

**Department of Molecular and Clinical Pharmacology  
Institute of Translational Medicine  
University of Liverpool**



**The Pharmacological Manipulation of the  
Nrf2 Pathway and Its Therapeutic  
Significance**

Thesis submitted in accordance with the requirements of the University of Liverpool  
for the degree of Doctor in Philosophy

**Adedamola Oladeji Olayanju**

August 2014

## **DECLARATION**

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any degree or other qualification.

**Adedamola Oladeji Olayanju**

This research was carried out in the MRC Centre for Drug Safety Science,  
Department of Pharmacology and Therapeutics,  
The University of Liverpool.

## **TABLE OF CONTENTS**

	<b><u>PAGE</u></b>
<b>ABSTRACT</b> .....	i
<b>ACKNOWLEDGEMENTS</b> .....	ii
<b>PUBLICATIONS</b> .....	iv
<b>ABBREVIATIONS</b> .....	v
<b><u>1.0 CHAPTER ONE: GENERAL INTRODUCTION</u></b>	
1.1 The Nrf2:Keap1 pathway .....	3
1.2 Keap1-dependent regulation of Nrf2.....	8
1.2.1 Nrf2 ubiquitination and proteasomal degradation.....	8
1.2.2 Keap1 as a sensor of oxidative stress.....	9
1.3 Models of Nrf2 regulation.....	11
1.3.1 Sequester and release.....	11
1.3.2 Dissociation of Keap1 and Cul3.....	13
1.3.3 Hinge and Latch model.....	15
1.3.4 Keap1 nucleocytoplasmic shuttling.....	18
1.3.5 Keap1 ubiquitination.....	20
1.3.6 The Cyclic and Sequential attachment and regeneration model....	22
1.3.7 Nrf2 as a sensor of stress.....	24
1.4 Keap1-independent regulation of Nrf2.....	26
1.4.1 Transcriptional control of Nrf2.....	26
1.4.2 Post-transcriptional control of Nrf2 and microRNAs.....	27
1.4.3 Role of microRNAs in regulating the Nrf2 pathway.....	28
1.4.4 microRNAs and reactive oxygen species (ROS) induction.....	29
1.4.5 Translational control of Nrf2.....	31
1.4.6 Post-translational control of Nrf2 – Phosphorylation.....	31
1.4.7 Cross-talk between Nrf2 and other pathways.....	34
1.5 Role of Nrf2 in protection against disease and its therapeutic significance in different organs.....	37
1.5.1 Liver.....	38
1.5.2 Kidney.....	38

1.5.3 Brain.....	39
1.5.4 Lipid metabolism.....	40
1.6 Activation of Nrf2 by small molecules.....	41
1.7 Cancer and Inhibition of Nrf2.....	41
1.8 Thesis aims.....	44

## **2.0 CHAPTER TWO: MATERIALS AND METHODS**

2.1 Materials and Reagents.....	47
2.2 Antibodies.....	48
2.3 Mice.....	49
2.4 Treatment of animals with CDDO-Me.....	50
2.5 Liver homogenisation.....	50
2.6 Cell lines.....	50
2.7 Cell culture.....	51
2.8 Peripheral blood mononucleocytes isolation (PBMCs) protocol.....	51
2.9 Primary human hepatocytes isolation.....	52
2.10 Cell counting and cell seeding.....	52
2.11 Treatment of cells with chemicals or compounds.....	53
2.12 Treatment of PBMCs with chemicals or compounds.....	54
2.13 Cell viability assay.....	54
2.14 RNA Interference.....	55
2.15 Isolation of RNA / DNase treatment.....	55
2.16 RNA quality control.....	56
2.17 cDNA protocol.....	57
2.18 Real-time Polymerase Chain Reaction (RT-PCR).....	57
2.19 Primer design for PCR amplification.....	58
2.20 Microfluidic cards.....	58
2.21 Preparation of Hepa1c1c7 and HepG2 cell lysate samples.....	59
2.22 Preparation of PBMCs cell lysate samples.....	59
2.23 Protein content determination of whole cell lysates.....	60
2.24 Protein content determination of liver homogenates.....	60
2.25 Western Blotting.....	61
2.26 Measurement of Proteasome activity.....	63

2.27	Liquid chromatography-mass spectrometric analysis of brusatol and other quassinoids.....	63
2.28	Data and Statistical analysis.....	64

**3.0 CHAPTER THREE: INVESTIGATING THE EFFECTS OF NRF2 MODULATION IN MOUSE *IN VIVO* MODELS AND PRIMARY HUMAN CELLS**

3.1	Introduction.....	68
3.2	Results.....	70
3.2.1	Induction of the Nrf2 pathway by CDDO-Me <i>in Vivo</i> .....	70
3.2.2	Investigation into the induction of Nrf2 signalling by CDDO-Me at the transcriptional level.....	71
3.2.3	Use of alternative strain of mouse to investigate the regulation of fatty acid synthesis genes by Nrf2.....	76
3.2.4	Investigation of the role of Nrf2 in the regulation of fatty acid synthesis genes.....	77
3.2.5	Investigation of Nrf2 response to CDDO-Me in human cells.....	78
3.3	Discussion.....	81

**4.0 CHAPTER FOUR: CHARACTERIZATION OF THE MECHANISM OF ACTION OF CDDO-Me**

4.1	Introduction.....	87
4.2	Results.....	88
4.2.1	Concentration- and time-dependent Induction of Nrf2 by CDDO-Me <i>in Vitro</i> .....	88
4.2.2	Functional consequences of Nrf2 induction by CDDO-Me.....	90
4.2.3	CDDO-Me induces Nrf2 via a post-transcriptional mechanism.....	92
4.2.4	Activation of Nrf2 by CDDO-Me is not dependent on the activation/inhibition of protein kinases and other signalling pathways.....	93
4.2.5	CDDO-Me does not stabilize Nrf2 mRNA.....	94
4.2.6	CDDO-Me stabilizes Nrf2 protein.....	96
4.2.7	CDDO-Me does not inhibit the catalytic activity of the proteasome <i>in vitro</i> .....	97
4.3	Discussion.....	101

## **5.0 CHAPTER FIVE: INHIBITION OF NRF2 BY BRUSATOL**

5.1 Introduction.....	106
5.2 Results.....	108
5.2.1 Brusatol inhibits Nrf2 in a dose- and time-dependent manner....	108
5.2.2 Decreased Levels of Nrf2 Regulated genes in Cells treated with brusatol.....	110
5.2.3 Brusatol can stimulate the depletion of pharmacologically induced Nrf2.....	111
5.2.4 Brusatol-mediated inhibition of Nrf2 is not associated with a reduction in cell viability.....	116
5.2.5 Brusatol sensitizes cells to chemical stress.....	117
5.2.6 Brusatol depletes Nrf2 in primary human Hepatocytes.....	119
5.2.7 Brusatol has a high specificity for the Nrf2 pathway and not a universal protein synthesis inhibitor.....	121
5.2.8 Uptake and cellular stability of brusatol.....	122
5.3 Discussion.....	129

## **6.0 CHAPTER SIX: CHARACTERISATION OF THE MECHANISM OF ACTION OF BRUSATOL**

6.1 Introduction.....	136
6.2 Results.....	137
6.2.1 Brusatol inhibits Nrf2 post-transcriptionally.....	137
6.2.2 Brusatol Inhibits Nrf2 independently of Keap1 and the proteasome.....	141
6.2.3 Brusatol does not enhance the catalytic activities of the proteasome in vitro.....	143
6.2.4 Brusatol initiates the depletion of Nrf2 independently of the autophagy pathway.....	146
6.2.5 Brusatol clears Nrf2 independently of protease and caspases.....	148
6.2.6 Brusatol induces the phosphorylation of p38 MAPK, SAPK JNK 1/2 AND PI3K/AKT pathways, but the depletion of Nrf2 is independent of the activation of these pathways.....	149
6.2.7 Inhibition of Nrf2 by Brusatol may be partially dependent on calcium modulation.....	154
6.3 Discussion.....	157

**7.0 CHAPTER SEVEN: OVERALL DISCUSSION**

7.1 Introduction.....	162
7.2 Activation of Nrf2 and its pharmacological significance.....	162
7.3 Inhibition of Nrf2 and its pharmacological significance.....	164
7.4 Concluding remarks and future investigations.....	166
<b>BIBLIOGRAPHY.....</b>	<b>167</b>

## **LIST OF FIGURES AND TABLE**

### **PAGE**

#### **1.0 CHAPTER ONE: GENERAL INTRODUCTION**

Figure 1.1 - Schematic diagram of the Keap1:Nrf2 pathway.....	5
Figure 1.2 - Schematic illustrating the structure of Nrf2.....	7
Figure 1.3 - Schematic illustrating sequester and release model of Nrf2 regulation.....	12
Figure 1.4 - Schematic illustrating dissociation of Keap1 and Cul3 model of Nrf2 regulation.....	14
Figure 1.5 - Schematic illustrating hinge and latch model of Nrf2 regulation.....	17
Figure 1.6 - Schematic illustrating Keap1 nucleocytoplasmic shuttling model of Nrf2 regulation.....	19
Figure 1.7 - Schematic illustrating ubiquitination of Keap1 model of Nrf2 regulation.....	21
Figure 1.8 - The cyclic sequential attachment and regeneration model of Nrf2 regulation..	23
Figure 1.9 - Schematic illustrating Nrf2 senses inducers directly model of Nrf2 regulation.....	25
Table 1: microRNAs that has been illustrated in regulating the Nrf2 pathway.....	30
Figure 1.10 - Schematic illustrating Keap1-independent mechanism of Nrf2 regulation....	36
Figure 1.11 – Role of Nrf2 in protection against disease.....	37

#### **2.0 CHAPTER TWO: MATERIALS AND METHODS**

Table 2: Antibodies table .....	49
Table 2.2 – Chemicals or compounds and their appropriate solvent .....	53
Table 2.3 – Primer Table .....	58

#### **3.0 CHAPTER THREE: INVESTIGATING THE EFFECTS OF NRF2 MODULATION IN MOUSE *IN VIVO* MODELS AND PRIMARY HUMAN CELLS**

Figure 3.1 - Induction of Nrf2 pathway by CDDO-Me <i>in vivo</i> .....	70
Figure 3.2 - Induction of Nqo1 protein expression by CDDO-Me is dependent on Nrf2.....	71



Figure 3.3 - Investigation into the induction of Nrf2 signalling by CDDO-Me at the transcriptional level.....	73
Table 3.1: Genes on custom-made Microfluidic Taqman Low Density Array cards .....	74
Figure 3.4 Induction of the Nrf2 pathway by CDDO-Me in CD1 mice.....	77
Figure 3.5: Investigation of the role of Nrf2 in the regulation of fatty acid synthesis genes.....	78
Figure 3.6 - Induction of Nrf2 pathway in freshly isolated human cells.....	80

#### **4.0 CHAPTER FOUR: CHARACTERIZATION OF THE MECHANISM OF ACTION OF CDDO-Me**

Figure 4.1 – Concentration-dependent induction of Nrf2 by CDDO-Me in vitro.....	89
Figure 4.2 - Time-dependent induction of Nrf2 in Hepa1c1c7 cells exposed to CDDO-Me.....	90
Figure 4.3 - Time-dependent induction of Nqo1 in Hepa1c1c7 cells exposed to CDDO-Me.....	91
Figure 4.4 - CDDO-Me does not provoke acute changes in Nrf2 or Keap1 mRNA expression.....	92
Figure 4.5 - Activation of Nrf2 by CDDO-Me is not dependent on the activation/inhibition of protein kinase and other signalling pathways.....	94
Figure 4.6 CDDO-Me does not stabilize Nrf2 mRNA.....	95
Figure 4.7 - CDDO-Me stabilizes Nrf2 protein in Hepa1c1c7 cells.....	96
Figure 4.8 – CDDO-Me does not inhibit proteasomal activity.....	100

#### **5.0 CHAPTER FIVE: INHIBITION OF NRF2 BY BRUSATOL**

Figure 5.1 – Depletion of Nrf2 in Hepa1c1c7 cells exposed to brusatol.....	109
Figure 5.2 – Brusatol reduced mRNA levels of Nrf2 downstream target genes.....	111
Figure 5.3 Brusatol inhibits the pharmacological induction of Nrf2.....	115
Figure 5.4 - Hepa1c1c7 cell viability following exposure to brusatol.....	117
Figure 5.5 – Brusatol sensitizes cells to chemical stress.....	119

Figure 5.6 – Brusatol depletes Nrf2 in primary human hepatocytes.....	120
Figure 5.7 – Brusatol is not a universal protein synthesis inhibitor.....	121
Figure 5.8 - Development of LC-MS/MS methods for analysing brusatol.....	125
Figure 5.9 - Analysis of brusatol stability in incubations of Hepa1c1c7 cells.....	128

**6.0 CHAPTER SIX: CHARACTERISATION OF THE MECHANISM OF ACTION OF BRUSATOL**

Figure 6.1 - Brusatol reduces Nrf2 via a post-transcriptional mechanism.....	140
Figure 6.2 - Brusatol depletes Nrf2 independently of Keap1.....	142
Figure 6.3 - Brusatol depletes Nrf2 independently of the proteasomal degradation pathway.....	145
Figure 6.4 - Brusatol decreases Nrf2 independently of the autophagic protein degradation machinery.....	147
Figure 6.5 - Brusatol does not deplete Nrf2 via proteases and caspases.....	149
Figure 6.6 - Brusatol depletes Nrf2 independently of the activation of protein kinase signalling pathways.....	153
Figure 6.7 - Brusatol-mediated depletion of Nrf2 is partially dependent on the modulation of calcium signalling.....	156

**7.0 CHAPTER SEVEN: OVERALL DISCUSSION**

Figure 7.1 – Proposed mechanism of action of brusatol .....	165
---	-----

## **ABSTRACT**

Nrf2 (Nuclear factor erythroid 2-related factor 2), a redox-sensitive transcription factor, plays a critical role in the regulation of cellular defence and contributes to a number of cellular processes. Nrf2 is regulated through an interplay of complex transcriptional and post-translational mechanisms that modulates its activity during cellular perturbations or other biological processes thereby ensuring cellular homeostasis is maintained through the orchestration of adaptive responses. However, there is mounting evidence that constitutive upregulation of the Nrf2 pathway drives the enhanced proliferation and chemoresistance of various cancers. Therefore, an ability to modulate the activity of the Nrf2 pathway holds promise as a therapeutic strategy in certain disease settings.

The work presented in this thesis showed that CDDO-Me provoked the induction of the Nrf2 pathway in C57BL6J WT and Nrf2 KO mice and CD1 WT mice. Analysis of CDDO-Me induced gene expression changes in both WT and Nrf2 KO mice showed a significant increase in the relative mRNA levels of ARE-dependent genes in the livers of CDDO-Me treated WT animals. Notably, CDDO-Me also provoked the accumulation of Nrf2 and NQO1 in human PBMCs and PHHs demonstrating its translational relevance. The mechanism of action of CDDO-Me as an inducer of Nrf2 is poorly understood. It was shown here that CDDO-Me post-transcriptionally evoked concentration and time-dependent, accumulation of Nrf2 protein in Hepa1c1c7 cells. Furthermore, CDDO-Me was shown to stabilize Nrf2 protein independently of the modulation of protein kinases and other signalling pathways that are purported to regulate Nrf2 activity.

The work here also provides *in vitro* insights into the molecular mechanism of Nrf2 inhibition by the quassinoid brusatol. Brusatol post-transcriptionally evoked concentration- and time-dependent, yet transient, depletion of basal and inducible protein levels of Nrf2 in Hepa-1c1c7 cells. Furthermore, the ability of brusatol to inhibit Nrf2 was not affected by siRNA depletion of Keap1. In keeping with the latter observation, brusatol induced the depletion of Nrf2 independently of the proteasome and autophagic degradation machineries. Thus, these findings indicate that brusatol exploits a previously unknown mechanism of Nrf2 degradation. By examining the molecular mechanisms underlying the activation of Nrf2 by CDDO-Me and its inhibition by brusatol, this work reveals novel aspects of regulation within this important cellular pathway, and informs the design of new pharmacological inducers and inhibitors, which hold promise as therapeutic agents in a number of diseases.

## **ACKNOWLEDGEMENTS**

First, I would like to thank the Almighty God for making this work a success and giving me the ability and the grace to complete this PhD programme. Many thanks go to my primary supervisor Dr. Chris Goldring for his supervision throughout my PhD programme. You indeed supervised me like a son rather like a student. This, coupled with your continued enthusiastic approach made me a better student and I have learnt so much from you to boost my confidence and prepare me for the future. I have learnt to become an independently thinking scientist and thanks for giving me the freedom to explore my own scientific ideas. I will always be indebted to you for your teachings, guidance, advice and support throughout my PhD and I couldn't have asked for a better person to supervise me. You are indeed a star and I am very grateful. I will also like to show my sincere gratitude to my second supervisor Dr. Neil Kitteringham. You are a fantastic person and many thanks for the wonderful teachings and jokes. It's amazing to know the amount of knowledge that is in your brain. How I wish I could have some of those cells transferred to me. I owe you so much particularly when I become the president of Nigeria and thanks for believing in me. Your motivation, support, guidance and advice kept me going throughout my stay in Liverpool. Infact, I feel extremely lucky having two geniuses to supervise me. My heartfelt gratitude also goes to a mentor Dr. Ian Copple, who taught me so much and shaped my thinking. It's been a great pleasure working with you on several projects. You showed so much kindness and patience in taking your time to share your knowledge and build me up more scientifically. I have no doubt seeing you as a Nobel Prize winner in the nearest future.

In addition, I will like to thank Dr. Jo Walsh and Lorna Kelly for taking their time to build my foundation. You were so approachable and you made the work look so easy. You encouraged me when I felt like giving up and I am indeed very grateful. My thanks go to my brusatol partner (lol), Holly K. Brian. Thanks for the laughs and support. It's been an honour working alongside you. You encouraged me and showed sincere help when I needed them. It was a pleasure working on those publications with you. Remember to visit Nigeria during your world trip. To Dr. James Maggs and Dr. Roz Jenkins, thanks for helping me with the mass spectrometric analysis of my work. Experts like you guys are rare and it was an honour to work alongside you. To Dr. George Edge, thanks for your kind help on the proteasome work. It was an honour working with you. My thanks also go to Dr Mike Cross and his team for his help and teachings on the kinases studies. Big shout out to Ahmad Alghanem, you are indeed a friend and a brother. You kept the motivation going in the lab

and thanks for the help with the kinases and PCR work. I would like to extend my thanks to the other people in my lovely group (Dr. P Starkey, J. Heslop, R. Hornsby, Dr. R. Kia, Dr. C Rowe, Dr. R. Sison-young, R. Eakins, Dr. P Sutton, Dr. L. Kamalian, Dr F. Zang, Dr J. Evans, Dr. C Winiarski, M. Wong) for their support, love and laughter. It's been a pleasure working with each and every one of you. Without you guys, the work would have been difficult. To Min-Wei Wong, thanks for being a nice person to work with and I wish you all the luck in the world in the nearest future! Thanks to Dr. Jean Sattish and his entire group (Thilipan, Junnat, Laith, Sammy, Mike, Naif) for the help on the PBMC work. It was nice working with you guys and for making my stay in Liverpool enjoyable. There was always one social thing or the other to get invited to. Lol. To all my other friends here, those in my United Nations office and everyone else in the laboratory particularly Dr. Monday Ogese, Mohammed Amali, James Akingbasote, Mohammed Aljasir, it's been a great pleasure knowing you and working alongside all of you. You are all amazing and I see bright futures ahead of all of you! In addition, I would like to acknowledge the financial support of the ITM team. Thanks to Lisa Crimmins and ITM team for your advice and support.

I want to specially thank my gorgeous Doyinsola Alli for all her support and love during my programme. You are definitely a gem in the human frame. Thanks for your patience and always attending to my calls and incessant requests occasionally. This programme wouldn't have been successful without your full support and love. I am looking forward to spoiling you in the future. Many thanks also goes to Hon. Sunday Adepoju, Mr Gbola Ojediran, Dr. Matthew Adepoju, Mr Gbenga Adeyemi and their family for all their kind help financially and morally during my programme. My sincere thanks go to my pastors (Pastor J. Taiwo, Pastor G. Orakpo, Pastor R. Ibrahim, Pastor S. Adelumola, Pastor F. Olaoye, Pastor S. Peluola) and family and the entire members of my church. Your prayers and support is deeply appreciated. To my friends outside work and band members, particularly, Dr. O. Dosunmu, Mr. T. Adubi, Mr. S. Oduwaiye, Mr A. Ajagbe, Mr M. Omotosho, Mr. A. Osifeso, Mr. R. Famuyiwa, Mr. S. Adewole and Mr. L. Adewole, thanks for your motivation throughout this PhD.

Finally, I would like to sincerely thank my family Deacon and Hon (Mrs) Olayanju, Mr Lanre Olayanju and Miss Tola Olayanju for everything they have done for me. None of this would have been possible without you people. You have been my rock all these years and I will be eternally grateful. I say a BIG thank you to you and looking forward to a bright future to spend with all of you. I LOVE YOU ALL!

## PUBLICATIONS

### Papers

- **Olayanju A.\***, Copple I.M.\*, Bryan H.K., Edge G.T., Zhixiu L., Dunn K., Sanderson C.M., White M.R., Alghanem A.F., Cross M.J., Wong M.H.L., O'Neill P.M., Ellis E.C., Ingelman-Sundberg M., Fenwick S.W., Malik H.Z., Kitteringham N.R., Goldring C.E., Park B.K. (2014) Brusatol Provokes a Rapid and Transient Inhibition of Nrf2 Signalling and Sensitizes Mammalian Cells to Chemical Toxicity – Implications for Therapeutic Targeting of Nrf2. *Manuscript is currently under review with Free Radical Biology & Medicine.*
- Thaventhiran T., Alhumeed N., Aw Yeang H.X., Sethu S., Downey J., Alghanem A., **Olayanju A.** Smith E., Cross M.J., Webb S., Williams D., Bristow A., Ball C., Stebbings R., Sathish J (2014) Failure to upregulate cell surface PD-1 is associated with dysregulated stimulation of T cells by TGN1412-like CD28 superagonist. *mAbs.* 6(5).
- Walsh J., Jenkins R.E., Wong M., **Olayanju A.**, Powell H., Copple I., O'Neill P.M., Goldring C.E., Kitteringham N.R., Park B.K. (2014) Identification and quantification of the basal and inducible Nrf2-dependent proteomes in mouse liver: Biochemical, pharmacological and toxicological implications. *Journal of Proteomics.* 108C:171-187.

### Reviews

- Hamdam J., Sethu S., Smith T., Alfirevic A., Alhaidari M., Atkinson J., Ayala M., Box H., Cross M., Delaunois A., Dermondy A., Govindappa K., Guillon J., Jenkins R., Kenna G., Lemmer B., Meecham K., **Olayanju A.**, Pestel S., Rothfuss A., Sidaway J., Sison-Young R., Smith E., Stebbings R., Tingle Y., Valentin J., Williams A., Williams D., Park K., Goldring C.E. (2013). Safety Pharmacology – current and emerging concepts. *Toxicology and Applied Pharmacology.* 273(2):229-41
- Bryan H.K.\*, **Olayanju A.\***, Goldring C.E., Park B.k. (2012) The Nrf2 cell defence pathway; Keap1-dependent and -independent mechanisms of regulation. *Biochem Pharmacol.* 85(6):705-17.

### Abstract

- **Olayanju A.\***, Copple I., Kitteringham N., Goldring C.E. (2014) probing the mechanism of action of brusatol. *Signalling and Acquired Resistance to Targeted Cancer Therapeutics.* 5–7 January 2014. Robinson College, Cambridge, UK.

### \*Joint first authors

## **ABBREVIATIONS**

AHR: aryl hydrocarbon receptor

AKR: Aldo-keto reductase

AKT: Protein Kinase B

ANOVA: Analysis of variance

AP-1: Activator protein 1

APAP: N-*acetyl-para-aminopheno*

ARE: Antioxidant response elements

ATM: Ataxia telangiectasia mutated

ATP: Adenosine triphosphate

BACH1: BTB and CNC homolog 1

BCA: Bicinchoninic Acid

BHA: Butylated hydroxyanisole

b-NF: beta-naphthoflavone

BRG1: Brahma-related gene 1

BSA: Bovine serum albumin

BTB: Bric-a-brac, tram-track, broad complex

bZIP: Basic leucine Zipper

Ca<sup>2+</sup>: Calcium

Cav-1: Caveolin-1

CBP: CREB binding protein

CDDO: 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid

CDDO-Im: CDDO imidazole

CDDO-Me: CDDO methyl ester

cDNA: complementary DNA

CHX: Cyclohexamide

CT: Cycle threshold

CK2: Casein kinase 2

CPDT: 5,6-Dihydrocyclopenta-1,2-dithiole-3-thione

CREB: cAMP responsive element binding protein

Crm1: chromosomal region maintenance protein 1

Cul3: Cullin 3

Cys: Cysteine

D3T: 3H-1, 2-dithiole-3-thione

DJ-1: Parkinson's disease protein 1

DMEM: Dulbecco's modified Eagle's medium

DMF: Dimethyl fumarate

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DNCB: 2,4-dinitrochlorobenzene

dNTP: Deoxyribonucleotide triphosphate

ECH: Erythroid cell-derived protein with CNC homology

EDTA: ethylenediaminetetraacetic acid  
ENC1: Ectoderm neural cortex protein 1  
EPA: Eicosapentaenoic acid  
ERE: Electrophile response element  
ERK: Extracellular signal-regulated kinase

FIAsH: Fluorescent arsenic hairpin  
FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase  
GCL:  $\gamma$ -glutamylcysteine ligase  
GCLC: GCL, catalytic subunit  
GCLM: GCL, regulatory subunit  
GI: Gastrointestinal  
GSK3 $\beta$ : Glycogen synthase kinase 3 beta  
GSTs: Glutathione S-transferases  
GstPi: Glutathione S-transferase pi

H<sub>2</sub>O: water  
HBSS: Hanks balanced salt solution  
HCV: Hepatitis C virus  
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
HIF-1 $\alpha$ : Hypoxia-inducible factor 1-alpha  
HMOX: Heme oxygenase  
HO-1: Heme oxygenase 1  
Hr: Hour  
H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide  
HRP: horseradish peroxidase

IAA: Iodoacetamide  
IAB: N-iodoacetyl-N-biotinylhexylenediamine  
IKK $\beta$ : Inhibitor of  $\kappa$ B kinase beta  
IRES: internal ribosomal entry site  
IVR: Intervening Region

JNK: c-Jun N-terminal Kinase

Keap1: Kelch-like ECH-associated protein 1  
KO: Knock out  
Kras: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

L: Litre  
Lys: Lysine

m: Milli  
M: Molar  
Maf: masculoaponeurotic fibrosarcoma  
MAPK: mitogen-activated protein kinase



MEFs: Mouse embryonic fibroblasts  
min: Minutes  
miRNA: microRNAs  
MOPS: 3-(N-morpholino) propanesulfonic acid  
mRNA: Messenger RNA  
Mrp3: Multidrug resistance-associated protein 3  
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MS/MS: Mass spectrometry  
m/z: mass-to-charge ratio

NADPH: nicotinamide adenine dinucleotide phosphate  
NAPQI: N-acetyl-p-benzoquinoneimine  
NDGA: Nordihydroguaiaretic acid  
Neh: Nrf2-ECH homology  
NES: Nuclear export signal  
NF- $\kappa$ B: nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells  
NLS: Nuclear localisation signal  
NOX4: NADPH oxidase 4  
NQO1: NAD(P)H:quinone oxidoreductase 1  
Nrf2: Nuclear Factor (erythroid-derived2)-like factor 2

ORF: Open reading frame

P62: Sequestosome 1  
PAO: Phenylarsine oxide  
PBMC: Peripheral blood mononucleocytes  
PBS: Phosphate-buffered saline  
PCR: Polymerase chain reaction  
PDGF: Platelet derived growth factor  
PERK: Protein kinase RNA- like endoplasmic reticulum kinase  
PHH: Primary human hepatocytes  
PI3K: Phosphatidyl inositol 3-kinase  
PKC: Protein Kinase C

RAC: Receptor associated coactivator  
RBC: Red blood cells  
RIPA: Radioimmunoprecipitation assay  
RISC: RNA-induced silencing complex  
RNA: Ribonucleic acid  
RNAi: RNA interference  
ROC1/RBX1: Ring box protein 1  
ROS: Reactive oxygen species  
rpm: revolutions per minute  
RPMI: Roswell Park Memorial Institute-1640

SAPK: Stress activated protein kinase  
SD: Standard deviation of the mean  
SDS: Sodium dodecyl sulphate

siRNA: Short interfering RNA  
SMRT: Silencing Mediator of Retinoid Thyroid  
STAT: Signal Transducers and Activators of Transcription  
SQSTM1: Sequestosome 1

tBHQ: tert-butylhydroquinone  
TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin  
TNF- $\alpha$ : Tumour necrosis factor alpha

UK: United Kingdom  
USA: United States of America  
UTR: Untranslated region

v/v: volume/volume

WT: Wild type

XRE: Electrophile response element

2-ME: 2-mercaptoethanol  
 $\mu$ : micro  
 $\beta$ -TrCP:  $\beta$ -transducin repeat-containing protein

# **CHAPTER ONE**

## **GENERAL INTRODUCTION**

1.1 The Nrf2:Keap1 pathway .....	3
1.2 Keap1-dependent regulation of Nrf2.....	8
1.2.1 Nrf2 ubiquitination and proteasomal degradation.....	8
1.2.2 Keap1 as a sensor of oxidative stress.....	9
1.3 Models of Nrf2 regulation.....	11
1.3.1 Sequester and release.....	11
1.3.2 Dissociation of Keap1 and Cul3.....	13
1.3.3 Hinge and Latch model.....	15
1.3.4 Keap1 nucleocytoplasmic shuttling.....	18
1.3.5 Keap1 ubiquitination.....	20
1.3.6 The cyclic sequential and regeneration model .....	22
1.3.7 Nrf2 as a sensor of stress .....	24
1.4 Keap1-independent regulation of Nrf2.....	26
1.4.1 Transcriptional control of Nrf2.....	26
1.4.2 Post-transcriptional control of Nrf2 and microRNAs.....	27
1.4.3 Role of microRNAs in regulating the Nrf2 pathway.....	28
1.4.4 microRNAs and reactive oxygen species (ROS) induction.....	29
1.4.5 Translational control of Nrf2.....	31
1.4.6 Post-translational control of Nrf2 – Phosphorylation.....	31
1.4.7 Cross-talk between Nrf2 and other pathways.....	34
1.5 Role of Nrf2 in protection against disease and its therapeutic significance in different organs.....	37
1.5.1 Liver.....	38
1.5.2 Kidney.....	38
1.5.3 Brain.....	39
1.5.4 Lipid metabolism.....	40
1.6 Activation of Nrf2 by small molecules.....	41
1.7 Cancer and Inhibition of Nrf2.....	41
1.8 Thesis aims.....	44

## 1.0 INTRODUCTION

### 1.1 The Nrf2:Keap1 pathway

Cells are constantly being exposed to insults which result from various chemical and oxidative reactions such as those involving environmental toxicants, mutagens and carcinogens. Such insults pose a high risk to the cell, disrupting homeostasis and normal cellular function and, not surprisingly, have been associated with disease pathogenesis (Osburn *et al.*, 2008). Mammalian cells have thus evolved complex signaling pathways and defense systems that function synergistically in order to reduce the deleterious effects of such intrinsically and extrinsically generated insults. One such pathway is the Keap1-Nrf2 cell defense pathway.

Nuclear Factor (erythroid-derived2)-like factor 2 (henceforth referred to as Nrf2) is a redox sensitive bZip transcription factor that induces the expression of a variety of genes which serve to protect against the deleterious effects of oxidative and chemical stress, thus ensuring normal cellular functions are maintained or restored. Nrf2 is a member of the cap 'n' collar family of transcription factors; these include Nrf1, Nrf3, Bach1 and Bach 2 (Motohashi *et al.*, 2002). The physiological importance of Nrf2 has been highlighted by the use of transgenic Nrf2 knockout mice (KO), which show enhanced susceptibility to various drug-induced toxicities such as acetaminophen and dextran sulphate (McMahon *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Cho *et al.*, 2002).

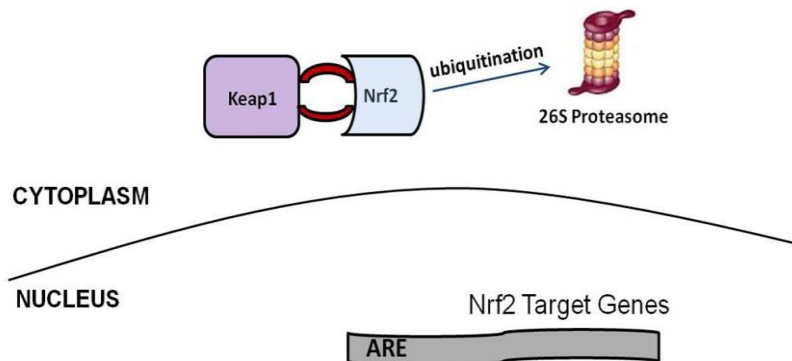
Nrf2 is a protein that is highly conserved across species and ubiquitously expressed throughout the body. Homologues have been identified in various species including fish, nematodes and flies (Moi *et al.*, 1994; Itoh *et al.*, 1995; Kobayashi *et al.*, 2002; Motohashi *et al.*, 2002; An *et al.*, 2003). The importance of this pathway in cell homeostasis is highlighted by the fact that Nrf2 is a ubiquitously expressed protein, and in particular, as shown in Nrf2 KO mice, plays a role not only in basal cell defense, but also in diverse cellular processes such as differentiation, proliferation and lipid metabolism (Kitteringham *et al.*, 2010; Bryan *et al.*, 2013) with its aberrant

expression playing a role in the pathogenesis of various diseases, such as neurodegeneration (Valko *et al.*, 2007; Bryan *et al.*, 2013).

The activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1 (Kelch-like ECH-associated protein 1), a cytosolic repressor protein that facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (Itoh *et al.*, 1999a; Zhang *et al.*, 2004) (figure 1.1a). Upon the exposure of cells to toxic insult and/or oxidative stress by electrophiles and oxidants, which cause an imbalance in the redox state of the cell, the Keap1-directed ubiquitination and degradation of Nrf2 is disrupted, leading to Nrf2 accumulation in the cytosol followed by its translocation to the nucleus. Here, Nrf2 binds to its transcriptional partner small musculoaponeurotic fibrosarcoma (Maf) proteins to form heterodimers (Motohashi *et al.*, 2004). These Nrf2-small Maf heterodimers then bind to the enhancer sequences (antioxidant response elements (ARE) or electrophile response element (ERE)) in the promoter regions of cytoprotective target genes (Friling *et al.*, 1990; Rushmore *et al.*, 1990b), which include a host of antioxidants, xenobiotic detoxification and DNA repair enzymes, molecular chaperones, anti-inflammatory response proteins and other cytoprotective enzymes (Hayes *et al.*, 2010). The coordinated expression of these genes converts toxic compounds to less harmful intermediates whilst repairing any damage caused within the cell (figure 1.1b).

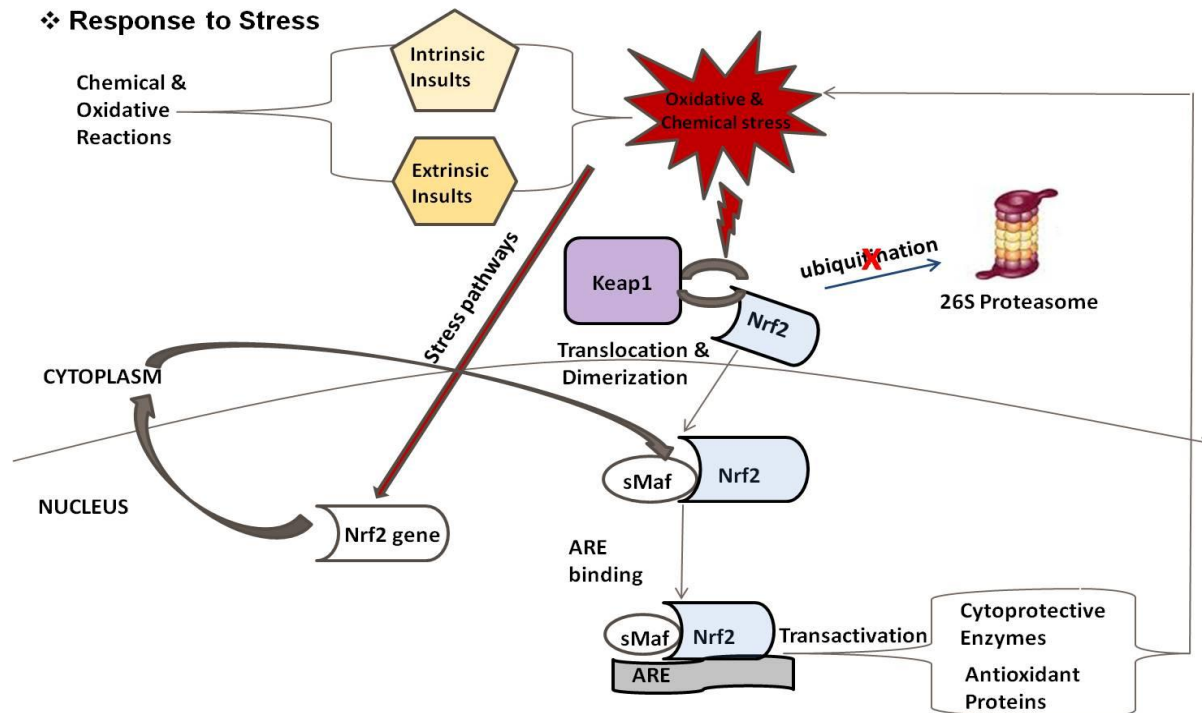
(A)

❖ Basal state



(B)

❖ Response to Stress

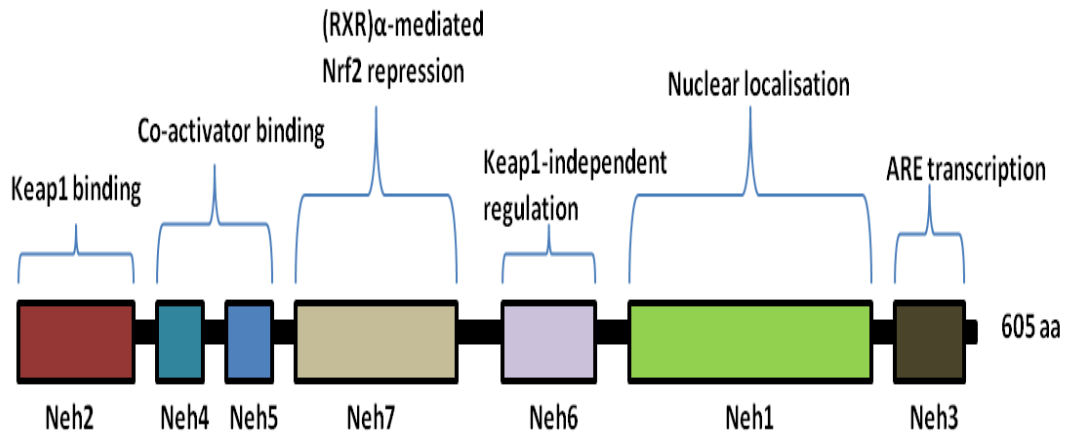


**Figure 1.1 - Schematic diagram of the Keap1:Nrf2 pathway:** In the absence of stress, the basal activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1, a cytosolic repressor protein that facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (A). In the presence of oxidative or chemical stress, the association between Nrf2 and Keap1 is disrupted, preventing the Keap1-directed ubiquitination of Nrf2. Nrf2 then translocates to the nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes augments numerous cell defence pathways and processes (B).

ARE sequences were first identified in the early 1990's prior to the discovery of Nrf2 itself (Friling *et al.*, 1990; Rushmore *et al.*, 1990b) and defined as 5'-gagTcACaGTgAGtCggCAaaatt-3' (Nioi *et al.*, 2003). The complex formed by Nrf2-small Maf heterodimers binding to the ARE sequence results in the recruitment of co-activators such as CREB binding protein (CBP) and receptor associated coactivator (RAC) which in turn, facilitate transcriptional processes (Katoh *et al.*, 2001; Lin *et al.*, 2006). Several other proteins have also been implicated in the regulation of Nrf2, including proteins of the cap 'n' collar family of proteins, AP-1 family, nuclear receptors, the chromatin remodeling factor BRG1 and the transcription co-repressor SMRT (Venugopal *et al.*, 1996; Zhang *et al.*, 2006; Iwasaki *et al.*, 2007; Wang *et al.*, 2007).

Structurally, Nrf2 is composed of 605 amino acids and categorized into six functional domains Neh1 - 7 (figure 1.2) (Itoh *et al.*, 1999b; Wang *et al.*, 2013). The Neh1 domain facilitates its nuclear localization, and contains a bZIP DNA binding domain through which interaction with transcriptional partners such as small Maf proteins occurs (Itoh *et al.*, 1995; Katsuoka *et al.*, 2005). The Neh2 domain functions as a negative regulator by providing a binding domain for Keap1. The Neh3 domain functions as a co-activator that facilitates the transcription of the ARE-dependent genes. The Neh4 and 5 domains function together to bind to co-activator such as CBP (Katoh *et al.*, 2001; Taguchi *et al.*, 2010). Neh6 is the domain that regulates the Keap1-independent regulation of Nrf2 pathway (McMahon *et al.*, 2004). Neh7 is the domain that facilitates retinoid X receptor alpha (RXR $\alpha$ )-mediated Nrf2 repression through a direct interaction between the two proteins (Wang *et al.*, 2013).





**Figure 1.2 - Schematic illustrating the structure of Nrf2.** The relative positions and main roles of each domain are shown in the diagram.

Several models of Nrf2 regulation have been suggested and can be categorized into Keap1- dependent and Keap1-independent regulation of Nrf2 which will be discussed below. For a detailed overview of these regulations, see reviews by (Baird *et al.*, 2011; Bryan *et al.*, 2013). Notably, none of the models suggested so far can fully address how constitutive activation of Nrf2-regulated genes is controlled basally. This could be as a result of auto-regulation of Nrf2, as suggested by Kwak *et al.*, or due to the constitutive generation of reactive oxygen species from normal cellular metabolic processes (Kwak *et al.*, 2002).

## 1.2 Keap1-dependent regulation of Nrf2

Keap1 is a cytoplasmic protein and a Cullin-3-based ubiquitin ligase adaptor. It is a protein rich in cysteine residues and interacts with the actin cytoskeleton through its double glycine repeat domain as revealed by immunocytochemistry and immunoprecipitation (Kang *et al.*, 2004; Watai *et al.*, 2007). It is composed of 624 amino acids and consists of three main functional domains; the Bric-a-brac, tram-track, broad complex (BTB), Intervening Region (IVR) and Kelch domains. The BTB region is involved in dimerization, the IVR is targeted by many thiol-reactive small molecule inducers of Nrf2, and the Kelch domain moderates Nrf2 binding (Baird *et al.*, 2011).

Following the discovery of Keap1 as an Nrf2 repressor (Itoh *et al.*, 1999b), the physiological importance of Keap1 was demonstrated by the genetic knock down of Keap1 gene which resulted in lethal deficiencies in new born mice most likely, as a result of hyperkeratosis of the upper GI tract leading to malnutrition (Wakabayashi *et al.*, 2003). In a recent study to support this, a graded depletion of Keap1 showed increased mortality in young mice (Taguchi *et al.*, 2010). Following hepatocyte-specific deletion of Keap1, there is a marked increase in the levels of Nrf2. This stimulation of Nrf2 reduces sensitivity of the cells to acetaminophen liver toxicity (Okawa *et al.*, 2006).

### 1.2.1 Nrf2 ubiquitination and proteasomal degradation

Under basal conditions, Keap1 directs Nrf2 ubiquitination and proteasomal degradation. In the presence of pharmacological inhibitors of the proteasome, Nrf2 is upregulated, as is the expression of ARE-dependent genes (Sekhar *et al.*, 2000; Itoh *et al.*, 2003; Stewart *et al.*, 2003). Keap1 acts as a substrate adaptor for a Cullin 3 (Cul3)-dependent E3 ubiquitin ligase complex, thereby bringing together Nrf2 and ROC1/RBX1 (Ring-box protein 1), a ring-box protein which recruits a ubiquitin charged E2 molecule. The ubiquitin molecule is conjugated to one of the lysine residues (lys-44,-50,-52,-53,-56,-64,-68) located on Nrf2 at the Neh2 domain

(Cullinan *et al.*, 2004b; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004; Furukawa *et al.*, 2005). A study by Zhang shows that mutation of these lysine residues causes an accumulation of Nrf2 whilst the reverse was seen when the mutated residues were substituted back to the initial lysine residues re-establishing the Keap1-directed ubiquitination and proteasomal degradation (Zhang *et al.*, 2004). Nrf2 has been shown to have a relatively short half-life of 10-20 minutes, hence contributing to a low basal expression in cells, most likely due to the continuous production of Nrf2 and its rapid degradation (Alam *et al.*, 2003; Itoh *et al.*, 2003). Keap1 is also a target for ubiquitination within the IVR domain under conditions of oxidative and chemical stress, however, this seems to be inducer specific. The Nrf2 inducer tert-butylhydroquinone (tBHQ) directs Keap1 for proteasomal degradation and using tandem mass spectrometry (MS/MS), the addition of an ubiquitin molecule onto ectopically expressed Keap1-V5 in cells at the Lys-298 residue was detected in the presence of tBHQ or Iodoacetamide (IAA). Notably, not all Nrf2 inducers cause Keap1 ubiquitination (Hong *et al.*, 2005; Zhang *et al.*, 2005).

### **1.2.2 Keap1 as a sensor of oxidative stress**

The high frequency of cysteine residues in the Keap1 sequence confers it with a relative high reactivity to electrophilic inducers (Miseta *et al.*, 2000). Several studies (Dinkova-Kostova *et al.*, 2002; Wakabayashi *et al.*, 2004) have shown the covalent modification of multiple cysteine residues by Nrf2 inducers, which is hypothesized to alter the conformation of Keap1, thus preventing its association with Nrf2, allowing Nrf2 to translocate to the nucleus. Dinkova-Kostova *et al.* identified the most reactive Keap1 cysteines using mass spectrometry in which dexamethasone mesylate modified C257, C273, C288 and C297 within the IVR domain in purified murine Keap1 (Dinkova-Kostova *et al.*, 2002).

Further to this discovery, mutagenesis analysis showed that C273 and C288 (mutants C273<sup>S/A</sup> and C288<sup>S/A</sup>) within the IVR domain are primarily responsible for repressing Nrf2 activity under basal conditions (Wakabayashi *et al.*, 2004). In support of this, it has been shown that in the presence of the mutant C273S<sup>A</sup>, Nrf2

activity was increased, in comparison to wild-type Keap1 (Zhang *et al.*, 2004; Kobayashi *et al.*, 2006). Recently, Yamamoto *et al.* further confirmed this *in vivo* using a transgenic complementation rescue model. The authors reported that transgenic expression of C273A or C288A mutant Keap1 was unable to reduce the constitutive high levels of Nrf2 in Keap1 null mice (Yamamoto *et al.*, 2008). In addition, the mutant forms of C273A and C288A were unable to prevent the lethality seen with the loss of Keap1, indicating the role of Nrf2/Keap1 in animal viability (Yamamoto *et al.*, 2008).

In addition to the aforementioned cysteines with roles in basal Nrf2 regulation, C151 found in the BTB domain has been shown to be important for the ability of various inducers (sulforaphane, nitric oxide, tBHQ, H<sub>2</sub>O<sub>2</sub>) to upregulate Nrf2 (Zhang *et al.*, 2003). In addition, it has been suggested that C151 may be important for the interaction between Keap1 and Cul3, the E3 ligase responsible for Nrf2 ubiquitination (Zhang *et al.*, 2003). In contrast to this, Yamamoto *et al.* saw the opposite, as tBHQ was able to induce Nrf2 in the presence of mutant C151 in isolated MEFs (Yamamoto *et al.*, 2008). This indicates that whilst C151 plays a role in the Keap1-dependent induction of Nrf2 by some compounds, it is not required for the action of all Nrf2 activators. This is also highlighted by the findings of C273 and C288 mutagenesis studies and that various electrophiles form covalent adducts with a subset of cysteine residues in Keap1, not just those mentioned above.

It is of major interest to know how the modifications of the cysteine residues confer a suppression of Keap1 and subsequent upregulation of Nrf2, thus providing vital information on the regulation of the Nrf2 pathway. Additionally, whilst it is plausible to state that a certain electrophile both modifies a subset of cysteine residues in Keap1 and induces Nrf2, this by no means links cause and effect due to the substantial number of other factors which may contribute- such as changes in redox state of the cell, changes in transcription and translation and alterations in protein degradation, among other things.

### 1.3 Models of Nrf2 regulation

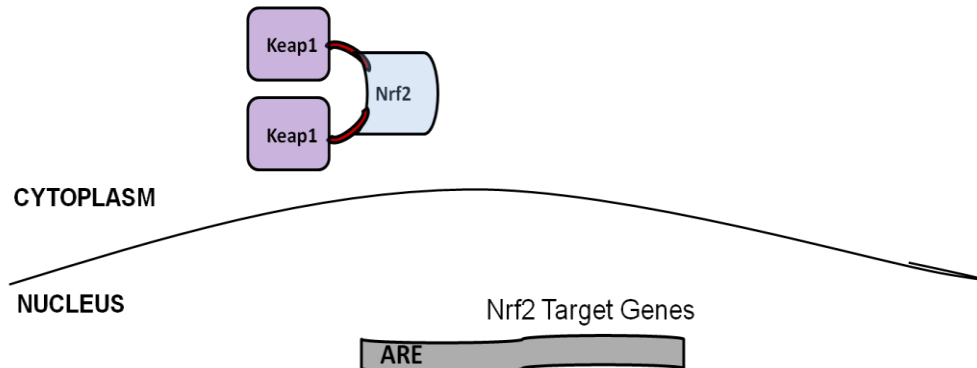
Different models of Nrf2 regulation have been proposed, and include the following: Sequester and release, Keap1 and Cul3 dissociation, Hinge and latch model, Keap1 nucleocytoplasmic shuttling, ubiquitination of Keap1, Nrf2 as a direct sensor. Each of these models is discussed in the following sections.

#### 1.3.1 Sequester and release:

This model proposes that Keap1, a predominantly cytoplasmic protein sequesters Nrf2 in the cytoplasm under basal conditions. In the presence of inducers, Nrf2 is released from Keap1 and translocates to the nucleus where it transactivates cytoprotective genes. The proposal for this model initiated from a study by Itoh *et al.* which showed that Nrf2 is localized both in the cytoplasm and the nucleus whereas Keap1 was found in the cytoplasm (Itoh *et al.*, 1999b). When Nrf2 is co-expressed with Keap1, the latter is able to sequester Nrf2 from the nucleus into the cytoplasm whereas in the absence of co-expressed Keap1, Nrf2 accumulates in the nucleus (Itoh *et al.*, 1999b)(figure 1.3). In support of this, sulforaphane and bis(2-hydroxybenzylidene)acetone have been shown to provoke a concentration-dependent dissociation of Keap1 from the Nrf2 Neh2 domain *in vitro* (Dinkova-Kostova *et al.*, 2002). The sequester and release model of Nrf2 regulation is summarised in figure 1.3.

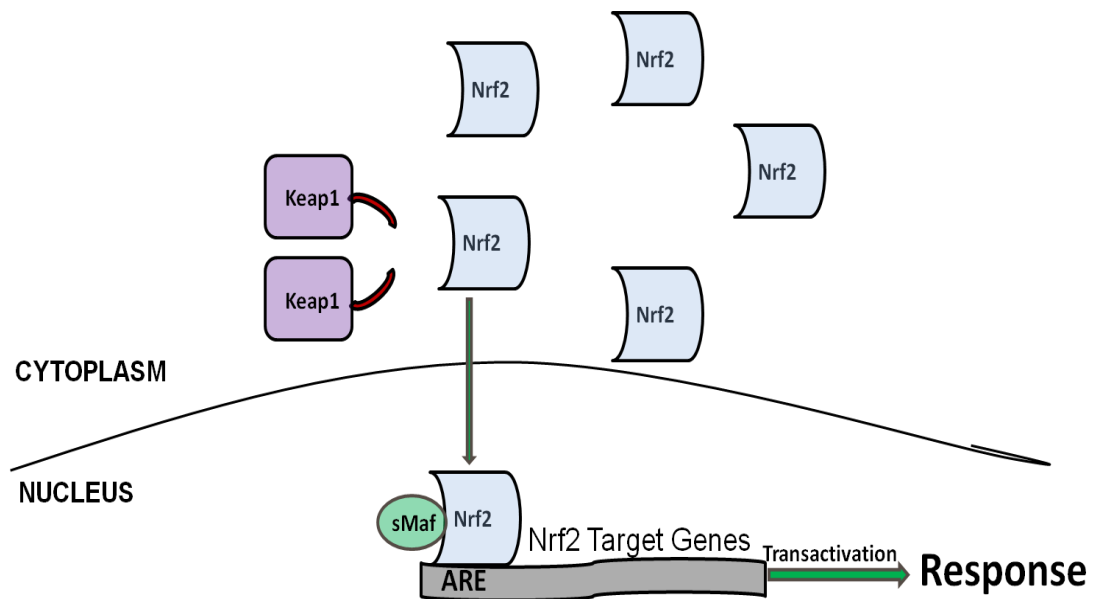
(A)

❖ Basal state



(B)

❖ Induced State



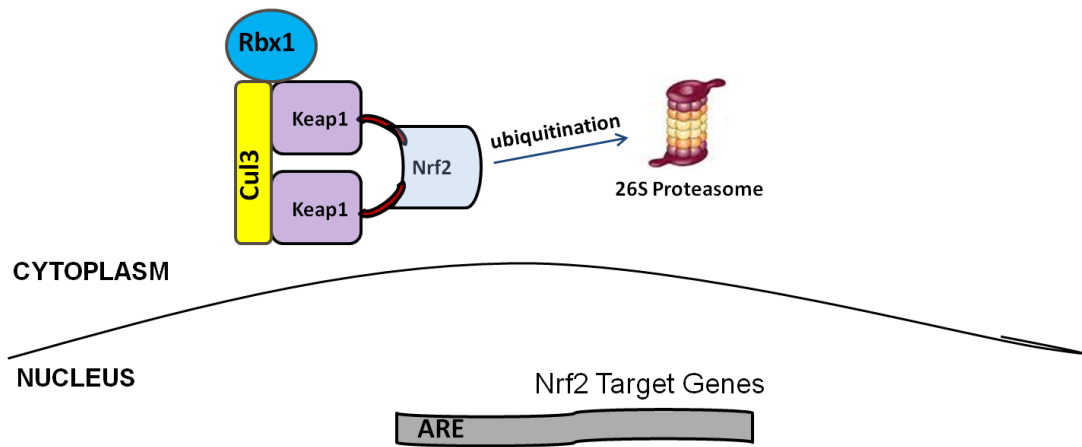
**Figure 1.3 - Schematic illustrating sequester and release model of Nrf2 regulation.** In the absence of stress, the basal activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1 by binding to the actin cytoskeleton in the cytoplasm (A). In the presence of inducers, the association between Nrf2 and Keap1 is disrupted, Nrf2 then translocates to the nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.

### 1.3.2 Dissociation of Keap1 and Cul3

Following studies showing the involvement of a Keap1-Cul3-Rbx1 complex in directing the proteasome-mediated degradation of Nrf2 (Cullinan *et al.*, 2004b; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004; Furukawa *et al.*, 2005), it was hypothesised that the dissociation of Keap1 and Cul3 leads to the stabilization of Nrf2 (figure 1.4). This was confirmed by Zhang and other researchers, who observed a dissociation of Keap1 from Cul3 in the presence of several Nrf2 inducers including tBHQ, sulforaphane, eicosapentaenoic acid (EPA) and N-iodoacetyl-N-biotinylhexylenediamine (IAB) (Zhang *et al.*, 2004; Gao *et al.*, 2007; Rachakonda *et al.*, 2008; Niture *et al.*, 2009). The dissociation of Keap1 and Cul3 prevents the ubiquitination and subsequent degradation of Nrf2. The dissociation of Keap1 and Cul3 was shown to be dependent on the presence of C151 within the BTB domain of Keap1. C151 is surrounded by four positively charged amino acids (K131, R135 and K150 and H154), theoretically making it highly reactive towards inducers. The modification of C151 by electrophilic Nrf2 inducers may provoke Cul3 dissociation via steric hindrance (Egglar *et al.*, 2009). However, it appears that not all Nrf2 inducers cause Keap1-Cul3 dissociation. The dissociation of Keap1 and Cul3 model of Nrf2 regulation is summarised in figure 1.4.

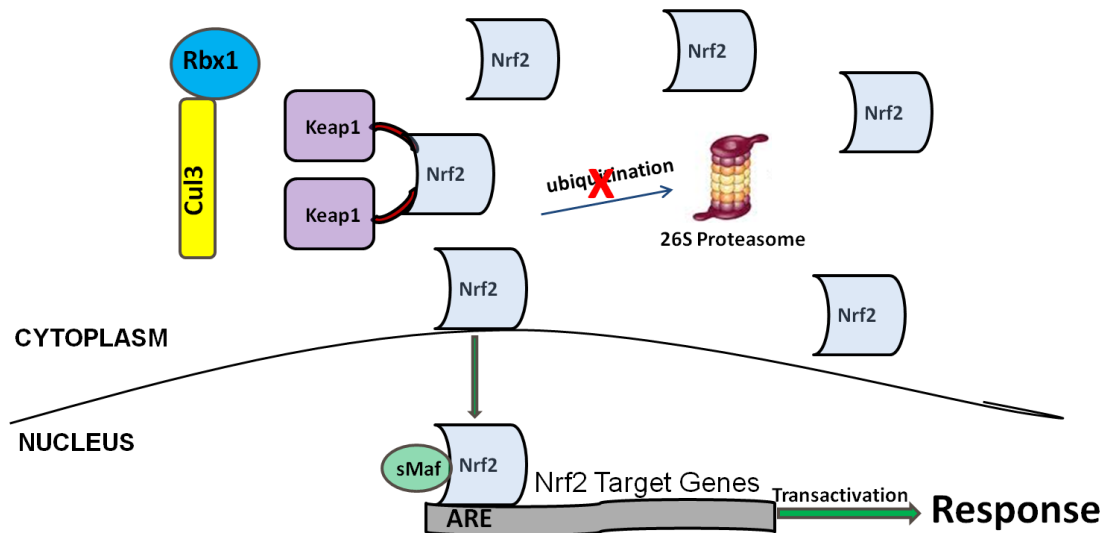
(A)

❖ Basal state



(B)

❖ Induced State



**Figure 1.4 - Schematic illustrating dissociation of Keap1 and Cul3 model of Nrf2 regulation.** In the absence of stress, the basal activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1, a cytosolic repressor protein that facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (A). In the presence of inducers, the association between Keap1 and Cul3 is disrupted, preventing the Keap1-directed ubiquitination of Nrf2. Nrf2 then translocates to the nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.



### 1.3.3 Hinge and Latch Model

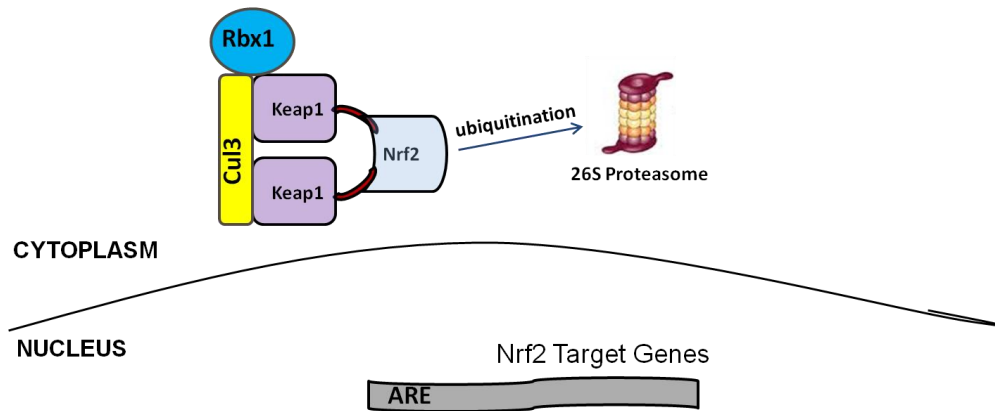
The homodimer Keap1 binds to a single molecule of Nrf2 at the Neh2 domain (Lo *et al.*, 2006; McMahon *et al.*, 2006). The ETGE motif within the Neh2 domain provides a binding site for the beta propeller formed by the Kelch repeats of Keap1 (Kobayashi *et al.*, 2004). Notably, a binding still occurs in the absence of this motif (McMahon *et al.*, 2006), however, it is insufficient to facilitate the ubiquitination of Nrf2 suggesting a second association is needed to initiate ubiquitination, hence the involvement of DLG motif (McMahon *et al.*, 2006). The ETGE and DLG motifs have different affinities for Nrf2 due to different electrostatic interactions. The ETGE motif has 13 electrostatic interactions with Keap1, and almost double of the DLG motif, which has 8 interactions with Keap1. Without the binding of both motifs with Keap1, no ubiquitination or degradation of Nrf2 is observed (McMahon *et al.*, 2006; Tong *et al.*, 2006). A recent study showed that the Keap1-DLGex binding is both enthalpy and entropy driven while Keap1-ETGE binding is enthalpy driven only (Fukutomi *et al.*, 2014). Further kinetic analysis therefore showed Keap1-DLGex binding to occur through fast-association and fast-dissociation complex (association rate constant ( $ka$ ) =  $6.1 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ ) and dissociation rate constant ( $kd$ ) =  $0.196 \text{s}^{-1}$ ) whereas Keap1-ETGE displayed a slow-association complex, hence a stable complex (association rate constant ( $ka$ ) =  $1.20 \times 10^{-3} \text{M}^{-1} \text{s}^{-1}$ ) and dissociation rate constant ( $kd$ ) =  $1.22 \times 10^{-4} \text{s}^{-1}$ ) (Fukutomi *et al.*, 2014).

Further exploration showed that Nrf2 forms an alpha helix with six lysine residues between the ETGE and DLG motifs. Deletion analysis showed that the lysine residues were necessary for Keap1-mediated Nrf2 degradation, as mutation of the lysine residues increased the stability of Nrf2 (Zhang *et al.*, 2004). Under basal conditions, one Nrf2 protein is sequestered by two Keap1 proteins via the DLG and ETGE motifs. Binding via the high-affinity ETGE motif provides the hinge which allows the free movement of Nrf2, whilst binding via the DLG acts as a latch positioning the lysine residues for ubiquitin conjugation (McMahon *et al.*, 2006; Tong *et al.*, 2006; Padmanabhan *et al.*, 2008). In the presence of inducers, it is thought that a conformational change of Keap1 inhibits binding via the DLG motif

(the latch) but does not affect binding via the ETGE motif (the hinge) of Nrf2. This impedes the ability of Keap1 to direct the ubiquitination of Nrf2, which is no longer degraded. Keap1 becomes saturated with Nrf2 and newly synthesized Nrf2 proteins are free to accumulate in the nucleus, bind to small Maf proteins and transactivate ARE regulated genes (figure 1.5). The destabilization of the Keap1-Nrf2 interaction is suggested to be due to a conformational change in the IVR domain of Keap1, as a result of cysteine modification. For a review, see (Copple, 2012; Bryan *et al.*, 2013). Although the hinge and latch model explains the mechanism by which certain inducers might activate Nrf2, it does not explain how compounds such as arsenic and chromium can completely dissociate the Nrf2-Keap1 complex. Taken together, this suggests that the Nrf2 response to different inducers is controlled by different mechanisms (He *et al.*, 2006; He *et al.*, 2008). However, currently the hinge and latch model is the most widely accepted mechanism of those proposed to date (Tong *et al.*, 2006). The hinge and latch model of Nrf2 regulation is summarised in figure 1.5.

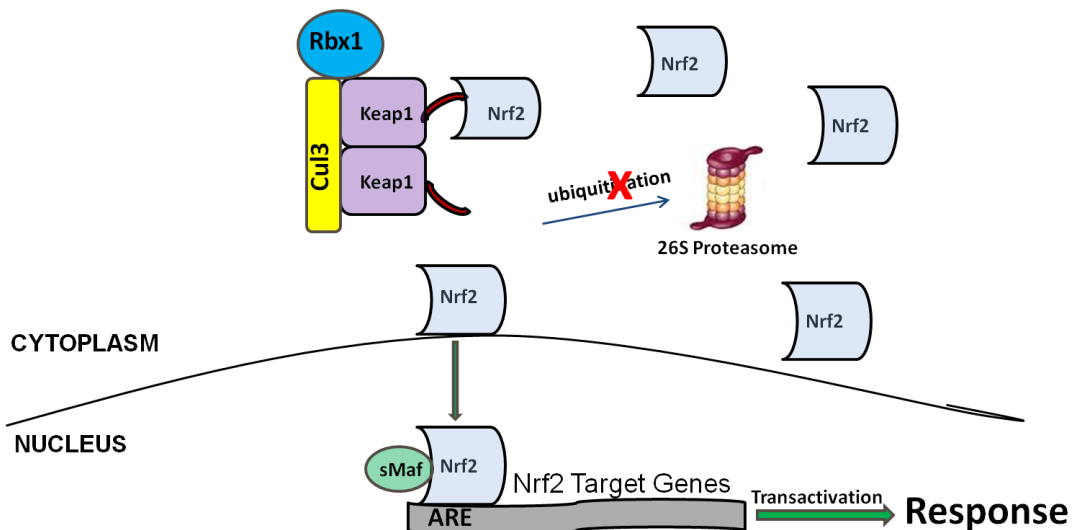
(A)

❖ Basal state



(B)

❖ Induced State



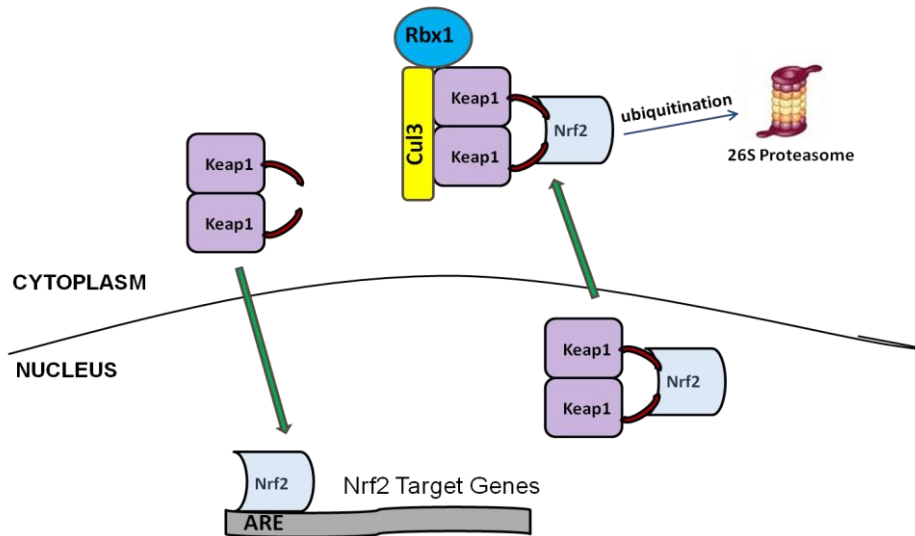
**Figure 1.5 - Schematic illustrating hinge and latch model of Nrf2 regulation.** In the absence of stress, the basal activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1 at the high affinity ETGE and low affinity DLG motif. This binding stabilizes Nrf2 orientation and facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (A). In the presence of inducers, the association between Keap1 and Nrf2 is disrupted at the weaker DLG motif, leading to a conformational change in Keap1, thus preventing Keap1-directed ubiquitination of Nrf2. Newly synthesized Nrf2 then translocates to the nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.

#### **1.3.4 Keap1 nucleocytoplasmic shuttling**

Studies using Nrf2 knockout mice have highlighted the role of Nrf2 in the basal regulation of ARE-dependent genes (McMahon *et al.*, 2001). This illustrates that some Nrf2 must be localised in the nucleus under basal conditions. Since the regulation of Nrf2 is primarily controlled by Keap1, it is possible that Keap1 somehow regulates Nrf2 levels in the nucleus or that Nrf2 can evade Keap1 under resting conditions. The identification of the nuclear export signal Crm1 within the IVR domain of Keap1 supported this view (Nguyen *et al.*, 2005; Velichkova *et al.*, 2005) and the chemical inhibition of Crm1 led to an increase in the nuclear levels of Keap1. In contrast, (Watai *et al.*, 2007) concluded that the vast majority of Keap1 is localised in the cytoplasm and does not translocate to the nucleus, although a small fraction was located in the nucleus with unknown physiological relevance. This model has several discrepancies and will need further elucidation. For a review see (Baird *et al.*, 2011). In summary, Keap1 nucleocytoplasmic shuttling model of Nrf2 regulation is summarised in figure 1.6.

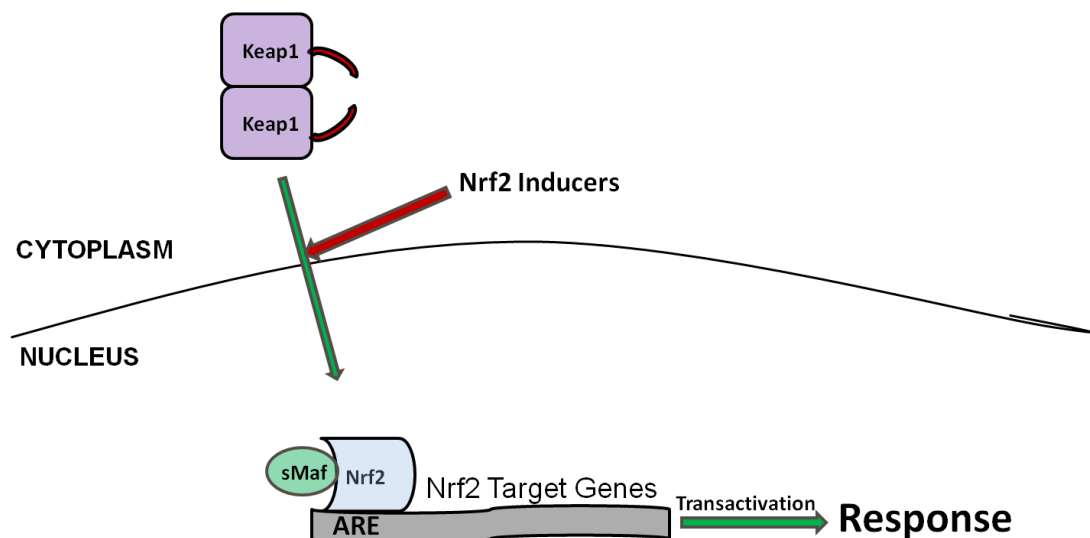
(A)

❖ Basal state



(B)

❖ Induced state



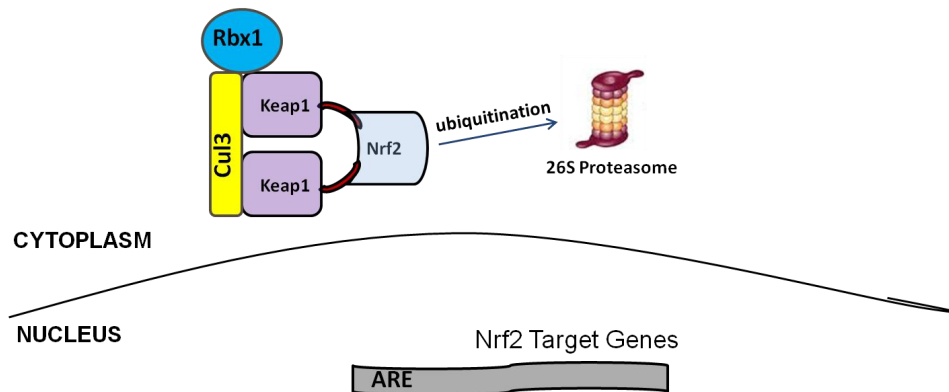
**Figure 1.6 - Schematic illustrating Keap1 nucleocytoplasmic shuttling model of Nrf2 regulation.** In the absence of stress, the basal activity of Nrf2 is primarily governed by an entry of Keap1 into the nucleus which regulates it (A). In the presence of inducers, the entry of Keap1 into the nucleus is prevented, allowing Nrf2 to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.

### **1.3.5 Keap1 ubiquitination**

Another proposed mode of Nrf2 regulation is Keap1 auto-ubiquitination. Keap1 can be ubiquitinated in response to the inducer tBHQ (Hong *et al.*, 2005; Zhang *et al.*, 2005). The authors confirmed this by mass spectrometry, with ubiquitination of Keap1 shown to occur at lysine 298 residue of the IVR domain in response to the Nrf2 inducer IAB (Hong *et al.*, 2005; Zhang *et al.*, 2005). The role of Keap1 ubiquitination in the mechanism of action of other Nrf2 inducers has yet to be examined. In summary, Keap1 ubiquitination model of Nrf2 regulation is summarised in figure 1.7.

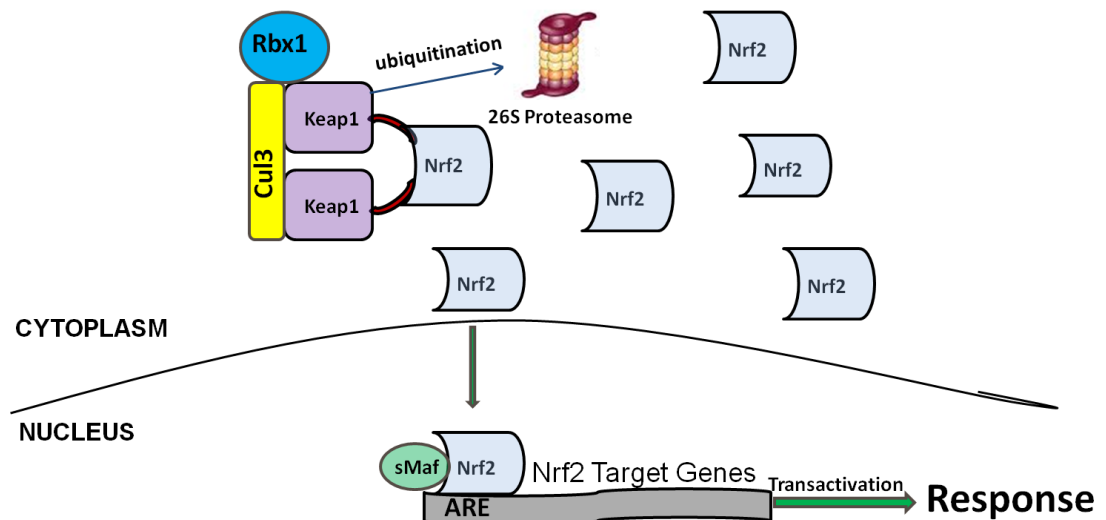
(A)

❖ Basal state



(B)

❖ Induced State

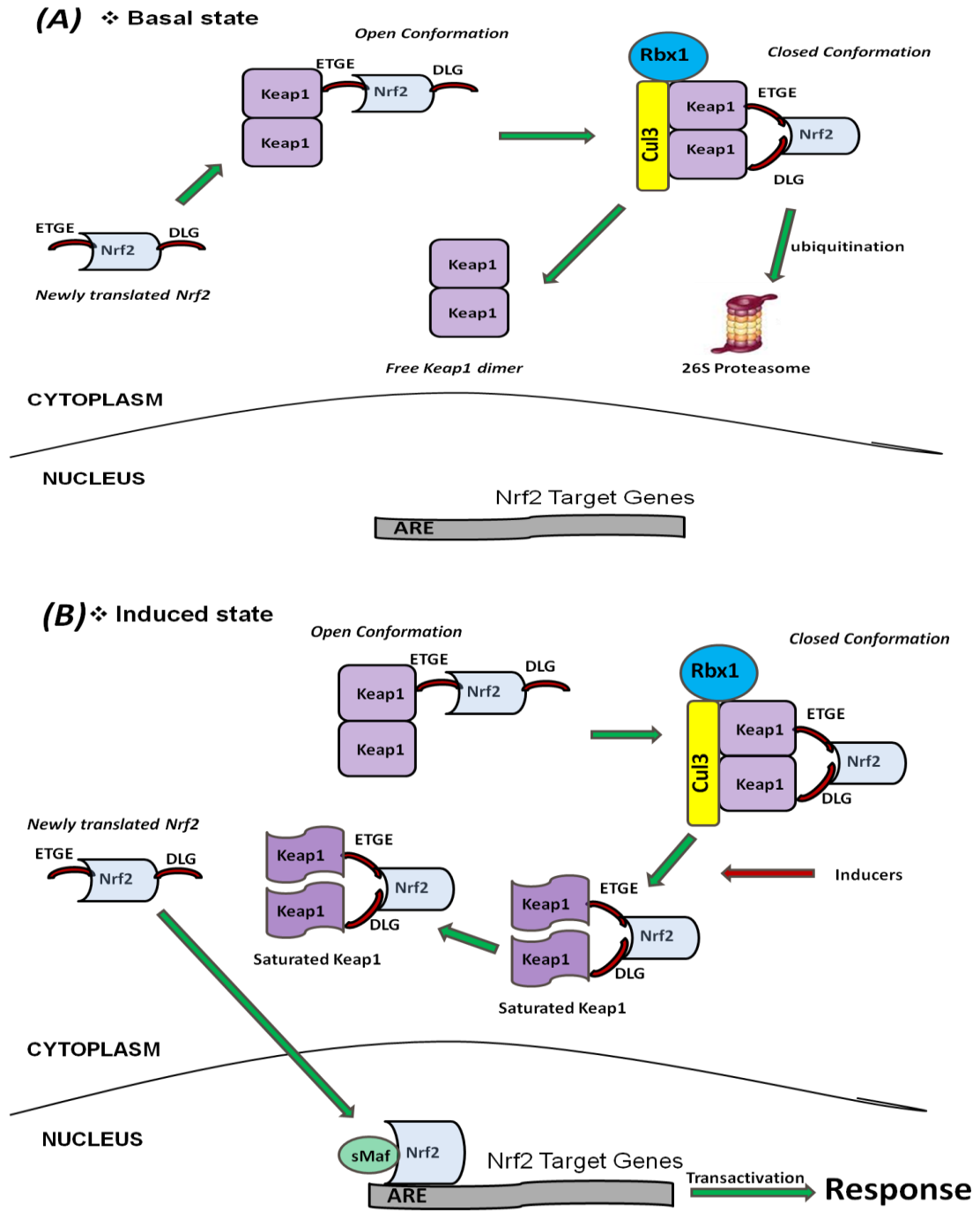


**Figure 1.7 - Schematic illustrating ubiquitination of Keap1 model of Nrf2 regulation.** In the absence of stress, the basal activity of Nrf2 is primarily governed by an interaction with a homodimer of Keap1, which facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (A). In the presence of inducers, Keap1 becomes a target of ubiquitination and subsequent degradation via the Cul3 ubiquitin ligase complex. Nrf2 becomes stabilized and then translocates to the nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.

### 1.3.6 The cyclic sequential attachment and regeneration model

Another recently proposed model of Keap1-mediated degradation of Nrf2 is the cyclic sequential attachment and regeneration model (Baird *et al.*, 2013)(figure 1.8). Using the techniques of quantitative FRET (Forster resonance energy transfer)-based system to investigate the interaction of Keap1 and Nrf2 in single live cells, it was shown that under basal conditions, Nrf2 and Keap1 exhibit two conformations; the open and closed conformations which occurs through binding of ETGE motif and DLG motif respectively (Baird *et al.*, 2013). Thus, at the basal state, the open conformation is formed first through the binding of Nrf2 to Keap1 at the ETGE motif followed by the binding of DLG to form the closed conformation. Hence, in the closed conformation, Nrf2 is correctly aligned with the E2 ubiquitin-conjugating enzyme bound to E3 and ubiquitination occurs. The released ubiquitinated Nrf2 is further degraded by the proteasome. This then frees up Keap1 dimer to be regenerated and then ready to bind newly translated Nrf2 and the whole cycle repeats again. In the presence of inducers, the cycle is disrupted in which Nrf2 is not correctly positioned for ubiquitination due to a conformational change in Keap1 occurring as a result of its cysteine modification by the inducers. Whilst Nrf2 is still bound to Keap1, it is no longer aligned appropriately with the E2 ubiquitin-conjugating enzyme bound to initiate ubiquitination. Thereby, Nrf2 is not released from Keap1 in the closed conformation and Keap1 becomes saturated. Newly translated Nrf2 is then free and able to bind to ARE on the cytoprotective genes and initiate their transcription (Baird *et al.*, 2013). In summary, the cyclic sequential attachment and regeneration model of Nrf2 regulation is summarised in figure 1.8.





**Figure 1.8 - The cyclic sequential attachment and regeneration model of Nrf2 regulation.** In the basal state, newly translated Nrf2 binds to Keap1 at the ETGE motif to form the open conformation followed by the DLG binding to form the closed conformation. This binding stabilizes Nrf2 orientation and facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cul3 ubiquitin ligase complex (A). In the presence of inducers, the closed conformation is stabilized as a result of the conformational change in Keap1, thus preventing Keap1-directed ubiquitination of Nrf2. Newly synthesized Nrf2 then translocates to the nucleus to illicit a response (B). Adapted from Baird et al., 2013.

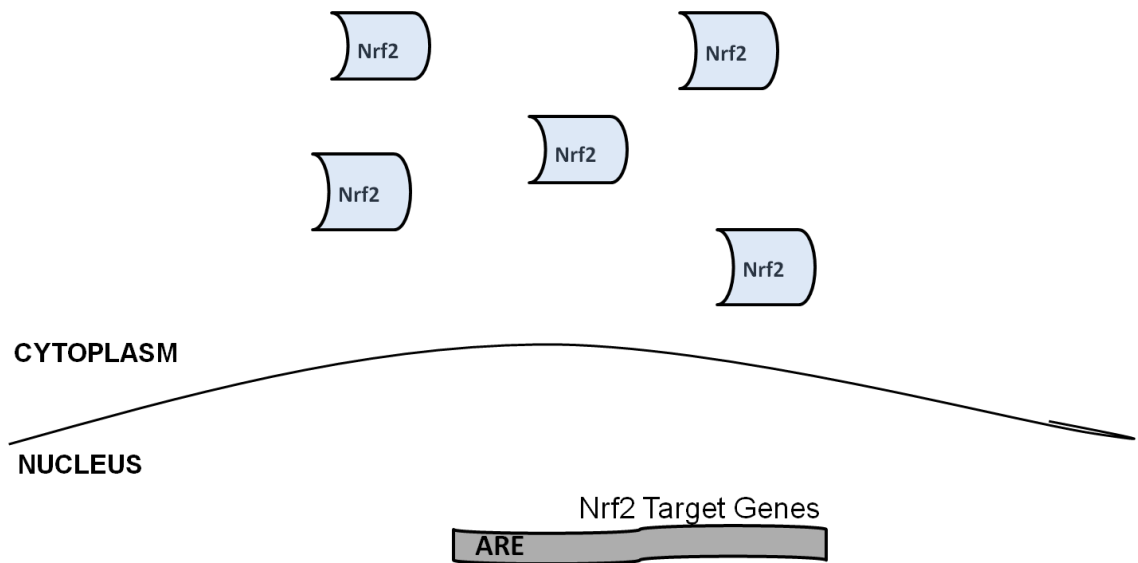
### 1.3.7 Nrf2 as a sensor of stress

Using recombinant proteins and Nrf2 purified from cell lysates, it has been shown that Nrf2 itself could be a sensor of the inducers FIAsh (an arsenic-based fluorophore) and phenylarsine oxide (PAO), which were able to bind directly to Nrf2 (He *et al.*, 2009). The authors confirmed that the binding of these inducers occurs at Cys235, Cys311, Cys414 and Cys506 of Nrf2, whilst mutation of these residues impeded the ability of Nrf2 to bind to the ARE of target genes (He *et al.*, 2009).

In addition, Nrf2 also contains nuclear import and export signals within the Neh5 domain, and these provide a means for auto-regulation of Nrf2 through its cellular localisation (Li *et al.*, 2005; Li *et al.*, 2006). Under basal conditions, there is an homeostatic balance between the NES and the NLS signals, maintaining the levels of Nrf2 in the nucleus. In the presence of an Nrf2 inducer, there is an imbalance through which the NES is deactivated, thereby causing an accumulation of the protein in the nucleus, as confirmed by studies in which mutation of the NES triggers an increase in the nuclear levels of Nrf2 (Li *et al.*, 2006) (figure 1.9). However, it has been shown that typical Nrf2 inducers can not potentiate the nuclear accumulation of Nrf2 that is associated with genetic loss of Keap1 (Wakabayashi *et al.*, 2003). Therefore, the inhibition of the nuclear export of Nrf2 does not appear to be a universal mechanism of Nrf2 activation.

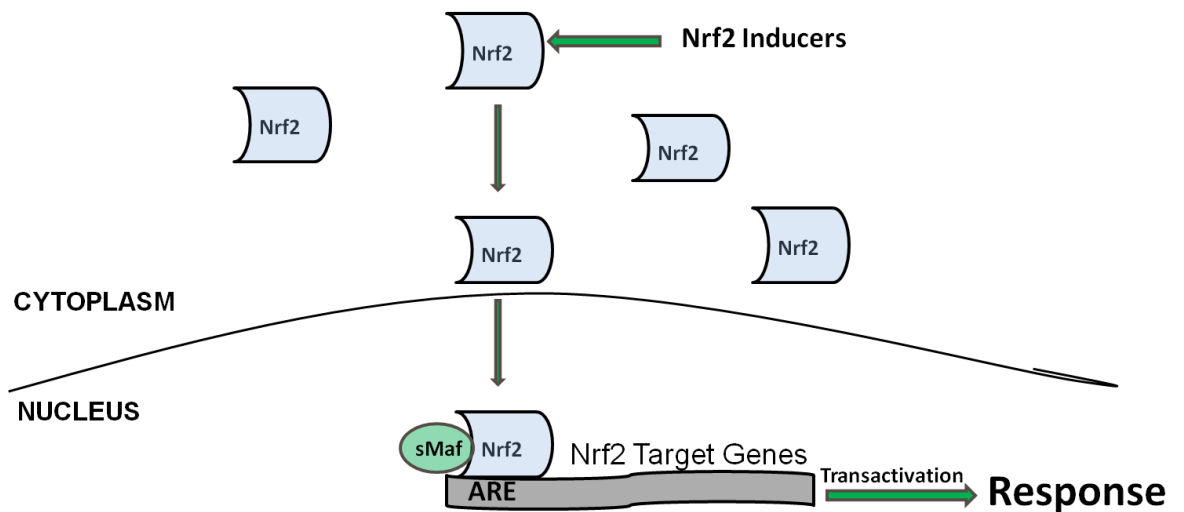
(A)

❖ Basal



(B)

❖ Induced



**Figure 1.9 - Schematic illustrating Nrf2 senses inducers directly model of Nrf2 regulation.**

*In the absence of stress, the basal activity of Nrf2 is primarily regulated by the nuclear export signal (NES) which controls the cytoplasmic distribution of Nrf2 (A). In the presence of inducers, which reacts with the cysteine residues within Nrf2, the NES becomes inactivated allowing the entry of Nrf2 through its nuclear localization signal (NLS) into nucleus where it binds to sMaf proteins to transactivate ARE-dependent genes. The coordinated induction of these genes then illicit a response (B). Adapted from Baird et al., 2011.*

## 1.4 Keap1-independent Regulation of Nrf2

There is also mounting evidence that demonstrates that Nrf2 activity can be regulated independently of Keap1. This will be further discussed below.

### 1.4.1 Transcriptional control of Nrf2

The possibility of Nrf2 being regulated at the transcriptional level is a sound hypothesis. In contrast to cellular work, where most effect on Nrf2 induction was seen at the protein level, (Nguyen *et al.*, 2003; Stewart *et al.*, 2003), certain inducers like D3T (3H-1, 2-dithiole-3-thione), Oltipraz, b-NF initiated an increase in the Nrf2 mRNA levels *in vivo* using mouse samples (Kwak *et al.*, 2001; Ramos-Gomez *et al.*, 2001). In addition, ARE and XRE sequences are found in the promoter region of genes such as glutathione S-transferases (GSTs) that are regulated by Nrf2 and AHR (aryl hydrocarbon receptor) respectively (Rushmore *et al.*, 1990a; Miao *et al.*, 2005). The intermediates generated as a result of the activation of the AHR pathway have been shown to induce ARE as seen in a study conducted by Miao *et al.* using TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), which induced AHR, ROS and Nrf2 simultaneously (Miao *et al.*, 2005). Analysis of the promoter sequence of Nrf2 shows that XRE-like elements are found at position -712 and at positions +755 and +850 within the mRNA initiation site (Miao *et al.*, 2005). Microarray data from the study by Kwak *et al.*, 2012 identified the presence of ARE sequences in Nrf2 promoter regions suggesting an auto-regulatory mechanism (Kwak *et al.*, 2002). Notably in this study, it was shown that D3T increased both Nrf2 protein and mRNA levels. This effect was inhibited in the presence of a protein synthesis inhibitor, cyclohexamide (CHX) indicating this compound functions at the mRNA level preventing Nrf2 transcription and translation. Interestingly, in a luciferase reporter assay, in which the direct binding of Nrf2 to the ARE sequences is assessed within its own promoter showed, an enhanced activity is seen whilst this effect was repressed using a mutant form (Kwak *et al.*, 2002).

It is also plausible for a cross-talk to exist between Nrf2 and other transcription factors that combat oxidative stress such as NF- $\kappa$ B (nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells). A study confirmed the presence of an NF- $\kappa$ B binding region on Nrf2's promoter region highlighting the possibility of NF- $\kappa$ B regulating Nrf2's activity (Nair *et al.*, 2008). Taken together, this suggests that the regulation of Nrf2 pathway is complex and regulated at multiple levels.

#### **1.4.2 Post-transcriptional control of Nrf2 - Nrf2 and microRNAs**

Evidences are mounting up about the involvement of microRNAs (miRNAs) in cellular homeostasis but require further investigation for it to be fully elucidated. miRNAs are also implicated in different pathways such as aging processes and their associated proteins that cross-talk with the Nrf2 pathway (Narasimhan *et al.*, 2012; Papp *et al.*, 2012; Stachurska *et al.*, 2013). miRNAs are short single stranded non-coding regulatory RNAs of about 18-24 nucleotides in length that coordinate the regulation of various physiological and pathological pathways such as atherosclerosis (Droge, 2002; Stocker *et al.*, 2004; Filipowicz *et al.*, 2008). Following their transcription by RNA polymerase II from genetic loci, they are exported as short hair pins to the cytoplasm where they are cleaved by cytoplasmic RNAses (e.g. DICER) which then yield a single stranded miRNA which can bind to the protein complexes in the RNA-induced silencing complex (RISC)-complexes and elicit function. miRNAs function by binding to the mRNA at the UTR (3' untranslated region) to initiate degradation or inhibition of the translational process (Brodersen *et al.*, 2009; Krol *et al.*, 2010). miRNAs could be regulating the Nrf2 pathway by two processes: they could either be regulated by the redox itself as seen with the down-regulation of Dicer protein, ribonuclease III, a regulatory enzyme in miRNAs synthesis with hydrogen peroxide (Ungvari *et al.*, 2013), or they are regulating the antioxidant pathway directly (Cheng *et al.*, 2013).

### 1.4.3 Role of microRNAs in regulating the Nrf2 pathway

The role of miRNAs in regulating the Nrf2 pathway and the associated Nrf2 binding partners is a focus of investigation in several laboratories. *In silico* analysis of Nrf2 mRNA identified 85 binding sites for miRNAs (Papp *et al.*, 2012), suggesting the potential role of miRNAs in regulating the Nrf2 pathway. It has been suggested that the majority of the miRNAs regulate the Nrf2 pathway in a negative feedback loop (Cheng *et al.*, 2013). miRNAs identified so far in direct Nrf2 down-regulation by binding to its mRNA 3'UTR include the following: miR-153, miR-27a, miR-142-5p, miR-144, miR-28, miR-122, miR-34a (Lu, 2009; Sangokoya *et al.*, 2010; Li *et al.*, 2011; Yang *et al.*, 2011; Narasimhan *et al.*, 2012) (table 1). miR-144 was the first micro-RNA shown to be involved in the regulation of Nrf2. miR-144 has shown a negative regulation of the Nrf2 pathway targeting the 3' UTR at the 265-271 and 370-377 sites (Sangokoya *et al.*, 2010).

Independently of Keap1, a study saw the down-regulation of Nrf2 through mRNA degradation and the alteration of Nrf2 protein stability by miR-28 in human epithelial and MCF-7 cells (Yang *et al.*, 2011). This was further supported by other microRNAs summarized in table 1 that saw similar findings in neuronal cell SH-SY5Y (Narasimhan *et al.*, 2012). Importantly, miR-200a on the other hand has been seen to show a positive regulation of Nrf2 by targeting the Keap1 mRNA. miR-200a has been shown to down regulate Keap1 which caused an increase in Nrf2 levels in MDA-MB-231 and Hs578T breast cancer cell lines (Eades *et al.*, 2011). The reverse was seen when miR-200a was silenced leading to a down-regulation of the Nrf2 pathway (Eades *et al.*, 2011).

MicroRNAs may also regulate the Nrf2 pathway response by targeting the downstream targets of Nrf2 directly as seen in a study in which miR-34 targets the glutathione S-transferase 1 gene in HEK293 cells (Li *et al.*, 2011). Notably, miR-34 also targets sirtuin 1 (Sirt1), a key protein involved in maintaining the antioxidant pathway during aging processes (Houtkooper *et al.*, 2012). The regulation of Nrf2 by Sirt1 still remains to be elucidated.

Furthermore, an association between microRNAs and other Nrf2 binding partners such as Bach1, DJ-1 and small Maf protein has been revealed (Minones-Moyano *et al.*, 2011; Hou *et al.*, 2012). Hence the microRNAs let-7b, let-7c, miR-98, miR-196, miR-155 and miR-34b/c have thus been shown to regulate Nrf2 through the regulation of the aforementioned binding partners (Alam *et al.*, 2007; Hou *et al.*, 2010; Hou *et al.*, 2012; Wagner *et al.*, 2012).

Notwithstanding, the regulation of the Nrf2 pathway by microRNAs through direct binding or indirectly via Nrf2 binding partners or Nrf2 target genes is plausible but further understanding of the individual functions of microRNAs is needed (Kulkarni *et al.*, 2014). For a summary of the microRNAs that have been shown to have a direct/indirect interaction with the Nrf2 pathway, see table 1.

#### **1.4.4 MicroRNAs and reactive oxygen species (ROS) induction**

It is also of interest to note that miRNAs may also regulate genes such as p47<sup>phox</sup>, subunits of NADPH oxidase complex, HMG box-containing protein 1, NOX4 which coordinate the generation of reactive oxygen species. A knock down studies on Dicer showed the down-regulation of reactive oxygen species when treated with known inducers such as TNF- $\alpha$ , phorbol ester and vascular endothelial growth factor (Berasi *et al.*, 2004; Shilo *et al.*, 2008; Vasa-Nicotera *et al.*, 2011; Jung *et al.*, 2012).

microRNA	Target			Regulation	Reference
	Protein	Interaction	Cell Type		
miR144	Nrf2	Direct	K562 &	Negative	Sangokoya <i>et al.</i> , 2010
	Nrf2	Direct	Reticulocytes	Negative	Ferreira <i>et al.</i> , 2011
miR-28	Nrf2	Direct	MCF-7 cells	Negative	Yang <i>et al.</i> , 2011
miR-153	Nrf2	Direct	SH-SY5Y	Negative	Narasimham <i>et al.</i> , 2012
miR-27a	Nrf2	Direct	SH-SY5Y	Negative	Narasimham <i>et al.</i> , 2012
miR-142-5p	Nrf2	Direct	SH-SY5Y	Negative	Narasimham <i>et al.</i> , 2012
miR-122	Nrf2	Direct	HEK293	Negative	Lu, 2009; Li <i>et al.</i> , 2011
miR-34a	Gst1	Indirect	HEK293	Negative	Li <i>et al.</i> , 2011
miR-200a	keap1	Indirect	MDA-MB-231 & Hs578T	Positive	Eades <i>et al.</i> , 2011
let-7b	Bach 1	Indirect	Huh-7 cells	Positive	Hou <i>et al.</i> , 2010; Hou <i>et al.</i> , 2012
let-7c	Bach 1	Indirect	Huh-7 cells	Positive	Hou <i>et al.</i> , 2010; Hou <i>et al.</i> , 2012
miR-98	Bach 1	Indirect	Huh-7 cells	Positive	Hou <i>et al.</i> , 2010; Hou <i>et al.</i> , 2012
miR-199	Bach 1	Indirect	Huh-7 cells	Positive	Hou <i>et al.</i> , 2010; Hou <i>et al.</i> , 2012
miR-155	Bach 1	Indirect	HUVECS	Positive	Pulkkinen <i>et al.</i> , 2011
miR-34b/c	DJ-1	Indirect	SH-SY5Y	Positive	Minones-Moyano <i>et al.</i> , 2011

**Table 1: microRNAs that has been illustrated in regulating the Nrf2 pathway**



#### **1.4.5 Translational control of Nrf2**

In addition to the transcriptional control of Nrf2, it may also be regulated at the translational level as seen in a study using rat cardiomyocytes (Purdom-Dickinson *et al.*, 2007) in which Nrf2 mRNA showed no significant increase following H<sub>2</sub>O<sub>2</sub> treatment. However, in the presence of the RNA synthesis inhibitor actinomycin D, there was no inhibition of Nrf2 protein accumulation and the measurement of new protein synthesis confirmed an increase in the translation of Nrf2 protein in response to H<sub>2</sub>O<sub>2</sub> (Purdom-Dickinson *et al.*, 2007). Analysis of Nrf2 mRNA identified the presence of a redox-sensitive internal ribosomal entry site (IRES) in the 5'-UTR (Li *et al.*, 2010). In the presence of H<sub>2</sub>O<sub>2</sub> and sulforaphane, there was an increase in the Nrf2 protein level resulting from an increased translation from the IRES in the Nrf2 transcript (Li *et al.*, 2010). Recently, a study by Perez-Leal *et al.* Suggested that an as-yet unidentified molecular process represses the translation of Nrf2 within the open reading frame (ORF) of the gene under basal condition (Perez-Leal *et al.*, 2013). Thus, it was identified by the same authors that the repressor mechanism involves the mRNA nucleotide sequences or tertiary structure of the 3' ORF, however, the mechanism of repressing Nrf2 at these sequences requires further investigation (Perez-Leal *et al.*, 2013). Due to the speed of activation seen with most Nrf2 inducers, it is not likely that translational control plays a major role, but it could enhance the stability of Nrf2 protein following induction.

#### **1.4.6 Post-translation control of Nrf2 - Phosphorylation**

The involvement of additional signalling pathways in regulating the Nrf2 pathway is also plausible and there is mounting evidence implicating their roles (Bryan *et al.*, 2013). Most inducers that have been shown to covalently modify Keap1 have additional effects on several signalling pathways which could ultimately induce Nrf2. Not surprisingly, several kinases have been identified to have a contribution to Nrf2 pathway regulation. These include the PI3K/AKT, ERK1/2 MAPK, P-SAPK/JNK and the p38 MAPK pathways. PKC was identified to have a role in the Nrf2's regulation as its induction increases Nrf2-dependent genes and vice versa (Huang *et*

*al.*, 2000). In addition, its inhibition attenuates tBHQ-mediated induction of Nrf2. Whilst Serine-40 was identified to be the site of phosphorylation on Nrf2 (Huang *et al.*, 2000), its mutation did not completely abolish the induction of Nrf2-dependent genes. In light of this, it could be possible that PKC's involvement is in conjunction with Keap1 (Huang *et al.*, 2002; Bloom *et al.*, 2003). Further exploration showed that it's the PKC-delta isoform that mediates the release of Nrf2 from Keap1 supporting findings where oxidative stress activates Src and the ATM family of the PKC pathways (Li *et al.*, 2004; Guo *et al.*, 2010). Using PKC-delta knock out cells, inducers were unable to initiate Nrf2's full response illustrating its significance in regulating the Nrf2 pathway.

Mitogen-activated protein kinase (MAPK) signalling has also been a focus of several studies associated with Nrf2 regulation as it is activated by oxidative stress. Some Nrf2 inducers such as tBHQ, sulforaphane and cadmium are known modulators of this pathway (Yu *et al.*, 1999; Alam *et al.*, 2000). The inhibition of key proteins in this pathway including ERK, MEK or p38 attenuate the expression of Nrf2 regulated genes (Yu *et al.*, 1999; Alam *et al.*, 2000). However, this is not conclusive as the opposite effect has been seen in that the inhibition of the p38 induced Nrf2 showed a negative regulation. These findings would therefore require further investigation (Yu *et al.*, 2000), however, the effect seen could be as a result of phosphorylation of Nrf2, Keap1 or both proteins at specific residues. Also importantly, it could also be as a result of an influence from an alternative pathway. Screening the Nrf2 sequence identified the presence of different threonine and serine residues (S215, S408, S558, T559, S577) corresponding to the MAPK consensus sequence (Sun *et al.*, 2009). Mutational studies on all residues had a slight decrease on the expression of Nrf2-regulated genes suggesting that the involvement of MAPK is limited, but may be involved in fine-tuning.

Additionally, PI3K has been shown to be a contributor to Nrf2 regulation. The Nrf2 inducer tBHQ has been shown to activate the PI3K pathway showing a positive regulation by increasing ARE-dependent genes (Kang *et al.*, 2001; Lee *et al.*, 2001). PI3K also contributes to ARE-dependent genes constitutive expression. A study by Healy *et al.*, 2005 saw an induction of NQO1 with the constitutive activation of PI3K

(Healy *et al.*, 2005). Interestingly, Nrf2 also regulates some kinase pathways as Nrf2 KO mice attenuated the expression of AKT and ERK1/2 to PDGF and insulin illustrating a complex feedback mechanism existing between pathways (Reddy *et al.*, 2008). Furthermore, Nrf2 has been shown to be phosphorylated by other proteins CK2, PERK and GSK3 $\beta$  suggesting a complex interplay of different mechanisms in Nrf2 regulation (Cullinan *et al.*, 2003; Cullinan *et al.*, 2004a; Salazar *et al.*, 2006; Pi *et al.*, 2007).

Studies by Jain and Jaswal *et al.*, suggested the involvement of GSK3- $\beta$  and Fyn kinase, a member of Src family, in Nrf2 regulation. These authors hypothesised that these kinase proteins reduce the accumulated Nrf2 levels in the nucleus upon the removal of Nrf2 inducers. This in turn phosphorylates GSK3 $\beta$  which increases the nuclear accumulation of Fyn, phosphorylating Nrf2 at Y568. Nrf2 is exported upon phosphorylation and targeted for ubiquitination by Keap1 (Jain *et al.*, 2006; Jain *et al.*, 2007). Further exploration of the involvement of GSK3 $\beta$  in Nrf2 stability suggests it to have an important role. GSK3 $\beta$  is involved in the regulation of several metabolic processes such as glycogen metabolism, Wnt signalling and apoptosis (Salazar *et al.*, 2006). Hayes group showed that the GSK3 $\beta$  phosphorylates the Neh6 domain of Nrf2, directing it for ubiquitination through the adaptor protein  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) which acts as a substrate receptor for Skp1-Cul1-Rbx1/Roc1 ubiquitin ligase complex (Rada *et al.*, 2011). It was also shown that Nrf2 has two binding sites, DSGIS<sup>338</sup> and DSAPGS<sup>378</sup> for  $\beta$ -TrCP as confirmed by biotinylated-peptide pull-down assays (Chowdhry *et al.*, 2013). GSK3 $\beta$  is a common downstream target of several signalling pathways such as PI3K and MAPK. A study by Rojo *et al.*, propose that the inhibition of GSK $\beta$  stabilizes Nrf2 due to a reduction in the GSK3 $\beta$ -directed ubiquitination (Rojo *et al.*, 2012) as seen with nordihydroguaiaretic acid (NDGA), an Nrf2 activator. The inhibition of GSK3 $\beta$  in Keap1 knock out cells also stabilizes Nrf2 and vice versa (Rojo *et al.*, 2012; Chowdhry *et al.*, 2013). In support of this, similar effects were seen in Keap1 knockout mice and in mutant Nrf2<sub>DETGE</sub> where the ability of Keap1 to repress Nrf2 is inhibited (Rada *et al.*, 2011; Chowdhry *et al.*, 2013) suggesting that the involvement of GSK3 $\beta$  in Nrf2 regulation is in a Keap1-independent manner.

#### 1.4.7 Cross-talk between Nrf2 and other pathways:

A further mode of Nrf2 regulation is through its interaction with other proteins. This could be as a result of direct binding with Nrf2 or through competing with its negative regulator Keap1. A number of proteins have been proposed to compete with Keap1 including fetal Alz-50 clone, prothymosin and caveolin-1 (Cav-1) (Strachan *et al.*, 2004; Karapetian *et al.*, 2005; Li *et al.*, 2012). Cav-1, a scaffold protein functions in signal transduction and uptake of lipophilic compounds and has also been shown to interact with a number of regulatory proteins such as Toll-like receptor 4; LC3B, Fas and survivin (Li *et al.*, 2012; Zheng *et al.*, 2012). The knock-down of Cav-1 using siRNA resulted in Nrf2-Keap1 dissociation whilst Cav-1 overexpression had no significant effect on their association but impeded the transcriptional activation of ARE-dependent genes by Nrf2 (Li *et al.*, 2012). Further analysis showed that the Nrf2-Keap1 association was increased following mutation of the Cav-1 binding motif on Nrf2 (Li *et al.*, 2012).

NF- $\kappa$ B, another transcription factor important in regulating cellular homeostasis (Oeckinghaus *et al.*, 2011), has been shown to cross-talk with Nrf2, negatively regulating its activity (Liu *et al.*, 2008). The p65 subunit of NF- $\kappa$ B binds to Keap1 to enhance ubiquitination of Nrf2, thus affecting the ability of Nrf2 to regulate the expression of downstream ARE-dependent genes (Liu *et al.*, 2008). Conversely, Keap1 has been shown to regulate the NF- $\kappa$ B pathway by binding inhibitor of  $\kappa$ B kinase beta (IKK $\beta$ ) thereby enhancing its ubiquitination and degradation (Lee *et al.*, 2009; Kim *et al.*, 2010). Elucidating the molecular mechanism of interaction between IKK $\beta$  and Keap1 and the subsequent effect on the Nrf2 pathway will have particular value in validating the potential of Nrf2 as a therapeutic target.

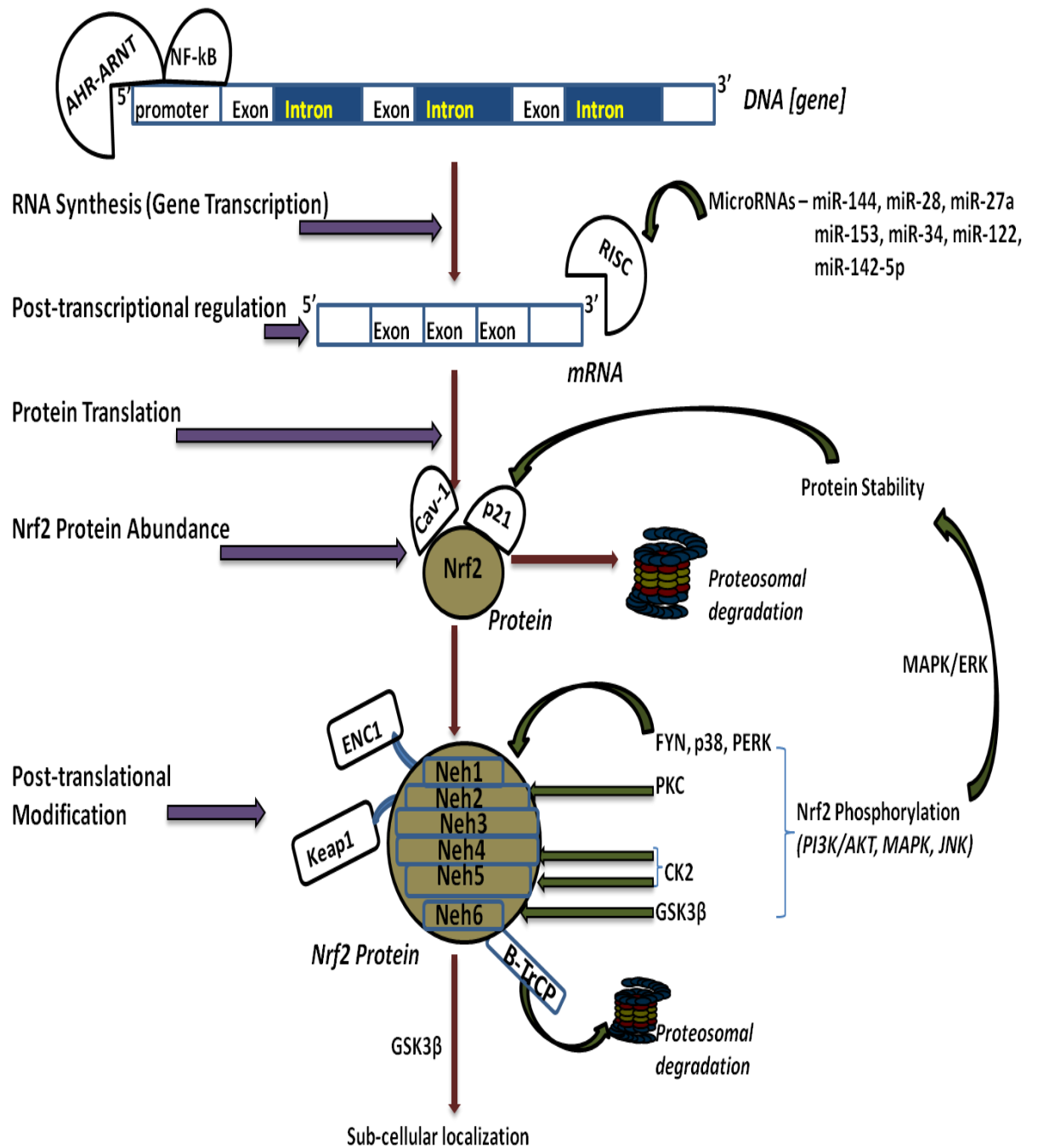
P62/sequestosome is a poly-ubiquitin binding protein and a selective substrate for autophagy that can also cross-talk with both Nrf2 and NF- $\kappa$ B (Moscat *et al.*, 2009). Studies have shown that binding occurs between p62 and Keap1 at the consensus sequence, (D/N)X(E/S)(T/S)GE, preventing the binding of Nrf2 to Keap1 (Cople *et al.*, 2010; Fan *et al.*, 2010; Jain *et al.*, 2010; Komatsu *et al.*, 2010; Lau *et al.*, 2010). Of recent, it was also shown that phosphorylation of P62 on serine 351 enhances

the binding between P62 and Keap1, hence increasing the expression of Nrf2-dependent genes. It was thus shown that the phosphorylation of P62 occurs in an mTORC1-dependent manner (Ichimura *et al.*, 2013). P62 also promotes the ubiquitination and degradation of Keap1 which in turn stabilizes Nrf2, and thus increases its activity. Notably, the p62 gene has an ARE binding consequence in its promoter region, highlighting the fact that it is also regulated by Nrf2 (Jain *et al.*, 2010). This results in a positive feedback mechanism, increasing the levels of p62 under conditions of cellular and environmental stress.

Other proteins that has been found to have a role in the regulation of Nrf2 activity is p21, a cyclin-dependent kinase inhibitor and the ectoderm neural cortex protein 1 (ENC1), a neural matrix protein that functions in neuronal differentiation (Chen *et al.*, 2009c; Wang *et al.*, 2009). P21 is able to bind directly to Nrf2 at the DLG and ETGE motif, thus preventing binding of Keap1 to the transcription factor (Chen *et al.*, 2009c). On the other hand, ENC1 negatively regulates Nrf2 protein levels and reduces expression of downstream genes. The downregulation of Nrf2 by ENC1 does not appear to occur at the mRNA level, nor does it result from an effect on protein stability, rather ENC1 has been shown to down-regulate synthesis of the Nrf2 protein (Wang *et al.*, 2009).

Regulation of the Nrf2 pathway may also be mediated by proteins that do not bind directly to either Nrf2 or Keap1. It has been suggested that DJ-1, a Parkinson's disease associated protein belonging to the Thi/Pfpl family could be one such protein. The protein has been shown to regulate Nrf2 processes but has not been found to bind to either Nrf2 or Keap1. (Clements *et al.*, 2006; Yang *et al.*, 2007).

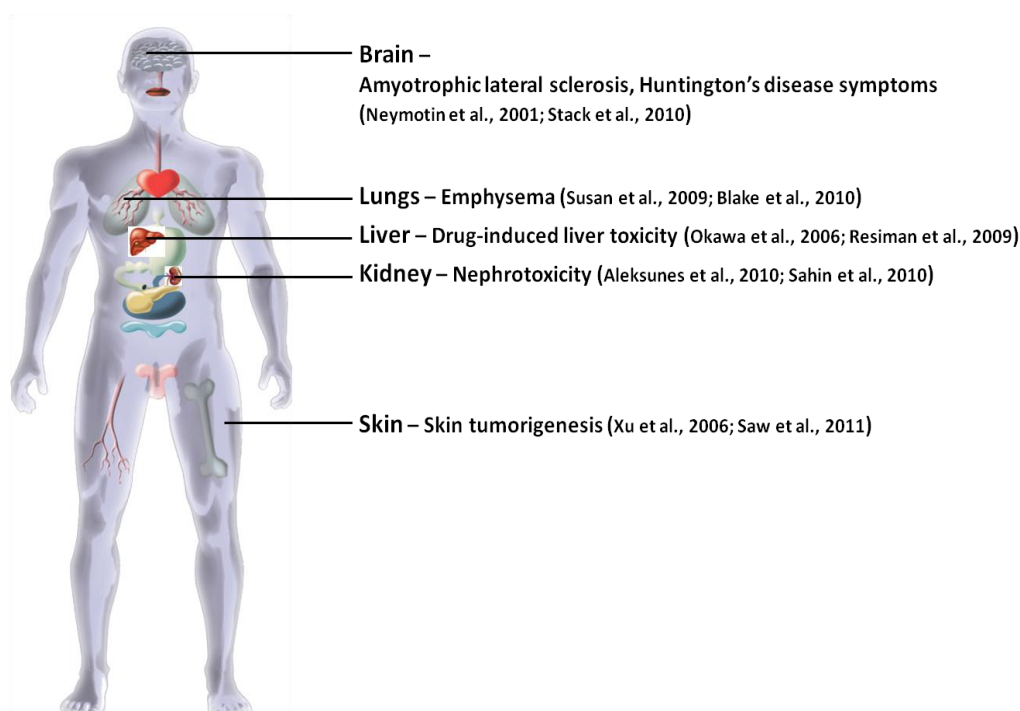
In summary, the Keap1-independent regulation of Nrf2 is summarised in figure 1.9



**Figure 1.10 - Schematic illustrating Keap1-independent mechanism of Nrf2 regulation.** Nrf2 activity has been shown to be regulated independently of Keap1, including via transcriptional control by AHR-ARNT and NF-κB, post-transcriptional control by micro-RNAs, translation control by increasing translation of the Nrf2 transcript, the IRES, and post-translational control by phosphorylation mediated by a variety of kinases. Nrf2 binding partners including p21, Cav-1 have also been shown to regulate Nrf2 activity.

### 1.5 Role of Nrf2 in protection against disease and its therapeutic significance in different organs.

Evidence suggesting that Nrf2 plays a role in regulating several metabolic processes in organs including the liver, kidney and brain is mounting (figure 1.10) (Copple, 2012). Dysregulation of the Nrf2 pathway has also been implicated in the pathogenesis of diverse diseases such as neurodegeneration, cancer and chronic kidney diseases (Zhao *et al.*, 2011b; Copple, 2012). Transgenic Nrf2 null mice have been shown to experience enhanced susceptibility to various drug-induced toxicities including those associated with acetaminophen (Chan *et al.*, 2001; Enomoto *et al.*, 2001), carbon tetrachloride (Xu *et al.*, 2008) and butylated hydroxyl toluene (Chan *et al.*, 1999). Notably, a recent study saw the opposite in which the intrahepatic shunt present in the Nrf2 KO mice may affect acetaminophen metabolism and hence reduce the sensitivity of these animals to the toxicological effects of acetaminophen (Skoko *et al.*, 2014). These observations suggest Nrf2 as a potential therapeutic target in a variety of pathological contexts. This will be further discussed in the following sub sections.



**Figure 1.11 – Role of Nrf2 in protection against disease.** Selected examples of implications of Nrf2 in diseases and its therapeutic significance in various organs

### **1.5.1 Liver:**

Drug-induced liver toxicities are a major cause of liver failure and a key factor in drug attrition (Ostapowicz *et al.*, 2002; Park *et al.*, 2011). Early studies noted that Nrf2 KO mice were more susceptible to acetaminophen-induced liver injury (Chan *et al.*, 2001; Enomoto *et al.*, 2001). Conversely, Keap1 KO mice show a high resistance to the drug's toxicity (Okawa *et al.*, 2006). Studies have shown the induction of Nrf2 and regulated genes by acetaminophen and its reactive metabolite within the non-toxic and toxic dose ranges (Goldring *et al.*, 2004; Copple *et al.*, 2008a). Other compounds have also been associated with enhanced hepatotoxicity in Nrf2 KO animals, such as pentachlorophenol, carbon tetrachloride, pyrazole and arsenic (Umemura *et al.*, 2006; Lu *et al.*, 2008; Xu *et al.*, 2008; Jiang *et al.*, 2009).

Pharmacological activation of Nrf2 using the potent inducer CDDO-Im has been shown to attenuate the liver injury provoked by acetaminophen in Nrf2 WT mice (Reisman *et al.*, 2009). In Nrf2 KO mice, the effect of CDDO-Im was lost, highlighting the involvement of Nrf2 in CDDO-Im induced protection against acetaminophen liver injury. Further to this, a similar effect was observed when sauchinone, an antioxidant lignan and inducer of Nrf2, was used to pre-treat mice prior to administration of toxic doses of APAP (Kay *et al.*, 2011). Other small molecule inducers of Nrf2 such as oltipraz and BHA (butylated hydroxyanisole) have been shown to protect against the oxidative and electrophilic stress associated with various drug-induced liver injuries (for a review see (Klaassen *et al.*, 2010). These findings together suggest the possibility of using Nrf2-inducing agents as an adjuvant/co-treatment to prevent drug toxicities in the liver.

### **1.5.2 Kidney:**

Kidney toxicity is a major concern in the development of new therapeutic drugs. Therefore, an understanding of the mechanisms that underlie kidney injury will inform the development of safe and effective drug candidates. Aged female Nrf2 KO mice develop lupus-like autoimmune nephritis, suggesting that Nrf2 regulates



homeostasis in the aging kidney (Yoh *et al.*, 2001). In addition, Nrf2 KO mice exposed to streptozotocin show enhanced susceptibility to hyperglycemia-induced diabetic nephropathy (Yoh *et al.*, 2008; Jiang *et al.*, 2010b). Elucidating the role of Nrf2 in the kidney could prove useful in the management of acute kidney injury occurring as a result of disease or drug administration. The role of Nrf2 in conferring protection against kidney injury has been highlighted in animal models of renal ischaemic-reperfusion (Yoon *et al.*, 2008; Wu *et al.*, 2011b) and also in models in which nephrotoxicity is induced by heavy metals (Chen *et al.*, 2009a; Molina-Jijon *et al.*, 2011; Prabu *et al.*, 2012), cyclosporine A (Louhelainen *et al.*, 2006) and ochratoxin A (Cavin *et al.*, 2007; Cavin *et al.*, 2009). The pre-induction of Nrf2 in these models ameliorates the deleterious effects of the nephrotoxins for example, treatment of WT mice with CDDO-Im has been shown to reduce the nephrotoxic effects of cisplatin (Aleksunes *et al.*, 2010). Furthermore, a phase II trial of CDDO-Me for the treatment of patients with lymphomas noted an enhanced glomerular filtration rate and reduced levels of creatinine and blood urea nitrogen in the CDDO-Me treatment group indicative of an improvement of renal function (Jiang *et al.*, 2010b). These studies together highlight an important role for Nrf2 in the kidney and identify the transcription factor as a promising target for treating a number of renal diseases. For a review, see (Shelton *et al.*, 2013)

### **1.5.3 Brain:**

Mao *et al.* and Jin *et al.* reported several nervous system dysfunction phenotypes such as neuronal apoptosis; tissue swelling in Nrf2 KO mice (Jin *et al.*, 2009; Mao *et al.*, 2011). Using Nrf2 activators CDDO-ethyl amide (CDDO-EA) and CDDO-trifluoroethyl amide (CDDO-TFEA), the dysfunctional effect was found to be alleviated in Nrf2 WT mice, but not in Nrf2 KO mice, indicating that the effect is attributable to the induction of Nrf2 and its target genes. This highlights the important role of Nrf2 in the brain and nervous system. In support of this, CDDO-Me has been shown to confer protection against Huntington's disease in a transgenic mouse, improving the behavioural phenotype and brain pathology (Stack *et al.*,

2010). In addition, Cullinan *et al.*, 2003 highlighted the role of Nrf2 in protecting cells against the deleterious effects associated with misfolded proteins as a result of stress induced in the endoplasmic reticulum (Cullinan *et al.*, 2003). This is of importance as a number of disorders in the nervous system are associated with misfolded protein. In Parkinson's disease models, Nrf2 has been shown to confer cytoprotection against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)(Chen *et al.*, 2009b; Jazwa *et al.*, 2011). Induction of the Nrf2 pathway (either by over expressing Nrf2, silencing Keap1 or using an inducer of Nrf2 such as CDDO-Me) inhibited the progression of disease in Parkinson's (Barone *et al.*, 2011) and Alzheimer's disease models (von Otter *et al.*, 2010), suggesting the modulation of the Nrf2 pathway as a novel therapeutic strategy. For a review, see (Zhang *et al.*, 2013).

#### **1.5.4 Lipid Metabolism:**

Recently, studies have highlighted a role for Nrf2 in regulating lipid metabolism. The proteomic profiling of Nrf2 KO mice revealed a significant upregulation of fatty-acid synthetic pathway proteins highlighting a role for Nrf2 in the regulation of fatty acid synthesis in the liver (Kitteringham *et al.*, 2010). In keeping with this, Nrf2 KO mice fed a high fat diet show an elevated total hepatic lipids and polyunsaturated fats (Shin *et al.*, 2009). In addition, overexpression of Nrf2 in Keap1 KO mice or the treatment of the WT mice with inducers of Nrf2 (CDDO-Im or oltipraz) depleted lipid-synthesis genes (Shin *et al.*, 2009; Chowdhry *et al.*, 2010). Although these findings illustrate a negative control of lipid synthesis by Nrf2, there are conflicting reports showing the opposite. For a review, see (Schneider *et al.*, 2013). Nonetheless, these findings highlight Nrf2 as a novel therapeutic target in lipid-related disorders, such as obesity and metabolic syndrome (Yu *et al.*, 2011; Schneider *et al.*, 2013).

### **1.6 Activation of Nrf2 by small molecules:**

Given that a large number of studies have highlighted the important roles of Nrf2 in disease pathology and drug toxicity, a number of groups have worked to develop small molecules that can activate the Nrf2 pathway. The semi-synthetic triterpenoids, which are derived from oleanic acid and include CDDO, are potent activators of the Nrf2 pathway and have been shown to have anti-cancer properties (Dinkova-Kostova *et al.*, 2005b; Liby *et al.*, 2005; Sporn *et al.*, 2011). Indeed, the methyl derivative of CDDO, CDDO-Me, is known to be one of the most potent activators of Nrf2, inducing the pathway at nanomolar concentrations in cellular systems (Dinkova-Kostova *et al.*, 2005b). CDDO-Me entered clinical trials for the treatment of chronic kidney disease in patients with type 2 diabetes but was withdrawn due to adverse events however, a new trial in pulmonary arterial hypertension patients is currently being carried out (Pergola *et al.*, 2011a; Pergola *et al.*, 2011b; de Zeeuw *et al.*, 2013).

Another Nrf2 activator, dimethyl fumarate (DMF) was recently licensed for treatment of relapsing-remitting multiple sclerosis (Bar-Or *et al.*, 2013; de Zeeuw *et al.*, 2013), while others including oltipraz, D3T, sulforaphane, and CPDT (5,6-Dihydrocyclopenta-1,2-dithiole-3-thione) are also under clinical investigation (for a review, see (Zenkov *et al.*, 2013)). While Nrf2 activating compounds hold promise as novel therapeutic tools, a comprehensive understanding of their mechanisms of action and the pathways they stimulate is required before they can be fully utilised in clinical settings. Due to the varied number of Nrf2 activators known, much focus of investigation is on the most-specific inducer of Nrf2 presently known (CDDO-Me).

### **1.7 Cancer & Inhibition of Nrf2**

There is an increasing body of evidence pointing towards the dark side of Nrf2 in cancer initiation and progression. In normal and premalignant tissues, Nrf2 holds an important role in cytoprotection and cancer prevention. However, in malignant cells, overexpression of Nrf2 has been implicated in chemoresistance and is thought

to contribute to the progression of cancer (Sporn *et al.*, 2012). Mechanisms leading to the enhanced levels of Nrf2 in cancer have been described in several studies and include (1) Keap1 somatic mutations, including those identified in pancreatic cancers (Takahashi *et al.*, 2010; Lister *et al.*, 2011); (2) Epigenetic silencing of Keap1 (Eades *et al.*, 2011) (3) Altered expression of Nrf2 binding partners such as p62 (Nezis *et al.*, 2012) (4) Transcriptional induction of Nrf2 by oncogenes such as K-Ras, B-Raf and Myc, as seen in mouse fibroblasts and *in vivo* models of pancreatic cancer (DeNicola *et al.*, 2011) (5) Keap1 post translational modifications leading to its altered expression (DeNicola *et al.*, 2011; Taguchi *et al.*, 2011; Sporn *et al.*, 2012), and (6) Nrf2 gene mutations (Shibata *et al.*, 2008b). The increased level of Nrf2 in cancer cells results in increased expression of genes encoding detoxification enzymes, antioxidant proteins and xenobiotic transporters, thus conferring protection against chemotherapeutic drugs and radiotherapy and contributing to the treatment-resistant phenotype of the cells. Furthermore, Nrf2 has also been shown to enhance cancer cell proliferation (Lister *et al.*, 2011; Mitsuishi *et al.*, 2012). This could be related to the role of the transcription factor in the regulation of Notch1 signalling (Wakabayashi *et al.*, 2010), given that the pathway is important in proliferation, as well as differentiation and apoptosis.

In addition to the aforementioned mechanisms by which Nrf2 contribute to cancer progression, Nrf2 has also been shown to regulate both the oxidative and non-oxidative pathways of the pentose phosphate pathway (PPP), a biochemical reaction that generates NADPH and pentoses. Hence, Nrf2 was shown to regulate the PGD (6-phosphogluconate dehydrogenase) and G6PD (glucose-6-phosphate dehydrogenase), critical genes in the regulation of glucose which primarily occurs through glycolysis or the oxidative phase of pentose phosphate pathway. Nrf2 was also shown to regulate the non-oxidative phase of the PPP by positively modulating the expression of transaldolase (TALDO)1 and transketolase (TKT), hence regulating the entry of carbon to the PPP (Wu *et al.*, 2011a; Mitsuishi *et al.*, 2012; Singh *et al.*, 2013). The analysis of the promoter region of TALDO1 showed the presence of functional ARE sequence, hence a direct target of Nrf2, however, the other studied genes in the PPP namely G6PD, PGD and TKT seems to be regulated indirectly by

Nrf2. Studies have shown the involvement of Nrf2-micro-RNAs mediated regulation of these genes through redox-sensitive histone deacetylase (HDAC)4 (Singh *et al.*, 2013). miR-1 and miR-206 have been shown to negatively regulate the expression of G6PD, PGD and TKT and since Nrf2 was shown to regulate the miR-1 and miR-206, then Nrf2 indirectly regulates the expression of G6PD, PGD and TKT (Singh *et al.*, 2013). In addition, Nrf2 has also been shown to regulate the expression of PK, a protein that catalyses the final step in glycolysis aiding the conversion of PEP (phosphoenolpyruvate) to pyruvate. Aberrant expression of these genes has been implicated in cancer progression thereby supporting role of Nrf2 in cancer progression (Chaneton *et al.*, 2012; Rabinowitz *et al.*, 2012; Hayes *et al.*, 2014a).

All these factors taken together lead to a poor prognosis for cancer patients with tumours in which Nrf2 is overexpressed. Consequently, the inhibition of Nrf2 may be a promising strategy in the treatment of such patients. Indeed, Nrf2 siRNA has been shown to increase the sensitivity of cancer cells to chemotherapeutic drugs including cisplatin and etoposide (Shibata *et al.*, 2008a; Homma *et al.*, 2009; Jiang *et al.*, 2010a; Lister *et al.*, 2011). However, only a limited number of small molecule inhibitors of Nrf2 have been developed to date. The most recently described small molecule inhibitors of Nrf2 include: retinoic acid receptor  $\alpha$  agonists that inhibit Nrf2 activity through the formation of complexes between the retinoic acid receptor  $\alpha$  and Nrf2 (Wang *et al.*, 2007); brusatol, a quassinoid isolated from the plant *Brucea javanica* that depletes Nrf2 in a rapid and reversible manner (Ren *et al.*, 2011); leutolin that has been shown to inhibit Nrf2 in human A549 lung cancer cells (Tang *et al.*, 2011); and 4-methoxychalcone, which has also been shown to inhibit Nrf2 in A549 cells (Lim *et al.*, 2013).

A recently developed approach involves the use of cancer suicide gene therapy, which utilises Nrf2-driven lentiviral vectors containing thymidine kinase. The lentiviral vectors, in conjunction with a prodrug ganciclovir, are transfected into cancer cells. The prodrug is then metabolized via a phosphorylation cascade into a toxic metabolite which actively kills the cancer cells, as well as neighbouring cells due to a bystander effect (Moolten, 1986; Leinonen *et al.*, 2012). This approach has significant potential given that it could be combined with conventional therapies

(Leinonen *et al.*, 2012). These factors together demonstrate the important role of Nrf2 in a cancer setting, and highlight the transcription factor as a potentially useful target in treating associated diseases.

### **1.8 Thesis aims**

On the basis that:

- The Nrf2 pathway plays a critical role in maintaining homeostasis under conditions of cellular stress,
- The Nrf2 pathway plays an important role in the pathogenesis of various diseases, and
- The Nrf2 pathway could be a useful therapeutic target,

The key aim of this thesis is to better define the chemical and molecular mechanisms that are required for the pharmacological manipulation of Nrf2, and the likely therapeutic significance of modulating the activity of this important pathway. This thesis will therefore examine:

- The responsiveness of the Nrf2 pathway to an inducer across a number of mammalian test systems, in order to understand the translational relevance of the *in vivo* and *in vitro* findings,
- Platforms for assessing inter-individual variability in activity of the Nrf2 pathway and its relevance to disease,
- The mechanism of action of CDDO-Me as an inducer of Nrf2,
- The inhibition of Nrf2 by the small molecule brusatol and its significance for defence against chemical stress, and
- The mechanism of action of brusatol as an inhibitor of Nrf2.

## **CHAPTER TWO**

### **MATERIALS AND METHODS**

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
2.1 Materials and Reagents.....	47
2.2 Antibodies.....	48
2.3 Mice .....	49
2.4 Treatment of animals with CDDO-Me.....	50
2.5 Liver homogenisation .....	50
2.6 Cell lines.....	50
2.7 Cell culture.....	51
2.8 Peripheral blood mononucleocytes isolation (PBMCs) protocol.....	51
2.9 Primary human hepatocytes isolation.....	52
2.10 Cell counting and cell seeding.....	52
2.11 Treatment of cells with chemicals or compounds.....	53
2.12 Treatment of PBMCs with chemicals or compounds.....	54
2.13 Cell viability assay.....	54
2.14 RNA Interference.....	55
2.15 Isolation of RNA / DNase treatment.....	55
2.16 RNA quality control.....	56
2.17 cDNA protocol.....	57
2.18 Real-time Polymerase Chain Reaction (RT-PCR).....	57
2.19 Primer design for PCR amplification.....	58
2.20 Microfluidic cards.....	58
2.21 Preparation of Hepa1c1c7 and HepG2 cell lysate samples.....	59
2.22 Preparation of PBMCs cell lysate samples.....	59
2.23 Protein Content Determination of whole cell lysates.....	60
2.24 Protein content Determination of liver homogenates.....	60
2.25 Western Blotting.....	61
2.26 Measurement of Proteasome Activity.....	63
2.27 Liquid Chromatography-Mass Spectrometry Analyses of Brusatol and other Quassinoids.....	63
2.28 Data and Statistical Analysis.....	64



## 2.1 Materials and Reagents

Unless otherwise stated, all other reagents were purchased from Sigma (Poole, Dorset, UK). CDDO-Me was synthesised by Michael Wong (Department of Chemistry, University of Liverpool, UK). Brusatol and Brucein D were synthesised by the School of Chinese Medicine, The Chinese University of Hong Kong.

20S human proteasome fragments (Enzo Life Sciences, UK), 7-amido-4-ethylcoumarin (AMC) standard (Enzo Life Sciences, UK), Absolute optically clear QPCR adhesive seal sheets (ABgene, Epsom, UK), Actinomycin D, AEBSF, Aproptinin, A23187, Ammonium Chloride, Bafilomycin A1, Bapta-AM, Bestatin Methyl Ester, Boc-LSTR-AMC, Brucein D, Calpeptin, CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega, UK), Collagenase A/IV (Roche, Basel, Switzerland/Sigma Aldrich, St. Louis, MO), Collagen-I coated plates (BD Beckinson, San Jose, CA), Cyclohexamide, Cyclosporin A, Dexamethasone, DNase/RNase free water, DMEM, DMSO, E64d, EGTA, Ethanol (Fisher, UK), Gel loading buffer (70 % (v/v) NUPAGE sample loading buffer, 30 % (v/v) NUPAGE reducing agent), Geneamp 9700 PCR system (Applied Biosystems, Warrington, UK), GF109203X, Glycine, GM6001, Go6976, GS-800 calibrated imaging densitometer (Biorad, Hemel Hempstead, UK), Hanks balanced salt solution, Hepatocyte growth factor (hGF), HEPES-buffered saline (HBS), HEPES buffer (Sigma, UK) Hybond nitrocellulose membrane (GE Healthcare, Little Chalfont, UK), Hyperfilm ECL (Amersham, Little Chalfont, UK), ImProm-II reverse transcription system (Promega, Southampton, UK), Insulin-transferrin-selenium (Life Technologies, Carlsbad, CA), Iodoacetamide, KCl, Kodak developer and fixer solutions, Lipofectamine RNAimax (Invitrogen, Paisley, UK), L-glutamine, Lymphoprep (Axis-Shield, UK), MG132, Microfluidic TaqMan Low Density Array cards (Applied Biosystems, Paisley, UK), Mini TRANS-Blot Cell System (Invitrogen, Paisley UK), Molecular weight markers (PrecisionPlus protein kaleidoscope standards) (Biorad, Hemel Hempstead, UK), MOPS, MRX microplate reader (Dynatech Laboratories, Billingshort, UK), NaCl (Fisher, Loughborough, UK), Nano-drop ND1000 Spectrophotometer (Labtech, East Sussex, UK), N-Ethylmaleimide, Non-fat dry milk (Biorad, UK), U0126, PBS, PCR primers (Sigma-Genosys, Haverhill, UK), Penicillin/Streptomycin, Pepstatin A Methyl Ester, Ponceau S solution, PP2,

Pre-cast 4-12 % NuPAGE Novex bis-tris polyacrylamide gels (Invitrogen, Paisley, UK), Rapamycin, RIPA Buffer, RNA extraction kit RNeasy Mini-kit (Qiagen, West Sussex, UK), The RNA 6000 Nano Kit was from Agilent (Berkshire UK), SDS, siRNA targeted against Keap1 and Nrf2 (Dharmacon, Lafayette, USA), SB203580, SP600125, 10x TBE, Tris-HCl (Fisher, Loughborough, UK), Suc-LLVY-AMC (Enzo Life Sciences, UK), Triton-X 100, U0126, Varioscan fluorescent plate reader, Williams E medium (Sigma Aldrich, St. Louis, MO), Western lightening chemiluminescence reagents (PerkinElmer, Beaconsfield, UK), Wortmanin, X-Cell Surelock mini-cell system (Invitrogen, Paisley, UK), Z-LLG-AMC (Enzo Life Sciences, UK), ZVAD.

**2.2 Antibodies:** Antibodies raised against Nrf2 (EP1808Y; Abcam, UK), ATP citrate lyase (ACL) (EP704Y; Abcam, UK), NQO1 (ab234; Abcam, UK), beta-actin (AC-15; Abcam, UK), Keap1 (E-20; Santa Cruz Biotechnology, Germany), Cyclin A (H-432; Santa Cruz Biotechnology), p62/SQSTM1 (P0067; Sigma-Aldrich, UK), phospho-p38 MAPK (#4511S, Cell Signaling Technology), phospho-AKT473 (#4060S; Cell Signaling Technology), phospho-Erk1/2 (#4377S, Cell Signaling Technology), phospho-SAPK (#9251S; Cell Signaling Technology) and phospho-CREB (#9198; Cell Signaling Technology), HIF-1 $\alpha$  (610959; BD Biosciences, UK) and Horseradish peroxidase-linked anti-rabbit (A9169; Sigma-Aldrich), mouse (A9044; Abcam) and goat (P0449; Dako, UK) secondary antibodies were purchased from the indicated companies.

**Table 2.1 Antibodies Table**

<b>Name</b>	<b>Code</b>	<b>Blocking</b>	<b>Primary dilution</b>	<b>Mol weight</b>
<b>Nrf2</b>	EP1808Y	10% milk TBST	1:400 in 2% milk TBST	75 kDa
<b>Keap1</b>	E-20	10% milk TBST	1:2000 in 2% milk TBST	75 kDa
<b>NQO1</b>	ab2346	10% milk TBST	1:5000 in 2% milk TBST	31 kDa
<b>ACL</b>	EP704Y	10% milk TBST	1:5000 in 2% milk TBST	125 kDa
<b>p-AKT473</b>	#4060S	5% BSA TBST	1:1000 in 2% BSA TBST	62 kDa
<b>p-JNK1/2</b>	#9251S	5% BSA TBST	1:1000 in 2% BSA TBST	46/54 kDa
<b>p-p38 MAPK</b>	#4511	5% BSA TBST	1:1000 in 2% BSA TBST	38 kDa
<b>p-Erk1/2</b>	#4377S	5% BSA TBST	1:1000 in 2% BSA TBST	42/44 kDa
<b>p-Creb</b>	#9198	5% BSA TBST	1:1000 in 2% BSA TBST	43 kDa
<b>HIF-1<math>\alpha</math></b>	610959	5% BSA TBST	1:1000 in 2% BSA TBST	120 kDa
<b>Cyclin A</b>	H-432	5% milk PBST	1:1000 in 5% milk PBST	54 kDa
<b>P62</b>	P0067	10% milk TBST	1:10,000 in 2% milk TBST	62 kDa
<b>B-Actin</b>	AC-15	10% milk TBST	1:20,000 in 2% milk TBST	42 kDa

### 2.3 Mice

All animal experiments were conducted under the Animals (Scientific Procedures) Act 1986 guideline and approved by the University of Liverpool Animal Ethics Committee. The transgenic Nrf2 null mice and genotyping of progeny have been previously described (McMahon *et al.*, 2001; Itoh *et al.*, 1997). Non-fasted male CD1 wild-type, C57BL6J wild-type and C57BL6J Nrf2 Knock-out mice aged 10-12 weeks old were fed on a chow diet and housed at 19 °C – 23 °C, under 12 h light/dark cycles with free access to water.

## **2.4 Treatment of animals with CDDO-Me**

For the pilot study to determine the optimum concentration of CDDO-Me that would give a maximum Nrf2 activation and subsequent downstream protein expression after 24 hours, a single dose of CDDO-Me (0, 0.1, 0.3, 1, 3 or 10 mg/kg in 100  $\mu$ L DMSO; (n=2)) was administered to the animals by intraperitoneal injection in the morning. At 24 hour post-dosing, the animals were culled by exposure to a rising concentration of CO<sub>2</sub> in a chamber. This was then followed by cardiac puncture and the livers were removed using sterile scissors and tweezers. The isolated livers were immediately snap-frozen in liquid nitrogen and stored at 80 °C.

For the subsequent studies, wild type and Nrf2 knockout C57BL6J mice and wild type CD1 mice were dosed with 3mg/kg CDDO-Me or DMSO vehicle control (n=6), and the livers were isolated according to the protocol described above, snap-frozen and stored at -80 °C.

## **2.5 Liver homogenisation**

100 mg of isolated liver from the treated mice was homogenised using a hand held glass-teflon homogeniser. The tissue was homogenized using 10 passes in phosphate buffered saline (PBS). The resulting homogenate was centrifuged at 10,000 g for 5 minutes. The supernatant was transferred to a fresh eppendorf while the unhomogenised tissue pellet containing the cell debris was discarded.

## **2.6 Cell lines:**

The mouse hepatoma Hepa1c1c7 cell line (ATCC CRL-2026), and human HepG2 cell line (ATCC HB-8065) were purchased from American Type Culture Collection (ATCC).

## **2.7 Cell culture**

Hepa-1c1c7 and HepG2 cell lines were maintained in a Dulbecco modified eagles medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin under a humidified environment at 37 °C in a 5 % CO<sub>2</sub>. Cells were allowed to grow in 75 cm<sup>2</sup> Nunclon culture flasks and passaged when about to reach full confluency. Briefly, when reached about 80 % confluency, cells were washed with DMEM without FBS, discarded and trypsinized at room temperature for few minutes. The trypsin was discarded and cells incubated for about 5 minutes to allow detachment. Cells were blasted off and resuspended in DMEM supplemented with FBS, broken down with a 21 gauge needle and a syringe couple of times to break cell clumps. The cells were then resuspended at appropriate ratio in a new 75 cm<sup>2</sup> Nunclon culture flask.

## **2.8 Pheripheral blood mononucleocytes Isolation (PBMCs) protocol**

Blood was donated from 8 voluntary healthy donors aged 20-40 years; 4 males and 4 females following a signed informed consent as approved by the Local Research Ethics Committee. Human peripheral blood mononuclear cells (PBMCs) were then isolated by density gradient centrifugation. Briefly, 100 mL of heparinised venous whole blood was transferred into 50 mL falcon tubes and equal volume of Lymphoprep<sup>TM</sup> (density of 1.077 g/mL, 9.1 % (w/v) sodium diatrizoate and 5.7 % (w/v) polysaccharide) was added to another falcon tube. The heparinised blood is carefully overlaid on top of the lymphoprep using 50 mL pipettes. The mixture was then centrifuged at 700 g (2000 rpm) for 20 minutes at room temperature with no brakes and acceleration set at 3. Following centrifugation, layers were formed according to the density of the cells. Cells of higher density like red blood cells (RBCs) and granulocytes form a layer at the bottom of the tube, followed by the lymphoprep<sup>TM</sup> layer while PBMCs with lower density form a layer above the lymphoprep<sup>TM</sup> layer. Using a sterile Pasteur pipette, the PMBC layer (whitish interface) was aspirated into a new falcon tube and made up to 50 mL with Phosphate buffered saline (PBS), Hanks balanced salt solution (HBSS) or culture

medium. This was centrifuged at 700 g (1800 rpm) for 10 minutes with brakes set to 9. The supernatant was discarded and then pellet was then resuspended in 1 mL of culture media. This was then made up to 25 mL total volume using the culture media and the total cell number was counted using the haemocytometer.

## **2.9 Primary human hepatocytes isolation**

Primary human hepatocytes were isolated according to a protocol previously described which involved a 2-step collagenase method (LeCluyse et al., 2005; Seglen et al., 1976; Berry et al., 1969). Briefly, patients' liver resections were donated as surgical waste from Aintree hospital, Liver United Kingdom following their full consent as approved by the Local Research Ethics Committee. The resections were perfused with HEPES-buffered saline (HBS) and thereafter digested with Collagenase A/IV (Roche, Basel, Switzerland/Sigma Aldrich, St. Louis, MO) in HBS containing calcium. The capsule was opened and the detached cells following their digestion were separated with the use of gauze. The cell suspension went through 2 steps of centrifugation at 80 x g for 5 minutes at a temperature of 4 °C. The cells were resuspended in Williams E medium (Sigma Aldrich, St. Louis, MO). Cells were counted accurately using a haemocytometer and seeded onto Collagen-I coated plates (BD Beckinon, San Jose, CA) at cell density  $2.5 \times 10^5$  cells/cm<sup>2</sup> in Williams E supplemented with 1% insulin-transferrin-selenium (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Sigma Aldrich, St. Louis, MO),  $10^{-7}$ M dexamethasone and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO). After 3 hours of incubation, non-attached cells were isolated from the attached cells by performing a series of washes and the culture medium was replaced.

## **2.10 Cell counting and cell seeding**

Cells were counted accurately using a haemocytometer. Briefly, cells were detached from the flasks and resuspended as described above and 10 µL of the cells was

added onto the haemocytometer. Cells at the central grid (5 x 5 squares) were visualised using the 20X objective lens of Nikon T1-SM light microscope.

### 2.11 Treatment of cells with chemicals or compounds

Hepa-1c1c7 or HepG2 cells were seeded on a 12 well Nunclon plate at a density of 300,000 cells/well in growth media unless otherwise stated and incubated for 16-18 hours. The chemicals/compounds are dissolved in their appropriate solvent (Table 2.2) and a stock concentration of 200x the required concentration. Thereafter, the cells were treated with the appropriate volume to get the overall concentration of DMSO/solvent in the cell culture media to maximum of 0.5 % (v/v). The cells were transferred to an incubator for the required time periods at 37 °C post-treatment.

**Table 2.2: Chemicals or compounds and their appropriate solvent**

Chemical/Compound	Solvent	Chemical/Compound	Solvent
<i>A23187</i>	DMSO/ethanol	<i>EGTA</i>	DMSO
<i>Actinomycin D</i>	DMSO/ACN	<i>GF109203X</i>	DMSO
<i>AEBSF</i>	H <sub>2</sub> O	<i>GM6001</i>	DMSO
<i>Ammonium Chloride</i>	H <sub>2</sub> O	<i>Go6976</i>	DMSO
<i>Aproptinin</i>	H <sub>2</sub> O/DMSO	<i>Hepatocyte growth factor (hGF)</i>	H <sub>2</sub> O/PBS+ 0.1% BSA
<i>Bafilomycin A1</i>	DMSO	<i>Iodoacetamide</i>	DMSO
<i>Bapta-AM</i>	DMSO	<i>MG132</i>	DMSO
<i>Bestatin Methyl Ester</i>		<i>N-Ethylmaleimide</i>	DMSO
<i>Brucein D</i>	DMSO	<i>Pepstatin A Methyl Ester</i>	DMSO

<i>Brusatol</i>	DMSO	<i>PP2</i>	DMSO
<b><i>Chemical/Compound</i></b>	<b>Solvent</b>	<b><i>Chemical/Compound</i></b>	<b>Solvent</b>
<i>Calpeptin</i>	DMSO	<i>Rapamycin</i>	DMSO
<i>CDDO-Me</i>	DMSO	<i>SB203580</i>	DMSO
<i>Cyclohexamide</i>	DMSO	<i>U0126</i>	DMSO
<i>Cyclosporin A</i>	DMSO/ethanol	<i>Wortmanin</i>	DMSO
<i>E64d</i>	DMSO/ethanol	<i>ZVAD</i>	DMSO

### 2.12 Treatment of PBMCs with chemicals or compounds

Freshly isolated PBMCs cells were seeded on a 6 well Nunclon plate at a density of  $10 \times 10^6$  cells/well in a total volume of 3 mL of growth media and incubated for 1 or 24 hours. CDDO-Me or DMSO was dissolved in the culture media at 100 x the required concentration for the required time periods. 30  $\mu$ L of CDDO-Me or DMSO was added to the required wells and the overall concentration of DMSO in the cell culture medium is 0.1 % (v/v). The cells were transferred to an incubator for the required time periods at 37 °C following treatment.

### 2.13 Cell viability assay

Cell viability was measured using the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega, UK) which determines the number of viable cells based on the quantification of ATP present. Briefly, Hepa-1c1c7 cells were seeded into 96 well plates at  $3 \times 10^4$  cells per well in 200  $\mu$ L supplemented-DMEM and the appropriate compound was added. Each well was then treated with 10  $\mu$ L CellTiter-Glo<sup>®</sup> Reagent and mixed for 1 minute on an orbital shaker at 45 g (700 rpm) to induce cell lysis. Thereafter, the sample was left for 5 minutes to equilibrate. 150  $\mu$ L of each sample from each well was then transferred to a white 96-well plate and the plate was left in the dark for additional 5-10 minutes at the room temperature to



equilibrate and stabilize the luminescent signal. Luminescence was then measured at 570 nm wavelength using a Varioscan flash fluorescent plate reader.

#### **2.14 RNA Interference**

Short Interfering RNA (siRNA) duplexes targeted against mouse Keap1 (subsequently referred to as si-KEAP1) and a scrambled, non-targeting control duplex (D-001210-03; subsequently referred to as si-CON) were purchased from Dharmacon's (Lafayette, CO) siGENOME library. Hepa1c1c7 cells were seeded into 12 well plates at  $1.25 \times 10^5$  cells per well and transfected for 48 hours with 10 nM siRNA using Lipofectamine RNA iMAX (Invitrogen, UK) according to the manufacturer's instructions. Cells were then dosed with appropriate doses of brusatol or DMSO for 2 hours and 24 hours and lysed in RIPA buffer.

#### **2.15 Isolation of RNA / DNase treatment**

After incubation, the media in the cells were removed and washed twice with ice-cold phosphate buffered saline (PBS) and discarded. The cells were then digested using RLT lysis buffer and transferred to RNase-free eppendorfs. Lysates were centrifuged for 3 minutes at 17000 g (14000 rpm) and the supernatant was removed and put into new RNase-free eppendorfs. 420  $\mu$ L of 100 % ethanol or 1 volume of 70 % ethanol was added to the supernatant and mixed gently. Up to 700  $\mu$ L of the sample including any precipitate formed was transferred into an RNeasy mini column placed in 2 mL collection tube. This was centrifuged for 15 seconds at 8000 g (9500 rpm) to wash. The flow-through was discarded. 350  $\mu$ L of buffer RW1 was added to the spin column, centrifuged again for 15 seconds at 8000 g (9500rpm) to wash. 10  $\mu$ L of DNase I stock solution was added to 70  $\mu$ L buffer RDD per sample. This was gently mixed by inverting the tube and added directly onto the RNase silica-gel membrane and was left for 15 minutes at the room temperature (20-30°C). 350  $\mu$ L of buffer RW1 was added to the spin column, centrifuged again for 15 seconds at 8000 g (9500 rpm) to wash and the flow through was discarded.

500 µL of buffer RPE was added to the mini spin column, centrifuged again for 15 seconds at 8000 g (9500 rpm) to wash and the flow through was discarded. 500 µL of buffer RPE was added to the mini spin column, centrifuged again for 2 minutes at 8000 g to wash and the flow through was discarded. The column was placed into a new 2 mL collection tube and centrifuged at 17000 g (14000 rpm) for 1 minute to dry the column. Finally, 30-50 µL of RNase free water was added to the column placed in a new collection tube and centrifuged at 9500 rpm for 1 minute.

### 2.16 RNA quality control

The concentration and quality of the RNA was measured using the NanoDrop ND-1000 spectrophotometer. 1.5 µL of DNase/RNase free water was added to the sensor and measured as a blank. Thereafter, 1.5 µL of each RNA was added to the sensor and then the concentration was measured based on the 'Beer's Law' equation as shown below:

$$C = (A * e) / b$$

Where:

C = Nucleic acid concentration in ng/µL

A = Absorbance in AU

e = wavelength-dependent extinction coefficient in ng-cm/µL

b = path length in cm

Note: path length is 1, this means the equation now becomes  $C = (A * e)$

The RNA quality is then assessed from two parameters:

1. 260/280 nM sample absorbance ratio: This indicates the purity of the RNA in relation to the protein. A value of > 1.8 is accepted. Values below this is regarded as contaminated by the protein and discarded.

2. 260/230 nM sample absorbance ratio: This indicates the purity of the RNA in relation to the solvents. A value of >1.8 is accepted. Values below this is regarded as contaminated by the solvents e.g. ethanol and discarded.

### **2.17 cDNA protocol**

cDNA synthesis from the sample RNA was carried out using the Promega ImProm-II™ Reverse Transcription System according to the manufacturer's instructions. Briefly, 2 µg of RNA was combined with 1 µL of random hexamer/oligo primer solution and was made to a volume of 20 µL with nuclease-free dH<sub>2</sub>O in RNase-free tubes. The mixture was incubated at 70 °C for 5 minutes and cooled on ice immediately. A reverse transcription reaction mixture was made up containing 8 µL of ImProm-II™ 5 x reaction buffer, 6.4 µL 25 mM MgCl<sub>2</sub>, 1 µL 0.5 mM dNTP mix and 2 µL ImProm-II™ reverse transcriptase in a final volume of 20 µL was added to the RNA solution of each sample. Reverse transcription was performed using GeneAmp 9700 polymerase chain reaction (PCR) system with the following parameters: strands were annealed (25 °C; 5 min) and extended (42 °C; 1 hour), before the reverse transcriptase was inactivated (70 °C; 15 min) and was cooled on ice immediately. Samples not containing random primers, dNTPS and reverse transcriptase were used as controls. Finally, 160 µL of nuclease-free dH<sub>2</sub>O was added to each tube to give a final cDNA concentration of 10 ng/µL.

### **2.18 Real-time Polymerase Chain Reaction (RT-PCR)**

RT-PCR was performed according to the manufacturer's protocol using the SYBR Green JumpStart Taq Ready Mix Kit. Briefly, 4 µL of cDNA (50 ng), 10 µL of 2x SYBR Green JumpStart *Taq* ReadyMix, 0.25 µL reference dye, 2 µL each of 250 nM of both forward and reverse primers and 2 µL of DNase/RNase-free water was combined in a 96 well PCR plates. Optically clear QPCR adhesive seal sheets were then used to seal the plate and centrifuged for 15 seconds at 700 g (1800 rpm) at 4°C. The plate was then placed in ABI PRISM 7000 Sequence Detection System to carry out the RT-PCR with the following protocol: Initial denaturation stage for 2 minutes at 94 °C.

Thereafter, there is a 40 cycle of 94°C for 15 seconds, 60 °C for 1 minute. Dissociation protocol was also set up to check the specificity of the primers and the accuracy of the SYBR Green I fluorescence with the following protocol: 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. The data was analyzed using the cycle threshold (CT) generated from the amount of template from each sample.

### 2.19 Primer design for PCR amplification

The following primers sequences for Nrf2, Keap1, Gapdh, Nqo1, Mrp3, Hmox, Gstpi, Gclc and Gclm are outlined below:

**Table 2.3 - Primer Table**

Primer Name	Forward primer (5' – 3')	Reverse Primer (3' – 5')
Nrf2	GACATCCTTTGGAGGCAAGA	AGGCATCTTGTTTGGGAATG
Keap1	CACAGCAGCGTGGAGAGA	CAACATTGGCGCGACTAGA
Gapdh	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG
Nqo1	AGCGTTCGGTATTACGATCC	AGTACAATCAGGGCTCTTCTCG
Mrp3	GCTGAGGGTGGGGATAATCT	GGCTCGGGCTAGGCATAC
Hmox	GTCAAGCACAGGGTGACAGA	ATCACCTGCAGCTCCTCAAA
Gstpi	TGTCACCCTCATCTACACCAAC	GACAGCAGGGTCTCAAAAGG
Gclc	ATGATAGAACACGGGAGGAGAG	TGATCCTAAAGCGATTGTTCTT
Gclm	TGACTCACAATGACCCGAAA	GATGCTTTCTGAAGAGCTTCC

### 2.20 Microfluidic cards

Microfluidic TaqMan Low Density Array cards were custom-made by Applied Biosystems (Paisley, UK). On each card is a combination of 48 several Nrf2 target genes to be amplified simultaneously. These 48 genes were selected based on the findings of the study by Kitteringham *et al.*, which reported some Nrf2-regulated proteome or those that function in the Nrf2-regulated pathway as identified by MetaCore analysis (Kitteringham *et al.*, 2010). The reverse transcribed cDNA as described in the section above was made up to a concentration of 2 ng/μL in

nuclease-free water. cDNA was pooled from all the samples (n=8) and then included on each plate to be amplified by the custom-designed genes and used as a reference marker. Samples were added across 5 TaqMan array cards in a randomised order, which was determined, using a programme in random.org (<http://www.random.org/>). The expressions of all other genes were expressed relative to the pool and normalised against the housekeeping gene, 18s ribosomal RNA as analysed by 7900HT Fast Real-Time PCR System (ABSciex) according to the manufacturer's instructions. The amplified data was analysed based on the fold increase using the comparative  $C_T$  method ( $\Delta\Delta C_T$ ) method.  $C_T$  values were determined using the RQ manager 1.2 component of the 7900HT Fast System software using a threshold manually set to a value of 0.3 for all plates.

## **2.21 Preparation of Hepa1c1c7 and HepG2 cell lysate samples**

After incubation, the media in the cells were removed and washed twice with ice-cold PBS and discarded. The cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulphate) or directly in LDS with  $\beta$ -mercaptoethanol and transferred to an eppendorf tube. Lysates were centrifuge at 17,000 g (14,000 rpm) for 5 minutes at 4 °C and the supernatant containing the whole cell contents were collected in a new eppendorf. All samples were stored at -80°C prior to protein content determination and western blotting.

## **2.22 Preparation of PBMCs cell lysate samples**

After incubation, cells were scraped and transferred using a pipette into 5 mL glass or 15 mL falcon tubes and centrifuged at 700 g (1800 rpm) for 5 minutes with acceleration and brake set to 9. The supernatant was discarded and the cells were resuspended in 1 mL HBSS and then transferred to 1.5 mL eppendorf. This was centrifuged again at 1800 rpm for 5 minutes with acceleration and brake set to 9. The supernatant was discarded and the cell pellet was lysed in 70  $\mu$ L of

radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulphate). Lysates were centrifuged at 14,000 rpm for 5 minutes at 4 °C and the supernatant containing the whole cell contents were collected in a new eppendorf. All samples were stored at -80 °C prior to protein content determination and western blotting.

### **2.23 Protein content determination of whole cell lysates**

The total protein content of the whole cell lysates was measured using the BCA (Bicinchoninic Acid; Sigma UK) assay according to the manufacturer's instructions. Briefly, the assay relies on the principle of the colour change as a result of the biochemical reactions between the concentration of the protein and the BCA reagents. Firstly, the peptide bonds in the protein cause a reduction of the  $\text{Cu}^{2+}$  ions to  $\text{Cu}^+$  ions in a temperature dependent manner at 37 °C. Secondly, the reduced  $\text{Cu}^+$  ions then bind to two molecules of bicinchoninic acid, which causes a colour change. The colour of the solution changes from green to purple depending on the concentration of the protein in the lysates. The increase in absorbance at 570 nm, which is proportional to the colour change, was measured using a MRX microplate reader. The total amount of the protein was quantified from a standard curve generated from known concentrations (0-2  $\mu\text{g}/\mu\text{L}$ ) of bovine serum albumin (BSA).

### **2.24 Protein content determination of liver homogenates**

The total protein content of the liver homogenates was performed as described by Lowry (Lowry *et al.*, 1951). Briefly, the assay relies on the principle of the colour change as a result of the biochemical reactions between the concentration of the protein and the Lowry reagents. Firstly, the peptide bonds in the protein react with the copper generating  $\text{Cu}^+$  ions in a temperature dependent manner at 37 °C which then reacts with the Folin reagent which causes a colour change. The protocol was therefore as follows: Briefly, a set of standards (0-2  $\mu\text{g}/\mu\text{L}$ ) were prepared using

BSA. A series of dilutions of the sample were made up to a volume of 200  $\mu$ L. Following this, 1 mL of Lowry's reagent was added to the samples and the standards, and left for 10 minutes at room temperature. Thereafter, 0.1 mL of diluted Folin's reagent was added to the mixture and the mixture was left at room temperature for 30 mins. The increase in abundance at 750 nm which is proportional to the colour change, was measured using a MRX microplate reader. The total amount of the protein is quantified from a standard curve generated from the known concentration of BSA.

## **2.25 Western blotting**

20  $\mu$ g of whole cell lysates were added to 5  $\mu$ L of the Nupage sample loading buffer (containing 30 % (v/v) NuPAGE reducing agent and 70 % (v/v) NuPAGE loading buffer; Invitrogen). The sample was then heated up for 5 minutes at 80  $^{\circ}$ C to allow protein denaturing. 4  $\mu$ L of the molecular weight marker (PrecisionPlus protein kaleidoscope standards) and the whole denatured samples were loaded on a 4-12% Novex bis-tris polyacrylamide gels (Life Technologies, Uk) using XCell Surelock mini-cell electrophoresis tanks and MOPS running buffer (50 mM MOPS, 50 mM Tris base, 3.5 mM sodium dodecyl sulphate, 1 mM EDTA, 0.25 % (v/v) NuPAGE antioxidant ). Samples were run at 90 V for 10 minutes to allow samples to settle down into the wells properly. Following this, the voltage was increased to 170 V for a further 60-70 minutes. The separated proteins in the gels are transferred onto hybond nitrocellulose paper (GE Healthcare, UK) at 230 mA current for 60 minutes using a Mini TRANS-Blot Cell System. To check if the proteins were transferred appropriately, Ponceau S solution was added to the membrane for few minutes and then washed off using tris-buffered saline (TBS; pH 7.0). Membranes were blocked for 30 minutes or overnight in tris-buffered saline (TBS; 0.15 mM NaCl, 25 mM Tris base, 3 mM KCl, pH 7.0) containing 0.1 % Tween 20 (TBS-T) and 10 % (weight/volume; (v/v)) non-fat milk (Bio-Rad) on an orbital shaker.

Membranes were probed for 3 hours with anti-Nrf2 (1:400 in TBS-Tween containing 2 % (w/v) non-fat dry milk; EP1808Y; Abcam, UK), overnight with anti-Keap1 (1:200

in TBS-Tween containing 2 % (w/v) non-fat dry milk; E-20; Santa Cruz, UK), anti-NQO1 (1:5,000 in TBS-Tween containing 2 % (w/v) non-fat dry milk; ab2346; Abcam, UK), anti-ACL (1:5,000 in TBS-Tween containing 2 % (w/v) non-fat dry milk; EP704Y; Abcam, UK), anti-phospho-AKT473 (1:1,000 in TBS-Tween containing 2 % (w/v) BSA; #4060S; Cell signaling technology, UK), anti-phospho-JNK1/2 (1:1,000 in TBS-Tween containing 2 % (w/v) BSA; #9251S; Cell signaling technology, UK), anti-phospho-p38 MAPK (1:1,000 in TBS-Tween containing 2 % (w/v) BSA; #4511; Cell signaling technology, UK), anti-p-Erk1/2 (1:1,000 in TBS-Tween containing 2 % (w/v) BSA; #4377S; Cell signaling technology, UK), anti-phospho-CREB (1:1,000 in TBS-Tween containing 2 % (w/v) BSA; #9198; Cell signaling technology, UK), anti-HIF-1 $\alpha$  (1:1000 in TBS-Tween containing 2 % (w/v) BSA; 610959; BD Biosciences, UK), anti-Cyclin A (1:1000 in PBS-Tween containing 5 % (w/v) milk; H-432; Santa Cruz, UK) or for 1 hr with anti-p62 (1:10,000 in TBST-Tween containing 2% (w/v) milk, P0067; Sigma-Aldrich, UK) primary antibodies. Mouse  $\beta$ -Actin (1:20,000 in TBS-Tween containing 2% (w/v) non-fat dry milk; AC-15; Abcam, UK) was used as a loading control. Membranes were washed in 6 x 10 minutes in TBS-Tween and probed for 1 hour with the appropriate HRP-linked secondary antibody. The following secondary antibodies were used: goat anti-rabbit (1:10,000 in TBS-Tween containing 2 % (w/v) non-fat dry milk; A9169; Sigma-Aldrich, UK), rabbit anti-goat (1:5,000 in TBS-Tween containing 2 % (w/v) non-fat dry milk; P0449; Dako, UK ), anti-mouse (1:10,000 in TBS-Tween containing 2 % (w/v) non-fat dry milk; A9044; Abcam, UK).

Membranes were washed in 6 x 10 minutes in TBS-Tween and visualised in the dark by the addition of enhanced western lightening chemiluminescence reagents Hyperfill ECL (Perkin Elmer, UK) to the membrane, a film placed on the membrane to pick up bands and exposed to Kodak developer and fixer solutions in ratio 1:1. Immunoreactive bands were scanned and quantified using GS-800 calibrated imaging densitometer and the Quantity one software according to the manufacturer's protocol and all blots normalised to  $\beta$ -actin



## **2.26 Measurement of Proteasome activity**

1 µg 20S human proteasome fragments (Enzo Life Sciences, UK) were added to each well of a 96 well plate. 5 mg of the Fluorogenic peptides Boc-LSTR-AMC (Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin, Sigma UK), Suc-LLVY-AMC (Suc-Leu-Leu-Val-Tyr-AMC, Enzo Life Sciences, UK) and Z-LLG-AMC (Z-Leu-Leu-Glu-AMC, Enzo Life Sciences, UK) were each dissolved in DMSO to give a concentration of 10 mM. This was diluted in assay buffer (50 mM HEPES, pH 7.8) to give a concentration of 500 µM for Boc-LSTR and Suc-LLVY-AMC and 5 mM for ZLLG-AMC and 20 µL was added to each well of a 96 well plate to give a final concentration of 50 µM for Boc-LSTR-AMC and Suc-LSTR-AMC and 500 µM for ZLLG-AMC. The tested compounds CDDO-Me, Brusatol, MG132 and epoxomicin were diluted in DMSO to give a concentration of 10 mM each. 0.3728 g potassium chloride (KCl) was dissolved in assay buffer to give a concentration of 20 mM. Each compound was further diluted in assay buffer to give the final concentrations as follows; potassium chloride (KCl) 20 mM, MG132 (10 nM, 100 nM, 1 µM, 10 µM), CDDO-Me (10 nM, 100 nM, 1 µM, 10 µM), Brusatol (10 nM, 100 nM, 1 µM, 10 µM) and epoxomicin (10 nM, 100 nM, 1 µM, 10 µM) were incubated at 37°C for a 24 hr time period. Simultaneously, a standard curve for protein degradation was created using the 20S proteasome substrate AMC diluted in assay buffer from 0-2 µM. Proteolytic activity was monitored by measuring the release of the fluorescent group 7-amido-4-ethylcoumarin (AMC) with a Varioscan fluorescent plate reader with excitation at 360 nm and emission at 460 nm..

## **2.27 Liquid chromatography-mass spectrometric analyses of brusatol and other quassinoids**

### ***2.271 Sample processing***

Aliquots (10 µL) of brusatol solutions (100 µg/mL for the standard solution; DMSO-water, 1:9, v/v) and aliquots (25-80 µL) of the various brusatol incubations (10 or 100 µg/mL; whole incubated solutions or supernatants of protein-containing

incubations deproteinized with acetonitrile) were injected onto the HPLC column without further treatment.

### **2.272 Analysis**

Aliquots of the whole solutions and supernatants were chromatographed at room temperature on an Agilent ZORBAX Eclipse 5- $\mu$ m XDB-C18 column (4.6 mm  $\times$  150 mm; Agilent Technologies, Santa Clara, CA, USA) using a linear gradient of acetonitrile in aqueous acetic acid (0.25 %, v/v). Typically, the entire gradient profile was 5 % to 50 % over 20 min, 50 % for 5 min, 50 % to 5 % over 1 min and 5 % for 4 min. When the supernatants of deproteinized surfactant-containing incubations were analysed, the gradient profile was 5 % to 50 % over 20 min, 50 to 80 % over 1 min, 80 % for 5 min, 80 to 5 % over 1 min and 5 % for 3 min. The flow rate was 1 mL/min. Eluent was delivered by a PerkinElmer series 200 HPLC system (pump and autosampler; PerkinElmer, Norwalk, CT, USA).

The LC column was connected to the Turbo V electrospray source of an API 4000 Qtrap hybrid quadrupole mass spectrometer (AB Sciex, Warrington, UK) via a flow-splitting T-piece. The split flow of eluate to the mass spectrometer was approximately 150  $\mu$ L/min.

The standard operating parameters of the mass spectrometer (Q1 operation) were as follows: source temperature, 400  $^{\circ}$ C; ionspray (electrospray capillary) voltage, 4,500 V; desolvation potential, 100 V or 200 V; curtain gas setting, 15; source gas (Gas-1) setting, 50; heater gas (Gas-2) setting, 50. All of the source gas requirements were met by a nitrogen/zero-grade air generator. The instrument was set up for full-scanning acquisitions in the positive-ion mode over a scan range of  $m/z$  100-1,000 with a scan time of 5 s. Instrument management and data processing were accomplished through Analyst 1.5.1 software.

### **2.29 Data and Statistical analysis**

Data are expressed as mean  $\pm$  S.D from the 3 independent experiments. The significance of differences within the data was assessed with an unpaired student t

test. A two-sided  $p$  value of  $\leq 0.05$  was considered to be statistically significant. For TLDA data, the significance of differences within the relative expression of genes in wild type and Nrf2 knock out mice where  $C_T$  values were available for  $\geq 4$  animals in each group was assessed with a one way ANOVA with Tukey multiple comparison testing. The normality of the data was assessed with a Shapiro-Wilk test.

## **CHAPTER THREE**

### **INVESTIGATING THE EFFECTS OF NRF2 MODULATION IN MOUSE *IN VIVO* MODELS AND PRIMARY HUMAN CELLS**

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
3.1 Introduction.....	68
3.2 Results.....	70
3.2.1 Induction of the Nrf2 pathway by CDDO-Me <i>in Vivo</i> .....	70
3.2.2 Investigation into the induction of Nrf2 signalling by CDDO-Me at the transcriptional level.....	71
3.2.3 Use of alternative strain of mouse to investigate the regulation of fatty acid synthesis genes by Nrf2.....	76
3.2.4 Investigation of the role of Nrf2 in the regulation of fatty acid synthesis genes.....	77
3.2.5 Investigation of Nrf2 response to CDDO-Me in human cells.....	78
3.3 Discussion.....	81

### 3.1 INTRODUCTION

Nrf2 has been highlighted by various studies to have numerous roles in maintaining cell homeostasis especially in the area of cytoprotection against toxic insults (for a review see (Bryan *et al.*, 2013)). Transgenic Nrf2 null mice have been shown to experience enhanced susceptibility to various drug-induced toxicities including acetaminophen (Chan *et al.*, 2001; Enomoto *et al.*, 2001), carbon tetrachloride (Xu *et al.*, 2008) and butylated hydroxyl toluene (Chan *et al.*, 1999). The low basal expression of Nrf2-regulated proteins in Nrf2-null mice coupled with a reduced ability to induce cytoprotective genes, could account for the enhanced toxicity seen following induction of chemical stress in Nrf2-null mice.

Previous studies have shown differences in the Nrf2 knock out (KO) and wild type (WT) mouse using gene microarray studies and targeted protein analysis (Thimmulappa *et al.*, 2002; Hu *et al.*, 2006; Nair *et al.*, 2007). In a recent study conducted by Kitteringham *et al.*, the basal WT and Nrf2 KO liver proteome was compared. This study showed that there was a depletion of cytoprotective proteins in the Nrf2 KO mice, whilst the opposite was seen with lipid metabolism, with an increased expression of proteins that function in the fatty acid synthetic pathway (Kitteringham *et al.*, 2010).

In recent years, investigations into Nrf2's role in health and disease have primarily focussed on the potential value of manipulating this pathway in cancerous environments. In particular, the activation of Nrf2 was considered to be beneficial. Many naturally occurring phytochemicals with proven cancer chemopreventive abilities such as sulforaphane and resveratrol are also Nrf2 inducers (Kensler *et al.*, 2000; Wolf, 2001; Kode *et al.*, 2008). Additionally, synthetic compounds like the triperpenoid CDDO and its derivatives (CDDO-Me and CDDO-Im) have recently been shown to inhibit cancer cell proliferation (Bernstein *et al.*, 2012; Deeb *et al.*, 2012b; Qin *et al.*, 2013) whilst simultaneously being potent inducers of the Nrf2 pathway.

The triterpenoid CDDO and its derivatives are semi-synthetic compounds and have a high potency for the Nrf2:Keap1 pathway. This was first demonstrated in a study that saw the potent activation of phase-II response by CDDO in wild type, but not

Nrf2-deficient cells (Dinkova-Kostova *et al.*, 2005a). This was further confirmed in a study that demonstrated the induction of Nrf2 protein and an increase in its associated downstream target gene HO-1 by CDDO (Liby *et al.*, 2005). It was also demonstrated in mice *in vivo* that CDDO-Im and CDDO-Me induce Nrf2-target gene Nqo1, with induction seen in the liver, lung and small intestine (Yates *et al.*, 2007).

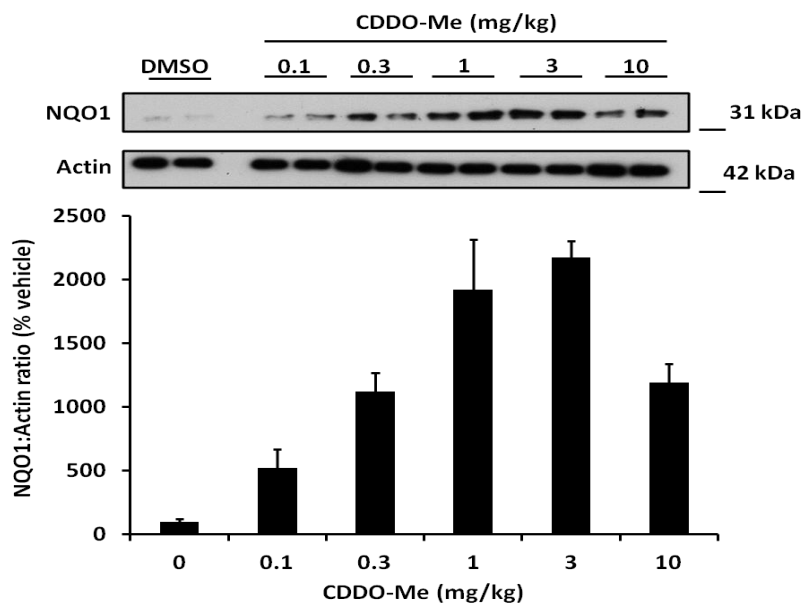
Recently, CDDO-Me has been tested in clinical trials for the treatment of chronic kidney disease in patients with type 2 diabetes (Pergola *et al.*, 2011b; de Zeeuw *et al.*, 2013) and it showed a high therapeutic potential. However, it was withdrawn in phase III due to adverse cardiovascular events in some patients (Tayek *et al.*, 2013). The mechanism of action of CDDO and its derivatives as inducers of Nrf2 remains unknown, but it has been suggested that these compounds are capable of modifying cysteine residues in Keap1 (Dinkova-Kostova *et al.*, 2005b; Ahn *et al.*, 2010; Takaya *et al.*, 2012). Due to the growing interest in investigating Nrf2 activators such as CDDO-Me as potential therapeutics and the adverse effects seen in clinical trials after chronic dosing, it is of interest to define the profile of Nrf2 induction at the mRNA and protein level and distinguish between the Nrf2-dependent and -independent effects of CDDO-Me. It is also important to define the expression of these proteins in different species and organs and investigate how these might translate to the susceptibility to or protection against drug-induced toxicities in humans. It will also be highly valuable to identify a surrogate marker of Nrf2 induction in humans, to allow the efficacy and safety of Nrf2 activators to be determined *in vivo* with minimal invasion.

Previous work conducted in the department has investigated the hepatic proteome in WT and Nrf2 KO mice, both at the basal level and following a single dose of CDDO-Me (Kitteringham *et al.*, 2010; Walsh *et al.*, 2014). These studies identified an important role for Nrf2 in the regulation of various aspects of cellular homeostasis. Therefore, the aim of the work described in this chapter was to establish the responsiveness of the Nrf2 pathway to CDDO-Me across a number of mammalian test systems, in order to understand the translational relevance of findings in the mouse, and establish a platform for assessing inter-individual variability in the activity of the pathway and its relevance to disease.

## 3.2 RESULTS

### 3.2.1 Induction of the Nrf2 pathway by CDDO-Me *in Vivo*

The constitutive proteomic study identified Nrf2 as a positive regulator of cytoprotection and negative regulator of fatty acid synthesis in the mouse liver (Kitteringham *et al.*, 2010). It was of interest to investigate the impact of Nrf2 induction on these pathways. To determine the optimum concentration of CDDO-Me that would induce the Nrf2 pathway, a pilot study was conducted in which C57BL6J WT mice were treated with CDDO-Me across a range of doses by intraperitoneal injection for 24 hours. Western blotting of liver lysates showed a dose-dependent increase in the level of NQO1 protein, a downstream target of Nrf2 with maximum induction seen at a concentration of 3mg/kg CDDO-Me (figure 3.1).

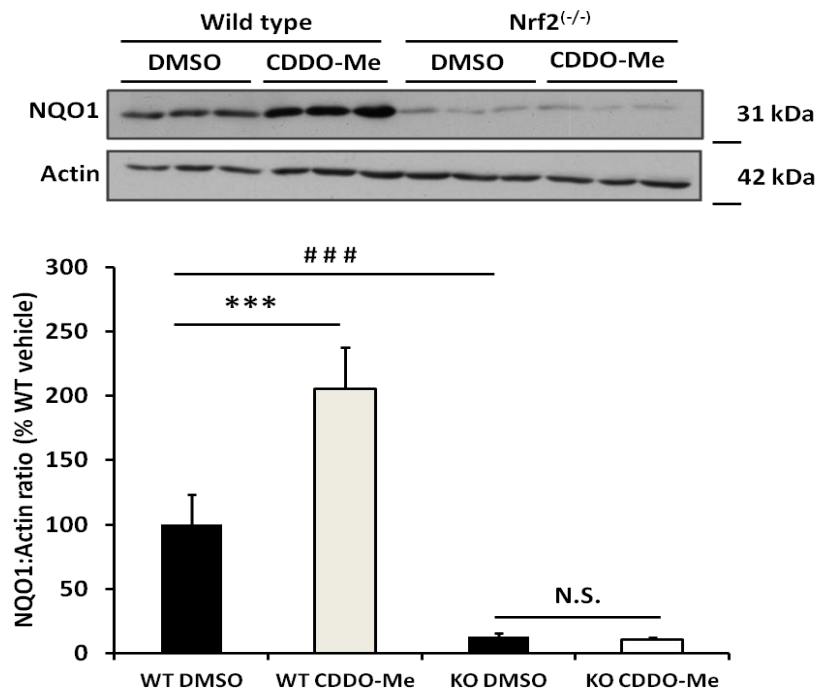


**Figure 3.1 - Induction of Nrf2 pathway by CDDO-Me *in vivo*.** Nqo1 protein levels in C57BL6J WT liver homogenates, following *in vivo* exposure to the indicated concentrations of CDDO-Me for 24h as determined by western blotting ( $n=2$ ). The mean Nrf2 values were plotted after normalization with actin + SEM.

This dose was therefore used in the subsequent study, in which WT and Nrf2 KO mice were exposed to CDDO-Me. In order to confirm the activation of Nrf2 at the selected dose of 3mg/kg CDDO-Me in WT animals, a western blot for Nqo1 was performed. CDDO-Me provoked a marked increase in hepatic Nqo1 in WT mice, but



had no effect in the livers of Nrf2 KO mice. The increased expression of Nqo1 confirms the activation of Nrf2 at the selected dose (figure 3.2). The work described in 3.2.1 has previously been reported in the PhD thesis of J. Henry (2012) and in our subsequent publication (Walsh *et al.*, 2014). It forms the basis for the *in vivo* work described in this chapter, and is included here with her permission.



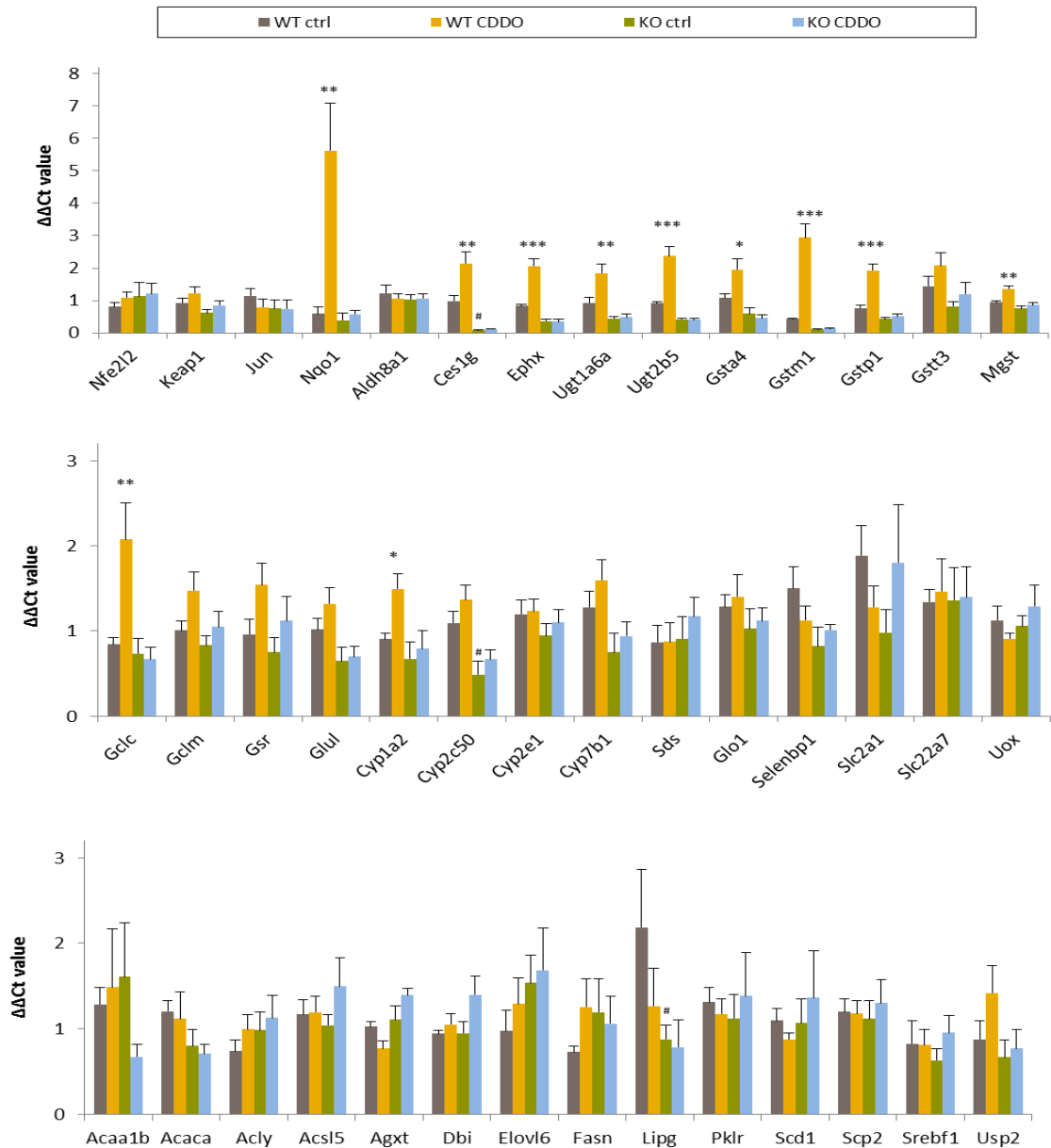
**Figure 3.2 - Induction of Nqo1 protein expression by CDDO-Me is dependent on Nrf2.** Nqo1 protein levels in C57BL6J mice liver homogenates following *in vivo* exposure to 3mg/kg CDDO-Me or DMSO for 24 hour, as determined by immunoblotting. The blots shown are a representative of six individual animals and quantified by densitometry. The mean Nqo1 values were plotted after normalization with actin + SEM. Statistical analysis was performed using a one way ANOVA with Tukey multiple comparison \*\*\*  $p \leq 0.001$  wild-type vehicle vs. wild-type CDDO-Me, ###  $p \leq 0.001$  wild-type vehicle vs. KO vehicle, N.S. non-significant difference.

### 3.2.2 Investigation into the induction of Nrf2 signalling by CDDO-Me at the transcriptional level

The study conducted by Kitteringham *et al.*, 2010, identified cytoprotection and lipid metabolism as key pathways modulated by Nrf2. It was therefore of interest to

investigate whether the basal differences in the protein levels of enzymes in these pathways were reflected at the mRNA level thus providing insight into the regulation of the Nrf2 pathway at the level of transcription. Furthermore, given that evidence for Nrf2-regulation of lipid metabolism has only recently emerged, an investigation into the differential expression of the lipid metabolism genes upon induction was also carried out using liver samples from the WT and Nrf2 knock-out C57BL6J mice dosed with 3mg/kg CDDO-Me. RNA was extracted from liver homogenates of vehicle and CDDO-Me treated WT and Nrf2 KO mice, and the associated cDNA was subjected to real-time PCR amplification using the custom-made Microfluidic TaqMan Low Density Array cards. The expression of all genes was determined relative to the pool and normalised against the house-keeping gene, 18S rRNA (figure 3.3)(Table 3.1).

Consistent with CDDO-Me's ability to induce Nrf2 signalling, there was a marked increase in the expression of 11 well characterized Nrf2 downstream target genes in the livers of CDDO-Me-treated WT animals, when compared to the vehicle control, including the following: Nqo1, Ces1g, Ephx, Ugt1a6a, Ugt2b5, Gsta4, Gstm1, Gstp1, Mgst, Gclc and Cyp1a2. The mRNA levels of 3 genes, Ces1g, Cyp2c50 and Lipg were shown to be significantly lower in the livers of vehicle control treated Nrf2 KO animals when compared to their WT counterparts. Importantly, CDDO-Me was unable to provoke changes in the expression of these genes in the livers of Nrf2 KO mice. Notably, Nrf2 mRNA was detected in Nrf2 KO mice. The Nrf2 probe used on the TLDA plate spans a region that includes part of exon 1 and exon 2. Since the KO mouse was generated through loss of exon 5, the probe was able to detect Nrf2 mRNA in the KO animal. This is consistent with the loss of exon 5 in the KO animals, which results in the translation of a non-functional protein (Lu *et al.*, 2011). The genes that were up-regulated by CDDO-Me in wild type animals are associated with drug metabolism and their regulation by Nrf2 has been well characterised. However, expression of the lipid metabolism genes that were included in the TLDA analysis was not significantly altered by CDDO-Me treatment.



**Figure 3.3 - Investigation into the induction of Nrf2 signalling by CDDO-Me at the transcriptional level.** Relative expression of mRNA levels in C57BL/6J WT and KO liver homogenates following *in vivo* exposure to 3mg/kg CDDO-Me or DMSO for 24 h, as determined by Microfluidic TaqMan low density array analysis. mRNA levels were normalised to the 18S ribosomal subunit. Levels of mRNA for *Nqo1*, *Ces1*, *Ephx*, *Ugt1a6a*, *Ugt2b5*, *Gsta4*, *Gstm1*, *Gstp1*, *Mgst*, *Gclc* and *Cyp1a2* were statistically significantly higher in CDDO-Me-treated wild type animals when compared to vehicle control (\*), while *Ces1g*, *Cyp2c50* and *Lipg* were significantly lower in *Nrf2*<sup>-/-</sup> vehicle control animals (#). There was no statistical difference in mRNA levels in CDDO-me and vehicle control *Nrf2*<sup>-/-</sup> animals. Data represent Mean + SEM of n=6 animals, and statistical significance was assessed using a one way ANOVA with Tukey multiple comparison testing.

**Table 3.1 – Genes on custom-made Microfluidic TaqMan Low Density Array cards**

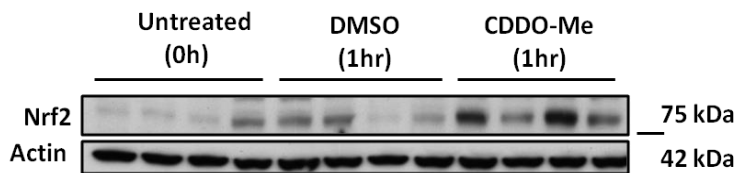
<b>Code</b>	<b>Name</b>	<b>General biochemical function</b>
<b>Nfe2l2</b>	Nuclear factor-erythroid 2-like 2	Transcription factor
<b>Keap1</b>	Kelch-like ECH-associated protein 1	Ubiquitin ligase substrate adaptor
<b>Jun</b>	Jun-proto-oncogene	Regulation of stimuli
<b>Nqo1</b>	NADPH:quinone oxidoreductase	Detoxification: Phase I reaction
<b>Aldh8a1</b>	Aldehyde dehydrogenase 8 family, member A1	Detoxification: Phase I reaction
<b>Ces1g</b>	Carboxyesterase 1G	Lipid metabolism: fatty acid oxidation
<b>Ephx</b>	Epoxide hydrolase 1, microsomal	Detoxification: Phase I reaction
<b>Ugt1a6a</b>	UDP glucuronosyltransferase 1 family, polypeptide A6	Detoxification: Phase II reaction
<b>Ugt2b5</b>	UDP glucuronosyltransferase 2 family, polypeptide B5	Detoxification: Phase II reaction
<b>Gsta4</b>	Glutathione S- transferase class Alpha 1	Detoxification: Phase II reaction
<b>Gstm1</b>	Glutathione S-transferase class Mu 1	Detoxification: Phase II reaction
<b>Gstp1</b>	Glutathione S- transferase class Pi 1	Detoxification: Phase II reaction
<b>Gstt3</b>	Glutathione S-transferase theta 3	Detoxification: Phase II reaction
<b>Mgst</b>	Microsomal glutathione S-transferase	Detoxification: Phase II reaction
<b>Gclc</b>	Glutamate-cysteine ligase, catalytic subunit	Antioxidant: GSH-based system
<b>Gclm</b>	Glutamate-cysteine ligase, modifier subunit	Antioxidant: GSH-based system
<b>Gsr</b>	Glutathione reductase	Antioxidant: GSH-based system
<b>Glul</b>	Glutamate-ammonia ligase	Metabolism of nitrogen
<b>Cyp1a2</b>	Cytochrome P450 1A2	Drug metabolism
<b>Cyp2c50</b>	Cytochrome P450 2c50	Drug metabolism
<b>Cyp2e1</b>	Cytochrome P450 2e1	Drug metabolism

<b>Cyp7b1</b>	Cytochrome P450 7b1	Drug metabolism
<b>Sds</b>	Serine dehydratase	Serine and glycine metabolism
<b>Glo1</b>	Glyoxalase	Catalyzes the conversion of hemimercaptal
<b>Selenbp1</b>	Selenium binding protein 1	Selenium regulation
<b>Slc2a1</b>	Solute carrier family 2, member A1	Glucose transport
<b>Slc22a7</b>	Solute carrier family 2, member A7	Glucose transport
<b>Uox</b>	Urate oxidase	Converts uric acid to allantoin
<b>Acaa1b</b>	Acetyl-CoA acyltransferase 1b	Peroxisome $\beta$ -oxidation of fatty acid
<b>Acaca</b>	Acetyl-CoA carboxylase alpha (ACC1)	Synthesis of malonyl-CoA
<b>Acly</b>	ATP-citrate lyase (ACL)	Synthesis of cytosolic acetyl CoA
<b>Acs15</b>	Acyl-CoA synthetase long-chain family member 5	Lipid biosynthesis
<b>Agxt</b>	Alanine-glyoxylate aminotransferase	Glyoxylate detoxification
<b>Dbi</b>	Diazepam binding inhibitor	Lipid metabolism: displacement of beta-carbolines and benzodiazepines
<b>Elovl6</b>	Elongation of very long-chain fatty acids 6	Synthesis: Fatty acid elongation
<b>Fasn</b>	Fatty acid synthase	Fatty acid synthesis
<b>Lipg</b>	Lipase, endothelial	Catabolism of fatty acid
<b>Pklr</b>	Pyruvate kinase, liver and RBC	Catalyses the production of phosphoenolpyruvate
<b>Scd1</b>	Stearoyl-CoA desaturase 1	Desaturation of fatty acid
<b>Scp2</b>	Sterol carrier protein 2	Oxidation of fatty acids
<b>Srebf1</b>	Sterol regulatory element-binding transcription factor 1	Transcription factor: regulates lipogenesis
<b>Usp2</b>	Ubiquitin specific peptidase 2	Protein breakdown regulation

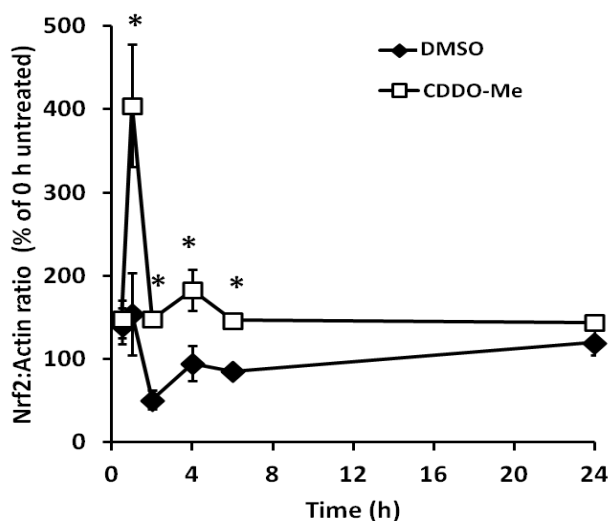
### 3.2.3 Use of alternative strain of mouse to investigate the regulation of fatty acid synthesis genes by Nrf2

Following the identification of Nrf2 as a negative regulator of fatty acid synthesis at the basal level using C57BL6 WT and Nrf2 KO mice, it was of interest to see if the effects of Nrf2 modulation were reproducible in another strain of mouse in order to demonstrate the responsiveness of the Nrf2 pathway in different mammalian test systems. To do this, a time-course study of Nrf2 pathway induction by CDDO-Me was conducted in CD1 WT mice, which were exposed to 3mg/kg CDDO-Me for up to 24 h. Western-blotting of liver homogenates showed a maximum increase in Nrf2 protein at 1 h in CD1 mice treated with CDDO-Me (figure 3.4a-b). Consistent with CDDO-Me's ability to induce Nrf2, the protein level of NQO1, a well characterized Nrf2 target was markedly increased in a time-dependent manner following the exposure of CD1 mice to CDDO-Me (figure 3.4c-d). These data indicate that CDDO-Me causes a functional activation of the Nrf2 pathway in CD1 mice.

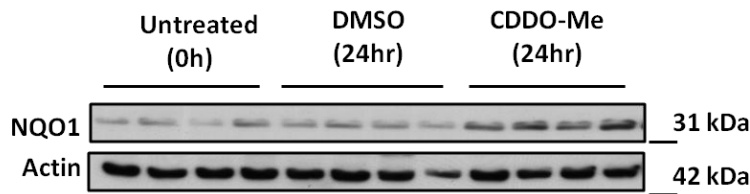
(A)



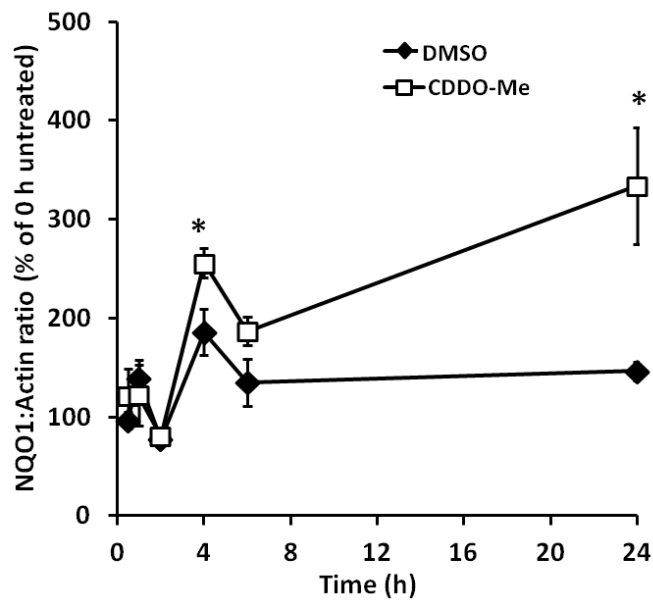
(B)



(C)



(D)

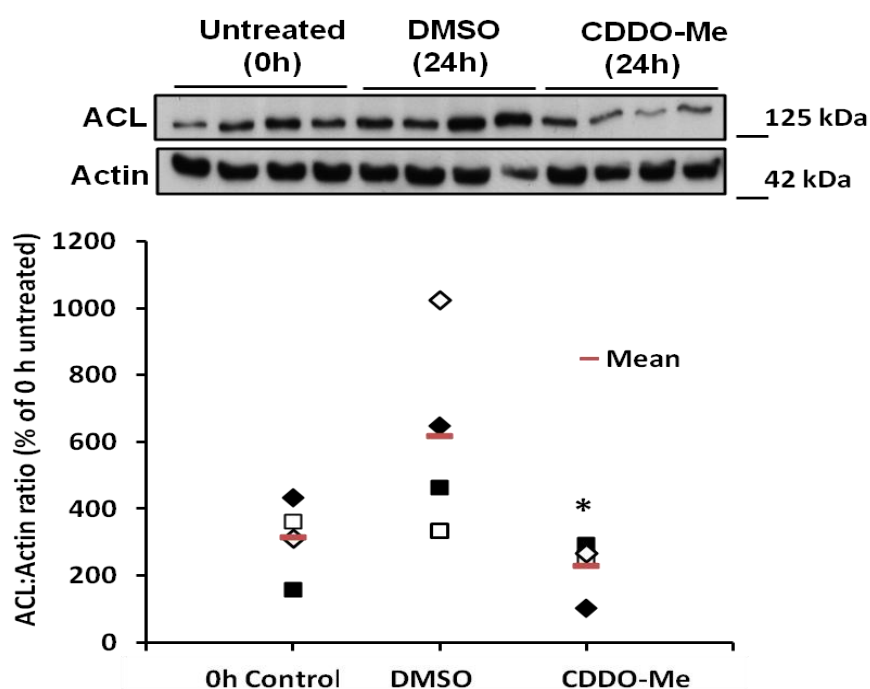


**Figure 3.4 Induction of the Nrf2 pathway by CDDO-Me in CD1 mice.** Nrf2 protein levels in liver homogenates of CD1 mice exposed to 3mg/kg CDDO-Me in DMSO for 1 hour (A)(n=4). The mean Nrf2 values were plotted after normalization with actin +/- SD (B). Nqo1 protein levels in liver homogenates of CD1 mice exposed to 3mg/kg CDDO-Me in DMSO for 24 hours (C). The mean Nqo1 values were plotted after normalization to actin +/- SD (D). Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$  wild-type CDDO-Me vs. wild-type vehicle).

### 3.2.4 Investigation of the role of Nrf2 in the regulation of fatty acid synthesis genes

Once it had been established that Nrf2 was functionally activated by CDDO-Me in the CD1 mouse model, the effect of the compound on the fatty acid synthesis pathway was investigated by determining hepatic levels of ATP citrate lyase (ACL)

protein expression by western blotting which is one of the key enzymes in fatty acid biosynthesis by catalyzing the metabolism of carbohydrates and the production of fatty acids. In addition, ACL was one of typical fatty acid synthesis proteins shown to be modulated in earlier studies (Kitteringham *et al.*, 2010). CDDO-Me provoked a time-dependent decrease in ACL expression in the livers of CD1 mice, when compared to the vehicle-treated mice (figure 3.5).



**Figure 3.5: Investigation of the role of Nrf2 in the regulation of fatty acid synthesis genes.** ACL protein levels in liver homogenates of CD1 mice exposed to 3mg/kg CDDO-me or DMSO for 24 h (n=4), as determined by immunoblotting. ACL values were plotted after normalization to actin (n=4). Statistical analysis was performed using an unpaired t-test (\*P<0.05 wild-type CDDO-Me vs. wild-type vehicle).

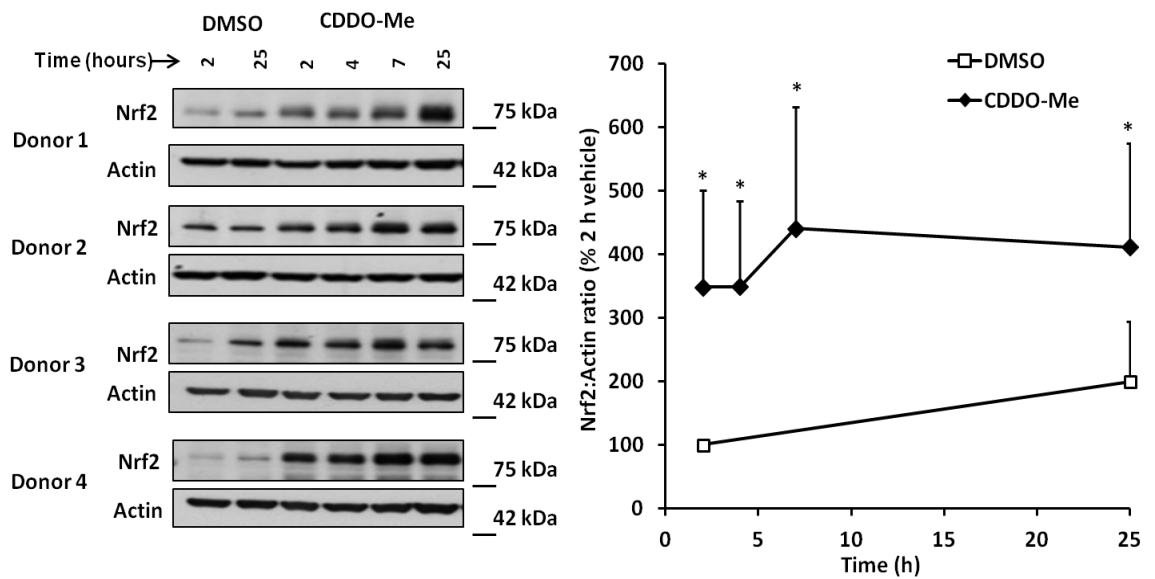
### 3.2.5 Investigation of Nrf2 response to CDDO-Me in human cells

In light of the previous data illustrating the role of CDDO-Me in the induction of Nrf2 signalling, and given the well characterized role of Nrf2 in cytoprotection, it was of interest to investigate the role of CDDO-Me in human cells, in order to understand the translational relevance of the findings in mice *in vivo*. To do this, the



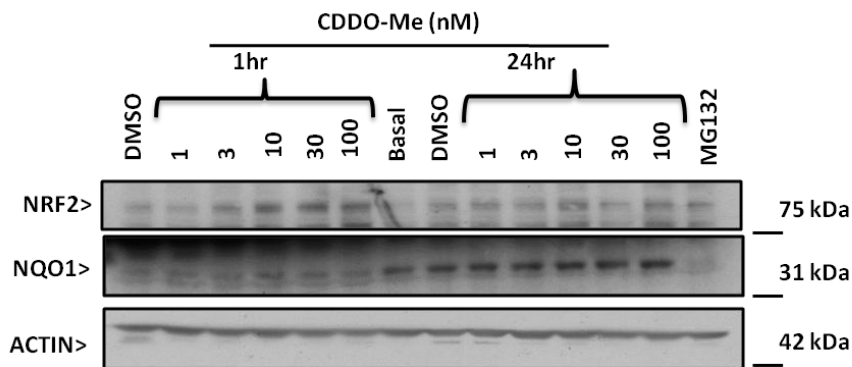
ability of CDDO-Me to induce Nrf2 in freshly isolated primary human hepatocytes (PHHs) and peripheral blood mononucleocytes (PBMCs) was tested. Freshly isolated PHHs or PBMCs were exposed to 100nM CDDO-Me ex vivo for 24 h, and whole-cell Nrf2 levels were determined by immunoblotting. CDDO-Me stimulated the accumulation of Nrf2 protein in PHHs and PBMCs (figure 3.6). Consistent with these observations, CDDO-Me caused an induction of Nqo1 in PBMCs, indicating that the induction of Nrf2 was functionally relevant (figure 3.6).

**(A)**

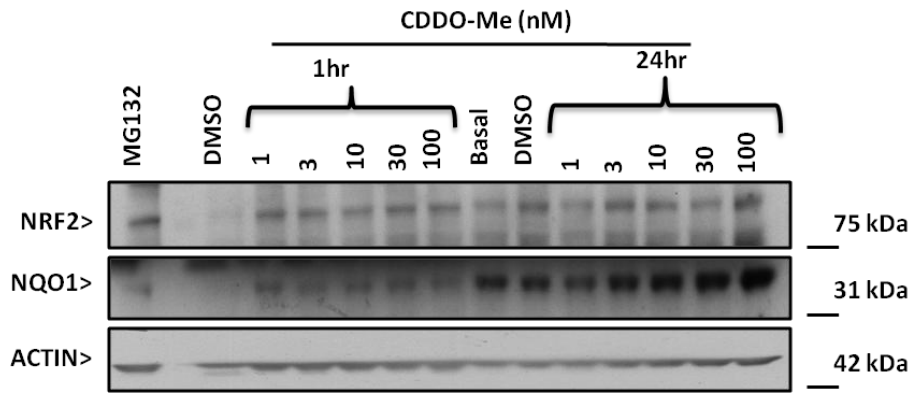


**(B)**

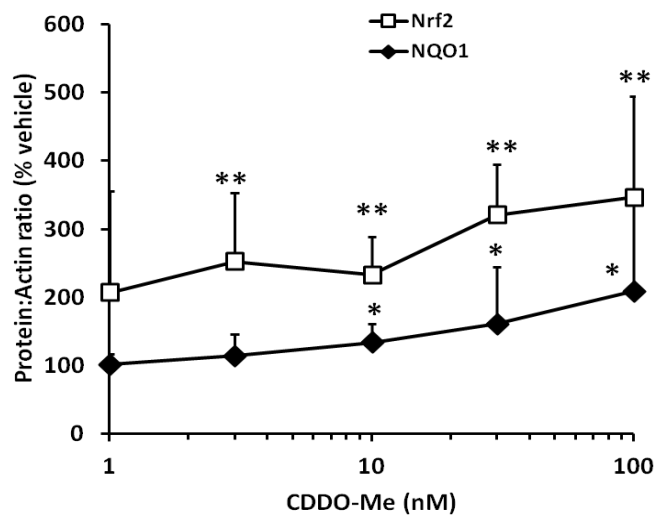
**Donor A**



**Donor B**



(C)



**Figure 3.6 - Induction of Nrf2 pathway in freshly isolated human cells.** Nrf2 protein levels in freshly isolated primary human hepatocytes exposed to 100nM CDDO-me or DMSO for the indicated times, as determined by western blotting (A). Nrf2 and Nqo1 protein levels in human PBMCs exposed to the indicated concentrations of CDDO-me for 1 or 24 h as determined by Western blotting (B-C). Human PBMCs treated with the proteasome inhibitor Mg132 for 2 h were used as a positive control. The blots shown are representative of experiments utilising cells from the 4 (PHH) and 6 (PBMC) separate donors and quantified by densitometry. The average Nrf2 and Nqo1 values of the independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*P<0.05, \*\*P<0.01 vs. vehicle ).

### **3.3 DISCUSSION**

A large number of studies have furthered our understanding of the mechanisms that regulate Nrf2 activity and the important functions of this pathway, especially in cytoprotection (Itoh *et al.*, 1997; Chan *et al.*, 2001; Copple *et al.*, 2008b; Bryan *et al.*, 2013). Due to the growing interest in investigating Nrf2 activators as therapeutic targets, gaining further information regarding the Nrf2-inducible genome, proteome and metabolome will be of utmost importance. In terms of drug safety science, identifying biomarkers of Nrf2 modulation and predicting the likely outcomes of sustained Nrf2 activation in man is crucial. It is thus important to characterize the proteins that are regulated by the Nrf2 pathway both basally and at the inducible levels. This will enable us to define the expression profiling of these proteins in different species and organs and how these might translate to the susceptibility to or protection against toxicities of various chemicals.

The findings of this chapter demonstrate that the triterpenoid CDDO-Me provokes an induction of the Nrf2 pathway in C57BL6J and CD1 mice. The induction of Nrf2 by CDDO-Me is associated with an increase in a classic Nrf2 target, Nqo1. Notably, CDDO-Me was shown not to activate Nqo1 in Nrf2-null mice.

A particular aim of the work described in this chapter was to define CDDO-Me induced gene expression changes in both wild type and Nrf2 knockout mice, to define any change in gene expression that might be observed as Nrf2-dependent or independent effects. In addition, as changes in mRNA are often not reflected at the protein level, it was also of interest to investigate whether the changes observed in the basal proteomic study (Kitteringham *et al.*, 2010) were reflected at the mRNA level.

The findings of the investigation into the hepatic genetic profile showed a significant increase in the relative mRNA levels of Nqo1, Ces1, Ephx, Ugt1a6a, Ugt2b5, Gsta4, Gstm1, Gstp1, Mgst, Gclc and Cyp1a2 in the livers of CDDO-Me treated wild type animals, when compared to vehicle-treated controls (figure 3.3). These genes are important in the regulation of phase II drug metabolism and glutathione synthesis. Whilst a positive role for Nrf2 in the regulation of

cytoprotective genes was seen in the mRNA study, in line with the findings at the protein level in the study by Kitteringham *et al.*, no negative regulation of fatty acid synthesis genes was identified at the mRNA level, in contrast to effects reported at the protein level (Kitteringham *et al.*, 2010). This lack of correlation between mRNA and protein could be indicative of a post-transcriptional role of Nrf2 in the regulation of lipid metabolism. However, other groups have reported an increase in the expression of genes involved in fatty acid synthesis in Nrf2 KO mice fed a high fat diet, when compared to WT mice (Tanaka *et al.*, 2008; Meakin *et al.*, 2014), as well as a down-regulation of lipid synthesis genes in Keap1 knockout mice and CDDO-Me treated WT mice (Yates *et al.*, 2009). The latter finding was corroborated here, with CDDO-Me shown to provoke a time-dependent inhibition of ACL protein expression in CD1 mice, under conditions of Nrf2 induction (figure 3.5). Therefore, whilst the weight of evidence indicates that Nrf2 negatively regulates lipid metabolism in the liver, the detailed underlying mechanisms require further investigation.

Of the tested genes that were up-regulated in CDDO-Me-treated WT mice, Nqo1 showed the greatest increase in mRNA expression. Nqo1 has previously been documented as a paradigmatic Nrf2-regulated gene (McWalter *et al.*, 2004; Yates *et al.*, 2007). Nqo1 is important for the reduction of quinones to hydroquinones. Altered expression of Nqo1 has also been implicated in the pathogenesis of cancers and Alzheimer's disease (Wang *et al.*, 2006; Kanagal-Shamanna *et al.*, 2012). The other tested genes that show significant upregulation of Nrf2 have also been identified as having a role in cytoprotection against chemical insults (Hayes *et al.*, 2014b). This data further confirms the ability of Nrf2 to regulate numerous cytoprotective processes in the mammalian liver.

It was also of interest to define the basal and inducible activity of Nrf2 in human cells and the differential activity of Nrf2 in discrete organs. This will be particularly useful when developing Nrf2-activators as therapeutic agents for the treatment of diseases where the aberrant expression of Nrf2 plays a role. There was a significant induction of Nrf2 by CDDO-Me in primary human hepatocytes and lymphocytes (figure 3.6). From the mouse hepatic profile, it was seen that Nqo1 had the highest

induction. This was therefore used as a surrogate marker of Nrf2 induction in the lymphocytes. Nqo1 induction in human Pheripheral blood mononucleocytes was first examined by Gordon et al., prior to the findings that saw Nqo1 gene induction by CDDO-Im and CDDO-me by Thimmulappa (Gordon *et al.*, 1991; Thimmulappa *et al.*, 2007) but the protein expression has not previously been defined, to our knowledge. Nqo1 protein levels were elevated in PBMCs following exposure to CDDO-Me for 24 h (figure 3.6b-c). Consistent with this, Boettler *et al.*, 2011 demonstrated induction of Nrf2 in PBMCs by the coffee constituent 5-O-caffeoylquinic acid (Boettler *et al.*, 2011). However, the above studies did not investigate inter-individual variation in the activity of the Nrf2 pathway between patients. Such variation could be due to differences in age, gender, genetic makeup, drug-drug interactions, lifestyle and environment (Severino *et al.*, 2004). This is of particular importance when considering idiosyncratic adverse drug reactions, a major limitation to drug development and approval. The findings here identify variation in the Nrf2 response to CDDO-Me amongst the six volunteers' PBMCs (figure 3.6).

As seen in the lymphocytes, there was also a clear indication of variation in Nrf2 and Nqo1 responses to CDDO-Me in the primary human hepatocytes of 4 donors (figure 3.6a). Nrf2 induction in hepatocytes has been described before (Keum *et al.*, 2006a) but inter-individual variation has not been fully explored. It will be useful to examine these variations and to investigate the effect of genetic influences, as polymorphisms have been identified in the promoter region of Nrf2 (Marzec *et al.*, 2007). It will therefore be of value to examine the role of Nrf2 pathway variation in determining susceptibility to drug-induced toxicity in humans. Future investigations could address this using a larger cohort of donors and by correlating inter-individual variability with susceptibility to adverse drug reactions. The expression levels of Nrf2 target genes in blood-derived immune cells could potentially be used as a marker to assess/predict the prognosis of drug-induced toxicity. It will also be valuable to assess which composition of the PBMCs are the most responsive to Nrf2 pathway modulation as there is variation in the composition of the macrophages, monocytes and lymphocytes among individuals.

In conclusion, this study supports a role for Nrf2 in the protection against chemical toxins through the induction of cytoprotective genes. Future work should focus on the characterization of the Nrf2-regulated proteome at the constitutive and inducible levels in lymphocytes in order to define the Nrf2-regulated proteins that are most sensitive to modulation of the pathway, and could therefore be used to assess inter-individual variation on a wider scale.

## **CHAPTER FOUR**

### **CHARACTERIZATION OF THE MECHANISM OF ACTION OF CDDO-Me**

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
4.1 Introduction.....	87
4.2 Results.....	88
4.2.1 Concentration- and time-dependent Induction of Nrf2 by CDDO-Me <i>in Vitro</i> .....	88
4.2.2 Functional consequences of Nrf2 induction by CDDO-Me.....	90
4.2.3 CDDO-Me induces Nrf2 via a post-transcriptional mechanism.....	92
4.2.4 Activation of Nrf2 by CDDO-Me is not dependent on the activation/inhibition of protein kinases and other signalling pathways.....	93
4.2.5 CDDO-Me does not stabilize Nrf2 mRNA.....	94
4.2.6 CDDO-Me stabilizes Nrf2 protein.....	96
4.2.7 CDDO-Me does not inhibit the catalytic activity of the proteasome <i>in vitro</i> .....	97
4.3 Discussion.....	101



## 4.1 INTRODUCTION

The findings of the previous chapter and those of studies by (Dinkova-Kostova *et al.*, 2005b; Liby *et al.*, 2005; Yates *et al.*, 2007) have shown that CDDO-Me has a high potency towards the Nrf2:Keap1 pathway across a number of mammalian test systems. It has also been shown to be an inhibitor of cellular inflammatory processes (Dinkova-Kostova *et al.*, 2005a) and have anti-proliferative properties, hence having chemopreventive and chemotherapeutic potentials (Bernstein *et al.*, 2012; Deeb *et al.*, 2012a; Qin *et al.*, 2013). CDDO-Me has been shown to have therapeutic potential in chronic kidney disease and cancer (Pergola *et al.*, 2011b; de Zeeuw *et al.*, 2013), although a recent clinical trial was terminated due to adverse cardiovascular events in some patients (Tayek *et al.*, 2013). However, CDDO-Me is currently going through another clinical trial in pulmonary arterial hypertension patients. CDDO-Me therefore holds promise as a novel therapeutic tool in settings where induction of Nrf2 is desirable.

The mechanism of action of CDDO-Me and its derivatives has yet to be fully characterized but studies have suggested that these compounds are capable of modifying cysteine residues in Keap1 (Dinkova-Kostova *et al.*, 2005b; Ahn *et al.*, 2010; Takaya *et al.*, 2012). A more detailed understanding of the chemical and molecular mechanisms that underlie the ability of CDDO-Me and its derivatives to induce Nrf2 would provide an opportunity to design and develop alternative small molecule inducers of Nrf2 for use in experimental and, potentially, clinical settings. In addition, the understanding of the mechanism of action of CDDO-Me will inform its clinical use and facilitates the fine-tuning of the compound to increase potency whilst preventing adverse drug reactions.

Therefore, the aim of the work described in this chapter was to provide *in vitro* insights into the mechanism of action of Nrf2 induction by CDDO-Me in hepatoma cells.

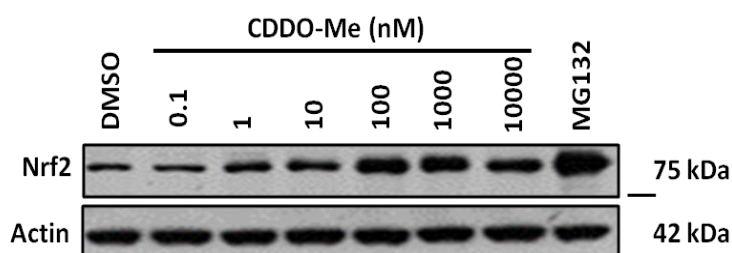
## 4.2 RESULTS:

### 4.2.1 Concentration- and time-dependent Induction of Nrf2 by CDDO-Me *in Vitro*

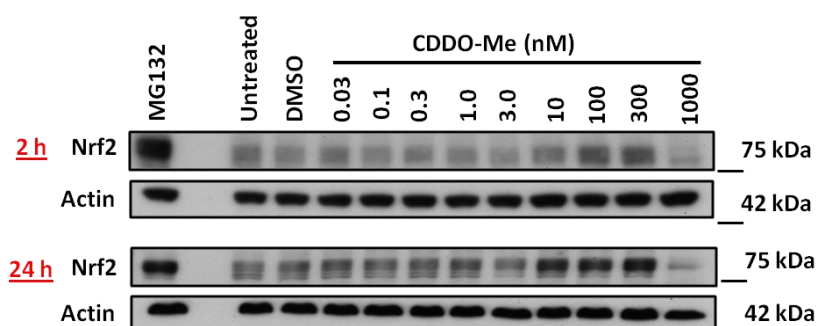
Western blotting on whole cell lysates from mouse hepatoma Hepa1c1c7 and human HepG2 cell lines exposed to CDDO-Me across a range of concentrations for 24 h, or 2 and 24 h respectively, was carried out in order to determine the optimal conditions for Nrf2 induction. CDDO-Me provoked a concentration-dependent increase in Nrf2 protein in Hepa1c1c7 cells and HepG2 cells with maximum induction seen at concentrations of 100 nM and 300 nM CDDO-Me at incubation times of 2 and 24 h, respectively (figure 4.1a-c). It was observed that beyond 300 nM CDDO-Me treatment, Nrf2 was reduced to lower than basal levels at both time points (figure 4.1b-c). This suggests that CDDO-Me may be toxic to the cells beyond 300 nM.

To determine the time course effect of CDDO-Me on Nrf2, Hepa1c1c7 cells were exposed to 100 nM CDDO-me for up to 24 h and whole-cell Nrf2 levels were determined by immunoblotting. Time course experiments demonstrated a rapid significant accumulation of Nrf2 within 15 minutes of exposure to 100 nM CDDO-Me, with maximum induction seen at 3 h of incubation with the compound (figure 4.2).

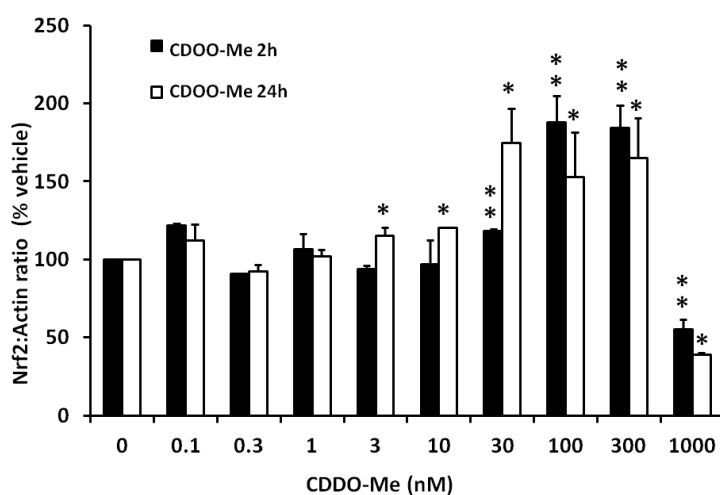
(A)



(B)

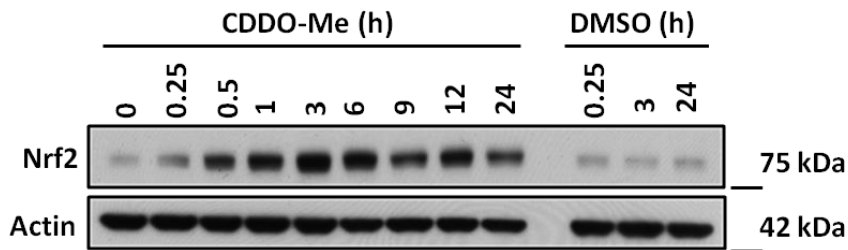


(C)

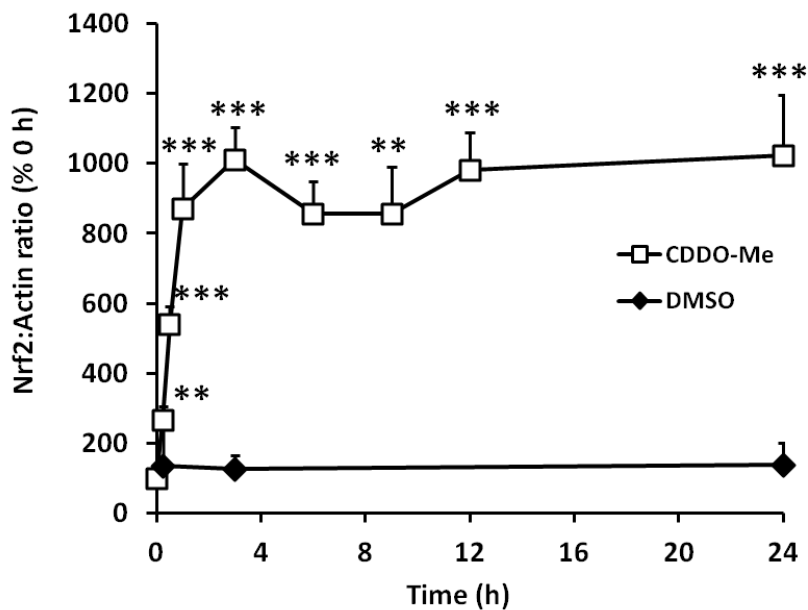


**Figure 4.1 – Concentration-dependent induction of Nrf2 by CDDO-Me in vitro.** Nrf2 protein levels in whole cell lysates prepared from Hepa1c1c7 cells treated with the indicated concentrations of CDDO-Me for 24 h (A) or from HepG2 cells treated with the indicated concentration of CDDO-Me for 2 or 24 h (B) as determined by western blotting. Hepa1c1c17 cells treated with 10  $\mu$ M Mg132 were used as a positive control. The blots shown are a representative of three independent experiments and quantified by densitometry (C). The average Nrf2 values of the three independent experiments represented in (B) were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*P<0.05, \*\*P<0.01 vs. vehicle).

(A)



(B)



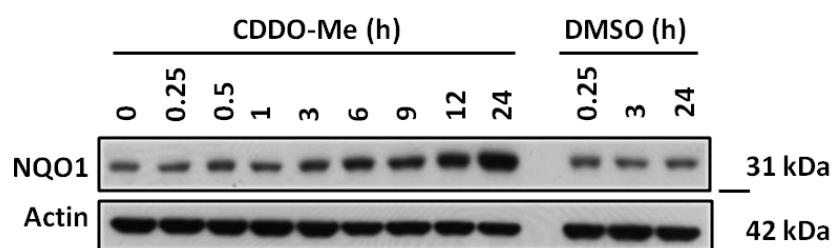
**Figure 4.2 - Time-dependent induction of Nrf2 in Hepa1c1c7 cells exposed to CDDO-Me.** Nrf2 protein levels in whole cell lysates prepared from Hepa1c1c7 cells exposed to 100 nM CDDO-Me for the indicated times as determined by western blotting. The blot shown (A) is representative of three independent experiments quantified by densitometry (B). The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. vehicle).

#### 4.2.2 Functional consequences of Nrf2 induction by CDDO-Me

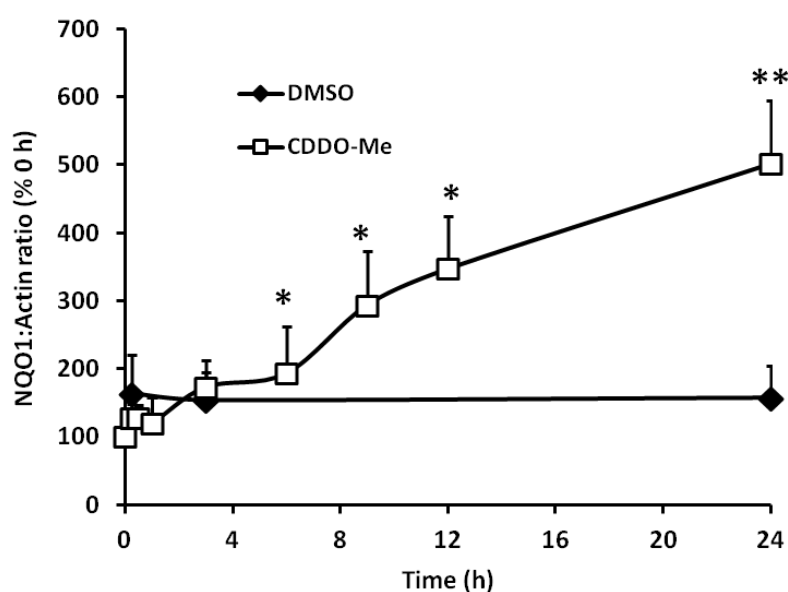
Nrf2 is known to regulate the basal and inducible expression of genes containing antioxidant response elements (ARE). Consistent with the ability of CDDO-Me to

provoke Nrf2 protein accumulation, NQO1, a well characterized Nrf2 target, showed a marked increase in a time-dependent manner following the exposure of Hepa1c1c7 cells to 100 nM CDDO-Me (figure 4.3). This result indicates that CDDO-Me causes a functional activation of Nrf2 cytoprotective pathway.

(A)



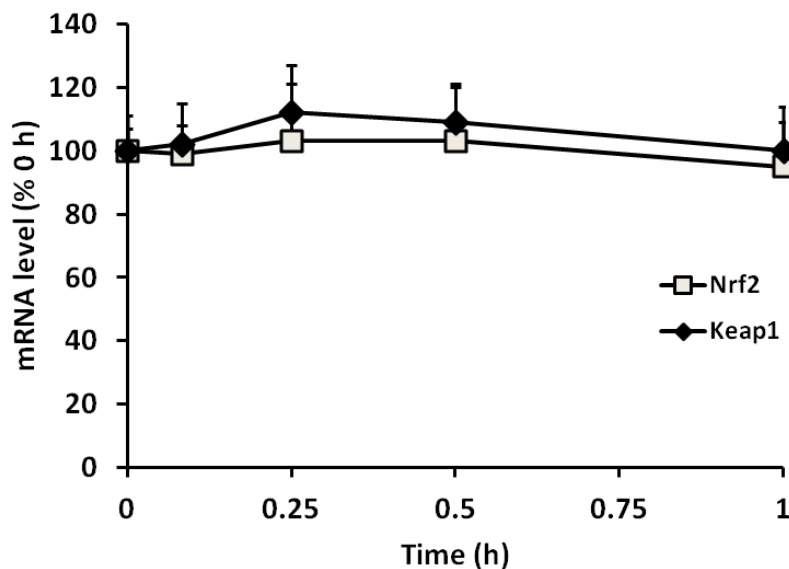
(B)



**Figure 4.3 - Time-dependent induction of Nqo1 in Hepa1c1c7 cells exposed to CDDO-Me.** NQO1 protein levels in whole cell lysates prepared from Hepa1c1c7 cells exposed to 100 nM CDDO-Me for the indicated times as determined by western blotting. The blot shown (A) is representative of three independent experiments quantified by densitometry (B). The average NQO1 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$  vs. vehicle).

#### 4.2.3 CDDO-Me induces Nrf2 via a post-transcriptional mechanism

It was hypothesized that CDDO-Me might induce Nrf2 protein accumulation at the transcriptional level by increasing the synthesis of Nrf2 mRNA. To examine this, total RNA was extracted from Hepa1c1c7 cells following exposure to CDDO-Me for up to 2 h. The cDNA was reverse transcribed and analysed by quantitative RT-PCR using oligonucleotide primers specific for Nrf2 and Keap1. The level of Gapdh mRNA was used to normalise the data. The findings of the experiment showed no significant increase in Nrf2 mRNA level within 1 h of exposure to CDDO-Me, despite these conditions provoking a maximal accumulation of Nrf2 protein (figure 4.4). This indicates that the increase in Nrf2 protein observed in Hepa1c1c7 cells following exposure to CDDO-Me is not a result of an increase in the expression of Nrf2 mRNA, and suggests that the compound exerts its effect on Nrf2 through a post-transcriptional mechanism. In addition, the level of Keap1 mRNA did not change significantly following CDDO-Me treatment (figure 4.4)

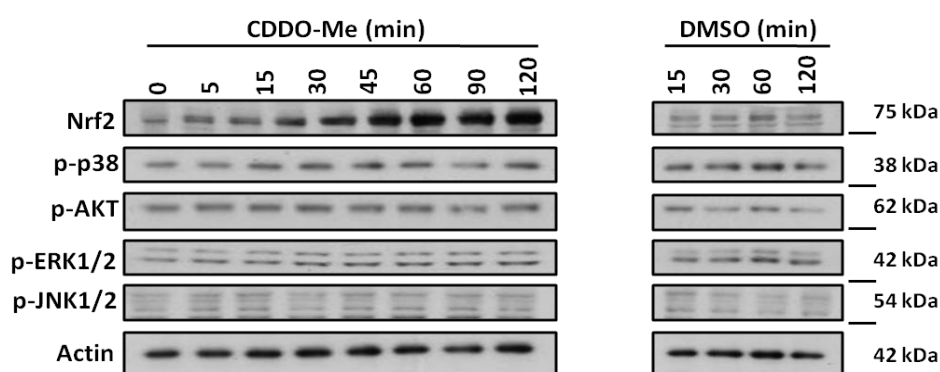


**Figure 4.4 - CDDO-Me does not provoke acute changes in Nrf2 or Keap1 mRNA expression.** Hepa1c1c7 cells were treated with 100 nM CDDO-Me for the indicated times, total RNA was isolated and the cDNA was synthesized. The cDNA was subjected to quantitative RT-PCR analysis using primers specific for Nrf2, Keap1 and Gapdh. The average Nrf2 and Keap1 mRNA levels were plotted after normalization with Gapdh mRNA levels + SD. No statistical significance was observed.

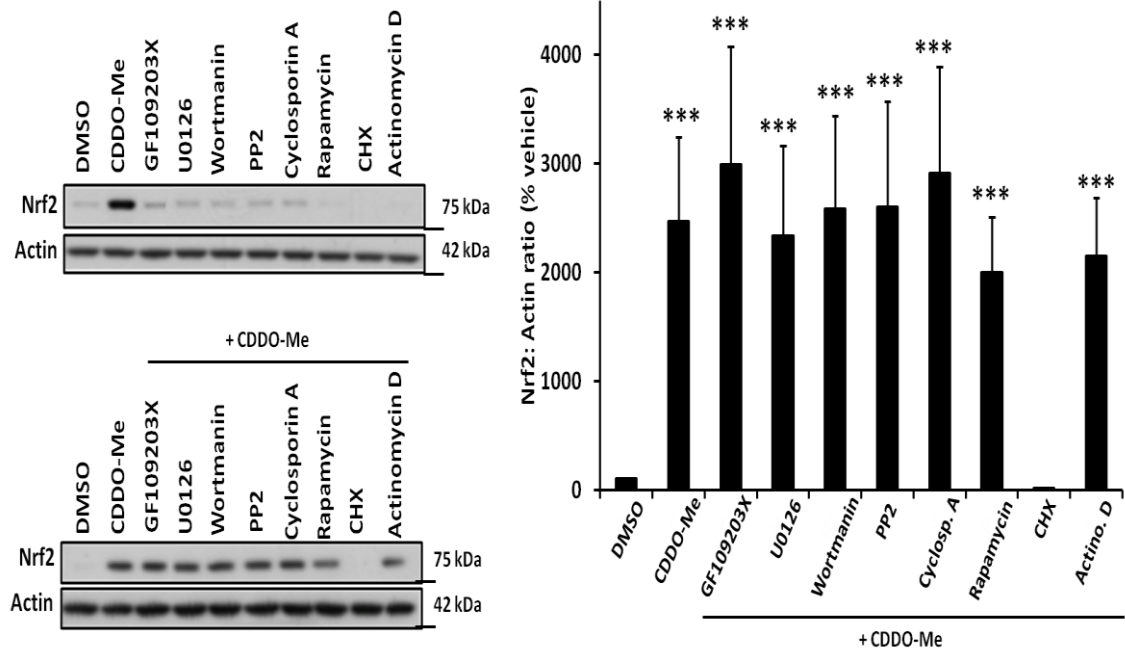
#### 4.2.4 Activation of Nrf2 by CDDO-Me is not dependent on the activation/inhibition of protein kinases and other signalling pathways

Protein kinases have important roles in various cellular processes; they are modulated upon exposure to chemicals which could lead to an imbalance in cellular homeostasis. It was therefore hypothesised that CDDO-Me could be inducing Nrf2 via the modulation of kinase signaling pathways previously implicated in the regulation of Nrf2 (Bryan *et al.*, 2013). To determine whether the ability of CDDO-Me to provoke Nrf2 was dependent on the modulation of these signaling pathways, Hepa1c1c7 cells exposed to CDDO-Me for up to 2 hours were analysed by western blotting for phosphorylation of AKT-473, p38 MAPK, ERK1/2 MAPK and JNK1/2. There was no significant effect on the phosphorylation of p38, AKT, ERK1/2 and JNK1/2 within the time-frame of Nrf2 induction with CDDO-Me (figure 4.5a). This was further confirmed using a panel of specific inhibitors GF109203X (PKC), U0126 (MEK1), Wortmanin (PI3K), PP2 (SRC), Cyclosporin A (Calcineurin) and Rapamycin (P70 S6). Hepa1c1c7 cells were pretreated for 1 h with these inhibitors before further treatment with CDDO-Me. The ability of CDDO-Me to induce Nrf2 was unaffected by the presence of these inhibitors (figure 4.5b). Taken together, the modulation of these signaling pathways does not appear to be an important element by which CDDO-Me induces Nrf2. Notably, Cyclohexamide, a protein synthesis inhibitor, prevented the activation of Nrf2 by CDDO-Me whilst CDDO-Me was still able to induce Nrf2 in the presence of actinomycin D (figure 4.5b). Taken together, this suggests that CDDO-Me could be stabilizing Nrf2 via a post-transcriptional mechanism.

(A)



(B)



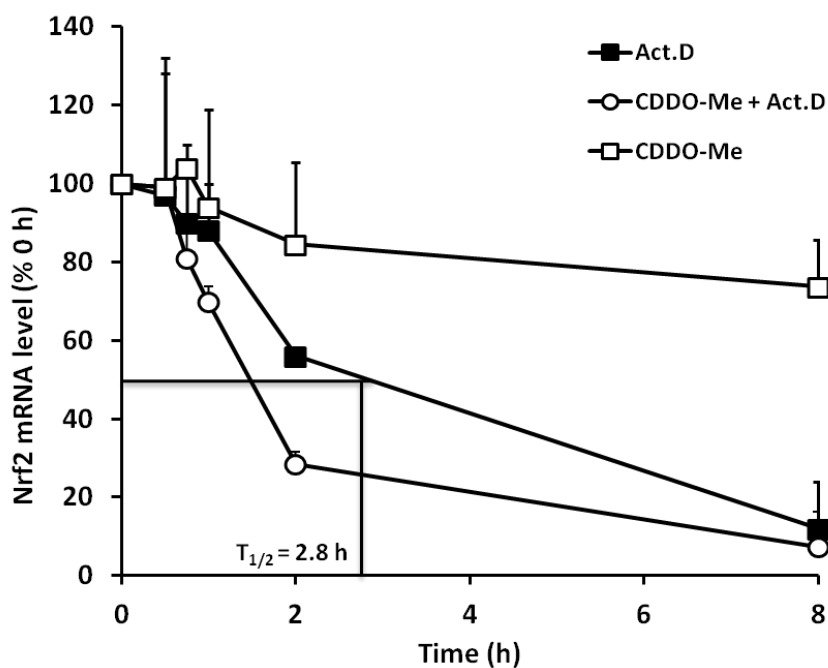
**Figure 4.5 - Activation of Nrf2 by CDDO-Me is not dependent on the activation/inhibition of protein kinase and other signalling pathways.** Nrf2 and phospho-p38 MAPK, AKT, ERK1/2 and JNK1/2 levels in total cell lysates of Hepa1c1c7 cells treated with 100 nM CDDO-Me for up to 2 h as determined by immunoblotting (A). Nrf2 protein levels in total cell lysates of Hepa1c1c7 cells pre-incubated with GF109203 (5  $\mu$ M), U0126 (5  $\mu$ M), Wortmanin (0.1  $\mu$ M), PP2 (5  $\mu$ M), Cyclosporin A (0.1  $\mu$ M), Rapamycin (0.1  $\mu$ M), cyclohexamide (5  $\mu$ g/mL) and actinomycin D (5  $\mu$ g/mL) for 1 h followed by a further treatment of 100 nM CDDO-Me for 1 h as determined by western blotting (B). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*\*\*) $P < 0.001$  vs. vehicle). (NB: please refer to section 6.2.6. for the positive controls for the inhibitors)

#### 4.2.5 CDDO-Me does not stabilize Nrf2 mRNA

From figure 4.5, the findings showed that the inhibition of general protein synthesis by CHX completely blocks the induction of Nrf2 by CDDO-Me, whilst CDDO-Me was able to induce Nrf2 protein in the presence of the RNA synthesis inhibitor actinomycin D. This suggests that CDDO-Me could be stabilizing the Nrf2 mRNA,



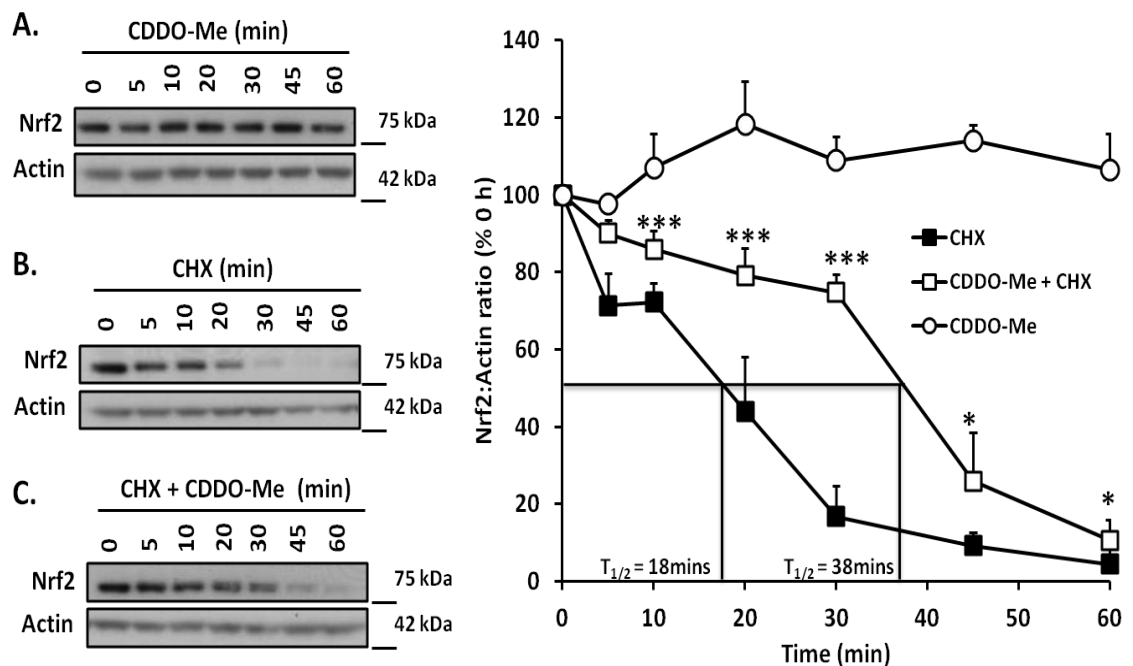
protein synthesis or the Nrf2 protein itself. To test that CDDO-Me works by stabilizing the Nrf2 mRNA, a time-course experiment was performed to characterize the effect of the inhibition of Nrf2 mRNA by actinomycin D in the presence or absence of CDDO-Me. To do this, Hepa1c1c7 cells were pretreated with 100 nM CDDO-Me or vehicle (DMSO) for 1 h, before further treatment with actinomycin D or vehicle for up to 8 h. Actinomycin D alone depleted Nrf2 mRNA over a time-course of 8 h (figure 4.6) with a half-life of approximately 2.8 h. Co-treatment of CDDO-Me with actinomycin D failed to increase the half-life of Nrf2 mRNA suggesting that CDDO-Me does not stabilize Nrf2 mRNA (figure 4.6).



**Figure 4.6 CDDO-Me does not stabilize Nrf2 mRNA.** Hepa1c1c7 cells were exposed to 100 nM CDDO-Me, treated with 5  $\mu$ g/mL Actinomycin D or pretreated with 100 nM CDDO-Me for 1 h followed by 5  $\mu$ g/mL Actinomycin D for the indicated time period. Total RNA was isolated and the cDNA was synthesized. The cDNA was subjected to quantitative RT-PCR analysis using primers specific for Nrf2 and Gapdh. The average Nrf2 mRNA levels of the two independent experiments were plotted after normalization with Gapdh mRNA levels + SD.

#### 4.2.6 CDDO-Me stabilizes Nrf2 protein

To test the possibility that CDDO-Me could be stabilizing the Nrf2 protein itself, a CHX chase experiment was performed to characterize the inhibition of Nrf2 protein synthesis by CHX in the presence/absence of CDDO-Me. To do this, Hepa1c1c7 cells were pretreated with 100 nM CDDO-Me or vehicle (DMSO) for 1 h before further treatment with CHX or vehicle for up to 1 h. CHX depleted Nrf2 over a time-course of 60 min (figure 4.4) giving Nrf2 a half-life of approximately 18 min which is consistent with findings of other studies (Nguyen *et al.*, 2003). A trace amount of Nrf2 was detected after 30 min exposure to CHX in Hepa1c1c7 cells. Co-treatment of CHX with CDDO-Me increased the half-life of Nrf2 protein from 18 to 38 min illustrating that CDDO-Me stabilizes the Nrf2 protein (figure 4.7)

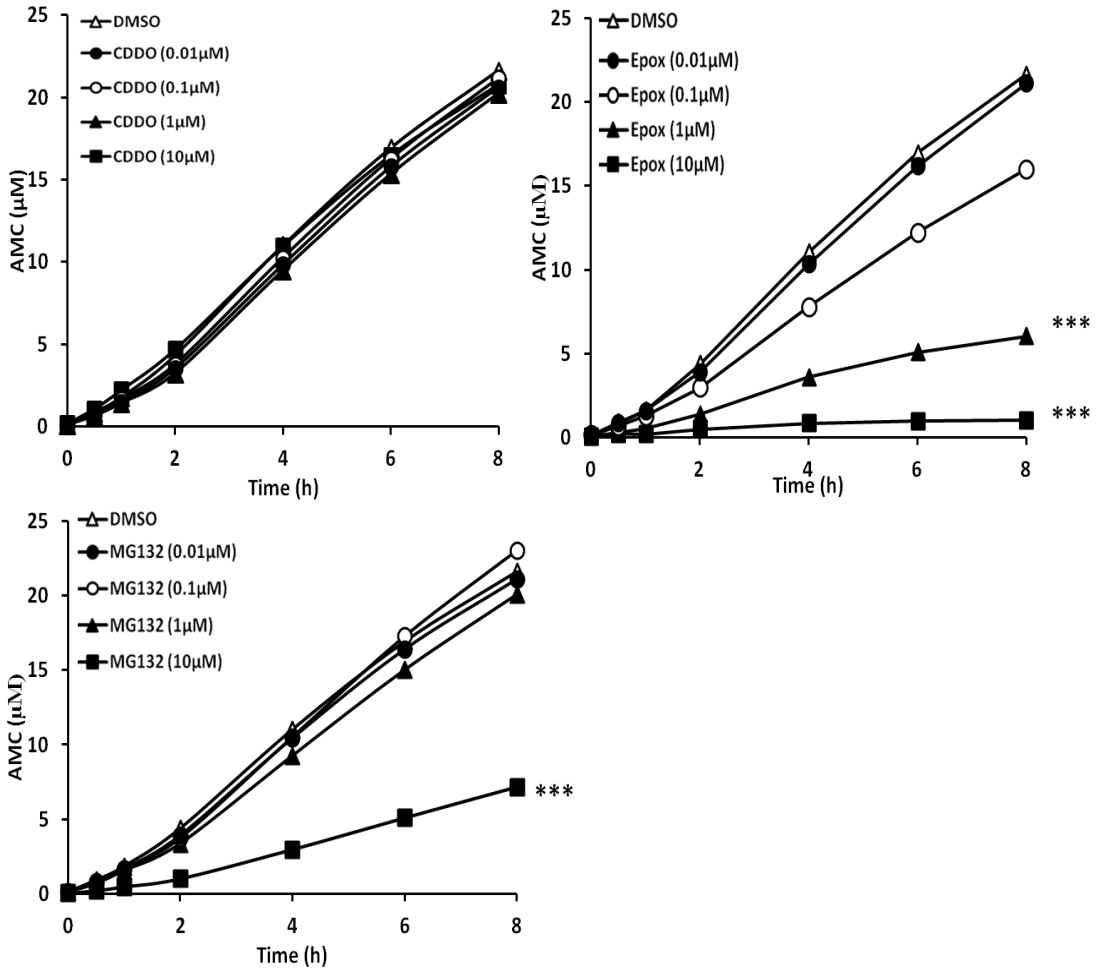


**Figure 4.7 - CDDO-Me stabilizes Nrf2 protein in Hepa1c1c7 cells.** Nrf2 protein levels in whole cell lysates of Hepa1c1c cells were treated with 100 nM CDDO-Me alone (A), treated with 5  $\mu$ g/ml CHX alone (B) or pretreated with 100 nM CDDO-Me for 1 h followed by CHX (C) for the indicated times as determined by immunoblotting. Nrf2 protein levels were normalised to beta-actin and quantified using densitometry. Data shown is a representative of three independent experiments + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\*\* $P < 0.01$  vs. CHX treated cells).

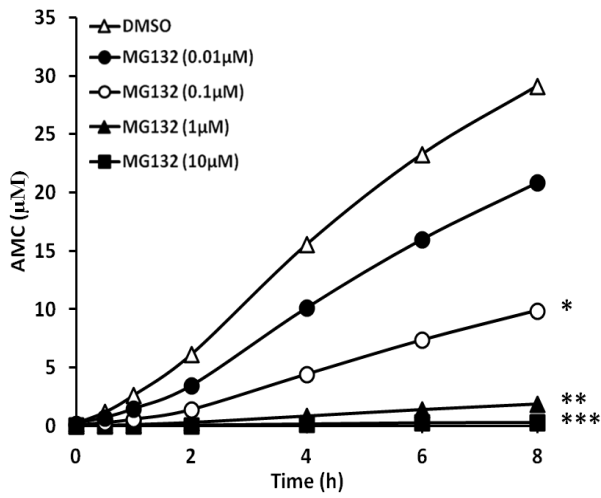
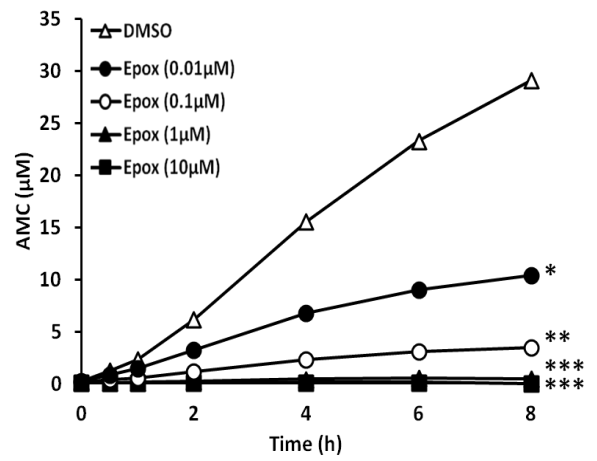
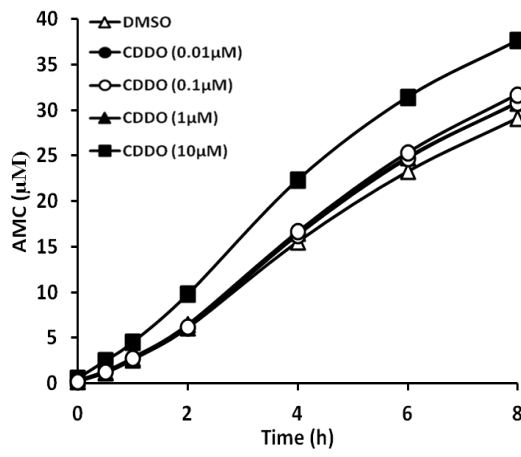
#### **4.2.7 CDDO-Me does not inhibit the catalytic activity of the proteasome *in vitro***

Given that the findings of figures 4.3-4.7 demonstrated that CDDO-Me induces Nrf2 in the absence of an effect on the Nrf2 mRNA, and has the ability to stabilize Nrf2 protein, it is plausible for CDDO-Me to be stabilizing the Nrf2 protein through the inhibition of its proteasomal degradation (Nguyen *et al.*, 2003). To investigate this, the effect of CDDO-Me on the catalytic activities of human 20S proteasome was examined *in vitro*. Subunits of the human 20S proteasome and the fluorescent peptide substrates Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (BOC-LSTR-AMC), Suc-Leu-Leu-Val-Tyr-AMC (SUC-LLCY-AMC) and Z-Leu-Leu-Glu-AMC (Z-LLG-AMC) corresponding to the trypsin, chymotrypsin and caspase-like activities respectively, were utilised to monitor the activities of the proteasome in the presence of CDDO-Me for up to 8 h *in vitro*. The hydrolysis of the fluorescent substrates BOC-LSTR-AMC, SUC-LLVY-AMC and Z-LLG-AMX was measured at an excitation of 360 nm and emission of 460 nm (figure 4.8). The findings demonstrate that CDDO-Me does not have an inhibitory effect on the catalytic activities of the 20S proteasome when compared to classical proteasome inhibitors MG132 and epoxomicin, which both exerted a concentration-dependent inhibition of each of the proteasomal catalytic sites (figures 4.8a-c). Therefore CDDO-Me appears to induce Nrf2 via a process that does not involve the inhibition of proteasomal activity.

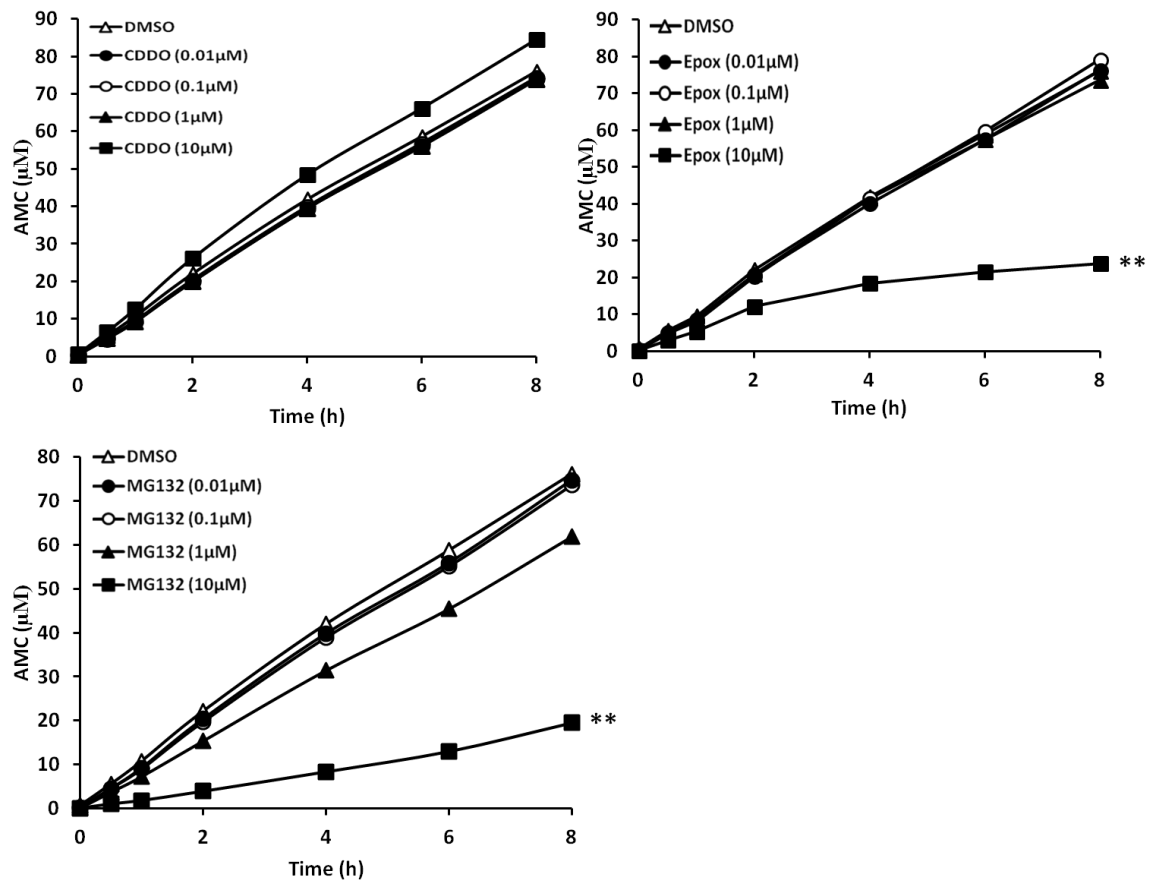
### A. Trypsin-like activity



## B. Chymotrypsin-like activity



### C. Caspase-like activity



**Figure 4.8 – CDDO-Me does not inhibit proteasomal activity.** Proteasome activity assay showing the effect of incubation with the indicated concentrations of CDDO-Me, epoxomicin or MG132 on the (A) trypsin (Boc-LSTR-AMC), (B) chymotrypsin (Suc-LLVY-AMC), and (C) caspase (Z-LLE-AMC)-like activities of human 20S proteasome at the indicated times. The average values of the three independent experiments were plotted. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. vehicle).

### **4.3 DISCUSSION**

The synthetic-triterpenoid CDDO and its derivatives (CDDO-Me and CDDO-Im) have proven anti-tumour, anti-viral and anti-inflammatory activities (Bernstein *et al.*, 2012; Deeb *et al.*, 2012; Qin *et al.*, 2013) and are some of the most potent inducers of Nrf2 described to date (Dinkova-Kostova *et al.*, 2005; Liby *et al.*, 2005). Due to the growing interest in the use of Nrf2 activators such as CDDO-Me as novel therapeutics, it was of interest to define the mechanism of action of this drug to optimise its therapeutic effects in disease settings.

The data presented in this chapter provides *in vitro* insights into the mechanism of Nrf2 activation by CDDO-Me in mouse Hepa-1c1c7 hepatoma cells. CDDO-Me provoked both concentration and time-dependent accumulation of Nrf2 protein. CDDO-Me showed a high specificity for the Nrf2 pathway at the nanomolar concentrations tested, as it did not induce the other panel of proteins tested such as p-p38, p-AKT473, p-JNK1/2 and p-ERK1/2. However, it has been reported that CDDO-Me modulates the activity of proteins such as NF- $\kappa$ B, STAT, ERK, SMAD and PPAR $\gamma$  at higher concentrations (Suh *et al.*, 2003; Ahmad *et al.*, 2006; Ji *et al.*, 2006; Liby *et al.*, 2006; Yore *et al.*, 2006; Wu *et al.*, 2011b).

Induction of Nrf2 by CDDO-Me was found to be independent of changes in the level of Nrf2 mRNA, indicating that the induction of Nrf2 by CDDO-Me proceeds through a post-transcriptional mechanism. Notably, the findings here suggest that CDDO-Me stabilizes Nrf2 protein, as seen from the CHX pulse chase experiment whereby the co-treatment of CDDO-Me with CHX increased the half-life of Nrf2 protein from 18 minutes to 38 minutes. CHX alone depleted Nrf2 protein over a time period of 60 minutes giving it a half-life of approximately 18 mins. This is consistent with the findings of other studies (Nguyen *et al.*, 2003). However, given that CDDO-Me had no effect on the level of Nrf2 mRNA and did not stabilize the Nrf2 mRNA, as the co-treatment of CDDO-Me with actinomycin D failed to increase the half-life of Nrf2 mRNA, these all point towards a post-transcriptional mechanism by which CDDO-Me stimulates Nrf2 accumulation.

It is plausible that CDDO-Me could be stabilizing Nrf2 protein by inhibiting its proteasomal degradation. However, when the ability of CDDO-Me to inhibit the human 20S proteasome was examined *in vitro*, the data showed CDDO-Me to have no significant effect on the proteasomal catalytic activities *in vitro*. Despite this result indicating that CDDO-Me does not inhibit the 20S proteasome *in vitro*, it is not conclusive enough to say CDDO-Me does not induce Nrf2 through this pathway, since it was examined in an *in vitro* system. Indeed, CDDO-Me could be metabolised in cells and the resulting metabolites might act as inhibitors of proteasomal activation.

Studies have suggested CDDO-Me and its derivatives induce Nrf2 through various mechanisms including the modification of Keap1 at critical cysteine residues although direct evidence is lacking (Dinkova-Kostova *et al.*, 2005; Ahn *et al.*, 2010; Takaya *et al.*, 2012). However, recent findings in our laboratory indicate that a chemically-tuned derivative of CDDO-Me modifies Keap1 *in vitro* and in cells. Therefore, triterpenoids are capable of directly modifying Keap1, although the mechanistic link between this and the activation of Nrf2 remains to be elucidated (data to be presented in the thesis of Holly Bryan).

It was also examined whether CDDO-Me stabilises Nrf2 protein through the modulation of intracellular signalling pathways implicated in the regulation of Nrf2 signalling, such as p38 (Keum *et al.*, 2006), PI3K (Nguyen *et al.*, 2003), MAPK/ERK (Nguyen *et al.*, 2003; Yuan *et al.*, 2006), JNK (Yuan *et al.*, 2006) and PERK (Cullinan *et al.*, 2003). The data demonstrated that CDDO-Me did not activate the mitogen-activated protein kinase p38, stress activated protein kinase JNK 1/2 and Phosphoinoside 3-kinase AKT within the time frame of Nrf2 accumulation. Using a panel of inhibitors of different signalling pathways such as GF109203X, U0126, Rapamycin, PP2 and Cyclosporin A, CDDO-Me still induced the activation of Nrf2 in the presence of these inhibitors. Although several studies have reported the modulation of some of these signalling pathways by CDDO-Me (Liby *et al.*, 2006; Ahmad *et al.*, 2006; Yore *et al.*, 2006; Ji *et al.*, 2006; Suh *et al.*, 2003; Wu *et al.*, 2011), experimental differences should be noted, mostly with regard to the concentrations used ( $\mu\text{M}$ ) which is higher than the nM concentrations that



provoked the maximum induction of Nrf2 here. In addition, the exposure time could also influence the biological effects of CDDO-Me in cells.

Taken together, whilst the data described here has furthered our understanding of the mechanisms that underlie the ability of CDDO-Me to induce Nrf2 in hepatoma cells, further information regarding the mechanism of action of CDDO-Me is needed to allow its potency and efficacy to be optimised for experimental and clinical use. Such insights will inform the design of safe and potent inducers of Nrf2 which hold promise as therapeutic agents in a number of disease contexts.

## **CHAPTER FIVE**

### **INHIBITION OF NRF2 BY BRUSATOL**

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
5.1 Introduction.....	106
5.2 Results.....	108
5.2.1 Brusatol inhibits Nrf2 in a dose- and time-dependent manner .....	108
5.2.2 Decreased Levels of Nrf2 Regulated genes in Cells treated with Brusatol.....	110
5.2.3 Brusatol can stimulate the depletion of pharmacologically induced Nrf2.....	111
5.2.4 Brusatol-mediated inhibition of Nrf2 is not associated with a reduction in cell viability.....	116
5.2.5 Brusatol Sensitizes Cells to chemical stress.....	117
5.2.6 Brusatol depletes Nrf2 in primary human Hepatocytes.....	119
5.2.7 Brusatol has a high specificity for the Nrf2 pathway and not a universal protein synthesis inhibitor.....	121
5.2.8 Uptake and cellular stability of brusatol.....	122
5.3 Discussion.....	129

## 5.1 INTRODUCTION

Nrf2 has been shown to contribute to a number of diverse cellular processes other than cell defence, such as differentiation, proliferation and lipid metabolism (Kitteringham *et al.*, 2010; Bryan *et al.*, 2013). In doing so, Nrf2 promotes the maintenance of cellular homeostasis under stress conditions, coordinating repair pathways in an attempt to return the cell to basal conditions. Transgenic Nrf2 knockout mice experience enhanced susceptibility to various drug-induced toxicities (McMahon *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Cho *et al.*, 2002) supporting the notion that Nrf2 has various roles in the pathogenesis of various diseases such as neurodegeneration (Valko *et al.*, 2007; Bryan *et al.*, 2013).

In recent years, studies have seen the involvement of Nrf2 in the pathogenesis of various diseases and how the manipulation of this pathway could be a therapeutic strategy in alleviating symptoms and potentially curing the disease of interest. This has led to the screening of many naturally occurring phytochemicals with proven cancer chemopreventive abilities (Wolf, 2001). Notably, most of these compounds are also potent Nrf2 inducers such as sulforaphane, resveratrol (Kensler *et al.*, 2000; Kode *et al.*, 2008) and triperpenoid CDDO and CDDO derivatives (CDDO-Im and CDDO-Me). These amongst many other Nrf2 inducers have shown potency in several disease settings like diabetes and cancer (Bernstein *et al.*, 2012; Deeb *et al.*, 2012b; Qin *et al.*, 2013). Whether there is a direct link between Nrf2 activation and cancer prevention is yet to be fully elucidated.

Interestingly, despite various findings showing the roles of Nrf2 in chemoprevention, there is mounting evidence to suggest that a variety of cancer cell types harbour a constitutive upregulation of the Nrf2 pathway, driving enhanced proliferation and chemoresistance (Ikeda *et al.*, 2004; Singh *et al.*, 2006; Wang *et al.*, 2008; Lister *et al.*, 2011). In this respect, Nrf2 seems to play a dual role by protecting cells from cancer promoting and chemotherapeutic agents (For a review see (Copple, 2012). Mutations within the Keap1 or Nrf2 genes have been identified in the domains responsible for protein dimerisation, resulting in de-regulation of Nrf2 by Keap1, thus high basal levels of Nrf2 in cancerous

environments (Padmanabhan *et al.*, 2006; Ohta *et al.*, 2008). A study by Kim *et al.*, 2007 showed the upregulation of Nrf2 and Prx1 in conditions of hypoxia, which is typical of a tumour microenvironment (Kim *et al.*, 2007). Wang *et al.*, 2008 also showed Nrf2 is expressed at the stage II and stage III of three cancer cell lines (neuroblastoma SH-SY5Y, breast adenocarcinoma MDA-MB-231 and non-small cell lung carcinoma A549), thus enhancing their chemoresistance to cisplatin, doxorubicin and etoposide (Wang *et al.*, 2008).

Therefore, an ability to modulate the activity of the Nrf2 pathway, through pharmacological inhibition of the transcription factor, holds promise as a therapeutic strategy in cancer settings. Studies on silencing Nrf2 or over-expressing Keap1 to reduce the basal levels of Nrf2 have shown positive results in overcoming chemoresistance in cultured cells (Cho *et al.*, 2008; Kim *et al.*, 2008). The effect is reversed when Nrf2 is over expressed, increasing resistance in various cancer cells (Wang *et al.*, 2008; Homma *et al.*, 2009; Zhang *et al.*, 2010).

The quassinoid brusatol, is a drug isolated from the plant *Brucea javanica* (Mata-Greenwood *et al.*, 2002) which has been shown to be a selective inhibitor of the Nrf2 pathway in various cell lines such as HeLa, MDA-MB-231 and Ishikawa and proven effective in reducing chemoresistance (Ren *et al.*, 2011). Brusatol showed specificity for the Nrf2 pathway as it had no effect on a panel of key modulators of various pathways including NF-kB and apoptotic pathways (Ren *et al.*, 2011). Brusatol sensitized A549 xenografts to cisplatin and this drug combination initiated apoptosis and reduced cell growth and tumour size significantly more than the individual drugs alone. Ren *et al.*, 2011 proposed that brusatol inhibits Nrf2 through enhanced ubiquitination and proteasomal degradation (Ren *et al.*, 2011) however its mechanism of action in this respect is yet to be fully elucidated.

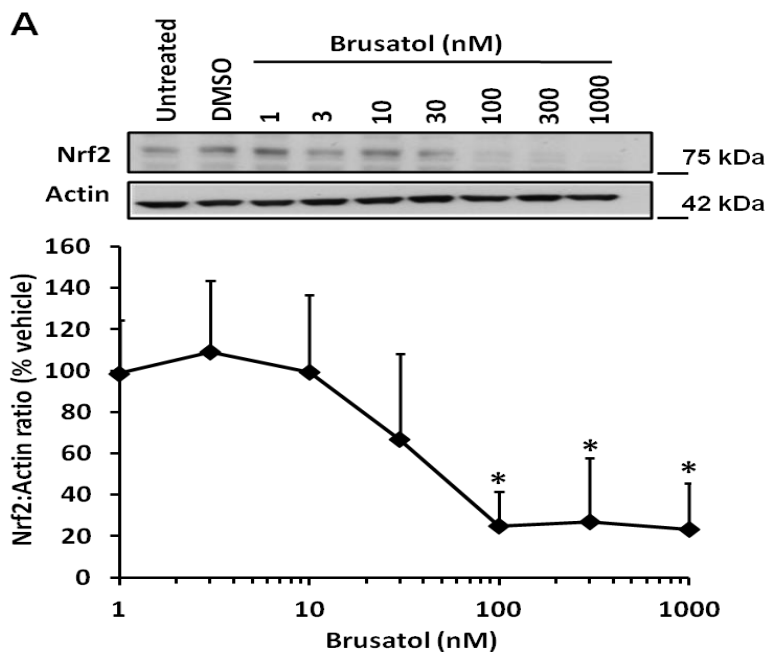
Therefore, the aim of the work described in this chapter is to provide *in vitro* insights into the molecular mechanism of Nrf2 inhibition by brusatol in mouse Hepa-1c1c7 hepatoma cells.

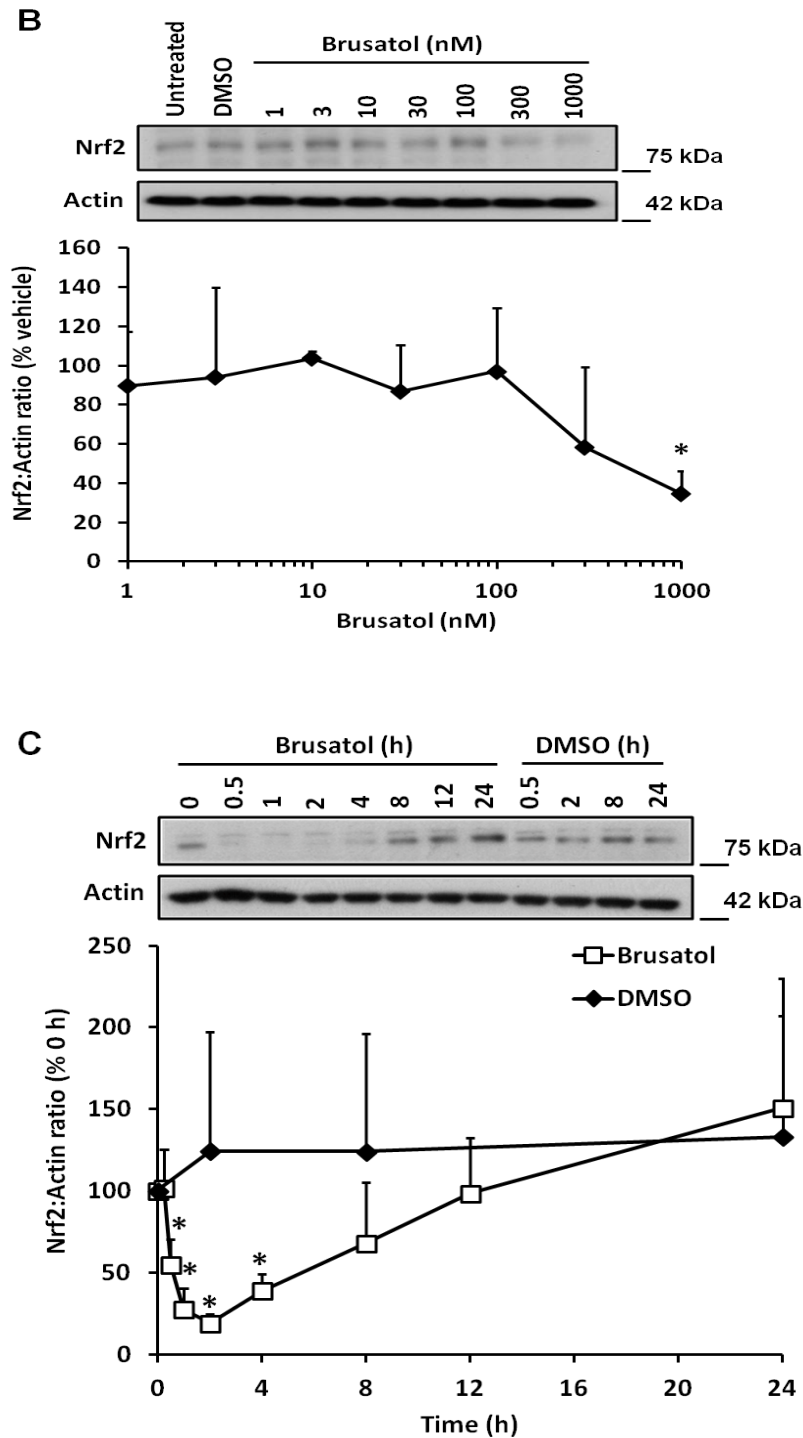
## 5.2 RESULTS

### 5.2.1 Brusatol inhibits Nrf2 in a dose- and time-dependent manner

In order to determine the optimal conditions of Nrf2 inhibition by brusatol, Hepa1c1c7 cells were treated with brusatol across a range of doses and incubation times, and the levels of Nrf2 protein in the cell lysates were determined by immunoblotting with an anti-Nrf2 antibody. It was observed that brusatol reduces the basal level of Nrf2 protein in Hepa1c1c7 cells in a dose-dependent manner with Nrf2 levels reduced to below the limit of detection following treatment with 100 nM brusatol for 2 hours (figure 5.1a). Interestingly, levels of Nrf2 protein were no lower following 24 hours treatment however protein levels follow the same pattern across the high doses at both time points (figure 5.1b)

Time course experiments show that treatment with 300 nM brusatol reduced Nrf2 levels to below the limit of detection of the assay within 30 minutes. Subsequently Nrf2 levels were restored at 4-8 hours post-treatment with 300 nM brusatol, following which Nrf2 levels reached basal levels (figure 5.1c)



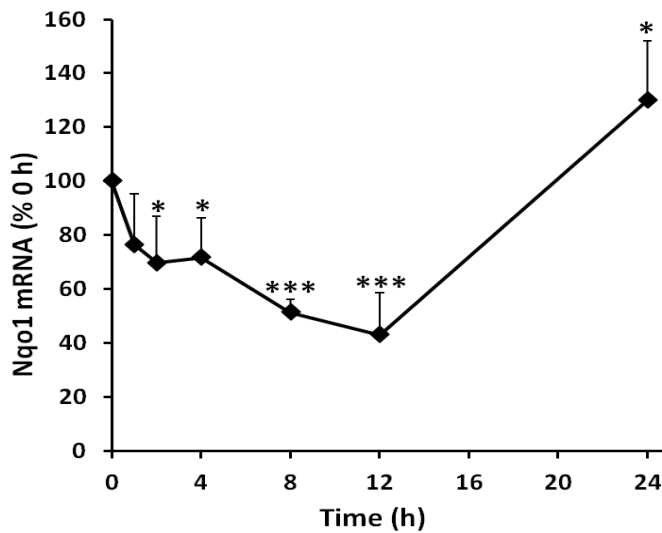


**Figure 5.1 – Depletion of Nrf2 in Hepa1c1c7 cells exposed to brusatol.** Nrf2 levels in total cell lysates prepared from Hepa1c1c7 cells treated with the indicated concentrations of brusatol for 2 (A) or 24 hours (B) or with 300 nM for the indicated times (C) as determined by western blotting. The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$  vs. Vehicle or 0 h control).

### 5.2.2 Decreased Levels of Nrf2 regulated genes in cells treated with brusatol

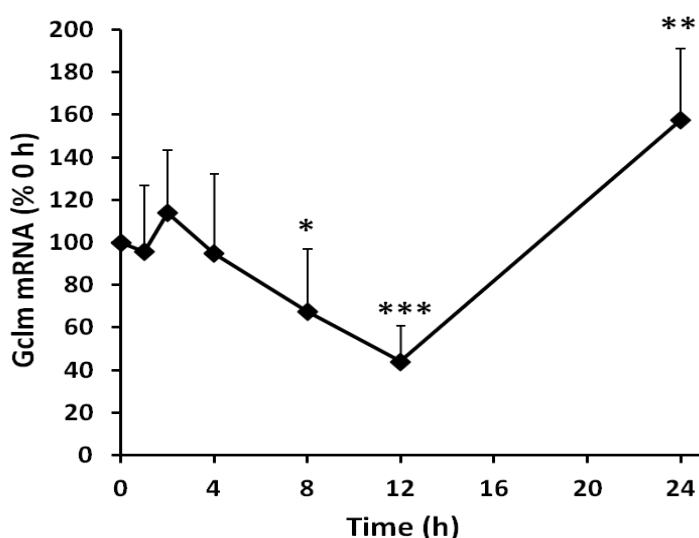
To determine whether the depletion of Nrf2 protein by brusatol is functionally relevant, the mRNA levels of a panel of Nrf2-regulated genes was determined by real time-PCR following the treatment of Hepa1c1c7 cells with 300 nM brusatol for up to 24 hours. Total RNA was extracted following brusatol treatment and the cDNA was analysed by quantitative RT-PCR using oligonucleotide primers specific for Nqo1 and Gclm. The levels of Gapdh mRNA were measured as a loading control to normalise the data. It was shown that the reduction in Nrf2 protein levels following brusatol treatment does in fact translate to a reduction in mRNA of downstream genes indicating a perturbed cell defence response (figures 5.2a-b)

(A)





(B)



**Figure 5.2 – Brusatol reduced mRNA levels of Nrf2 downstream target genes.** Hepa1c1c7 cells were treated with 300 nM brusatol for the indicated times, total RNA was isolated and the cDNA was synthesized. The cDNA was subjected to quantitative RT-PCR analysis, using a primers specific for Nqo1, Gclm and Gapdh. The average Nqo1 and Gclm mRNA levels were plotted after normalization with Gapdh mRNA levels + SD (A-B). Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. 0 h control).

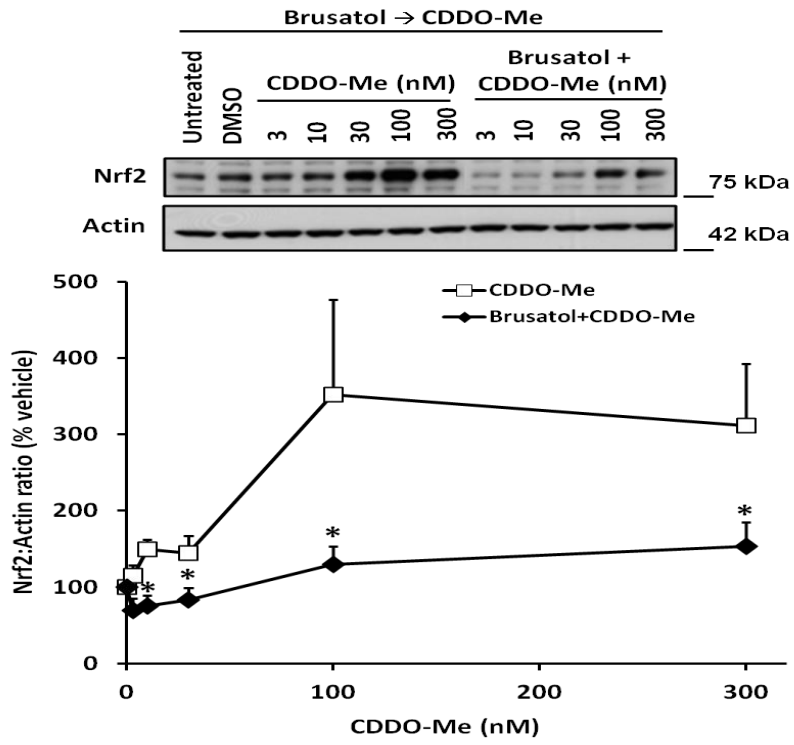
### 5.2.3 Brusatol can stimulate the depletion of pharmacologically induced Nrf2

Given the ability of brusatol to reduce basal Nrf2 levels, and the potential therapeutic value of Nrf2 inhibitors in cancer cells exhibiting constitutively high levels of Nrf2, we decided to determine whether brusatol can deplete pharmacologically induced Nrf2. To investigate this, cells were treated with the small molecule Nrf2 inducers, CDDO-Me, N-ethylmaleimide and iodoacetamide. Specifically, Hepa1c1c7 cells were pre-treated with 300 nM brusatol for 2 hours before being exposed to CDDO-Me (figure 5.3a) or N-ethylmaleimide (figure 5.3b) or iodoacetamide (figure 5.3c) for 1 hour and the level of Nrf2 protein in the cell lysates was determined by immunoblotting. Brusatol inhibited the ability of the small molecules to induce Nrf2.

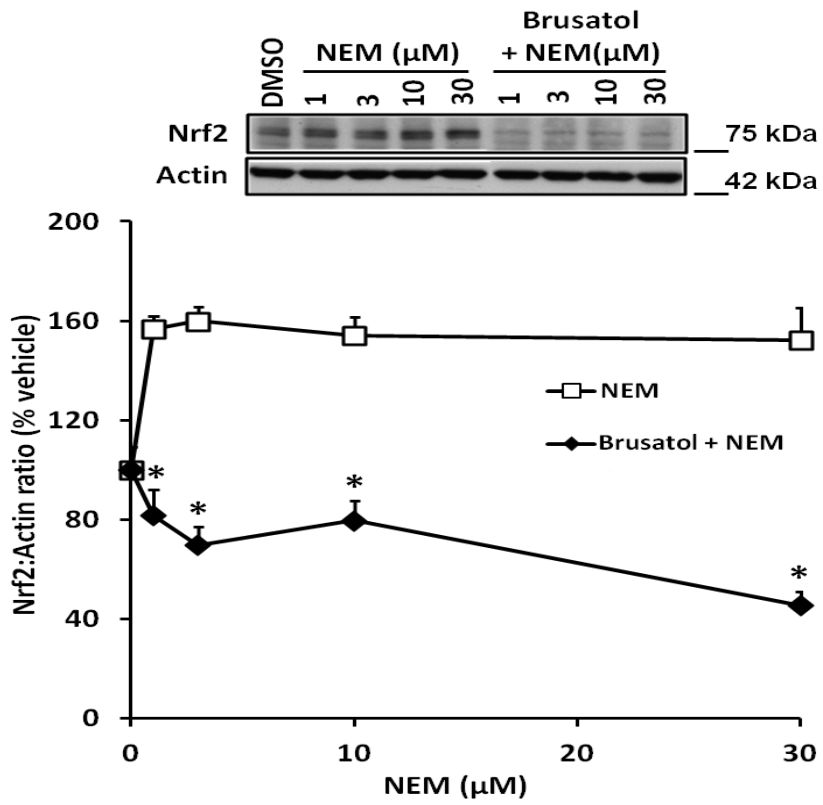
Additionally, cells were pre-treated with CDDO-Me for 1 hour prior to treatment with brusatol to determine whether brusatol can clear pharmacologically induced Nrf2 levels. Brusatol was able to attenuate the induction of Nrf2 seen with CDDO-Me, significantly reducing the maximal increase in Nrf2 seen with this compound (figure 5.3d).

To determine whether brusatol was preventing CDDO-Me from inducing Nrf2 due to a chemical interaction that may occur outside the cells, cells were washed with DMEM media following CDDO-Me pre-treatment for 1 hour before brusatol was added. Following the removal of CDDO-Me, there was an induction of Nrf2 protein in Hepa1c1c7 cells, however, as seen in the previous results, brusatol depleted the induced levels of Nrf2 (figure 5.3e) implying that brusatol-mediated depletion of CDDO-Me-induced Nrf2 is not due to a chemical interaction between the two small molecules.

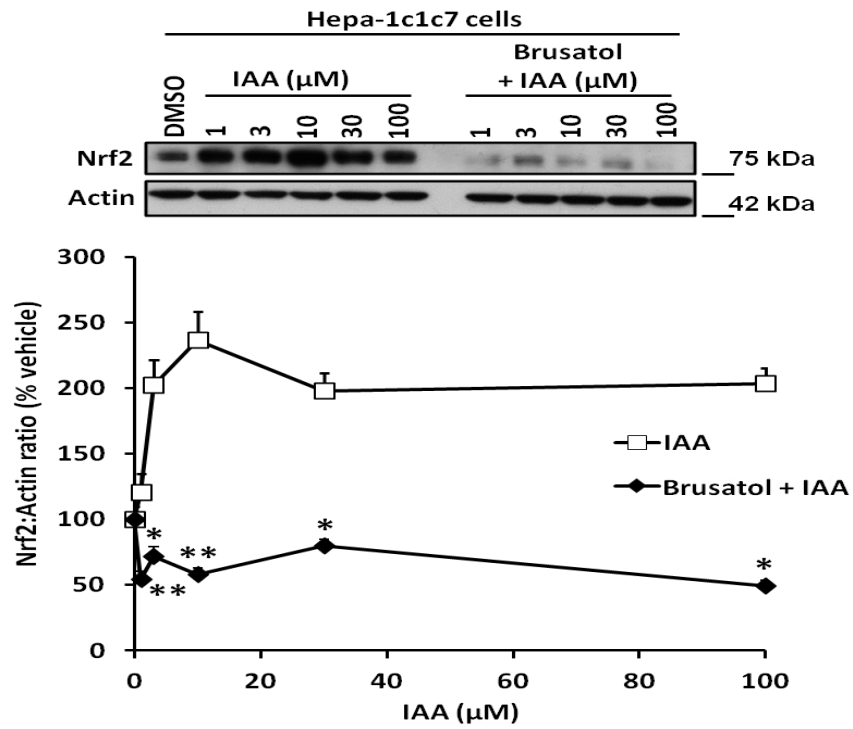
(A)



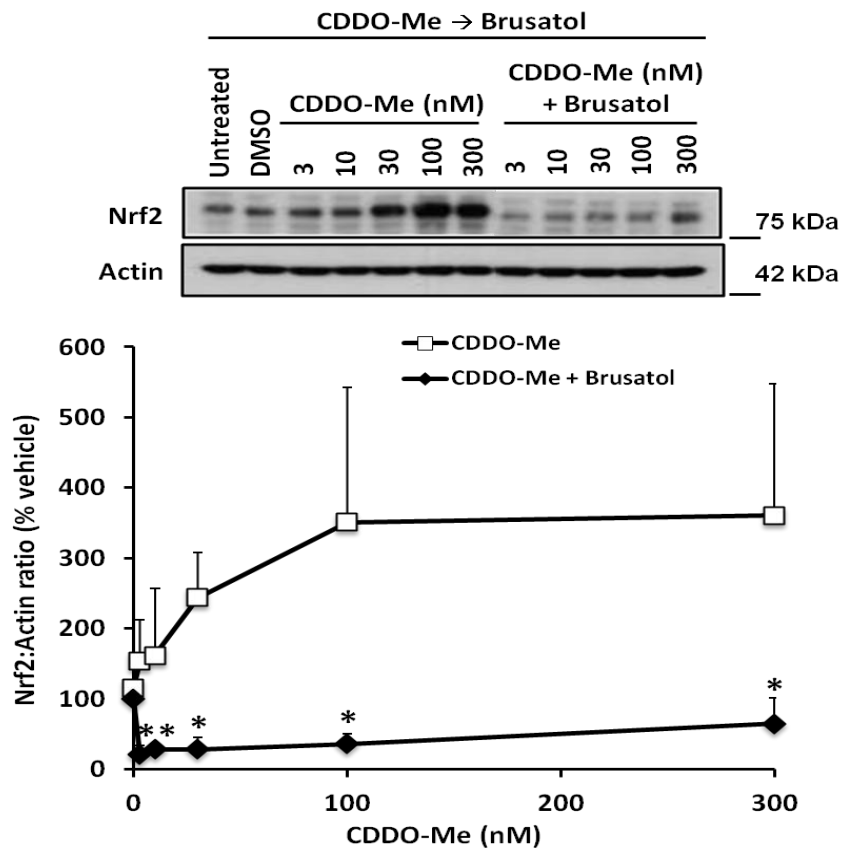
(B)



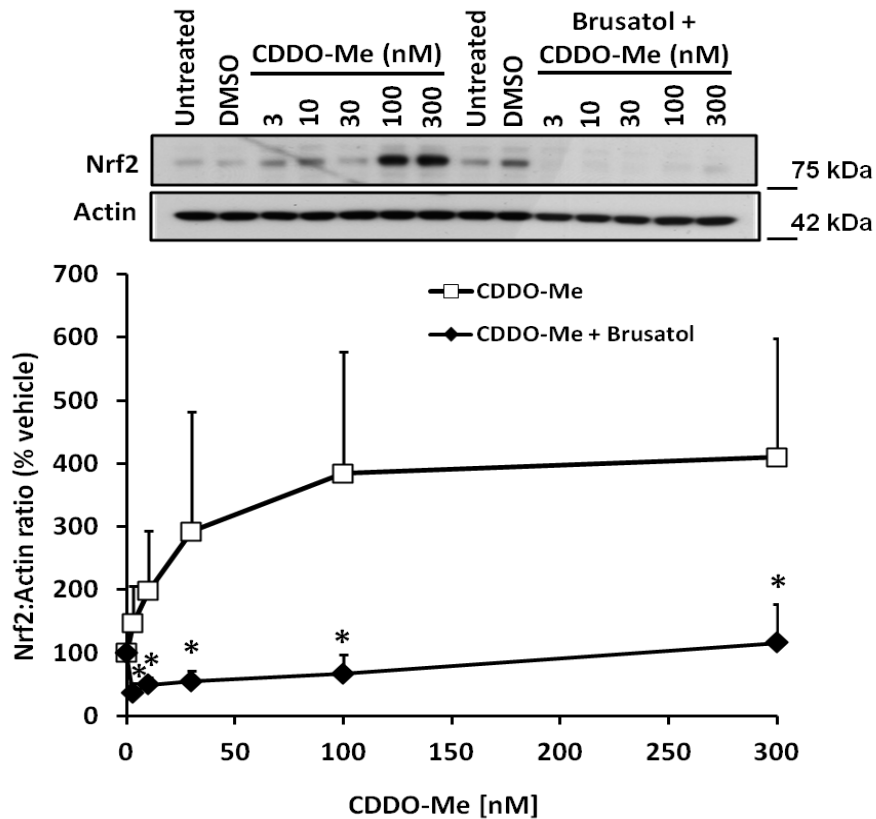
(C)



(D)



(E)

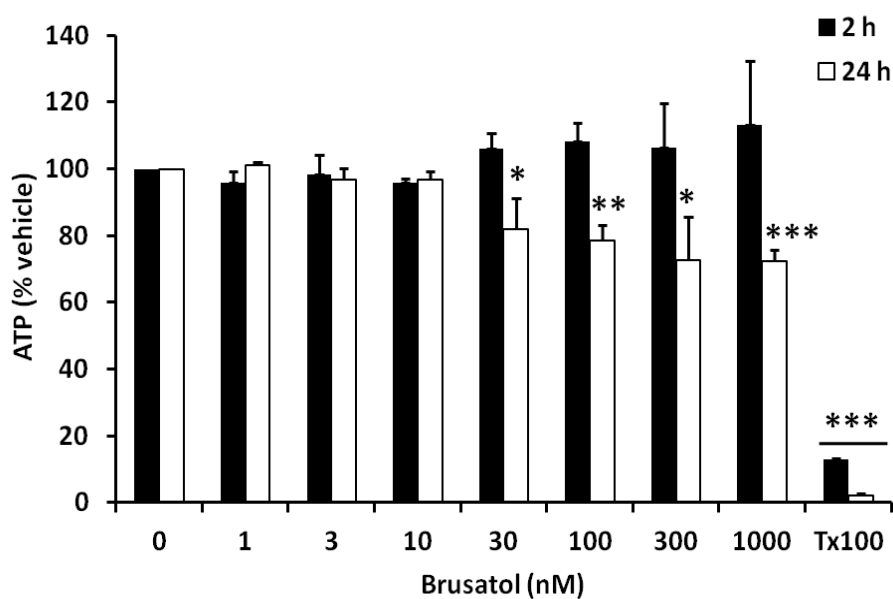


**Figure 5.3 Brusatol inhibits the pharmacological induction of Nrf2.** Nrf2 protein levels in whole cell lysates prepared from Hepa1c1c7 cells pretreated with 300 nM Brusatol for 2 hours respectively and then treated with the indicated concentrations of CDDO-Me (A) N-ethylmaleimide (B) or Iodoacetamide for 1 hour (C). Nrf2 protein levels in total cell lysates from Hepa1c1c7 cells pre-treated with 100 nM CDDO-Me for 1 hour followed by 300nM brusatol for 2 hours (D). Nrf2 protein levels in Hepa1c1c7 cells pre-treated with 100 nM CDDO-Me for 1hour, washed with DMEM and then treated with 300 nM brusatol for 2 hours (E). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization against actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$  vs. CDDO-Me, IAA or NEM alone).

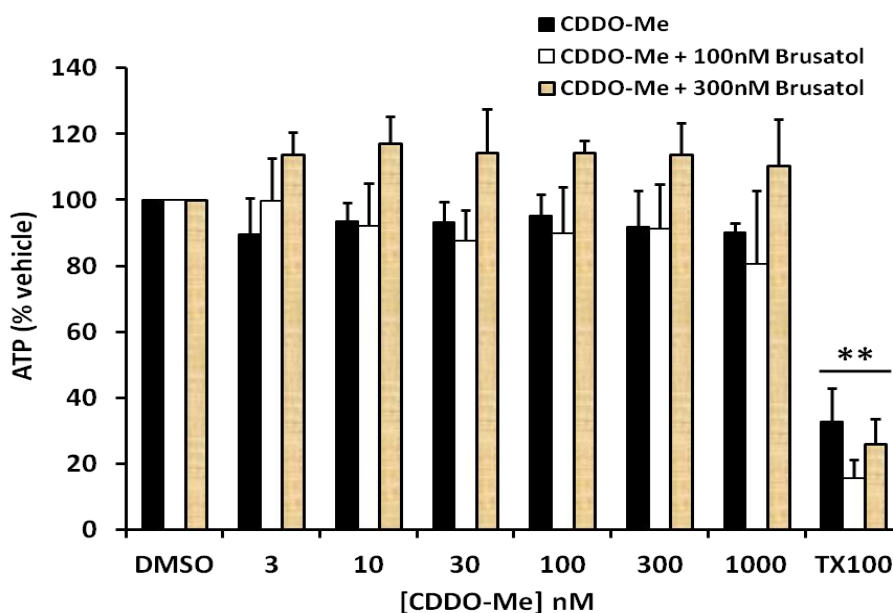
#### 5.2.4 Brusatol-mediated inhibition of Nrf2 is not associated with a reduction in cell viability

Using a cell viability assay, it was determined that Nrf2 levels were not being reduced due to brusatol provoking cell death. This experiment confirmed that 2 hours of treatment with brusatol from 0-1  $\mu$ M was not toxic to the cells when compared to 2% Triton X 100 that showed a significant reduction in cell viability. However at higher concentrations of brusatol, toxicity was observed after 24 hours (figure 5.4a). It was also confirmed that treatment with both CDDO-Me and brusatol simultaneously was not causing any significant cell death (figure 5.4b).

(A)



(B)

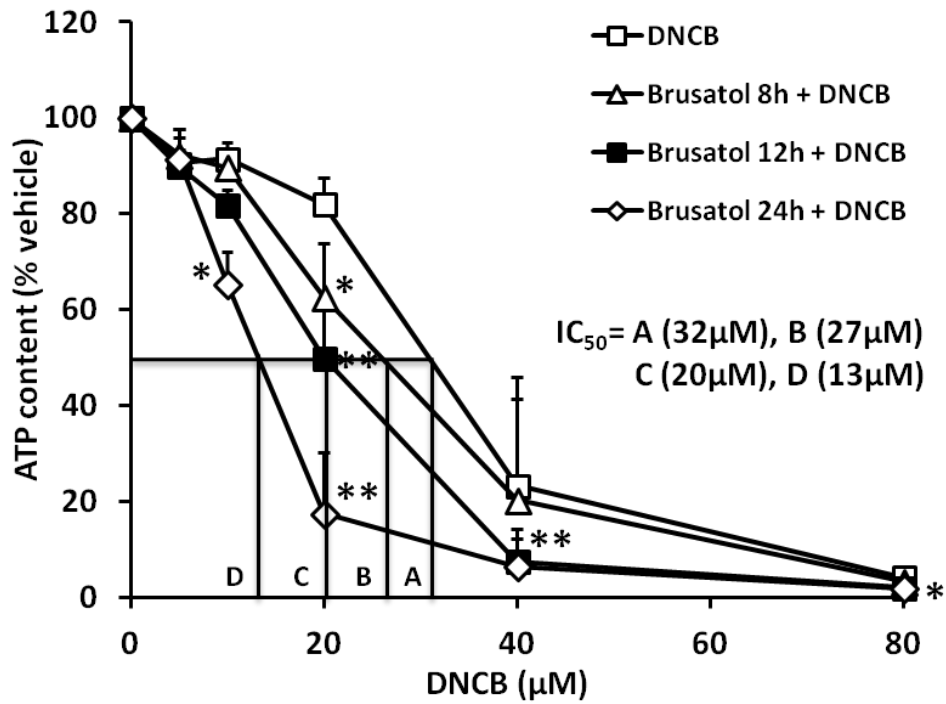


**Figure 5.4 - Hepa1c1c7 cell viability following exposure to brusatol** – ATP levels were measured in Hepa1c1c7 cells treated with the indicated concentrations of brusatol for 2 or 24 hours (A) or pre-treated with 100 nM or 300 nM Brusatol for 3 hours and 2 hours respectively, followed by exposure to the indicated concentrations of CDDO-Me for 1 hour (B). 2 % (w/v) triton X 100 (TX100) was used as a positive control. ATP levels are expressed as a percentage of those measured in vehicle exposed cells + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$  vs. Vehicle control).

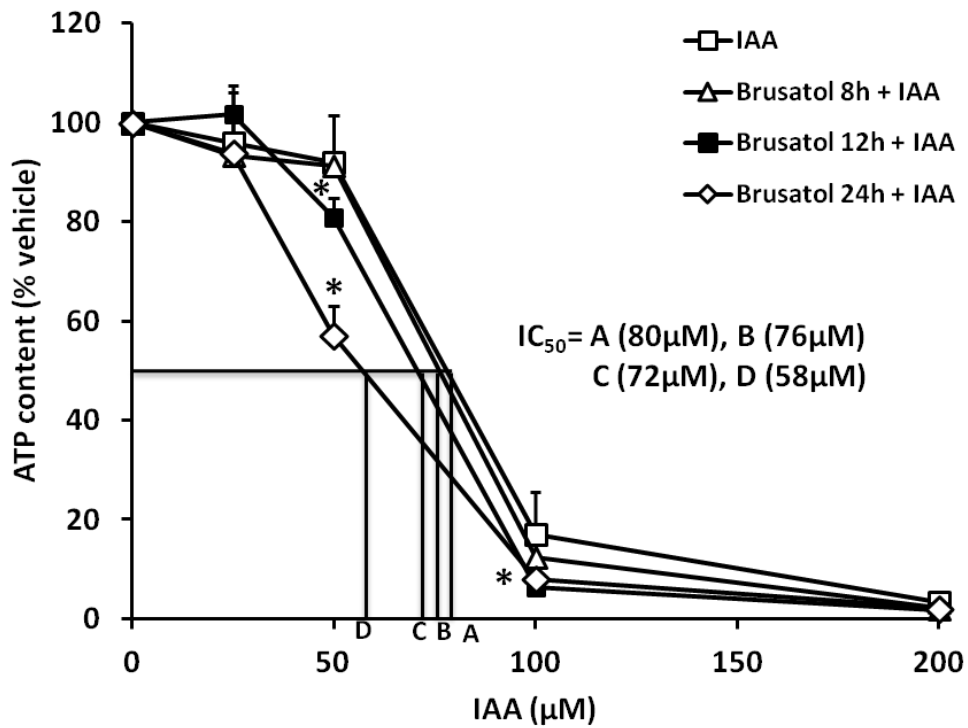
### 5.2.5 Brusatol sensitizes cells to chemical stress

Due to an increasing body of evidence highlighting the role of elevated levels of Nrf2 in chemo-resistance, and the ability of brusatol to reduce constitutive and induced Nrf2 levels, it was of interest to determine if brusatol could sensitize cells to the toxicity provoked by chemical stressors. To investigate this, Hepa1c1c7 cells were pretreated with 300 nM brusatol for up to 24 hours and then treated with DNCB or IAA for 6 hours or NAPQI for 12 hours. The toxicity of the compounds was determined using the ATP assay (figures 5.5a-d). Brusatol significantly sensitized Hepa1c1c7 cells to the stress generated by DNCB, IAA, NEM and NAPQI in both a concentration and time-dependent manner (figures 5.5a-d).

(A)

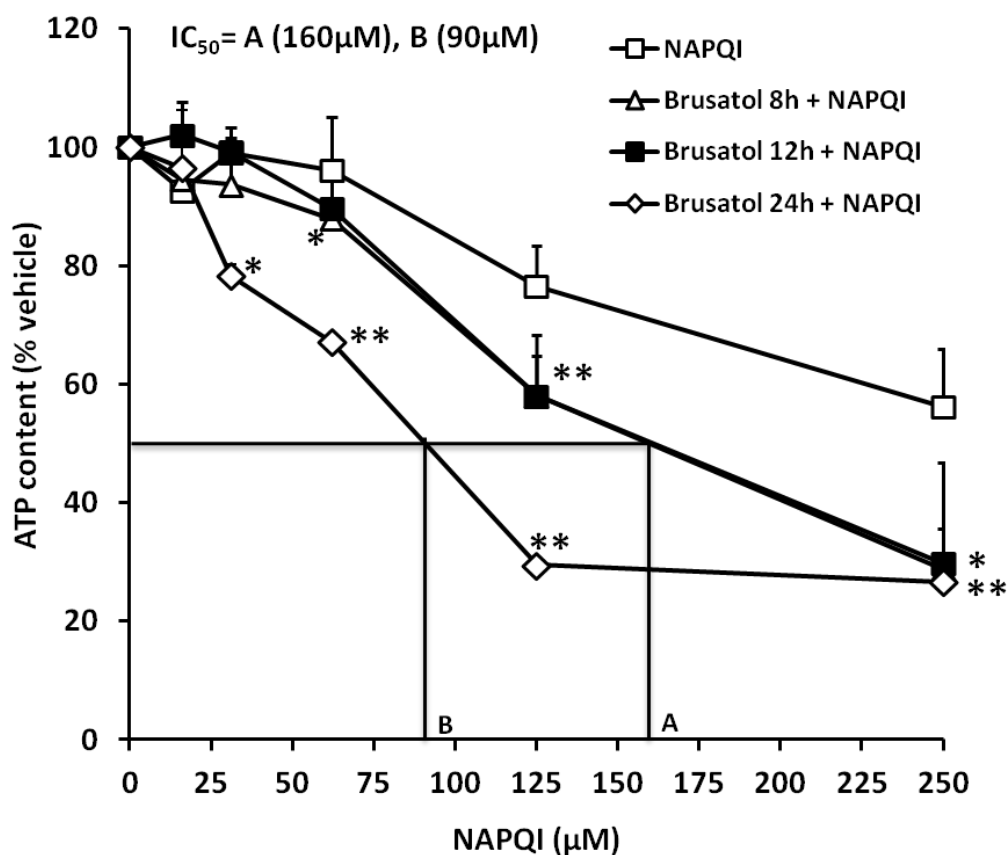


(B)





(C)

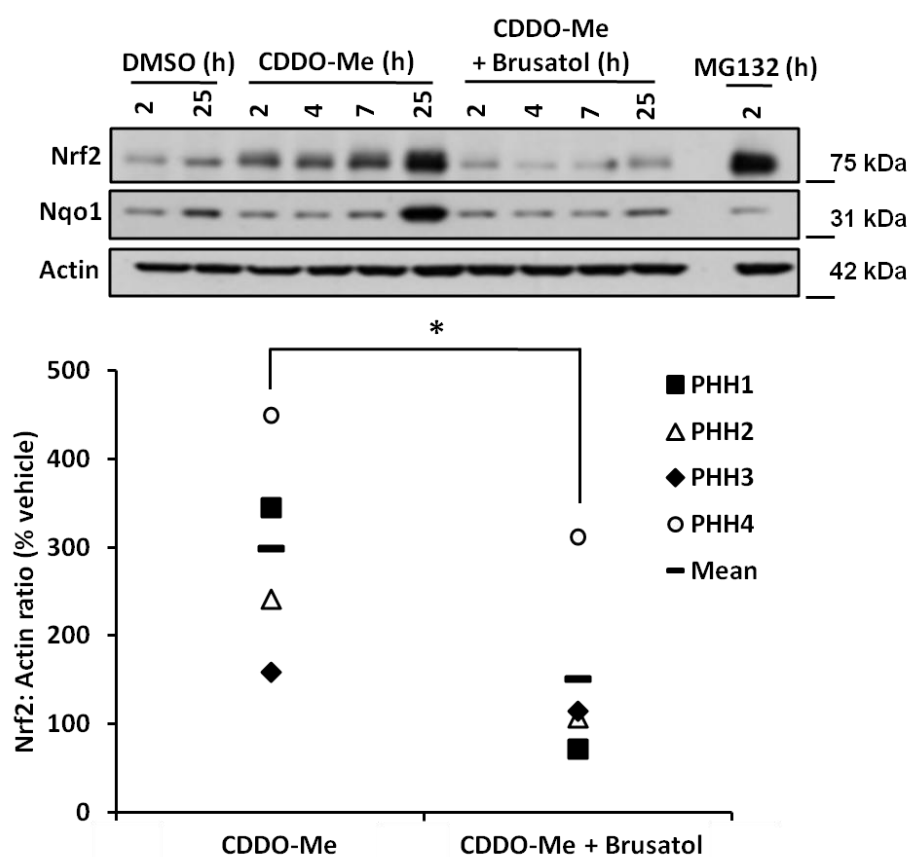


**Figure 5.5 – Brusatol sensitizes cells to chemical stress-** ATP levels were measured in Hepa1c1c7 cells treated with 300 nM brusatol 8,12,20 and 24 hours before being treated with the indicated concentrations of (A) DNCB for 6 hours (B) Iodoacetamide for 6 hours (C) NAPQI for 12 hours. ATP levels are expressed as a percentage of those measured in vehicle exposed cells + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$  vs. Vehicle control).

### 5.2.6 Brusatol depletes Nrf2 in primary human hepatocytes

In light of the ability of brusatol to deplete Nrf2 *in vitro* and *in vivo* (Ren *et al.*, 2011), it was of interest to determine its ability to inhibit the Nrf2 pathway in primary human cells. To do this, freshly isolated human hepatocytes were pre-treated with CDDO-Me, a potent Nrf2 inducer for 1 hour prior to treatment with brusatol. Brusatol depleted CDDO-Me induced Nrf2 protein in the cell lysates, as determined by immunoblotting (figure 5.6). Brusatol also prevented the induction

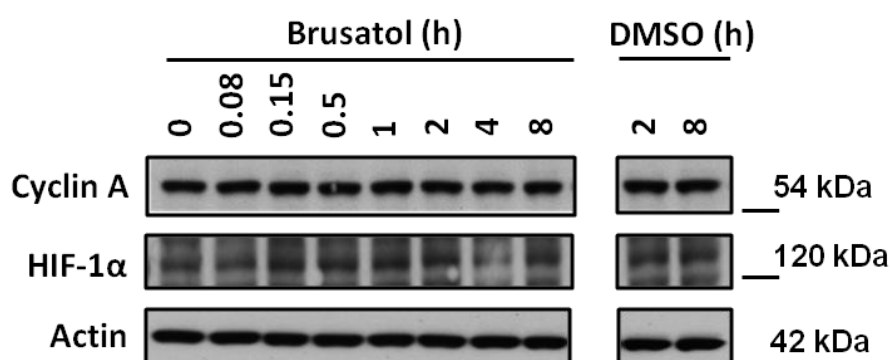
of Nqo1 by CDDO-Me in primary human hepatocytes indicating that the inhibition of Nrf2 was functionally relevant (figure 5.6).



**Figure 5.6 – Brusatol depletes Nrf2 in primary human hepatocytes.** Nrf2 levels in total cell lysates prepared from isolated primary human hepatocytes pre-treated with 100 nM CDDO-Me for 1 hour before being treated with 100 nM brusatol for the indicated times. Primary human hepatocytes treated with Mg132 for 2 hours were used as a positive control. The blots shown are a representative of independent experiments utilising cells from 4 different donors and quantified by densitometry. The average Nrf2 values of the four independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$  vs. CDDO-Me alone).

### 5.2.7 Brusatol has a high specificity for the Nrf2 pathway and not a universal protein synthesis inhibitor

In light of the above data demonstrating the ability of brusatol to inhibit Nrf2 signalling in different mammalian test systems, it was of interest to test whether brusatol was a universal protein synthesis inhibitor or it has a high specificity for the Nrf2 pathway. To do this, Hepa1c1c7 cells were treated with brusatol for up to 8 h and the levels of short-lived proteins cyclin A and HIF-1 $\alpha$ , in the cell lysates were determined by immunoblotting with an anti-cyclin A and anti-HIF-1 $\alpha$  antibodies. It was observed that brusatol does not reduce the basal level of cyclin A and HIF-1 $\alpha$  proteins in Hepa1c1c7 cells in a time-dependent manner (figure 5.7). Thus, brusatol has a high specificity for the Nrf2 pathway and that it is not a universal protein synthesis inhibitor as it had no effect on these proteins tested here at nanomolar concentrations of brusatol.



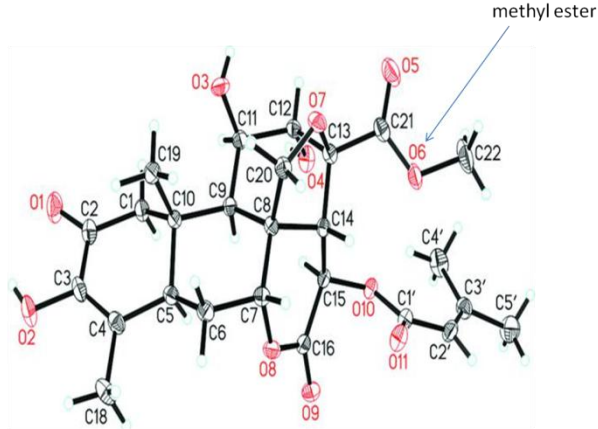
**Figure 5.7 – Brusatol is not a universal protein synthesis inhibitor.** Cyclin A and HIF-1 $\alpha$  protein levels in total cell lysates prepared from Hepa1c1c7 cells treated with 300 nM for the indicated times as determined by western blotting. The blots shown are a representative of three independent experiments and quantified by densitometry.

## 5.2.8 Uptake and cellular stability of brusatol

### *5.2.8i Development/Optimisation of LC-MS and LC-MS/MS methods for analysing brusatol*

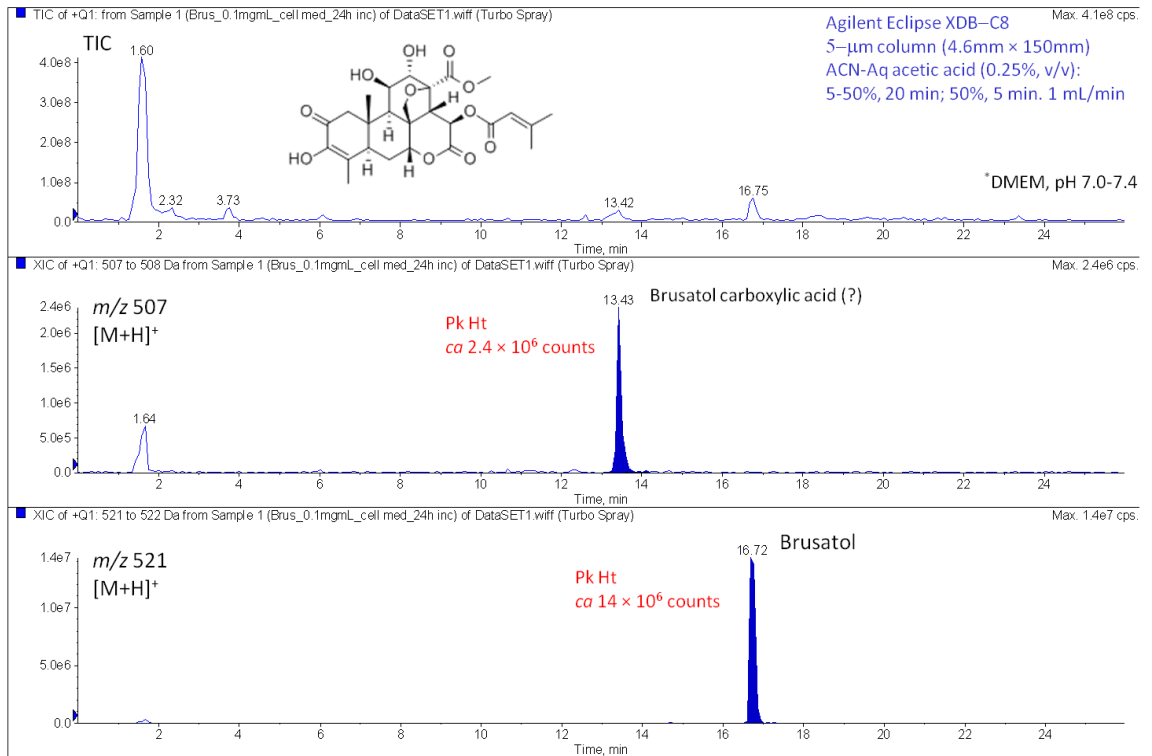
The findings of this chapter showed that the time-dependent reduction of Nrf2 protein levels occurred in a reversible manner. It was therefore of interest to investigate the uptake and cellular stability of brusatol. This could give a further insight into understanding the transient nature of brusatol-mediated depletion of Nrf2, which was possibly due to instability of the parent drug as a result of metabolism to one or more inactive metabolites. To investigate this, a mass spectrometric analysis of the uptake and stability of brusatol was developed (figure 5.8b). 100 µg/mL brusatol was incubated in cell culture medium containing phenol red +/- FCS over 24 hours under standard incubation conditions of 37 °C and O<sub>2</sub>/CO<sub>2</sub>. Aliquots of the incubation mixture were deproteinised with ice-cold ACN (1:1) and analysed by LC-MS (Q1). 100 µg/mL of brusatol (R<sub>t</sub> 17 min) yielded an abundant protonated molecule ([M+H]<sup>+</sup>) at *m/z* 521 (figure 5.8b-c). There was evidence of partial (*ca* 15%) hydrolysis of brusatol (*m/z* 521) to its putative carboxylic acid (*m/z* 507) in cell culture medium (± FCS) over 24 hours under standard incubation conditions of 37°C under O<sub>2</sub>/CO<sub>2</sub> (figure 5.8b-c). However, no evidence of protonated molecule ([M+H]<sup>+</sup>) at *m/z* 521 or partial hydrolysis *m/z* 507 were seen in aliquots of cell culture medium alone or with the vehicle (DMSO) incubated in cell culture medium at 0 and 24 hours confirming that the observed peaks seen with brusatol is due to the presence of the parent compound only (figure 5.8d-e). In addition, bruceine D (R<sub>t</sub> 8 min) yielded corresponding ions at *m/z* 411 and *m/z* 433, respectively (figure 5.8f).

### A. The Molecular Structure of Brusatol

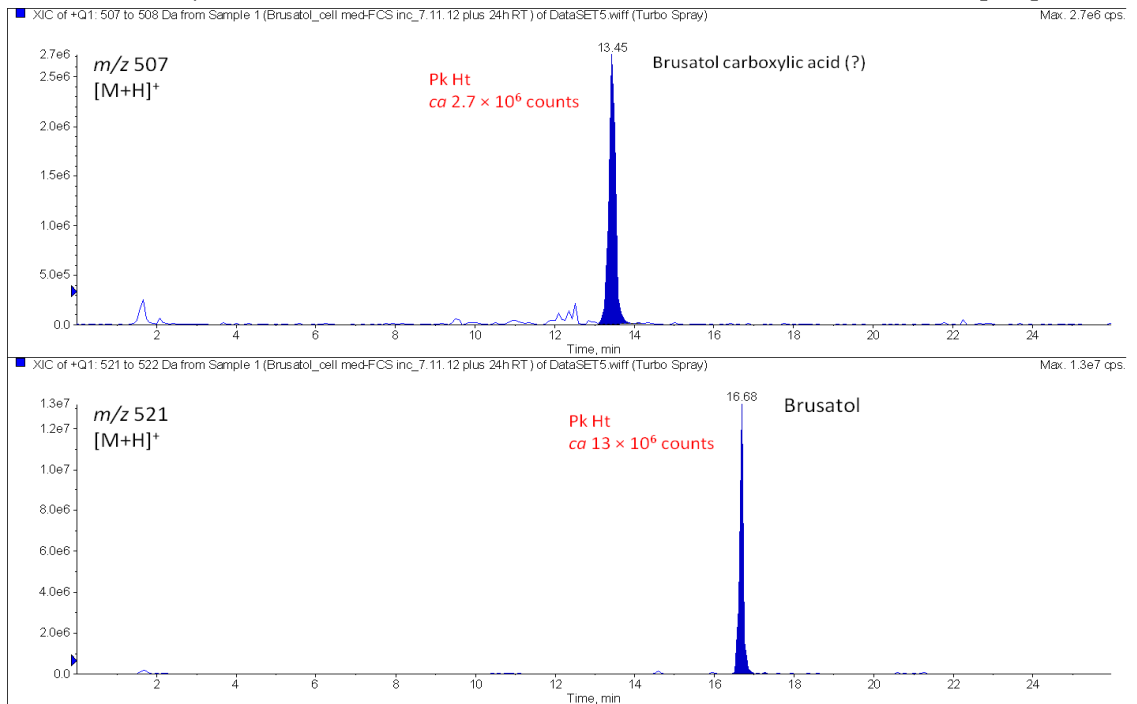


$C_{26}H_{32}O_{11}$   
MW=520

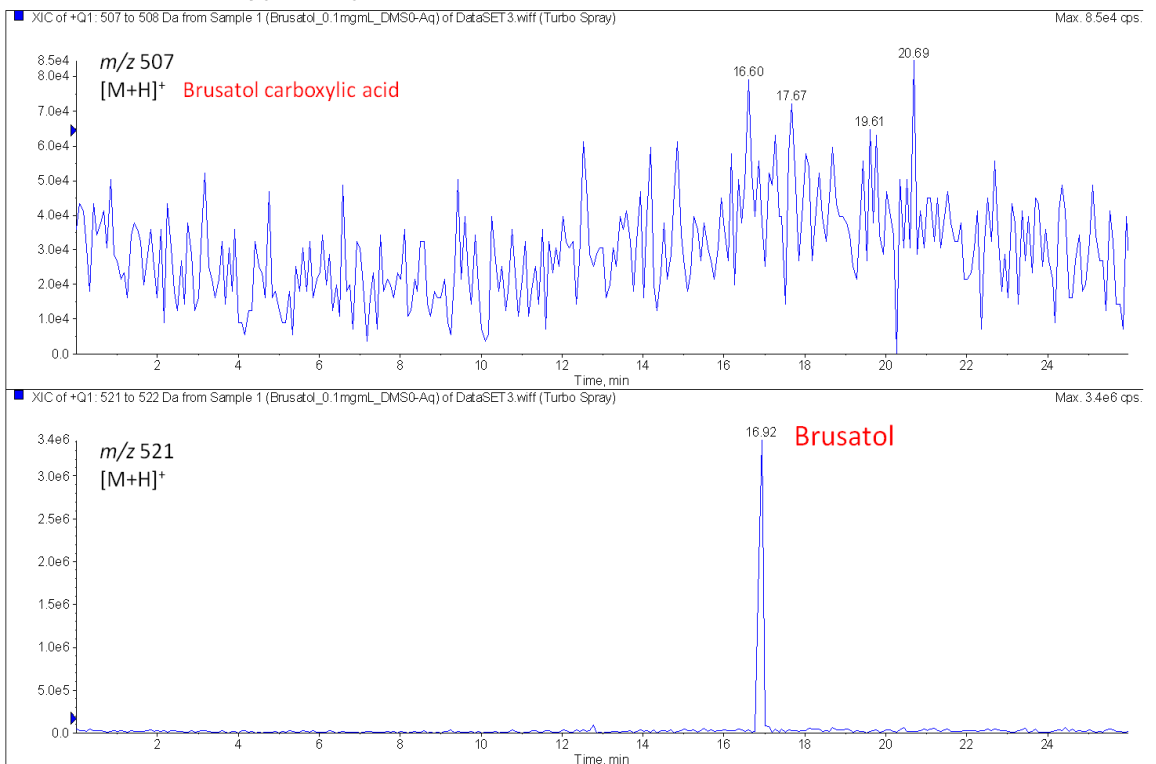
### B. Brusatol (100 µg/mL): incubated in cell culture medium\* for 24 h at 37°C under O<sub>2</sub>/CO<sub>2</sub>

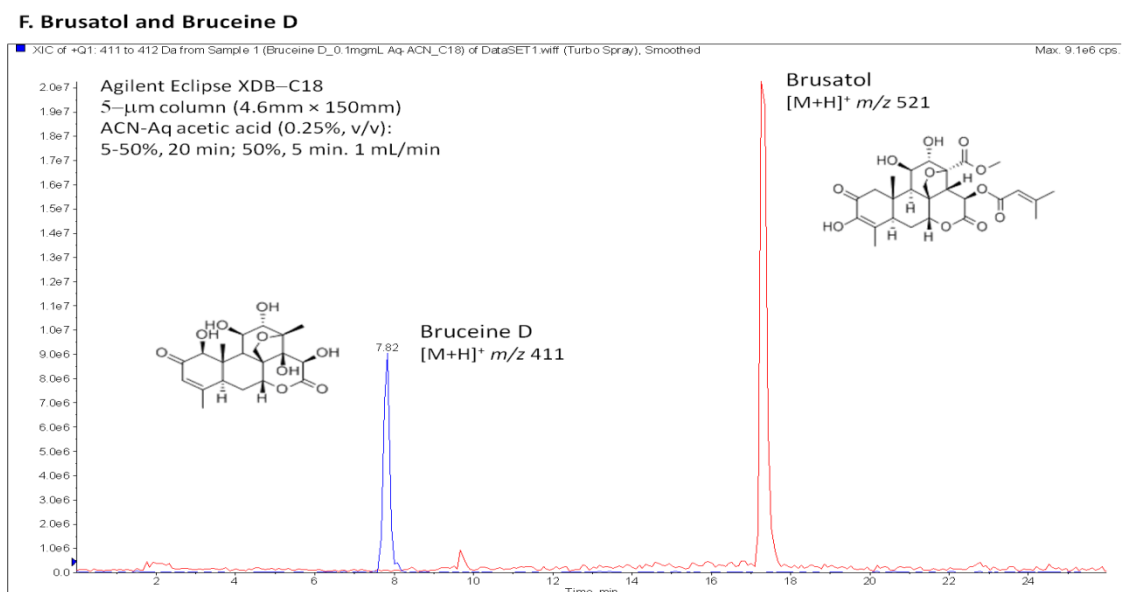
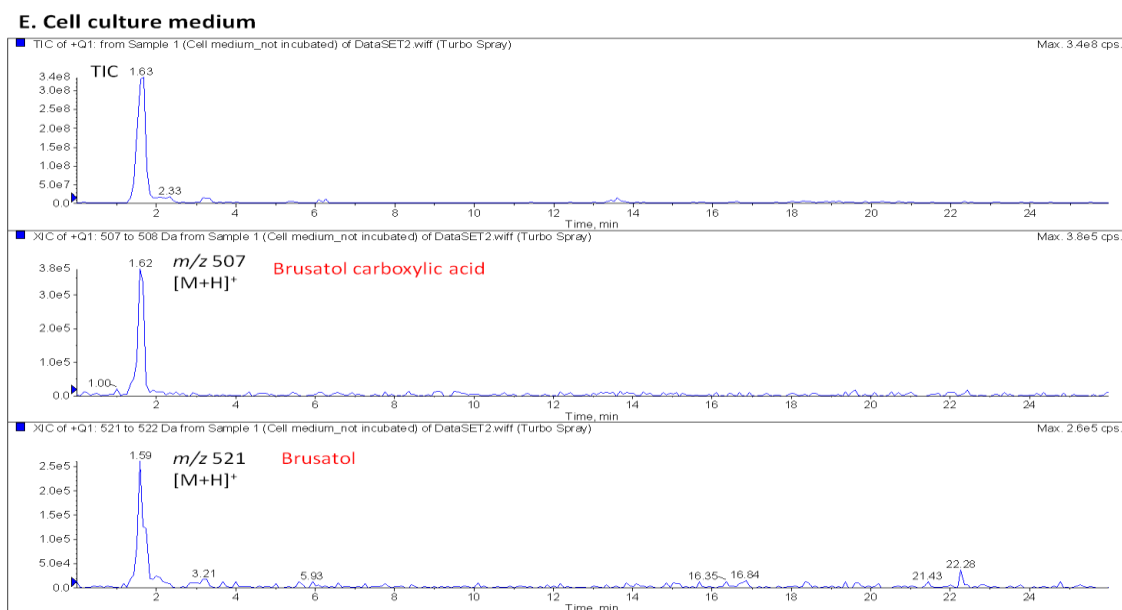


**C. Brusatol (100 µg/mL): incubated in cell culture medium + FCS for 24 h at 37°C under O<sub>2</sub>/CO<sub>2</sub>**



**D. Brusatol: DMSO-Aq (1:9, v/v)**





**Figure 5.8 - Development of LC-MS/MS methods for analysing brusatol.** The molecular structure of brusatol (A). LC-MS/MS chromatograms showing the presence/absence of brusatol ( $m/z$  521; retention time, 17 min), brusatol carboxylic acid ( $m/z$  507; retention time, 14 min) or brucein D signal ( $m/z$  433; retention time, 8 min) by co-eluting materials extracted from brusatol incubated in cell culture medium alone (B) brusatol incubated in cell culture medium + FCS (C) DMSO incubated in cell culture medium + FCS (D) cell culture medium alone (E) brusatol and brucein D incubated in cell culture medium + FCS (E) at a concentration of 100 µg/mL brusatol, 100 µg/mL or DMSO vehicle alone.

### **5.2.8ii Analysis of brusatol stability in incubations of Hepa1c1c7 cells**

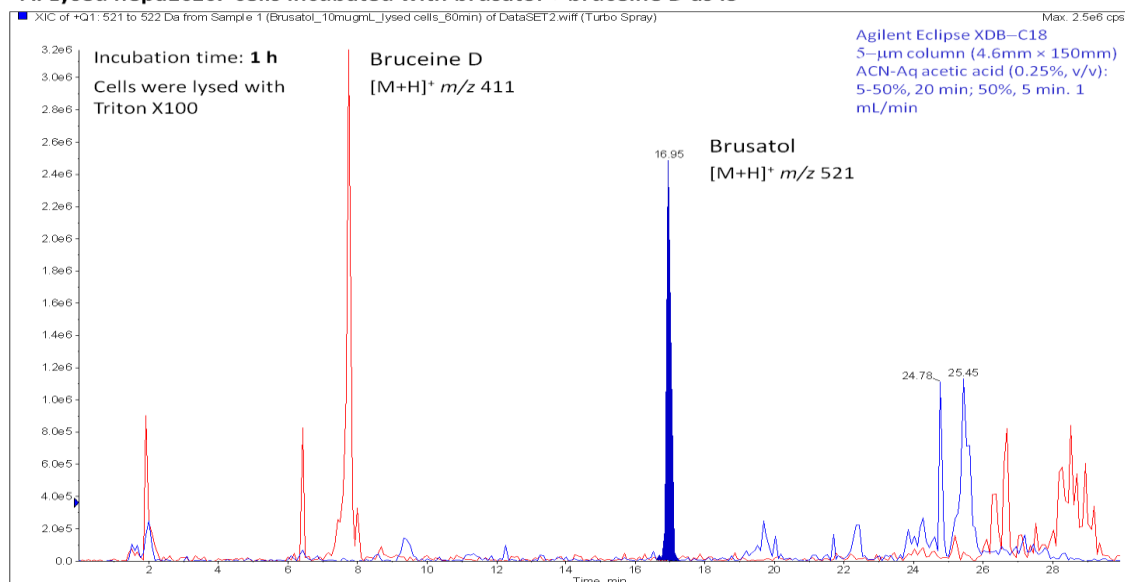
To check if the same results were observed under cellular conditions, brusatol (10 µg/mL) was incubated with triton X-100 lysed Hepa1c1c7 cells for a time period up to 24 hour under standard incubations. 50 µg/mL brucein D was added to the supernatant as internal standard (IS), and aliquots were deproteinised with ice-cold ACN (1:1) before being analysed by LC-MS (Q1). Brusatol ( $R_t$  17 min) yielded an abundant protonated molecule ( $[M+H]^+$ ) at  $m/z$  521 while bruceine D ( $R_t$  8 min) yielded a corresponding ion at  $m/z$  411 (figure 5.9a) with lysed Hepa1c1c7 cells at 1 hour of incubation time (figure 5.9a). At 24 hour time point, similar peaks were yielded, but no obvious hydrolysis of 10 µg/mL brusatol ( $m/z$  521) to its putative carboxylic acid ( $m/z$  507) was seen with lysed Hepa1c1c7 cells as had been observed with the 100 µg/mL brusatol incubated in cell medium alone (figure 5.9b). This may have been due to the concentration of the 10 µg/mL brusatol used instead of the 100 µg/mL used earlier or was possibly due to the method of lysing the cells. To investigate the result seen with the triton X 100 lysed Hepa1c1c7 cells, the same experiment was repeated with Hepa1c1c7 cells lysed by sonication and incubated with 10 µg/mL brusatol, using brucein D as the internal standard. Similar results were observed (figure 5.9c), with no evidence of the carboxyl brusatol metabolite.

To investigate whether the transient nature of Nrf2 depletion caused by brusatol was due to the instability of the parent compound, a time-course analysis of 10 µg/mL brusatol incubated with Hepa1c1c7 cells lysed by sonication in the cell culture medium under standard conditions was performed. 50 µg/mL bruceine D was added to the supernatant as internal standard (IS), and aliquots of the incubation were deproteinised with ice-cold ACN (1:1) and analysed by LC-MS (Q1). Analyte  $[M+H]^+$  peak areas were integrated and the ratio of brusatol:IS was analysed at each time point (figure 5.9d). The results showed that brusatol was stable over time until 24 hour when there was a decline in the relative peak area (figure 5.9d). However, it should be noted that the brucein D peak was unstable over the 24 hour time period indicating that further work is needed to confirm the findings seen with brusatol. Overall, these results suggest that the rapid loss of Nrf2 inhibition caused by brusatol in Hepa1c1c7 cells (0.5-2 h) is probably not due to

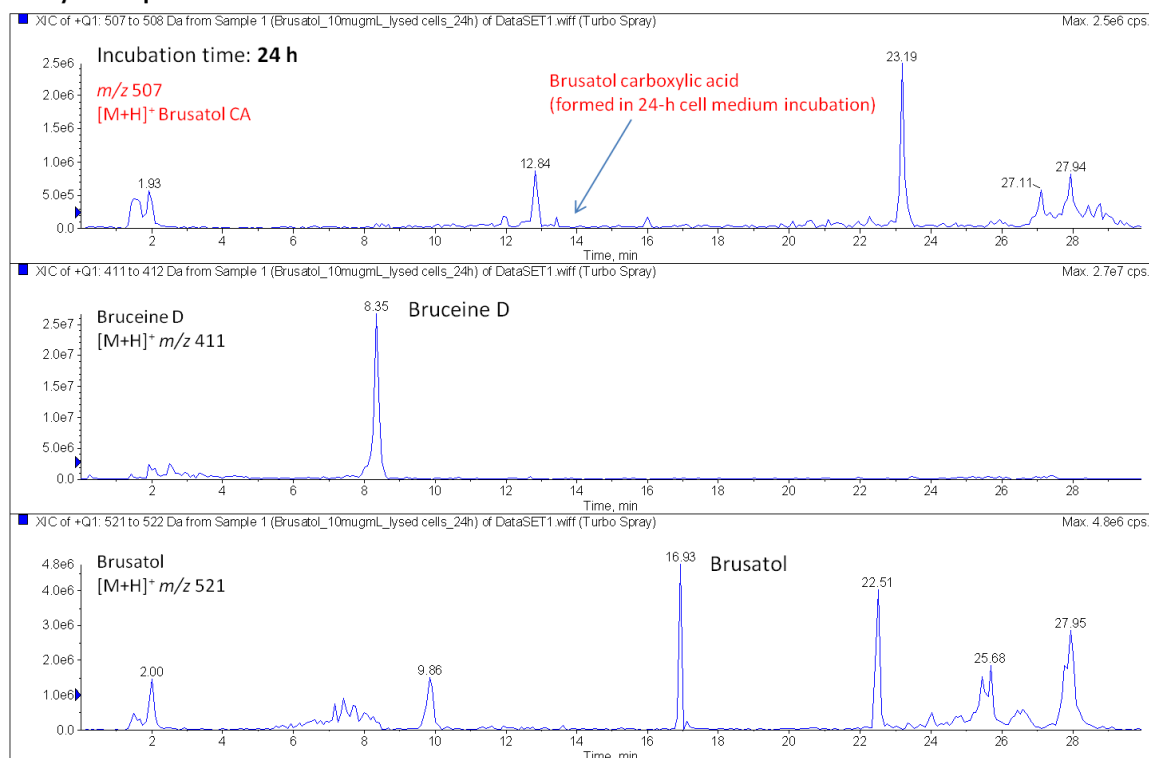


metabolism of the parent compound to an inactive derivative. Thus, the short-lived inhibition of Nrf2 by brusatol is still unexplained and requires further investigation.

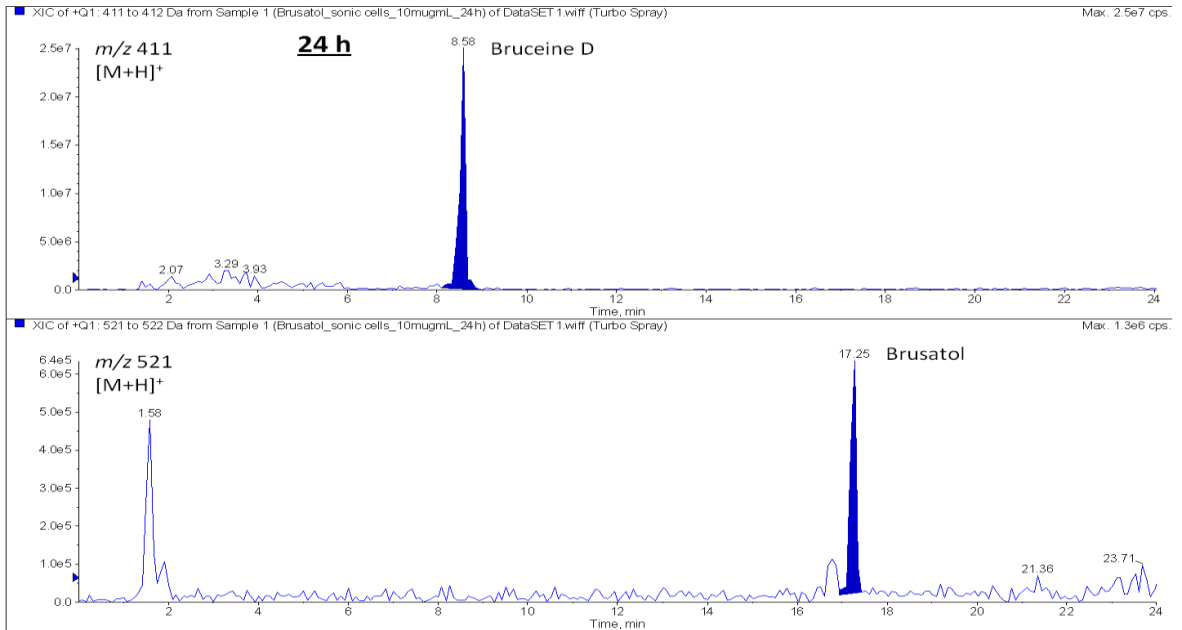
### A. Lysed hepa1c1c7 cells incubated with brusatol + bruceine D as IS



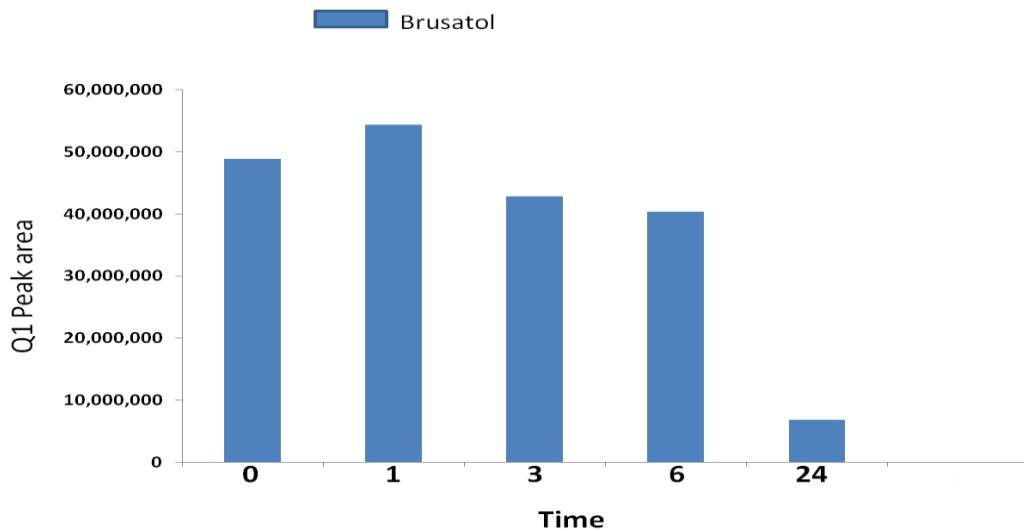
### B. Lysed hepa1c1c7 cells incubated with brusatol + (Bruceine D as IS



**C. Sonicated hepa1c1c7 cells incubated with brusatol (10 µg/mL) + bruceine D as IS**



**D. Q1 peak areas of brusatol (10 µg/mL; 19.2 µM) incubated with sonicated hepa1c1c7 cells & bruceine D added as IS**



**Figure 5.9 - Analysis of brusatol stability in incubations of Hepa1c1c7 cells.** LC–MS/MS chromatograms showing the presence/absence of brusatol ( $m/z$  521; retention time, 17 min), brusatol carboxylic acid ( $m/z$  507; retention time, 14 min) or brucein D signal ( $m/z$  433; retention time, 8 min) by co-eluting materials extracted from lysed Hepa1c1c7 cells treated with 10µg/mL brusatol for 1 h (A) or 24 h (B), sonicated Hepa1c1c7 cells treated with 10 µg/mL brusatol for 24 h (C). Q1 peak areas of 10 µg/mL brusatol incubated with sonicated Hepa1c1c7 (D). 50 µg/mL brucein D was used as IS.

### 5.3 Discussion

The naturally occurring quassinoid brusatol, one of few known inhibitors of Nrf2, has proven anti-tumour, anti-viral and anti-inflammatory activities (Hall *et al.*, 1983; Yan *et al.*, 2010; Zhao *et al.*, 2011a). Consistent with the findings of Ren *et al.*, brusatol has a high specificity for the Nrf2 pathway and it is not a universal protein synthesis inhibitor as it had no effect on a panel of proteins tested here and those in the study of Ren *et al.*, at nanomolar concentrations of brusatol. However, at micromolar concentrations, it was seen to be a general protein synthesis inhibitor (Willingham *et al.*, 1981; Willingham *et al.*, 1984). The importance of this compound as an inhibitor of Nrf2 has previously been highlighted by its use to augment the pharmacological activity of known chemotherapeutic agents in models of drug-resistant cancer (Ren *et al.*, 2011).

The aim of this chapter was to provide *in vitro* insights into the ability of brusatol to mediate Nrf2 inhibition in mouse Hepa-1c1c7 hepatoma cells. Brusatol evoked both concentration and time -dependent, yet transient, depletion of basal and inducible protein levels of Nrf2 (Figure 5.1 and 5.3). In addition, brusatol also reduced the mRNA levels of the Nrf2 targets Nqo1, and Gclm further indicating its inhibitory effect on the Nrf2 pathway.

Studies have highlighted the role of Nrf2 in conferring protection against the deleterious effects of oxidative stress and other insults generated by electrophiles (Copple *et al.*, 2008b). This is of particular interest in cancer settings as recent investigation into the roles that Nrf2 may play in cancer have shown that a number of cancer cells- both primary and immortalised cell lines- contain constitutively high Nrf2 levels. This up-regulation has been shown to enhance cell proliferation and confer a degree of chemo-resistance (Lister *et al.*, 2011).

Hence, various researchers have focused on developing a therapeutic strategy by the use of the combination approaches in which the knock-down of Nrf2 sensitizes cells to the action of chemotherapeutic drugs (Cho *et al.*, 2008; Kim *et al.*, 2008). Silencing of Nrf2 using siRNAs has also been shown to sensitize cells to oxidative stress and other chemical insults (Chia *et al.*, 2010). Therefore the use of brusatol

alongside chemotherapy could reduce Nrf2 levels in the cell allowing the active compound to take advantage of the compromised cell defence situation of the cancer cell. In line with this, brusatol was able to sensitize cells to oxidative stress induced by various electrophiles (DNCB, IAA and NAPQI) in a time-dependent manner, thus highlighting the role of Nrf2 in cytoprotection.

Depletion of Nrf2 by brusatol was associated with a decrease in expression of the classic Nrf2 targets Nqo1 and Gclm. A small number of studies have seen a correlation between an increase in drug transporters like Mrps and other Nrf2-regulated genes like Gclc and chemo-resistance as observed in cancer settings (Wang *et al.*, 2010; Mahaffey *et al.*, 2012; Siegel *et al.*, 2012). It is plausible that brusatol may be reducing the drug transporters and other associated proteins, thereby sensitizing cancer cells to chemotherapy but this requires further investigation.

Thus, it is of interest to develop small molecule inhibitors of Nrf2 as the genetic prolonged down-regulation of Nrf2 may initiate compensatory mechanisms due to the cells reacting to the reduction in Nrf2 levels and attempting to restore cell homeostasis, making the interpretation of data more complex and more difficult to understand.

It was observed that the inhibitory effect of brusatol on the Nrf2 pathway is reversible, and Nrf2 quickly recovers to basal levels after treatment with brusatol, which is consistent with findings of Ren *et al.* (2011). A recovery of Nrf2 protein levels was observed using Hepa1c1c7 cells after 2-4 hours of exposure to brusatol. In line with the study of Ren *et al.*, where brusatol was removed after 4 hours of incubation, the same effect of recovery was observed in this study using Hepa1c1c7 cells pretreated with brusatol without the removal of brusatol from the cell culture. There are three possible explanations for the recovery of Nrf2 levels. First, it could be by a natural regulatory process, which accelerates selectively the reading rate of Nrf2 mRNA, perhaps via a de-repression mechanism resulting in an increase of Nrf2 synthesis that counterbalances the enhanced degradation of Nrf2. Secondly, brusatol could be removed actively. It might be eliminated or bioinactivated by

metabolism processes. Lastly, if the second process is true i.e. brusatol being metabolised, the resulting metabolites might act as antagonists of Nrf2 ubiquitination.

As seen from section 5.2.8, there was evidence of partial (*ca* 15%?) hydrolysis of brusatol (*m/z* 521) to its putative carboxylic acid (*m/z* 507) in cell culture medium ( $\pm$  FCS) over 24 hours under standard incubation conditions. However, under cellular conditions using both Triton-x lysed cells and cells lysed by sonication, no evidence of hydrolysis was observed. This could be the result of the ten-fold difference from a concentration of 100  $\mu\text{g}/\text{mL}$  used in cell culture condition to 10  $\mu\text{g}/\text{mL}$  under cellular conditions. Notably, the hydrolysis observed was seen at the 24 hour time point after which the recovery of Nrf2 had occurred at earlier time points of 2-4 hours of drug incubation. In addition, a time-course analysis of 10  $\mu\text{g}/\text{mL}$  brusatol stability under cellular conditions also showed that brusatol was stable up until 24 h. However, this might not be conclusive yet as the internal standard (brucein D) showed inconsistent reading over time. Nonetheless, the rapidity of suppression of Nrf2 levels by brusatol in Hepa1c1c7 cells (0.5-2 h) suggests this effect is produced by the parent compound and the recovery in Nrf2 is not due to metabolism of the parent compound, as the recovery time is not within the time frame within which Nrf2 protein has gone to the basal levels and beyond. Future work should aim to investigate enzymology by considering the following Phase I pathways: hydrolysis of one or both of the ester linkages, hydrolysis of the lactone ring, reduction of one or both of the carbonyl functions, reduction of the enol function, dehydrogenation of one or both of the secondary alcohol functions, hydroxylations yielding primary or secondary alcohol functions and dehydrogenation of the dihydrodiol function. Furthermore, irrespective of whether brusatol undergoes metabolism in Hepa1c1c7 cells, analysis of brusatol stability in incubations of freshly isolated mouse/human hepatocytes should be considered as this will shed more insight into the safety aspects of the drug under clinical use.

Therefore, the recovery of Nrf2 seen with brusatol could be as a result of a natural regulatory process as indicated above which is discussed in the next chapter or a

novel mechanism, which will require further investigation. Future investigations should aim to better understand the chemical and molecular mechanisms that underlie the transient nature of Nrf2 inhibition provoked by brusatol. Such knowledge may allow chemical optimisation to generate analogues that have a long-lasting effect and overcoming the need to undertake repeated dosing to achieve a desired pharmacological effect.

Gaining further information regarding the pharmacology of brusatol will be of the utmost importance in terms of drug safety science, given its apparent value as a pharmacological and biochemical tool. Currently there are few small molecule compounds available to specifically and significantly reduce Nrf2 levels in the cell. Leutolin has been shown to inhibit Nrf2 in human A549 lung cancer cells (Tang *et al.*, 2011) but there has been discrepancies as to how specific it is in inhibiting Nrf2, as a study also saw an opposite effect where the Nrf2 pathway was induced at the same micromolar concentrations tested in both systems (Sun *et al.*, 2012). This is also true for another Nrf2 inhibitor 4-methoxychalcone described recently (Lim *et al.*, 2013). In addition, trigonelline has also been shown to be an inhibitor of Nrf2 in pancreatic cancer cell lines and sensitize these cells to chemotherapeutic drugs (Arlt *et al.*, 2013).

Other means to inhibit Nrf2 include the use of targeted siRNA, however this comes with its own caveats such as the toxicity of Lipofectamine used to transfect cells. Therefore the ability to deplete Nrf2 in a timely manner with a pharmacological agent will be invaluable in the study of the role of Nrf2 regarding cytoprotection *in vitro* and *in vivo*. Brusatol could also be used alongside siRNA experiments to confirm observations seen in the absence of Nrf2. In this case, it is important to note that brusatol had minimal effects within the cell and caused minimal toxicity. This will be of immense use when translated to the use of brusatol in clinical patients. Additionally, the depletion of Nrf2 in cells using brusatol could give insights into the mechanism of action of other compounds, which may work via Nrf2.

By further defining the pharmacological effects of brusatol on the Nrf2 pathway, brusatol could be developed into therapies where the inhibition of Nrf2 is needed. This may also reveal novel aspects of regulation within this important cellular pathway, and inform the design of new pharmacological inhibitors, which hold promise as therapeutic agents, particularly in the treatment of cancer.

## **CHAPTER SIX**

# **CHARACTERIZATION OF THE MECHANISM OF ACTION OF BRUSATOL**



<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
6.1 Introduction.....	136
6.2 Results.....	137
6.2.1 Brusatol inhibits Nrf2 post-transcriptionally.....	137
6.2.2 Brusatol Inhibits Nrf2 independently of Keap1 and the proteasome.....	141
6.2.3 Brusatol does not enhance the catalytic activities of the proteasome <i>in vitro</i> .....	143
6.2.4 Brusatol initiates the depletion of Nrf2 independently of the autophagy pathway.....	146
6.2.5 Brusatol clears Nrf2 independently of protease and caspases.....	148
6.2.6 Brusatol induces the phosphorylation of p38 MAPK, SAPK JNK 1/2 AND PI3K/AKT pathways, but the depletion of Nrf2 is independent of the activation of these pathways.....	149
6.2.7 Inhibition of Nrf2 by Brusatol may be partially dependent on calcium modulation.....	154
6.3 Discussion.....	157

## 6.1 INTRODUCTION

The findings of the previous chapter and those of a study by Ren *et al.*, 2011 have shown that brusatol selectively inhibits the Nrf2 pathway and sensitizes cells to the cytotoxic effects of chemotherapeutics drugs like cisplatin (Ren *et al.*, 2011) and model electrophiles in mammalian cells. Brusatol therefore holds promise as a novel therapeutic tool in disease settings where inhibition of Nrf2 is desirable e.g. to overcome chemoresistance in cancer.

The mechanism of action of brusatol has yet to be fully characterized. Ren *et al.*, 2011 proposed that brusatol inhibits Nrf2 by enhancing its ubiquitination and degradation but this requires further clarification (Ren *et al.*, 2011). A more detailed understanding of the chemical and molecular mechanisms that underlie the ability of brusatol to inhibit Nrf2 would provide an opportunity to design and develop alternative small molecule inhibitors for use in experimental and, potentially, clinical settings.

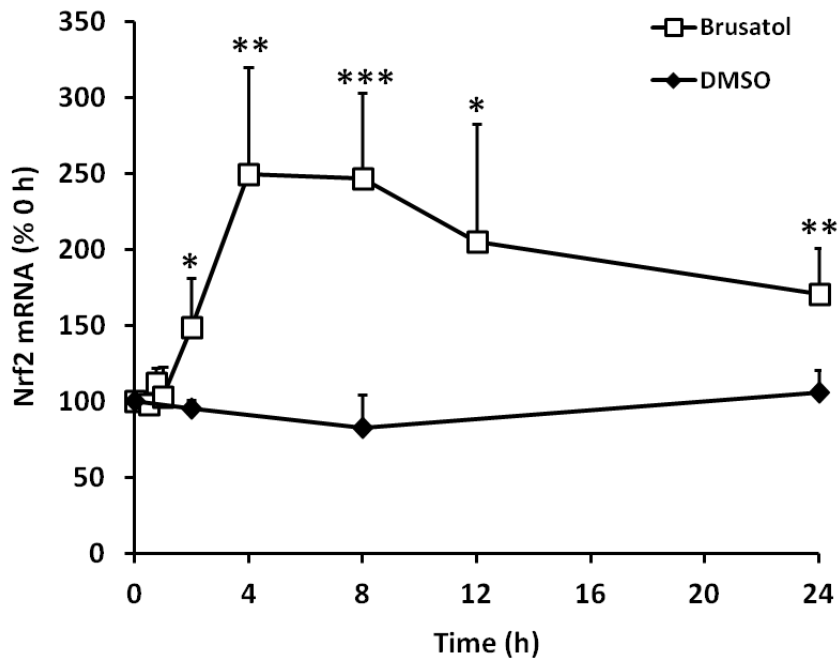
In this chapter, *in vitro* insights are provided into the mechanism of action of Nrf2 inhibition by brusatol in mouse Hepa1c1c7 hepatoma cells.

## **6.2 RESULTS**

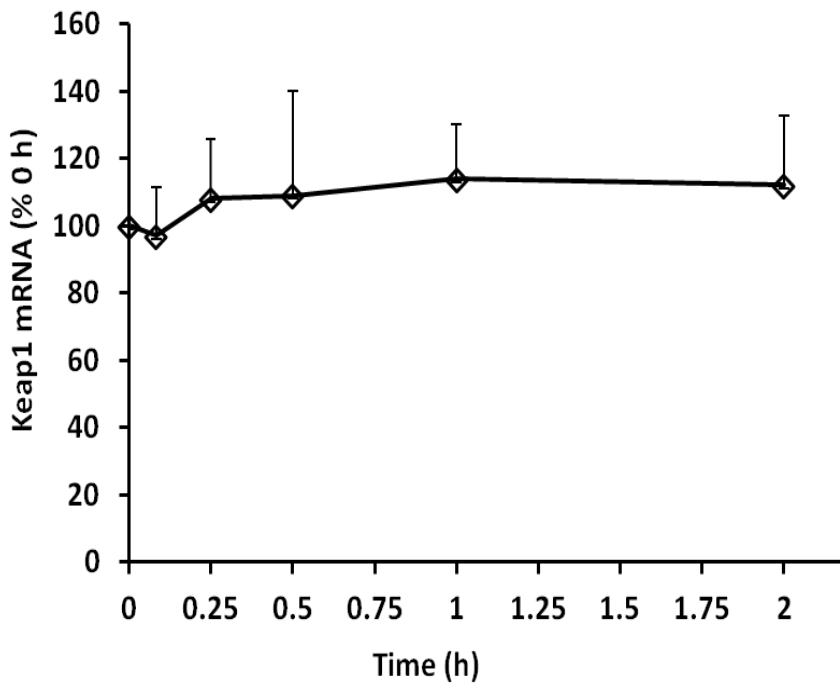
### **6.2.1 Brusatol inhibits Nrf2 post-transcriptionally**

To determine whether the decrease in Nrf2 protein expression with brusatol was due to an inhibition of the synthesis of Nrf2 protein, the levels of Nrf2 mRNA were measured. Total RNA was extracted from Hepa1c1c7 cells following exposure to brusatol for up to 24 h. The cDNA was then analysed by quantitative RT-qPCR using oligonucleotide primers specific for Nrf2 and Keap1. The level of Gapdh mRNA was used as a control to normalise the data. The mRNA levels of Nrf2 did not decrease within the early time points at which there was a depletion of Nrf2 protein following brusatol treatment (figure 6.1a & c); however, there was an increase in the Nrf2 mRNA level at later time points reaching a maximum between 4 to 8 h of exposure to brusatol (figure 6.1a). Indeed, Nrf2 protein was depleted to below the limit of detection of the western blotting assay within 30 minutes of exposure to brusatol, whilst the recovery of Nrf2 protein towards the resting level occurred after the significant increase in the level of Nrf2 mRNA as seen from the superimposition of the mRNA and protein data (figure 6.1d). This indicates that a mechanism via a reduction in the expression of Nrf2 mRNA is not responsible for the depletion of Nrf2 protein observed in Hepa1c1c7 cells following exposure to brusatol. In addition, the level of Keap1 mRNA did not change significantly following brusatol treatment (figure 6.1b); therefore an increase in Keap1-mediated Nrf2 degradation at the transcriptional level is unlikely to be responsible for the observed reduction in Nrf2 levels (figure 6.1b)

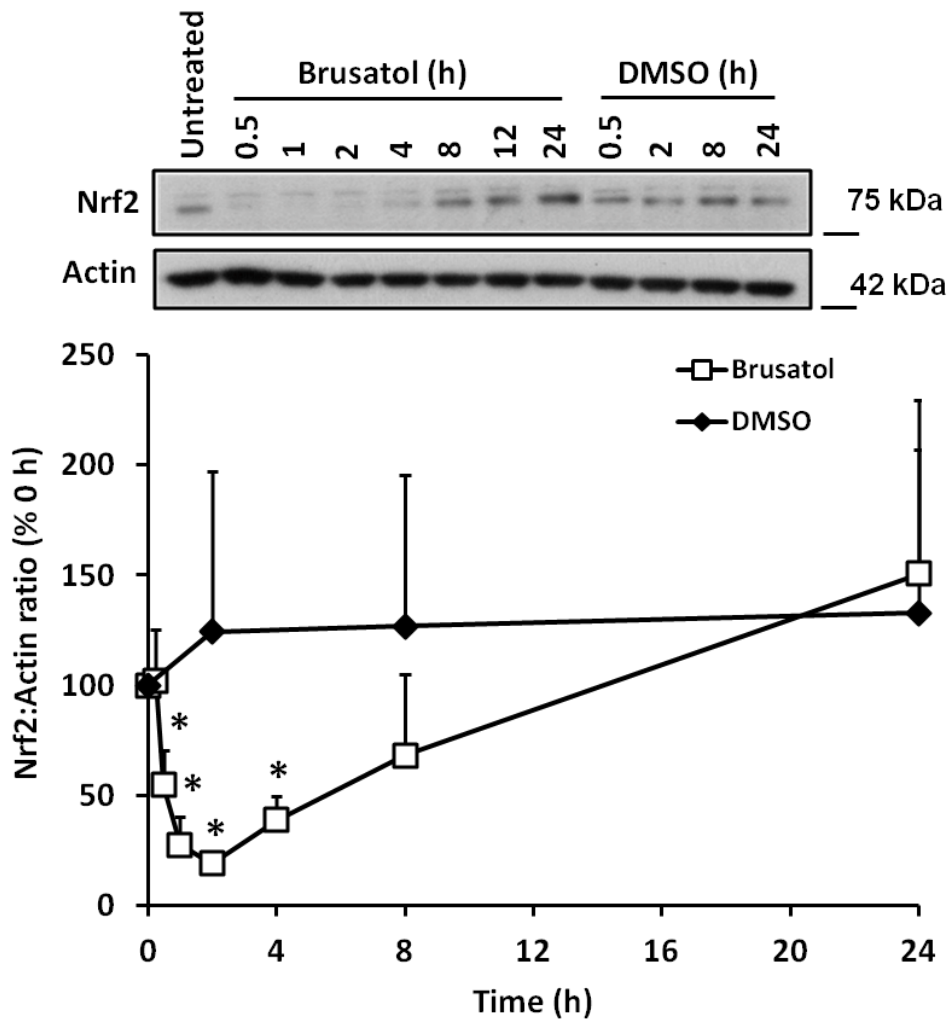
(A)



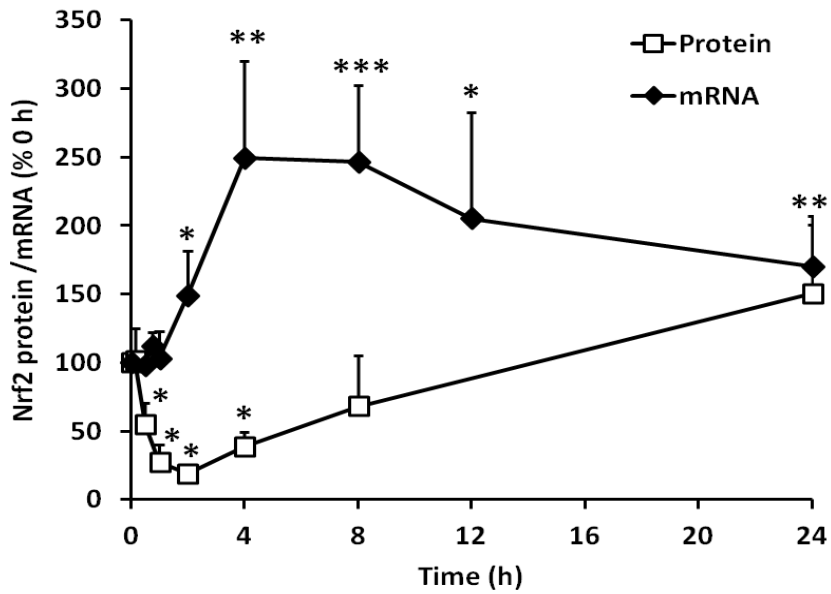
(B)



(C)



(D)

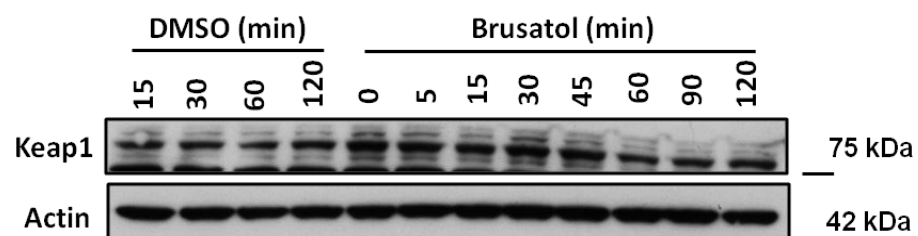


**Figure 6.1 - Brusatol reduces Nrf2 via a post-transcriptional mechanism.** Hepa1c1c7 cells were treated with 300 nM brusatol for the indicated times, total RNA was isolated and the cDNA was synthesized. The cDNA was subjected to quantitative RT-PCR analysis using primers specific for Nfr2, Gapdh and Keap1. The average Nrf2 and Keap1 mRNA levels were plotted after normalization with Gapdh mRNA levels + SD (A-B). Nrf2 protein levels in total cell lysates prepared from Hepa1c1c7 cells treated with the 300 nM brusatol for the indicated times as determined by western blotting (C). Superimposition of Nrf2 protein expression and Nrf2 mRNA expression to brusatol treatment for up to 24 h (D). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$ ).

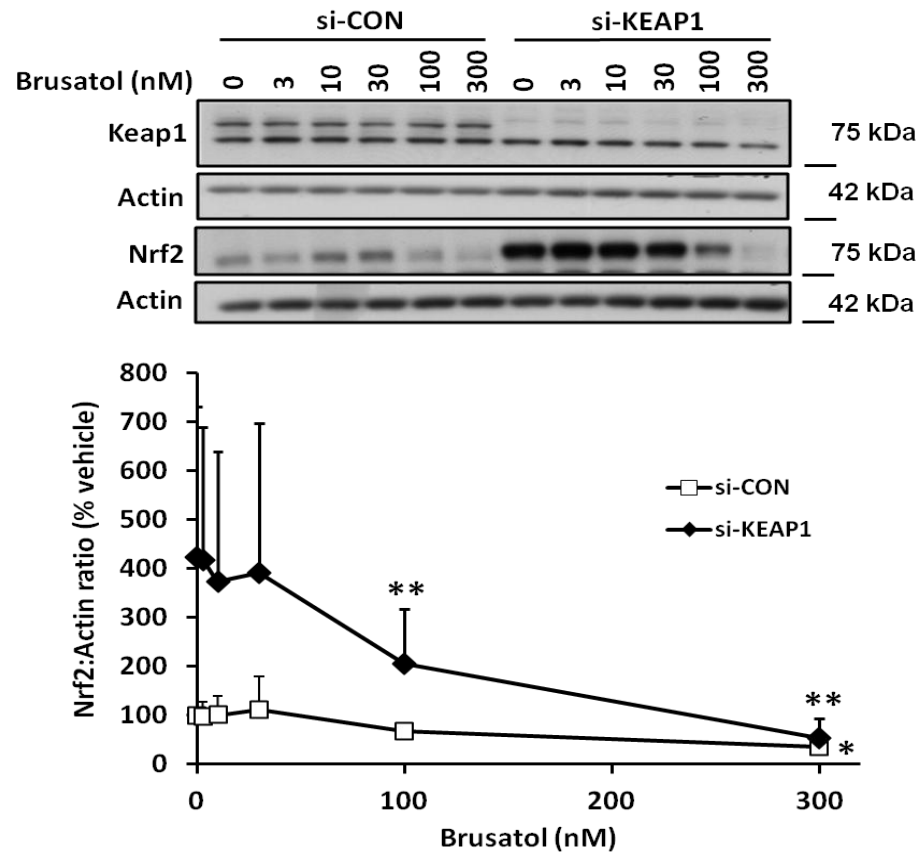
### 6.2.2 Brusatol Inhibits Nrf2 independently of Keap1 and the proteasome

Having determined that brusatol is capable of depleting basal and pharmacologically induced Nrf2 (chapter 5), it was hypothesised that it was doing so via increased proteasomal degradation of Nrf2, as this is one of the classical mechanisms whereby low cellular protein levels including Nrf2 are maintained in the cell (Nguyen *et al.*, 2003). As Keap1 is responsible for targeting Nrf2 to the proteasomes, and an increase in Keap1 has been associated with a decrease in Nrf2 (Zhang *et al.*, 2004), Hepa1c1c7 cells were exposed to with brusatol for up to 2 hours and the levels of Keap1 protein was analysed by immunoblotting. Keap1 protein levels did not change following treatment with brusatol (figure 6.2a). Additionally, siRNA mediated knock down of Keap1 in Hepa-1c1c7 cells did not abrogate the ability of brusatol to inhibit Nrf2 (figure 6.2b), whilst Keap1 siRNA reduced cellular Keap1 protein levels significantly (figure 6.2b).

(A)



(B)



**Figure 6.2 - Brusatol depletes Nrf2 independently of Keap1.** Keap1 protein levels in total cell lysates prepared from Hepa1c1c7 cells treated with 300 nM brusatol for the indicated times as determined by western blotting (A). Nrf2 and Keap1 protein levels in Hepa1c1c7 cells transfected with 10 nM keap1-targeting siRNA or non-targeting control siRNA for 48 hours followed by exposure to the indicated concentrations of brusatol for additional 2 hours as determined by western blotting (B). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$ ).

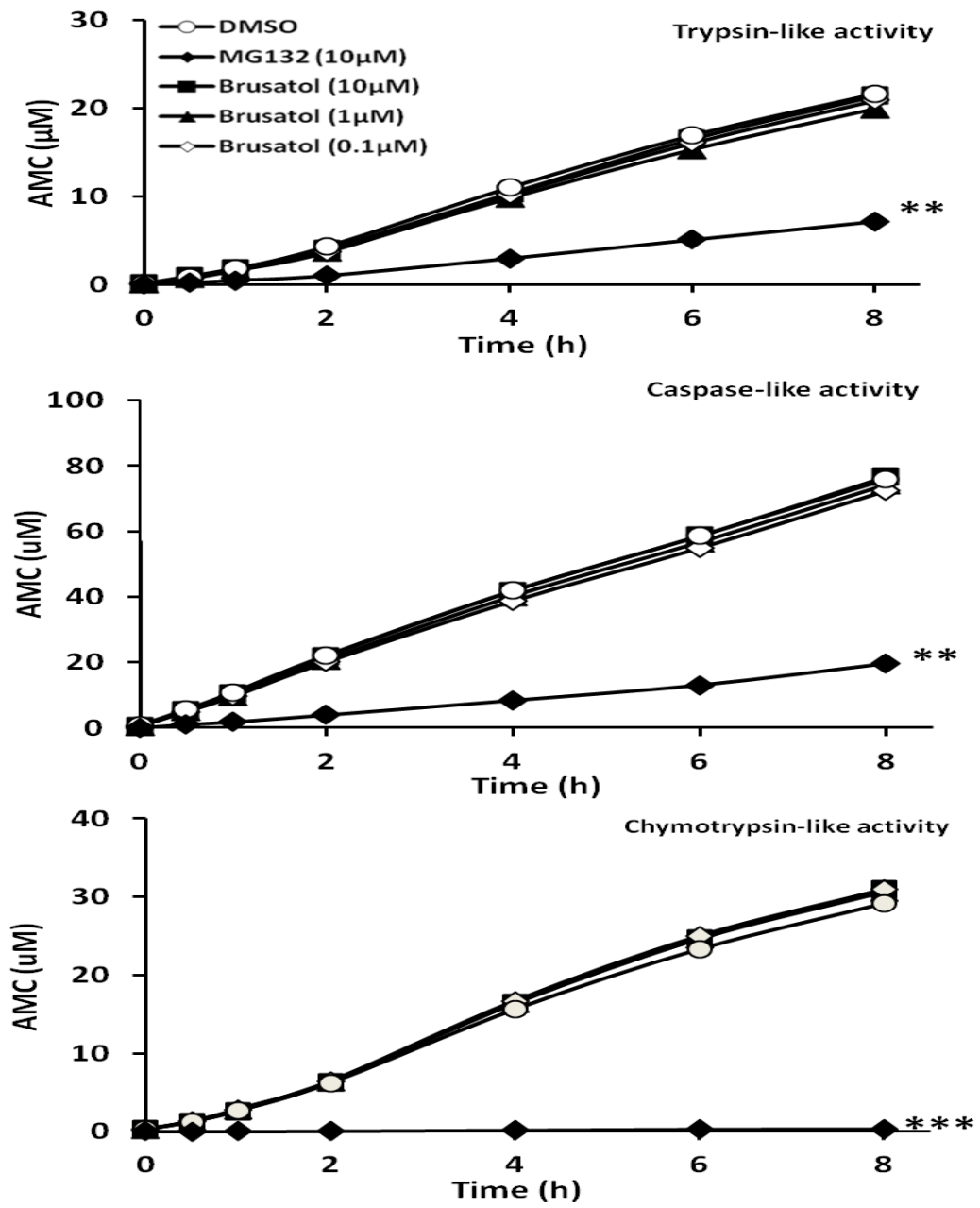


### 6.2.3 Brusatol does not enhance the catalytic activities of the proteasome *in vitro*

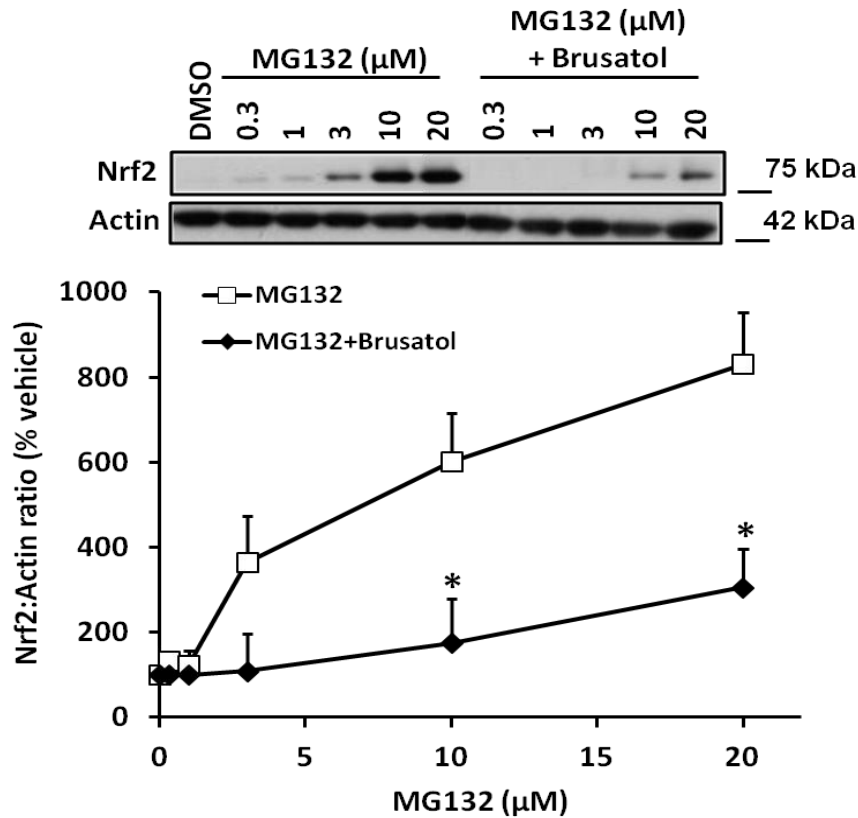
To investigate whether brusatol was able to activate Nrf2 degradation via the proteasome independently of Keap1, subunits of the human 20S proteasome and the fluorogenic peptide substrates Boc-LSTR-AMC (50 $\mu$ M), Suc-LLVY-AMC (50 $\mu$ M) and Z-LLG-AMC (400 $\mu$ M) corresponding to the trypsin, chymotrypsin and caspase-like activities respectively were utilised to monitor the activities of the proteasome in the presence of brusatol for up to 8 hours *in vitro*. The hydrolysis of the fluoregenic substrates BOC-LSTR-AMC, Suc-LLVY-AMC and Z-LLG-AMC was measured at excitation 360 nm and emission of 460 nm (figure 6.3a). The findings demonstrate that brusatol does not enhance the catalytic activities of the 20S proteasome; hence the ability of brusatol to deplete Nrf2 appears to work via a process that does not involve the enhancement of the proteasomal degradation of Nrf2. As Nrf2 is rapidly degraded by the proteasome, cells treated with the proteasomal inhibitor MG132 showed marked Nrf2 accumulation (figure 6.3b). However, brusatol was able to abrogate the accumulation of Nrf2 provoked by MG132 (figure 6.3b).

Taken together, this data suggests that brusatol does not alter the levels of Keap1 (section 6.2.2) thus targeting Nrf2 for proteasomal degradation, nor does it activate the proteasome. Brusatol is also capable of reducing Nrf2 protein levels in conditions when the proteasome is inhibited. Therefore brusatol is able to clear basal and pharmacologically induced Nrf2 protein via a mechanism that is independent of Keap1 and the proteasome.

(A)



(B)

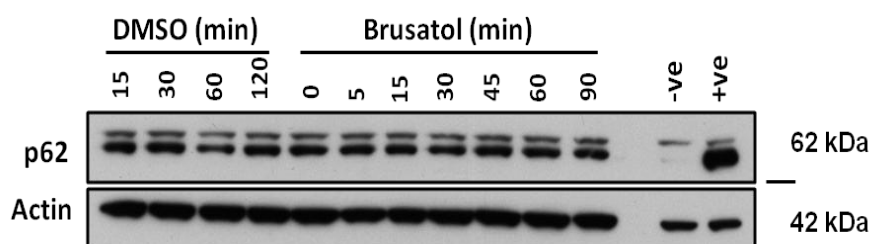


**Figure 6.3 - Brusatol depletes Nrf2 independently of the proteasomal degradation pathway.** Proteasome activity assay showing the effect of the indicated concentration of brusatol and MG132 on the trypsin (*Boc-LSTR-AMC*), chymotrypsin (*Suc-LLVY-AMC*), and caspase (*Z-LLE-AMC*)-like activities of human 20S proteasome at the indicated times (A). Nrf2 protein levels in total cell lysates prepared from Hepa1c1c7 cells treated with the indicated concentrations of MG132 for 2 hours followed by 300 nM brusatol for a further 2 hours, as determined by western blotting (B). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\* $P < 0.05$ , \*\* $P < 0.01$ ).

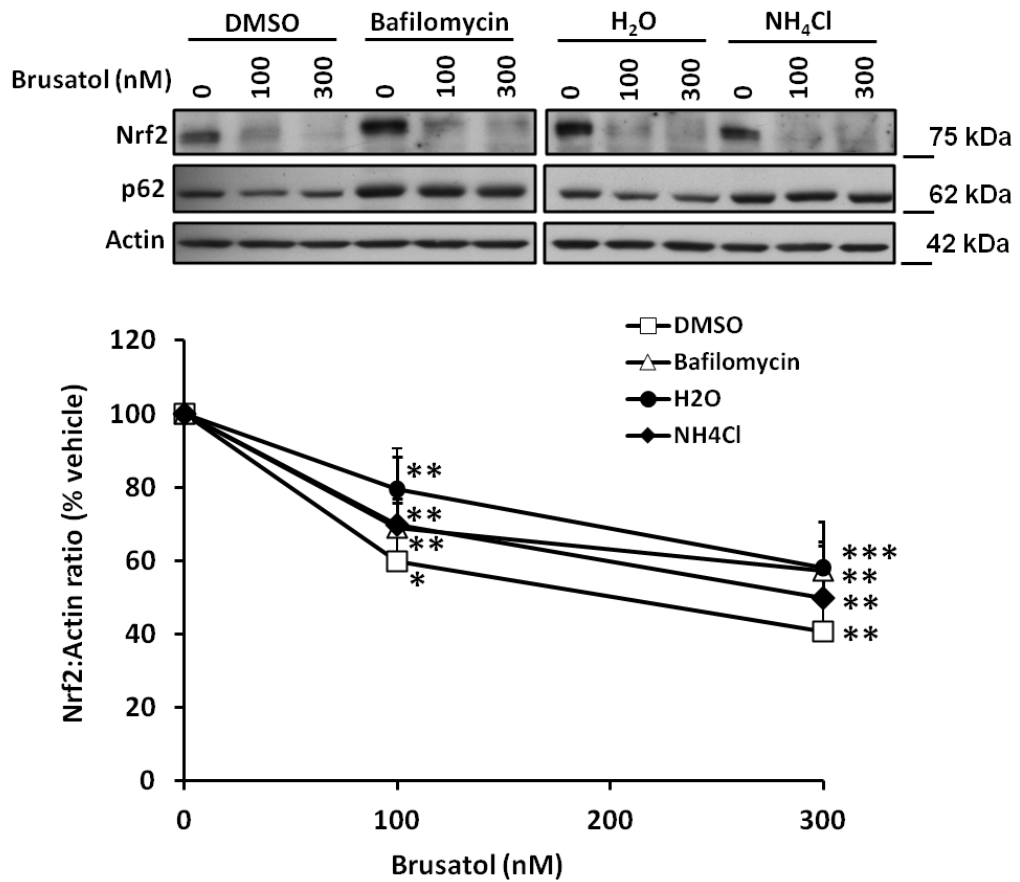
#### 6.2.4 Brusatol initiates the depletion of Nrf2 independently of the autophagy pathway

Having determined that brusatol inhibits Nrf2 independently of Keap1 and the proteasome, alternative mechanisms of protein degradation were investigated. Recently, the Keap1/Nrf2 pathway has been linked with autophagy via the scaffold protein sequestosome/p62 which binds Keap1 targeting it for autophagic degradation (Copples *et al.*, 2010). It was thus hypothesised that brusatol could promote the degradation of Nrf2 via this mechanism. To determine whether the decrease in Nrf2 protein expression was dependent on autophagy, Hepa1c1c7 cells were treated with brusatol for up to 2 hours and the levels of p62 protein were analysed by immunoblotting (p62 accumulation is indicative of a reduction in autophagy (Nezis *et al.*, 2012)). p62 protein levels did not change following treatment with brusatol (figure 6.4a). Additionally, Hepa1c1c7 cells were treated with the autophagy inhibitors Bafilomycin A1 or Ammonium chloride (NH<sub>4</sub>Cl) for 16 hours. Cells were then treated with 0, 100 or 300 nM brusatol for a further 2 hours. Autophagy inhibition was confirmed by the accumulation of p62 in cells exposed to Bafilomycin A1 and NH<sub>4</sub>Cl. In the presence of autophagy inhibitors, brusatol was still able to reduce Nrf2 protein levels (figure 6.4b). Therefore, these data indicate that brusatol does not activate the autophagic pathway as a means of depleting Nrf2 protein.

(A)



(B)

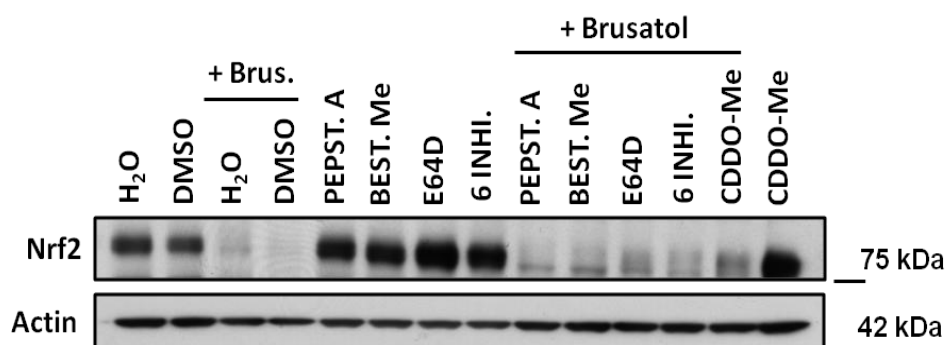


**Figure 6.4 - Brusatol decreases Nrf2 independently of the autophagic protein degradation machinery.** p62 levels in total cell lysates of Hepa1c1c7 cells treated with 300 nM brusatol or DMSO for the indicated times, as determined by western blotting. HeLa cells expressing p62-FLAG were used a positive control for p62 protein (A) Nrf2 and p62 protein levels in total cell lysates of Hepa1c1c7 cells pretreated with 30 nM Bafilomycin A1, 10 mM Ammonium chloride (NH<sub>4</sub>Cl) or vehicle control (DMSO/H<sub>2</sub>O) for 16 hours to inhibit autophagy followed by treatment with the indicated concentration of brusatol for a further 2 hours, as determined by western blotting (B). The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*P < 0.05, \*\*P < 0.01).

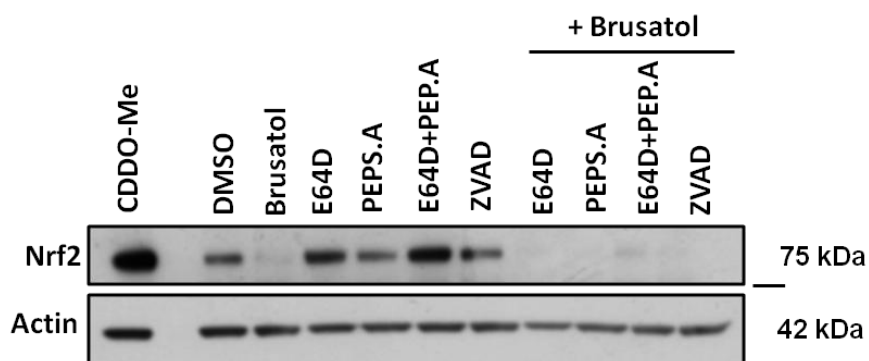
### 6.2.5 Brusatol clears Nrf2 independently of protease and caspases

To determine whether the decrease in Nrf2 protein expression by brusatol was dependent on the activation of proteases, Hepa1c1c7 cells were treated with a panel of protease inhibitors; AEBSF (serine), GM6001 (matrix metalloproteinase), Calpeptin (calpains), Pepstatin A (aspartyl peptidases), Bestatin Methyl Ester (aminopeptidase), E64D (thiol protease), ZVAD (caspases) or vehicle control (DMSO/H<sub>2</sub>O) for 1 hour. Cells were then treated with 300 nM brusatol for a further 2 hours and the levels of Nrf2 protein were analysed by immunoblotting. Brusatol was able to deplete Nrf2 protein in the presence of the protease and caspase inhibitors (Figures 6.5a & b) both individually and in combination. Interestingly, E64D on its own was able to induce Nrf2 protein expression suggesting a role for cysteine proteases in the regulation of Nrf2 (Figures 6.5a&b).

(A)



(B)



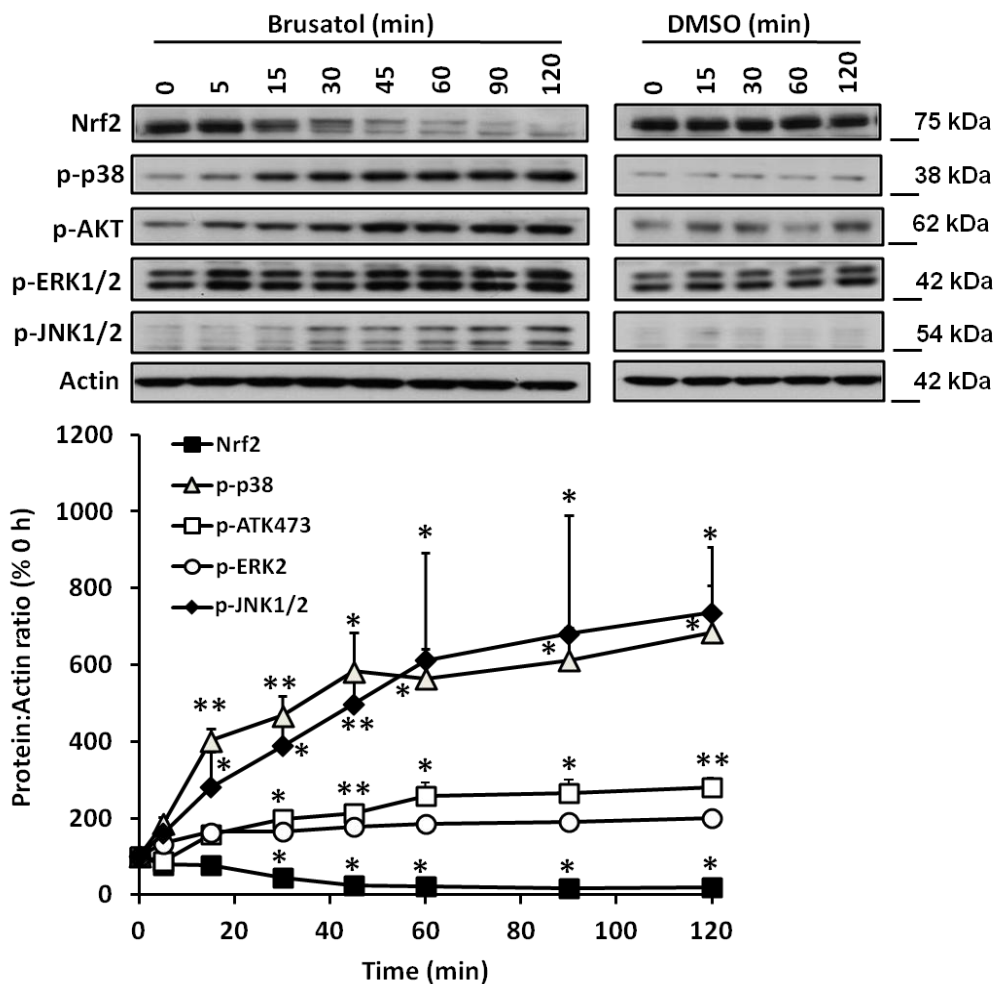
**Figure 6.5 - Brusatol does not deplete Nrf2 via proteases and caspases.** Nrf2 protein levels in total cell lysates of Hepa1c1c7 cells pretreated with 250  $\mu$ M AEBSF, 50  $\mu$ M GM6001, 50  $\mu$ M Calpeptin, 50  $\mu$ M Pepstatin Methyl Ester, 50  $\mu$ M Bestatin Methyl Ester, 50  $\mu$ M E64D, all 6 drugs combined, 50  $\mu$ M ZVAD or vehicle ( $H_2O$ /DMSO) for 1 h followed by 300 nM brusatol for 2 h as determined by immunoblotting (A-B). The blots shown are a representative of two independent experiments.

### 6.2.6 Brusatol induces the phosphorylation of p38 MAPK, SAPK JNK 1/2 AND PI3K/AKT pathways, but the depletion of Nrf2 is independent of the activation of these pathways

It was hypothesised that brusatol could be depleting Nrf2 via the modulation of kinase signalling pathways previously implicated in the regulation of Nrf2 (Bryan *et al.*, 2013). To determine whether the depletion of Nrf2 provoked by brusatol was dependent on the modulation of these kinase signaling pathways, Hepa1c1c7 cells exposed to brusatol for up to 2 hours were analysed by western blot for phosphorylation of AKT-473, p38 MAPK, ERK1/2 MAPK and JNK1/2 SAPK. In parallel with the depletion of Nrf2, brusatol provoked a marked increase in the phosphorylation of p38, AKT473, JNK1/2 and ERK1/2 (figure 6.6a). To test whether the activation of these kinases played a mechanistic role in the depletion of Nrf2, Hepa1c1c7 cells were pre-treated for 1 hour with the specific inhibitors SB203580 (p38), LY294002 (AKT), SP600125 (SAPK) and U0126 (ERK1/2) before further treatment with brusatol and analysed by immunoblotting. Brusatol's inhibitory

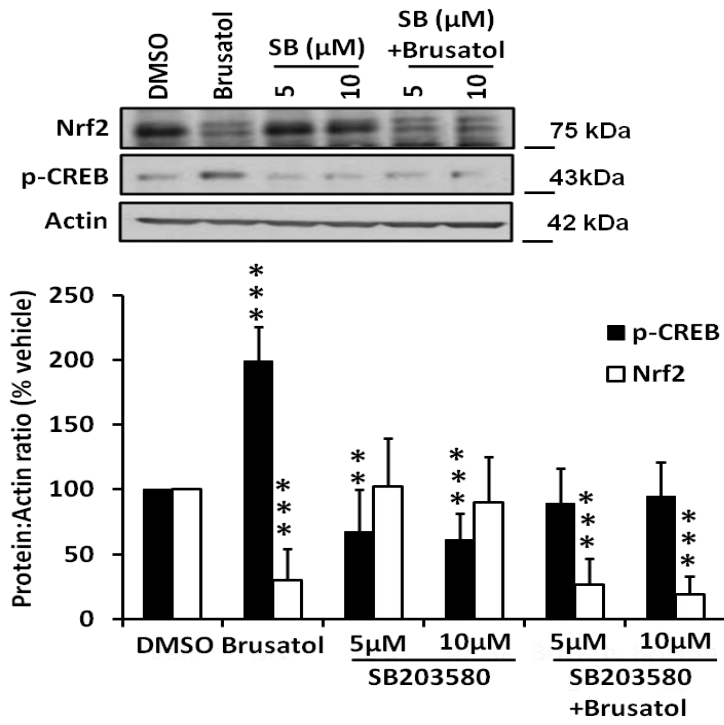
effect on Nrf2 was unaffected by the presence of these kinase inhibitors (figure 6.6b-d). Recent studies have also shown the involvement of GSK-3 $\beta$  in the degradation of Nrf2 (Chowdhry *et al.*, 2013), however brusatol's inhibitory effect on Nrf2 was found to be independent of GSK-3 $\beta$  activity as brusatol still provoked the depletion of Nrf2 protein in the presence of GSK-3 $\beta$  inhibitor CT99021 (figure 6.6c). Taken together, the increase in the phosphorylation of AKT, p38, ERK1/2 and JNK1/2 provoked by brusatol does not appear to be an important element of its mechanism of action as an inhibitor of Nrf2.

(A)

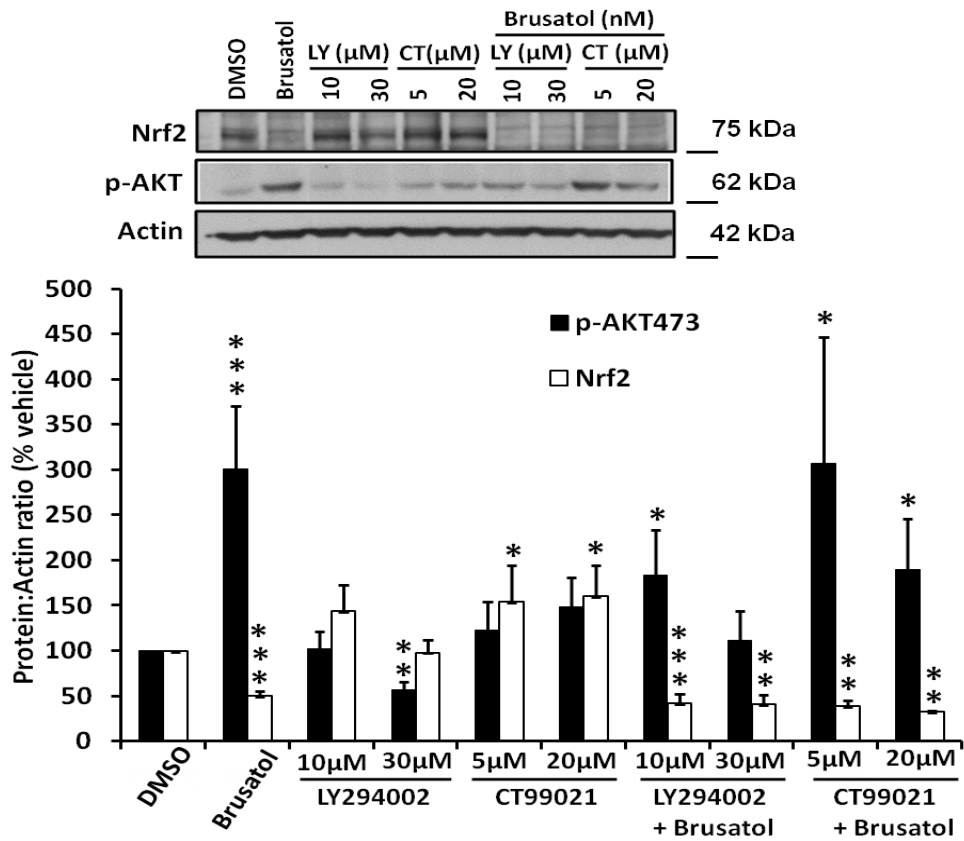




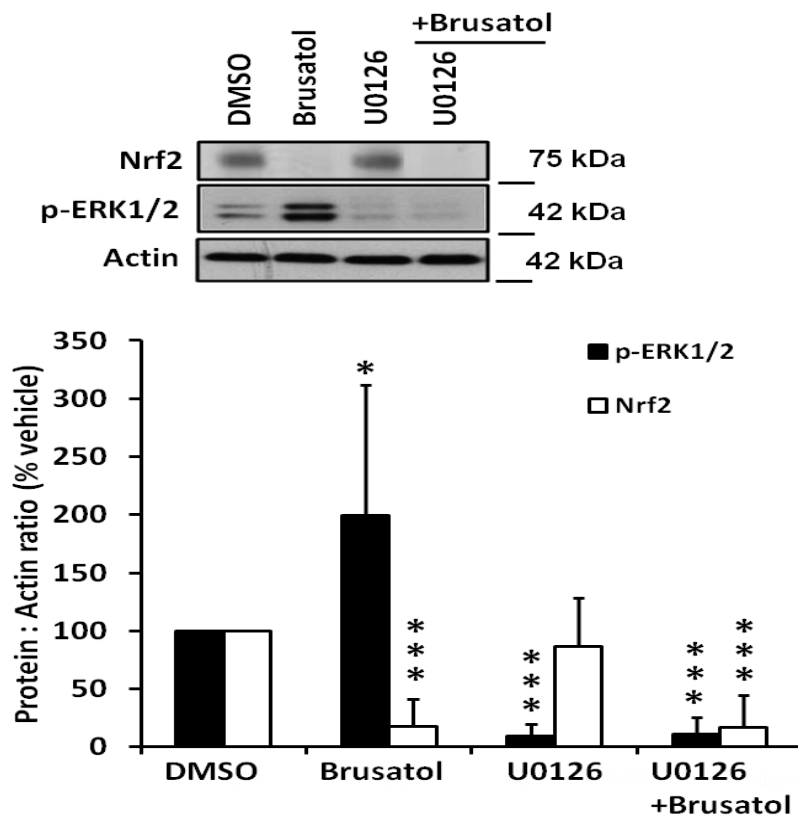
(B)



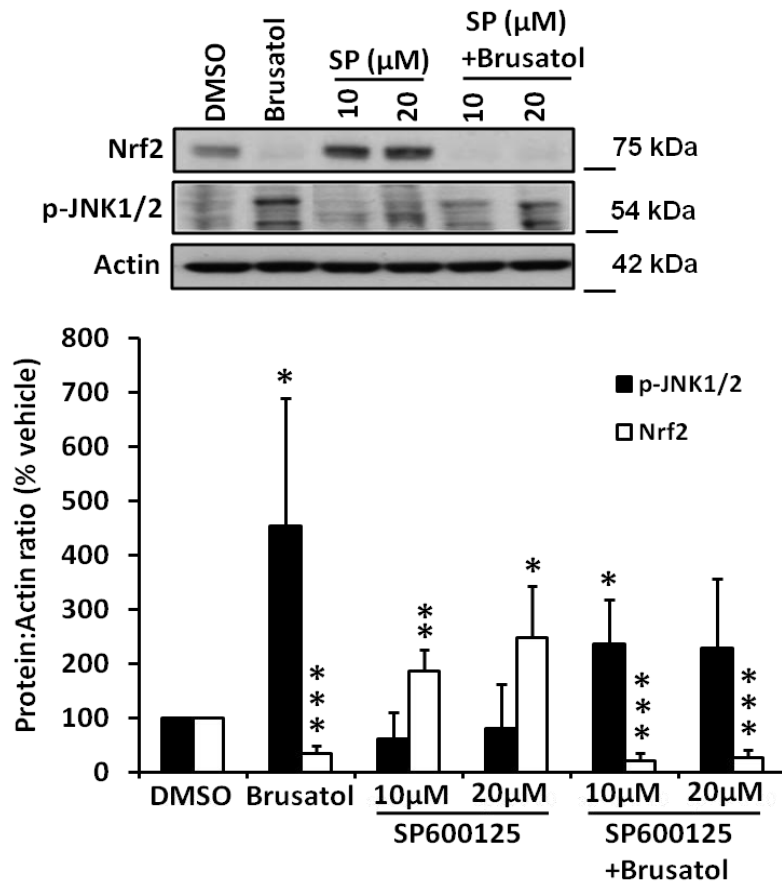
(C)



(D)



(E)

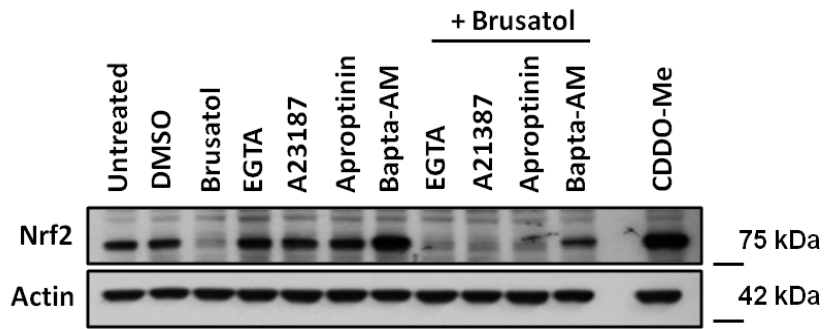


**Figure 6.6 - Brusatol depletes Nrf2 independently of the activation of protein kinase signalling pathways:** Nrf2 and phospho-p38 MAPK, AKT, ERK1/2 and SAPK levels in the total cell lysates of Hepa1c1c7 cells treated with 300 nM brusatol for up to 2 hours as determined by immunoblotting (A). Nrf2 and phospho-Creb, AKT and SAPK in Hepa1c1c7 cells pretreated with the indicated concentration of SB203580, LY2940002, CT99021, SP600125 or 5 μM for 1 h, before treatment with 300 nM brusatol for a further 2 hours as determined by immunoblotting (B-E). The average protein levels of the three independent experiments were plotted after normalization with actin + SD. Statistical analysis was performed using an unpaired t-test (\*P<0.05, \*\*P<0.01).

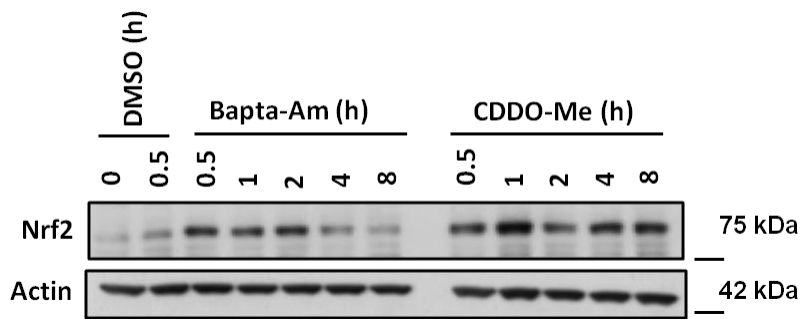
### **6.2.7 Inhibition of Nrf2 by Brusatol may be partially dependent on calcium modulation**

It was further hypothesized that the brusatol-mediated decrease in Nrf2 may involve a calcium-dependent process as some biological processes depend on calcium regulation. To test this, Hepa1c1c7 cells were treated with the following: EGTA, an extracellular calcium chelator, for 15 minutes; A23187, a calcium ionophore for 20 minutes, BAPTA-AM, an intracellular calcium chelator, for 60 minutes. Thereafter, cells were treated with 300 nM brusatol for 2 hours. Brusatol depleted Nrf2 protein in the presence of A23187 and EGTA (figure 6.7a). However, brusatol was unable to totally deplete Nrf2 in the presence of BAPTA-AM, suggesting the inhibition of Nrf2 by brusatol may be partly dependent on a calcium-regulated process (figure 6.7a). Surprisingly, BAPTA-AM induced Nrf2 protein expression suggesting that a calcium-regulated process could also be regulating the basal level of Nrf2 (figure 6.7a). To further test BAPTA-AM's ability to induce Nrf2, a time-course comparison between BAPTA-AM and the potent inducer of Nrf2, CDDO-Me was performed. BAPTA-AM induced Nrf2 transiently with induction seen by 0.5 hours and stabilized up until 2 hours, after which the level of Nrf2 returned to baseline (figure 6.7b). To further confirm whether brusatol's mechanism of action is partially dependent on a calcium-regulated process, a time-course comparison between BAPTA-AM and CDDO-Me in the presence of brusatol was performed. Brusatol was less able to reduce the BAPTA-AM-induced Nrf2 protein compared to Nrf2 that had been induced by CDDO-Me (figure 6.7c). The diminished ability of brusatol to deplete Nrf2 in the presence of BAPTA-AM could be attributed to the fact that the increase in Nrf2 protein provoked by BAPTA-AM is transient compared to the sustained effect of CDDO-Me (figures 6.7b & c). Taken together, this data suggests that brusatol-mediated degradation of Nrf2 could be partly mediated via a calcium-regulated process.

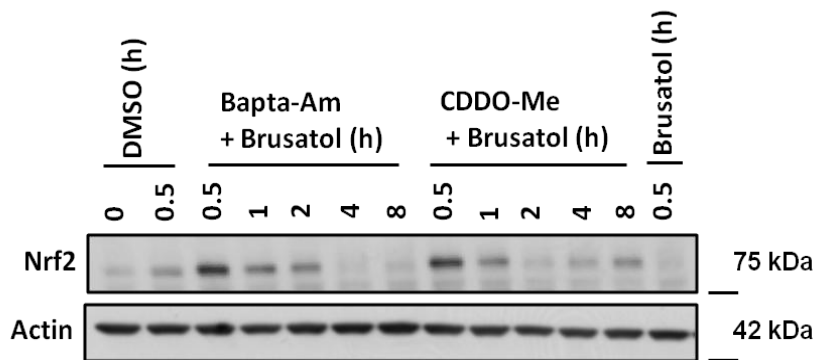
(A)



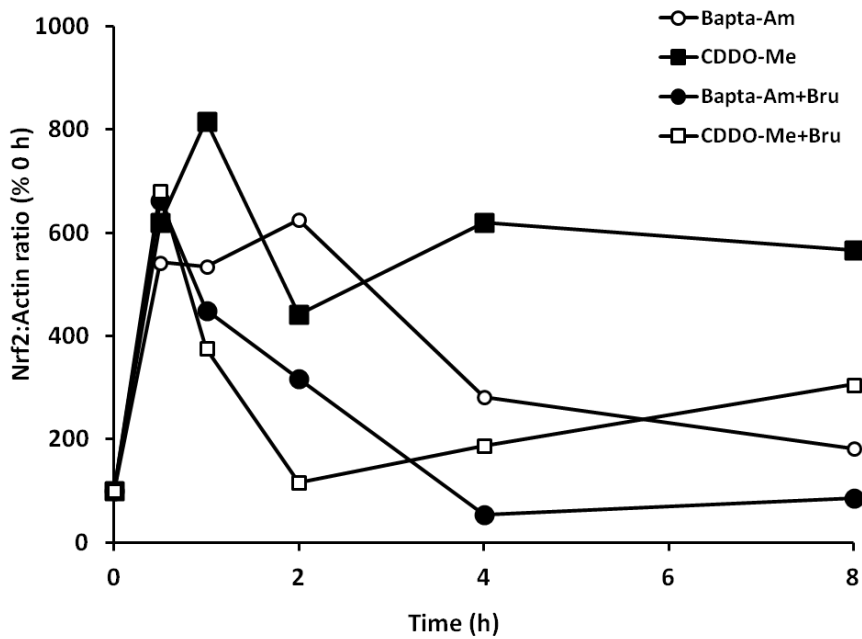
(B)



(C)



(D)



**Figure 6.7 - Brusatol-mediated depletion of Nrf2 is partially dependent on the modulation of calcium signalling.** Nrf2 protein levels in the total cell lysates of Hepa1c1c7 cells pretreated with 2.5 mM EGTA or 5  $\mu$ M A23187 for 15 and 20 mins respectively, or 10  $\mu$ g/mL aprotinin or 20  $\mu$ M BAPTA-AM for 60 minutes followed by 300 nM brusatol treatment for a further 2 hours (A), or in Hepa1c1c7 cells treated with 20  $\mu$ M BAPTA-AM or 100 nM CDDO-Me or vehicle for the indicated times (B) or in Hepa1c1c7 cells pretreated with 20  $\mu$ M BAPATA-AM or 100 nM CDDO-me or vehicle for 60 minutes before the addition of 300 nM of brusatol for the indicated times (C) as determined by western blotting. The blots shown are a representative of three independent experiments and quantified by densitometry. The average Nrf2 values of the two independent experiments for the result present in C were plotted after normalization with actin (D).

### **6.3 Discussion**

The data presented in this chapter provides *in vitro* insights into the mechanism of Nrf2 inhibition by brusatol in mouse Hepa-1c1c7 hepatoma cells. As data from chapter 5 had shown that brusatol provoked both concentration and time - dependent, yet transient, depletion of Nrf2, it was of interest to define the mechanism of action of this drug. Brusatol-mediated depletion of Nrf2 protein was found to be independent of changes in the level of Nrf2 mRNA, suggesting that brusatol works through a post-translational mechanism. Furthermore, the ability of brusatol to inhibit Nrf2 was not affected by siRNA depletion of Keap1 indicating that brusatol may by-pass the canonical mechanisms of Nrf2 regulation (Zhang *et al.*, 2004). Brusatol's lack of dependence on Keap1 indicates its potential utility in disease contexts, particularly cancer, where Keap1-mediated repression of Nrf2 has been by-passed. Brusatol did not enhance the catalytic activities of the human 20S proteasome *in vitro*, and was able to induce the depletion of Nrf2 in the presence of the proteasome inhibitor MG132 and the autophagy inhibitors bafilomycin A1 and ammonium chloride. Additionally, brusatol was able to induce the depletion of Nrf2 in the presence of various protease inhibitors. It should be noted that the concentration of protease inhibitors used here are consistent with those used routinely in the published literature. Further work is needed to confirm that the relevant proteases were indeed inhibited under the experimental conditions.

Several intracellular signalling pathways such as p38 (Keum *et al.*, 2006b), PI3K (Nguyen *et al.*, 2003), MAPK/ERK (Yuan *et al.*, 2006), JNK (Yuan *et al.*, 2006), PERK (Cullinan *et al.*, 2003) have been implicated in the regulation of Nrf2 signalling. Notably, brusatol stimulated a marked activation of the mitogen-activated protein kinase p-p38, stress activated protein kinase p-JNk1,2 and Phosphoinoside 3-kinase p-AKT within the time frame of Nrf2 depletion (figure 6.6a) however, in the presence of the p38 inhibitor SB203580, JNK inhibitor SP600125, AKT inhibitor LY294002, GSK-3 $\beta$  inhibitor CT99021 and ERK inhibitor U0126, the ability of brusatol to provoke the depletion of Nrf2 was unaffected. The increase in the phosphorylation of p-P38, p-AKT and p-JNk1/2 stimulated by brusatol could be a direct effect of the depletion of Nrf2 due to cross talk between Nrf2 and these

signaling pathways. The induction of these stress response pathways may also underlie, at least partly, the ability of brusatol to limit the proliferation of cancer cells *in vitro* and *in vivo* (Ren *et al.*, 2011).

Interestingly, BAPTA-AM appears to partly inhibit the inhibitory action of brusatol towards Nrf2s. Of note, BAPTA-AM alone induced Nrf2 transiently in a time-dependent manner, with maximum induction seen at 2 hours. Taken together this suggests a role of Ca<sup>2+</sup> signalling in the mechanism of action of brusatol. A role for Ca<sup>2+</sup> signalling in the Nrf2-activation process has been suggested previously (Burdette *et al.*, 2010; Reddy *et al.*, 2012; Lee *et al.*, 2013); Indeed, Burdette *et al.* reported the induction of Nrf2 with HCV (hepatitis C virus) infection, and that the HCV induction of Nrf2 was inhibited by BAPTA-AM (Burdette *et al.*, 2010). Reddy *et al* and Lee *et al* also showed that BAPTA-AM abrogates the induction of Nrf2 by antioxidants. It is possible that the time frames used in these studies overlaps with the time at which the effect of BAPTA-AM or Nrf2 has worn off. Nonetheless, it is plausible that Ca<sup>2+</sup> signalling has a role in the regulation of the Nrf2 pathway.

Taken together, these findings indicate that brusatol exploits a previously unknown mechanism of Nrf2 regulation and exhibits high specificity for the Nrf2 pathway. Indeed, it appears unlikely that the depletion of Nrf2 provoked by brusatol is due to universal inhibition of protein synthesis, as the compound had no effect on a panel of proteins tested here and by (Ren *et al.*, 2011).

In conclusion, brusatol's mode of action as an inhibitor of Nrf2 is not dependent on Keap1 or proteasomal degradation, nor does it work through alternative conventional protein degradation pathways such as autophagy and the activation of proteases. Brusatol induces the PI3K, MAPK and SAPK signaling pathways but its ability to deplete Nrf2 is independent of these effects. These findings point towards a novel mechanism by which brusatol inhibits the Nrf2 pathway and suggest that unknown processes are involved in the regulation of Nrf2 perhaps via a repression of the translation of the Nrf2 mRNA within the open reading frame of the gene (Perez-Leal *et al.*, 2013). Further exploration of the mechanism of action of this compound is needed to allow its potency and efficacy to be optimised for



experimental and clinical use. Such insights will also inform the design of new pharmacological inhibitors, which hold promise as therapeutic agents, particularly in the treatment of cancer.

## **CHAPTER SEVEN**

### **OVERALL DISCUSSION**

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
7.1 Introduction.....	162
7.2 Activation of Nrf2 and its pharmacological significance.....	162
7.3 Inhibition of Nrf2 and its pharmacological significance.....	164
7.4 Concluding remarks and future investigations.....	166

## 7.1 Introduction

Since the discovery of Nrf2, the role of this transcription factor has been highlighted in the maintenance of cellular homeostasis by combating toxic insults generated intrinsically and extrinsically (Itoh *et al.*, 1997; Chan *et al.*, 2001; Enomoto *et al.*, 2001; Copple *et al.*, 2008a; Xu *et al.*, 2008). In addition, Nrf2 has been shown to have a role in the pathogenesis of various diseases in discrete organs of the body (Copple, 2012). Many of these diseases have a low survival rate due to drug-resistance or a lack of appropriate therapies (Jemal *et al.*, 2009). For example, heightened cell defence, which is common in cancer settings as a result of intrinsically generated reactive oxygen species associated with oncogenic proliferation, ensures that cellular homeostasis is maintained (Bhat *et al.*, 2010). This, on the other hand, enhances chemoresistance, reducing the efficacy of cytotoxic cancer drugs. Thus, there is an urgent need for the identification of novel targets for pharmacological manipulation, to directly treat the disease of interest or increase the efficacy of currently used therapies. Since Nrf2 is a master regulator of cell defence and has been shown to have roles in different organs and associated diseases such as neurodegeneration, cardiovascular dysfunction and cancer (Copple, 2012; Bryan *et al.*, 2013), the main aims of the studies presented in this thesis were to explore the pharmacological induction and inhibition of Nrf2 and its therapeutic significance, to better understand the likely value of manipulating Nrf2 to treat disease and/or enhance the action of existing therapies.

## 7.2 Activation of Nrf2 and its pharmacological significance

Transgenic Nrf2 knockout mice show enhanced susceptibility to various toxicities and diseases such as chronic obstructive pulmonary disease and acute lung injury due to low basal expression of cell defence genes (Chan *et al.*, 2001; Enomoto *et al.*, 2001; Xu *et al.*, 2008; Copple, 2012). In addition, the pharmacological induction of Nrf2 or genetic activation by silencing of Keap1 reduces the deleterious effects of oxidative stress and reduces the progression of stress-induced diseases (Klaassen *et al.*, 2010; Ohkoshi *et al.*, 2013).

Many Nrf2 inducers have been identified so far, amongst which the triterpenoid CDDO & its derivatives CDDO-Im and CDDO-Me are potent inducers with anticancer properties (Liby *et al.*, 2005). CDDO-Me was tested in clinical trials for the treatment of chronic kidney disease but was withdrawn due to adverse cardiovascular effects (Tayek *et al.*, 2013). Notably, the mechanistic reasons behind these adverse reactions are yet to be determined but suggested recently that it could be as a result of CDDO-Me promoting fluid retention in patients with more advanced chronic kidney disease (Chin *et al.*, 2014; Van Laecke *et al.*, 2014), although the lethal effects of Keap1 knock out *in vivo* illustrate that constitutive activation of Nrf2 can have deleterious effects (Wakabayashi *et al.*, 2003; Taguchi *et al.*, 2010). Dimethyl fumarate (DMF), another Nrf2 activator was recently licensed for the treatment of multiple sclerosis (Linker *et al.*, 2011).

Therefore, triterpenoids continue to have potential for development into novel therapies but their pharmacological mechanisms need to be fully defined to ensure their safe and efficacious use. Here, CDDO-Me was found to activate the Nrf2 pathway in a number of mammalian test systems ranging from established mouse hepatoma cells to primary human cells. Notably, CDDO-Me stimulated the accumulation of Nrf2 and Nqo1 in human PBMCs and PHHs. These findings demonstrate the translational relevance of data generated in mouse and other test systems in published works. In addition, the work presented here also establishes a platform for assessing inter-individual variability in the activity of the Nrf2 pathway and its relevance to disease. It was found that the basal and inducible levels of Nrf2 and Nqo1 vary across a relatively small number of individuals. Further work is required to assess the robustness of these observations in a larger number of individuals, and the correlation between inter-individual variability in Nrf2 pathway activity and susceptibility to disease and drug-induced toxicities.

Due to the adverse effects associated with CDDO-Me during a recent clinical trial, it is crucial to understand the mechanism of action of this compound. The findings of this work have shown that CDDO-Me stabilizes Nrf2 via a post-transcriptional mechanism. This, coupled with kinase signalling pathways not having significant roles in CDDO-Me-mediated Nrf2 induction, provides a platform for current and

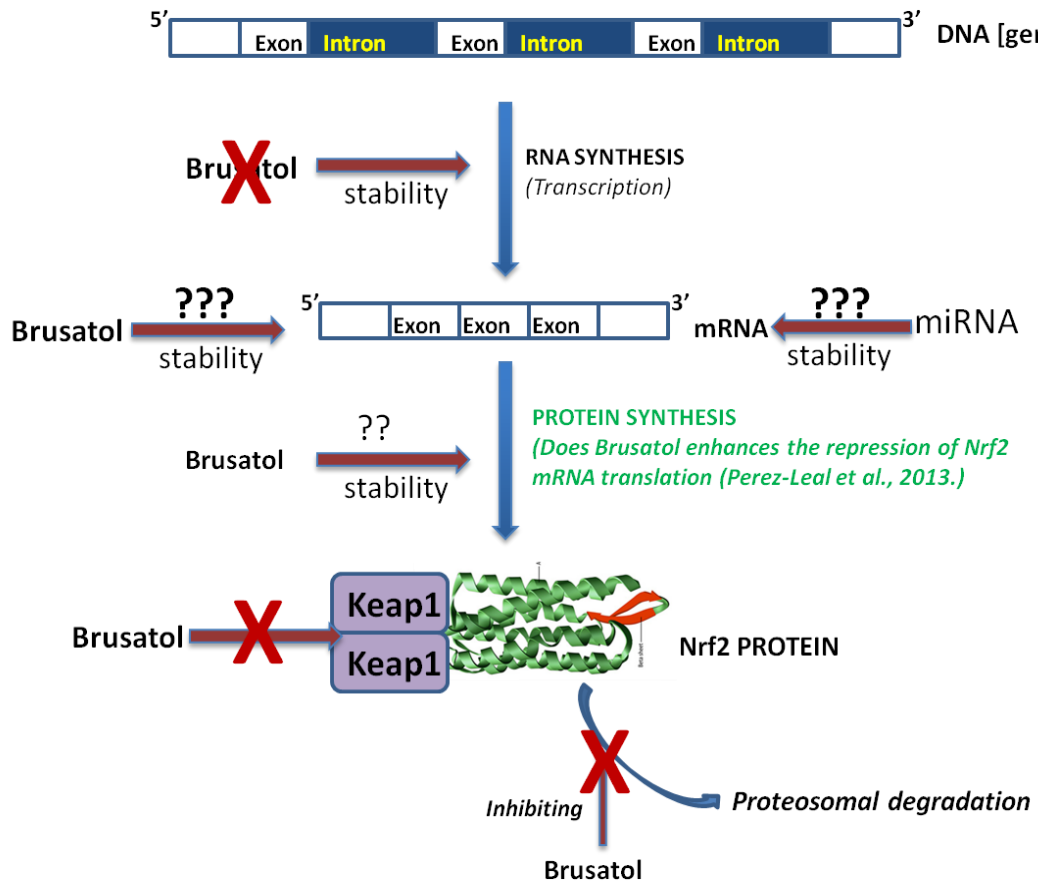
future work to build upon. Previous work has indicated that CDDO-Me and related triterpenoids modify Keap1 and its ability to repress Nrf2, thus stabilising the transcription factor. Further work will be necessary to prove this concept directly.

### **7.3 Inhibition of Nrf2 and its pharmacological significance**

Recent studies have illustrated a role for Nrf2 in the progression of cancer and resistance to chemotherapy (Ikeda *et al.*, 2004; Singh *et al.*, 2008; Wang *et al.*, 2008; Kensler *et al.*, 2010; Lister *et al.*, 2011; Taguchi *et al.*, 2011), as a result of somatic mutations in the Keap1 gene (Suzuki *et al.*, 2008), somatic mutations in the Nrf2 gene (Shibata *et al.*, 2008b), promoter methylation of Keap1 (Muscarella *et al.*, 2011) and other factors that dysregulate Keap1:Nrf2 signaling. The elevated Nrf2 level could contribute to chemoresistance by upregulating the expression of detoxification enzymes, antioxidant proteins and xenobiotic transporters thereby decreasing the potency of cytotoxic anticancer drugs (Wang *et al.*, 2008). Therefore there is an interest in developing compounds that selectively inhibit Nrf2 as adjuvants to conventional chemo- and radio-therapies.

Amongst the very few compounds identified to date, the quassinoid brusatol is a potent inhibitor of Nrf2. Brusatol has been reported in a study by (Ren *et al.*, 2011) to be a selective inhibitor of Nrf2 that enhances cisplatin treatment in xenografts. However, the mechanism of action of this compound is yet to be fully determined. The findings of (Ren *et al.*, 2011) and those in this thesis have shown brusatol to be a promising Nrf2 inhibitor. Brusatol was found to inhibit the Nrf2 pathway in a number of mammalian test systems ranging from mouse hepatoma cells to primary human cells. Brusatol inhibits Nrf2 in freshly isolated primary human hepatocytes indicating its potential utility in humans. Its mechanism of action still requires further investigation, but the findings presented here show that brusatol inhibits Nrf2 in a post-transcriptional manner and that the inhibition is independent of the conventional processes of Nrf2 regulation, including Keap1 mediated repression and degradation via the proteasomes and autophagic systems. This, coupled with kinase signalling pathways not having significant roles in Nrf2 inhibition by brusatol,

provides a platform for current and future work to build upon. Brusatol could therefore be enhancing the repression of Nrf2 mRNA translation but this requires further investigation (Figure 7.1)



**Figure 7.1 – Proposed mechanism of action of brusatol**

Knowing the significant role of Nrf2 in chemoprevention, there is a potential for Nrf2 inhibitors to sensitise cells to chemical stress. Here, it was shown that brusatol sensitises mammalian cells to chemical stress. Therefore, the inhibition of Nrf2 could increase adverse reactions to drugs. Thus, the ratio of risk and benefit of inhibiting Nrf2 needs to be determined in different disease and therapeutic contexts.

The search for novel Nrf2 activators and inhibitors will be aided by the use of high-throughput screening systems, such as cells expressing Nrf2-sensitive luciferase reporter transgenes that are responsive to Nrf2-inducing compounds (Linker *et al.*, 2011; Smirnova *et al.*, 2011; Hirotsu *et al.*, 2012; Oikawa *et al.*, 2012), and shown in our lab to respond to inhibitors of Nrf2 (data presented in the reports of Min-Wei Wong). Another approach involves the *in silico* screening of compounds that can modify Keap1 (Wu *et al.*, 2010), although such a strategy is currently limited by the absence of a complete crystal structure of the Keap1 protein.

#### **7.4 Concluding Remarks and future investigations**

The work in this thesis highlights Nrf2 as a potential therapeutic target and has furthered the understanding of the mechanisms that regulate Nrf2 activity and the important functions of this pathway, especially in cytoprotection, and forms the basis for several areas of future investigations. Despite the potential value of Nrf2 inducers, some concerns remain as to whether the activation of Nrf2 could promote chemoresistance and the progression of diseases such as atherosclerosis (Sussan *et al.*, 2008; Harada *et al.*, 2012). Additional unknown undesirable effects could also be associated with Nrf2 activation under clinical application, and the need to fully define the pharmacodynamic effects of these activators in patients is clear. A body of evidence has also indicated that elevated levels of Nrf2 aid cancer progression. Therefore, an understanding of the balance between the positive and negative roles of Nrf2 needs to be established, to enable the development of Nrf2 activators as safe and efficacious therapies. Therefore, future work should aim to address these concerns.

In conclusion, this work has added to the body of evidence that highlights the promising therapeutic significance of manipulating Nrf2 activity. It is hoped that it provides a foundation for future work that aims to develop small molecule inducers and inhibitors of Nrf2 with utility in a number of clinical scenarios.



## **BIBLIOGRAPHY**

Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D (2006). Triterpenoid CDDO-Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on Cys-179. *The Journal of biological chemistry* **281**(47): 35764-35769.

Ahn YH, Hwang Y, Liu H, Wang XJ, Zhang Y, Stephenson KK, *et al.* (2010). Electrophilic tuning of the chemoprotective natural product sulforaphane. *Proceedings of the National Academy of Sciences of the United States of America* **107**(21): 9590-9595.

Alam J, Cook JL (2007). How many transcription factors does it take to turn on the heme oxygenase-1 gene? *American journal of respiratory cell and molecular biology* **36**(2): 166-174.

Alam J, Killeen E, Gong P, Naquin R, Hu B, Stewart D, *et al.* (2003). Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. *American journal of physiology. Renal physiology* **284**(4): F743-752.

Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, *et al.* (2000). Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *The Journal of biological chemistry* **275**(36): 27694-27702.

Aleksunes LM, Goedken MJ, Rockwell CE, Thomale J, Manautou JE, Klaassen CD (2010). Transcriptional regulation of renal cytoprotective genes by Nrf2 and its potential use as a therapeutic target to mitigate cisplatin-induced nephrotoxicity. *The Journal of pharmacology and experimental therapeutics* **335**(1): 2-12.

An JH, Blackwell TK (2003). SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes Dev* **17**(15): 1882-1893.

Arlt A, Sebens S, Krebs S, Geismann C, Grossmann M, Kruse ML, *et al.* (2013). Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. *Oncogene* **32**(40): 4825-4835.

Baird L, Dinkova-Kostova AT (2011). The cytoprotective role of the Keap1-Nrf2 pathway. *Archives of toxicology* **85**(4): 241-272.

Baird L, Lleres D, Swift S, Dinkova-Kostova AT (2013). Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1-Nrf2 protein complex. *Proceedings of the National Academy of Sciences of the United States of America* **110**(38): 15259-15264.

Bar-Or A, Gold R, Kappos L, Arnold DL, Giovannoni G, Selmaj K, *et al.* (2013). Clinical efficacy of BG-12 (dimethyl fumarate) in patients with relapsing-

remitting multiple sclerosis: subgroup analyses of the DEFINE study. *Journal of neurology* **260**(9): 2297-2305.

Barone MC, Sykiotis GP, Bohmann D (2011). Genetic activation of Nrf2 signaling is sufficient to ameliorate neurodegenerative phenotypes in a *Drosophila* model of Parkinson's disease. *Disease models & mechanisms* **4**(5): 701-707.

Berasi SP, Xiu M, Yee AS, Paulson KE (2004). HBP1 repression of the p47phox gene: Cell cycle regulation via the NADPH oxidase. *Molecular and cellular biology* **24**(7): 3011-3024.

Bernstein SH, Venkatesh S, Li M, Lee J, Lu B, Hilchey SP, *et al.* (2012). The mitochondrial ATP-dependent Lon protease: a novel target in lymphoma death mediated by the synthetic triterpenoid CDDO and its derivatives. *Blood* **119**(14): 3321-3329.

Bhat V, McIntyre M, Meyers T (2010). Efficacy and safety of a lower-dose valganciclovir (valcyte) regimen for cytomegalovirus prophylaxis in kidney and pancreas transplant recipients. *P & T : a peer-reviewed journal for formulary management* **35**(12): 676-679.

Bloom DA, Jaiswal AK (2003). Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *The Journal of biological chemistry* **278**(45): 44675-44682.

Boettler U, Sommerfeld K, Volz N, Pahlke G, Teller N, Somoza V, *et al.* (2011). Coffee constituents as modulators of Nrf2 nuclear translocation and ARE (EpRE)-dependent gene expression. *The Journal of nutritional biochemistry* **22**(5): 426-440.

Brodersen P, Voinnet O (2009). Revisiting the principles of microRNA target recognition and mode of action. *Nature reviews. Molecular cell biology* **10**(2): 141-148.

Bryan HK, Olayanju A, Goldring CE, Park BK (2013). The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochemical pharmacology* **85**(6): 705-717.

Burdette D, Olivarez M, Waris G (2010). Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway. *The Journal of general virology* **91**(Pt 3): 681-690.

Cavin C, Delatour T, Marin-Kuan M, Fenaille F, Holzhauser D, Guignard G, *et al.* (2009). Ochratoxin A-mediated DNA and protein damage: roles of nitrosative and oxidative stresses. *Toxicological sciences : an official journal of the Society of Toxicology* **110**(1): 84-94.

- Cavin C, Delatour T, Marin-Kuan M, Holzhauser D, Higgins L, Bezencon C, *et al.* (2007). Reduction in antioxidant defenses may contribute to ochratoxin A toxicity and carcinogenicity. *Toxicological sciences : an official journal of the Society of Toxicology* **96**(1): 30-39.
- Chan K, Han XD, Kan YW (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proceedings of the National Academy of Sciences of the United States of America* **98**(8): 4611-4616.
- Chan K, Kan YW (1999). Nrf2 is essential for protection against acute pulmonary injury in mice. *Proceedings of the National Academy of Sciences of the United States of America* **96**(22): 12731-12736.
- Chaneton B, Gottlieb E (2012). Rocking cell metabolism: revised functions of the key glycolytic regulator PKM2 in cancer. *Trends in biochemical sciences* **37**(8): 309-316.
- Chen J, Shaikh ZA (2009a). Activation of Nrf2 by cadmium and its role in protection against cadmium-induced apoptosis in rat kidney cells. *Toxicology and applied pharmacology* **241**(1): 81-89.
- Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, *et al.* (2009b). Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. *Proceedings of the National Academy of Sciences of the United States of America* **106**(8): 2933-2938.
- Chen W, Sun Z, Wang XJ, Jiang T, Huang Z, Fang D, *et al.* (2009c). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Molecular cell* **34**(6): 663-673.
- Cheng X, Ku CH, Siow RC (2013). Regulation of the Nrf2 antioxidant pathway by microRNAs: New players in micromanaging redox homeostasis. *Free radical biology & medicine* **64**: 4-11.
- Chia AJ, Goldring CE, Kitteringham NR, Wong SQ, Morgan P, Park BK (2010). Differential effect of covalent protein modification and glutathione depletion on the transcriptional response of Nrf2 and NF-kappaB. *Biochemical pharmacology* **80**(3): 410-421.
- Chin MP, Reisman SA, Bakris GL, O'Grady M, Linde PG, McCullough PA, *et al.* (2014). Mechanisms contributing to adverse cardiovascular events in patients with type 2 diabetes mellitus and stage 4 chronic kidney disease treated with bardoxolone methyl. *Am J Nephrol* **39**(6): 499-508.
- Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, *et al.* (2002). Role of NRF2 in protection against hyperoxic lung injury in mice. *American journal of respiratory cell and molecular biology* **26**(2): 175-182.

Cho JM, Manandhar S, Lee HR, Park HM, Kwak MK (2008). Role of the Nrf2-antioxidant system in cytotoxicity mediated by anticancer cisplatin: implication to cancer cell resistance. *Cancer letters* **260**(1-2): 96-108.

Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, *et al.* (2010). Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. *Free radical biology & medicine* **48**(2): 357-371.

Chowdhry S, Zhang Y, McMahon M, Sutherland C, Cuadrado A, Hayes JD (2013). Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* **32**(32): 3765-3781.

Clements CM, McNally RS, Conti BJ, Mak TW, Ting JP (2006). DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proceedings of the National Academy of Sciences of the United States of America* **103**(41): 15091-15096.

Copple IM (2012). The Keap1-Nrf2 cell defense pathway--a promising therapeutic target? *Advances in pharmacology* **63**: 43-79.

Copple IM, Goldring CE, Jenkins RE, Chia AJ, Randle LE, Hayes JD, *et al.* (2008a). The hepatotoxic metabolite of acetaminophen directly activates the Keap1-Nrf2 cell defense system. *Hepatology* **48**(4): 1292-1301.

Copple IM, Goldring CE, Kitteringham NR, Park BK (2008b). The Nrf2-Keap1 defence pathway: role in protection against drug-induced toxicity. *Toxicology* **246**(1): 24-33.

Copple IM, Lister A, Obeng AD, Kitteringham NR, Jenkins RE, Layfield R, *et al.* (2010). Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *The Journal of biological chemistry* **285**(22): 16782-16788.

Cullinan SB, Diehl JA (2004a). PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *The Journal of biological chemistry* **279**(19): 20108-20117.

Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA (2004b). The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Molecular and cellular biology* **24**(19): 8477-8486.

Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Molecular and cellular biology* **23**(20): 7198-7209.

de Zeeuw D, Akizawa T, Agarwal R, Audhya P, Bakris GL, Chin M, *et al.* (2013). Rationale and trial design of Bardoxolone Methyl Evaluation in Patients with

Chronic Kidney Disease and Type 2 Diabetes: the Occurrence of Renal Events (BEACON). *Am J Nephrol* **37**(3): 212-222.

Deeb D, Gao X, Liu Y, Kim SH, Pindolia KR, Arbab AS, *et al.* (2012a). Inhibition of cell proliferation and induction of apoptosis by oleanane triterpenoid (CDDO-Me) in pancreatic cancer cells is associated with the suppression of hTERT gene expression and its telomerase activity. *Biochemical and biophysical research communications* **422**(4): 561-567.

Deeb D, Gao X, Liu YB, Gautam SC (2012b). Inhibition of cell proliferation and induction of apoptosis by CDDO-Me in pancreatic cancer cells is ROS-dependent. *Journal of experimental therapeutics & oncology* **10**(1): 51-64.

DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, *et al.* (2011). Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* **475**(7354): 106-109.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, *et al.* (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* **99**(18): 11908-11913.

Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, *et al.* (2005a). Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci USA* **102**(12): 4584-4589.

Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, *et al.* (2005b). Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proceedings of the National Academy of Sciences of the United States of America* **102**(12): 4584-4589.

Droge W (2002). Free radicals in the physiological control of cell function. *Physiological reviews* **82**(1): 47-95.

Eades G, Yang M, Yao Y, Zhang Y, Zhou Q (2011). miR-200a regulates Nrf2 activation by targeting Keap1 mRNA in breast cancer cells. *The Journal of biological chemistry* **286**(47): 40725-40733.

Eggler AL, Small E, Hannink M, Mesecar AD (2009). Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1. *The Biochemical journal* **422**(1): 171-180.

Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, *et al.* (2001). High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing

enzymes and antioxidant genes. *Toxicological sciences : an official journal of the Society of Toxicology* **59**(1): 169-177.

Fan W, Tang Z, Chen D, Moughon D, Ding X, Chen S, *et al.* (2010). Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. *Autophagy* **6**(5): 614-621.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews. Genetics* **9**(2): 102-114.

Friling RS, Bensimon A, Tichauer Y, Daniel V (1990). Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. *Proceedings of the National Academy of Sciences of the United States of America* **87**(16): 6258-6262.

Fukutomi T, Takagi K, Mizushima T, Ohuchi N, Yamamoto M (2014). Kinetic, thermodynamic, and structural characterizations of the association between Nrf2-DLGex degron and Keap1. *Molecular and cellular biology* **34**(5): 832-846.

Furukawa M, Xiong Y (2005). BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Molecular and cellular biology* **25**(1): 162-171.

Gao L, Wang J, Sekhar KR, Yin H, Yared NF, Schneider SN, *et al.* (2007). Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3. *The Journal of biological chemistry* **282**(4): 2529-2537.

Goldring CE, Kitteringham NR, Elsby R, Randle LE, Clement YN, Williams DP, *et al.* (2004). Activation of hepatic Nrf2 in vivo by acetaminophen in CD-1 mice. *Hepatology* **39**(5): 1267-1276.

Gordon GB, Prochaska HJ, Yang LY (1991). Induction of NAD(P)H:quinone reductase in human peripheral blood lymphocytes. *Carcinogenesis* **12**(12): 2393-2396.

Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT (2010). ATM activation by oxidative stress. *Science* **330**(6003): 517-521.

Hall IH, Lee KH, Imakura Y, Okano M, Johnson A (1983). Anti-inflammatory agents III: Structure-activity relationships of brusatol and related quassinoids. *Journal of pharmaceutical sciences* **72**(11): 1282-1284.

Harada N, Ito K, Hosoya T, Mimura J, Maruyama A, Noguchi N, *et al.* (2012). Nrf2 in bone marrow-derived cells positively contributes to the advanced stage of atherosclerotic plaque formation. *Free radical biology & medicine* **53**(12): 2256-2262.

Hayes JD, Dinkova-Kostova AT (2014a). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends in biochemical sciences* **39**(4): 199-218.

Hayes JD, Dinkova-Kostova AT (2014b). The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends in biochemical sciences*.

Hayes JD, McMahon M, Chowdhry S, Dinkova-Kostova AT (2010). Cancer Chemoprevention Mechanisms Mediated Through the Keap1-Nrf2 Pathway. *Antioxid Redox Sign* **13**(11): 1713-1748.

He X, Chen MG, Lin GX, Ma Q (2006). Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2 x Keap1 x Cul3 complex and recruiting Nrf2 x Maf to the antioxidant response element enhancer. *The Journal of biological chemistry* **281**(33): 23620-23631.

He X, Chen MG, Ma Q (2008). Activation of Nrf2 in defense against cadmium-induced oxidative stress. *Chemical research in toxicology* **21**(7): 1375-1383.

He X, Ma Q (2009). NRF2 cysteine residues are critical for oxidant/electrophile-sensing, Kelch-like ECH-associated protein-1-dependent ubiquitination-proteasomal degradation, and transcription activation. *Molecular pharmacology* **76**(6): 1265-1278.

Healy ZR, Lee NH, Gao X, Goldring MB, Talalay P, Kensler TW, *et al.* (2005). Divergent responses of chondrocytes and endothelial cells to shear stress: cross-talk among COX-2, the phase 2 response, and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **102**(39): 14010-14015.

Hirotsu Y, Katsuoka F, Funayama R, Nagashima T, Nishida Y, Nakayama K, *et al.* (2012). Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. *Nucleic acids research* **40**(20): 10228-10239.

Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, *et al.* (2009). Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**(10): 3423-3432.

Hong F, Sekhar KR, Freeman ML, Liebler DC (2005). Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. *The Journal of biological chemistry* **280**(36): 31768-31775.

Hou W, Tian Q, Steuerwald NM, Schrum LW, Bonkovsky HL (2012). The let-7 microRNA enhances heme oxygenase-1 by suppressing Bach1 and attenuates oxidant injury in human hepatocytes. *Biochimica et biophysica acta* **1819**(11-12): 1113-1122.

- Hou W, Tian Q, Zheng J, Bonkovsky HL (2010). MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. *Hepatology* **51**(5): 1494-1504.
- Houtkooper RH, Pirinen E, Auwerx J (2012). Sirtuins as regulators of metabolism and healthspan. *Nature reviews. Molecular cell biology* **13**(4): 225-238.
- Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, *et al.* (2006). Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray. *Life sciences* **79**(20): 1944-1955.
- Huang HC, Nguyen T, Pickett CB (2000). Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proceedings of the National Academy of Sciences of the United States of America* **97**(23): 12475-12480.
- Huang HC, Nguyen T, Pickett CB (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *The Journal of biological chemistry* **277**(45): 42769-42774.
- Ichimura Y, Waguri S, Sou YS, Kageyama S, Hasegawa J, Ishimura R, *et al.* (2013). Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Molecular cell* **51**(5): 618-631.
- Ikeda H, Nishi S, Sakai M (2004). Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis. *The Biochemical journal* **380**(Pt 2): 515-521.
- Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, *et al.* (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications* **236**(2): 313-322.
- Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M (1995). Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Molecular and cellular biology* **15**(8): 4184-4193.
- Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, *et al.* (1999a). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & development* **13**(1): 76-86.
- Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, *et al.* (1999b). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Gene Dev* **13**(1): 76-86.



Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T, Yamamoto M (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to cells : devoted to molecular & cellular mechanisms* **8**(4): 379-391.

Iwasaki K, Hailemariam K, Tsuji Y (2007). PIAS3 interacts with ATF1 and regulates the human ferritin H gene through an antioxidant-responsive element. *Journal of Biological Chemistry* **282**(31): 22335-22343.

Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Overvatn A, *et al.* (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *The Journal of biological chemistry* **285**(29): 22576-22591.

Jain AK, Jaiswal AK (2006). Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *The Journal of biological chemistry* **281**(17): 12132-12142.

Jain AK, Jaiswal AK (2007). GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *The Journal of biological chemistry* **282**(22): 16502-16510.

Jazwa A, Rojo AI, Innamorato NG, Hesse M, Fernandez-Ruiz J, Cuadrado A (2011). Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism. *Antioxid Redox Signal* **14**(12): 2347-2360.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ (2009). Cancer statistics, 2009. *CA: a cancer journal for clinicians* **59**(4): 225-249.

Ji Y, Lee HJ, Goodman C, Uskokovic M, Liby K, Sporn M, *et al.* (2006). The synthetic triterpenoid CDDO-imidazolide induces monocytic differentiation by activating the Smad and ERK signaling pathways in HL60 leukemia cells. *Molecular cancer therapeutics* **5**(6): 1452-1458.

Jiang T, Chen N, Zhao F, Wang XJ, Kong B, Zheng W, *et al.* (2010a). High levels of Nrf2 determine chemoresistance in type II endometrial cancer. *Cancer research* **70**(13): 5486-5496.

Jiang T, Huang Z, Chan JY, Zhang DD (2009). Nrf2 protects against As(III)-induced damage in mouse liver and bladder. *Toxicology and applied pharmacology* **240**(1): 8-14.

Jiang T, Huang Z, Lin Y, Zhang Z, Fang D, Zhang DD (2010b). The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. *Diabetes* **59**(4): 850-860.

Jin W, Wang H, Yan W, Zhu L, Hu Z, Ding Y, *et al.* (2009). Role of Nrf2 in protection against traumatic brain injury in mice. *Journal of neurotrauma* **26**(1): 131-139.

Jung HJ, Suh Y (2012). MicroRNA in Aging: From Discovery to Biology. *Curr Genomics* **13**(7): 548-557.

Kanagal-Shamanna R, Zhao W, Vadhan-Raj S, Nguyen MH, Fernandez MH, Medeiros LJ, *et al.* (2012). Over-expression of CYP2E1 mRNA and protein: implications of xenobiotic induced damage in patients with de novo acute myeloid leukemia with inv(16)(p13.1q22); CBFbeta-MYH11. *International journal of environmental research and public health* **9**(8): 2788-2800.

Kang KW, Cho MK, Lee CH, Kim SG (2001). Activation of phosphatidylinositol 3-kinase and Akt by tert-butylhydroquinone is responsible for antioxidant response element-mediated rGSTA2 induction in H4IIE cells. *Molecular pharmacology* **59**(5): 1147-1156.

Kang MI, Kobayashi A, Wakabayashi N, Kim SG, Yamamoto M (2004). Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**(7): 2046-2051.

Karapetian RN, Evstafieva AG, Abaeva IS, Chichkova NV, Filonov GS, Rubtsov YP, *et al.* (2005). Nuclear oncoprotein prothymosin alpha is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Molecular and cellular biology* **25**(3): 1089-1099.

Kato Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes to cells : devoted to molecular & cellular mechanisms* **6**(10): 857-868.

Katsuoka F, Motohashi H, Ishii T, Aburatani H, Engel JD, Yamamoto M (2005). Genetic evidence that small Maf proteins are essential for the activation of antioxidant response element-dependent genes. *Molecular and cellular biology* **25**(18): 8044-8051.

Kay HY, Kim YW, Ryu da H, Sung SH, Hwang SJ, Kim SG (2011). Nrf2-mediated liver protection by sauchinone, an antioxidant lignan, from acetaminophen toxicity through the PKCdelta-GSK3beta pathway. *British journal of pharmacology* **163**(8): 1653-1665.

Kensler TW, Curphey TJ, Maxiutenko Y, Roebuck BD (2000). Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins. *Drug metabolism and drug interactions* **17**(1-4): 3-22.

Kensler TW, Wakabayashi N (2010). Nrf2: friend or foe for chemoprevention? *Carcinogenesis* **31**(1): 90-99.

Keum YS, Han YH, Liew C, Kim JH, Xu C, Yuan X, *et al.* (2006a). Induction of heme oxygenase-1 (HO-1) and NAD[P]H: quinone oxidoreductase 1 (NQO1) by a

phenolic antioxidant, butylated hydroxyanisole (BHA) and its metabolite, tert-butylhydroquinone (tBHQ) in primary-cultured human and rat hepatocytes. *Pharmaceutical research* **23**(11): 2586-2594.

Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, *et al.* (2006b). Mechanism of action of sulforaphane: inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 cells. *Cancer research* **66**(17): 8804-8813.

Kim JE, You DJ, Lee C, Ahn C, Seong JY, Hwang JI (2010). Suppression of NF-kappaB signaling by KEAP1 regulation of IKKbeta activity through autophagic degradation and inhibition of phosphorylation. *Cellular signalling* **22**(11): 1645-1654.

Kim SK, Yang JW, Kim MR, Roh SH, Kim HG, Lee KY, *et al.* (2008). Increased expression of Nrf2/ARE-dependent anti-oxidant proteins in tamoxifen-resistant breast cancer cells. *Free radical biology & medicine* **45**(4): 537-546.

Kim YJ, Ahn JY, Liang P, Ip C, Zhang Y, Park YM (2007). Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology. *Cancer research* **67**(2): 546-554.

Kitteringham NR, Abdullah A, Walsh J, Randle L, Jenkins RE, Sison R, *et al.* (2010). Proteomic analysis of Nrf2 deficient transgenic mice reveals cellular defence and lipid metabolism as primary Nrf2-dependent pathways in the liver. *Journal of proteomics* **73**(8): 1612-1631.

Klaassen CD, Reisman SA (2010). Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. *Toxicology and applied pharmacology* **244**(1): 57-65.

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, *et al.* (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and cellular biology* **24**(16): 7130-7139.

Kobayashi A, Kang MI, Watai Y, Tong KI, Shibata T, Uchida K, *et al.* (2006). Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. *Molecular and cellular biology* **26**(1): 221-229.

Kobayashi M, Itoh K, Suzuki T, Osanai H, Nishikawa K, Katoh Y, *et al.* (2002). Identification of the interactive interface and phylogenetic conservation of the Nrf2-Keap1 system. *Genes to cells: devoted to molecular & cellular mechanisms* **7**(8): 807-820.

Kode A, Rajendrasozhan S, Caito S, Yang SR, Megson IL, Rahman I (2008). Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial

cells. *American journal of physiology. Lung cellular and molecular physiology* **294**(3): L478-488.

Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, *et al.* (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature cell biology* **12**(3): 213-223.

Krol J, Loedige I, Filipowicz W (2010). The widespread regulation of microRNA biogenesis, function and decay. *Nature reviews. Genetics* **11**(9): 597-610.

Kulkarni SR, Donepudi AC, Xu J, Wei W, Cheng QC, Driscoll MV, *et al.* (2014). Fasting induces nuclear factor E2-related factor 2 and ATP-binding Cassette transporters via protein kinase A and Sirtuin-1 in mouse and human. *Antioxid Redox Signal* **20**(1): 15-30.

Kwak MK, Itoh K, Yamamoto M, Kensler TW (2002). Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Molecular and cellular biology* **22**(9): 2883-2892.

Kwak MK, Itoh K, Yamamoto M, Sutter TR, Kensler TW (2001). Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1, 2-dimethiole-3-thione. *Molecular medicine* **7**(2): 135-145.

Lau A, Wang XJ, Zhao F, Villeneuve NF, Wu T, Jiang T, *et al.* (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Molecular and cellular biology* **30**(13): 3275-3285.

Lee DF, Kuo HP, Liu M, Chou CK, Xia W, Du Y, *et al.* (2009). KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta. *Molecular cell* **36**(1): 131-140.

Lee EK, Kim JA, Park SJ, Kim JK, Heo K, Yang KM, *et al.* (2013). Low-dose radiation activates Nrf1/2 through reactive species and the Ca(2+)/ERK1/2 signaling pathway in human skin fibroblast cells. *BMB reports* **46**(5): 258-263.

Lee JM, Hanson JM, Chu WA, Johnson JA (2001). Phosphatidylinositol 3-kinase, not extracellular signal-regulated kinase, regulates activation of the antioxidant-responsive element in IMR-32 human neuroblastoma cells. *The Journal of biological chemistry* **276**(23): 20011-20016.

Leinonen HM, Ruotsalainen AK, Maatta AM, Laitinen HM, Kuosmanen SM, Kansanen E, *et al.* (2012). Oxidative stress-regulated lentiviral TK/GCV gene therapy for lung cancer treatment. *Cancer research* **72**(23): 6227-6235.

Li B, Wang X, Rasheed N, Hu Y, Boast S, Ishii T, *et al.* (2004). Distinct roles of c-Abl and Atm in oxidative stress response are mediated by protein kinase C delta. *Genes Dev* **18**(15): 1824-1837.

Li N, Muthusamy S, Liang R, Sarojini H, Wang E (2011). Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1. *Mechanisms of ageing and development* **132**(3): 75-85.

Li W, Jain MR, Chen C, Yue X, Hebbar V, Zhou R, *et al.* (2005). Nrf2 Possesses a redox-insensitive nuclear export signal overlapping with the leucine zipper motif. *The Journal of biological chemistry* **280**(31): 28430-28438.

Li W, Liu H, Zhou JS, Cao JF, Zhou XB, Choi AM, *et al.* (2012). Caveolin-1 inhibits expression of antioxidant enzymes through direct interaction with nuclear erythroid 2 p45-related factor-2 (Nrf2). *The Journal of biological chemistry* **287**(25): 20922-20930.

Li W, Thakor N, Xu EY, Huang Y, Chen C, Yu R, *et al.* (2010). An internal ribosomal entry site mediates redox-sensitive translation of Nrf2. *Nucleic acids research* **38**(3): 778-788.

Li W, Yu SW, Kong AN (2006). Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. *The Journal of biological chemistry* **281**(37): 27251-27263.

Liby K, Hock T, Yore MM, Suh N, Place AE, Risingsong R, *et al.* (2005). The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer research* **65**(11): 4789-4798.

Liby K, Voong N, Williams CR, Risingsong R, Royce DB, Honda T, *et al.* (2006). The synthetic triterpenoid CDDO-Imidazolide suppresses STAT phosphorylation and induces apoptosis in myeloma and lung cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**(14 Pt 1): 4288-4293.

Lim J, Lee SH, Cho S, Lee IS, Kang BY, Choi HJ (2013). 4-methoxychalcone enhances cisplatin-induced oxidative stress and cytotoxicity by inhibiting the Nrf2/ARE-mediated defense mechanism in A549 lung cancer cells. *Molecules and cells* **36**(4): 340-346.

Lin W, Shen G, Yuan X, Jain MR, Yu S, Zhang A, *et al.* (2006). Regulation of Nrf2 transactivation domain activity by p160 RAC3/SRC3 and other nuclear co-regulators. *Journal of biochemistry and molecular biology* **39**(3): 304-310.

Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P, *et al.* (2011). Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain : a journal of neurology* **134**(Pt 3): 678-692.

- Lister A, Nedjadi T, Kitteringham NR, Campbell F, Costello E, Lloyd B, *et al.* (2011). Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy. *Molecular cancer* **10**: 37.
- Liu GH, Qu J, Shen X (2008). NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochimica et biophysica acta* **1783**(5): 713-727.
- Lo SC, Li X, Henzl MT, Beamer LJ, Hannink M (2006). Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *The EMBO journal* **25**(15): 3605-3617.
- Louhelainen M, Merasto S, Finckenberg P, Lapatto R, Cheng ZJ, Mervaala EM (2006). Lipoic acid supplementation prevents cyclosporine-induced hypertension and nephrotoxicity in spontaneously hypertensive rats. *Journal of hypertension* **24**(5): 947-956.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry* **193**(1): 265-275.
- Lu H, Cui W, Klaassen CD (2011). Nrf2 protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced oxidative injury and steatohepatitis. *Toxicol Appl Pharmacol* **256**(2): 122-135.
- Lu SC (2009). Regulation of glutathione synthesis. *Molecular aspects of medicine* **30**(1-2): 42-59.
- Lu Y, Gong P, Cederbaum AI (2008). Pyrazole induced oxidative liver injury independent of CYP2E1/2A5 induction due to Nrf2 deficiency. *Toxicology* **252**(1-3): 9-16.
- Mahaffey CM, Mahaffey NC, Holland W, Zhang H, Gandara DR, Mack PC, *et al.* (2012). Aberrant regulation of the MRP3 gene in non-small cell lung carcinoma. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* **7**(1): 34-39.
- Mao L, Wang H, Wang X, Liao H, Zhao X (2011). Transcription factor Nrf2 protects the spinal cord from inflammation produced by spinal cord injury. *The Journal of surgical research* **170**(1): e105-115.
- Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lancken PN, *et al.* (2007). Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21**(9): 2237-2246.

- Mata-Greenwood E, Cuendet M, Sher D, Gustin D, Stock W, Pezzuto JM (2002). Brusatol-mediated induction of leukemic cell differentiation and G(1) arrest is associated with down-regulation of c-myc. *Leukemia* **16**(11): 2275-2284.
- McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, *et al.* (2001). The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer research* **61**(8): 3299-3307.
- McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD (2004). Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *The Journal of biological chemistry* **279**(30): 31556-31567.
- McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD (2006). Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *The Journal of biological chemistry* **281**(34): 24756-24768.
- McWalter GK, Higgins LG, McLellan LI, Henderson CJ, Song L, Thornalley PJ, *et al.* (2004). Transcription factor Nrf2 is essential for induction of NAD(P)H:quinone oxidoreductase 1, glutathione S-transferases, and glutamate cysteine ligase by broccoli seeds and isothiocyanates. *The Journal of nutrition* **134**(12 Suppl): 3499S-3506S.
- Meakin PJ, Chowdhry S, Sharma RS, Ashford FB, Walsh SV, McCrimmon RJ, *et al.* (2014). Susceptibility of Nrf2-Null Mice to Steatohepatitis and Cirrhosis upon Consumption of a High-Fat Diet Is Associated with Oxidative Stress, Perturbation of the Unfolded Protein Response, and Disturbance in the Expression of Metabolic Enzymes but Not with Insulin Resistance. *Molecular and cellular biology* **34**(17): 3305-3320.
- Miao W, Hu L, Scrivens PJ, Batist G (2005). Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *The Journal of biological chemistry* **280**(21): 20340-20348.
- Minones-Moyano E, Porta S, Escaramis G, Rabionet R, Iraola S, Kagerbauer B, *et al.* (2011). MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. *Human molecular genetics* **20**(15): 3067-3078.
- Miseta A, Csutora P (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Molecular biology and evolution* **17**(8): 1232-1239.

Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, *et al.* (2012). Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer cell* **22**(1): 66-79.

Moi P, Chan K, Asunis I, Cao A, Kan YW (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences of the United States of America* **91**(21): 9926-9930.

Molina-Jijon E, Tapia E, Zazueta C, El Hafidi M, Zatarain-Barron ZL, Hernandez-Pando R, *et al.* (2011). Curcumin prevents Cr(VI)-induced renal oxidant damage by a mitochondrial pathway. *Free radical biology & medicine* **51**(8): 1543-1557.

Moolten FL (1986). Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer research* **46**(10): 5276-5281.

Moscat J, Diaz-Meco MT (2009). p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell* **137**(6): 1001-1004.

Motohashi H, Katsuoka F, Engel JD, Yamamoto M (2004). Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proceedings of the National Academy of Sciences of the United States of America* **101**(17): 6379-6384.

Motohashi H, O'Connor T, Katsuoka F, Engel JD, Yamamoto M (2002). Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* **294**(1-2): 1-12.

Muscarella LA, Barbano R, D'Angelo V, Copetti M, Coco M, Balsamo T, *et al.* (2011). Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome. *Epigenetics : official journal of the DNA Methylation Society* **6**(3): 317-325.

Nair S, Doh ST, Chan JY, Kong AN, Cai L (2008). Regulatory potential for concerted modulation of Nrf2- and Nfkb1-mediated gene expression in inflammation and carcinogenesis. *British journal of cancer* **99**(12): 2070-2082.

Nair S, Xu C, Shen G, Hebbar V, Gopalakrishnan A, Hu R, *et al.* (2007). Toxicogenomics of endoplasmic reticulum stress inducer tunicamycin in the small intestine and liver of Nrf2 knockout and C57BL/6J mice. *Toxicology letters* **168**(1): 21-39.

Narasimhan M, Patel D, Vedpathak D, Rathinam M, Henderson G, Mahimainathan L (2012). Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells. *PloS one* **7**(12): e51111.



- Nezis IP, Stenmark H (2012). p62 at the interface of autophagy, oxidative stress signaling, and cancer. *Antioxid Redox Signal* **17**(5): 786-793.
- Nguyen T, Sherratt PJ, Huang HC, Yang CS, Pickett CB (2003). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *The Journal of biological chemistry* **278**(7): 4536-4541.
- Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB (2005). Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *The Journal of biological chemistry* **280**(37): 32485-32492.
- Nioi P, McMahon M, Itoh K, Yamamoto M, Hayes JD (2003). Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *The Biochemical journal* **374**(Pt 2): 337-348.
- Niture SK, Jaiswal AK (2009). Prothymosin-alpha mediates nuclear import of the INrf2/Cul3 Rbx1 complex to degrade nuclear Nrf2. *The Journal of biological chemistry* **284**(20): 13856-13868.
- Oeckinghaus A, Hayden MS, Ghosh S (2011). Crosstalk in NF-kappaB signaling pathways. *Nature immunology* **12**(8): 695-708.
- Ohkoshi A, Suzuki T, Ono M, Kobayashi T, Yamamoto M (2013). Roles of Keap1-Nrf2 system in upper aerodigestive tract carcinogenesis. *Cancer prevention research* **6**(2): 149-159.
- Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, *et al.* (2008). Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer research* **68**(5): 1303-1309.
- Oikawa D, Akai R, Tokuda M, Iwawaki T (2012). A transgenic mouse model for monitoring oxidative stress. *Scientific reports* **2**: 229.
- Okawa H, Motohashi H, Kobayashi A, Aburatani H, Kensler TW, Yamamoto M (2006). Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. *Biochemical and biophysical research communications* **339**(1): 79-88.
- Osburn WO, Kensler TW (2008). Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutation research* **659**(1-2): 31-39.
- Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, *et al.* (2002). Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Annals of internal medicine* **137**(12): 947-954.

- Padmanabhan B, Tong KI, Kobayashi A, Yamamoto M, Yokoyama S (2008). Structural insights into the similar modes of Nrf2 transcription factor recognition by the cytoplasmic repressor Keap1. *Journal of synchrotron radiation* **15**(Pt 3): 273-276.
- Padmanabhan B, Tong KI, Ohta T, Nakamura Y, Scharlock M, Ohtsuji M, *et al.* (2006). Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Molecular cell* **21**(5): 689-700.
- Papp D, Lenti K, Modos D, Fazekas D, Dul Z, Turei D, *et al.* (2012). The NRF2-related interactome and regulome contain multifunctional proteins and fine-tuned autoregulatory loops. *FEBS letters* **586**(13): 1795-1802.
- Park BK, Laverty H, Srivastava A, Antoine DJ, Naisbitt D, Williams DP (2011). Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. *Chemico-biological interactions* **192**(1-2): 30-36.
- Perez-Leal O, Barrero CA, Merali S (2013). Translational control of Nrf2 within the open reading frame. *Biochemical and biophysical research communications* **437**(1): 134-139.
- Pergola PE, Krauth M, Huff JW, Ferguson DA, Ruiz S, Meyer CJ, *et al.* (2011a). Effect of bardoxolone methyl on kidney function in patients with T2D and Stage 3b-4 CKD. *Am J Nephrol* **33**(5): 469-476.
- Pergola PE, Raskin P, Toto RD, Meyer CJ, Huff JW, Grossman EB, *et al.* (2011b). Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *The New England journal of medicine* **365**(4): 327-336.
- Pi J, Bai Y, Reece JM, Williams J, Liu D, Freeman ML, *et al.* (2007). Molecular mechanism of human Nrf2 activation and degradation: role of sequential phosphorylation by protein kinase CK2. *Free radical biology & medicine* **42**(12): 1797-1806.
- Prabu SM, Muthumani M (2012). Silibinin ameliorates arsenic induced nephrotoxicity by abrogation of oxidative stress, inflammation and apoptosis in rats. *Molecular biology reports* **39**(12): 11201-11216.
- Purdum-Dickinson SE, Sheveleva EV, Sun H, Chen QM (2007). Translational control of nrf2 protein in activation of antioxidant response by oxidants. *Molecular pharmacology* **72**(4): 1074-1081.
- Qin Y, Deng W, Ekmekcioglu S, Grimm EA (2013). Identification of unique sensitizing targets for anti-inflammatory CDDO-Me in metastatic melanoma by a large-scale synthetic lethal RNAi screening. *Pigment cell & melanoma research* **26**(1): 97-112.
- Rabinowitz JD, Vastag L (2012). Teaching the design principles of metabolism. *Nature chemical biology* **8**(6): 497-501.

Rachakonda G, Xiong Y, Sekhar KR, Stamer SL, Liebler DC, Freeman ML (2008). Covalent modification at Cys151 dissociates the electrophile sensor Keap1 from the ubiquitin ligase CUL3. *Chemical research in toxicology* **21**(3): 705-710.

Rada P, Rojo AI, Chowdhry S, McMahon M, Hayes JD, Cuadrado A (2011). SCF/ $\beta$ -TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Molecular and cellular biology* **31**(6): 1121-1133.

Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, *et al.* (2001). Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **98**(6): 3410-3415.

Reddy NM, Kleeberger SR, Bream JH, Fallon PG, Kensler TW, Yamamoto M, *et al.* (2008). Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. *Oncogene* **27**(44): 5821-5832.

Reddy PV, Gandhi N, Samikkannu T, Saiyed Z, Agudelo M, Yndart A, *et al.* (2012). HIV-1 gp120 induces antioxidant response element-mediated expression in primary astrocytes: role in HIV associated neurocognitive disorder. *Neurochemistry international* **61**(5): 807-814.

Reisman SA, Buckley DB, Tanaka Y, Klaassen CD (2009). CDDO-Im protects from acetaminophen hepatotoxicity through induction of Nrf2-dependent genes. *Toxicology and applied pharmacology* **236**(1): 109-114.

Ren D, Villeneuve NF, Jiang T, Wu T, Lau A, Toppin HA, *et al.* (2011). Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **108**(4): 1433-1438.

Rojo AI, Medina-Campos ON, Rada P, Zuniga-Toala A, Lopez-Gazcon A, Espada S, *et al.* (2012). Signaling pathways activated by the phytochemical nordihydroguaiaretic acid contribute to a Keap1-independent regulation of Nrf2 stability: Role of glycogen synthase kinase-3. *Free radical biology & medicine* **52**(2): 473-487.

Rushmore TH, King RG, Paulson KE, Pickett CB (1990a). Regulation of glutathione S-transferase Ya subunit gene expression: identification of a unique xenobiotic-responsive element controlling inducible expression by planar aromatic compounds. *Proceedings of the National Academy of Sciences of the United States of America* **87**(10): 3826-3830.

Rushmore TH, Pickett CB (1990b). Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-

responsive element controlling inducible expression by phenolic antioxidants. *The Journal of biological chemistry* **265**(24): 14648-14653.

Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A (2006). Glycogen synthase kinase-3 $\beta$  inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *The Journal of biological chemistry* **281**(21): 14841-14851.

Sangokoya C, Telen MJ, Chi JT (2010). microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood* **116**(20): 4338-4348.

Schneider KS, Chan JY (2013). Emerging role of Nrf2 in adipocytes and adipose biology. *Advances in nutrition* **4**(1): 62-66.

Sekhar KR, Soltaninassab SR, Borrelli MJ, Xu ZQ, Meredith MJ, Domann FE, *et al.* (2000). Inhibition of the 26S proteasome induces expression of GLCLC, the catalytic subunit for gamma-glutamylcysteine synthetase. *Biochemical and biophysical research communications* **270**(1): 311-317.

Severino G, Del Zompo M (2004). Adverse drug reactions: role of pharmacogenomics. *Pharmacological research : the official journal of the Italian Pharmacological Society* **49**(4): 363-373.

Shelton LM, Park BK, Copple IM (2013). Role of Nrf2 in protection against acute kidney injury. *Kidney international* **84**(6): 1090-1095.

Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M, *et al.* (2008a). Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* **135**(4): 1358-1368, 1368 e1351-1354.

Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, *et al.* (2008b). Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proceedings of the National Academy of Sciences of the United States of America* **105**(36): 13568-13573.

Shilo S, Roy S, Khanna S, Sen CK (2008). Evidence for the involvement of miRNA in redox regulated angiogenic response of human microvascular endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **28**(3): 471-477.

Shin S, Wakabayashi J, Yates MS, Wakabayashi N, Dolan PM, Aja S, *et al.* (2009). Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. *European journal of pharmacology* **620**(1-3): 138-144.

Siegel D, Yan C, Ross D (2012). NAD(P)H:quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochemical pharmacology* **83**(8): 1033-1040.

Singh A, Boldin-Adamsky S, Thimmulappa RK, Rath SK, Ashush H, Coulter J, *et al.* (2008). RNAi-mediated silencing of nuclear factor erythroid-2-related factor 2 gene expression in non-small cell lung cancer inhibits tumor growth and increases efficacy of chemotherapy. *Cancer research* **68**(19): 7975-7984.

Singh A, Happel C, Manna SK, Acquah-Mensah G, Carrerero J, Kumar S, *et al.* (2013). Transcription factor NRF2 regulates miR-1 and miR-206 to drive tumorigenesis. *The Journal of clinical investigation* **123**(7): 2921-2934.

Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, *et al.* (2006). Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS medicine* **3**(10): e420.

Skoko JJ, Wakabayashi N, Noda K, Kimura S, Tobita K, Shigemura N, *et al.* (2014). Loss of nrf2 in mice evokes a congenital intrahepatic shunt that alters hepatic oxygen and protein expression gradients and toxicity. *Toxicological sciences: an official journal of the Society of Toxicology* **141**(1): 112-119.

Smirnova NA, Haskew-Layton RE, Basso M, Hushpalian DM, Payappilly JB, Speer RE, *et al.* (2011). Development of Neh2-luciferase reporter and its application for high throughput screening and real-time monitoring of Nrf2 activators. *Chemistry & biology* **18**(6): 752-765.

Sporn MB, Liby KT (2012). NRF2 and cancer: the good, the bad and the importance of context. *Nature reviews. Cancer* **12**(8): 564-571.

Sporn MB, Liby KT, Yore MM, Fu L, Lopchuk JM, Gribble GW (2011). New synthetic triterpenoids: potent agents for prevention and treatment of tissue injury caused by inflammatory and oxidative stress. *Journal of natural products* **74**(3): 537-545.

Stachurska A, Ciesla M, Kozakowska M, Wolfram S, Boesch-Saadatmandi C, Rimbach G, *et al.* (2013). Cross-talk between microRNAs, nuclear factor E2-related factor 2, and heme oxygenase-1 in ochratoxin A-induced toxic effects in renal proximal tubular epithelial cells. *Molecular nutrition & food research* **57**(3): 504-515.

Stack C, Ho D, Wille E, Calingasan NY, Williams C, Liby K, *et al.* (2010). Triterpenoids CDDO-ethyl amide and CDDO-trifluoroethyl amide improve the behavioral phenotype and brain pathology in a transgenic mouse model of Huntington's disease. *Free radical biology & medicine* **49**(2): 147-158.

Stewart D, Killeen E, Naquin R, Alam S, Alam J (2003). Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *The Journal of biological chemistry* **278**(4): 2396-2402.

- Stocker R, Keaney JF, Jr. (2004). Role of oxidative modifications in atherosclerosis. *Physiological reviews* **84**(4): 1381-1478.
- Strachan GD, Morgan KL, Otis LL, Caltagarone J, Gittis A, Bowser R, *et al.* (2004). Fetal Alz-50 clone 1 interacts with the human orthologue of the Kelch-like ECH-associated protein. *Biochemistry* **43**(38): 12113-12122.
- Suh N, Roberts AB, Birkey Reffey S, Miyazono K, Itoh S, ten Dijke P, *et al.* (2003). Synthetic triterpenoids enhance transforming growth factor beta/Smad signaling. *Cancer research* **63**(6): 1371-1376.
- Sun GB, Sun X, Wang M, Ye JX, Si JY, Xu HB, *et al.* (2012). Oxidative stress suppression by luteolin-induced heme oxygenase-1 expression. *Toxicology and applied pharmacology* **265**(2): 229-240.
- Sun Z, Huang Z, Zhang DD (2009). Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. *PLoS one* **4**(8): e6588.
- Sussan TE, Jun J, Thimmulappa R, Bedja D, Antero M, Gabrielson KL, *et al.* (2008). Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice. *PLoS one* **3**(11): e3791.
- Suzuki M, Betsuyaku T, Ito Y, Nagai K, Nasuhara Y, Kaga K, *et al.* (2008). Down-regulated NF-E2-related factor 2 in pulmonary macrophages of aged smokers and patients with chronic obstructive pulmonary disease. *American journal of respiratory cell and molecular biology* **39**(6): 673-682.
- Taguchi K, Maher JM, Suzuki T, Kawatani Y, Motohashi H, Yamamoto M (2010). Genetic analysis of cytoprotective functions supported by graded expression of Keap1. *Molecular and cellular biology* **30**(12): 3016-3026.
- Taguchi K, Motohashi H, Yamamoto M (2011). Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes to cells : devoted to molecular & cellular mechanisms* **16**(2): 123-140.
- Takahashi T, Sonobe M, Menju T, Nakayama E, Mino N, Iwakiri S, *et al.* (2010). Mutations in Keap1 are a potential prognostic factor in resected non-small cell lung cancer. *Journal of surgical oncology* **101**(6): 500-506.
- Takaya K, Suzuki T, Motohashi H, Onodera K, Satomi S, Kensler TW, *et al.* (2012). Validation of the multiple sensor mechanism of the Keap1-Nrf2 system. *Free radical biology & medicine* **53**(4): 817-827.
- Tanaka Y, Aleksunes LM, Yeager RL, Gyamfi MA, Esterly N, Guo GL, *et al.* (2008). NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet. *J Pharmacol Exp Ther* **325**(2): 655-664.

- Tang X, Wang H, Fan L, Wu X, Xin A, Ren H, *et al.* (2011). Luteolin inhibits Nrf2 leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs. *Free radical biology & medicine* **50**(11): 1599-1609.
- Tayek JA, Kalantar-Zadeh K (2013). The Extinguished BEACON of Bardoxolone: Not a Monday Morning Quarterback Story. *American journal of nephrology* **37**(3): 208-211.
- Thimmulappa RK, Fuchs RJ, Malhotra D, Scollick C, Traore K, Bream JH, *et al.* (2007). Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. *Antioxid Redox Signal* **9**(11): 1963-1970.
- Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer research* **62**(18): 5196-5203.
- Tong KI, Katoh Y, Kusunoki H, Itoh K, Tanaka T, Yamamoto M (2006). Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Molecular and cellular biology* **26**(8): 2887-2900.
- Umemura T, Kuroiwa Y, Kitamura Y, Ishii Y, Kanki K, Kodama Y, *et al.* (2006). A crucial role of Nrf2 in in vivo defense against oxidative damage by an environmental pollutant, pentachlorophenol. *Toxicological sciences : an official journal of the Society of Toxicology* **90**(1): 111-119.
- Ungvari Z, Tucsek Z, Sosnowska D, Toth P, Gautam T, Podlutzky A, *et al.* (2013). Aging-induced dysregulation of dicer1-dependent microRNA expression impairs angiogenic capacity of rat cerebrovascular endothelial cells. *The journals of gerontology. Series A, Biological sciences and medical sciences* **68**(8): 877-891.
- Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology* **39**(1): 44-84.
- Van Laecke S, Van Biesen W, Vanholder R (2014). The paradox of bardoxolone methyl: a call for every witness on the stand? *Diabetes, obesity & metabolism*.
- Vasa-Nicotera M, Chen H, Tucci P, Yang AL, Saintigny G, Menghini R, *et al.* (2011). miR-146a is modulated in human endothelial cell with aging. *Atherosclerosis* **217**(2): 326-330.

Velichkova M, Hasson T (2005). Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Molecular and cellular biology* **25**(11): 4501-4513.

Venugopal R, Jaiswal AK (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proceedings of the National Academy of Sciences of the United States of America* **93**(25): 14960-14965.

von Otter M, Landgren S, Nilsson S, Zetterberg M, Celojovic D, Bergstrom P, *et al.* (2010). Nrf2-encoding NFE2L2 haplotypes influence disease progression but not risk in Alzheimer's disease and age-related cataract. *Mechanisms of ageing and development* **131**(2): 105-110.

Wagner AE, Boesch-Saadatmandi C, Dose J, Schultheiss G, Rimbach G (2012). Anti-inflammatory potential of allyl-isothiocyanate--role of Nrf2, NF-(kappa) B and microRNA-155. *Journal of cellular and molecular medicine* **16**(4): 836-843.

Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, *et al.* (2004). Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proceedings of the National Academy of Sciences of the United States of America* **101**(7): 2040-2045.

Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S, *et al.* (2003). Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nature genetics* **35**(3): 238-245.

Wakabayashi N, Shin S, Slocum SL, Agoston ES, Wakabayashi J, Kwak MK, *et al.* (2010). Regulation of notch1 signaling by nrf2: implications for tissue regeneration. *Science signaling* **3**(130): ra52.

Walsh J, Jenkins RE, Wong M, Olayanju A, Powell H, Copple I, *et al.* (2014). Identification and quantification of the basal and inducible Nrf2-dependent proteomes in mouse liver: Biochemical, pharmacological and toxicological implications. *Journal of proteomics*.

Wang B, Jin F, Xie Y, Tang Y, Kan R, Zheng C, *et al.* (2006). Association analysis of NAD(P)H:quinone oxidoreductase gene 609 C/T polymorphism with Alzheimer's disease. *Neuroscience letters* **409**(3): 179-181.

Wang H, Liu K, Geng M, Gao P, Wu X, Hai Y, *et al.* (2013). RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer research* **73**(10): 3097-3108.

Wang J, Zhang M, Zhang L, Cai H, Zhou S, Zhang J, *et al.* (2010). Correlation of Nrf2, HO-1, and MRP3 in gallbladder cancer and their relationships to



clinicopathologic features and survival. *The Journal of surgical research* **164**(1): e99-105.

Wang XJ, Hayes JD, Henderson CJ, Wolf CR (2007). Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha. *Proceedings of the National Academy of Sciences of the United States of America* **104**(49): 19589-19594.

Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, *et al.* (2008). Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* **29**(6): 1235-1243.

Wang XJ, Zhang DD (2009). Ectodermal-neural cortex 1 down-regulates Nrf2 at the translational level. *PLoS one* **4**(5): e5492.

Watai Y, Kobayashi A, Nagase H, Mizukami M, McEvoy J, Singer JD, *et al.* (2007). Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes to cells : devoted to molecular & cellular mechanisms* **12**(10): 1163-1178.

Willingham W, 3rd, Considine RT, Chaney SG, Lee KH, Hall IH (1984). Reversibility of protein synthesis inhibition by quassinoid antineoplastic agents in a rabbit reticulocyte system. *Biochemical pharmacology* **33**(2): 330-333.

Willingham W, Jr., Stafford EA, Reynolds SH, Chaney SG, Lee KH, Okano M, *et al.* (1981). Mechanism of eukaryotic protein synthesis inhibition by brusatol. *Biochimica et biophysica acta* **654**(2): 169-174.

Wolf CR (2001). Chemoprevention: increased potential to bear fruit. *Proceedings of the National Academy of Sciences of the United States of America* **98**(6): 2941-2943.

Wu JH, Miao W, Hu LG, Batist G (2010). Identification and characterization of novel Nrf2 inducers designed to target the intervening region of Keap1. *Chemical biology & drug design* **75**(5): 475-480.

Wu KC, Cui JY, Klaassen CD (2011a). Beneficial role of Nrf2 in regulating NADPH generation and consumption. *Toxicological sciences : an official journal of the Society of Toxicology* **123**(2): 590-600.

Wu QQ, Wang Y, Senitko M, Meyer C, Wigley WC, Ferguson DA, *et al.* (2011b). Bardoxolone methyl (BARD) ameliorates ischemic AKI and increases expression of protective genes Nrf2, PPARgamma, and HO-1. *American journal of physiology. Renal physiology* **300**(5): F1180-1192.

Xu W, Hellerbrand C, Kohler UA, Bugnon P, Kan YW, Werner S, *et al.* (2008). The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. *Laboratory investigation; a journal of technical methods and pathology* **88**(10): 1068-1078.

Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H, *et al.* (2008). Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. *Molecular and cellular biology* **28**(8): 2758-2770.

Yan XH, Chen J, Di YT, Fang X, Dong JH, Sang P, *et al.* (2010). Anti-tobacco mosaic virus (TMV) Quassinoids from *Brucea javanica* (L.) Merr. *Journal of agricultural and food chemistry* **58**(3): 1572-1577.

Yang M, Yao Y, Eades G, Zhang Y, Zhou Q (2011). MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. *Breast cancer research and treatment* **129**(3): 983-991.

Yang W, Chen L, Ding Y, Zhuang X, Kang UJ (2007). Paraquat induces dopaminergic dysfunction and proteasome impairment in DJ-1-deficient mice. *Human molecular genetics* **16**(23): 2900-2910.

Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T, *et al.* (2007). Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Molecular cancer therapeutics* **6**(1): 154-162.

Yates MS, Tran QT, Dolan PM, Osburn WO, Shin S, McCulloch CC, *et al.* (2009). Genetic versus chemoprotective activation of Nrf2 signaling: overlapping yet distinct gene expression profiles between Keap1 knockout and triterpenoid-treated mice. *Carcinogenesis* **30**(6): 1024-1031.

Yoh K, Hirayama A, Ishizaki K, Yamada A, Takeuchi M, Yamagishi S, *et al.* (2008). Hyperglycemia induces oxidative and nitrosative stress and increases renal functional impairment in Nrf2-deficient mice. *Genes to cells : devoted to molecular & cellular mechanisms* **13**(11): 1159-1170.

Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, Kobayashi M, *et al.* (2001). Nrf2-deficient female mice develop lupus-like autoimmune nephritis. *Kidney international* **60**(4): 1343-1353.

Yoon HY, Kang NI, Lee HK, Jang KY, Park JW, Park BH (2008). Sulforaphane protects kidneys against ischemia-reperfusion injury through induction of the Nrf2-dependent phase 2 enzyme. *Biochemical pharmacology* **75**(11): 2214-2223.

Yore MM, Liby KT, Honda T, Gribble GW, Sporn MB (2006). The synthetic triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole blocks nuclear factor-kappa B activation through direct inhibition of I kappa B kinase beta. *Molecular cancer therapeutics* **5**(12): 3232-3239.

Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, *et al.* (1999). Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *The Journal of biological chemistry* **274**(39): 27545-27552.

Yu R, Mandlekar S, Lei W, Fahl WE, Tan TH, Kong AN (2000). p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. *The Journal of biological chemistry* **275**(4): 2322-2327.

Yu Z, Shao W, Chiang Y, Foltz W, Zhang Z, Ling W, *et al.* (2011). Oltipraz upregulates the nuclear factor (erythroid-derived 2)-like 2 [corrected](NRF2) antioxidant system and prevents insulin resistance and obesity induced by a high-fat diet in C57BL/6J mice. *Diabetologia* **54**(4): 922-934.

Yuan X, Xu C, Pan Z, Keum YS, Kim JH, Shen G, *et al.* (2006). Butylated hydroxyanisole regulates ARE-mediated gene expression via Nrf2 coupled with ERK and JNK signaling pathway in HepG2 cells. *Molecular carcinogenesis* **45**(11): 841-850.

Zenkov NK, Menshchikova EB, Tkachev VO (2013). Keap1/Nrf2/ARE redox-sensitive signaling system as a pharmacological target. *Biochemistry. Biokhimiia* **78**(1): 19-36.

Zhang DD, Hannink M (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Molecular and cellular biology* **23**(22): 8137-8151.

Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Molecular and cellular biology* **24**(24): 10941-10953.

Zhang DD, Lo SC, Sun Z, Habib GM, Lieberman MW, Hannink M (2005). Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway. *The Journal of biological chemistry* **280**(34): 30091-30099.

Zhang J, Ohta T, Maruyama A, Hosoya T, Nishikawa K, Maher JM, *et al.* (2006). BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress. *Molecular and cellular biology* **26**(21): 7942-7952.

Zhang M, An C, Gao Y, Leak RK, Chen J, Zhang F (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in neurobiology* **100**: 30-47.

Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, *et al.* (2010). Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. *Molecular cancer therapeutics* **9**(2): 336-346.

Zhao M, Lau ST, Leung PS, Che CT, Lin ZX (2011a). Seven quassinoids from Fructus Bruceae with cytotoxic effects on pancreatic adenocarcinoma cell lines. *Phytotherapy research : PTR* **25**(12): 1796-1800.

Zhao Z, Chen Y, Wang J, Sternberg P, Freeman ML, Grossniklaus HE, *et al.* (2011b). Age-related retinopathy in NRF2-deficient mice. *PloS one* **6**(4): e19456.

Zheng Y, Morris A, Sunkara M, Layne J, Toborek M, Hennig B (2012). Epigallocatechin-gallate stimulates NF-E2-related factor and heme oxygenase-1 via caveolin-1 displacement. *The Journal of nutritional biochemistry* **23**(2): 163-168.