGL4.32:pGL4.73, peaking at 6.8-fold for a ratio of 19:1 (Fig 3.3A). However, the data produced by samples with a plasmid ratio of

4:1 was much more consistent while still giving a strong induction of NF- κ B-RE activity (5.1-fold; $p < 0.01$). Furthermore, the luminescence produced by the renilla luciferase was much more variable with other plasmid ratios than with 4:1 (Fig 3.3B). For this reason, a plasmid ratio of 4:1 was selected for future experiments.

A



B

Plasmid Ratio (PGL4.32:PGL4)	Stimulus	Average Renilla Luminescence	Range	% variability
1:1	Unstimulated	46023	19638	42.66996936
	LPS	52072	35871	68.88797135
2:1	Unstimulated	23621	18579	78.65625198
	LPS	32888	28238	85.86110435
4:1	Unstimulated	21167	2767	13.07254388
	LPS	18088	2619	14.47961299
9:1	Unstimulated	8427	8906	105.6841106
	LPS	8429	11582	137.4065725
10:1	Unstimulated	5701	7341	128.7781774
	LPS	4286	3479	81.1807257

Fig 3.3. pGL4.32 and pGL4.73 are most reliable at a ratio of 4:1. (A) THP-1 cells were transfected with various ratios of pGL4.32:pGL4.73 totalling 500ng plasmid for each sample. 24h after transfection cells were incubated with 10 μ g/mL LPS for 7h. Luminescence was read using the Promega Dual-Glo Luciferase Assay System, and firefly luminescence was normalised to renilla luminescence. (B) Table showing variance in renilla luminescence readings. N=3, mean \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; One way ANOVA with Tukey's multiple comparison test.

A concentration response experiment was conducted to find the optimal concentration of LPS to use for the assay. THP-1 cells were transfected with 400ng pGL4.32 and 100ng pGL4.73. After 24h cells were stimulated with various concentrations of LPS for 7h. LPS induced NF- κ B luciferase activity at every concentration except 0.001 μ g/mL. Two concentrations caused a peak 5.6-fold induction of NF- κ B-RE driven luciferase activity. These were 0.1 and 1 μ g/mL ($p < 0.05$). Lower concentrations are preferable, so the recommended LPS concentration for use is 0.1 μ g/mL.

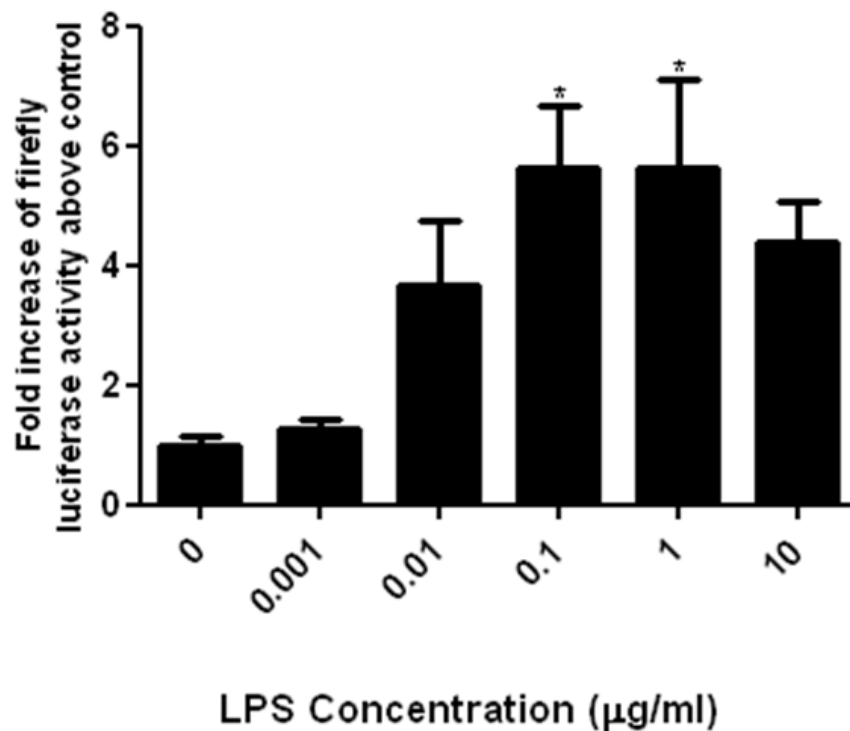


Fig 3.4. 0.1 μ g/mL and 1 μ g/mL are the most effective concentrations of LPS for NF- κ B-RE activity. THP-1 cells were transfected with 400ng pGL4.32 and 100ng pGL4.73. 24h after transfection cells were incubated with the indicated concentration of LPS for 7h. Luminescence was read using the Promega Dual-Glo Luciferase Assay System, and firefly luminescence was normalised to renilla luminescence. N=3, mean \pm SEM; * $p < 0.05$; One way ANOVA with Tukey's multiple comparison test.

The conditions optimised here (Fig 3.5) provide a functional LPS-induced NF- κ B-RE luciferase assay, which causes a consistent, strong induction of luciferase activity using 0.1 μ g/mL LPS for 7h. The relevant assay conditions used in this assay were used as a starting point for the ARE-luciferase assay.

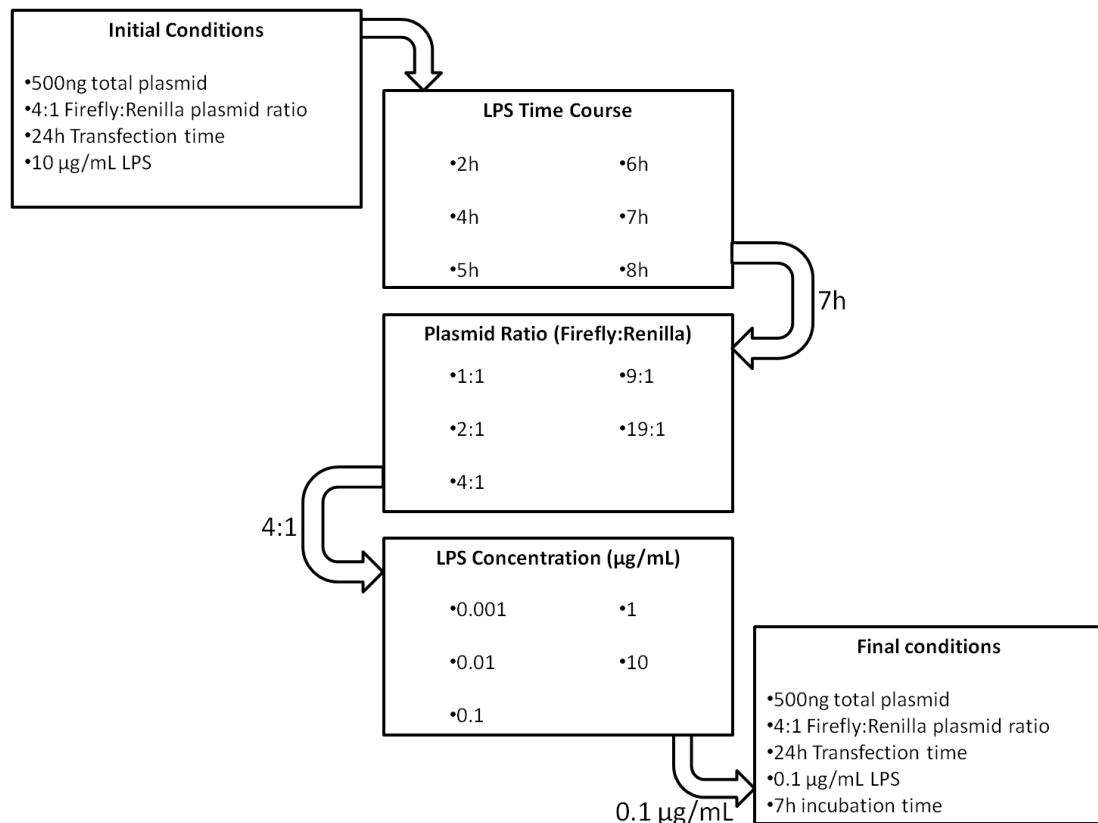
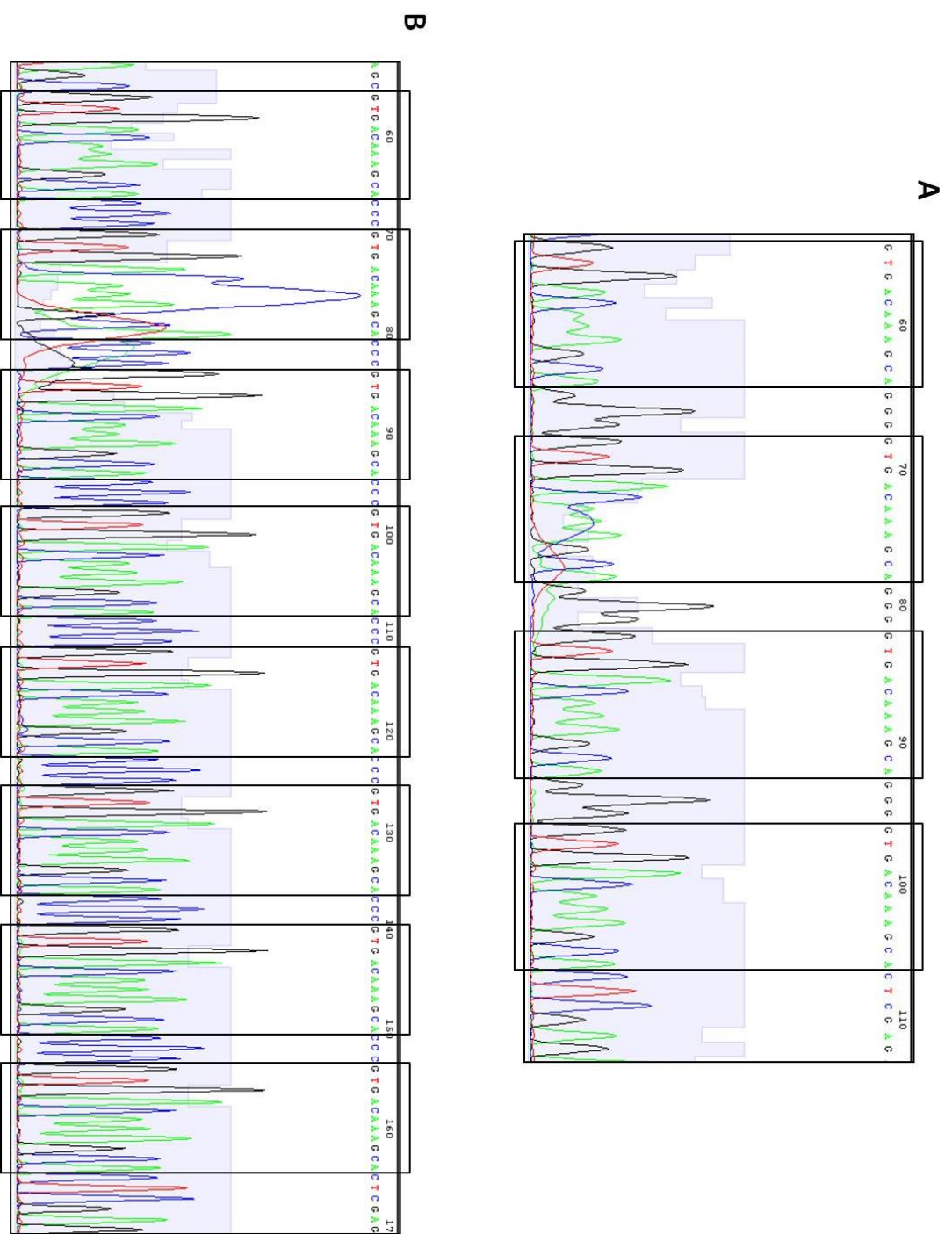


Fig 3.5. A summary of the optimisation of LPS-induced NF- κ B-RE-driven luciferase activity. Initial conditions were decided upon using manufacturer’s advice and instructions. 10 μ g/mL LPS has been used previously by our lab to induce NF- κ B-driven target genes in THP-1 cells (Rushworth et al., 2008).

3.4.2 Optimisation of an ARE luciferase assay

Two ARE-luciferase plasmids, 4xARE and 8xARE (fully characterised and functionally verified in human cells in: Wang et al., 2006) were a gift from Prof Roland Wolf (University of Dundee, UK). Firstly, before further experimentation, they were sent to The Genome Analysis Centre (Norwich, UK) for sequence verification. The core ARE sequence is (A/G)TGACnnnGC(A/G), and these plasmids contain the sequence GTGACAAAGCA from the murine GSTA2 promoter. The sequence is repeated 4 times in the promoter of the 4xARE plasmid, and 8 times in the 8xARE plasmid. Promoter sequences for both plasmids were confirmed (Fig 3.6).

Fig 3.6. Promoter sequences of 4xARE and 8xARE plasmids. Plasmid samples were sent to TGAC for sequencing. Files were analysed using A Plasmid Editor. Promoter sequences are shown.



The 8xARE plasmid was used for early optimisation because it was expected to produce a stronger luminescence signal. Initially, THP-1 cells were transfected with 8xARE and pGL4.73 in a 4:1 ratio, as carried out for the NF- κ B luciferase assay. 24h after transfection they were stimulated with 50 μ M tBHQ for 16h or 24h. The results demonstrated that tBHQ resulted in an increase in luciferase activity, as expected (Fig. 3.7A). However, upon closer examination, the change in luciferase was due to a change in Renilla, the control, rather than firefly luciferase activity. This may be due to instability of the transfected plasmids over time. To test this, THP-1 samples were transfected with 8xARE and pGL4.73 for multiple time periods up to 48h. The luciferase activity for both plasmids dropped off after about 30h (Fig 3.7B and C), suggesting that the plasmids are not stable for the period of time needed for the cells to recover post-transfection and to stimulate ARE-luciferase activity.

A

Stimulus	Firefly	Renilla	Normalised to Renilla	Fold Induction over DMSO
DMSO	2355	15177	0.16	1.00
16hr tBHQ	2048	4849	0.42	2.72
24hr tBHQ	3964	12834	0.31	1.99

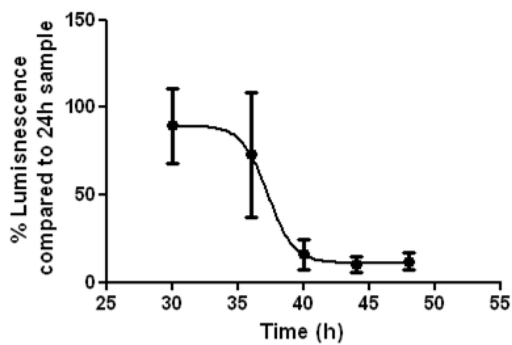
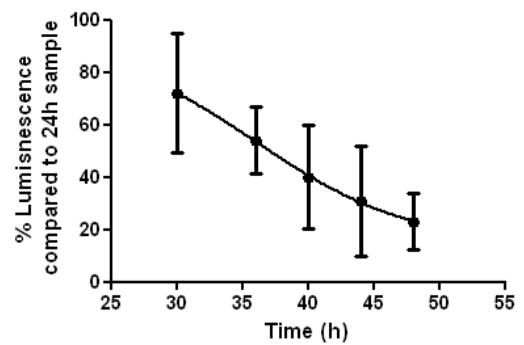
B**C**

Fig 3.7. Plasmids are unstable in THP-1 cells over extended periods of time. THP-1 cells were transfected with 400ng 8xARE and 100ng pGL4.73. **(A)** 24h after transfection cells were incubated with 50 μ M tBHQ or DMSO for 16 or 24h. Firefly and renilla luciferase activity was measured using the Promega Dual-Glo luciferase Assay System. **(B)** Firefly and **(C)** Renilla activity was recorded in unstimulated cells at various time points up to 48h. N=3, mean \pm SEM.

Having shown that THP-1 cells are an unsuitable cell line for ARE-luciferase plasmids, different cell lines were selected. Wolf and colleagues have previously reported tBHQ-induced ARE activity using these plasmids in the MCF-7 breast cancer cell line and this was therefore selected as a positive control (Wang et al., 2006). HepG2 hepatocytes were selected because they are commonly used for ARE driven luciferase assays (Huang et al., 2011; Kim et al., 2011; Li et al., 2014b). 24h after transfection the cells were stimulated with 50 μ M tBHQ for 18h. tBHQ induced a significant induction of 3.7-fold ($p < 0.01$) in MCF-7 cells (Fig 3.8A) and 4.3-fold ($p < 0.001$) in HepG2 cells (Fig 3.8B). This time, only the firefly luciferase activity was affected by tBHQ, whereas renilla readings were constant. Because the renilla readings were so high in the MCF-7 cells, the entire plate was read in order to check for signal bleeding into surrounding wells. The luminescence signal does bleed into adjacent and diagonal wells (Fig 3.8C). Although this is minimal (0.26% for adjacent and 0.06% for diagonal wells), it was decided to keep all samples at least one well apart on the plates for luminescence readings.

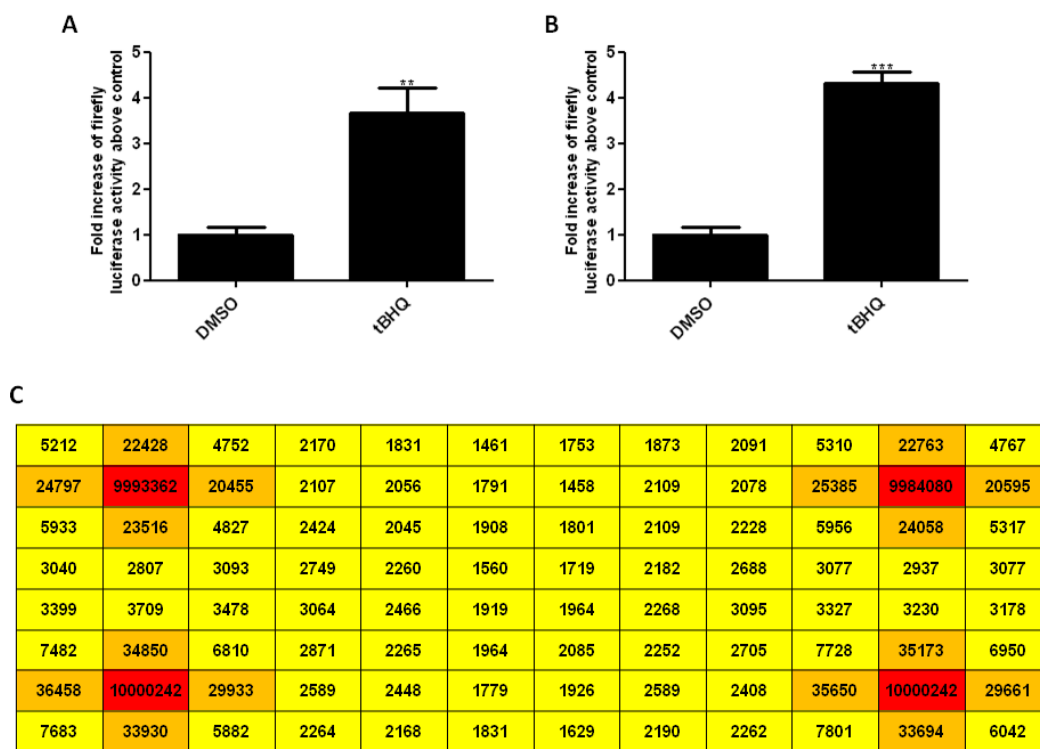


Fig 3.8. tBHQ activates ARE-driven luciferase activity in MCF-7 cells and HepG2 cells. (A) MCF-7 or **(B)** HepG2 cells were transfected with 400ng 8xARE and 100ng pGL4.73. 24h after transfection cells were incubated with DMSO or 50 μ M tBHQ for 18h. Luminescence was read using the Promega Dual-Glo Luciferase Assay System, and firefly luminescence was normalised to renilla luminescence. N=3, mean \pm SEM; **p<0.01, ***p<0.001; Student's t-test. **(C)** Renilla luminescence readings from MCF-7 cells. Red indicates wells containing the lysate from transfected cells, orange indicates empty wells with signal bleeding, yellow indicates unaffected empty wells.

The 8xARE plasmid was compared to the 4xARE plasmid. 24h after transfection HepG2 cells were stimulated with 50 μ M tBHQ for 18h. The results show no significant difference between the two plasmids (Fig 3.9).

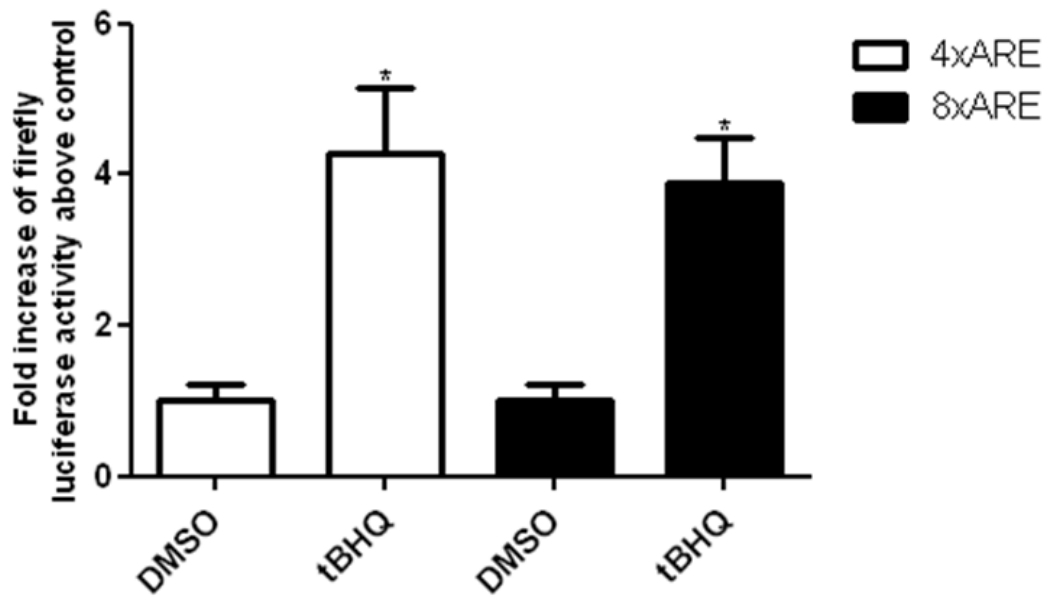


Fig 3.9. The 4xARE plasmid and 8xARE plasmid respond similarly in HepG2 cells. HepG2 cells were transfected with 400ng 4xARE or 8xARE and 100ng pGL4.73. 24h after transfection cells were incubated with 50 μ M tBHQ or DMSO for 18h. Luminescence was read using the Promega Dual-Glo Luciferase Assay System, and firefly luminescence was normalised to renilla luminescence. N=3, mean \pm SEM; * p <0.05; One way ANOVA with Tukey's multiple comparison test.

3.5 Discussion

Reporter assays are useful tools for measuring regulation of promoter-driven transcription. This can be particularly relevant for transcription factors that use the same promoter sequence to regulate a large number of functionally similar genes, such as Nrf2 and the p65 subunit of NF- κ B. Nrf2 targets have anti-inflammatory effects, and NF- κ B regulates a large number of pro-inflammatory cytokines. Reporter assays can be used to identify molecular regulators of these transcription factors, and may also have some predictive utility in identifying whether they are likely to have a broader pro- or anti-inflammatory effects.

This study has produced working experimental procedures to examine transcriptional activity of the transcription factors NF- κ B and Nrf2 *in vitro*, which are both suitable for preliminary screening for compounds that may provide anti-inflammatory protection via Nrf2. These protocols were optimised taking into account ease of use, assay sensitivity, and reliability. The NF- κ B-RE luciferase assay was set up in THP-1 cells, and was developed to produce a consistent response when stimulated with the TLR4 ligand LPS. The ARE-luciferase assay was shown to be poorly suited to THP-1 cells, but was established in MCF-7 and HepG2 cells.

THP-1 cells were chosen for both luciferase assays. This is because there is considerable evidence provided by our group and others that Nrf2 plays an important role in the resolution of inflammation, and NF- κ B is an important pro-inflammatory mediator (Rushworth et al., 2005; Rushworth et al., 2008; Reddy et al., 2011; Steel et al., 2012; Soetikno et al., 2013). When THP-1 cells were found to be unsuitable for the ARE-luciferase assay, HepG2 cells and MCF-7 cells were selected instead. There are several reasons for this. The first is that HepG2 cells are hepatocytes, which play a key role in detoxification processes. Nrf2, being a key regulator of detoxification

enzymes, is particularly relevant in the liver (Sahi et al., 2010; Cornejo et al., 2013). In addition both cell lines are commonly used for luminescence assays, and therefore uncertainty over the effect of the cell line on the assay was minimized (Chen et al., 2004; Niture et al., 2009; Do et al., 2013; Jiang et al., 2014; Wu et al., 2014). MCF-7 cells were specifically selected because they have been previously found to be a good cell line for use with the ARE plasmids in this study (Wang et al., 2006).

Stable transfections were considered, but transient transfections were chosen for the assays because they allow for more versatility in assay conditions, and because several reporter plasmids were used. There are several transfection methods that can be used to transfect THP-1 cells. Electroporation and DEAE-dextran can be used and were considered, but both were rejected in favour of Lipofectamine (Maess et al., 2011; Makon-Sebastien et al., 2014). Lipofectamine was chosen for several reasons. The first is that it is well-suited to optimising assays. A base protocol for each cell line used in this study has been published by Life Technologies, which is easy to adapt for optimisation purposes if necessary. The transfection protocol itself doesn't take very long, which makes it suitable for use with a high number of samples. In addition, it did not have an effect on cell morphology and has been used to transfect the ARE plasmids used in the development of this assay in HepG2 cells and MCF-7 cells (Wang et al., 2006).

The ARE reporter assay was established in HepG2 and MCF-7 cells. This is because the plasmids were not stable for long enough in THP-1 cells. The eventual conditions that were optimised are very similar to other luciferase assays that have been used for research into Nrf2. The 18h time point for tBHQ stimulation was decided upon because it has been used before in these cell lines (Wang et al., 2006). There is some variability with this among published assays. 16h and 24h have also been used to induce ARE-driven

luciferase activity with tBHQ in HepG2 cells (Niture et al., 2009; Do et al., 2013). While this shows that tBHQ-induced luciferase activity in this assay is very similar to published assays, different inducers may require different time points and this may need to be optimised on a case-by-case basis. For example, diallyl polysulfides induce ARE-driven luciferase activity at 24h (Chen et al., 2004).

The scale of induction of ARE-driven luciferase activity caused by 50 μ M tBHQ in HepG2 cells is also comparable to assays in the literature. 4.3-fold in this study compares favourably to the 2.5-fold and 2.8-fold inductions reported by Niture and co-workers, and Wang and co-workers respectively (Wang et al., 2006; Niture et al., 2009). A higher induction of 8-fold was achieved by Do and co-workers (2013), and interestingly they used a concentration of 30 μ M. In MCF-7 cells, the assay is less favourable. This study showed a 3.7-fold induction using tBHQ, whereas Wang et al. (2006) show a 50-fold induction. The reasons for this are unclear, and may have to do with the quantities of the plasmids used, because this is not made clear in the publication.

There have been a few LPS-induced NF- κ B-RE-driven luciferase assays set up in THP-1 cells. They are similar to the one developed in this study. They use 1 μ g/mL LPS as an inducer, which was found to be equal to 0.1 μ g/mL LPS in this study. They also use similar LPS stimulation times, with one using 4h and the other using 6h. The 4h and 6h time points were not significantly different from 7h in this study, suggesting that the variance in LPS stimulation time periods has a negligible effect on the assay (Spellerberg et al., 1996; Baumann et al., 2005). However, the induction of reporter gene activity was lower in this study than it was in either published study, which reported inductions of approximately 10-12-fold. The differences may be to do with the plasmids used. One luciferase assay was driven by a cloned TNF promoter (Baumann et al., 2005). The other assay was a bit different, in that a dose

response assay was shown. 0.1 $\mu\text{g}/\text{mL}$ LPS caused approximately 5-fold induction. However, this is difficult to assess due to the scaling on the graph. Similarly to this study, there is not much difference between the higher concentrations used – 20, 10, and 1 $\mu\text{g}/\text{mL}$ LPS all cause a similar induction of NF- κ B-RE-driven luciferase activity. Unlike this study it does not extend to 0.1 $\mu\text{g}/\text{mL}$. This may be because of the type of LPS used or the plasmids used, which use a 2x murine NF- κ B promoter sequence, and a minimal *c fos* promoter (Spellerberg et al., 1996).

There are some limitations that must be considered. Luciferase assays use a known promoter to induce gene expression, but some promoter sequences are known to be regulated by several transcription factors, and some promoter sequences (including the ARE) are known to overlap (Friling et al., 1992; Wasserman and Fahl, 1997; Kurokawa et al., 2009; Kaspar and Jaiswal, 2010). Therefore, other experiments are required to verify that Nrf2 or NF- κ B are the driving forces of transcription whenever a molecule affects luciferase expression. EMSAs, Western blots and PCRs along with inhibitor experiments (if one is available) can be used to back up data obtained from these assays. Furthermore, although biological context is provided by the environment of living cells, the very presence of the luciferase plasmid changes that biological context. Therefore, preliminary data gained from this assay must be backed up with complimentary experiments such as those described above.

This assay is also fairly cumbersome. Adherent cells thus far have been grown in a standard tissue culture plate, and transferred to a solid white plate after cell lysis. Aside from being slow, this leads to pipetting errors and loss of sample. Now that the protocol has been shown to work, there should not be a problem growing the cells in a tissue culture treated solid white plate to cut out this step, but it has not yet been tried. The main disadvantage of doing

this is that it becomes difficult to monitor the state of the cells. The biggest issue, however, is that THP-1 cells need a high cell number to deliver a high signal. This means that they need to be seeded in a 24-well plate, and then pelleted at the time of harvesting before transferring to a solid white plate. Much like with the adherent cells, this is time consuming and leads to errors.

This assay provides a framework upon which to develop a high-throughput assay, but it will require adapting if it is to be used as such. High throughput assays require efficiency in addition to scientific rigour. There are several ways in which these assays can become more efficient. The first, as mentioned above, is to seed directly onto a tissue culture treated white plate. This serves to cut down on errors in pelleting and transferring the cells, and also cuts down the time and reagents spent doing so. The second is to use 96-well plates whenever possible. More samples per plate is time-efficient, and the size of the wells makes it cost-efficient. However, this would exclude certain cell lines from being suitable for high throughput research, such as THP-1 cells. Perhaps the biggest modification for high throughput is transferring the assay to stable transfections. Cutting out on the transfection step reduces errors and is both resource and time-effective, but is only viable if the assay conditions don't need further optimisation.

Expanding the assays into different cell lines may also be an option, especially if they can be established in a cell line that is relevant to both Nrf2 and NF- κ B in inflammation. One potential benefit to having both luciferase assays set up in the same cell line is that inhibitors of the NF- κ B-RE assay can be linked with activators of the ARE assay. RAW 264.7 cells, for example, are a murine macrophage cell line in which Nrf2 activation has been linked to anti-inflammatory activity (Han et al., 2013; Lee et al., 2014a).

Improving the current model is also important. Further experiments with the NF- κ B assay can be improved by using an established NF- κ B inhibitor to

compare experimental inhibitors to. Other inflammatory mediators such as TNF also activate NF- κ B in THP-1 cells, and can be used to expand the assay to study other pathways (Desai et al., 2012).

In conclusion, this study has developed two useful luciferase assays to investigate transcription driven by the ARE or NF- κ B-RE promoters. The NF- κ B-RE assay has been optimised to use LPS as a model of pro-inflammatory signalling in THP-1 cells. The ARE assay was developed in HepG2 and MCF-7 cells after the plasmids were found to degrade rapidly in THP-1 cells. Both assays provide a framework for future development of a high-throughput reporter assay, and can be used to identify novel activators of ARE-driven genes and anti-inflammatory drug candidates.

Chapter 4: The effects of long chain diallyl polysulfides from garlic on the Nrf2 pathway in human monocytic cells

Acknowledgements:

Miriam Arbach and Dr Chris Hamilton synthesised the diallyl sulfides and provided the garlic oils. Miriam also performed the HPLC chromatography.

Arthur Mallett (undergraduate project student) performed some of the experiments in Figs 4.2, 4.3, 4.4 and 4.7.

4.1 Introduction

A diet rich in fruit and vegetables is widely accepted to have a positive effect on health and improve protection against a variety of diseases including coronary heart disease, stroke, rheumatoid arthritis, and COPD (Dauchet et al., 2006; Boeing et al., 2012). The nutrients that cause these effects are a key area of interest for both nutrition and medical researchers because they may have implications for disease prevention and drug design. For example, common fruits and vegetables such as onions and cranberries contain quercetin, which has been associated with anti-inflammatory activity (Askari et al., 2012; Boots et al., 2011), and diets high in fibre decrease risk of cardiovascular disease (Threapleton et al., 2013).

For centuries it has been believed that garlic has therapeutic properties. Modern research has produced evidence to support these beliefs. Garlic consumption has been linked with protection against risk factors of cardiovascular disease, including lowering cholesterol and vascular calcification (Warshafsky et al., 1993; Durak et al., 2004). In addition, a number of human trials have shown that garlic lowers biomarkers of various conditions, including hypertension, inflammation and atherosclerosis (Zeb et al., 2012; Ried et al., 2013).

Garlic contains high concentrations of organosulfur compounds which have been the subject of medical research *in vitro* and *in vivo* (Yoshida et al., 1999; Ichikawa et al., 2006; Quintero-Fabian et al., 2013). In particular, Allicin may play an important role in the bioactivity of garlic. Garlic itself does not contain high concentrations of allicin. Instead, allicin is produced when garlic is crushed. When this happens the enzyme alliinase is released from the vacuole, and converts alliin into allicin (Fig 4.1A) (Wang et al., 2011). However, allicin itself is unstable, and degrades into diallyl polysulfides (Munchberg et al., 2007). Both Allicin and its polysulfide degradation

products have been linked with many of the health benefits attributed to garlic *in vivo*. These include anti-inflammatory, antiproliferative, and antibacterial properties (Tsao and Yin, 2001; Kalayarasan et al., 2009; Li et al., 2012c; Gu et al., 2013; Shin et al., 2013a).

Diallyl polysulfides contain a polysulfide chain flanked by two allyl groups, giving the overall formula $\text{CH}_2\text{CHCH}_2\text{-S}_x\text{-CH}_2\text{CHCH}_2$ (Fig 4.1B). They vary in the length of their polysulfide chain; 1-4 sulfurs are common, whereas longer chains containing 5-7 sulfurs are increasingly less so (Taucher et al., 1996). Most research to date has focused on short chain ($x \leq 3$) polysulfides, and an increasing body of literature demonstrates that these compounds activate the cytoprotective transcription factor Nrf2 in a variety of tissue types.

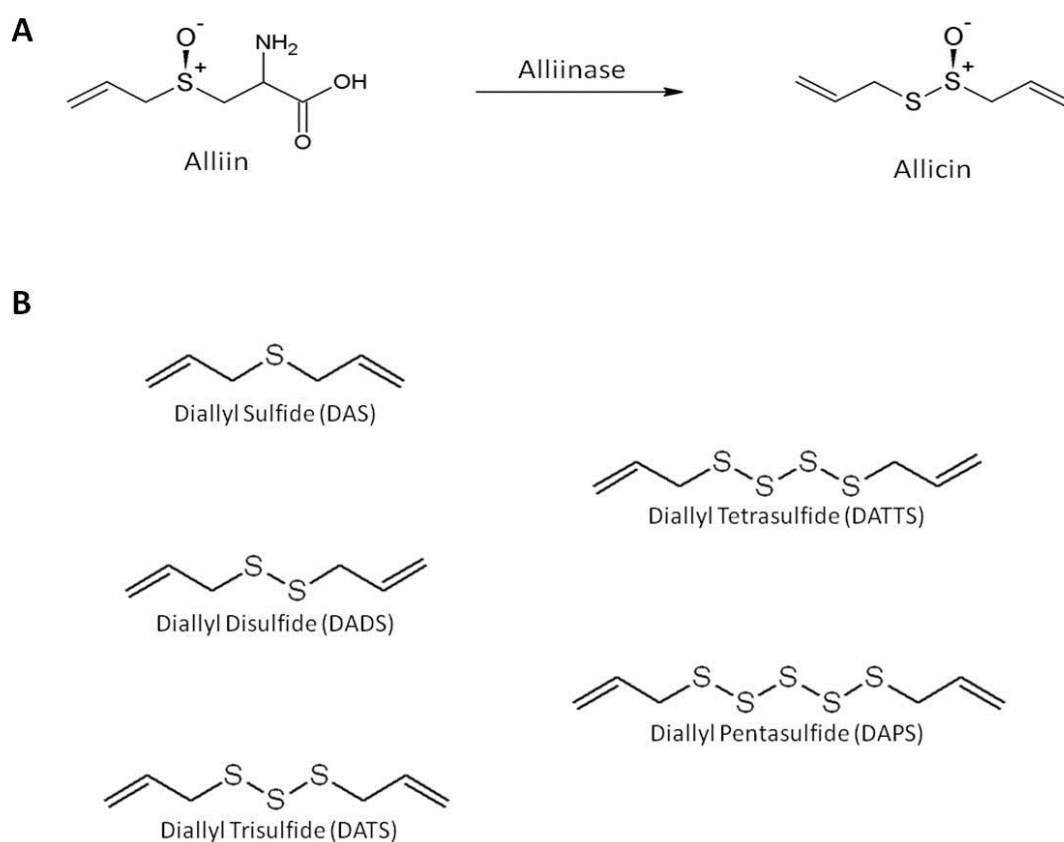


Fig 4.1. Garlic organosulfur compounds. (A) Alliinase converts alliin to allicin when garlic is damaged. **(B)** Chemical structures of diallyl polysulfides.

Diallyl Sulfide (DAS) shows upregulation of Nrf2 and inhibition of gentamicin-induced TNF and NF- κ B expression in rat kidneys (Kalayarasan et al., 2009). Diallyl disulfide and diallyl trisulfide upregulate Nrf2 and inhibit inflammatory mediators in RAW264.7 macrophages (Shin et al., 2013b; You et al., 2013).

Long chain polysulfides are widely hypothesised to be more biologically active than short chain polysulfides (Chen et al., 2004; Munchberg et al., 2007; Saidu et al., 2013), but this has not been confirmed with respect to Nrf2 activation. Recent data show that diallyl tetrasulfide (DATTS) induces Nrf2 and HO-1 in human colorectal cells (Saidu et al., 2013), suggesting that longer chain diallyl polysulfides are biologically active, but due to the fact that DATTS was used in isolation it is difficult to draw conclusions about its relative activity. This study focused on the effects of the long chain polysulfides DATTS and diallyl pentasulfide (DAPS) on the Nrf2 pathway in human monocytes. DAPS was characterised as a novel inducer of the Nrf2 pathway, and its activity was directly compared to shorter chain diallyl polysulfides.

4.2 Aims:

Hypothesis: Long chain diallyl polysulfides activate Nrf2 expression more powerfully than short chain diallyl polysulfides. Long chain diallyl polysulfides confer protective effects to monocytes.

Aims:

1. To investigate whether the long chain polysulfides DATTS and DAPS induce the Nrf2 pathway in THP-1 monocytes.
2. To examine/identify mechanisms involved in Nrf2 activation by diallyl polysulfides.
3. To elucidate the relationship between polysulfide chain length and its effectiveness in inducing Nrf2 pathway activation.
4. To investigate potential beneficial effects of long chain polysulfides.

4.3 Results

4.3.1 Garlic oils activate the Nrf2 pathway in THP-1 monocytes.

Previous work has shown that garlic oils contain high concentrations of diallyl polysulfides, but whether they activate the Nrf2 pathway in monocytes is unknown. To investigate this, two garlic oils were selected for experiments in human THP-1 monocytes. The garlic oils were selected based on their polysulfide content. Naturex was chosen because it had the highest amount of long chain polysulfides (DATTS, DAPS, DAHS) of all the garlic oils, and Stringer was chosen because it has comparatively low concentrations of long chain polysulfides (Table 1). Diallyl polysulfides can be synthesised by reacting diallyl disulfide with liquid sulfur (Wang et al., 2012b). This method was used to make a polysulfide mixture (PS Mix) which was used as a positive control for polysulfide activity.

Garlic Oil	DADS	DATS	DATTS	DAPS	DAHS	Unknown*
Naturex	27.45%	31.83%	16.50%	4.79%	2.46%	16.97%
Stringer	18.29%	42.90%	11.17%	1.39%	0.76%	25.49%
PS Mix	6.39%	19.55%	41.81%	17.07%	4.04%	10.40%

*Bioactive organosulfur constituents of garlic oils are reviewed by Lawson (1993).

Table 4.1. Diallyl polysulfide composition of garlic oils. Garlic oil components were separated by HPLC, and diallyl polysulfide content was measured by absorbance at 240nm.

THP-1 cells were incubated with 10 µg/mL of each garlic oil for various time points up to 8h. Cells were lysed and total cell extracts were measured for Nrf2 protein expression by Western blot. Fig 4.2 demonstrates that Nrf2 is induced by both garlic oils and PS mix. All three upregulated Nrf2 after 4h of exposure. PS Mix induced Nrf2 expression more powerfully than either garlic

oil, which may be due to the higher concentration of diallyl polysulfides present. Naturex induced Nrf2 earlier than Stringer, but neither garlic oil caused a sustained Nrf2 activation after 4h.

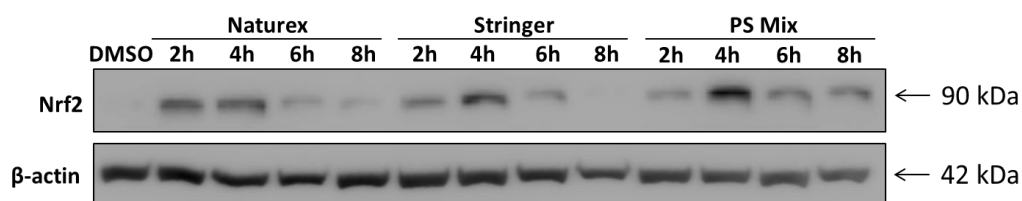


Fig 4.2. Garlic oils induce Nrf2 accumulation in THP-1 cells. THP-1 cells were incubated with 10 µg/mL Naturex, Stringer or PS Mix for the indicated time point. DMSO was used as a vehicle control. Whole cell lysates were prepared and Nrf2 expression measured by western blot analysis. β-actin expression is shown as a loading control. Results are representative of three independent experiments; replicates are shown in Appendix II.

To investigate whether the induced Nrf2 is transcriptionally active in THP-1 cells, the effect of the garlic oils on expression of two downstream target genes, HO-1 and NQO1, was measured. THP-1 cells were incubated with 10 µg/mL of the indicated garlic oil for up to 12h and HO-1 and NQO1 mRNA expression were measured by RT-qPCR. Fig 4.3A demonstrates that the garlic oils significantly induced HO-1 mRNA expression in THP-1 cells, with a peak induction at 8 hr. Furthermore, Stringer was twice as effective at inducing HO-1 as Naturex (18.4-fold vs 9.1 fold induction over control), although neither was as effective as the PS mix control. Both garlic oils also significantly induced NQO1 mRNA expression in THP-1 cells (Fig 4.3B). Stringer induced a more powerful and prolonged activation of NQO1, with expression still increasing at 12 h post stimulation, topping even that of PS mix at 12 h (Stringer, 5.4-fold over control vs Naturex, 4.0 fold vs PS Mix, 3.8 fold).

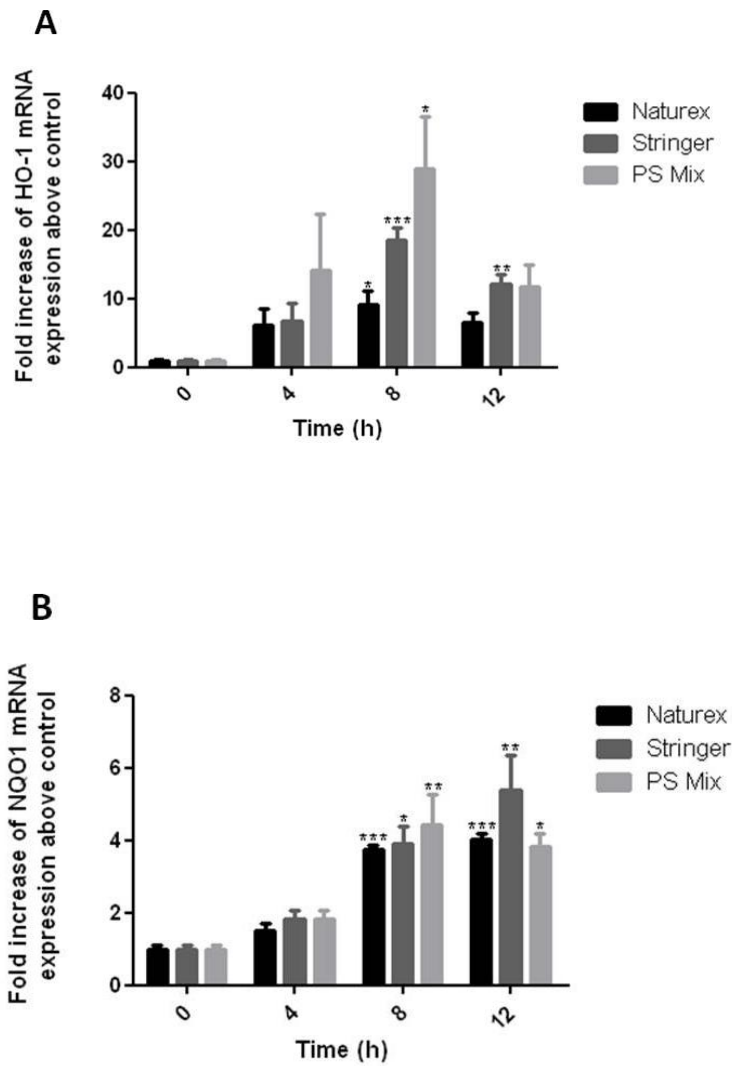


Fig 4.3. Garlic oils induce HO-1 and NQO1 mRNA expression in THP-1 cells. THP-1 cells were incubated with 10 $\mu\text{g}/\text{mL}$ Naturex, Stringer or PS Mix for the indicated time point. In the 0h samples the cells were incubated with the vehicle control, DMSO, for 12h. RNA was extracted and mRNA expression of **(A)** HO-1 or **(B)** NQO1 was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, $n=3$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; one way ANOVA with Tukey's multiple comparison test.

In order to verify that transcriptional activity correlates with protein production, THP-1 cells were incubated with 10 µg/mL of the garlic oils or PS mix. HO-1 protein expression was analysed. Both garlic oils increased HO-1 protein expression by 16h, which remained increased at 24h (Fig 4.4). Consistent with the mRNA expression data, Stringer was again more powerful than Naturex, but not as powerful as PS mix.

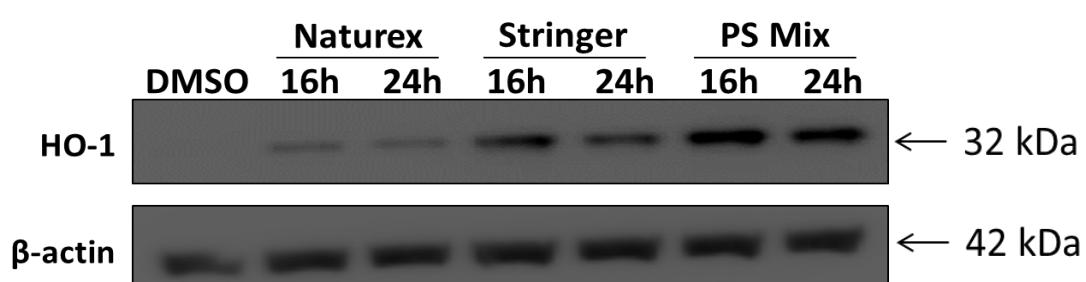


Fig 4.4. Garlic oils Induce HO-1 protein expression in THP-1 cells. THP-1 cells were incubated with 10 µg/mL Naturex, Stringer or PS Mix for the indicated time point. DMSO was used as a vehicle control. Whole cell lysates were collected and HO-1 expression was measured by Western blot. β-actin expression is shown as a loading control. Results are representative of three independent experiments; replicates are shown in Appendix II.

4.3.2 Long chain polysulfides activate HO-1 in THP-1 cells.

Taken together the results show that garlic oils activate the Nrf2 pathway in monocytes. Interestingly Stringer was the more effective of the two despite Naturex containing higher concentrations of long chain diallyl polysulfides. However, conclusions based on this evidence would be flawed because Stringer contains 8.5% more unidentified compounds. Therefore, a direct comparison between the diallyl polysulfides was performed. THP-1 cells were incubated with 20 µM DADS (2S), DATS (3S), DATTS (4S) or DAPS (5S) for 8h. Total RNA was extracted and HO-1 expression measured by RT-qPCR. The results revealed a strong correlation between chain length and the amount

of HO-1 produced (Fig 4.5). DAPS was the strongest inducer with a 47-fold induction over control ($p < 0.001$), over twice as effective ($p < 0.01$) as DATTS, which showed a 20.7-fold induction over control ($p < 0.01$). DATS caused a 16-fold induction of HO-1 mRNA when compared to DMSO alone ($p < 0.05$), whereas DADS caused a non-significant 3.1-fold increase in HO-1 production. Increase in activity from DADS to DATS and from DATTS to DAPS was larger than that from DATS to DATTS.

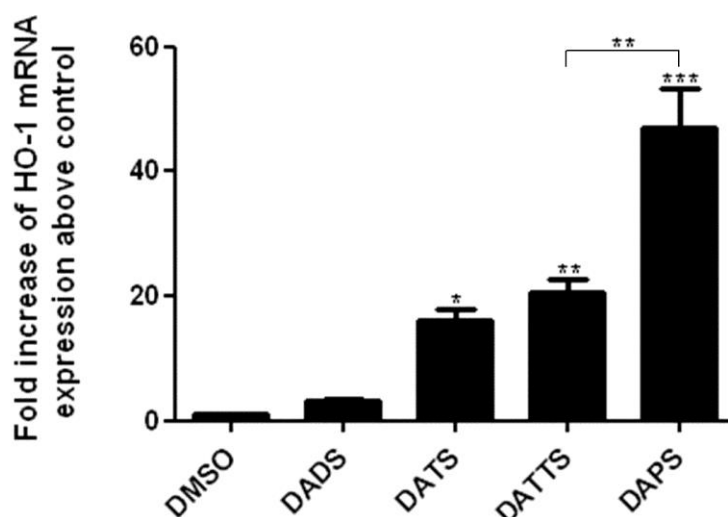


Fig 4.5. Diallyl polysulfides induce HO-1 mRNA expression in THP-1 cells. Cells were incubated with 20 μ M DADS, DATS, DATTS, or DAPS for 8h. DMSO was used as a vehicle control. Total RNA was extracted and HO-1 expression was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, $n=3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; One way ANOVA with Tukey's multiple comparison test.

The differences in effectiveness could be due to a difference in kinetics rather than activity. To account for this possibility, the long chain polysulfides, DATTS and DAPS, were incubated with THP-1 cells for up to 24h. HO-1 mRNA and protein expression was measured. A difference in kinetics

between the two compounds was observed at both mRNA and protein level. DATTS induced HO-1 mRNA and protein expression compared to DMSO alone, peaking at 8h ($p<0.05$) and 12h respectively (Fig 4.6A and C). In contrast DAPS produces a later, more sustained induction of HO-1 mRNA, peaking at 8-12h ($p<0.01$, 0.01 respectively), with protein peaking at 16h (Fig 4.6B and C). At no point, however, does DATTS induce HO-1 more strongly than DAPS, confirming that DAPS is the more effective diallyl polysulfide of the two, despite the difference in kinetics.

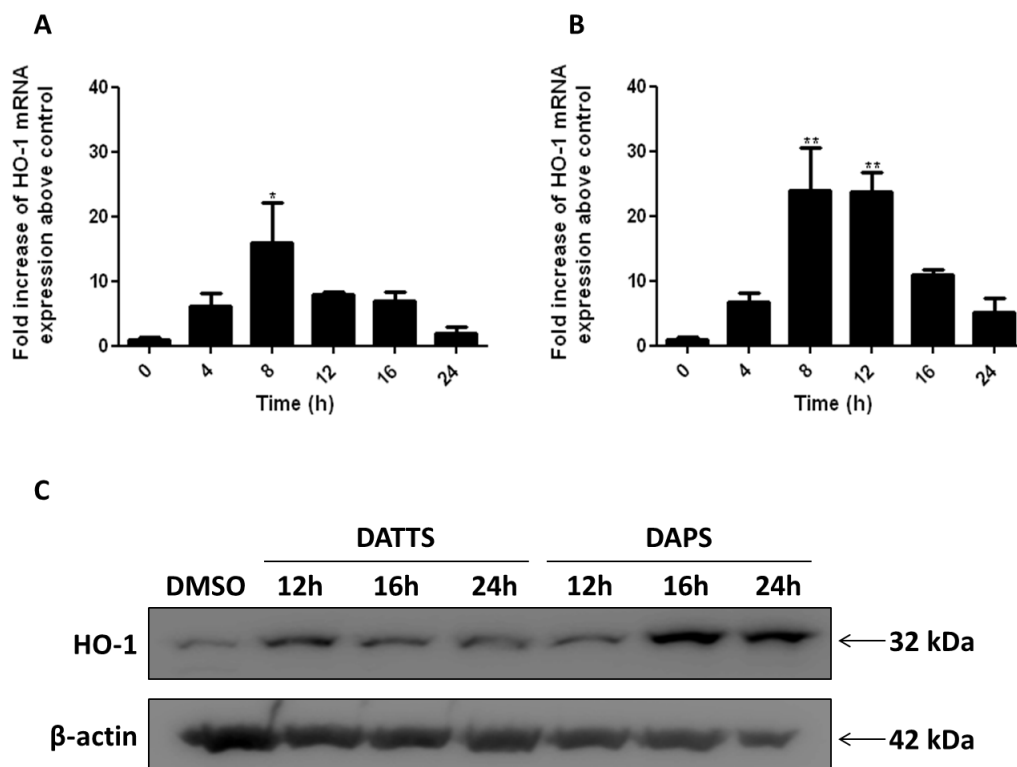


Fig 4.6. DATTS and DAPS-induced HO-1 expression in THP-1 cells. Cells were incubated with 20 μ M DATTS or DAPS for the indicated time points. DMSO was used as a vehicle control in the 0h samples. Total RNA was extracted and HO-1 expression in response to **(A)** DATTS and **(B)** DAPS was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, $n=3$. * $p<0.05$, ** $p<0.01$; One way ANOVA with Tukey's multiple comparison test. **(C)** Whole cell lysates were prepared and HO-1 expression measured by Western blot Analysis. Representative of three independent experiments; replicates are shown in Appendix II.

To test the ability of DAPS to induce HO-1 at lower concentrations, cells were treated for 8h with various concentrations of DAPS ranging from 0.5 μ M – 20 μ M and HO-1 mRNA expression was examined. The lowest concentration of DAPS to induce HO-1 was 5 μ M (Fig 4.7), which gave a 4.2-fold induction over control (not significant; $p>0.05$). The highest concentration tested, 20 μ M, gave the highest induction (61.1-fold; $p<0.001$), which was approximately 4-times more powerful than 10 μ M (not significant; $p>0.05$).

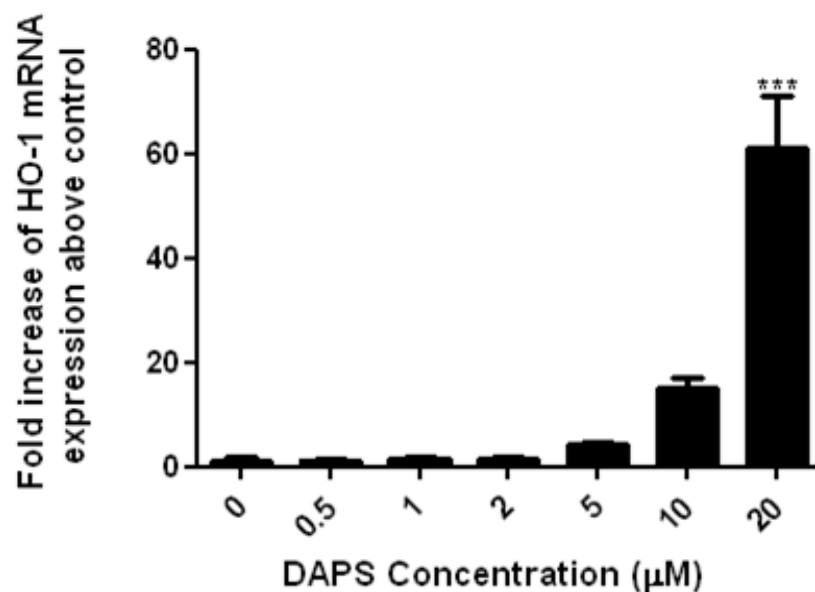


Fig 4.7. DAPS-induced HO-1 mRNA is concentration dependent. THP-1 cells were incubated with 0-20 μ M DAPS for 8h. DMSO was used as a vehicle control in the 0 μ M sample. Total RNA was extracted and HO-1 mRNA expression measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, $n=3$. *** $p<0.001$; One way ANOVA with Tukey's multiple comparison test.

4.3.3 Long chain polysulfides activate the Nrf2 pathway.

To determine if other ARE-driven genes are also upregulated in response to long chain polysulfides in monocytes, THP-1 cells were incubated with either DATTS or DAPS for up to 24h and NQO1 and ferritin H mRNA expression examined. DATTS caused a non-significant induction of NQO1 over control (2.8-fold; $p>0.05$) which peaked between 12 and 16h after stimulation (Fig 4.8A). In contrast, after DAPS stimulation NQO1 expression was high between 8-16h (Fig 4.8B), with the peak induction at 16h (3.7-fold; $p<0.01$). DAPS provoked a more sustained induction than DATTS; NQO1 mRNA levels were still over 2-fold above basal after 24h. Ferritin mRNA expression showed a similar pattern. DATTS caused an induction at 4h (Fig 4.8C), and mRNA levels peak at 16h (3.3 fold over control, $p<0.05$). DAPS caused a more powerful 4.3-fold induction ($p<0.01$) which peaked at 16h (Fig 4.8D). Similar to HO-1 and NQO1, Ferritin H is still raised at 24h in cells stimulated with DAPS, but not with DATTS. Interestingly, both NQO1 and ferritin H mRNA expression were induced later than HO-1 and DAPS caused a higher, more sustained induction than DATTS for all three genes.

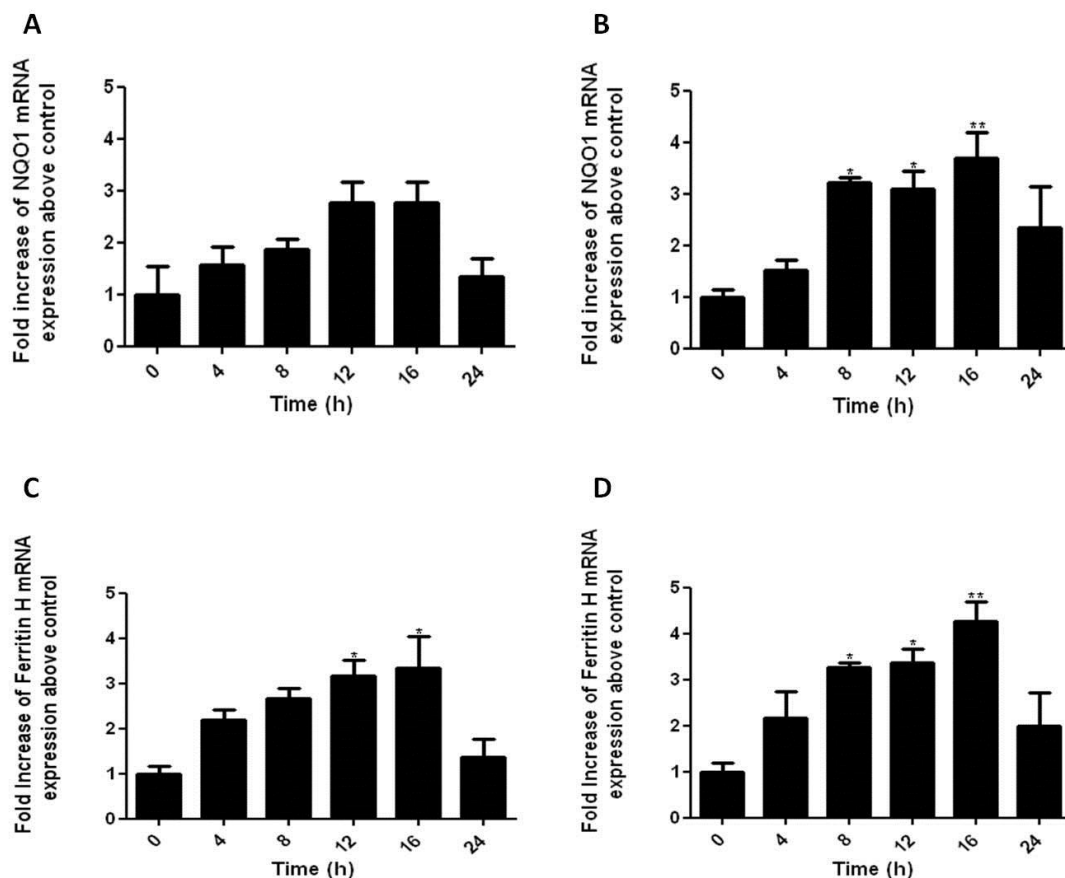


Fig 4.8. Long chain polysulfide induction of NQO1 and Ferritin H mRNA expression. THP-1 cells were stimulated with 20 μ M (A) DATTs or (B) DAPS. After the indicated time period RNA was extracted and NQO1 mRNA expression was measured by RT-qPCR. RNA from cells stimulated with (C) DATTs and (D) DAPS was also analysed for ferritin expression. Results shown are normalised to GAPDH, and show the mean \pm SEM of three independent experiments. * p <0.05, ** p <0.01; One way ANOVA with Tukey's multiple comparison test.

All of the genes tested in this study were chosen because they contain ARE sequences in their promoters (Li and Jaiswal, 1992; Prestera et al., 1995; Iwasaki et al., 2007). To verify that long chain polysulfides activate this promoter region, a luciferase reporter assay was performed. HepG2 cells were transfected with an 8xARE firefly luciferase reporter and a renilla control vector, PGL4. 18h after transfection, cells were stimulated with DAPS for 18h and luciferase activity was measured using the Promega Dual-Glo[®] luciferase assay system. Fig 4.9 shows that DAPS significantly induces ARE-

driven luciferase activity over DMSO ($p < 0.001$), confirming that DAPS induces ARE promoter activity.

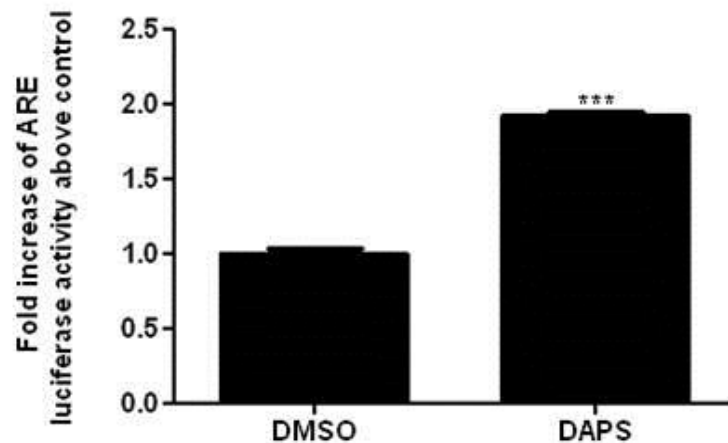
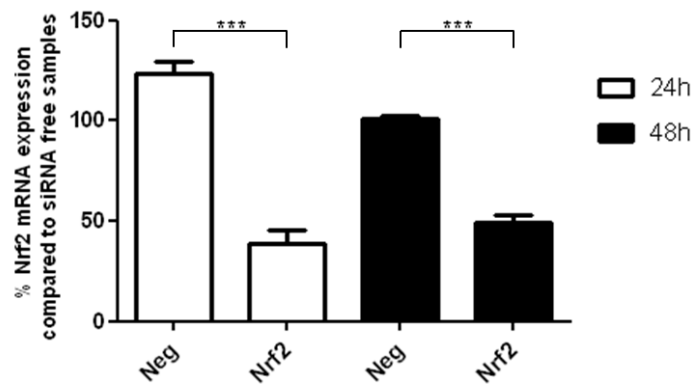


Fig 4.9. DAPS induced ARE luciferase activity in HepG2 cells. HepG2 cells were transfected with ARE-luciferase and PGL4 plasmids for 18h prior to incubation with DAPS for 18h. DMSO was used as a vehicle control. Luminescence was read using the Dual-Glo luciferase assay, and firefly luciferase was normalised to renilla luciferase activity. Mean \pm SEM, $n=3$; *** $p < 0.001$; student's t-test.

Nrf2 is the key regulator of ARE-driven gene transcription (Itoh et al., 1997). Silencing Nrf2, therefore, should inhibit DAPS induced HO-1 expression in THP-1 cells. THP-1 cells were transfected with either Nrf2 or negative control siRNA using a validated protocol (Fig 4.10A). An electroporated THP-1 sample without any siRNA was used as a transfection control. After 24h, cells were

treated with 20 μ M DAPS for 24h and whole cell lysates were harvested and probed for HO-1 expression by Western blot analysis. Nrf2 siRNA suppressed DAPS-induced HO-1 protein expression compared with negative control siRNA (Fig 4.10B).

A



B

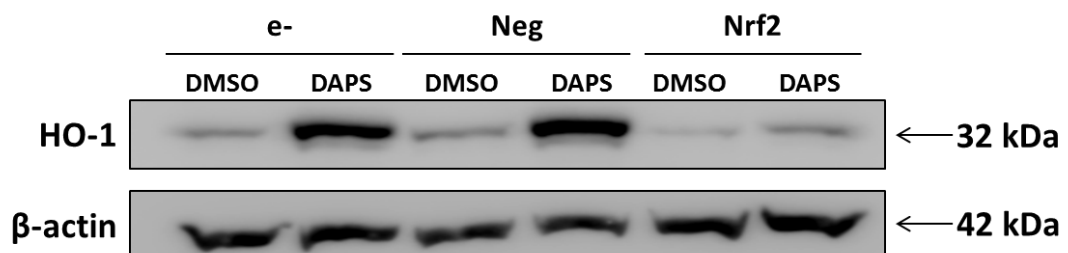


Fig 4.10. Nrf2 siRNA suppresses DAPS-induced HO-1 expression. THP-1 cells were transfected with 200nM Nrf2 siRNA or negative control siRNA. An electroporated sample with no siRNA was used as a control. **(A)** Nrf2 mRNA expression was measured 24 and 48h after transfection by RT-qPCR and normalised to GAPDH. Mean \pm SEM, n=3;***p<0.001; One way ANOVA with Tukey's multiple comparison test. **(B)** After 24h, cells were incubated with 20 μ M DAPS for 24h. DMSO was used as a vehicle control. Cell lysates were collected and HO-1 expression was measured by Western blot. β -actin expression is shown as a loading control. Results are representative of three independent experiments; replicates are shown in Appendix II.

When Nrf2 is activated, its interaction with Keap1 is disrupted, resulting in nuclear accumulation of newly synthesised Nrf2. Therefore, Nrf2 protein expression in DATTS and DAPS-stimulated THP-1 cells was examined. Fig 4.11 demonstrates that Nrf2 accumulated in cells incubated with DATTS or DAPS over the 8h period, with highest expression between 4 and 6 h for both molecules.

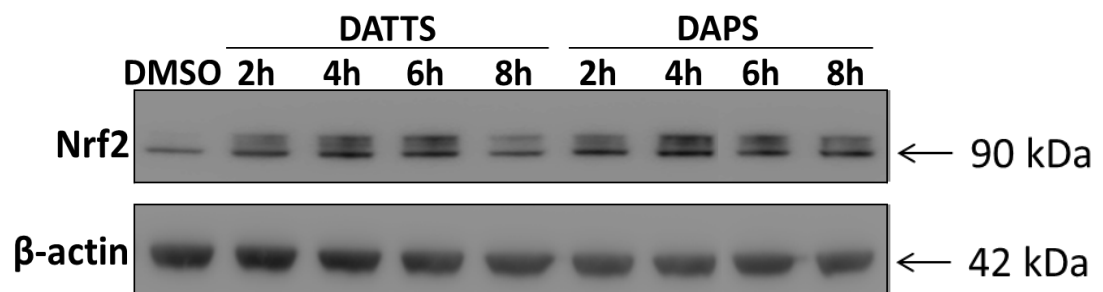
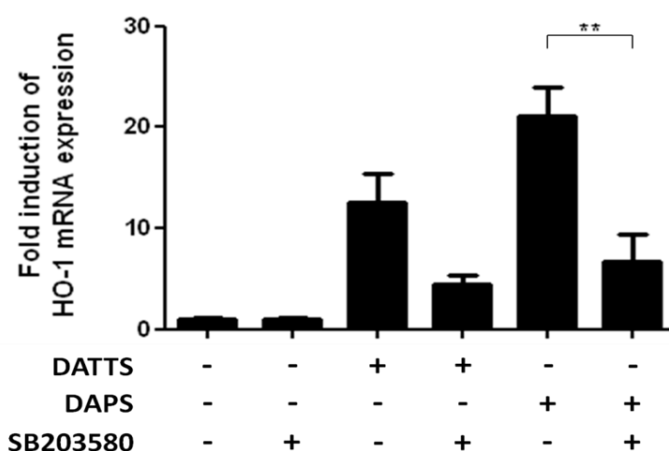


Fig 4.11. DATTS and DAPS induce Nrf2 protein expression in THP-1 cells. THP-1 cells were incubated with 20 μ M DATTS or DAPS for the indicated time period. DMSO was used as a vehicle control. Cell lysates were collected and Nrf2 expression was measured by western blot. β -actin expression is shown as a loading control. Results are representative of three independent experiments; replicates are shown in Appendix II.

4.3.4 Signalling mechanisms upstream of DAPS-induced Nrf2

Short chain diallyl polysulfide compounds have been shown to induce HO-1 via p38 MAP kinase (Gong et al., 2004; Ho et al., 2012). Whether this is a common form of activation for all diallyl polysulfides is unknown. THP-1 cells were pre-incubated with the p38 MAP kinase inhibitor SB203580 for 30min prior to addition of 20 μ M DATTS or DAPS for 8h, and HO-1 mRNA expression measured. SB203580 inhibited HO-1 mRNA expression by both DATTS (64.1%; not significant) and DAPS (67.9%; $p < 0.01$) (Fig 4.12A). To confirm that p38 is part of an Nrf2 dependent signalling mechanism, THP-1 cells were incubated with SB203580 for 30min prior to 20 μ M DAPS for 4h. Cell extracts were probed for Nrf2 protein expression by Western blot. SB203580 blocked DAPS-induced Nrf2 protein expression, suggesting that p38 MAP kinase acts upstream of Nrf2 (Fig 4.12B).

A



B

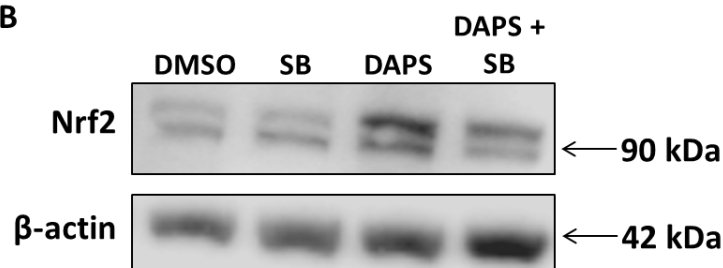


Fig 4.12. p38MAP kinase regulates DATTS and DAPS-induced Nrf2 and HO-1 expression. **(A)** THP-1 cells were incubated with 10 μ M SB203580 for 30min prior to addition of 20 μ M DATTS or DAPS for 8h. In samples lacking DATTS, DAPS, and/or SB203580, DMSO was used as a vehicle control. Total RNA was extracted and HO-1 expression measured by RT-qPCR and normalised to GAPDH. Results show the mean \pm SEM of three independent experiments. **(B)** THP-1 cells were incubated with 10 μ M SB203580 for 30min prior to incubation with DAPS for 4h. Cell lysates were probed for Nrf2 expression by western blot. Data shown is representative of three independent experiments; replicates are shown in Appendix II. ** $p < 0.01$; One way ANOVA with Tukey's multiple comparison test.

ROS are an established mechanism of activating Nrf2 (Numazawa et al., 2003) and diallyl polysulfide compounds have previously been found to induce production of ROS in erythrocytes (Munday et al., 2003). To investigate whether ROS play a role in DAPS-induced Nrf2 activation, THP-1 cells were treated with the ROS scavenger N-acetyl-cysteine (NAC) prior to

DAPS incubation for 16 hr. Analysis of HO-1 protein expression revealed that NAC suppressed DAPS induced HO-1 protein production (Fig 4.13A). Similarly, NAC inhibited Nrf2 expression after 4h DAPS incubation (Fig 4.13B). These results indicate that DAPS induces ROS prior to Nrf2 activation.

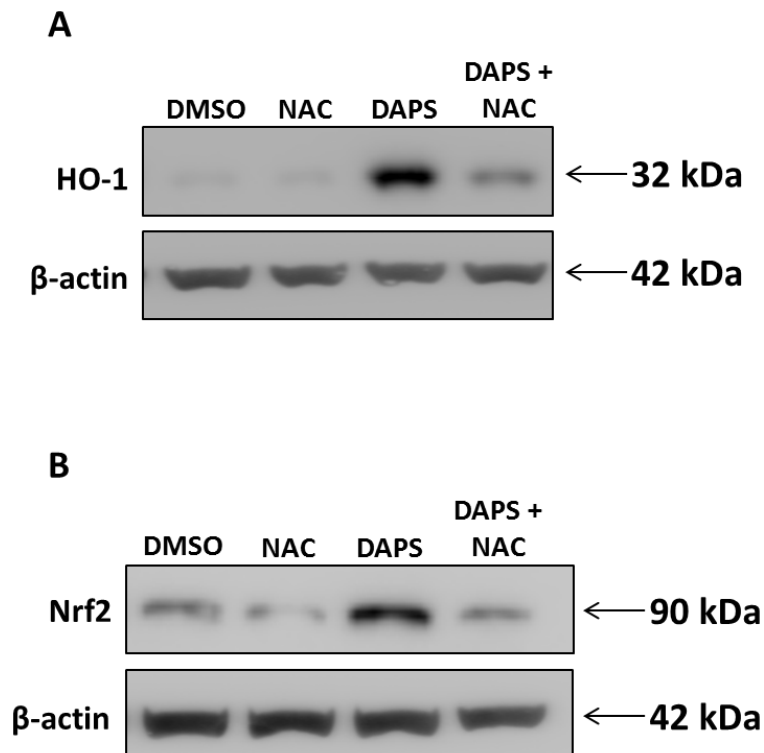


Fig 4.13. DAPS induces Nrf2 via ROS production. (A) THP-1 cells were incubated with 1mM NAC for 30min prior to addition of 20 μ M DAPS for 24h. DMSO was used as a vehicle control. Cell lysates were probed for HO-1 expression by western blot. **(B)** THP-1 cells were incubated with 1mM NAC for 30min prior to incubation with DAPS for 4h. Cell lysates were probed for Nrf2 expression by Western blot. β -actin is shown as a loading control. Data shown is representative of three independent experiments; replicates are shown in Appendix II.

4.3.5 Effect of DAPS on inflammation and cytotoxicity in THP-1 cells

Short length diallyl polysulfides have been shown to downregulate inflammatory mediators (Kalayarasan et al., 2009; You et al., 2013). It is unknown whether long chain polysulfides have the same effect. Therefore, THP-1 cells were incubated with DAPS for either 4h or 20h prior to addition of 100 pg/mL LPS for 2h and TNF mRNA expression was measured. The results (Fig 4.14) show that DAPS had no effect on LPS-induced TNF expression after 4h pre-incubation, and even appears to increase LPS induced TNF after 20h pre-incubation although this is not significant.

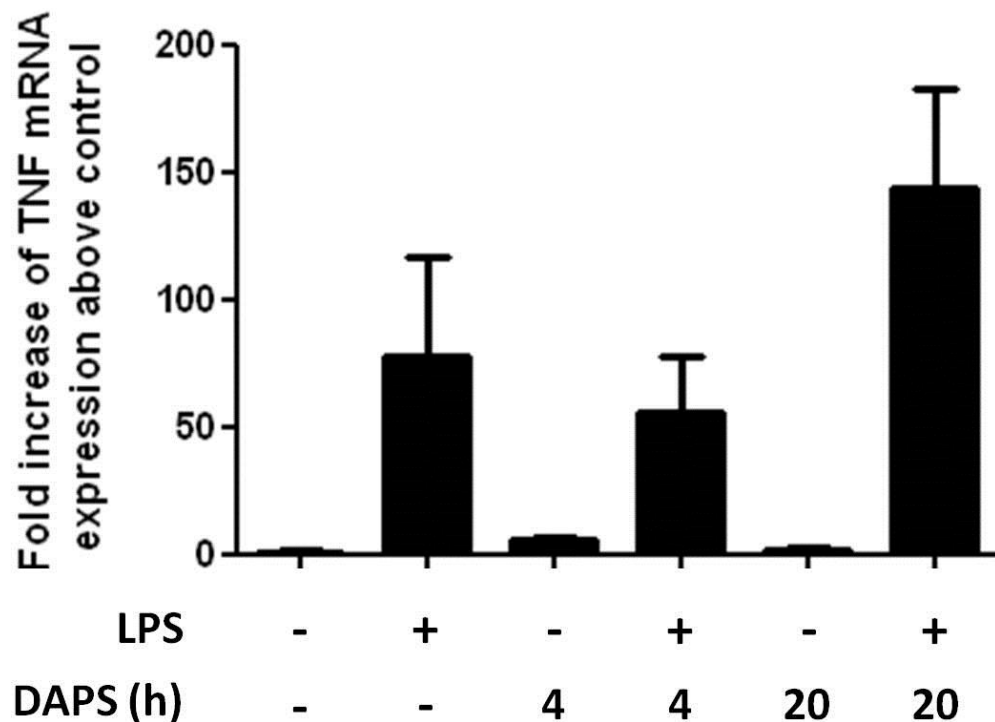


Fig 4.14. DAPS does not inhibit LPS-induced TNF. THP-1 cells were incubated 20 μ M DAPS for 4 or 24h before stimulation with 100 pg/mL LPS. DMSO was used as a vehicle control for DAPS in unstimulated cells. TNF expression was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, n=3; One way ANOVA with Tukey's multiple comparison test.

Nrf2 is known to protect against damage caused by oxidative stress, and lower cellular concentrations of free radicals (Ruiz et al., 2013; Zhang et al., 2013d). DAS and DADS have been shown to protect against excessive hydrogen peroxide induced necrosis (Gong et al., 2004; Koh et al., 2005). DADS has also been shown to protect against H₂O₂-induced genotoxicity (Belloir et al., 2006). The effect of DAPS on H₂O₂ induced cell death was measured by MTS assay. In order to ensure that cytotoxicity rather than proliferation was measured, cells were seeded at a confluent density (0.5 x 10⁴ cells per well) and cell morphology was recorded at the end of the experiment (data not shown). DAPS was unable to prevent H₂O₂-induced cell death in THP-1 cells (Fig 4.15). Interestingly, two-Way ANOVA analysis revealed that although the differences between the cytotoxicity of THP-1 cells with and without DAPS at each concentration of H₂O₂ are not significant, when the datasets were treated as a whole, DAPS exacerbated H₂O₂-induced cell death (p<0.05).

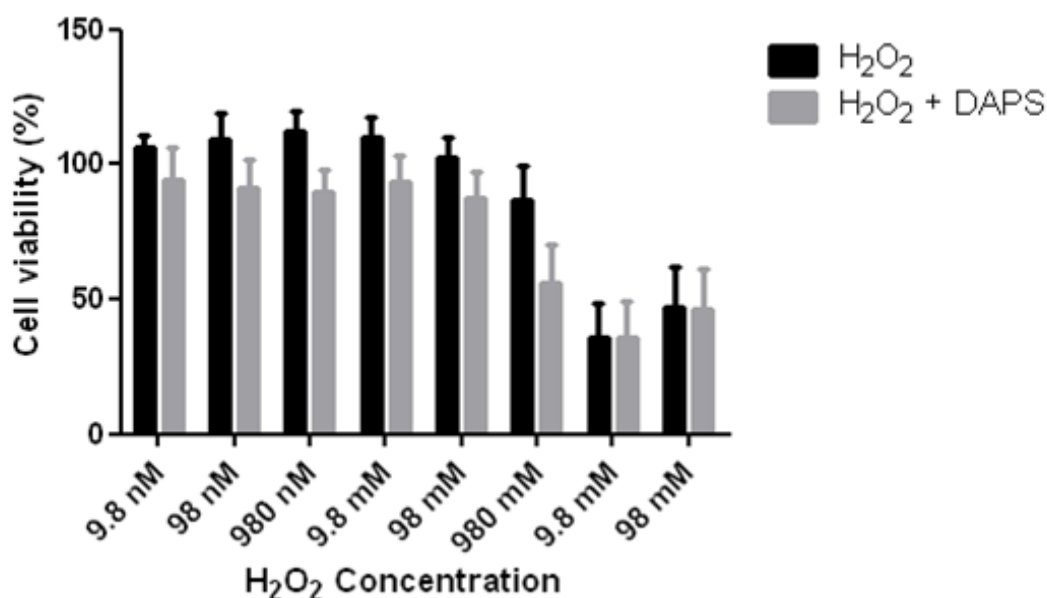


Fig 4.15. DAPS does not protect THP-1 cells from hydrogen peroxide-induced toxicity. 20 μ M DAPS or DMSO was added to THP-1 cells for 4h before addition of H₂O₂ at various concentrations for 24h. MTS was added to the cells and absorbance readings were taken at 492nm to calculate cell viability. Mean \pm SEM, n=3; Two way ANOVA with Bonferroni post test.

4.4 Discussion

Garlic has been linked with numerous health benefits (Warshafsky et al., 1993; Durak et al., 2004; Zeb et al., 2012; Ried et al., 2013). Some evidence suggests that diallyl polysulfides, a major component of crushed garlic and garlic oils, may be responsible for these effects by activating the Nrf2 pathway (Kalayarasan et al., 2009; Shin et al., 2013). This study focused on the effects of garlic oil and long chain diallyl polysulfides in THP-1 human monocytes. For the first time, it was shown that diallyl polysulfides activate the Nrf2 pathway in human monocytic cells. DAPS was identified as a novel activator of the Nrf2 pathway, which is much more effective than shorter diallyl polysulfides. However, the increased activation of the Nrf2 pathway did not inhibit LPS-induced TNF expression, or improve cell viability after incubation with H₂O₂.

Garlic has been associated with activation of the Nrf2 pathway *in vitro* and *in vivo*. Aged red garlic extract induces HO-1 and Nrf2 in RAW 264.7 cells, and inhibits LPS-induced COX-2 expression in mouse lung while inducing HO-1 expression (Park et al., 2012), and garlic reduces oxidative stress in Wistar rats (Kalayarasan et al., 2008). In contrast, Fisher and co-workers (2007) report that garlic oil does not activate the Nrf2 pathway, although the conclusions of this study are debatable because although garlic oil induced Nrf2 and ARE gene products are not statistically significantly increased, they are generally higher than in untreated cells. In this project two independently sourced garlic oils were added to THP-1 human monocytic cells. Both garlic oils induced ARE-driven genes HO-1 and NQO1 and their primary regulator, Nrf2. HO-1 was far more powerfully activated by the garlic oils than NQO1. This may be due to activation via different pathways that act synergistically to induce HO-1 but not NQO1, but it has been shown that Nrf2-induced HO-1 can be more powerfully increased than other ARE-driven genes (Rushworth

et al., 2006; Donovan et al., 2012), including by a specific Nrf2 activating peptide detailed in chapter 5 of this thesis. In this study it is explainable by the fact that the raw PCR data shows that basal levels of HO-1 are much lower than NQO1 in THP-1 cells, so while the fold induction of HO-1 higher, the actual production of both genes' mRNA is very similar after incubation with the garlic oils.

This data is consistent with the consensus that garlic oils do activate the Nrf2 pathway. Interestingly Stringer, the garlic oil containing a higher total percentage of diallyl polysulfides, was more effective than Naturex in every experiment. The rest of this study focused on the effects of these diallyl polysulfides on the Nrf2 pathway in monocytes.

Diallyl polysulfides are thought to be a major biologically active component of aged garlic extract and garlic oils. Many studies have noted the effects of diallyl sulfides on ARE dependent gene expression (Chen et al., 2004; Ho et al., 2012; Saidu et al., 2013), but none have done so in monocytes. This study identifies DATTS and DAPS as powerful inducers of the Nrf2 pathway in monocytes. Both were shown to induce Nrf2 protein expression and NQO1, Ferritin H, and HO-1 mRNA expression. A role for Nrf2 in the upregulation of some of these genes was confirmed by the upregulation of ARE-luciferase by DAPS, and inhibition of DAPS-induced HO-1 by Nrf2 siRNA. The most interesting aspect of these compounds was that comparing different diallyl polysulfides revealed a clear relationship between chain length and the size of HO-1 induction. In particular DAPS was over twice as powerful as DATTS. DAPS was also shown to be a more powerful inducer of NQO1 and ferritin than DATTS. This pattern is consistent with work showing that DATS is a more powerful activator of HO-1 and NQO1 mRNA than DADS and DAS in HepG2 cells (Chen et al., 2004), but there are other studies showing the opposite. Fisher and co-workers (2007) found that DAS was an effective activator of

NQO1 in WKY rat liver and ARE-luciferase activity in C57BL6/J mice, but DADS and DATS were ineffective. The differences in effect may be due to the species used, or because Fisher and co-workers carried out their experiments *in vivo*. However, it should be noted that in the latter study the dose of DAS administered to the rats and mice was 3.75 times higher than DADS or DATS, and was also measured in mg/ kg body weight, which does not give a per molecule comparison of activity. In the present study, and that carried out by Chen and co-workers (2004), the diallyl polysulfide compounds were directly compared using equivalent molar concentrations.

One notable advantage of this study is that it uses low concentrations of diallyl polysulfide compounds compared to some others, which have used concentrations greater than 100 μ M (Chen et al., 2004; Saidu et al., 2013; You et al., 2013). In this study DAPS induced HO-1 expression at concentrations as low as 5 μ M, which is important because long chain polysulfides are not as prevalent in garlic as short chain polysulfides. It is unknown why the long chain polysulfides are so effective, but there are a number of possibilities, and the most likely hypotheses involve ROS production, an established mechanism of Nrf2 activation (Numazawa et al., 2003; Li et al., 2014b), although other signalling mechanisms can't be discounted.

ROS are known to induce Nrf2 and p38 activation (Peus et al., 1999; Numazawa et al., 2003). Furthermore, diallyl polysulfides are inducers of ROS (Na et al., 2012; Chandra-Kuntal et al., 2013), and have been shown to induce hydrogen peroxide production in a manner that revealed a positive correlation between activity and polysulfide chain length in rat erythrocytes (Munday et al., 2003). NAC, a ROS scavenger blocks Nrf2 induction by DAS (Gong et al., 2004), DATS (Chen et al., 2004), and DATTS (Saidu et al., 2013), suggesting that this is a general mechanism by which diallyl polysulfides activate Nrf2. In this study NAC blocked DAPS-induced Nrf2 and HO-1

expression, showing that ROS act upstream of Nrf2. ROS induction by diallyl polysulfides is thought to be induced by metabolism of the parent compounds (reviewed in Munchberg et al., 2007). Therefore, ROS production is very likely to be a source of variation in the activity of the compounds, because the metabolism is known to differ for longer polysulfides.

ROS production likely occurs due to the creation of hydropersulfide (RSSH) and hydropolysulfides (RS_xH ; $x > 2$) when diallyl polysulfides containing three or more sulfurs react with a thiol (for example, GSH). RS_xH ($x \geq 2$) can set off a chain of reactions with transition metal ions and O_2 to produce $O_2^{\bullet-}$. Furthermore, a perthiyl radical (RS_x^{\bullet}) is produced (Chatterji et al., 2005). These can react with GSH to initiate a chain of reactions that not only produce $O_2^{\bullet-}$ but also recycle a polysulfide molecule. Longer diallyl polysulfides may produce longer hydropolysulfides, which are considered more reactive (Munchberg et al., 2007), although production of hydropersulfides is considered more likely (Munday et al., 2003). Diallyl polysulfides with ≥ 4 sulfurs are also susceptible to homolytic bond cleavage, which produces yet more perthiyl radicals.

If long chain polysulfides produce more perthiyl radicals and longer hydropolysulfides it is very likely that they produce more ROS, and therefore higher Nrf2 activation than short chain polysulfides. This has yet to be determined experimentally, however.

Another possible mechanism which cannot be ignored is post-translational modification of the sulfurs in cysteine residues of Keap1. Keap1 is regulated by cysteine residues, which can be modified to prevent Nrf2 degradation (Zhang and Hannink, 2003; Wakabayashi et al., 2004; Wu et al., 2010; Hu et al., 2011). Diallyl polysulfides have been shown to modify cysteines in transient receptor potential cation channel, A1 (TRPA1) and β -tubulin (Hosono et al., 2005; Hinman et al., 2006). Recently Diallyl trisulfide has been

shown to modify cysteine residues in Keap1 (Kim et al., 2014). This reaction would likely produce a hydropolysulfide, which would cause ROS production. This mechanism of biological activity is considered an oversimplification of the complex chemistry capable by diallyl polysulfides (Munchberg et al., 2007) but cannot be ruled out as a potential source of ROS production. This is almost certainly not the primary mechanism, however, because the ROS scavenger NAC and the p38 inhibitor SB203580 inhibited DAPS induced Nrf2 expression. Instead, this suggests that there are signalling intermediates between the diallyl polysulfides and Keap1 cysteine modification.

p38 is activated upstream of Nrf2 activation by a number of small molecules, including quercetin (Yao et al., 2007) and procyanidins (Bak et al., 2012). Importantly, p38 has also been shown to act upstream of Nrf2 in response to DAS in MRC-5 cells (Ho et al., 2012) and partially in HepG2 cells. This suggests that diallyl polysulfide compounds could act via a common pathway, although this hypothesis is challenged by Chen and co-workers (2004) who found that SB203580 did not affect DAPS induced ARE-luciferase activity in HepG2 cells.

It is apparent that many Nrf2 activators cause therapeutic effects via a negative feedback mechanism. The activator induces oxidative stress which activates the Nrf2 pathway. Nrf2 and its target genes then remove the stress caused by the inducer, and also provide other therapeutic effects that are required. This approach appears to work. Sulforaphane has anti-inflammatory properties (Zhao et al., 2013b) and has been shown to activate Nrf2 via ROS production (Lee and Lee, 2011; Lee et al., 2012a). Quercetin and tBHQ have both been reported to induce oxidative stress (Lapidot et al., 2002; Imhoff and Hansen, 2010) but are widely accepted to protect against oxidative stress (Eftekharzadeh et al., 2010; Ramyaa and Padma, 2013). Whether DAPS has a therapeutic effect is unknown. Short chain polysulfides

have all been shown to dampen inflammation. DAS inhibits gentamicin-induced iNOS and TNF in Wistar rats (Kalayarasan et al., 2009), DADS inhibits LPS induced IL-1 β and IL-6 in RAW264.7 cells (Shin et al., 2013), and DATS has been shown to inhibit LPS-induced I κ B α phosphorylation and degradation, which leads to an inhibition of a number of pro-inflammatory mediators including TNF, IL-6 and MCP-1 in RAW 264.7 cells (You et al., 2013).

In this study DAPS had no effect on LPS-induced TNF production and hydrogen peroxide induced cytotoxicity in THP-1 cells. This suggests that in this case Nrf2 activation caused by DAPS is insufficient to cause protection. This is an interesting result because clearly an increase in Nrf2 activation is not necessarily a guarantee of protective activity. It is possible that there is an optimal chain length that causes the highest ratio of Nrf2 activation to the stress required to achieve it. A lot of work has documented anti-cancer effects of diallyl polysulfides and while they protect against apoptosis via Nrf2 in some situations (Tsai et al., 2013b), they are also cytotoxic in others (Choi and Park, 2012; Chandra-Kuntal et al., 2013; Shin et al., 2013a). It may be that long chain polysulfides are more cytotoxic than short chain polysulfides, and it is entirely possible that despite the powerful effect on Nrf2 activation caused by long chain polysulfides, they are more suited for stress-inducing therapeutics such as anti-cancer therapy. Future work should focus on this relationship between chain length and activity because it is clearly more complex than the relationship seen with Nrf2 activation suggests. It is also very possible that DAPS has potential protective effects not investigated in this study, or that monocytes are a poor target for polysulfides. Finally, it is very interesting that almost all studies revealing an inhibitory effect of diallyl polysulfides on inflammatory mediators are conducted on mice and cell lines. Only a single study, which demonstrates an inhibition of COX-2 induced by IL-1 β or monosodium urate crystals in OA

synovial cells and arterial chondrocytes, was conducted on human cells (Lee et al., 2009b).

In conclusion, garlic oils mainly composed of diallyl polysulfides activate the Nrf2 pathway in THP-1 cells. Comparing the diallyl polysulfides directly revealed that longer chain length resulted in a stronger induction of HO-1. DAPS is identified as a novel Nrf2 inducer, which signals via ROS and p38. However, it was unable to protect against LPS induced TNF or hydrogen peroxide induced cytotoxicity revealing a need to further investigate the relationship between polysulfide chain length and protective activity.

Chapter 5: Characterisation of the Nrf2/Keap1 interaction as a drug target for inflammatory disease using a Neh2 peptide mimetic

Acknowledgements:

Richard Steel synthesised the peptides, and carried out the experiments described in figs 5.2 and 5.6.

5.1 Introduction

Nrf2 is regulated by Keap1, an adaptor protein of the E3 ubiquitin ligase complex. When Nrf2 is bound to Keap1 it is targeted for degradation; if it is not continually degraded, it translocates to the nucleus to initiate transcription of its target genes (Cullinan et al., 2004). Nrf2 activation has been shown to inhibit pro-inflammatory mediators *in vivo* and *in vitro*, and relieve symptoms of inflammatory diseases in animal models, making it a promising therapeutic target (Rushworth et al., 2008; Kudoh et al., 2014). Indeed, bardoxolone methyl has recently been used in clinical trials for stage 4 chronic kidney disease (de Zeeuw et al., 2013). The trial was stopped due to patient deaths, which is thought to be caused by increased fluid retention, leading to increased blood pressure and cardiovascular complications (Chin et al. 2014). These effects have not been attributed to Nrf2, and may be due to off-target effects of bardoxolone methyl. Most activators of the Nrf2 pathway tend to do so via modification of cysteine residues in Keap1 and/or phosphorylation of Nrf2, but mechanistically this is not specific and can affect other proteins (Niture et al., 2009; Holland and Fishbein, 2010; Magesh et al., 2012; Levonen et al., 2014; Wall et al., 2014). Therefore, inhibiting the Nrf2/Keap1 interaction using a competitive inhibitor may reduce side effects, and therefore lethality, of Nrf2-targeted therapeutics.

The sequences of Nrf2 that bind Keap1 are highly conserved. One molecule of Nrf2 binds a Keap1 homodimer using two sequences in the Neh2 domain. One binding sequence is a strong binding ETGE motif, whereas the other is a weaker binding DLG motif. Both use the same binding site in the DGR region of Keap1's Kelch domain (Tong et al., 2006).

Designing a small molecule to inhibit the Nrf2/Keap1 interaction is complex, even before time and cost are even considered. The binding site is large, polar and in a recent screen of 337,116 compounds *in vitro*, only two were shown to inhibit Nrf2 binding with an IC50 value of <2 μ M, and one of those is a predicted Michael acceptor (Wang et al., 2012a). A simple approach to validate the Nrf2/Keap1 interaction as a drug target before the academic field becomes too invested in the problem would be to use a peptide mimetic. The effect of peptides on the Nrf2/Keap1 interaction has been investigated in a chitin pull-down assay. Sequences of 10, 14, or 16 amino acids long (Table 5.1) were added to samples containing isolated Keap1/Nrf2. The 16mer and 14mer displaced Nrf2 much more strongly than the 10mer (Lo et al., 2006), suggesting that they may act as effective activators of the Nrf2 pathway.

Peptide	Sequence
10mer	LDEETGEFLP
14mer	LQLDEETGEFLPIQ
16mer	AFFAQLQLDEETGEFL
14mer – Scrambled	EFGTDIQLLEPQLE
TAT	YGRKKRRQRRR

Table 5.1 Keap1-targeting peptide sequences. For TAT-conjugated peptides, the TAT sequence is bound to the N-terminus.

Peptides are too large to diffuse across the cell membrane. However, specific peptide sequences can activate membrane transport mechanisms in order to shuttle large molecules in or out of the cell. One such mechanism is that used by the HIV protein Tat (transcription-transactivating protein). This protein contains an 11 residue sequence that causes cell penetration by endocytosis (Deshayes et al., 2005). By adjoining this TAT sequence to the Neh2 peptides, they should enter the cell by the same mechanism. The design of a cell-penetrating peptide targeted to the Nrf2/Keap1 interaction will not only validate Nrf2 as a drug target, but will also be a valuable molecular biology tools to study Nrf2 activation. This study investigates cell-penetrating Neh2 mimetic peptides in THP-1 monocytes.

5.2 Aims

Hypothesis: Peptides mimicking the Keap1 binding sequence of the Neh2 domain induce expression of Nrf2 and its target genes, and inhibit LPS-induced cytokine production.

Aims:

- To verify that mimetic peptides matching the Keap1 binding sequence of the Neh2 domain of Nrf2 are biologically active.
- To find the optimum peptide length for biological activity.
- To provide a tool to activate Nrf2 with minimal non-specific activity.
- To validate the Nrf2-Keap1 interaction as a potential drug target.

5.3 Results

5.3.1 TAT-14 is a cell penetrating peptide that binds to Keap1

To investigate the effects of Neh2 mimetic peptides on the Nrf2 pathway in THP-1 cells, they were conjugated to the TAT cell penetration sequence and tested for induction of HO-1 mRNA. HO-1 is a well characterised Nrf2 target gene, which can be induced by Nrf2 activation in THP-1 cells, and RT-qPCR can quantify the differences between the peptides (Rushworth et al., 2008). THP-1 cells were incubated with 75 μ M TAT-10, TAT-14, or TAT-16 for either 3h or 6h. Total RNA was extracted and measured for HO-1 expression by qRT-PCR. Of the three peptides, the most active was TAT-14 (Fig 5.1A), causing a rapid induction of HO-1 mRNA as early as 3h (3.5 fold; $p < 0.01$), and a peak 20.3 fold induction at 6h ($p < 0.01$). TAT-16 also induced HO-1 expression, which was also highest at 6h. However, TAT-16 only induced HO-1 mRNA by 6.3 fold ($p < 0.001$), indicating that TAT-14 was three times more powerful ($p < 0.001$). TAT-10 did show mild activity, but was by far the least active of the peptides. TAT-10 was the only peptide to have no effect at 3h, and showed a modest 2.6 fold induction at 6h (Not significant; $p > 0.05$).

Due to the fact that the 16mer has been previously shown to be the strongest disruptor of the Nrf2/Keap1 interaction (Lo et al., 2006), THP-1 cells were incubated with TAT-16 for an extended time course to find out whether it acts more slowly than TAT-14. The results show that TAT-16 was not as effective as TAT-14 at any time point (Fig 5.1B). The most active peptide, TAT-14, was taken forward for further experiments.

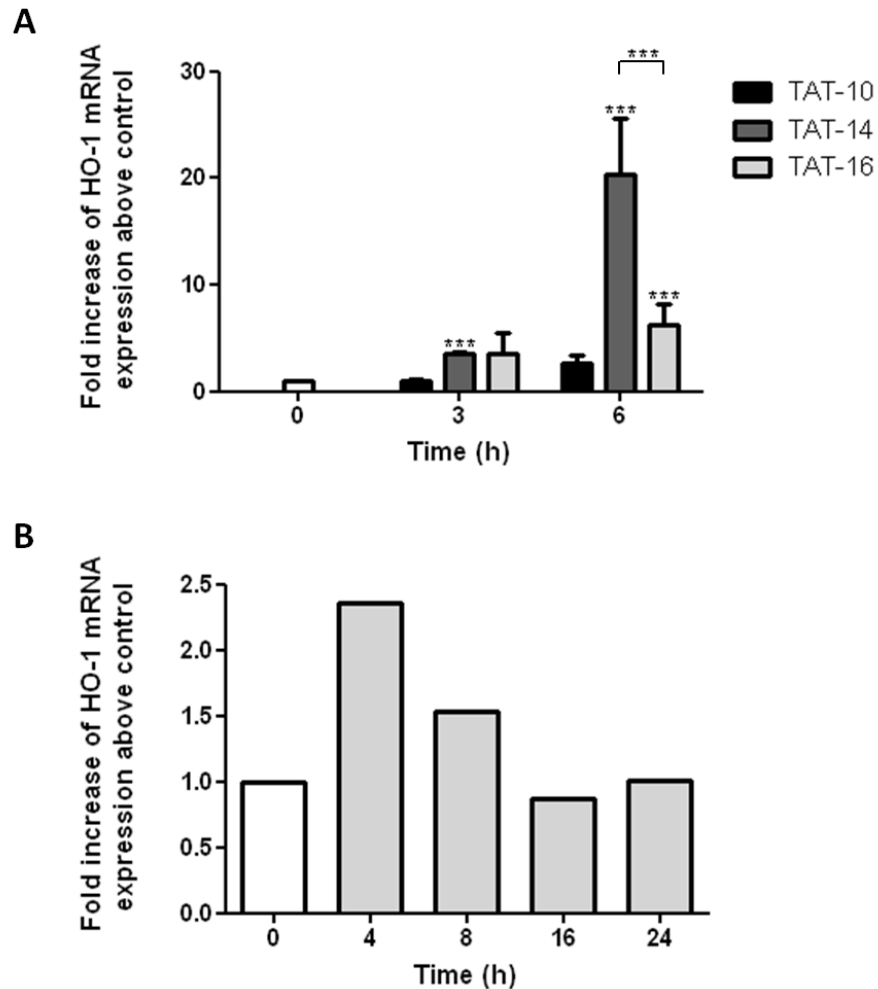


Fig 5.1 TAT-14 is the most active disruptor of the Nrf2/Keap1 interaction. (A) 75 μ M TAT-10, TAT-14, and TAT-16 were incubated with THP-1 cells for 3 or 6h. (B) THP-1 cells were incubated with 75 μ M TAT-16 for up to 24h. PBS was used as a vehicle control. HO-1 mRNA was measured by RT-qPCR. Mean \pm SEM, n=3 (A) or n=1 (B); ***p<0.001; One way ANOVA with a Tukey's multiple comparison test.

The interaction between two molecules of different sizes can be measured *in vitro* by fluorescence polarization. To verify that TAT-14 and 14mer can bind to Keap1, they were added to a solution containing Keap1, and a fluorescent 14mer (F-14). Measuring the fluorescence polarization revealed that both peptides were able to displace F-14, showing that they are capable of binding Keap1 (Fig 5.2). K_i was calculated for both peptides, which revealed that TAT-14 bound Keap1 more strongly than the 14mer ($K_i = 3.628$ and 12.24 nM respectively).

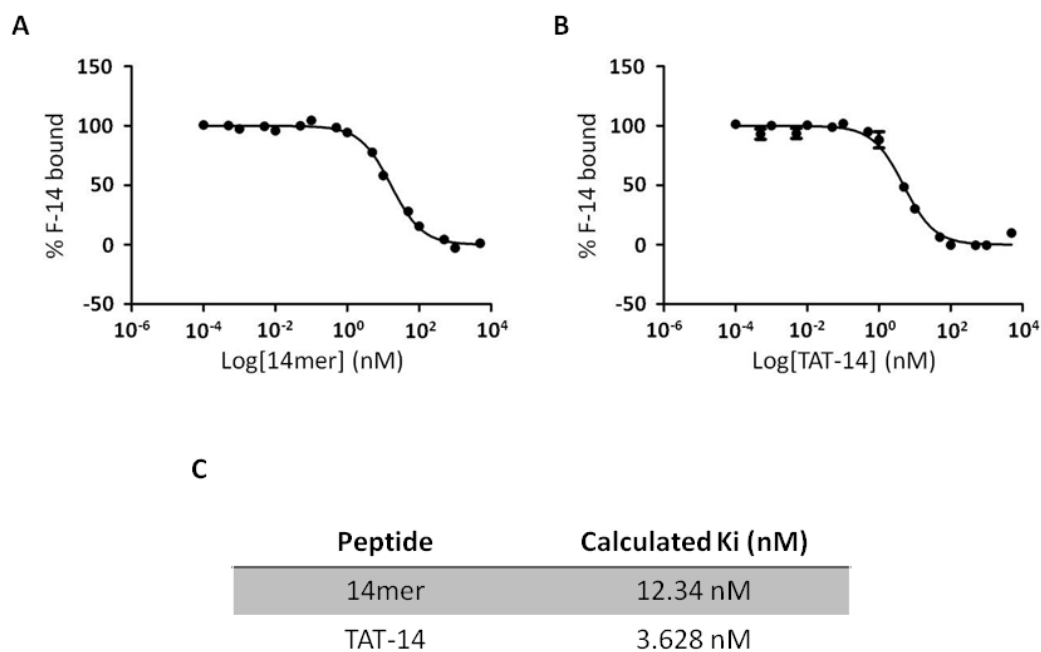


Fig 5.2 14mer and TAT-14 both bind Keap1. Varying concentrations of the **(A)** 14mer or **(B)** TAT-14 was added to a complex of Keap1 and F-14. Fluorescence polarization was recorded as a measure of Keap1 binding. K_i was calculated using non-linear regression **(C)**. Results show $n=3$.

5.3.2 TAT-14 activates the Nrf2 pathway

Fig 5.1 and 5.2 suggest that TAT-14 induces HO-1 by binding Keap1. In order to verify that this is a specific mechanism, a control peptide was created. The control peptide, TAT-sc, consists of the TAT cell penetration sequence linked to a scrambled arrangement of the 14mer peptide and therefore this peptide should enter the cell, but not bind Keap1. The ability of the peptides to disrupt the Nrf2/Keap1 interaction in THP-1 cells can be shown by measuring Nrf2 protein expression because the Nrf2/Keap1 interaction is required to prevent accumulation of Nrf2. THP-1 cells were incubated with 75 μ M TAT-14 or TAT-sc for 1, 3 or 6h. Cell lysates were prepared and probed for Nrf2

expression by Western immunoblotting. TAT-14 induced Nrf2 by 1h, which peaked at 3h and returned to basal levels by 6h (Fig 5.3). In contrast TAT-sc did not induce Nrf2 protein. This suggests that the induction of Nrf2 is due to the specific sequence of the 14mer, rather than a non-specific stress response caused by the addition of a cell penetrating peptide.

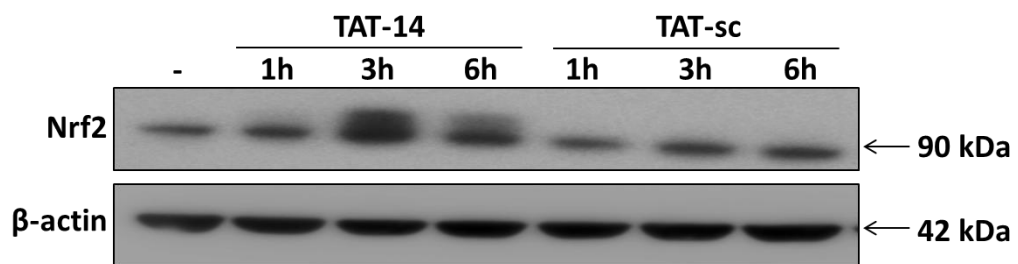


Fig 5.3 TAT-14 induces Nrf2 protein expression. THP-1 cells were incubated with 75 μ M TAT-14 or TAT-sc for 1, 3, or 6h. Nrf2 expression in cell lysates was measured by Western immunoblotting. β -actin was used as a loading control. Data shown is representative of three independent experiments; replicates are shown in Appendix II.

Due to the fact that Nrf2 protein peaked at 3h, and the time delay between the peaks in Nrf2 protein and HO-1 mRNA can be as much as 9h (Park et al., 2011b), a more thorough time course was conducted for HO-1 mRNA expression. TAT-sc was used as a control for Nrf2 independent HO-1 expression. The 14mer without TAT was also added to show whether the peptide was able to penetrate the cell membrane alone.

THP-1 cells were incubated with each peptide for 2, 4, 6, or 8h and HO-1 mRNA was measured by qRT-PCR. TAT-14 powerfully induced HO-1 mRNA expression (Fig. 5.4) with a rapid 5-fold induction at 2h ($p < 0.001$) and peaked at 6h with a 23.9-fold induction ($P < 0.001$). HO-1 levels decreased again at 8h (11.7-fold; $p = 0.01$). Although TAT-sc caused a small induction at 6h, it was not significant (2.9 fold; $p > 0.05$), and was 8 fold less effective than TAT-14

($p < 0.001$). This indicates that the induction of HO-1 by TAT-14 is sequence specific. The 14mer did not induce HO-1 at all at any time point, revealing that the TAT sequence is necessary for activity.

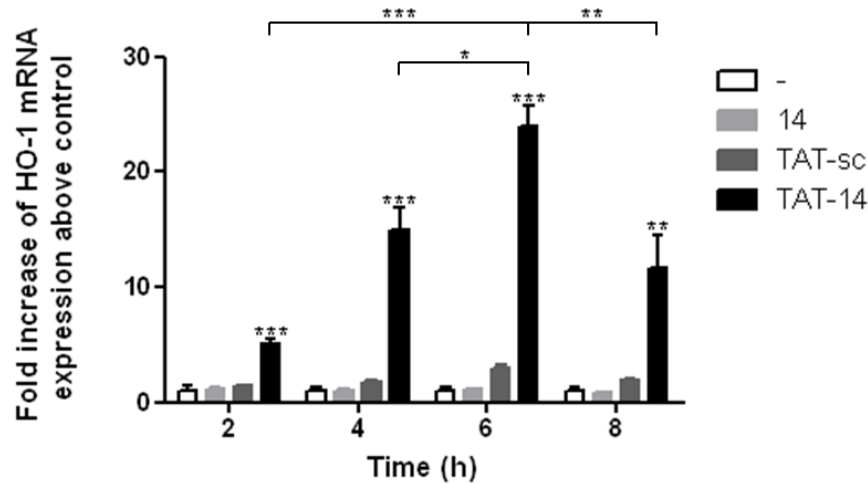


Fig 5.4 TAT-14 induced HO-1 mRNA expression peaks at 6h. THP-1 cells were incubated with 75 μM of the indicated peptide for up to 8h. PBS was used as a vehicle control. HO-1 mRNA expression was measured by RT-qPCR. Mean \pm SEM, $n=3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; One way ANOVA with Tukey's multiple comparison test.

To test whether the increased HO-1 mRNA translated into protein, THP-1 cells were incubated with either TAT-14 or TAT-sc. Cell lysates were prepared and probed for HO-1 protein expression by Western immunoblotting. Incubation with TAT-14 caused an increase in HO-1 protein expression at 9h, which peaked at 12h (Fig 5.5). HO-1 protein returned to basal levels by 16h. TAT-sc did not induce HO-1 protein at any time point tested.

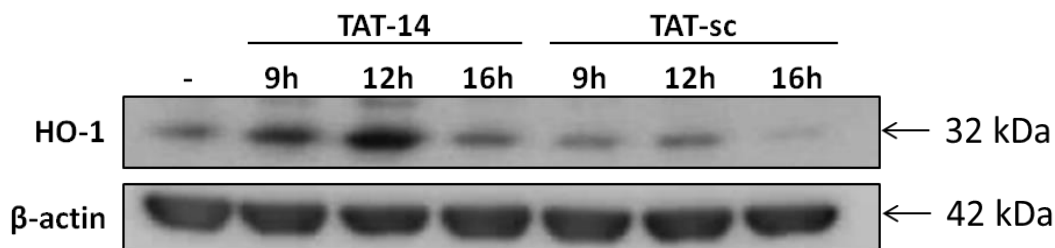


Fig 5.5 TAT-14 induces HO-1 protein expression. THP-1 cells were incubated with TAT-14 or TAT-sc for 9, 12, or 16h. HO-1 expression in cell lysates was measured by Western immunoblotting. β -actin was used as a loading control. Data shown is representative of three independent experiments; replicates are shown in Appendix II.

TAT-14 is effective at $75\mu\text{M}$, but compounds effective at lower concentrations are advantageous because they are cheaper to use, and are less likely to cause side effects. Therefore, TAT-14 was tested at a range of concentrations. THP-1 cells were incubated for 6h with $7.5\text{-}75\mu\text{M}$ TAT-14. HO-1 expression was measured by qRT-PCR.

$75\mu\text{M}$ TAT-14 was disproportionately more powerful than the lower concentrations (Fig 5.6). With a 32.5-fold induction over control ($p < 0.05$), it is 4.4 fold more powerful than $37.5\mu\text{M}$ despite only having double the number of peptide molecules, although the difference between the two is not statistically significant ($p > 0.05$). The lowest concentration of TAT-14 that induced HO-1 is $18.75\mu\text{M}$, which gave a 2.3-fold induction over control cells (not significant; $p > 0.05$).

Due to its effectiveness at $75\mu\text{M}$, TAT-14 was used at this concentration for the remainder of the project.

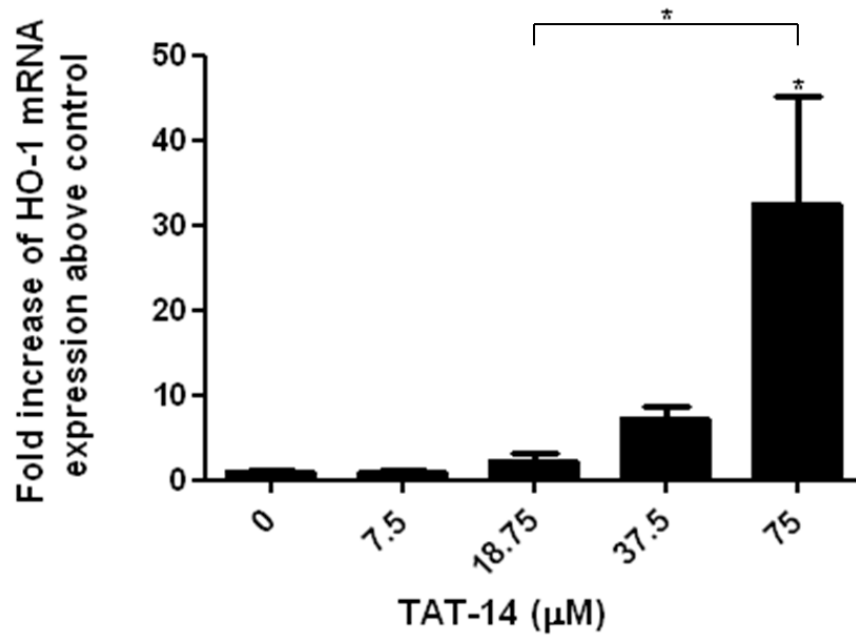


Fig 5.6 TAT-14 activates HO-1 mRNA in a concentration dependent manner. THP-1 cells were incubated with varying concentrations of TAT-14 for 6h. HO-1 expression was measured by RT-qPCR. Mean±SEM, n=3; *p<0.05; One way ANOVA with Tukey's multiple comparison test.

HO-1 is one of many Nrf2 driven genes. Other Nrf2-driven genes NQO1 and ferritin are also well documented to be responsible for cytoprotective effects (Kleszczynski et al., 2013; Liang et al., 2013; Versellotti et al., 2014). The effects of TAT-14 and TAT-sc on NQO1 and Ferritin H mRNA expression were tested by incubating THP-1 cells with 75μM of each peptide for up to 8h. Following TAT-sc incubation neither gene was significantly induced, whereas after incubation with TAT-14 both NQO1 and Ferritin H mRNA levels were significantly increased over control with a 5.5-fold (P<0.01) and 3.2-fold (p<0.001) induction respectively (Fig 5.7). These results show that TAT-14 activates multiple Nrf2-driven genes.

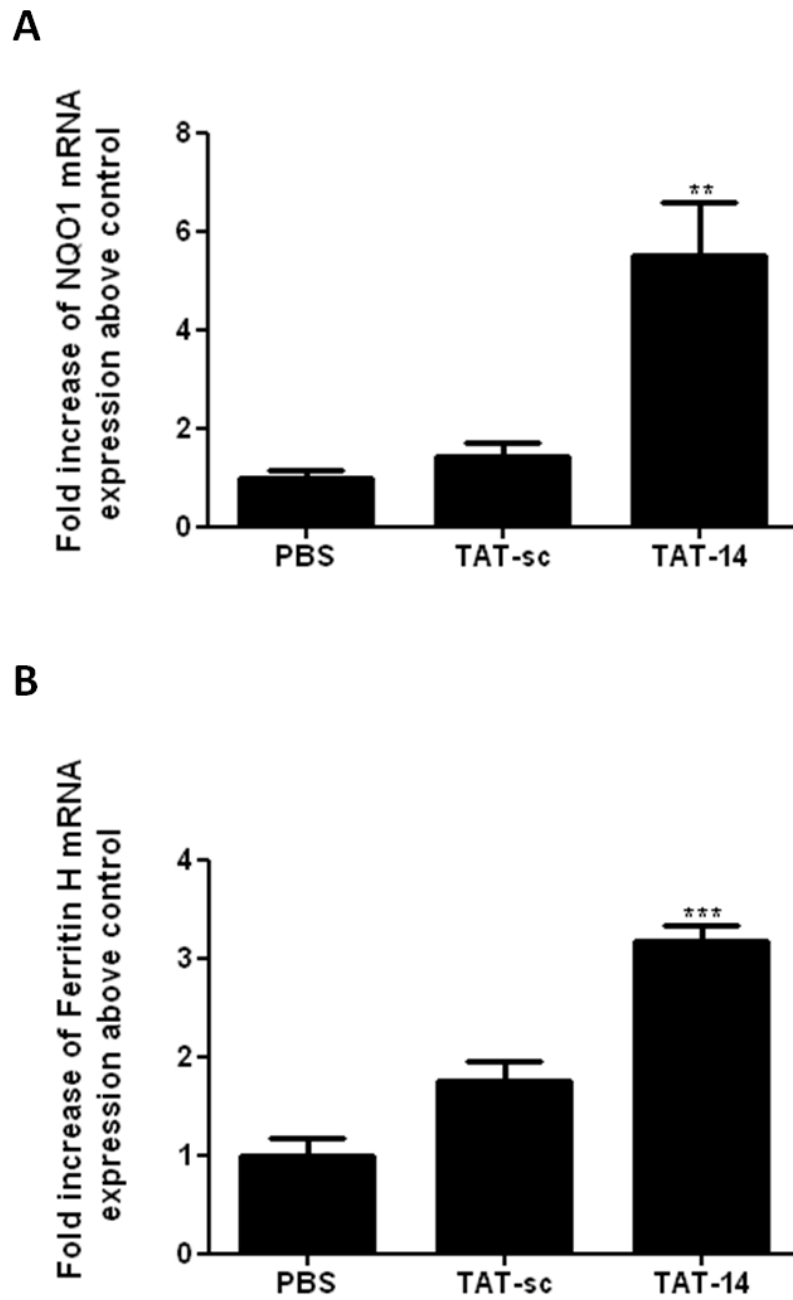


Fig 5.7 TAT-14 induces NQO1 and Ferritin mRNA. THP-1 cells were incubated with 75 μ M of the indicated peptide for 8h. RNA was extracted and RT-qPCR was used to measure **(A)** NQO1 expression, or **(B)** Ferritin expression. Mean \pm SEM, n=3; **p<0.01, ***p<0.001; One way ANOVA with Tukey's multiple comparison test.

5.3.3: Anti-inflammatory effects of TAT-14 in THP-1 cells

Activation of Nrf2 results in suppression of inflammation, making the Nrf2/Keap1 interaction a potential drug target. However, the effects of activating Nrf2 using an inhibitor of the Nrf2/Keap1 interaction to dampen the inflammatory response are unknown. Therefore, THP-1 cells were stimulated with 75 μ M TAT-14 or TAT-sc for 4h before addition of 10 μ g/mL LPS for 4h. Supernatants were prepared and soluble TNF levels were measured by ELISA. Fig 5.8 demonstrates that LPS induced TNF production (524 pg/mL TNF), which was significantly inhibited by the presence of TAT-14 (339 pg/mL TNF, $p=0.0438$; t-test). Interestingly, TAT-sc slightly increased TNF expression, although this was not statistically significant, suggesting that although it does not activate the Nrf2 pathway, there is a chance that it is not biochemically inert.

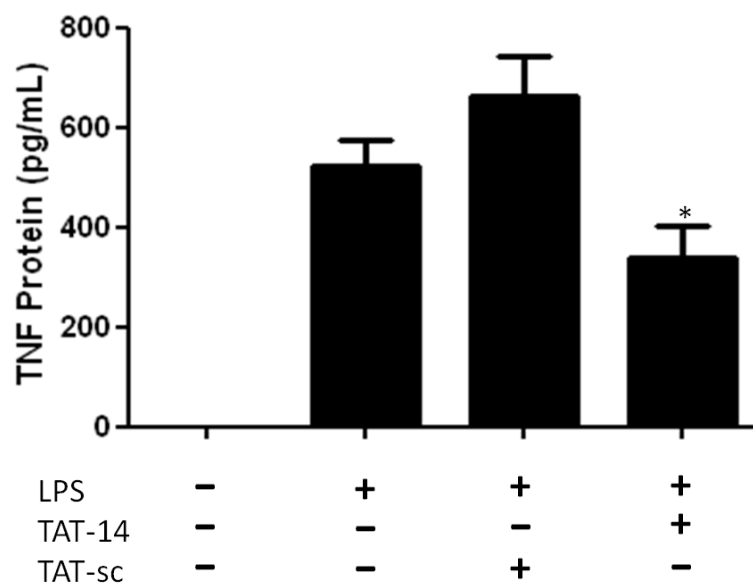


Fig 5.8 TAT-14 inhibits LPS induced soluble TNF protein expression. THP-1 cells were incubated with TAT-14 or TAT-sc for 4h prior to 50ng/mL LPS for 4h. Supernatants were prepared, and TNF expression was measured by ELISA. Mean \pm SEM, $n=3$; * $p<0.05$; student's t-test.

In order to test whether TAT-14 inhibits LPS induced TNF at the mRNA level, THP-1 cells were incubated for 4h prior to addition of LPS for 4h. TNF mRNA expression was measured by qRT-PCR. Fig 5.9a shows that TNF mRNA is decreased by approximately 66% when LPS is added in the presence of TAT-14 compared to LPS alone (Fig 5.9A; $p=0.05$). LPS-induced TNF in monocytes is driven by NF- κ B (Yao et al., 1997). Therefore, the effect of TAT-14 on LPS-induced IL-1 β mRNA was also tested. THP-1 cells were incubated with 75 μ M TAT-14 or TAT-sc for 4h before addition of LPS for 4h. IL-1 β mRNA was measured by qRT-PCR. TAT-14 inhibited LPS induced IL-1 β mRNA by 39% (not significant; Fig 5.9B).

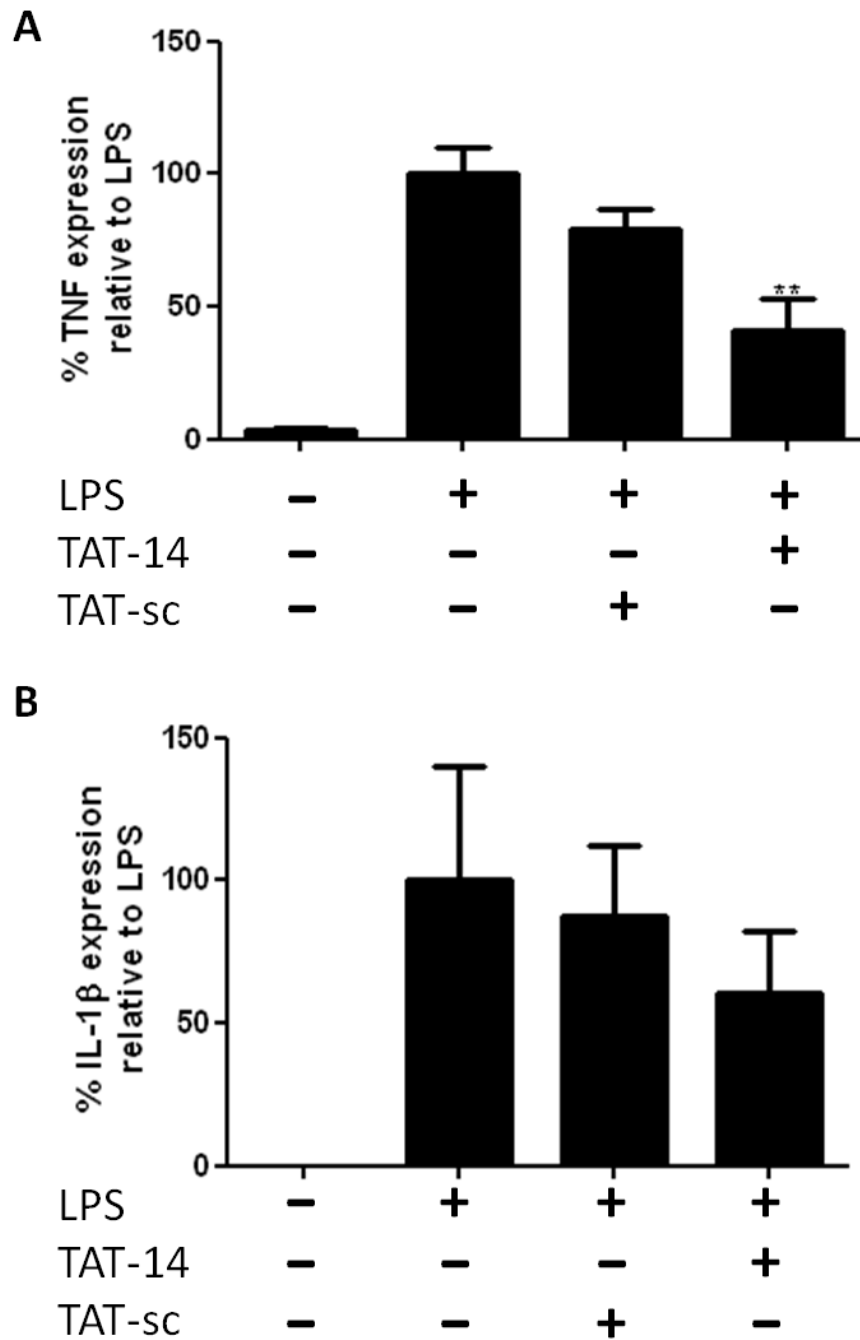


Fig 5.9 TAT-14 inhibits TNF mRNA expression. THP-1 cells were incubated with 75 μ M TAT-14 or TAT-sc for 4h before addition of 10 μ g/mL LPS. **(A)** TNF mRNA expression and **(B)** IL-1 β expression were measured by RT-qPCR. Mean \pm SEM, n=3; **p<0.01; One Way ANOVA with Tukey's multiple comparison test.

5.4 Discussion

Nrf2 activity is primarily regulated by the Nrf2/Keap1 interaction. Disruption leads to expression of Nrf2, and upregulation of cytoprotective genes (Itoh et al., 1999). However, the Keap1 interaction is redox sensitive, and the vast majority of Nrf2 activators do so through modification of Keap1 cysteine residues by electrophiles and oxidative stress. These are non-specific reactive molecules that can cause off-target effects (Levonen et al., 2014). Competitive inhibition of the Nrf2 binding site on Keap1 may increase specificity for Nrf2 activation. This study identified TAT-14 as the most biologically active of three cell-penetrating Neh2 mimetic peptides. TAT-14 was shown to directly bind the Kelch domain of Keap1 *in vitro*, and induce Nrf2 protein expression in THP-1 monocytes. TAT-14 induced ARE-driven genes, HO-1, NQO-1, and ferritin H. In addition, TAT-14 downregulates LPS-induced TNF expression in THP-1 monocytes. This study shows for the first time that a competitive inhibitor of Keap1 activates Nrf2 in monocytes, and has anti-inflammatory properties. This suggests that TAT-14 is a useful tool in studying Nrf2 activation, and that Keap1 inhibition might be a viable strategy in combating inflammatory diseases.

Inhibiting the Nrf2/Keap1 interaction was attempted by producing peptide sequences homologous to the Keap1 binding sequence in the Neh2 domain of Nrf2. Testing the three peptides TAT-10, TAT-14, and TAT-16 in THP-1 cells revealed that TAT-14 was the most effective activator of HO-1. TAT-10 was the least effective – it is possible that it does not bind as well to Keap1, and this is confirmed by Lo et al (2006) who purified the Nrf2/Keap1 complex by chitin pull-down assay, and then added the 10mer, 14mer, and 16mer peptides to show that they displace Nrf2 from the complex (measured by Western blotting). This *in vitro* assay showed that the 10mer is the least active peptide, showing inhibition of Nrf2/Keap1 after addition of 1 μ g of the

peptide to the sample, where both the 14mer and 16mer show similar activity with 100ng. The effect of the 10mer is probably exaggerated in this experiment because it is the smallest peptide, meaning that there are more molecules per gram. TAT-16 was less effective than expected. The 16mer is the strongest binding peptide *in vitro*, but in THP-1 cells it is unable to produce the same effect. TAT-16 is very different from TAT-14. It contains an extra AFFAQ on the N-terminus, and misses PIQ on the C-terminus of the Keap1-binding sequence. It is possible that there is interference between the TAT and 16mer caused by the N-terminal amino acids or lack of the PIQ sequence, which could affect either cell penetration or Keap1 binding. TAT-14 is the only peptide that shows strong effects in both THP-1 cells and *in vitro* binding assays. Due to the high bioactivity of TAT-14, neither TAT-10 nor TAT-16 was investigated further.

Induction of HO-1 does not indicate that TAT-14 is a competitive inhibitor of Nrf2. Therefore, fluorescence polarization was used to show that TAT-14 binds to the kelch domain of Keap1. This was measured by using TAT-14 or the 14mer to displace a FITC-conjugated 14mer from the Kelch domain *in vitro*. TAT-14 bound Keap1 strongly, with a K_i of 3.628 nM. Interestingly this is stronger than the 14mer. This is not undesirable – an improvement on the binding affinity of the native sequence is a good thing, but from a drug design perspective it is important to understand why it occurs. It is possible that the TAT sequence stabilises the ETGE β -turn structure required for Keap1 binding.

Under basal conditions, Nrf2 is constitutively produced and degraded (Zhang and Hannink, 2003). The Nrf2/Keap1 interaction is necessary for degradation of Nrf2. Therefore, when the Nrf2/Keap1 interaction is inhibited, the amount of cellular Nrf2 increases. TAT-14 increases cellular Nrf2, whereas TAT-sc does not. Coupled with the FP data, this suggests that TAT-14 is disrupting

the Keap1/Nrf2 interaction. Importantly, TAT-sc did not produce the same effects, showing that TAT-14 activity is sequence specific. There are two mechanisms by which the Nrf2/Keap1 interaction can be inhibited. The first is disruption of the DLG/Kelch interaction, which inhibits Keap1-induced degradation of Nrf2 (Kato et al., 2005; Baird et al., 2013). The other is disruption of the ETGE/Kelch interaction, which is the high affinity binding sequence of Nrf2 (Tong et al., 2006b). TAT-14 almost certainly acts through inhibiting the ETGE motif because the Keap1 protein used in the FP assay contains no dimerization site, and the F-14 peptide only contains an ETGE motif. Therefore, the displacement of F-14 caused by TAT-14 in the fluorescence polarization assay is an inhibition of the strong-binding ETGE motif. There is no evidence to suggest that it doesn't use the same mechanism to displace Nrf2 in THP-1 cells.

Induction of HO-1, Ferritin and NQO1 mRNA indicates that the increased cellular Nrf2 is functional. All three genes are Nrf2-driven, suggesting that Nrf2 transcription partners are either already present in the nucleus, or that their presence is Nrf2-dependent. TAT-14 is an effective inducer of HO-1 expression in THP-1 cells; less effective per molecule than curcumin, but more effective than alpha-lipoic acid and epigallocatechin (Ogborne et al., 2005; Rushworth et al., 2006; Ogborne et al., 2008). At low concentrations, however, TAT-14 was ineffective. There could be a number of reasons for this. In cellular assays Keap1 is accessible only by bypassing cell membranes. Even with a cell penetration sequence TAT-14 may not enter cells as efficiently as small molecules. Furthermore, in cellular assays the Keap1 concentration is unknown. Cells also have thousands of molecules that can potentially interfere with binding efficiency, and it is already well-known that short peptides are rapidly degraded by proteases (Pernot et al., 2011). The ratio of TAT-14 to Keap1 molecules could be quite low once these factors have been taken into account. Another factor that could lower the efficiency

of TAT-14 is its mode of action. One of the key features of signalling pathways, especially those that involve degradation products and ROS, is signal amplification. TAT-14 should not activate Nrf2 via signalling pathways, and therefore there will be no amplification of the signal upstream of this. One molecule of TAT-14 binds one molecule of Keap1. However, Nrf2 binds Keap1 in a 1:2 ratio, meaning that for every two molecules of TAT-14 only one molecule of Nrf2 is activated. It is possible that, to some extent, lack of reactivity is the price to pay for target specificity.

Nrf2 knockout mice are susceptible to inflammatory diseases such as sepsis (Thimmulappa et al., 2006; Kong et al., 2010), which has led to research into small molecules that may activate Nrf2 and inhibit inflammation. Although there has been some measure of success with small molecules (Kalayarasan et al., 2009; Kim et al., 2013; Zhao et al., 2013b; Ramyaa et al., 2014), none of these are specific activators of Nrf2. In this study the effect of TAT-14 on inflammatory mediators was investigated in THP-1 cells. The results showed that TAT-14 inhibits TNF mRNA production by 66%. This does have an effect on protein production; soluble TNF protein was inhibited by 35%. It is possible that the discrepancy between mRNA and protein production is due to the fact that the ELISA does not measure membrane-bound TNF. IL-1 β was also inhibited at the mRNA level, although the effect was not statistically significant. These data suggest that Nrf2 plays a large role in dampening the immune response upstream of gene transcription, and therefore support the hypothesis that the Nrf2/Keap1 interaction is a good drug target.

This study shows that competitive inhibition of the Nrf2/Keap1 interaction using TAT-14 is a viable strategy in inhibiting production of inflammatory mediators. Non-competitive mechanisms of inhibiting the Nrf2/Keap1 interaction tend to have a common problem. This is that Nrf2 is a stress sensor which acts as part of a negative feedback system in which cellular

stress pathways activate Nrf2, causing a subsequent decrease in stress. Many of the well-characterised small molecules that activate Nrf2 do so by activating cell signalling pathways, or increasing ROS production (Rushworth et al., 2006; McNally et al., 2007; Lee and Lee, 2011), which are common activators of many cell signalling pathways. This is highlighted by the fact that the Nrf2 activator bardoxolone methyl has just been pulled from clinical trials for type-2 diabetes and stage 4 chronic kidney disease because patients given the drug were found to be more susceptible to serious cardiovascular side-effects, including heart failure compared to those given a placebo (de Zeeuw et al., 2013). Whether this is caused by Nrf2 is unknown because bardoxolone methyl is not a specific activator of the Nrf2 pathway (Gao et al., 2013), and many have argued that the adverse effects could have been predicted (Thomas, 2012; Rossing, 2013; Zhang, 2013). TAT-14 is designed to bind Keap1 specifically in order to keep non-specific interactions to a minimum. Therefore, any negative side effects should be a direct result of Nrf2 activation. This means that further work involving TAT-14 will either validate Nrf2 as a drug target and maybe even TAT-14 as a viable drug, or it will be a valuable tool that can provide insight into any disadvantages of activating Nrf2.

One limitation with this study is that, short of running experiments on every protein in the human genome, it is almost impossible to prove that TAT-14 is a specific activator of Nrf2. Measures have been taken to ensure its specificity as much as possible. It is homologous to the Keap1-binding region of Nrf2, it has been shown to bind Keap1, and the scrambled peptide does not exhibit similar activity. However, this study does not account for the fact that there are other proteins, including IKK β and Bcl-2, which use the same binding pocket of Keap1 as Nrf2. The effect of TAT-14 on these mediators is unknown, and is the minimal amount of future work required to verify its specificity. It is likely that because TAT-14 contains the exact binding

sequence of Nrf2, any other mediators that are affected by it will also be affected by any other drug that inhibits the Nrf2/Keap1 interaction by the same mechanism. This does not apply to the TAT sequence. TAT is assumed throughout this study to have little biological activity beyond cell penetration. There is no evidence to suggest that it should have adverse effects, but the FP assay revealed that TAT-14 binds Keap1 more strongly than the 14mer alone suggesting that it is not entirely inert.

Although THP-1 cells are a good model cell line for inflammation, TAT-14 needs to be validated in primary human monocytes in order to confirm that its effects are physiologically relevant (Rushworth et al., 2005; Rushworth et al., 2008). Testing it in other tissue types would also be advantageous because Nrf2 is a key regulator implicated in protecting against a wide range of diseases including neurodegenerative diseases (Ryu et al., 2013), and cancer (Bishayee et al., 2011). Whether TAT-14 has potential to be used in animal research is unknown. One study has shown that TAT conjugated peptides similar to the ones used in this study improve brain injury in mice after being directly injected into the affected tissue, but more thorough work could be done on different delivery methods and bodily distribution (Zhao et al., 2011). The final limitation is that TAT-14 may only be useful in cells with fully functional Keap1. Cell lines with Keap1 mutations include NCI-H1184 and NCI-H1648 lung cancer cells (Padmanabhan et al., 2006), which contain mutations in the Kelch domain that reduces Nrf2 binding. TAT-14 may not be able to activate Nrf2 in these cells because the same mechanisms that prevent sequestration of Nrf2 should also apply to TAT-14, although this has not been determined experimentally. In addition other cell lines, including A549 cells and NCI-H460 cells, have silenced Keap1 expression (Wang et al., 2008b). A Keap1 targeting peptide would likely be ineffective in these cells.

The current work shows that TAT-14 activates the Nrf2 pathway, binds Keap1, and inhibits LPS-induced inflammatory mediators in THP-1 cells. Future work can expand on this research in two broad directions. The first is finding a more drug-like molecule to target the Nrf2/Keap1 interaction. The second is to use TAT-14 to further characterise the effects of targeting the Nrf2/Keap1 interaction.

The long term goal of inhibiting the Nrf2/Keap1 interaction is to produce therapeutics to increase cytoprotection and decrease toxic insult. Diseases in which these are major factors include inflammatory and neurodegenerative diseases (Hsieh and Yang, 2013; Marques et al., 2014; Wu et al., 2014). Classically, there have been criticisms of peptides as drugs. Aside from being expensive to make peptides have poor oral uptake and have issues entering tissues *in vivo* (especially crossing the blood-brain barrier). Small peptides are also prone to degradation. In cell culture studies this isn't huge problem, but *in vivo* the drug has to reach its target tissue. This poses a particular problem with oral delivery of a drug because it must bypass the digestive tract without degradation (Gupta et al., 2013).

Small molecules may overcome some or all of these issues, but finding a small molecule inhibitor of the Nrf2/Keap1 interaction is a difficult task. The main approaches have been to modify Nrf2 mimetic peptides to find improved binding sequences, or to screen libraries of compounds by FP assay. The former approach was undertaken by Hancock and co-workers (2012), who formed a fusion peptide from the 7-amino acid long Keap1 binding sequences of Nrf2 (DEETGEF) and sequestosome-1 (DPSTGEL). The resulting peptide (DPETGEL) showed stronger binding than either of the native peptides by FP assay. Interestingly the core ETGE sequence could not be improved upon, suggesting that any small molecules that could be made should at least be large enough to mimic this sequence. The same group

went on to show that activity could be improved further by modifying n-terminal conjugates to become more lipophilic (Hancock et al., 2013). Although these studies offer important insights into the chemistry of the Nrf2-Keap1 interaction, progress towards a small molecule inhibitor using this method has not progressed quickly. Screening libraries of compounds is another method, which has shown more promise. In a recent screen of 337,116 compounds a single molecule has been considered to be a promising lead (Wang et al., 2012a) with Keap1 binding activity and predicted non-reactivity. An independent study of a similar scale found several compounds that bind Keap1, but only one showed biological activity (Zhuang et al., 2014). Although these are important discoveries, such a low hit-rate is worrying considering the amount of work that still needs to be done before the molecule can be considered anywhere near ready for therapeutic use.

Despite the focus on small molecules, there is still hope for a peptide based solution. Despite the criticisms of peptides, these arguments are made almost redundant by the fact that there are over 100 peptide drugs licensed for medical use for the treatment of a wide range of medical conditions including type 2 diabetes and hypertension (Craik et al., 2013). Keap1 targeting peptides have been shown to reduce damage to the blood-brain barrier after traumatic brain injury in mice (Zhao et al., 2011). The peptides in the study were TAT-14 with an extra proline residue between the TAT and 14mer, and TAT-10 with a calpain cleavage sequence between the TAT and 10mer. The calpain cleavage site was added to take advantage of the ubiquitously expressed cleavage protein, calpain, and was intended to remove the TAT from the 10mer to prevent any interference with its ability to bind Keap1. Interestingly TAT-14 with proline did not show any biological effects but TAT-CAL-10 induced ARE driven gene expression and protected against tissue damage from traumatic brain injury. Although the work is promising for the use of peptides as drugs, it also highlights two weaknesses

with the approach. Firstly, altering the peptide sequence to achieve cell penetration is both necessary and has the potential to block activity of the peptide. This is highlighted by the differences in the results between the present study and the work by Zhao and co-workers. The 10mer has biological activity in the mouse study, but not in this study. This is probably because in this study TAT is still attached at the point of Keap1 binding. Secondly, the peptides were injected directly into the brain in order to bypass the blood brain barrier. This is not a practical method of drug delivery. However, these problems are not insurmountable. Optimisation of peptide sequences and cell penetration motifs have not been extensively studied with Keap1 inhibitors in cells or animal models. In addition, drug delivery research into using nanoparticles as drug delivery mechanisms is ongoing, and may solve problems with bioavailability in the future (Chatterjee et al., 2014).

In addition to potential therapeutic advantages, TAT-14 can be used as a tool to study specific activation of the Nrf2 pathway. This study has directly led to the commercial production of TAT-14 by several biotechnology companies including Tocris (catalog number: 4811), and Merck Millipore (catalog number: 492042). TAT-14 can be used to extend this project in several ways. Most importantly, it can be used to simulate the effects of a future drug targeting the Nrf2/Keap1 interaction. Diseases where one such drug could be of therapeutic benefit, side effects, and mechanisms of action can all be predicted with TAT-14. There are also immediate questions about Nrf2 signalling in inflammation that TAT-14 can help answer. Testing the effect of TAT-14 on other Keap1 substrates will help to verify the specificity of Keap1 as a drug target. It is possible that the effect on some of these mediators will be marginal because their binding affinity for Keap1 is orders of magnitude lower than Nrf2 (Hancock et al., 2013). Once this is established further experiments can be done to elucidate the source of the anti-inflammatory

activity of TAT-14. There are several proposed mechanisms by which Nrf2 could dampen the inflammatory response. The first is via its target genes, which have been implicated in protection against various inflammatory diseases (Yeligar et al., 2010; Shyur et al., 2011). This can be considered unlikely in the case of TAT-14 because the kinetics of ARE gene induction and anti-inflammatory activity don't match. The second is by affecting Keap1 binding partners, which include IKK β and NF- κ B itself (Lee et al., 2009a; Kim et al., 2010; Yu et al., 2011). The third is by cross talk between Nrf2 and NF- κ B signalling, potentially involving competition with the transcriptional co-factor CBP (Liu et al., 2008; Ziady et al., 2012). Studying upstream activators of TNF should be the focus of this future study. Confirming a role for NF- κ B, and finding out which part of the pathway the inhibition occurs at could help to understand how Nrf2 confers anti-inflammatory activity. Using co-immunoprecipitation to test the effects of TAT-14 on the binding between IKK β or NF- κ B and Keap1 would be a good initial experiment to confirm whether these interactions play a role in the activity of TAT-14.

Another interesting project would be to research the effects of long-term treatment with a Keap1 inhibitor. The effects of TAT-14 are transient, but either repeated stimulations or expressing it in a plasmid could provide sustained activation. Finally, Nrf2 is a promising target for lots of diseases. This project has focused on inflammatory effects, and a similar peptide has shown benefits in traumatic brain injury (Zhao et al., 2011). However, neurodegenerative diseases, cancer, and diabetes (Lastres-Becker et al., 2012; Kavitha et al., 2013; Xu et al., 2014a) all show roles for Nrf2, and TAT-14 could be used to verify the Nrf2/Keap1 interaction as a drug target in these diseases.

In conclusion, TAT-14 is a peptide which specifically activates Nrf2, causing powerful induction of ARE-containing genes. It is anti-inflammatory in THP-1

monocytes, suggesting that developing the Nrf2/Keap1 interaction is a good drug target worthy of further research. TAT-14 itself may have potential as an anti-inflammatory drug, and could be used heavily in the future development of Keap1 inhibitors. Furthermore, TAT-14 is commercially available for use as a tool to study the Nrf2 pathway before more drug-like molecules are developed.

Chapter 6: The effects of Nrf2 over-expression on chemoresistance in malignant melanoma

Acknowledgements:

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6.1 Introduction

Malignant melanoma is a form of skin cancer that affects melanocytes, which are cells that produce the pigment melanin. Although melanocytes are most commonly found in the stratum basale of the skin, they are also located in other areas of the body such as the bones, inner ear and eyes (Wakamatsu et al., 2008; Zhang et al., 2012b; Ye et al., 2013). Over 13, 000 people were diagnosed with malignant melanoma in 2011 in the UK. In comparison to other skin cancers it is rare (over 100,000 people were diagnosed with non-melanoma skin cancer in 2011), but far more deadly; out of 2794 deaths caused by skin cancer in the UK in 2011, 2209 (79%) were melanoma patients (CRUK., 2014c).

Early diagnosis of melanoma almost always results in successful recovery from the disease. The usual treatment is surgery, which has an extremely high success rate. 20-year survival is about 90% for stage I melanomas and 80% for stage II melanomas (Lee et al., 2013d). Unfortunately, melanoma has a tendency to aggressively metastasise and once this has occurred, surgery is often no longer an option. Melanoma is highly resistant to chemotherapy and radiotherapy, and patients that have unresectable stage IV (Table 6.1) melanoma have approximately 5-10% survival rate for 5 years after diagnosis, and even those whose melanomas are operable still only have about a 15-30% 5-year survival rate (Wevers et al., 2013).

Stage	Melanoma Description	Primary Treatment
0	On surface of skin	Surgery
1	A <1mm thick	Surgery
	B 1-2mm thick or ulcerated	
2	A 2-4mm thick or 1-2mm thick and ulcerated	Surgery. Occasionally chemotherapy to reduce chances of melanoma returning.
	B >4mm thick or 2-4 mm thick and ulcerated	
	C >4mm thick and ulcerated	
3	A Not ulcerated and has spread to up to three lymph nodes	Surgery. Radiotherapy. Immunotherapy. Chemotherapy. Clinical trials.
	B Ulcerated and has spread to up to three nearby lymph nodes or Not ulcerated, spread into 1-3 lymph nodes, which are enlarged or Spread into areas of skin or lymphatic channels, not spread into lymph nodes	
	C Ulcerated and has spread into 1-3 lymph nodes, which are enlarged, or Spread into ≥4 lymph nodes	
4	Metastasised into other parts of the body	Surgery. Chemotherapy (dacarbazine, temozolomide, combination treatments BRAF inhibitors, immunotherapy, antibody treatments) . Radiotherapy. Clinical trials. Considered incurable.

Table 6.1. Stages of melanoma and treatment options (NHS, 2012; ACS, 2013).

A common problem with cancer treatment is that mechanisms which normal cells use to protect themselves from damage are hijacked by cancer cells, providing resistance to chemotherapy and radiotherapy. This is almost certainly the case in melanoma cells, which contain high concentrations of melanin. Melanin is a blanket term to describe a group of tyrosine-derived pigment compounds in a variety of organisms that are responsible for skin colour, freckles, and play a role in eye colour (Stamatas et al., 2004; Wakamatsu et al., 2008). Although the function of melanin is under investigation, a primary function is likely to protect against UV damage.

Melanin is noted for its ability to absorb UV light, and a high density of melanin has been linked with protection against UV-induced DNA damage. In agreement with this line of thought, people with dark skin are less susceptible to melanoma than those with fair skin (Meredith and Riesz, 2003; Tadokoro et al., 2003). The chances of African Americans getting melanoma in their lifetime is 0.09% compared to 1.93% for the American population as a whole (Altekruse et al., 2010).

Melanoma cells, therefore, are believed to be highly resistant to radiotherapy. There has also been little success with chemotherapy. Dacarbazine is the standard treatment, but even the most optimistic studies show that it has a poor overall response rate at around 10-12% (Patel et al., 2011; Bedikian et al., 2014). While it may have a small effect, its median response duration is measured in months, and it does not cure the disease (Velho, 2012). Targeted therapies have had better response rates, but are still underwhelming. About 65-70% of melanomas contain a mutation in BRAF, with the most common being V600E (older studies report this as V599E) (Davies et al., 2002; Kumar et al., 2003). BRAF is involved in inducing cell proliferation via MAP kinase pathways. The V600E mutation commonly found in melanoma patients activates BRAF in these cells. BRAF inhibitors have shown favourable improvements over dacarbazine treatment. Unfortunately, BRAF inhibitors are no use to patients without mutated BRAF, and the cancer grows resistant after about 8-9 months of treatment in most patients, who then relapse (Villanueva et al., 2010; Chapman et al., 2011; Sun et al., 2014).

Currently, the most promising form of therapy against melanoma is immunotherapy. Ipilimumab inhibits a regulator of cytotoxic T-cell activity, allowing them to target tumours. Trials show a significant improvement in patients treated with ipilimumab, but also had high rates of severe adverse

effects that were linked to some patient deaths. Most side effects are related to immune system activation (Hodi et al., 2010; Robert et al., 2011; Ribas, 2012). A new drug, pembrolizumab, is another immunotherapeutic agent targeted against an immunosuppressive receptor, programmed death receptor 1 (PD-1) to perform a similar function. It has recently been used in phase I clinical trials and was found to exhibit a overall response rate of 20-26%. No patient deaths were linked to the drug, and side effects were mild (Robert et al., 2014).

One possible method of melanoma treatment is to increase susceptibility of melanoma cells to chemotherapeutics. The transcription factor Nrf2 is often credited for protecting cells against various types of damage. It has been implicated in protecting cells from DNA damage and has been identified to play an important role in chemoprevention (Khor et al., 2008; Kim et al., 2012b; Rodriguez et al., 2013; Singh et al., 2013a; Yang et al., 2014c). Like melanin, the mechanisms associated with Nrf2 that protect normal cells from damage also protect cancerous cells from chemo and radiotherapy. Silencing Nrf2 activity *in vitro* and *in vivo* has been shown to increase the effectiveness of chemotherapeutic drugs in several cancer cell types including lung, breast and pancreatic cancer cells (Wang et al., 2008b; Singh et al., 2010b; Arlt et al., 2013).

Skin cells are thought to express high concentrations of protective enzymes in order to deal with increased exposure to UV light and other toxins that are likely to be encountered by the body's exterior. Therefore, it is very possible that Nrf2 plays a strong role in the resistance of melanoma to chemotherapy. This study investigated Nrf2 expression in melanoma cells and whether silencing Nrf2 can enhance the effectiveness of chemotherapy.

6.2 Aims

Hypothesis: Melanoma cells express higher levels of Nrf2 than melanocytes. Cell lines with high levels of Nrf2 expression are more resistant to toxicity caused by chemotherapeutic drugs. Nrf2 siRNA sensitises melanoma cells to the toxic effects of chemoptherapeutic drugs.

Aims:

1. To analyse 11 melanoma cell lines for expression of Nrf2 and downstream target genes compared to melanocytes.
2. To investigate any correlation between Nrf2 expression and chemoresistance.
3. To study the effects of silencing Nrf2 on chemotherapeutic drug induced toxicity in melanoma cells.
4. To investigate potential mechanisms of Nrf2-depedent chemoresistance.

6.3 Results

6.3.1 Expression of Nrf2 pathway members in melanoma cell lines

Overexpression of Nrf2 has been noted in a number of cancers, including lung cancer, breast cancer and endometrial cancer (Jiang et al., 2010; Chen et al., 2012; Zhan et al., 2012). In this study Nrf2 protein expression was investigated in 11 human melanoma cell lines and human melanocytes by Western blot analysis. All 11 melanoma cell lines contained more Nrf2 protein than melanocytes (Fig 6.1A). Analysing densitometry of the melanoma samples revealed that Colo829 contained the least Nrf2 protein. Nrf2 expression in other melanoma cells was expressed in relation to Colo829 cells to show the differences between the cell lines. M202, UACC1273, A2058, and M263 cells contained higher amounts of Nrf2 protein than the other melanoma cell lines (6.2, 6.3, 8.5 ($p < 0.01$), and 5.9-fold more than Colo829 cells respectively) (Fig 6.1B). Nrf2 mRNA expression was also examined by RT-qPCR. M202, UACC1273, and M238 cells had higher Nrf2 mRNA expression than other cell lines (Fig 6.1C), but the differences were not large, and there was no significant difference between any samples. A375 cells had markedly low Nrf2 expression at both mRNA and protein level.

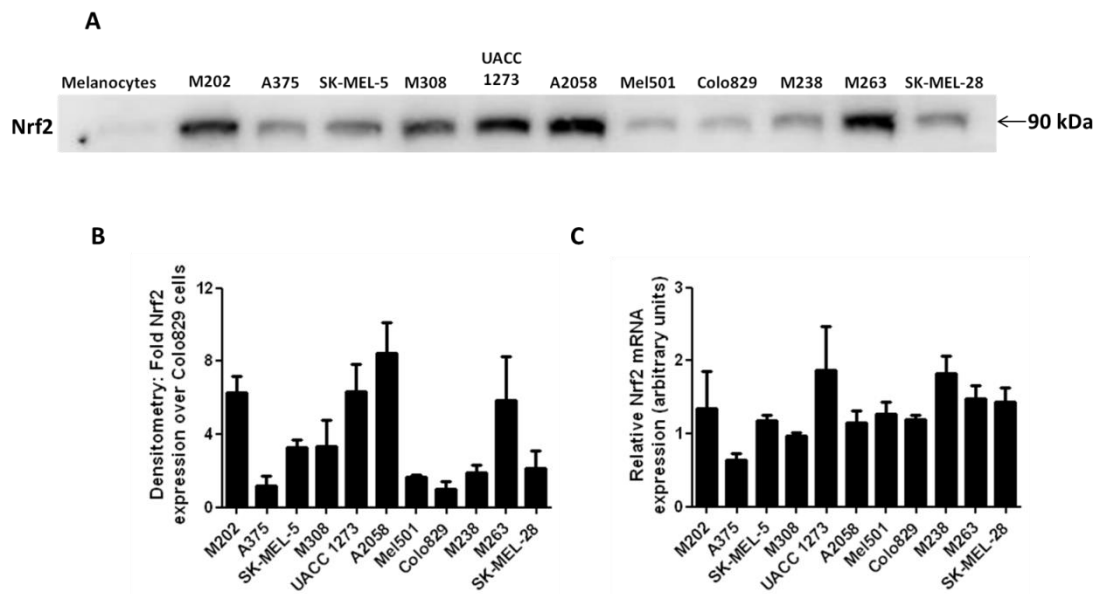


Fig 6.1. Nrf2 expression in melanoma cell lines. (A) Protein extracts of 1×10^6 cells were prepared for each cell line. 50 μ g protein was loaded onto a polyacrylamide gel, and Nrf2 expression was measured by Western blot analysis. Image shown is representative of 3 independent experiments. **(B)** Densitometry was measured for each of the three replicate experiments and normalised to the lowest expressing cell line, Colo829 cells. **(C)** 1×10^6 cells of each cell line were isolated and RNA was extracted. Nrf2 mRNA expression was measured by RT-qPCR. Mean \pm SEM, $n=3$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; one way ANOVA with Tukey's multiple comparison test.

Nrf2 activity is inhibited by its binding partner, Keap1. Therefore, transcriptional activity of Nrf2-regulated genes was examined. HO-1 and NQO1 were chosen because they have been linked with chemoresistance. M202 cells expressed higher levels of both HO-1 and NQO1 mRNA compared to other melanoma cell lines. Other cell lines with high expression of HO-1 mRNA are SK-MEL-5 and SK-MEL-28 (Fig 6.2A), which had modest Nrf2 expression. In contrast, UACC1273 and A2058 cells had high NQO1 mRNA expression (Fig 6.2B), which correlates with the high Nrf2 protein observed in these cells. M263 cells and M308 cells did not have particularly high expression of either gene relative to the other melanoma cell lines.

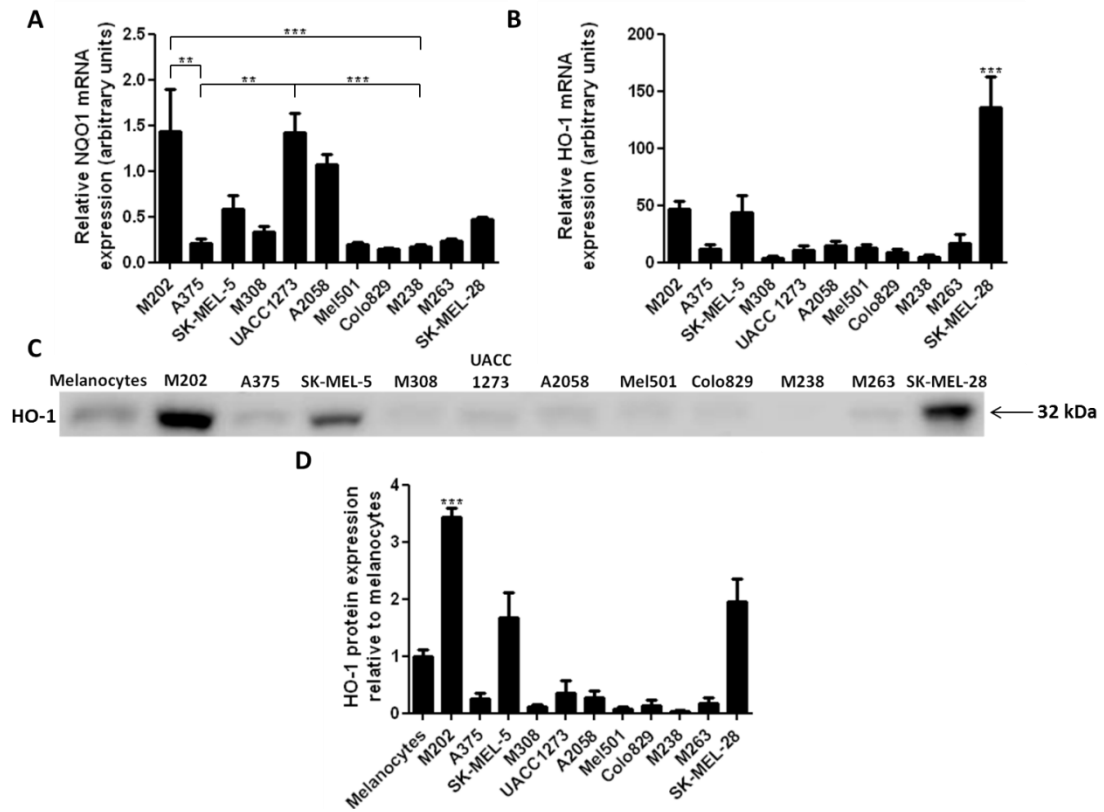


Fig 6.2. NQO1 and HO-1 expression in melanoma cell lines. 1×10^6 cells were collected for each cell line and RNA was extracted. Samples were analysed by RT-qPCR for **(A)** NQO1 and **(B)** HO-1 mRNA expression. **(C)** Protein extracts of 1×10^6 cells were prepared for each cell line, and 50 μ g protein was loaded onto a polyacrylamide gel. HO-1 expression was measured by Western blot analysis. Image shown is representative of 3 independent experiments. **(D)** Densitometry was measured for each of the three replicate experiments and expressed as fold expression relative to melanocytes. Mean \pm SEM, $n=3$; ** $p < 0.01$, *** $p < 0.001$; one way ANOVA with Tukey's multiple comparison test.

6.3.2 Effect of chemotherapeutic drugs on cytotoxicity in melanoma cells

The following cell lines were selected for further study based on the expression data (Table 6.2): M202, UACC 1273 and SK-MEL-5 cells were selected for their high expression of Nrf2 and its downstream targets. M238 and A375 were selected because they had low expression of these same genes. Each cell line was examined for its resistance against three anti-cancer drugs: dacarbazine, doxorubicin and cisplatin. IC₅₀ values are summarised in Table 6.4.

Cell Line	Nrf2	HO-1	NQO1
M202	High	High	High
UACC 1273	High	Low	High
SK-MEL-5	Medium	High	Medium
A375	Low	Low	Low
M238	Low	Low	Low

Table 6.2. Summary of Nrf2 pathway expression in selected melanoma cell lines.

Dacarbazine is a drug commonly prescribed to melanoma patients, although it has poor efficacy in the clinic (Patel et al., 2011; Bedikian et al., 2014). To date, resistance to dacarbazine has not been linked to Nrf2 activity. The effects of dacarbazine on cytotoxicity in these 5 cell lines was measured by MTS assay. M202 cells and UACC 1273 cells were highly resistant to dacarbazine, and even the highest concentration used (100 µM) did not significantly affect cell viability (Fig 6.3A and D). In contrast, cell viability of SK-MEL-5 and A375 cells was inhibited by 39% (p<0.05) and 46% (p<0.001) respectively after incubation with 100 µM dacarbazine (Fig 6.3B and C). Only M238 cells were affected by more than 50% by any concentration of

dacarbazine. Cell viability was decreased by 36% after incubation with 100 μ M dacarbazine ($p < 0.001$; Fig 6.3E).

Cell Line	Seeding Density (Cells Per Well)
M202	8×10^3
UACC 1273	8×10^3
SK-MEL-5	1×10^4
A375	5×10^3
M238	1×10^4

Table 6.3. Seeding density of cell lines in 96-well plates. Cells were seeded in 100 μ L RPMI in 96-well plates. These seeding densities allow optimum growth over 72h.

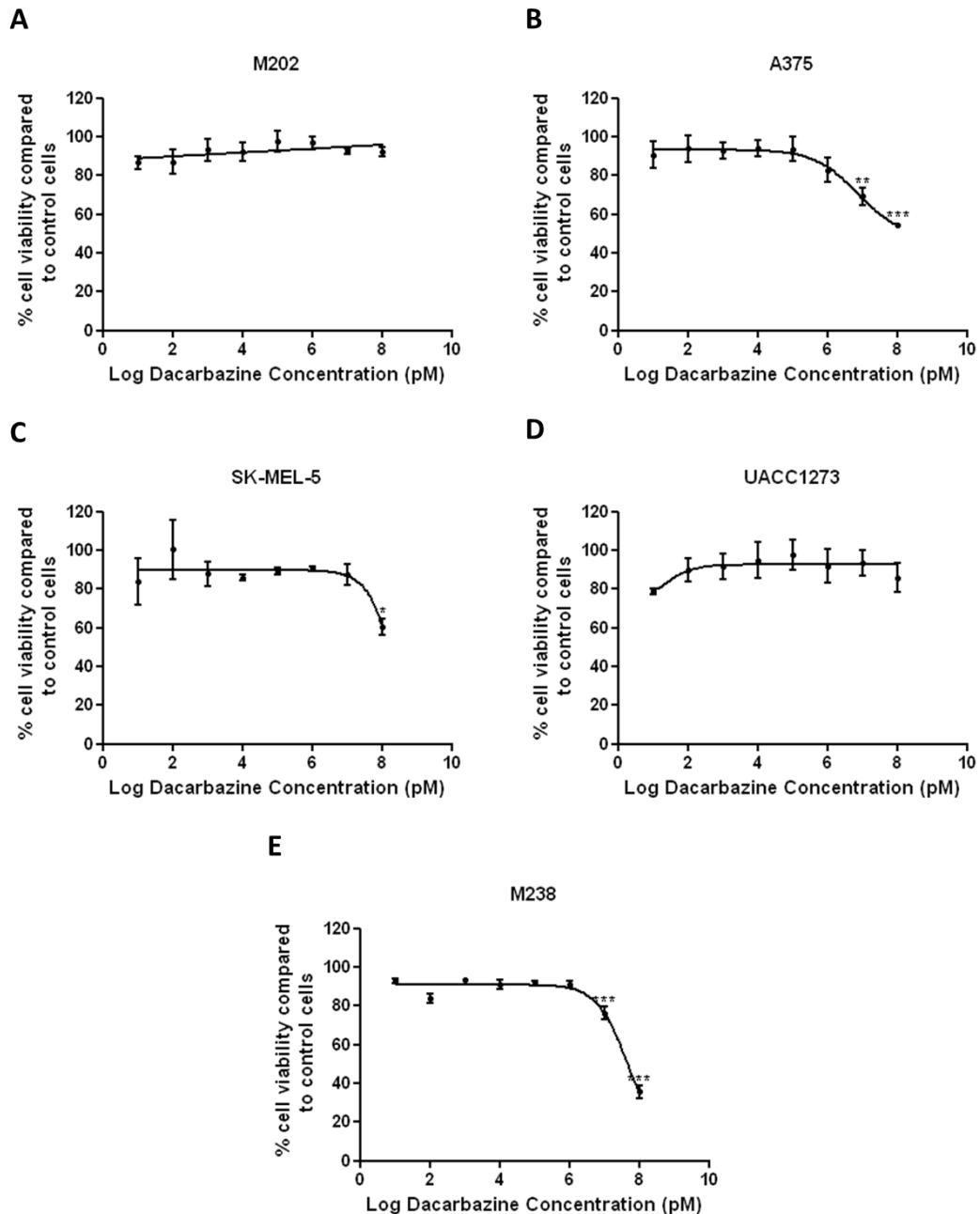


Fig 6.3. The effects of dacarbazine on cytotoxicity of melanoma cells. Cells were seeded in 96-well plates and incubated overnight at 37°C. Dacarbazine or DMSO was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 10 pM to 100 μM. 48h after incubation cytotoxicity was measured by MTS assay and expressed as a percentage compared to vehicle control cells. Mean ± SEM, n=3; Mean ± SEM, n=3; *p<0.05, **p<0.01, ***p<0.001; one way ANOVA with Tukey's multiple comparison test.

The results with dacarbazine support the clinical evidence, which shows that dacarbazine is not a very effective drug. Doxorubicin may be a more effective drug than dacarbazine, but is not used to treat skin cancer presumably because its rapid clearance from the circulatory system and toxicity to non-cancerous cells mean that the skin an unsuitable target for the drug (Zheng et al., 2010). However, doxorubicin is still involved in melanoma research, as drug delivery methods are researched (Pegoraro et al., 2013).

Doxorubicin strongly inhibited cell viability in all melanoma cell lines. The most resistant cell lines were M202, in which doxorubicin had an IC_{50} of 1.1 μ M, and UACC 1273, in which doxorubicin had an IC_{50} of 1.0 μ M (Fig 6.4A and D; Table 6.4). In contrast the most susceptible cell lines, A375 and M238 doxorubicin had an IC_{50} of 0.2 μ M (Fig 6.4 B and E), which is almost a magnitude lower than in UACC 1273 cells. SK-Mel-5 cells (IC_{50} : 0.3 μ M) were more resistant than A375 and M238 cells. Interestingly, at the highest concentrations of doxorubicin, SK-MEL-5 cells were less susceptible to doxorubicin. 10 μ M doxorubicin caused at least an 80% inhibition of cell viability in every cell line except SK-MEL-5, which was a 65% reduction ($P < 0.001$).

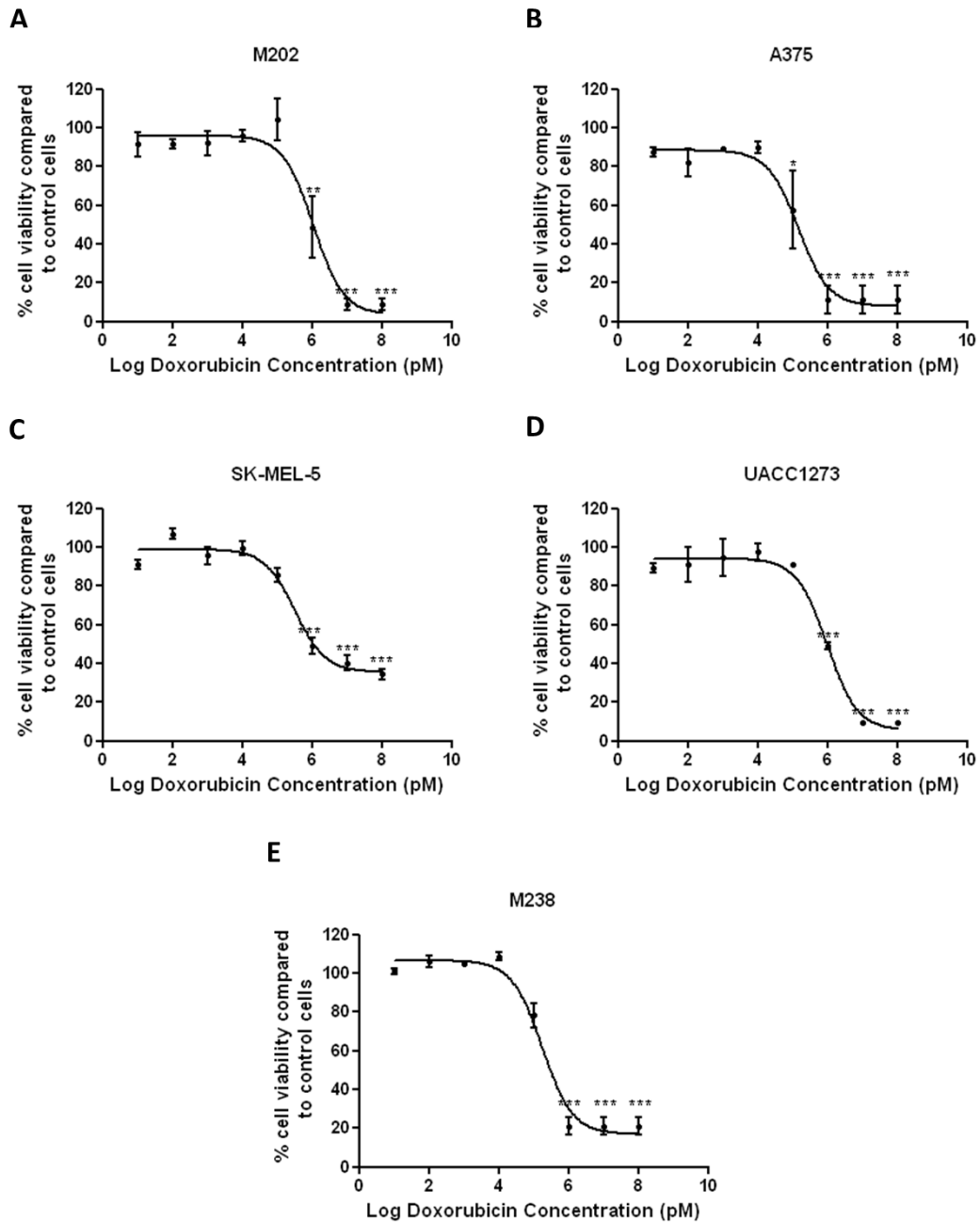


Fig 6.4. The effects of doxorubicin on cytotoxicity in melanoma cells. Cells were seeded in 96-well tissue culture plates and incubated overnight at 37°C. Doxorubicin or DMSO was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 10 pM to 100 μM. 48h after incubation cell viability was measured by MTS assay and expressed as a percentage compared to vehicle control cells. Mean ± SEM, n=3; *p<0.05, **p<0.01, ***p<0.001; one way ANOVA with Tukey's multiple comparison test.

Clinical trials have indicated that cisplatin alone has a response rate of 15-20%, in melanoma, although as high as 50% has been reported. Cisplatin is often used in clinical trials as part of combination therapies with dacarbazine (Chapman et al., 1999; Luke and Schwartz, 2013). The effects of cisplatin on cell viability was investigated and compared with the effects of the other drugs. Cisplatin had the least effect in M202 cells, where it had an IC_{50} of 39.7 μ M (Fig 6.5A). In contrast, the most susceptible cell lines were A375 cells (IC_{50} = 2.3 μ M; Fig 6.5B) and M238 cells (IC_{50} = 7.1 μ M; Fig 6.5E). Cisplatin had an IC_{50} of 11.9 μ M in UACC 1273 cells (Fig 6.5D), and 12.8 μ M in SK-MEL-5 cells (Fig 6.5C). Interestingly, although cisplatin had a low IC_{50} in M238 cells, cell viability was only blocked by 68% using the highest concentration of cisplatin ($p < 0.001$).

Interestingly, M202, UACC 1273, and SK-MEL-5 cells are more resistant than A375 and M238 cells to all three drugs. These three cell lines are those with the highest Nrf2 pathway expression.

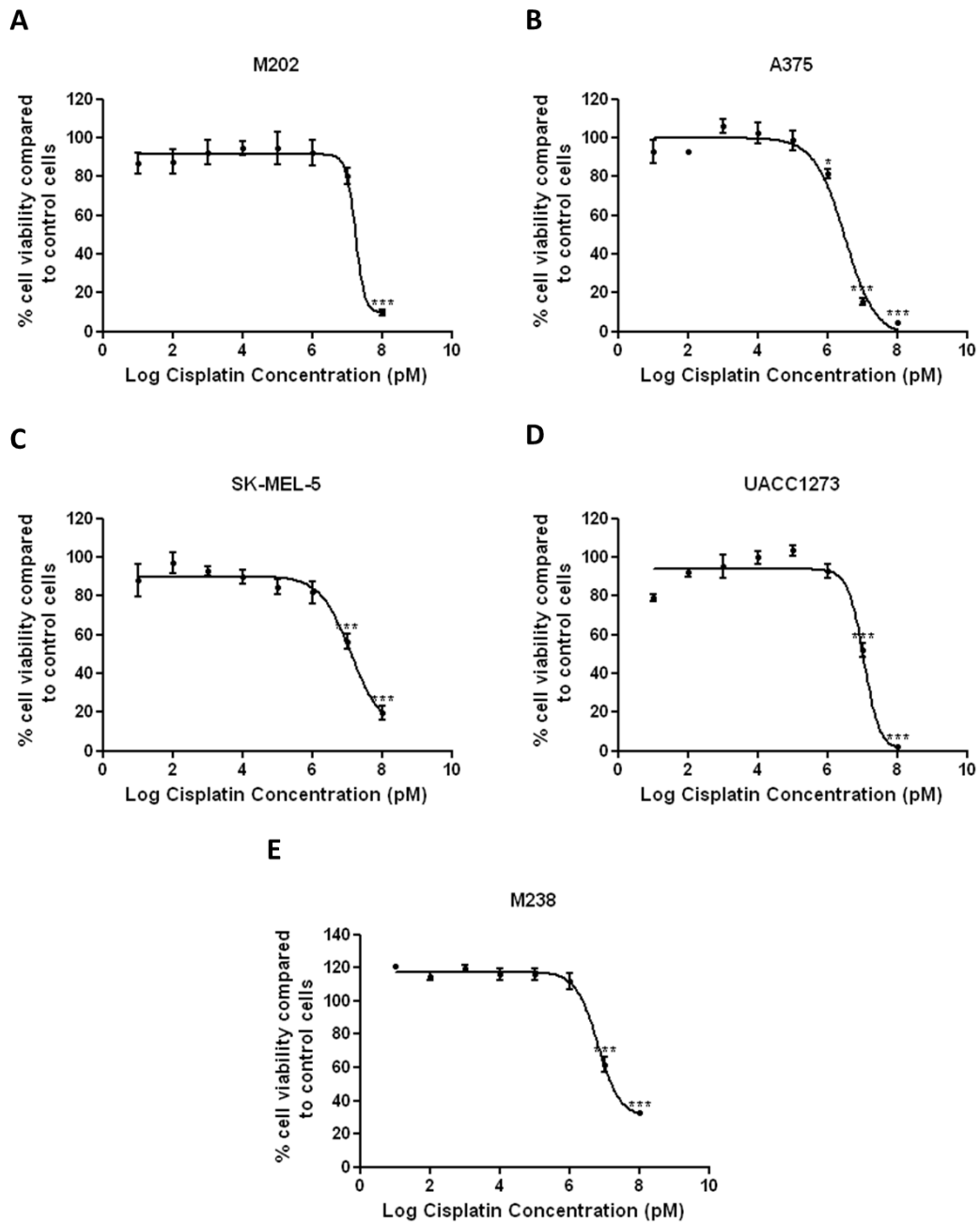


Fig 6.5. The effects of cisplatin on cytotoxicity in melanoma cells. Cells were seeded in 96-well tissue culture plates and incubated overnight at 37°C. Cisplatin or DMSO was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 10 pM to 100 μM. 48h after incubation cell viability was measured by MTS assay and expressed as a percentage compared to vehicle control cells. Mean ± SEM, n=3; . Mean ± SEM, n=3; *p<0.05, ***p<0.001; one way ANOVA with Tukey's multiple comparison test.

Cell Line	IC ₅₀ (μM)		
	Dacarbazine	Doxorubicin	Cisplatin
M238	46.6	0.2	7.1
UACC 1273	>100	1.0	11.9
SK-MEL-5	>100	0.3	12.8
A375	>100	0.2	2.3
M202	>100	1.1	39.7

Table 6.4. IC₅₀ of drugs in melanoma cells. Cytotoxicity data from Fig 6.3, 6.4, and 6.5 were analysed by non-linear regression, and IC₅₀ values were calculated.

6.3.3 The role of Nrf2 in chemoresistance in melanoma cells.

Although there is a correlation between Nrf2 expression and chemoresistance in the selected cell lines, this alone does not mean that Nrf2 is responsible, nor does it give any indication of how powerful an effect Nrf2 would have if it was responsible. Therefore, the three cell lines containing high amounts of Nrf2 (M202, UACC 1273, and SK-MEL-5) were taken forward for further experiments.

First, siRNA knockdown was verified in the melanoma cell lines. Nrf2 siRNA and a negative control (Neg) siRNA were transfected into the three cell lines, and Nrf2 mRNA expression was measured after 48 and 72h by RT-qPCR. All cell lines were very resistant to siRNA knockdown and Nrf2 expression could not be knocked down in UACC 1273 cells at all (data not shown). 200 nM siRNA caused a 57% knockdown of Nrf2 siRNA after 48h in M202 cells ($p < 0.01$; Fig 6.6A), which remained reduced by 40% after 72h. In SK-MEL-5 cells, a higher concentration of Nrf2 siRNA was required to ensure that the Nrf2 siRNA remained low at 72h. 250 nM siRNA ensured a knockdown of Nrf2 mRNA by 57% at 48h ($P < 0.05$), which remained knocked down by 55% at 72h ($p < 0.05$; Fig 6.6B).

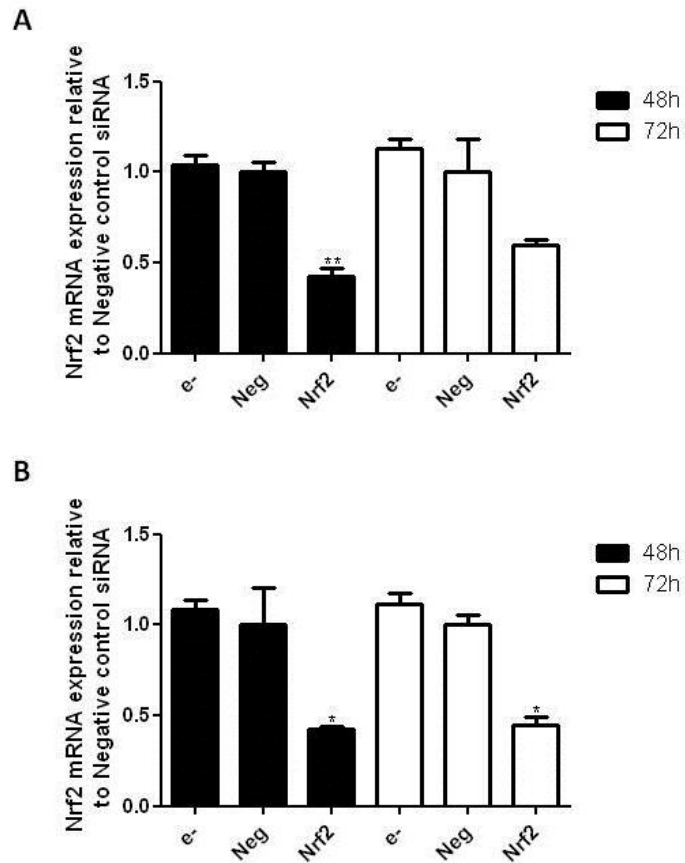


Fig 6.6. Nrf2 expression in melanoma cell lines. (A) 1×10^6 M202 cells were transfected with 200ng or **(B)** 1×10^6 SK-MEL-5 cells were transfected with 250 ng of Nrf2 or negative control (Neg) siRNA by electroporation. Electroporated cells with no siRNA (e-) were used as a transfection control. Transfected cells were seeded in 6-well plates and incubated at 37°C for 48 or 72h. Total RNA was extracted and Nrf2 mRNA expression was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, n=3; *p<0.05, **p<0.01; one way ANOVA with Tukey’s multiple comparison test.

High Nrf2 expression does not guarantee its transcriptional activation. Nrf2 is regulated at the protein level by Keap1. Furthermore, a correlation between Nrf2 and HO-1 is not necessarily indicative of Nrf2 activity, because HO-1 has other transcription factors that regulate its activity independently of Nrf2. Therefore, both M202 cells and SK-MEL-5 cells were transfected with siRNA as previously described and HO-1 mRNA expression was measured after 48h by RT-qPCR. M202 cells showed a 47% (p<0.05) inhibition of HO-1 mRNA (Fig 6.7A), and SK-MEL-5 cells showed a 39% (p<0.05) inhibition of HO-1 mRNA

(Fig 6.7B). Considering the low rates of Nrf2 knockdown achieved, this data suggests that Nrf2 is a major factor in the regulation of HO-1 in these cell lines, and confirms that Nrf2 is transcriptionally active in addition to being over-expressed.

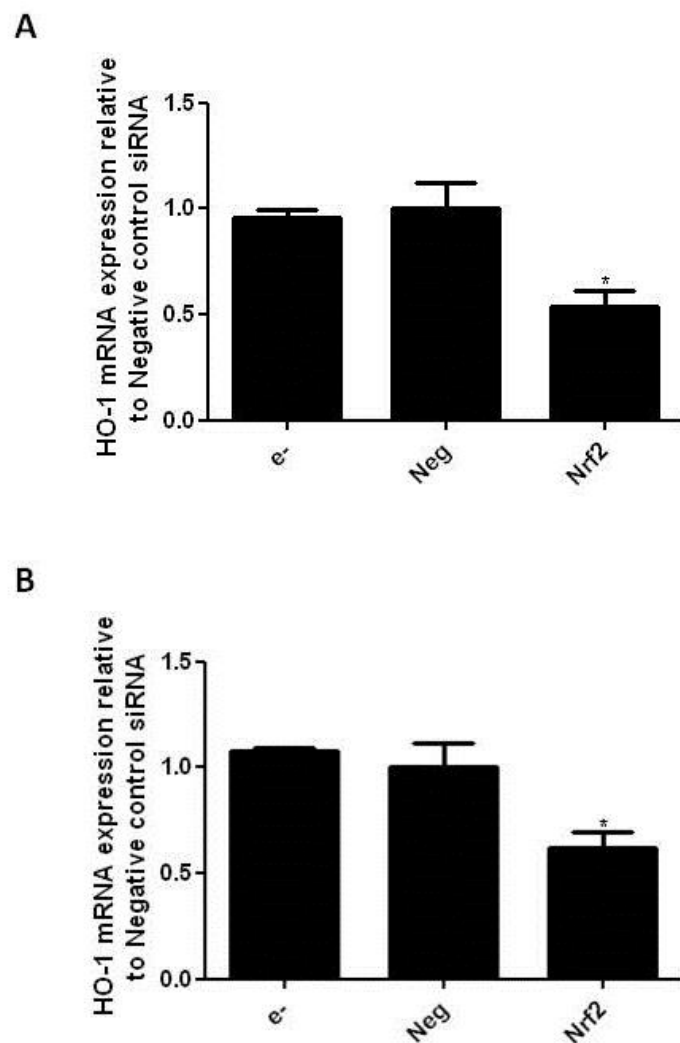


Fig 6.7. Nrf2 is transcriptionally active in M202 and SK-MEL-5 cells. (A) 1×10^6 M202 cells were transfected with 200ng or (B) 1×10^6 SK-MEL-5 cells were transfected with 250ng of Nrf2 or negative control (Neg) siRNA by electroporation. Electroporated cells with no siRNA (e-) was used as a transfection control. Transfected cells were seeded in 6-well plates and incubated at 37°C for 48 or 72h. Total RNA was extracted and HO-1 expression was measured by RT-qPCR and normalised to GAPDH. Mean \pm SEM, n=3; *p<0.05; one way ANOVA with Tukey's multiple comparison test.

These results suggest that Nrf2 in M202 and SK-MEL-5 cells is transcriptionally active, inducing target genes, which means that it may contribute to chemoresistance by upregulation of cytoprotective genes. Therefore, the effect of Nrf2 siRNA on chemoresistance was tested. M202 cells and SK-MEL-5 cells were transfected with Nrf2 or control siRNA as described above, and seeded in 96-well plates for 24h followed by dacarbazine for 48 h and cytotoxicity measured by MTS assay. There was little difference between cytotoxicity in cells that contain control siRNA or Nrf2 siRNA in either cell line, although in M202 cells cytotoxicity was slightly higher in cells containing Nrf2 siRNA (Fig 6.8A). In SK-MEL-5 cells cell viability was almost identical regardless of the siRNA used (Fig 6.8B). Two-way ANOVA analysis showed that Nrf2 siRNA was not a significant factor in the differences in cell viability in M202 or SK-MEL-5 cells. IC_{50} values could not be calculated for either cell line because cell viability was not inhibited by more than 50%. Despite the minor differences in M202 cells, cytotoxicity was not significantly increased by Nrf2 siRNA, which suggests that Nrf2 is not involved in chemoresistance to dacarbazine.

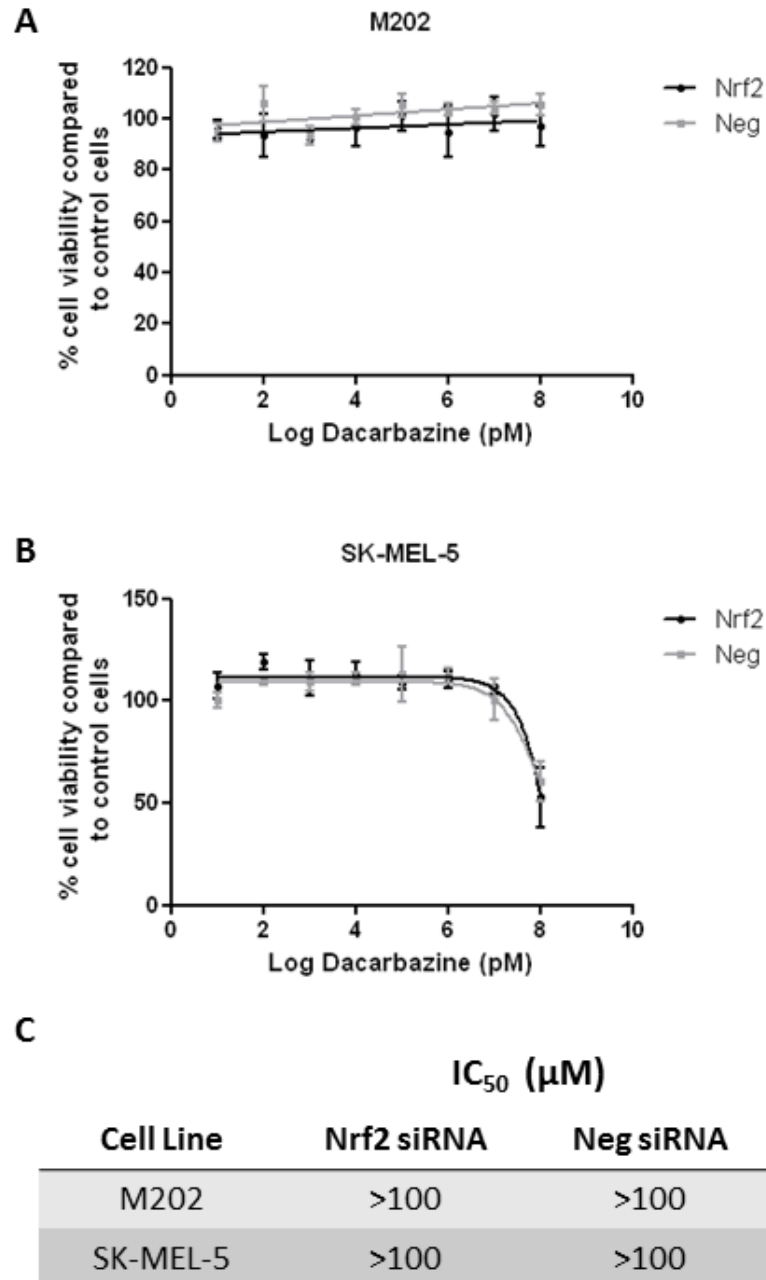


Fig 6.8. The effects of Nrf2 siRNA on dacarbazine-induced toxicity. 1×10^6 (A) M202 cells or (B) SK-MEL-5 cells were transfected with Nrf2 siRNA or negative control siRNA as previously described. Cells were seeded in 96-well tissue culture plates and incubated overnight at 37°C. Dacarbazine or DMSO was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 10 pM to 100 μM. 48h after incubation cell viability was measured by MTS assay and expressed as a percentage compared to vehicle control cells. Mean ± SEM, n=3; datasets were statistically analysed by two-way ANOVA

The role of Nrf2 in protecting melanoma cells from doxorubicin was then examined in M202 cells and SK-MEL-5 cells. In M202 cells there was little difference in cytotoxicity at concentrations of doxorubicin lower than 0.1 μM . At 0.1 μM and 1 μM cell viability was inhibited by 16% and 74% respectively in cells transfected with Nrf2 siRNA, whereas in cells containing negative control siRNA this was just 7% and 58% (Fig 6.9A). IC_{50} values for doxorubicin were calculated to be 0.72 μM in negative control cells, but 0.32 μM in Nrf2 siRNA cells, a reduction of 56% (Fig 6.9C). However, two-way ANOVA analysis of the data revealed that the differences observed in cells containing Nrf2 siRNA were not significant. In SK-MEL-5 cells a similar pattern occurred, but the effect was not as strong. IC_{50} s were calculated as 0.3 μM in negative control cells and 0.16 μM in cells containing Nrf2 siRNA. This is a smaller reduction than in M202 cells, but still stands at 41% (Fig 6.9C). As with M202 cells, two-way ANOVA analysis revealed that these differences were not statistically significant.

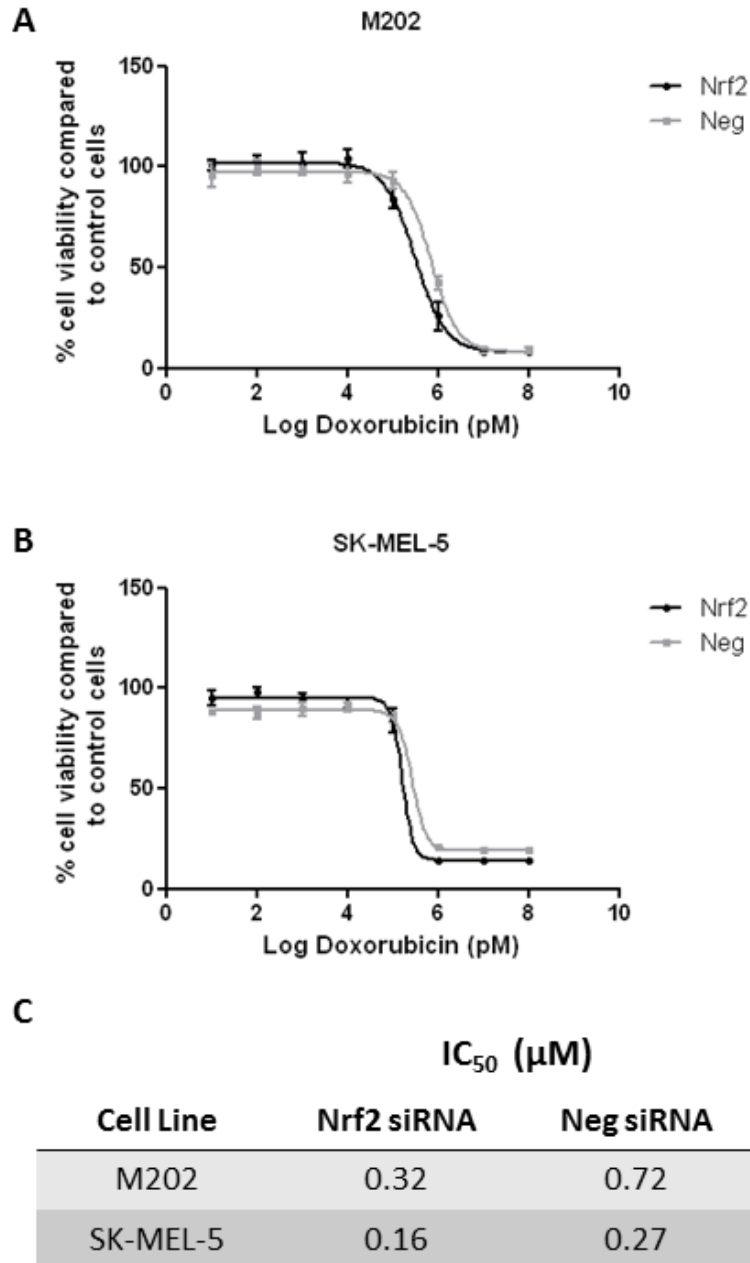


Fig 6.9. The effects of Nrf2 siRNA on doxorubicin-induced toxicity. 1×10^6 (A) M202 cells or (B) SK-MEL-5 cells were transfected with Nrf2 siRNA or negative control siRNA as previously described. Cells were seeded in 96-well tissue culture plates and incubated overnight at 37°C. Doxorubicin or H₂O was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 10 pM to 100 μM. 48h after incubation cell viability was measured by MTS assay and expressed as a percentage compared to vehicle control cells. (C) IC₅₀ values were calculated by non-linear regression. Mean ± SEM, n=3; datasets were statistically analysed by two-way ANOVA.

Due to the range of efficacy that cisplatin had, and its clinical relevance over the other active drug, doxorubicin, a tighter concentration range was used to examine the effect of Nrf2 siRNA on cisplatin-induced toxicity. This was to increase the sensitivity of the assay, and obtain more accurate IC₅₀ values for cisplatin. In both M202 cells, cisplatin was more effective in causing cytotoxic effects in cells transfected with Nrf2 siRNA than in cells transfected with negative control siRNA. Two-way ANOVA analysis showed that the reduced resistance to cisplatin in M202 cells was significant ($p < 0.01$). Nrf2 siRNA lowered the IC₅₀ of cisplatin in M202 cells by 46%, bringing the IC₅₀ of cisplatin down from 26.2 μM to 14.2 μM (Fig 6.10A and C). In SK-MEL-5 cells Nrf2 siRNA did not significantly affect cisplatin-induced toxicity. The IC₅₀ of cisplatin was 40.8 μM in control cells and 27.9 μM in cells containing Nrf2 siRNA (Fig 6.10B and C).

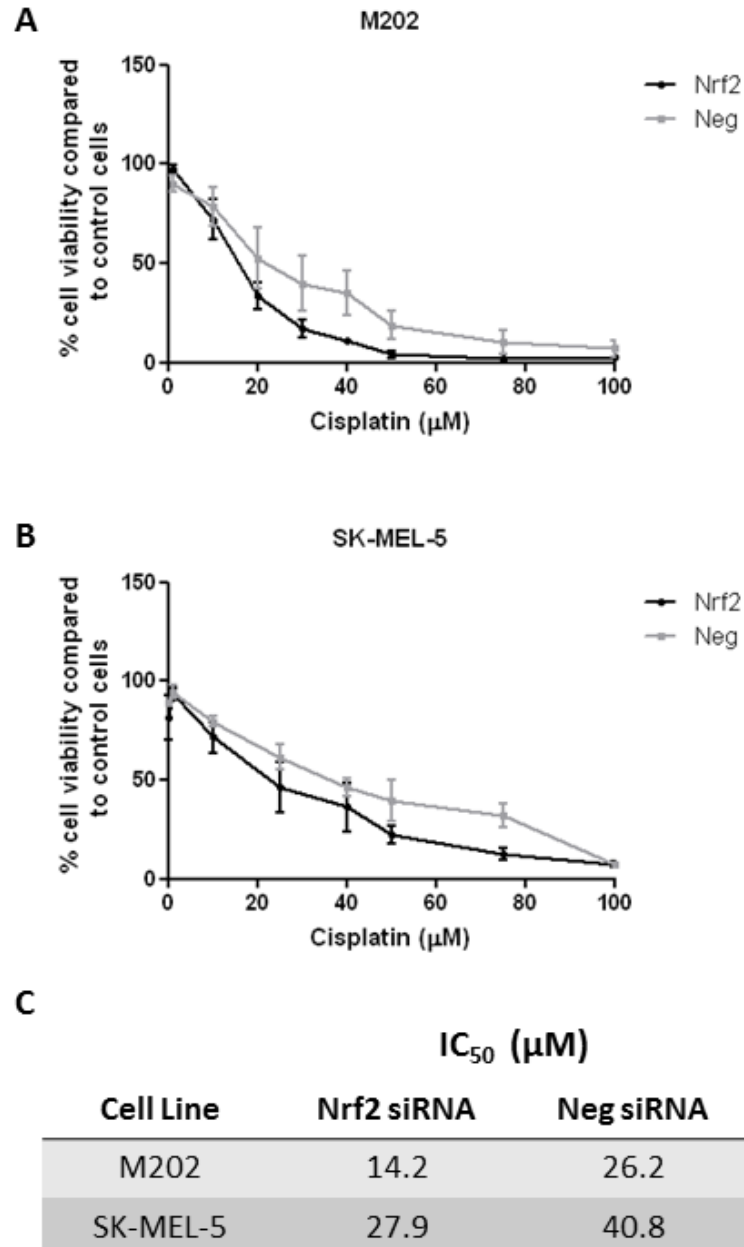


Fig 6.10. The effects of Nrf2 siRNA on cisplatin-induced toxicity. 1×10^6 (A) M202 cells or (B) SK-MEL-5 cells were transfected with Nrf2 siRNA or negative control siRNA as previously described. Cells were seeded in 96-well tissue culture plates and incubated overnight at 37°C. Cisplatin or PBS was added to the cells in triplicate at 8 concentrations in 10-fold increments ranging from 0.1 µM to 100 µM. 48h after incubation cell viability was measured by MTS assay and expressed as a percentage compared to vehicle control cells. (C) IC₅₀ values were calculated by non-linear regression. Mean ± SEM, n=3; datasets were statistically analysed by two-way ANOVA.

6.3.4 Cisplatin upregulates ROS and the Nrf2 pathway in M202 cells.

This data suggests that Nrf2 plays a role in protecting M202 cells from cisplatin chemotherapy. However, the mechanisms behind this role of Nrf2 are unknown. One possibility is that cisplatin induces Nrf2 expression via ROS induction. Therefore, H₂DCFDA was used as a probe to measure ROS production in M202 cells in response to cisplatin stimulation. 50 μ M cisplatin induced ROS by 3.2-fold over control cells ($p < 0.001$), and at 100 μ M ROS was increased by 4.1-fold (Fig 6.11; $p < 0.001$).

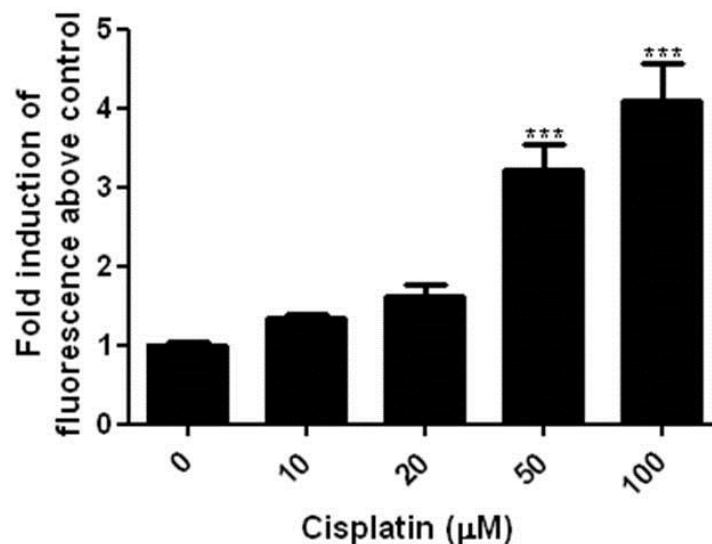


Fig 6.11. Cisplatin induces ROS in M202 cells. M202 cells were seeded in a black 96-well plate. Cells were allowed to grow to 80% confluency over 48h. Media was removed and replaced with H₂DCFDA in PBS for 40 min. H₂DCFDA was removed and replaced with media for 20 minutes. Fluorescence was read at 485/520 nm. The indicated concentration of cisplatin was added to the cells for 4h. Fluorescence was measured at 485/520 nm. Mean \pm SEM, n=3; *** $p < 0.001$; one way ANOVA with Tukey's multiple comparison test.

ROS are known to signal upstream of Nrf2 (Numazawa et al., 2003). It is possible that although Nrf2 is overexpressed in M202 cells, it can also be induced by cisplatin. In addition to basal levels of Nrf2, upregulation of Nrf2

could also occur, which would contribute to chemoresistance observed in M202 cells. Therefore, M202 cells were incubated with 5 μ M cisplatin for up to 8h. Samples stimulated by cisplatin had elevated Nrf2 after 6h, and Nrf2 expression remained high at 8h (Fig 6.12A). To test whether HO-1 expression was also increased, M202 cells were stimulated with cisplatin by the same method for up to 24h. HO-1 expression was measured by Western blot. The results showed that HO-1 was elevated at 24h (Fig 6.12B). These results support the idea that cisplatin activity is combated by Nrf2 activity that is both present at high concentrations basally, but also induced by the drug itself.

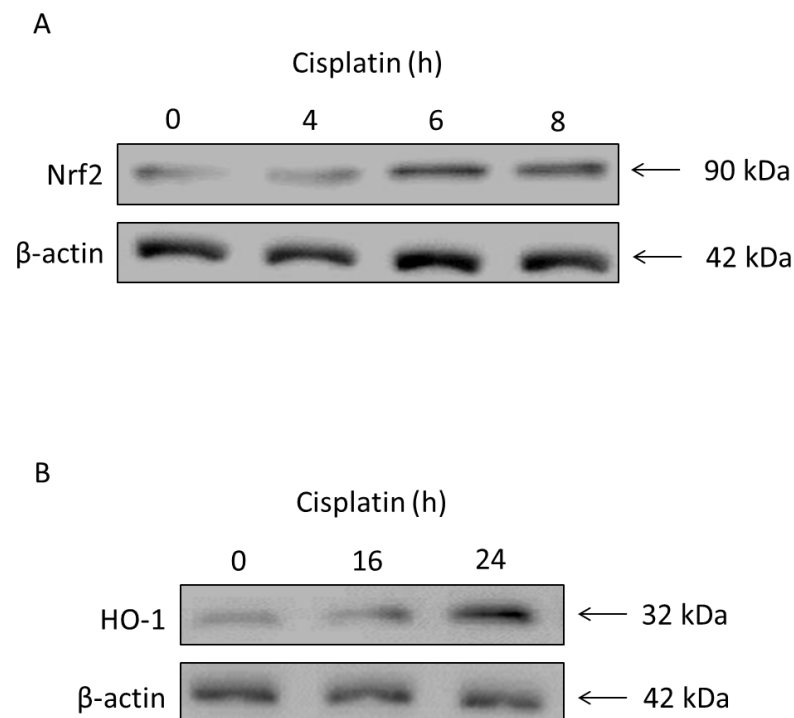


Fig 6.12. Cisplatin induces Nrf2 and HO-1 in M202 cells. M202 cells were seeded in 6 well plates at a density of 0.5×10^6 per ml and allowed to grow to 80% confluency. 5 μ M cisplatin was added for the indicated time period. Cell lysates were prepared and samples were analysed by western blot for **(A)** Nrf2 or **(B)** HO-1. β -actin was used as a loading control. Data shown is representative of three independent experiments; replicates are shown in Appendix II.

6.4 Discussion

Malignant melanoma is the most deadly form of skin cancer, largely because it is resistant to non-surgical forms of therapy. Nrf2 has been linked with chemoresistance in several cancers including lung, breast and endometrial cancer (Jiang et al., 2010; Chen et al., 2012; Zhan et al., 2012). In this study Nrf2 expression, activity, and its effect on chemoresistance was investigated in a selection of 11 melanoma cell lines. Nrf2 was found to be expressed highly in all cell lines compared to melanocytes. The Nrf2 target gene HO-1 was found to be over-expressed in three cell lines at mRNA and protein level. 5 cell lines with varying levels of Nrf2 expression were selected for toxicity experiments using anti-cancer drugs. Chemoresistance to dacarbazine, doxorubicin and cisplatin correlated with Nrf2 expression. Doxorubicin was the most effective drug in all 5 cell lines, whereas dacarbazine was barely effective at all. Nrf2 siRNA increased the susceptibility of M202 cells to cisplatin, suggesting that Nrf2 plays a role in chemoresistance in melanoma. However, Nrf2 siRNA did not significantly affect melanoma cells treated with doxorubicin or the standard treatment, dacarbazine. The mechanisms by which Nrf2 might protect against cisplatin are unknown. This study showed that cisplatin induces ROS production in M202 cells and it also upregulates Nrf2 and HO-1 protein expression, although more work is required to put these experiments into context.

Nrf2 was found to be highly expressed in all melanoma cell lines, as measured by Western blot. Interestingly, none of the cell lines had lower Nrf2 expression than the melanocytes, which would be an expected result if Nrf2 depletion was a significant cause in the onset of melanoma. No loading control for the Western blots was provided because its expression cannot be assumed to be constant between different cell lines. However, cell numbers and total protein loaded onto the gel was controlled for, and the results were

repeatable by densitometry, indicating that the data obtained is reliable. In addition the data correlated well with NQO1 mRNA expression, which was measured by PCR. HO-1 expression did not correlate well, although this may be because of other factors in its regulation. SK-MEL-28 cells, for example, were rejected for further work because, although they had high HO-1 expression, they have a known mutation in the HO-1 promoter that enhances its expression (Okamoto et al., 2006).

The cells that were selected for further research were chosen mainly for their Nrf2 expression, but also for their ability to induce NQO1 (UACC 1273), HO-1 (SK-MEL-5), or both (M202). A375 and M238 cells were selected because they both had low expression of Nrf2 and its target genes.

Dacarbazine is the current standard chemotherapeutic treatment for metastatic melanoma. It had poor efficacy in the melanoma cell lines, although was least effective in those that expressed Nrf2. Despite this, Nrf2 siRNA had no effect on dacarbazine-induced cytotoxicity, which suggests that if an Nrf2 inhibitor does have a role to play in the treatment of melanoma it is not as a supplement to dacarbazine. It is possible that selection bias could be a reason for these findings. If these cell lines were derivatives of those taken from melanoma patients, it is possible that the patients have already received a failed dacarbazine treatment regime. Furthermore, the fact that the treatment regime has failed may be the very reason the cells were gathered in the first place. In all probability, however, it is more likely that the resistance to dacarbazine observed in this study is an accurate representation of the current state of melanoma treatment. All clinical evidence points to the fact that dacarbazine is a poor drug. Patients who are treated with dacarbazine have a median survival of about 9 months, and the objective response rate is reported to be 10-12% (Patel et al., 2011; Bedikian et al., 2014).

Doxorubicin was a much more effective drug than dacarbazine in these assays. IC_{50} values were below 1 μ M in all cell lines. The cell lines showed the same pattern, with those expressing high amounts of Nrf2 being more resistant than those that do not. Nrf2 siRNA transfected cells were not significantly more susceptible to doxorubicin than negative control siRNA. This suggests that Nrf2 does not play a role in protecting the cells against doxorubicin. However, it may be prudent to repeat the experiment using a tighter concentration range of doxorubicin between 0.1-1 μ M because there is a slight variation between Nrf2 siRNA and negative control siRNA at these concentrations. Unlike dacarbazine, doxorubicin is not currently used to treat melanoma, which brings into question the clinical significance of this portion of the study. The reasons for the limited use of doxorubicin are that it has serious toxic effects on non-cancerous tissue, and poor delivery to target tissue due to clearance from the circulatory system (Zheng et al., 2010). However, a lot of research is currently being done on drug delivery mechanisms which have been shown to improve the effectiveness of doxorubicin in melanoma *in vivo*, while decreasing non-specific toxicity (Zheng et al., 2010; Pegoraro et al., 2013; Wang et al., 2014e). If these advancements overcome the weaknesses of doxorubicin, evidence in this study and others suggests that it is a more promising drug than dacarbazine in terms of effectiveness against melanoma cells. Combining drug delivery research with mechanisms to increase the effect of doxorubicin may be a realistic option for the future of chemotherapy against melanoma.

The IC_{50} of cisplatin, like doxorubicin and dacarbazine, was lower in cells containing less Nrf2. Nrf2 siRNA had lowered the IC_{50} by 46% in M202 cells, showing that Nrf2 plays a role in protecting the cells from cisplatin. Cisplatin was more effective than dacarbazine, but less so than doxorubicin. Despite this, cisplatin is a more clinically relevant drug than doxorubicin. It can be used in combination therapy with dacarbazine or temozolamide (Chapman et

al., 1999; Alrwas et al., 2014), although clinical trials have not shown any benefit over dacarbazine treatment alone. Despite this, clinical trials involving cisplatin are still occurring (Lian et al., 2013; Alrwas et al., 2014), and increasing susceptibility of melanoma cells to cisplatin may be a valid strategy for the future treatment. *In vivo* studies in other cancers support this method of chemotherapy (Ji et al., 2013b; Jiang et al., 2013), and this study suggests that inhibiting Nrf2 activity may be one way of achieving it in melanoma.

Cisplatin is believed to work by several methods. The classical mechanism is by crosslinking DNA, and the others include production of ROS and activation of inflammatory mediators (Casares et al., 2012; Enoiu et al., 2012). The mechanism by which Nrf2 protects against cisplatin induced toxicity is unknown but, as a broad protective transcription factor it could conceivably block any of the known mechanisms.

Nrf2 is known to be induced by, and protect against ROS (Numazawa et al., 2003; Kombairaju et al., 2014). The data in this study that shows an induction of the Nrf2 pathway and ROS in M202 cells correlates with the idea that this is the primary mechanism. In addition recent research on Nrf2 and melanoma suggests that Nrf2 siRNA increases cisplatin-induced ROS, although a causal relationship between ROS inhibition and increased susceptibility to cisplatin was not shown (Miura et al., 2013).

As detailed in previous chapters, Nrf2 plays an important role in protecting against inflammation. A study in mice has linked Nrf2 and HO-1 to cisplatin resistance by inhibiting induced inflammatory mediators (So et al., 2008)

Nrf2 has also been shown to increase DNA repair in response to UV damage in skin cells (Rodriguez et al., 2013), and can upregulate the base excision repair enzyme, OGG1 (8-oxoguanine DNA glycosylase) (Singh et al., 2013a).

Furthermore, well-established blockers of apoptosis are also connected with the Nrf2 pathway. One of these is Bcl-2, which is a target of Nrf2 transcription (Niture and Jaiswal, 2012).

In addition to blocking mechanisms of action by cisplatin, Nrf2 also upregulates multidrug resistance transporters; cisplatin may be neutralised by these before its downstream mechanisms begin (Vollrath et al., 2006; Aleksunes et al., 2008).

A major issue with Nrf2 as a cancer target is the lack of compounds that inhibit its activity, and the few that do are non-specific. Both brusatol and trigonelline have been shown to increase susceptibility of cells to chemoresistance through Nrf2 inhibition (Boettler et al., 2011; Ren et al., 2011; Arlt et al., 2013). Perhaps the most important future work is to elucidate the mechanisms by which Nrf2 is over-expressed, because this will largely determine the kinds of drugs that would work. For example, brusatol is thought to work by increasing Nrf2 ubiquitination, but this mechanism of Nrf2 inhibition may not work in cells where Keap1 does not bind Nrf2 in the first place.

Current research suggests that Nrf2 can become over-expressed by numerous mechanisms. These mechanisms are still under investigation, but they include mutations of Nrf2 and Keap1, and silencing Keap1, disrupting Nrf2 degradation and resulting in elevated Nrf2 expression.

Although our laboratory has previously shown that Nrf2 can be regulated at the mRNA level, this study shows little difference in Nrf2 mRNA expression between the melanoma cell lines (Rushworth et al., 2008). Although Nrf2 mRNA regulation cannot be ruled out as a hypothetical possibility, there is currently no evidence to suggest that it plays a role in the over-expression of Nrf2 in cancer. Nrf2 has been shown to be mutated, however. Mutations of

the DLG and ETGE motif of Nrf2 have been found in around 10% of lung cancer patients, resulting in its increased expression (Shibata et al., 2008).

There has been much work on the role of Keap1 in cancers, and it has been found that Keap1 can be silenced or mutated, both of which result in a decrease in Nrf2 degradation. Mutations in the IVR/kelch domain of Keap1 have been identified in a number of cancers including adenocarcinoma, ovarian, and endometrial cancers (Padmanabhan et al., 2006; Konstantinopoulos et al., 2011; Wong et al., 2011). In addition, a recent paper identified a Keap1 mutation in melanoma cells (Miura et al., 2013). Silencing of Keap1 has been shown to occur by microRNA and by hypermethylation of its promoter. The latter has been observed in lung cancer cells and prostate cancer cells (Wang et al., 2008b; Zhang et al., 2010; Eades et al., 2011).

Sequestosome 1 is a scaffold protein involved in inducing degradation of ubiquitinated proteins. It contains an STGE motif that binds to Keap1 in the same binding pocket as Nrf2's ETGE sequence. Overexpression of sequestosome 1 is believed to result in overexpression of Nrf2 by competing for Keap1, and inducing Keap1 degradation. Evidence for this has been provided by silencing of sequestosome 1, which caused increased Keap1 expression and a reduction of Nrf2 activity. There is not yet evidence that sequestosome 1 plays a role in Nrf2 upregulation in cancer (Cople et al., 2010).

The numerous mechanisms by which Nrf2 can be over-expressed limit the number of possibilities for effective drug targets. Ignoring difficulty and monetary factors, the most obvious solution is to simply block Nrf2/ARE binding. However, Nrf2/MAF binding or Nrf2 mRNA production are also potentially targets for drug design.

There were several limitations in this study. The use of cell lines provided a broad range of data, but require confirmation in patient samples. Furthermore, cancers are known to adapt to chemotherapy, becoming resistant to treatment over time, and the treatment regimen of the patients these cells were derived from is unknown (Duru et al., 2014; Sun et al., 2014). This is especially relevant with dacarbazine, which was the least effective of the three drugs and the most used in the treatment of melanoma.

Future work investigating the role of Nrf2 in melanoma could go in several directions. Firstly, the work in this study can be extended and verified. There are other drugs used in melanoma chemotherapy, such as temozolomide which Nrf2 may have an effect on. The effect of the Nrf2 pathway on immunotherapy is also unknown. Further investigation into the role of Nrf2 target genes is also important. There is not currently an Nrf2 inhibitor, but if specific target genes can be identified as drug targets, inhibiting them may have the same effect. The work can be verified by extending the study into human tissue to examine Nrf2 expression, and mutations in the Nrf2 pathway. Animal studies may also be useful, although they would be a better option if a specific Nrf2 inhibitor was widely available. Tangential, but still relevant to this project would be the development of such an inhibitor.

In conclusion, Nrf2 has been found to be over-expressed by varying degrees in 11 melanoma cell lines. Cell lines with more Nrf2 are more chemoresistant to dacarbazine, doxorubicin, and cisplatin than those with less Nrf2. Knocking down Nrf2 using siRNA increases susceptibility of M202 cells to cisplatin, but not dacarbazine or doxorubicin. Therefore, this study suggests that Nrf2 plays a role in the high resistance to chemotherapy observed in malignant melanoma treatment. These conclusions support the need for research into inhibition of the Nrf2 pathway as a potential therapeutic target.

Chapter 7: General Discussion

Nrf2 is an important subject of medical research. The protective effects of Nrf2 are extremely well documented. Nrf2 knockout mice are more susceptible to oxidative damage (Kurzatkowski et al., 2013), diabetic retinopathy (Xu et al., 2014b), tumourigenesis, inflammation (Cheung et al., 2014), and bacterial infection (Reddy et al., 2009). The functions of Nrf2 are simultaneously diverse and monotonous. On one hand Nrf2 induces transcription of a wide variety of genes, regulating a wide variety of processes. On the other hand almost all of these processes, even those that are harmful to the organism, are cytoprotective (Joshi and Johnson, 2012; Gupte et al., 2013; Turpaev, 2013; Xiang et al., 2014). In addition to being protective, the way in which it is regulated makes it ideal for targeting. It is primarily regulated by its interaction with Keap1, and is highly inducible. Therefore, Nrf2 activators are of interest for their therapeutic potential. Cancer is an exception to the benefits of Nrf2. In cancer, aberrant activation of Nrf2 can lead to protection of cancer cells from chemo- and radiotherapy (Itoh et al., 1999; Shibata et al., 2008a; Singh et al., 2010b).

This study investigated the protective role of Nrf2, mechanisms of targeting it in the diet and inflammation, and the consequences of its overexpression in cancer. Diallyl polysulfides are examined in THP-1 monocytes to examine HO-1 induction, and it was found that long-chain diallyl polysulfides are more effective than short chain polysulfides. DAPS was identified and characterised as a novel activator of the Nrf2 pathway. However, it failed to prevent H₂O₂-induced cytotoxicity or LPS-induced TNF expression. This suggests that powerful Nrf2 activation is not sufficient to provide Nrf2-mediated protection, perhaps due to lack of target specificity. TAT-14 is a 14 amino acid long peptide that mimics the ETGE Keap1-binding sequence of Nrf2 conjoined to a TAT cell penetration sequence. It was shown to bind Keap1 using a fluorescence polarization assay, and activate the Nrf2 pathway in THP-1 monocytes. Unlike DAPS, it did inhibit LPS-induced TNF and IL-1 β

expression. This suggests that targeting the Nrf2/Keap1 interaction by competitive inhibition is a preferable mechanism of activation. To aid these studies into the protective role of Nrf2, two luciferase assays were developed to identify inducers of Nrf2 and inhibitors of the inflammatory mediator, NF- κ B. Finally, this study examined Nrf2 expression in melanoma, and found that Nrf2 and its target genes can be overexpressed in melanoma cell lines. 5 cell lines were carried forward, and it was found that cells that overexpress Nrf2 were more resistant to chemotherapeutic drugs than those with low Nrf2 expression. Furthermore, Nrf2 siRNA rendered M202 cells susceptible to cisplatin, and had a similar effect in SK-MEL-5 cells. These results suggest that an Nrf2 inhibitor may be useful to use in combination with chemotherapy.

The role of Nrf2 in inhibiting inflammation is well documented (Ma et al., 2006; Osburn et al., 2008; Rushworth et al., 2008; Kong et al., 2010). There have been plenty of studies investigating the effects of natural products on Nrf2 activation in inflammation, and *in vitro* studies have been promising. Quercetin, sulforaphane and curcumin are all Nrf2 activators that have been used to inhibit inflammation *in vivo* and *in vitro* (Carmona-Ramírez et al., 2013; Soetikno et al., 2013; Wu et al., 2013; Zhao et al., 2013b; Ramyaa et al., 2014). However, there is little evidence for the anti-inflammatory capability of these compounds when they are used in clinical trials. Quercetin has shown mild effects on blood pressure, but did not affect inflammatory mediators in two studies (Pfeuffer et al., 2013; Zahedi et al., 2013). In clinical trials the role of curcumin remains unclear, and is better noted for studies into cardiovascular conditions. In one study, 500mg/day curcumin over seven days was shown to decrease serum cholesterol levels and increase plasma antioxidant capacity. However, 6g/day curcumin did not have a strong protective effect (Pungcharoenkul and Thongnopnua, 2011). Another study in elderly patients showed no change in cholesterol levels after 6 months of curcumin supplementation (Baum et al., 2007).

Currently, the most promising is the organosulfur compound sulforaphane, which has been linked with positive antioxidant effects in humans, and may protect human gastric mucosa against *helicobacter pylori* infection. In the latter case, a mouse model was used to show that this may be due to anti-oxidative and anti-inflammatory mechanisms which are dependent on Nrf2 (Yanaka et al., 2009; Kensler et al., 2012).

There are other promising compounds however, that warrant further research. These are the organosulfur compounds found in garlic. Allicin is the precursor of diallyl polysulfides, and has been used in human studies. It has been shown to reduce inflammatory mediators involved in exercise-induced muscle damage, including IL-6, in athletes (Su et al., 2008). It has also been shown to reduce the size of mouth ulcers (Jiang et al., 2012). Diallyl polysulfides are thought to be the bioactive components of crushed garlic, and may exert their activity via Nrf2 activation (Chen et al., 2004). Diallyl polysulfides are fairly stereotypical Nrf2-activating natural products. They are reported to inhibit oxidative stress (Kuo et al., 2013; Zeng et al., 2013), but also signal through ROS production (Gong et al., 2004; Chandra-Kuntal et al., 2013). Animal studies suggest that consumption of these molecules through the diet may be highly beneficial to long term health (Kalayarasan et al., 2008; Kalayarasan et al., 2009).

This study showed that with the lengthening of the polysulfide chain, diallyl polysulfides activate the Nrf2 pathway more effectively, but this does not translate to increased protection against ROS induced cytotoxicity or LPS-induced TNF. This does not mean that DAPS has no positive effects whatsoever – untested effects that have been associated with diallyl polysulfides include protection against cardiac dysfunction (Huang et al., 2013b), lung disease (Sapkota et al., 2014), and inhibition of angiogenesis (Thejass and Kuttan, 2007). The fact that diallyl polysulfides have been

associated with these effects indicates that they are molecules worth pursuing, but this research suggests that the correlation between chain length and Nrf2 activation does not predict any correlation between chain length and cytoprotection. Research should continue to compare polysulfides to find which ones have the most beneficial effects. There is probably an optimal chain length that activates protective pathways while not causing too much damage. Furthermore, longer diallyl polysulfides may have a greater anti-bacterial or anti-cancer effect, which is likely to work via pro-apoptotic pathways rather than cytoprotective pathways (Ma et al., 2014).

Garlic contains many compounds in addition to diallyl polysulfides (Lawson et al., 1993), so while it is important to find the active ingredients, dietary studies are more reliable if they use garlic or garlic oils instead of the individual constituents. Testing this *in vivo* can ensure that the experiment is representative of the diet. Some of the constituents of garlic may have additive effects, or may interfere with the health benefits. Another factor that this methodology takes into account is that oral delivery is possible, so bioavailability can be measured.

One way of taking high amounts of specific molecules is dietary supplements. These have their own problems. In principle they may be a good idea, and would be a more practical way of getting specific nutrients. They solve the problem that food contains other molecules, and allow for optimisation of individual components to maximise health benefits. The problem with them is that current laws determining their regulation are poor. The problems customers face can include mislabelling of products, contaminants, and incorrect health claims which are often based upon shoddy evidence or miscommunication between researchers and laypeople (Petroczi et al., 2011).

Although there have been mixed results for Nrf2 activators in nutritional research, Nrf2 itself has consistently been shown to have value as a protective mediator. Therefore, there has been much interest in targeting it therapeutically. Factors that matter in drug design are slightly different to nutritional research. Drugs are designed to do specific jobs during diseases to alleviate symptoms and combat the source of the disease. Unlike compounds consumed through the diet, drugs are desired to have a strong acute effect, and are delivered as purified molecules at known concentrations. The most important difference between medicine and the diet, therefore, is targeting.

Nrf2 is a good target because of both its regulation and function. It has a very clear primary regulator in Keap1, and the Nrf2/Keap1 interaction has been very well characterised, even if the mechanisms of exactly how Nrf2 is regulated by the interaction are still under investigation (Tong et al., 2006; Baird et al., 2013). There are two notable examples of drugs that target the Nrf2/Keap1 interaction. One is dimethyl fumarate, and the other is bardoxolone methyl. Dimethyl fumarate is thought to have neuroprotective effects via Nrf2 activation. It was approved this year by the Food and Drug Administration in the USA for treatment of multiple sclerosis after successful clinical trials (Linker et al., 2011; Ruggieri et al., 2014). In contrast, when bardoxolone methyl was used in clinical trials for stage 4 chronic kidney disease, the trial was stopped due to patient deaths (de Zeeuw et al., 2013), despite early work showing beneficial effects (Tran et al., 2008). The adverse events were cardiovascular in nature, and may be due to increased fluid retention and increased blood pressure (Chin et al., 2014). The problem with bardoxolone methyl is that it is not a specific activator of the Nrf2 pathway, and is therefore liable to have unpredictable side effects (Gao et al., 2013). It has even been argued that although the specific effects may have been unpredictable, the fact that bardoxolone methyl induces ROS and cellular stress indicates that harmful side effects were extremely likely (Thomas,

2012; Rossing, 2013; Zhang, 2013). It is important to note that there is no reason to believe that dimethyl fumarate is a specific Nrf2 activator, either (Chen et al., 2013; Zhao et al., 2014). These trials show that Nrf2 is clearly a good drug target, but there is a tendency for Nrf2 activators to activate non-specific pathways, and the side effects can be lethal.

An alternative approach is to design competitive inhibitors of the Nrf2/Keap1 interaction. By mimicking the features of the Keap1-binding sequence of Nrf2, it should reduce off-target effects barring any that Nrf2 would have itself. Targeting Nrf2 by inhibiting its interaction with Keap1 can be carried out with reasonable specificity, and low toxicity (Hancock et al., 2012; Tian et al., 2012). This study tested a cell-penetrating peptide mimicking the Keap1-binding sequence of the Neh2 domain in THP-1 monocytes. The data shows that the peptide induces Nrf2 expression and subsequent transcription of known Nrf2 target genes. It also inhibited LPS-induced TNF production. The most important insight that can be gained from this research is that targeting the Nrf2/Keap1 interaction works in cell culture, and lowers expression of an inflammatory mediator. TAT-14 can be used as a tool for future research to simulate the effects of a more drug-like molecule. Another aspect of future work is to enhance drug delivery mechanisms, which may enhance the effectiveness of a peptide-drug. It will not solve the problem of poor metabolic stability of peptides, however, so a small molecule inhibitor is still a preferable option. It should be noted, however, that although it is often thought that peptides make poor drugs, they do reach the market, and a similar Nrf2-targeting peptide has been shown to protect against brain damage *in vivo* (Zhao et al., 2011; Craik et al., 2013).

Since this work started there has been a lot of progress in the search for a small molecule inhibitor of the Nrf2/Keap1 interaction. The peptide sequence of Keap1 binding proteins has been tested, and a hybrid sequence combining

elements of the Nrf2 and SQSM1 binding domains has been shown to improve binding efficiency over both native sequences *in vitro* (Hancock et al., 2012). The best success, however, has been from large scale screening studies. So far three small molecules have been identified that can displace the native Nrf2 sequence in a fluorescence polarization assay and induce ARE-dependent gene expression in cell culture models. Two of these came from screening libraries of 300, 000 compounds and the other from a library of 250, 000 compounds (Wang et al., 2012a; Jiang et al., 2014; Zhuang et al., 2014). There has been no clinical success for these molecules, and beneficial effects have not been shown.

This study has shown that an effect of blocking the Nrf2/Keap1 interaction with TAT-14 is to inhibit pro-inflammatory cytokine production. A long term prospect is the possibility of using TAT-14 or a similarly constructed peptide as a drug to use in inflammatory diseases. Nrf2 deficiency causes lupus-like symptoms and increases susceptibility to sepsis in mice, so a murine model of sepsis may be a good starting point for such a project (Ma et al., 2006; Kong et al., 2010).

However, this barely scratches the surface of the numerous benefits targeting Nrf2 may have. Future work can be done with TAT-14 to investigate these effects. This work can study the protective role of Nrf2 in other diseases, such as airway diseases or cardiovascular diseases. There is also the issue of other Keap1 binding proteins. Inhibiting the Nrf2/Keap1 interaction may have effects on IKK β , Bcl-2, or SQSM1 signalling, and this warrants further experimentation (Cople et al., 2010; Tian et al., 2012). Arguably the most pressing concern is the bardoxolone methyl clinical trial (de Zeeuw et al., 2013). It is assumed that the adverse side effects were due to its non-specificity, and although this is very likely, it is not certain. TAT-14 should have none of these side effects, and could be used alongside bardoxolone

methyl in studies *in vitro* and *in vivo* to investigate likely causes of the side effects, and whether Nrf2 activation is likely to be involved. If Nrf2 activation is the cause of the side effects, TAT-14 will mimic the effects of bardoxolone methyl and it is extremely important to find this out before further clinical trials take place.

The current state of the field is quite promising. Small molecules are being found, and attempts to validate Nrf2 as a drug target using peptides have been successful. To continue, targeting Nrf2 activation for medicinal purposes should focus on direct inhibitors and move away from molecules that induce Nrf2 expression by reacting with cysteine residues on Keap1 in order to maximise the effectiveness and safety of the drug.

There is an exception to the protective roles of Nrf2, and this is during cancer. In a healthy individual Nrf2 protects against the onset of cancer by detoxifying carcinogens. During cancer its protective utility is hijacked, and used to protect cancer cells from damage. This can cause radio- and chemoresistance. This study investigated the role of Nrf2 in malignant melanoma, and showed that all 11 cell lines tested contained more Nrf2 than melanocytes. Some of these cell lines also showed high levels of transcription of HO-1 or NQO1. Others did not. 3 cell lines with high Nrf2 expression and HO-1/NQO1 expression were carried forwards, as were 2 cell lines which had comparatively low expression. The cells highly expressing Nrf2 were more resistant than those that don't to dacarbazine, cisplatin, and doxorubicin. Nrf2 was silenced in two of the cell lines that have high Nrf2 expression, M202 and SK-MEL-5 cells. Nrf2 siRNA sensitised M202 cells to cisplatin, and had similar effects in SK-MEL-5 cells that didn't reach statistical significance. These results suggest that Nrf2 protects melanoma cells against chemotherapy, but it does not protect against the standard treatment dacarbazine, or doxorubicin.

There are a number of mechanisms by which Nrf2 may be contributing to chemoresistance against cisplatin, and future work could focus on establishing what these are (Fig 7.1).

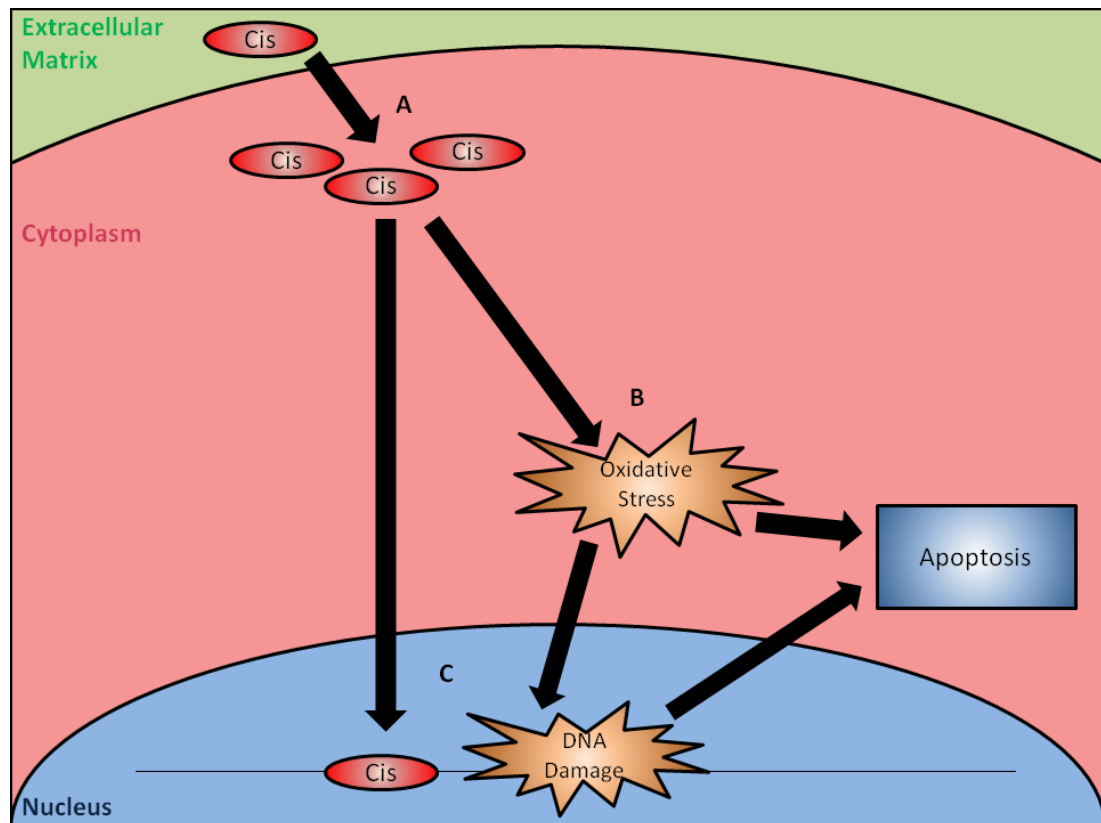


Fig 7.1. Cisplatin-induced apoptosis. Cisplatin induces apoptosis by crosslinking DNA directly, or by inducing oxidative stress. Nrf2 could potentially inhibit cisplatin-induced apoptosis at many places along the pathway, but current knowledge of Nrf2 activity suggests that the most likely mechanisms are by **A)** inhibiting cisplatin cellular accumulation, **B)** inhibition cisplatin-induced oxidative stress, or **C)** inhibiting cisplatin-induced DNA damage.

There is some indirect evidence that Nrf2 may inhibit cisplatin by facilitating its cellular export. Nrf2 is known to induce multidrug-associated resistance proteins, and their role in inhibiting the effects of cisplatin is controversial. For example, one study shows that MRP2 protects against cisplatin-induced kidney damage in mice, and inhibits build-up of platinum (Wen et al., 2014).

In direct contrast, another study has shown that MRP2^{-/-} mice did not have any effect on cisplatin activity and that in human cancer patients there was no correlation between MRP2 polymorphisms and cisplatin toxicity (Sprowl et al., 2012). Cisplatin exerts its toxic effects by two key methods. The first is by crosslinking DNA directly, and the second is by producing ROS, which can cause both DNA and general cellular damage. Enough damage causes apoptosis. Unfortunately, Nrf2 inhibits apoptosis, and there are several mechanisms by which the Nrf2 pathway may protect cancer cells from cisplatin-induced apoptosis. Nrf2 is a known antioxidant, and there is some evidence suggesting that Nrf2-induced target genes, such as HO-1, block cisplatin-induced ROS (Kim et al., 2008; Kim et al., 2009). Nrf2 is also known to upregulate some DNA repair enzymes, such as OGG1 (Singh et al., 2013a) and anti-apoptotic mediators, such as Bcl-xL and Bcl-2 (Niture and Jaiswal, 2012; Niture and Jaiswal, 2013).

Inhibition of Nrf2 likely does not have an effect on its own, but the evidence provided in this study and others suggests that Nrf2 inhibition may be a valid strategy to enhance chemotherapy. Combination treatments are fairly common (Daponte et al., 2013; Robert et al., 2013; Lazaryan et al., 2014; Suzuki et al., 2014), and could be considered a long term target for the use of a drug targeting Nrf2.

If Nrf2 inhibition is ever to be used in chemotherapy the most important project that needs to be done is to find inhibitors of the Nrf2 pathway. Melanoma is not the only cancer to be protected by the Nrf2 pathway, suggesting that Nrf2 inhibition could be a way of increasing the effectiveness of chemotherapy in many cancers. There are no Nrf2 inhibitors currently available, and the little research into them has used molecules that are not specific. Hypothetically, there are several possible sites of Nrf2 inhibition that could make good drug targets.

The first possibility is to block Nrf2 production. Little is known about regulation of Nrf2 production, but there are interesting leads, which could merit further investigation. The Nrf2 promoter contains a sequence that can form a G-quadruplex, and it is possible that regulation of this structure could affect Nrf2 mRNA production (Waller et al., 2014). MicroRNAs have been shown to regulate Nrf2 translation (Singh et al., 2013b; Yang et al., 2014d), so it may be possible to either mimic the effects of these sequences or to alter the regulation of the microRNAs to inhibit Nrf2 production.

Brusatol is thought to inhibit Nrf2 by increasing its ubiquitination and degradation (Ren et al., 2011). This is currently the only method of inhibiting Nrf2 using a small molecule that has been experimentally verified. It is also an extremely difficult mechanism to design a drug for because the interaction that increases Nrf2 ubiquitination is unknown. Some cancers overexpress Nrf2 due to silenced Keap1, which can be caused by hypermethylation of its promoter (Guo et al., 2012). Demethylation of DNA is difficult to target specifically, but Chen et al (2014) have successfully reactivated epigenetically silenced ICAM-1 using a targeted mechanism in HEK293 cells. A similar approach to Keap1 may help reduce aberrant Nrf2 expression.

There are several ways to inhibit Nrf2 nuclear activity. Currently Nrf2 nuclear import is probably not a viable option because not enough is known about the regulation of its nuclear localisation sequences (Theodore et al., 2008). However, there are two key interactions required for Nrf2-dependent gene transcription. These are the Nrf2/Maf interaction (Li et al., 2008), and the Nrf2/ARE interaction. Of these the Nrf2/Maf interaction is probably more viable because blocking the ARE may also block other sites, such as the TRE, and MARE. The Neh3, Neh4, and Neh5 domains in Nrf2 are also known to be required for transcriptional activation (Kato et al., 2001; Nioi et al., 2005;

Apopa et al., 2008). Currently they are not well enough understood to be considered potential drug targets.

There are a lot of possibilities for Nrf2 inhibitors, and a lot of research is still required to make them a reality. The benefits to Nrf2 inhibition in cancer are becoming increasingly evident, and further research into potential inhibitors would be welcome.

In conclusion, this study has identified DAPS as a novel dietary activator of Nrf2, and has used TAT-14 to validate the use of Keap1 inhibitors to inhibit inflammatory cytokine production. This research also failed to link a powerful activation of the Nrf2 pathway by DAPS with a cytoprotective effect, highlighting the superiority of non-reactive activators of the Nrf2 pathway. TAT-14 can be used as a tool to investigate the effects of Nrf2 activation before a small molecule activator is fully characterised. In addition, the protective effects of Nrf2 are hijacked by malignant melanoma, and protect melanoma cells from chemotherapeutic drugs, such as cisplatin and doxorubicin. This, combined with research in other cancers, suggests that Nrf2 inhibitors may be effective when used in conjunction with chemotherapy. Targeting Nrf2 in cancer requires a better understanding of its regulation if an effective drug is to be found.

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Appendix I: Buffer contents

Cell culture

Freezing medium

Per vial:

900 µL sterile foetal bovine serum

100 µL sterile DMSO

Western immunoblotting (Novex)

1x running buffer (800 mL)

40ml NuPage 20x MOPS SDS running buffer

760ml deionised H₂O

1x transfer buffer

680mL deionised H₂O

80mL methanol

40mL 20X Transfer Buffer

1 ml NuPAGE Antioxidant

Western immunoblotting (Bio-rad)

SDS PAGE gels

The following components provide enough for two SDS PAGE Gels

Bottom gel stock

90.75 g Tris Base

Top up to 500 mL with deionised H₂O

pH to 8.8

10ml 20% SDS

Upper gel stock

12.11 g Tris Base

Top up to 200 mL deionised H₂O

pH to 6.8

4 mL 20% SDS

10% APS

23 mg APS crystals

230 µL deionised H₂O

Resolving gel

	Gel Concentration		
	7.5%	10%	12%
Bottom gel stock	3.7 mL	3.7 mL	3.7 mL
30% acrylamide	3.7 mL	5 mL	6.5 mL
Deionised H₂O	7.4 mL	6 mL	4.5 mL
10% APS	130 µL	130 µL	130 µL
TEMED	13µL	13µL	13µL

Stacking gel

600µL upper gel stock

1 mL 30% acrylamide

4.25 mL ddH₂O

75 µL 10% APS

7.5 µL TEMED

5x running buffer (1 L)

30 g Tris Base

140 g Glycine

5 g SDS

Top up to 1 L with deionised H₂O

10x transfer buffer (1 L)

24.26g Tris Base

112.6g Glycine

1g SDS

Make up to 1L with deionised H₂O

1x transfer buffer (1L)

100ml 10x Transfer Buffer

700ml H₂O

200ml Methanol

Immunoblotting

20x TBST (1 L)

48.4g Tris Base

160g NaCl

62ml 5M HCl

20ml Tween-20

pH to 7.6

Top up to 1 L in deionised H₂O

ELISA

Coating buffer – 0.2M sodium phosphate

11.8 g Na₂HPO₄

16.1 g NaH₂PO₄

Make up to 1.0 L with deionised H₂O

pH to 6.5.

PBS

16.0 g NaCl

2.32 g Na₂HPO₄

0.4 g KH₂PO₄

0.4 g KCL

Make up to 2 L with deionised H₂O

pH to 7.0

Wash buffer

2.0 L PBS

100 µL Tween-20

Assay diluent

90% PBS

10% foetal bovine serum

pH 7.0.

Fluorescence polarization assay***Sodium phosphate buffer***

1.5 mL 100 mM Sodium Phosphate

150 µL Tween-20

13.35 mL water

Interaction solution

11.1 μL F-14 (5 μM stock)

17.9 μL Keap1(18.57 μM stock)

9971 μL sodium phosphate buffer

Appendix II: Western blot replicates

Fig 4.2

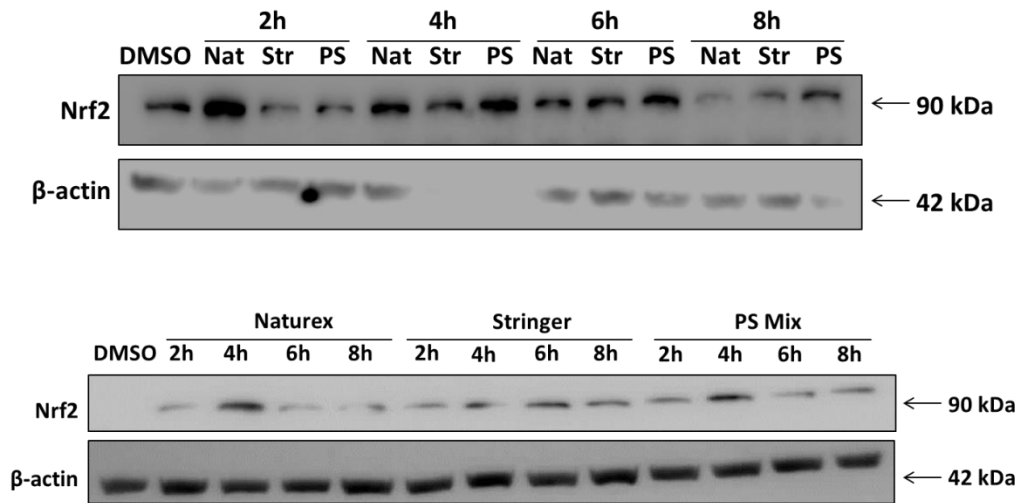


Fig 4.2. Garlic oils induce Nrf2 accumulation in THP-1 cells. THP-1 cells were incubated with 10 μ g/mL Naturex, Stringer or PS Mix for the indicated time point. DMSO was used as a vehicle control. Whole cell lysates were prepared and Nrf2 expression measured by western blot analysis. β -actin expression is shown as a loading control. Results are representative of three independent experiments.

Fig 4.4

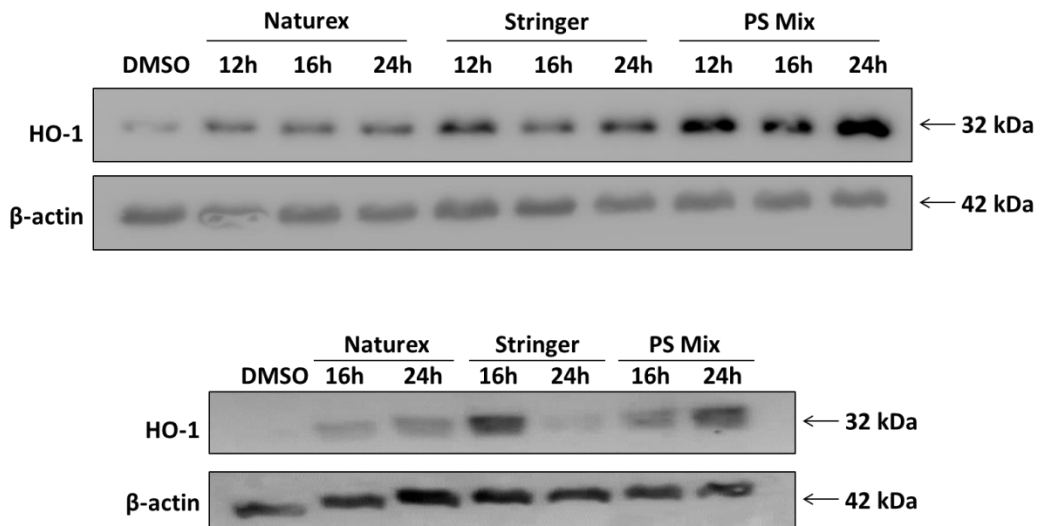


Fig 4.4. Garlic oils induce HO-1 protein expression in THP-1 cells. THP-1 cells were incubated with 10 μ g/mL Naturex, Stringer or PS Mix for the indicated time point. DMSO was used as a vehicle control. Whole cell lysates were collected and HO-1 expression was measured by Western blot. β -actin expression is shown as a loading control.

Fig 4.6C

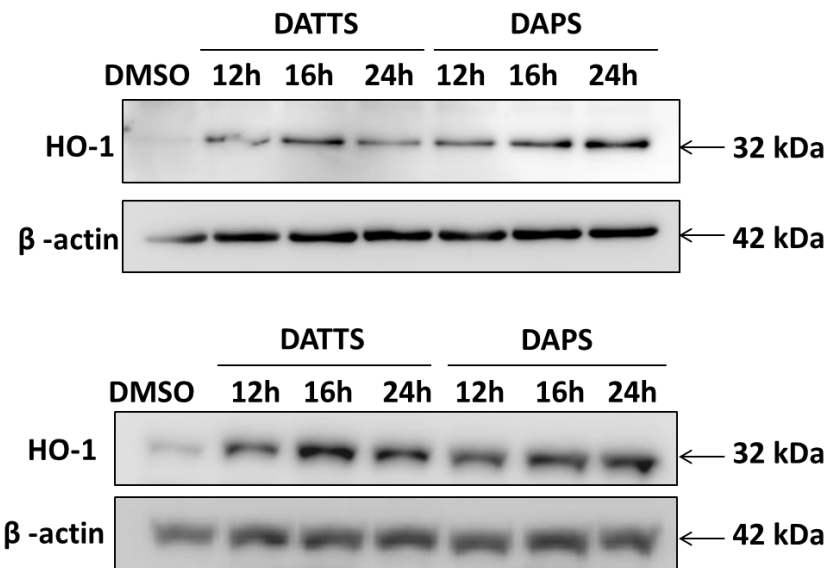


Fig 4.6C. DATTs and DAPS-induced HO-1 expression in THP-1 cells. Cells were incubated with 20 μ M DATTs or DAPS for the indicated time points. 24h DMSO was used as a vehicle control. Whole cell lysates were prepared and HO-1 expression measured by Western blot Analysis.

Fig 4.10B

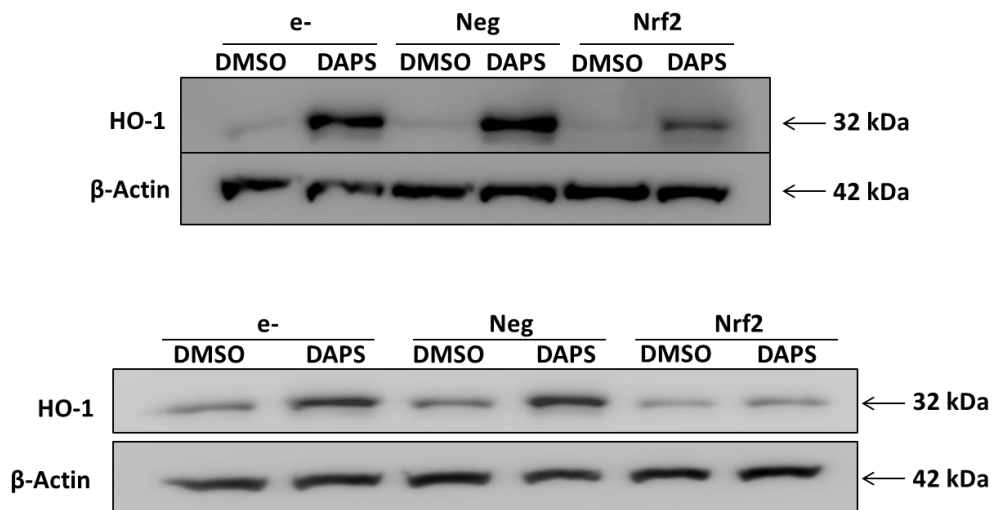


Fig 4.10B. Nrf2 siRNA suppresses DAPS-induced HO-1 expression. THP-1 cells were transfected with 200nM Nrf2 siRNA or negative control siRNA. An electroporated sample with no siRNA was used as a control. After 24h, cells were incubated with 20 μ M DAPS for 24h. DMSO was used as a vehicle control. Cell lysates were collected and HO-1 expression was measured by Western blot. β -actin expression is shown as a loading control.

Fig 4.11

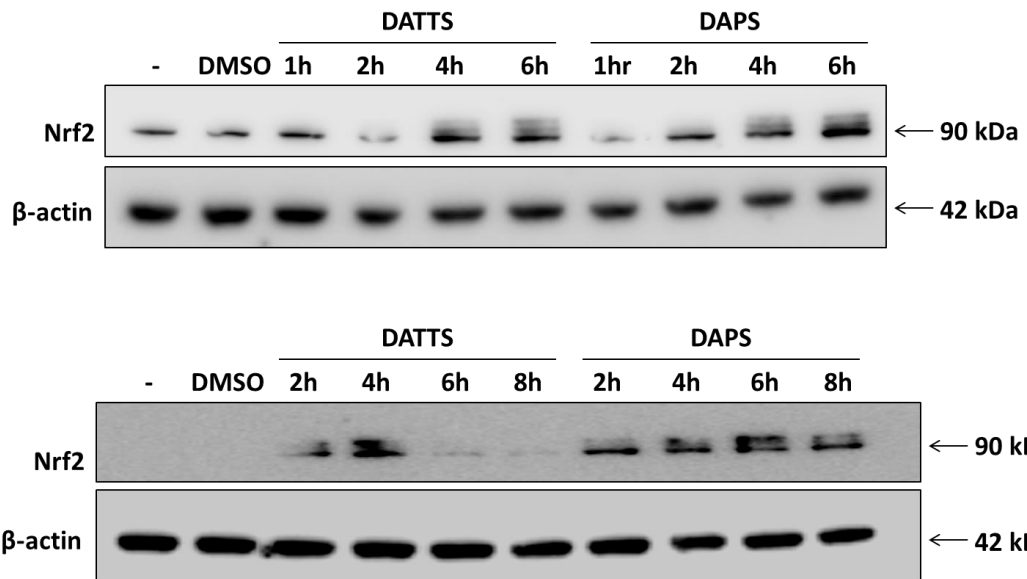


Fig 4.11. DATTS and DAPS induce Nrf2 protein expression in THP-1 cells. THP-1 cells were incubated with 20 μ M DATTS or DAPS for the indicated time period. DMSO was used as a vehicle control. Cell lysates were collected and Nrf2 expression was measured by western blot. β -actin expression is shown as a loading control.

Fig 4.12B

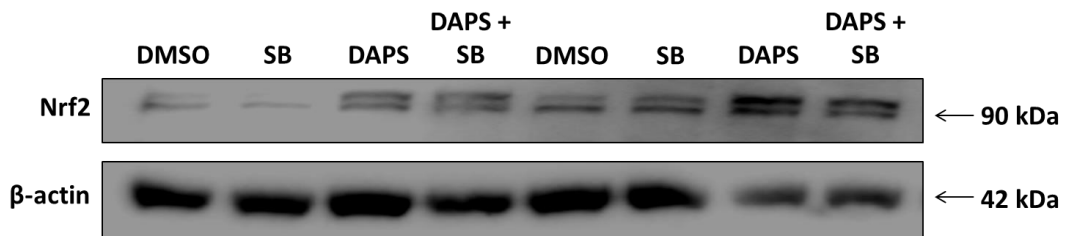


Fig 4.12B. p38MAP kinase regulates DATTS and DAPS-induced Nrf2 expression. THP-1 cells were incubated with 10 μ M SB203580 for 30min prior to incubation with DAPS for 4h. Cell lysates were probed for Nrf2 expression by western blot. β -actin expression is shown as a loading control.

Fig 4.13A

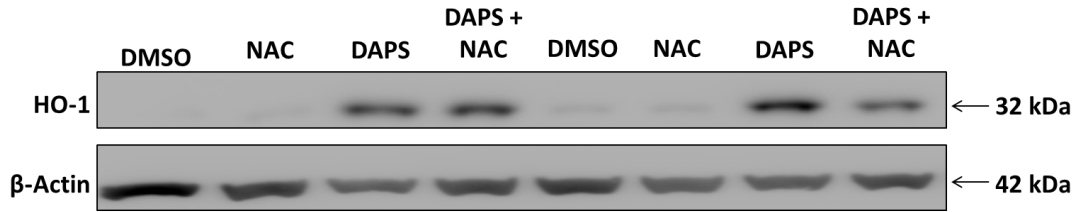


Fig 4.13A. DAPS induces HO-1 via ROS production. THP-1 cells were incubated with 1mM NAC for 30min prior to addition of 20 μ M DAPS for 24h. DMSO was used as a vehicle control. Cell lysates were probed for HO-1 expression by western blot. β -actin is shown as a loading control.

Fig 4.13B

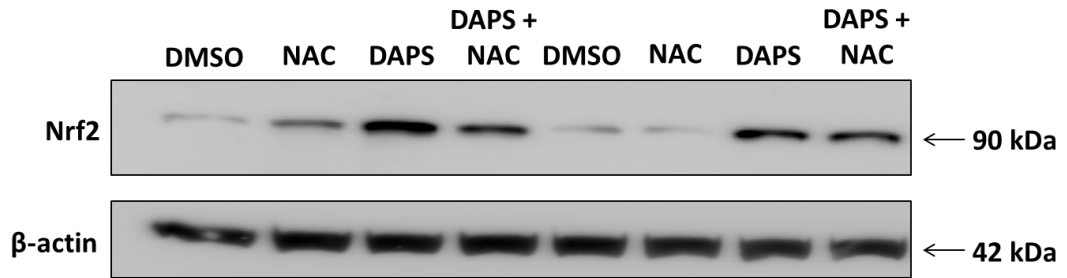


Fig 4.13B. DAPS induces Nrf2 via ROS production. THP-1 cells were incubated with 1mM NAC for 30min prior to incubation with DAPS for 4h. Cell lysates were probed for Nrf2 expression by Western blot. β -actin is shown as a loading control.

Fig 5.3

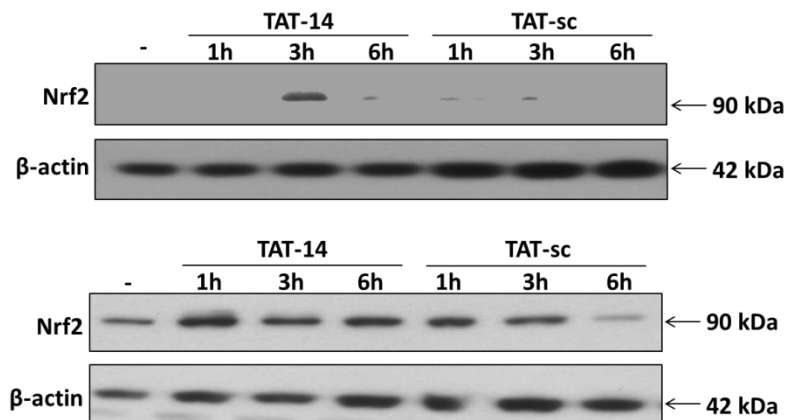


Fig 5.3 TAT-14 induces Nrf2 protein expression. THP-1 cells were incubated with 75 μ M TAT-14 or TAT-sc for 1, 3, or 6h. Nrf2 expression in cell lysates was measured by Western immunoblotting. β -actin was used as a loading control.

Fig 5.5

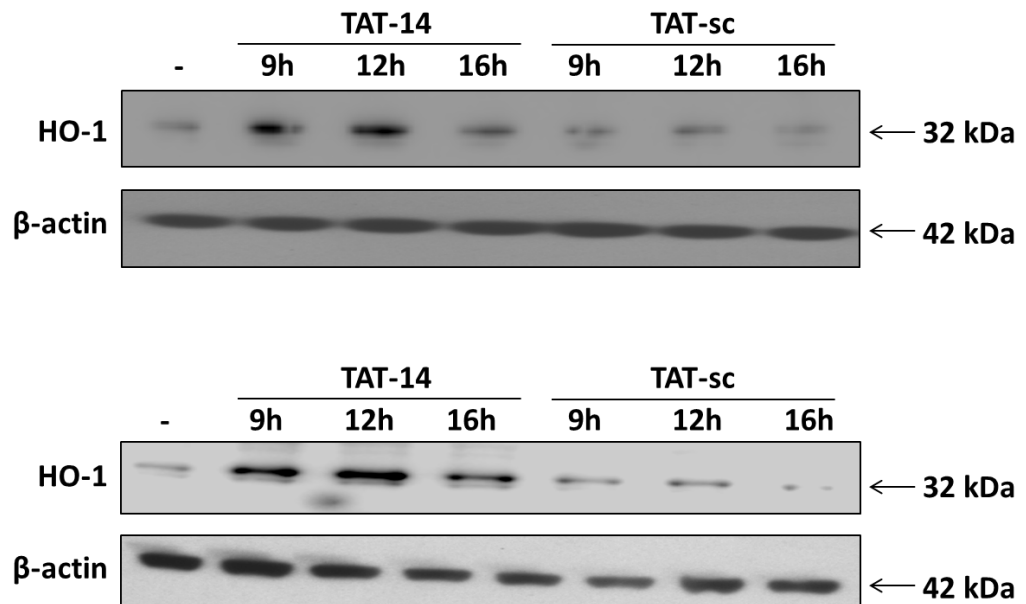


Fig 5.5 TAT-14 induces HO-1 protein expression. THP-1 cells were incubated with TAT-14 or TAT-sc for 9, 12, or 16h. HO-1 expression in cell lysates was measured by Western immunoblotting. β -actin was used as a loading control.

Fig 6.12A

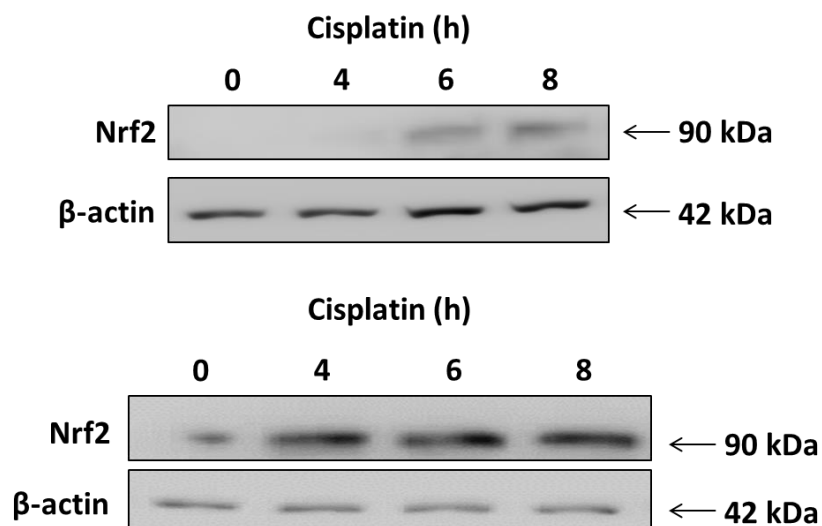


Fig 6.12A. Cisplatin induces Nrf2 in M202 cells. M202 cells were seeded in 6 well plates at a density of 0.5×10^6 per ml and allowed to grow to 80% confluency. 5 μ M cisplatin was added for the indicated time period. Cell lysates were prepared and samples were analysed by western blot for Nrf2 expression. β -actin was used as a loading control.

Fig 6.12B

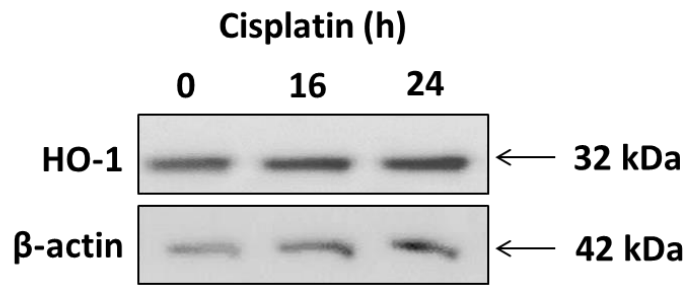


Fig 6.12B. Cisplatin induces HO-1 in M202 cells. M202 cells were seeded in 6 well plates at a density of 0.5×10^6 per ml and allowed to grow to 80% confluency. 5 μ M cisplatin was added for the indicated time period. Cell lysates were prepared and samples were analysed by western blot for HO-1 expression. β -actin was used as a loading control.