INVESTIGATING THE PUTATIVE THERAPEUTIC EFFECTS OF SULFORAPHANE IN THE HUMAN LENS

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By

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Abstract

Purpose: Cataract (a clouding of the lens) and PCO, a lens wound-healing response that occurs following cataract surgery, are important medical problems affecting millions worldwide. Reducing the incidence of these conditions would have a marked effect on the personal well being of millions of people. The isothiocyanate, SFN is known to have dose-dependent cytoprotective and cytotoxic properties. The present study had three aims. Firstly, to determine if the isothiocyanate, SFN could yield protection to lens cells against oxidative stress. Secondly, to identify the mechanisms by which SFN can elicit cytoprotection to lens cells against oxidative stress. The final aim was to establish the ability of SFN to initiate death of lens cells and prevent PCO formation.

Methods: The human lens epithelial cell line FHL 124 was used in combination with whole porcine lens culture (to assess opacity) and human capsular bags (as a PCO model) were employed as the experimental systems. Whole lens cultures were monitored using brightfield and darkfield imaging; quantification was performed with ImageJ analysis software. Capsular bags were monitored using phase-contrast microscopy. The ApoToxGlo Triplex assay was used to assess FHL 124 cell survival, cytotoxicity and apoptosis. The MTS assay was used to assess cell populations. To determine levels of DNA strand breaks, the alkaline comet assay was performed and quantified. Lactate dehydrogenase levels in the medium (of FHL 124 cells and porcine lenses) were evaluated to reflect cell damage/death. To assess level of gene expression an Illumina whole genome HT-12 v4 beadchip microarray was employed. Real-time PCR was used to assess ER stress gene expression. Protein expression was validated by Western blot and immunocytochemistry (FHL 124 cells and capsular bags).

Results: 30 μM H_2O_2 exposure to FHL 124 cells caused a significant reduction in cell viability and increased cytotoxicity/apoptosis; these effects were significantly inhibited (~80%) by 24 hours pre-treatment with 1 μM SFN. In addition, 1 μM SFN significantly reduced H_2O_2-induced DNA strand breaks. When applied to cultured porcine lenses, SFN protected against H_2O_2-induced opacification. Illumina whole genome HT-12 v4 beadchip microarray data revealed 8 genes up-regulated following 24 hours exposure to 1 μM or 2 μM SFN. These included NQO1 and TXNRD1, which also demonstrated upregulation at the protein level. Nrf2 was found to actively translocate to the cell nucleus in response to a four hour exposure to 0.5, 1 and 2 μM SFN. SFN was found to induce apoptosis and ER stress in FHL 124 cells at SFN concentrations 10 μM and higher. Application of these concentrations of SFN to capsular bag cultures demonstrated a retardation of cell growth at 10 μM and complete ablation with 100 μM SFN

Conclusions: The dietary component SFN, at low concentrations (≤5 μM) demonstrates an ability to protect human lens cells against oxidative stress and thus could potentially delay the onset of cataract. It is likely that this protection is mediated by SFN induced Nrf2 signalling. Higher concentrations (≥10 μM) of SFN are capable of inducing ER stress and cell death in human lens epithelial cells and thus provide a novel agent to be used in the prevention of PCO.

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List of Abbreviations

AC	anterior chamber
ACO	anterior capsule opacification
AITC	ally isothiocyanate
AIF	apoptosis inducing factor
ANOVA	analysis of variance
APL	acute promyelocytic leukemia
ARE	antioxidant response element
As_2O_3	arsenic trioxide
BITC	benzyl isothiocyanate
BSA	bovine serum albumin
CB	capsular bag
CDK	cyclin-dependent kinase
CDKIs	cyclin dependent kinase inhibitors
CGase	cysteinylglycinase
CRY	cytochrome P450
CT	cycle threshold
CYP	cytochrome P450
d	days
dH_2O	double distilled water
DMEs	drug-metabolising enzymes
DSBs	double strand breaks
EAEB	East Anglia Eye Bank
ECCE	extracapsular cataract extraction
ECM	extracapsular matrix
EMEM	eagle's minimal essential medium
ER	endoplasmic reticulum
FCS	foetal calf serum
FHL	foetal human lens
FTL	ferritin
Fz	wnts fizzled
h	hours
HIF-1	hypoxia-inducible factor-1

HO-1	heme oxygenase-1
H_2O_2	hydrogen peroxide
ICCE	intracapsular cataract extraction
INT	iodotetrazolium chloride
IOL	intraocular lens
ITC	isothiocyanate
G6PD	glucose-6-phosphate dehydrogenase
GEO	gene expression omnibus
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSTs	glutathione S-transferases
GTP	γ-glutamyl transpeptidase
Keap 1	kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
LECs	lens epithelial cells
LENF	lens epithelial necrosis factor
lfc	log fold change
LOCH	lens organ culture with H_2O_2
mRNA	messenger RNA
MM	multiple myeloma
MRPs	multidrug resistant proteins
NAT	n-acetyltransferase
NDMA	n-nitrosodimethylamine
NHEJ	non-homologous end joining
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PC	posterior chamber
PCO	posterior capsule opacification
PCR	polymerase chain reaction
P/ECCE	phacoemulsification
PEITC	phenethyl isothiocyanate
PIR	pirin
PSC	posterior subcapsular cataract

PMMA	polymethylmethacrylate
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene fluoride
QR	quinine oxidoreductase
RIN	RNA integrity number
RNA	ribonucleic
RNase	ribonuclease
ROS	reactive oxygen species
SCGE	single cell gel electrophoresis
SDS	sodium dodecyl suphate
SFN	SFN
SH	thiol
siRNA	small interfering RNA
SOD	superoxide dismutase
Tg	thapsigargin
Ttase	thioltransferase
UGT	UDP glucuronosyltransferases
γ-GT	γ-glutamyl transferase

CHAPTER 1
GENERAL INTRODUCTION

1.1 The Eye

As a conscious sense organ, the eye allows vision. The human eye is enormously complicated - a perfect and interrelated system of about 40 individual subsystems, including the retina, pupil, iris, cornea, lens and optic nerve (Andrzejewska-Buczko and Buczko 1996) (Figure 1.1).

The cornea, which is a clear lamellar structure that admits light to the interior of the eye, bends the light rays, which then pass through the pupil, which is formed by the iris, a thin contractile tissue. The iris also divides the front section of the eye into the anterior and posterior chambers (Forrester et al., 2002). Contraction as a result of the action of the sphincter pupillae and dilation via the dilator pupillae muscle of the iris regulates the amount of light that is conveyed to the lens. The lens is situated behind the aqueous humour and the iris. The aqueous humour is a clear and colourless fluid which is actively and continually secreted by the ciliary body (2-3µl/min) (Forrester et al., 2002). It is essential because it provides nutrition to several avascular ocular tissues including the lens and the corneal endothelium. The lens actually changes shape through a process known as accommodation, which requires relaxation or contraction of the ciliary muscle a sphincter). The lens is attached to the ciliary body by suspensory ligaments known as the zonules of Zinn. Contraction of the ciliary muscle rslackens the zonules and results in rounder lens. Relaxation of the ciliary muscle forms tension on the zonules and makes the lens flatter. These modifications

allow objects near and far to be focused on the retina at the back of the eye (Kahan et al. 1999). Behind the lens is the vitreous body. The vitreous humour is a transparent viscoelastic gel. Unlike the aqueous humour, it is not produced rapidly and its turnover is extremely slow. Its composition is 98% water but its viscosity is two to four times higher than of water (Forrester et al., 2002). Aqueous and vitreous humours are not mixed because the lens and its suspensory ligaments separate the two.

The retina is responsible for converting the light signal received from the lens into neural impulses that are then transmitted to the brain. The retina is composed of two layers, the inner neurosensory retina and the retinal pigmented epithelium (RPE) (Forrester et al., 2002). The neurosensory retina has been specially adapted to be stimulated by light and transfers neural impulses to the brain via the retinal ganglion axons that form the optic nerve. The light sensitive layer is composed of rods and cones that contain the photosensitive pigment rhodopsin that converts light into nerve impulses. The retina is supported by the blood vessels of the choroid as well as the retinal vessels and contains millions of light-sensing cells called rods and cones, which are named for their distinct shapes. The cone and rod cells are stimulated by light and convert the image to electrical impulses which are ultimately sent via the optical nerve to the brain where an image is produced (Kopel 1980).

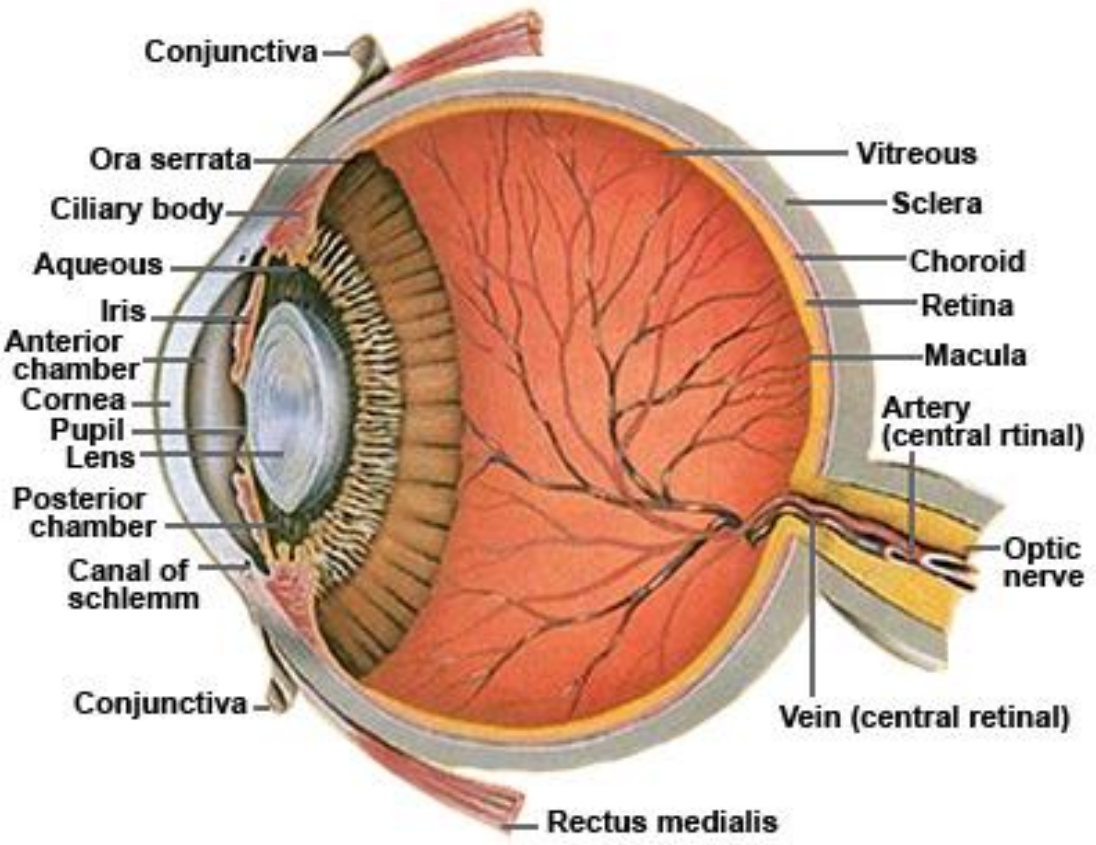

Figure 1.1 Cross section of a human eye
(http://www.mastereyeassociates.com/eye-anatomy-eye-problems/)

1.2 The Lens

1.2.1 Lens development

The human lens begins to form when the embryo is approximately 4 mm long, at 3-4 weeks gestation. The pattern of cellular differentiation is tightly regulated by several transcription factors such as PAX6, SIX3, SIX5 and PITX3 (Francis and Moore 1999). PAX6 is often referred to as the eye “master gene” as it plays a pivotal role in eye development. However, it should be noted that PAX6 alone is insufficient for lens differentiation as PAX6 has been found in tissues other than the eye.

During embryo development an outgrowth forms from the anterior neural plate of the forebrain called the optic vesicle (OV), and PAX6 has been shown to be expressed in the anterior neural plate in the cells that are the origin of the OV. PAX6 activates a second transcription factor, Sox2, in the surface ectoderm as well as maintaining its own expression. Additionally, a member of the transforming growth factor family of proteins, bone morphogenetic protein 4 (BMP4) is secreted by the OV in the mouse and upregulates Sox6 and a further transcription factor, Lmaf. At this stage, PAX6 and Sox 2 expression is maintained by BMP7 (Wawersik et al. 1999). Lens organogenesis begins with a thickening of the surface ectoderm and under the combined function of PAX6 and Sox2 crystallin expression is intitated (Kamachi et al. 2000, Kamachi et al. 1995) . This layer of cells secretes fibroblast growth factor (FGF), which promotes neuronal retinal cell differentiation to form the eye (Pittack et al. 1997).

Furthermore, surrounding tissues release transforming growth factor β (TGF β) to initiate retinal pigmented epithelium (RPE) formation. The thickening surface ectoderm overlays the optic vesicle to form the lens placode (Figure 1.2 a and b). The lens placode invaginates, forming the lens pit (Figure 1.2 c), closing over to form a hollow lens vesicle that temporarily remains attached to the surface ectoderm (which later forms the cornea) by the lens stalk (Figure 1.3 d). Immediately following the formation of the lens vesicle, cells on the posterior surface elongate towards the anterior face and form lens fibre cells. The resultant fibre cells form the optically clear nucleus of the mature lens.

As the lens continues to develop, mitosis in the cells of the posterior segment of the lens declines and stops. Conversely the cells forming the anterior segment of the lens continue to divide in a narrowly defined area which lies anterior to the equator, called the germinative, or proliferative region. This developmental segregation occurs where the optic cup anatomically separates the anterior and posterior segments of the lens (McAvoy et al. 1981). The fibre cells elongate towards both anterior and posterior poles, inserting themselves beneath the undifferentiated epithelial cells and below the posterior capsule thus forming highly organized concentric shells (Davson et al. 1990). It is at this stage in development that the new fibre cells begin to express β and γ crystallins.

In concert with transcription factors, lens growth is tightly controlled by a variety of growth factors, defined by Nicola (1994) as “a group of polypeptides with the ability to modulate growth, survival, differentiation and effector functions *in vivo* and *in vitro*”. In 1963, Coulombre and Coulombre carried out a lens inversion experiment on

embryonic chick lens. At the time when the primary fibres were formed they inverted the lens (180°) such that the epithelial cells were facing the retina. In this new environment, after six days these cells, which do not normally differentiate, elongated and differentiated into fibre cells. Further, in 1989, McAvoy and Chamberlain removed fluid from the aqueous and vitreous humours of the rat. Using the ELISA technique they found that the vitreous humour had a higher level of FGF suggesting that a change in FGF concentration could be responsible for lens cell differentiation, giving rise to the "FGF gradient hypothesis". To test this, they dissected off the primary fibre cells from a rat lens leaving a clean monolayer of lens epithelial cells. These cells were maintained in either serum free medium or serum free medium supplemented with FGF. It was shown that at low concentrations of FGF epithelial cell proliferation was observed whereas at higher concentrations migration was also observed. Also, based on the expression of α crystallin, the epithelial cells differentiated into fibre cells. However, in another study, using human lens cells, Ibaraki et al (1995) found that epidermal growth factor (EGF) stimulated proliferation in a dose dependent manner and also stimulated cell differentiation. Both results are interesting as both growth factors operate through the Ras/MAPKinase pathway.

Other growth factors have been implicated in lens development, and include platelet derived growth factor (PDGF), transforming growth factor β (TGFβ), insulin and insulin-like growth factor 1 (IGF-1). With the exception of TGF β, all the growth factors are able to stimulate lens epithelial cell DNA synthesis in a number of species (Gao et al. 1997), although in the human eye EGF may be the more important factor in lens cell proliferation (Majima 1995, 1998).

It can be seen that growth and development of the lens is tightly regulated by a number of transcription and growth factors, and any changes in the activity of these factors may be cataractogenic.

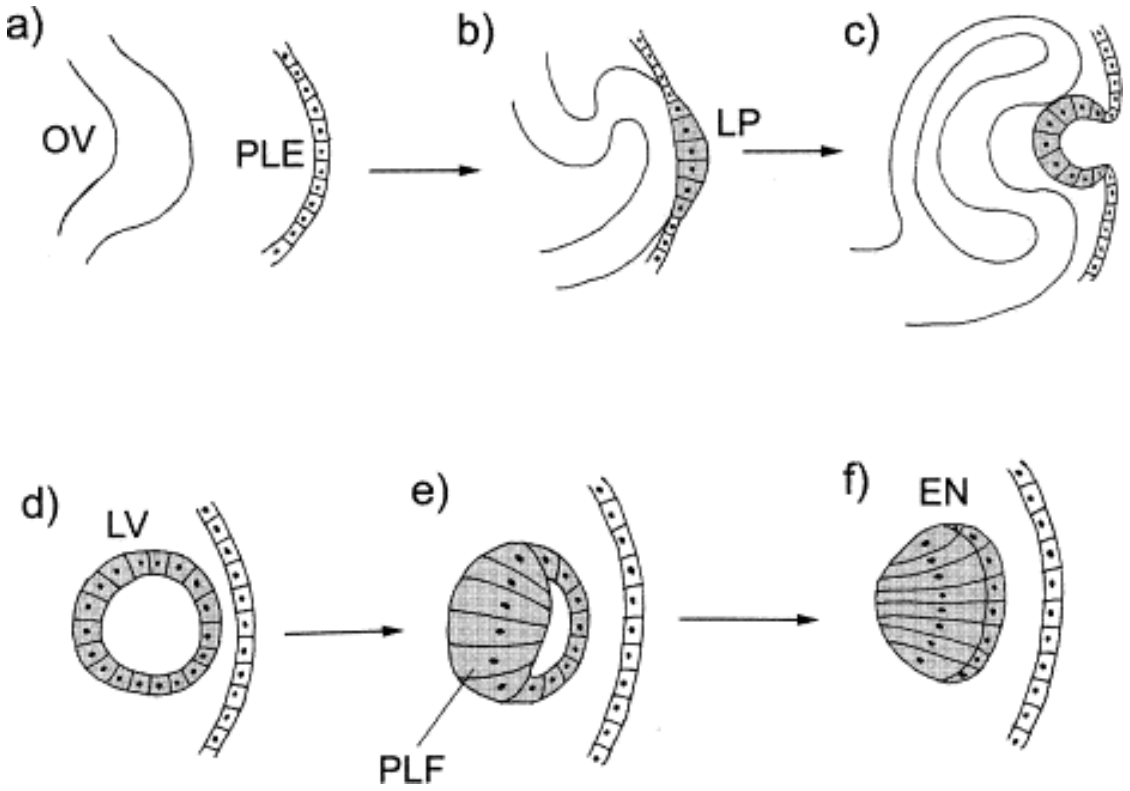

Figure 1.2 *a:* The optic vesicle (OV) approaches the presumptive lens ectoderm (PLE), human age 1 week, 3 mm embryo length, Carnegie stage 4. *b:* Contact between OV and PLE results in lens placode (LP) formation, human age 2 week, 4 mm embryo length, Carnegie stage 6. *c:* LP invagination produces a lens pit. *d:* Lens pit detaches from the surface ectoderm and the lens vesicle (LV) is formed, human age 4 weeks, 10 mm embryo length, Carnegie stage 12. *e:* Posterior lens vesicular cells elongate toward the anterior epithelial cells to form primary lens fibers (PLF). *f:* The embryonal nucleus is formed following the obliteration of the lens vesicle lumen, human age 6 weeks, 16 mm embryo length, Carnegie stage 16 (Reddy et al. 2004).

1.2.2 Mature lens

The lens is comprised of three main structures, the lens capsule, lens epithelium and lens fibres (Figure 1.3). The lens capsule, forming the outermost layer, is a smooth, transparent basement membrane that completely surrounds the lens. It is important during the process of accommodation for modifying the shape of the lens. The capsule varies from 2- 28 micrometers in thickness, being thickest near the equator and thinnest near the posterior pole (John Forrester 1996). The anterior and posterior capsule surfaces are produced by the lens epithelium and the lens fibers respectively. The anterior lens capsule is in immediate contact with a single layer of lens epithelial cells that lie at the anterior and equatorial regions.

These are two cells types in the lens. The lens epithelium, located in the anterior portion of the lens between the lens capsule and the lens fibers, is a simple cuboidal epithelium, which regulates most of the homeostatic functions of the lens (Candia 2004). The cells of the lens epithelium also serve as the progenitors for new lens fibres. The lens fibres are prismatic and very long highly specialized cells. Their length can be up to 12 mm. The fibres stretch lengthwise from the posterior to the anterior poles and the middle of each fibre cell lies on the equator. They produce the predominant water-soluble proteins of the lens called crystallins. Transparency of the lens is maintained by the crystallins, a group of soluble proteins produced by the lens fibre cells. The crystallins form approximately 90% of the dry weight of the lens and create the refractive index necessary to focus light onto the retina. Any disturbance in the ordered packing of the crystallins reduces the transmission of light.

Lens growth persists throughout life by continual epithelial cell proliferation, migration and differentiation into fibre cells. In the post natal lens the anterior epithelia is quiescent and proliferation mainly occurs at the germinative zone, located just anterior to the lens equator. Cell division gives rise to either new anterior epithelia or cells which migrate posteriorly into the transitional zone where they undergo differentiation into fibre that elongate and become hexagonal in cross section. From the lens equators newly formed fibre cells extend their two apices toward the lens anterior and posterior poles and the lens sutures, which in the adult lens assume a four-pointed star arrangement. Part of the transmission properties of the lens can be attributed to the fibre cells, which are densely and regularly packed into an organized cellular arrangement. These cells are intimately connected by moderate interdigitions in superficial regions and distinct interlocking processes father into the nuclear region. Low resistance gap junctions are particularly important to permit rapid intercellular movement of small molecules and ions via the lens gap-junction like protein, known as the major intrinsic protein of lens fibre, which is a member of the water-transporting aquaporins.

The highly refractive index of the lens is due in part to the accumulation of lens fibre specific crystallin proteins, which are embedded in a complex cytoskeletal matrix and ultimately comprise 90% of the total cellular protein. The lens contains α, β and γ-crystallin as a result of gene duplication and divergent evolution from ancestors with different functions (Wistow and Piatigorsky 1988). The α-crystallin are members of the small heat-shock protein (α-Hsp) surperfamily and function as molecular chaperones as well as structural proteins. Each contains a characteristic α-crystallin domain that is highly conserved.

Considerable evidence in rat studies has determined that lens cell polarity and patterns of growth are regulated by growth factors found in the aqueous and vitreous humours (VH) in the anterior segment of the eye (Lovicu and McAvoy 2005). In particular, FGF family members present in a regulatory anterior-posterior gradient, determine qpithelial cell proliferation, migration and differentiation. Other factors, including inhibitory influences, may also contribute to the proposed FGF gradient, such as the Wnts. Locatisation of the Wnts Fizzled (Fz) receptors suggests that Wnt signaling is involved in the formation and maintenance of the epithelial phenotypes, as illustrated in figure 1.4. there is evidence for the involvement of growth factors in the various stages of development such as, transforming growth factor beta (TGFβ), which seems to regulate aspects of fibre differentiation and mitogens such as, IGF, EGF and PDGF are thought to induce epithelial cell proliferation (Lovicu and McAvoy 2005)

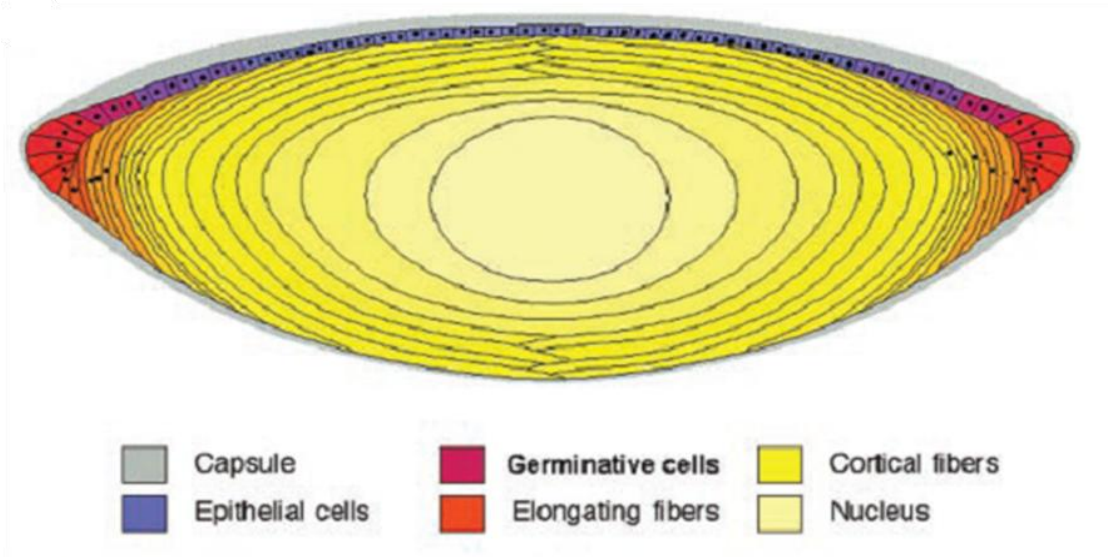

Figure 1.3 **A** diagram of the adult human lens in cross-section (Maidment et al. 2004)

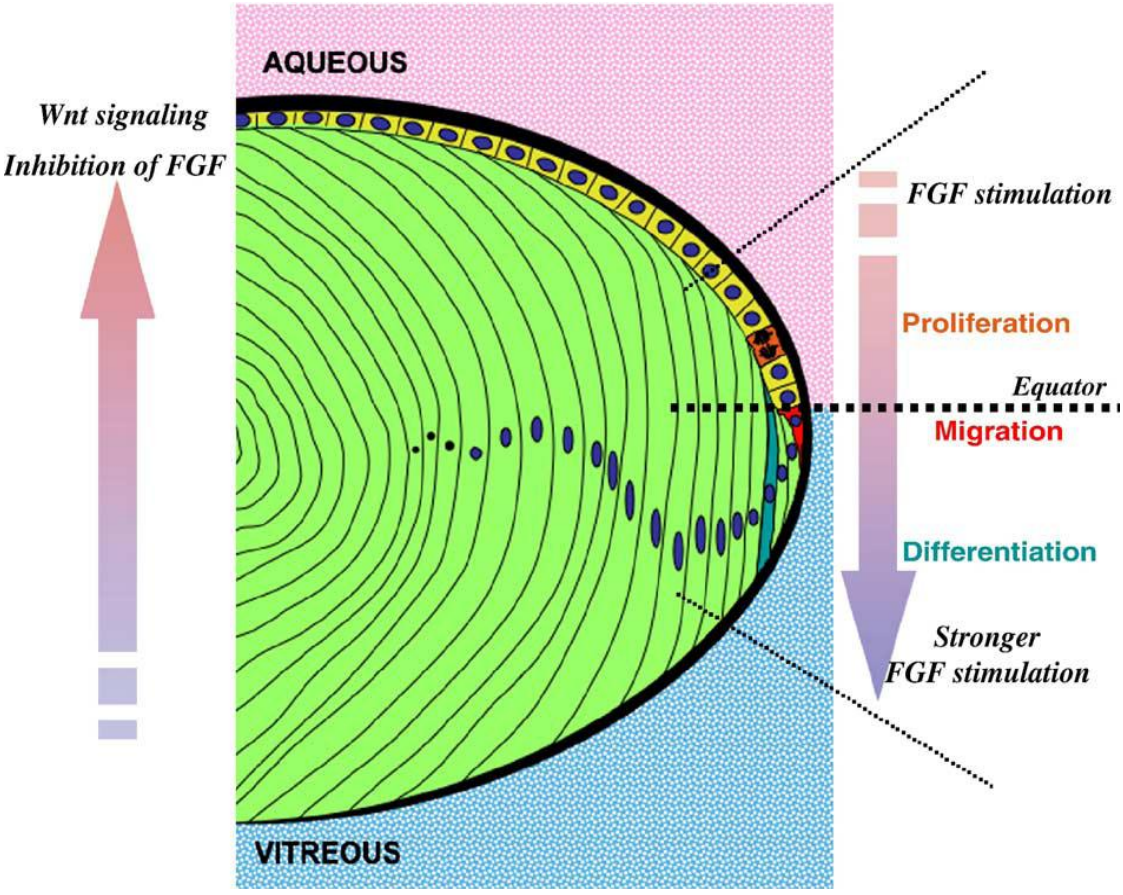

Figure 1.4 Diagram indicating how the ocular media and a gradient of FGF stimulation may determine antero-posterior patterns of lens cell behavior. In the postnatal lens, cell proliferation is restricted to the epithelium and predominantly occurs in a band of cells above the equator known as the germinative zone. Progeny of proliferative activity migrate (or become displaced) below the equator where they initiate fiber elongation. These zones coincide with compartments defined by the anatomy of the eye: the epithelial cells are exposed to aqueous (pink background) and the fiber cells are exposed to vitreous (blue background). The cellular behaviors indicated, proliferation (orange), migration (red), and fiber differentiation (blue), are observed both *in vivo* (in an antero-posterior direction) and in lens epithelial explants. The right-hand arrow indicates the gradient of FGF stimulation that is proposed to govern this antero-posterior pattern of cell behaviour *in vivo*. The left-hand arrow indicates that other factors, including inhibitory influences, may also contribute to the proposed FGF gradient.(Lovicu and McAvoy 2005)

1.3 Cataract

1.3.1 Definition and onset

Cataract can be defined as any loss of transparency of the lens (Quillen 1999). It is the major cause of blindness worldwide (Resnikoff et al. 2004a). In cataract, the lens becomes cloudy or opaque which is often age related (Goodenough et al. 1998). Estimates by the World Health Organisation suggest that cataract accounted for 48% of world blindness in 2002 (Resnikoff et al. 2004). Blindness due to cataract is increasing due to world population growth (6,000 million in the year 2000 forecasted to incease to 8,000 million in 2020) and increasing human longevity, which is true for the developing and industrialized world (2000). Cataract prevalence increase with age (Keeffe and Taylor 1996a, McCarty and Taylor 2001). In the United States, the prevalence of cataract is approximately 5% at age 65. This percentage increases to about 50% for the persons older than 75 years (Klein 1993, Klein et al. 1992, Leibowitz et al. 1980). The prevalence of cataract has been shown to double with each decade (Keeffe and Taylor 1996). The situation is different in developing countries where the average age of onset is fifty-five years. Considering the short current life expectancy in these countries (sixty-three years), delaying cataract of six months would decrease the number of blind people by approximately one million (Watkins 2002). Cataract consititues a major health issue. Cataract occurs in different compartments of the lens. Typically progression of cataracts is slow, but if left untreated is potentially blinding. Cataracts generally present in both eyes, but one eye is generally affected earlier than the other (Quillen 1999).

1.3.2 Types, classification and risk factors of cataract

The classification by aetiology distinguishes age-related cataract, congenital cataract, and secondary cataract. Age-related cataract is the most common type of cataract affecting both men and women (Thylefors et al. 2002). Congenital cataract is present at or develops shortly after birth and is possibly caused by galactosemia or maternal infection in early pregnancy. In secondary cataract, systemic metabolic disturbances result in the loss of transparency of the lens. Many diseases like diabetes and hypocalcaemia all could increase the risk of this type of cataract. Cataract is classified by morphology and divided in to four types corresponding to the location of opacity, including nuclear sclerosis cataract, cortical cataract, anterior sub-capsular cataract and posterior sub-capsular cataract. The first two forms are most closely associated with aging. Nuclear cataract occurs in the nucleus or central part of the lens. There is an associated deposition of (pale and deep) yellow or brown pigment within the lens (Bollinger and Langston 2008). Cortical cataract forms in the lens cortex, which is the outer shell of the lens. It extends its white spokes from the outside of the lens to the center, and its symptoms often include problems with transmission of light and glare (Bollinger and Langston 2008). Posterior subcapsular cataract starts as a small opacity at the back of the lens, under the capsule often in the visual axis. Risk factors of cataract, such as ageing and diabetes have been mentioned previously. Some other factors also contribute to cataract, such as UV-B exposure, smoking, and ionizing radiation (Allen and Vasavada 2006).

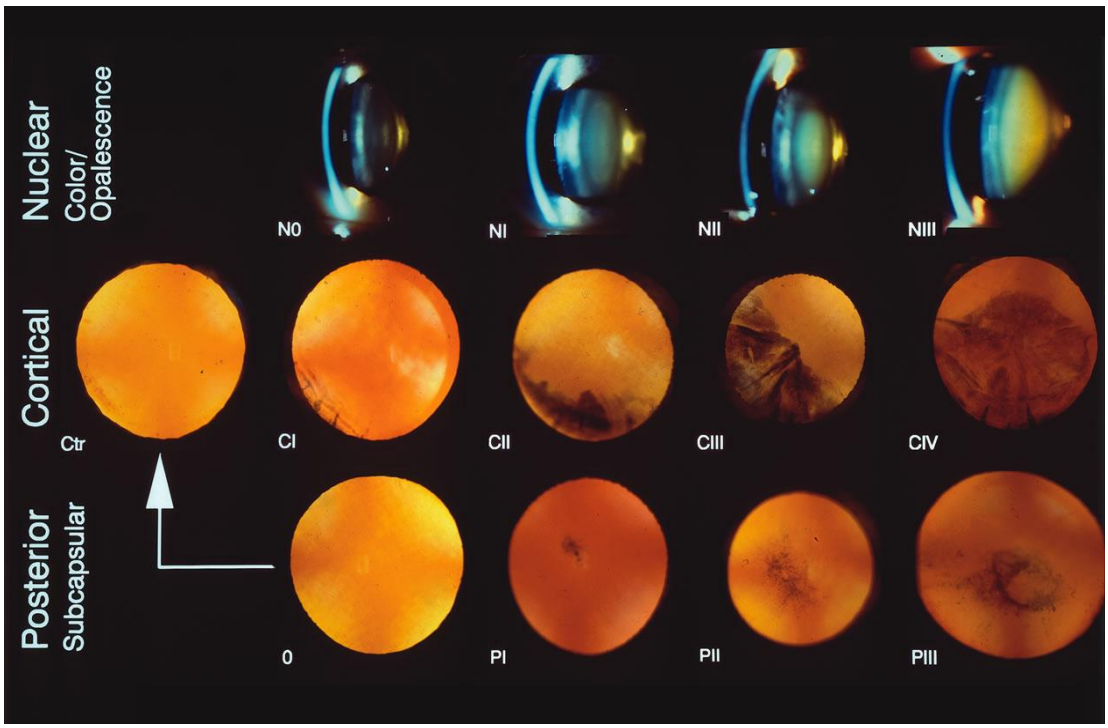

Figure 1.5 The Lens Opacity Classification System II (LOCS II) photographic grading standards. N = Nuclear photographs. Stage 0 = normal; I–III = various stages of nuclear cataract. For nuclear opalescence, the average opalescence across the entire nuclear region is used. An opalescence that is less than or equal to Photographic Standard 0 = grade 0; if the opalescence is less than or equal to Standard I, the grade is 1, and so on. For Color Grading of the nucleus, only the N2 standard is used. (<N2 for color=0, equal to N2 in color=1, and N2 for color = 2) C = Cortical photographs. 0-Trace (Tr) = normal; I–IV = various steps of cortical cataract (roughly CII ¼, CIII ½, CIV ¾. CV > ¾). P = Posterior subcapsular photographs. 0 = normal; I–III = various stages of posterior subcapsular cataracts (Chylack et al. 1989)

1.3.3 Cataract surgery

At present, the only way to treat cataract is by surgery. Cataract surgery has become the most commonly performed surgical procedure in the world (Keeffe and Taylor 1996b). More than 156 000 cataract operations were performed in 1995 by the National Health Service in England and Wales (Minassian et al. 2001). There are intra- and extra-capsular cataract extraction (ICCE or ECCE) methods. The more historical of the two; ICCE, involves the removal of the entire lens and capsule. This technique is no longer in use in the developed world unless there are extenuating circumstances. The visual outcomes of this method are pooor and complications are common, however ICCE remains in use in the developing world where the benefits are outweighed by cost and simplicity.

Since 1982 the main surgical practice has been ECCE (Minassian et al. 2001), which requires significantly increased financial investment. Inthis technique the lens cataractous material is removed, whilst the majority of the capsule is left in place. In more detail a small incision of 2 to 3 mm is made at the edge of the cornea. Then a curvilinear continuous tear, known as a capsulorhexis (Figure 1.6), is created in the anterior lens capsule to allow access to the opacified region of the lens, which generally concerns lens fibre cells. As before, the lens capsular bag that remains intact provides a barrier between the anterior and posterior segments of the eye and is the site for IOLs implantation to restore visual acuity. This technique was initially developed using a manual lens nucleus extraction method that required a larger incision and more working space within the eye. Nowadays howeverultrasiound is used to emulsify cataractous lens material, using a technique called

phacoemulsification (phako or P/ECCE) and it is simultaneously removed by means of a dual system irrigation-aspiration. Therefore, cataract extraction can occur through a small incision of 2 to 3 mm. following phako more viscoelastic substance is injected into the capsule in order to maintain the capsular shape for the implantation of a foldable IOL that unfurls unaided within the capsular in a manner analogous to that of an opening umbrella; an illustration of an IOL seated within the capsular bag is shown in Figure 1.6. The use of smaller incisions is paramount because there is less alteration to the shape of the cornea, which is responsible for approximately two thirds of the focusing in the eye.

An IOL central optic is usually a 6 mm disc with dual supporting side struts called haptics that hold it in position within the lens capsule. Traditionally they were constructed of an inflexible material called polymethylmethacrylate (PMMA) which has since been superseded by numerous flexible polymers. They are usually monofocal allowing for distance vision only and visual correction is still required for near visual acuity. Increasingly however, multi-focal IOLs are also now available. The use of flexible polymers has allowed the invention of adaptive IOLs that are now beginning to allow limited visual accommodation. Several factors determine the success of cataract surgery. These include surgical experience, the choice of surgical procedure, environmental and social conditions and the presence of ocular comorbidities (McCarty and Taylor 2001).

Intraoperative complications include retrobulbar hemorrhage, perforation of the globe, allergic reactions to the anesthetic agent, or hypotension. Postoperative complications

include postoperative pain, elevated intraocular pressure, or endophthalmitis (inflammatory condition of the intraocular cavities) (Ou and Ta 2006). Endophthalmitis, one of the more serious postoperative complications of lens surgery, is currently very rare (0.05 %) when very strict antiseptic precautions are observed. Other rare complications include wound dehiscence with bulbar hypotension, epithelial growth into the wound cleft, allergic scleral reactions to the eye drops, or subluxation of the IOL. The most common complication is posterior capsule opacification (PCO), also called “after-cataract” and will be discussed in more detail later. The incidence of PCO is influenced by many factors including surgeon, surgical technique and age of the patient (Wormstone 2002). In the latter case PCO occurs in almost 100% of children undergoing cataract surgery and in adults those under the age of 40 have a significantly greater chance of developing PCO than patients over the age of 60 years. While the frequency of PCO has now been reduced by improvements in surgical technique and the use of modern, sharp-edged flexible lenses PCO is an unresolved problem (Wormstone et al. 2009). Preservation of a closed posterior capsule helps prevent postoperative complications such as retinal detachment and macular edema, which implies that a further reduction of the PCO rate would be achievable (Kohnen et al. 2009).

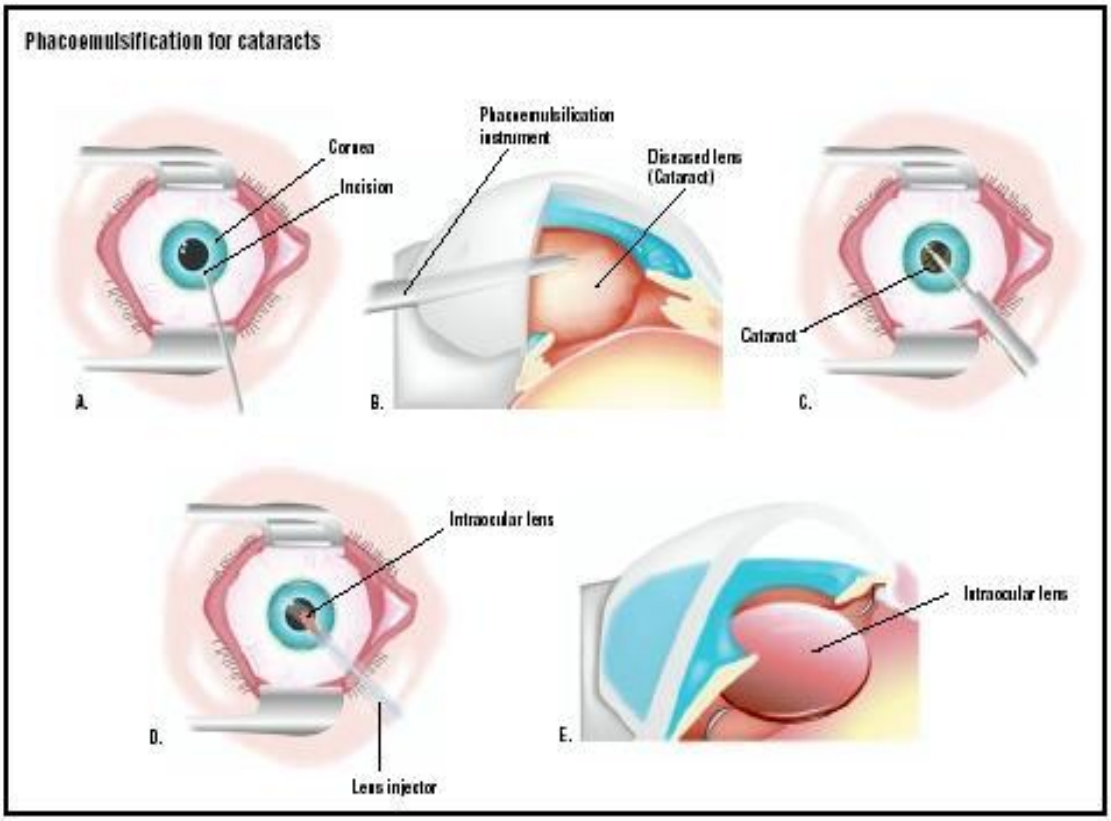

Figure 1.6 In a phacoemulsification procedure, an incision is first made in the cornea, the outer covering of the eye (A). A phacoemulsification instrument uses ultrasonic waves to break up the cataract (B). Pieces of the cataract are then suctioned out (C). To repair the patient's vision, a folded intraocular lens is pushed through the same incision (D) and opened in place (E) (http://www.surgeryencyclopedia.com/Pa-St/Phacoemulsification-for-Cataracts.html#ixzz2QUVOA2Wt)

1.4 Oxidation and Cataract

1.4.1 Reactive oxygen species (ROS) in the lens

Any free radical involving oxygen can be referred to as a reactive oxygen species (ROS). Free radicals are atomic or molecular species with at least one unpaired electron in the outermost shell. These unpaired electrons cause free radicals to be highly reactive and likely to take part in chemical reactions (Huang et al. 2006). ROS molecules include the superoxide anion (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) (Figure 1.4). Contrary to O_2^- and •OH, which are extremely unstable and react at or near their site of formation, H_2O_2 is less reactive, freely diffusible and relatively long-lived (Linetsky and Ortwerth 1996).

ROS can be generated either endogenously or exogenously (Andley et al. 2000). Endogenous sources include mitochondria, peroxisomes, lipooxygenases, NADPH oxidase and cytochrome P450. Exogenous sources include ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins (Andley et al. 2000). It is widely believed that a rise in the intracellular levels ROS will damage various cell components, interrupt physiological functions and lead to ageing and various oxidative-stress-associated cataracts (Finkel and Holbrook 2000, Spector 1995). The damage can consist of protein modification, lipid peroxidation and DNA fragmentation, all of which have been proposed to contribute to cataractogenesis. The lens is able to defend itself against oxidation using antioxidants from either enzymatic or non-enzymatic systems (Kise et al. 1994).

1.4.2 Antioxidant system in the lens

The lens has a well-designed system of defence against oxidation just like other organs (Augusteyn et al. 1987). Primary antioxidants include non-enzymatic (e.g., glutathione, vitamin C, vitamin E and carotenoids) and enzymatic (e.g., superoxide dismutase, glutathione peroxidase and catalase) systems. Lens cells contain enzymes that can degrade damaged proteins through proteolysis (Hodges and Scott 1992) or repair damaged nucleic acids. Two protein/enzyme repair systems have been identified in recent years (Figure 1.7). One is the glutathione-dependent (GSH-dependent) thioltransferase system (Chrestensen et al. 1995, Holmgren 1989, Raghavachari and Lou 1996) which may be critical in maintaining the lens in a reduced state by cleaving protein–thiol mixed disulfide bonds formed on the oxidation of lens proteins. The other is the NADPH-dependent thioredoxin / thioredoxin reductase system (Hansson et al. 1989), which is very effective in reducing protein–protein disulfide bonds. The ROS and the antioxidant systems are summarised in Figure 1.7.

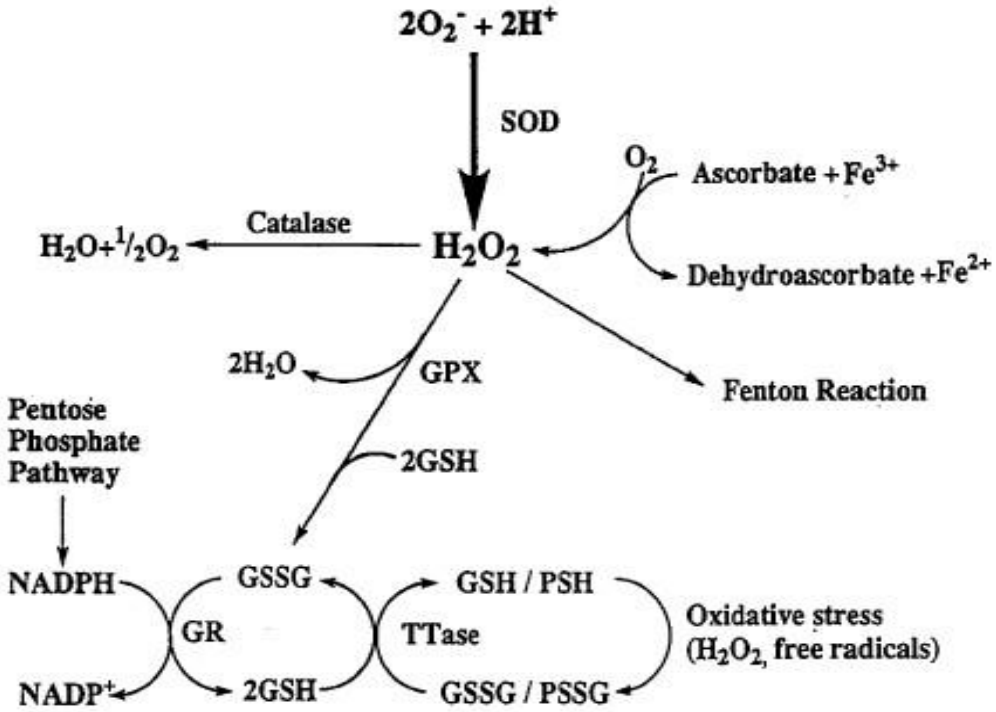

Figure 1.7 The reactive oxygen species and the primary antioxidant system in the lens. H_2O_2, generated by the dismutation of superoxide anion or by the reaction between ascorbate and Fe^{+3} can be degraded by several pathways. These include catalase, glutathione peroxidase (GPX), and the Fenton reaction. The decrease in the SH/S-S ratio by oxidation can be reversed by the glutathione reductase (GR)-pentose phosphate shunt cycle and by thioltransferase (TTase). These mechanisms protect the lens from oxidative damage. GR: glutathione reductase; GPx:glutathione peroxidase; SOD: superoxide dismutase; TTase: thioltransferase. Reprinted with permission from (Lou et al., 1998).

1.4.3 Association between oxidation with age-related cataract formation

Maintaining transparency so that light can be transmitted and focused on the retina is the major function of the lens. The lens has an unusually high protein concentration, which can be as high as 50% in the nuclear region of the lens (Eldred et al. 2011, Gunhaga 2011). Crystallins, the structural proteins of lens, contain a high level of thiol groups that are necessary to be in the reduced state to maintain clarity of the lens (Beebe et al. 2011, Beswick and Harding 1984, Michael and Bron 2011). In the normal young human lens, they did not observe any oxidation of cytosolic or membrance proteins. In addation, a healthy lens utilizes its various antioxidants and oxidation defence enzymes to protect crystallins against oxidation. However, in the ageing lens, some differnces can already be seen. Some oxidation of the membrane proteins is present and only half of the protein thiols remain buried. Protection and repair mechanisms against oxidative stress slowly deteriorate or become ineffective so that the lens is less able to counteract the effects of H_2O_2 or other oxidants; transparency is lost and cataract occurs (Lou and Dickerson 1992). Identification of molecules and systems that can modulate oxidative stress are therefore of great interest to cataract research in relation to identifying strategies to delay cataract onset (Wride 2011).

1.5 Posterior Capsule Opacification (PCO)

1.5.1 Pathogenesis and prevalence of PCO

Posterior capsule opacification (PCO) is the most common long term complication of cataract surgery and it occurs once the continuity of the lens capsule has been

breached (Wormstone et al. 2002). In a meta-analysis of 49 studies published between 1979 and 1996, more than 25% of patients developed visually significant PCO within 5 years of standard extracapsular cataract extraction (ECCE) cataract surgery and the incidence increased with time (Schaumberg et al. 1998). The age at which the patient presents cataract for surgery is an additional and profound influencing factor. The PCO event in children is far more ferocious than in adulthood, with almost 100% developing visually significant PCO (Knight-Nanan et al. 1996). In a study of children, who received a small incision, foldable, posterior chamber acrylic lens implantation, 100% of children ≤ 4 year developed PCO in the mean follow up period of 2.75 year after surgery (Stager et al. 2002). Likewise PCO prevalence in adults shows the same trend with increasing age (Moisseiev et al. 1989). Therefore, a clear understanding of the pathological mechanisms involved therapies and/or improve surgical practices for PCO inhibition.

PCO results from the resilient growth onto the posterior capsule of residual lens epithelial cells (LECs) (De Groot et al. 2005) (Figure 1.8 B). LECs of anterior origin can access and grow onto the posterior capsule if the anterior rhexis size is larger than the optic of the intraocular lens (IOL) (Apple et al. 1992b). When this occurs, the cells ultimately encroach on the visual axis and light scattering can occur. Clinically, these changes are shown by the formation of Elschnig pearls (hypertrophic epithelial cells at the margin of the rhexis, likely to include the visual axis), the Sommerring ring (fibrotic changes in the more peripheral regions of the capsular bag) and the wrinkling of the capsular bag itself (Marcantonio and Vrensen 1999). Inflammatory cells can also play a role in PCO, especially in patients who experience significant postoperative inflammation following cataract surgery. In these cases, PCO appears as

a whitish haze over the capsule and usually develops within the first month following surgery. This type of PCO usually decreases with time after the first 3 months but can cause significant loss of vision (Duncan and Wormstone 1999).

Treatment of PCO is usually is relatively straightforward and often effective using the neodymium:YAG (Nd:YAG) laser to cut an opening in the posterior lens capsule, thus clearing the visual axis and restoring vision (Vock et al. 2009). However, disruption of the posterior capsule after phacoemulsification or standard ECCE results in a relative increase in the occurrence of complications. In particular, Nd:YAG capsulotomy has been associated with damage to IOLs, postoperative increase in intraocular pressure, cystoid macular edema, disruption of the anterior vitreous face and increased incidence of retinal complications including retinal detachment (Dewey 2006, Murrill et al. 1995, Steinert et al. 1991). Several attempts have been made to find an appropriate therapeutic concept to significantly lower the rate of PCO.

Various surgical methods can negatively influence the development of PCO, such as atraumatic surgery and thorough cortical cleaning (Apple et al. 1992). A recent advancement in surgical method and IOL design has shown great promise to reduce the progression of visually disruptive PCO and may be especially applicable to patients in which PCO is a particular problem such as in paediatrics (De Groot et al. 2005). At surgery twin rhexis excisions are carried out, such that in addition to the usual anterior rhexis, a posterior rhexis is performed for “bag-in-a-lens” IOL implantation. A twin-capsulorhexis IOL accommodates the capsule in a groove at its circumference, capturing the lens cells within the remaining equatorial region of the

capsular bag. Initial studies have been positive however, the high degree of technical skill required for this procedure and the likelihood of further complications, may negate widespread adoption of this technology.

Numerous studies have investigated the influence that the IOL material has on PCO development. A common material used was polymethylmethacrylate (PMMA) and this has subsequently been associated with the highest rates of PCO in comparison to modern materials. A general consensus shows that PMMA is inferior to silicone and acrylic (Auffarth 2004, Hayashi et al. 2001, Hollick et al. 1999). Current IOL design rationale is to form a physical barrier to the migration of LECs by using IOL designs that maximize contact between the IOL optic and the posterior capsule so that no cells can reach the visual axis. A sharp optic edge induces contact inhibition and prevents epithelial cell migration. It is clear that IOL design plays an important role in PCO inhibition (Nishi 1999) and a sharp edge is associated with a significant reduction role in PCO (Buehl et al. 2005, Buehl et al. 2002, Buehl et al. 2004, Kruger et al. 2000). Sharp edge IOL implantation is however also associated with the incidence of anterior capsule contraction and opacification. Recent studies report that the sharp edge design silicone IOL caused more capsulorhexis contraction than the round edge design (Sacu et al. 2004), however this phenomenon is still a matter of much controversy (Miyata et al. 2007).

Theoretically, the most efficient approach to address this problem is to eliminate all the cells within the capsular bag at surgery and a range of cytotoxic agents have been tested in a number of human and animal model systems such as, mitomycin-C (Jordan

et al. 2001), 5-fluorouracil (Fernandez et al. 2004) and thapsigargin (Duncan et al. 1997). None of these treatments have yet reached the clinic; therefore there is scope to develop further agents to prevent PCO. A major problem when administering drugs into the capsule bag is the leakage of the drug into the anterior segment, such that non-target ocular tissue damage may be caused (Behar-Cohen et al. 1995). One approach adopted to address this problem and prevent these side effects is to coat the implanted IOL with cytotoxic agents. In a range of models, including the human capsule bag model and an *in vivo* animal model, drugs such as thapsigargin and a plant anti-mitotic bFGF- conjugate (bFGF-saporin) (Behar-Cohen et al. 1995), have shown the potential to target the capsule bag. However, it was been pointed out that the potential of toxic side effects has effects has prevented their use in human clinical trials (Duncan et al. 2007).

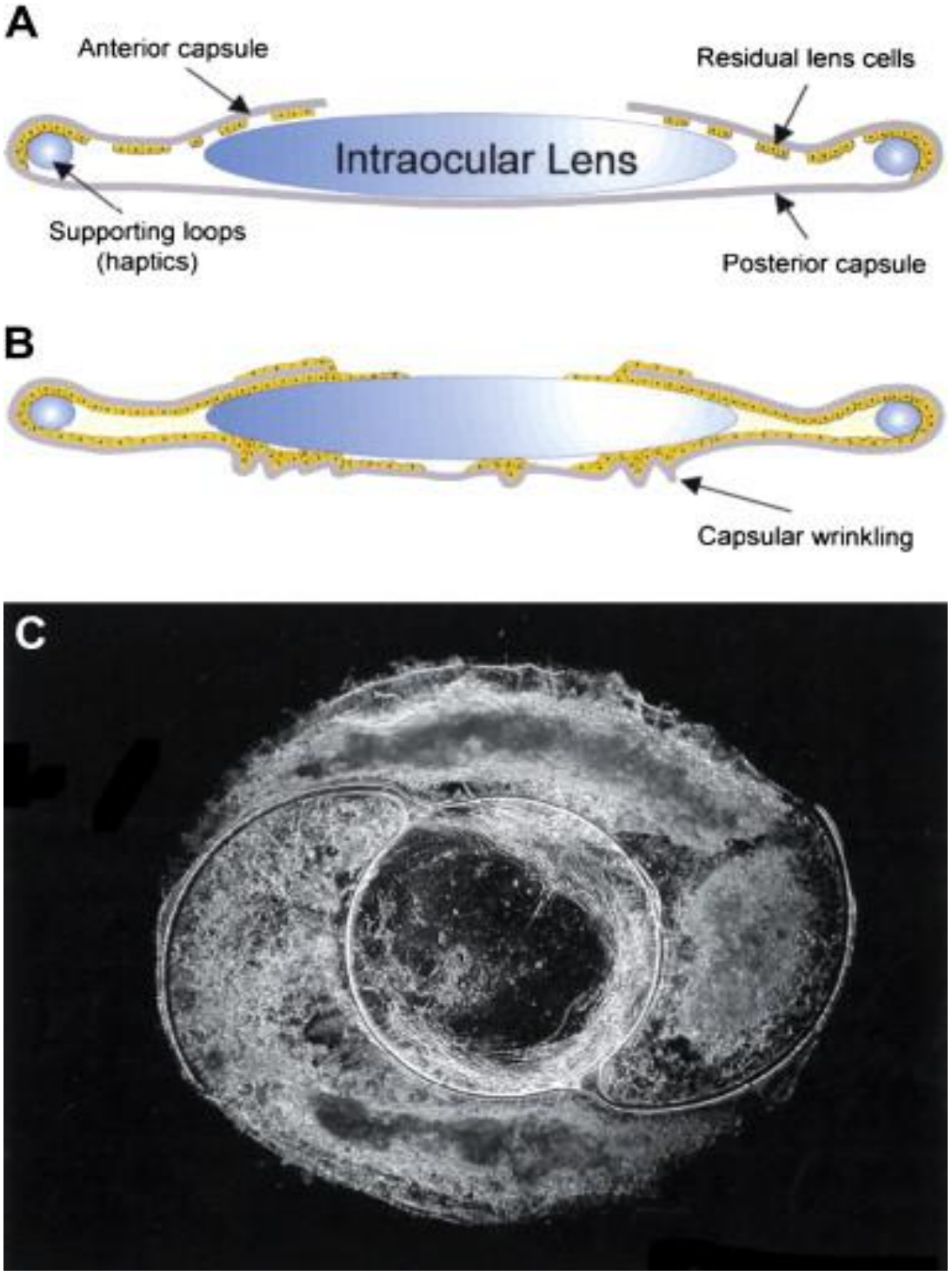

Figure 1.8 A schematic representation of (A) the post-surgical capsular bag and (B) the extensive growth and modification that gives rise to Posterior Capsule Opacification. (C) A dark-field micrograph of a capsular bag removed from a donor eye that had undergone cataract surgery prior to death that exhibits light scattering regions beneath an intraocular lens (Wormstone 2002).

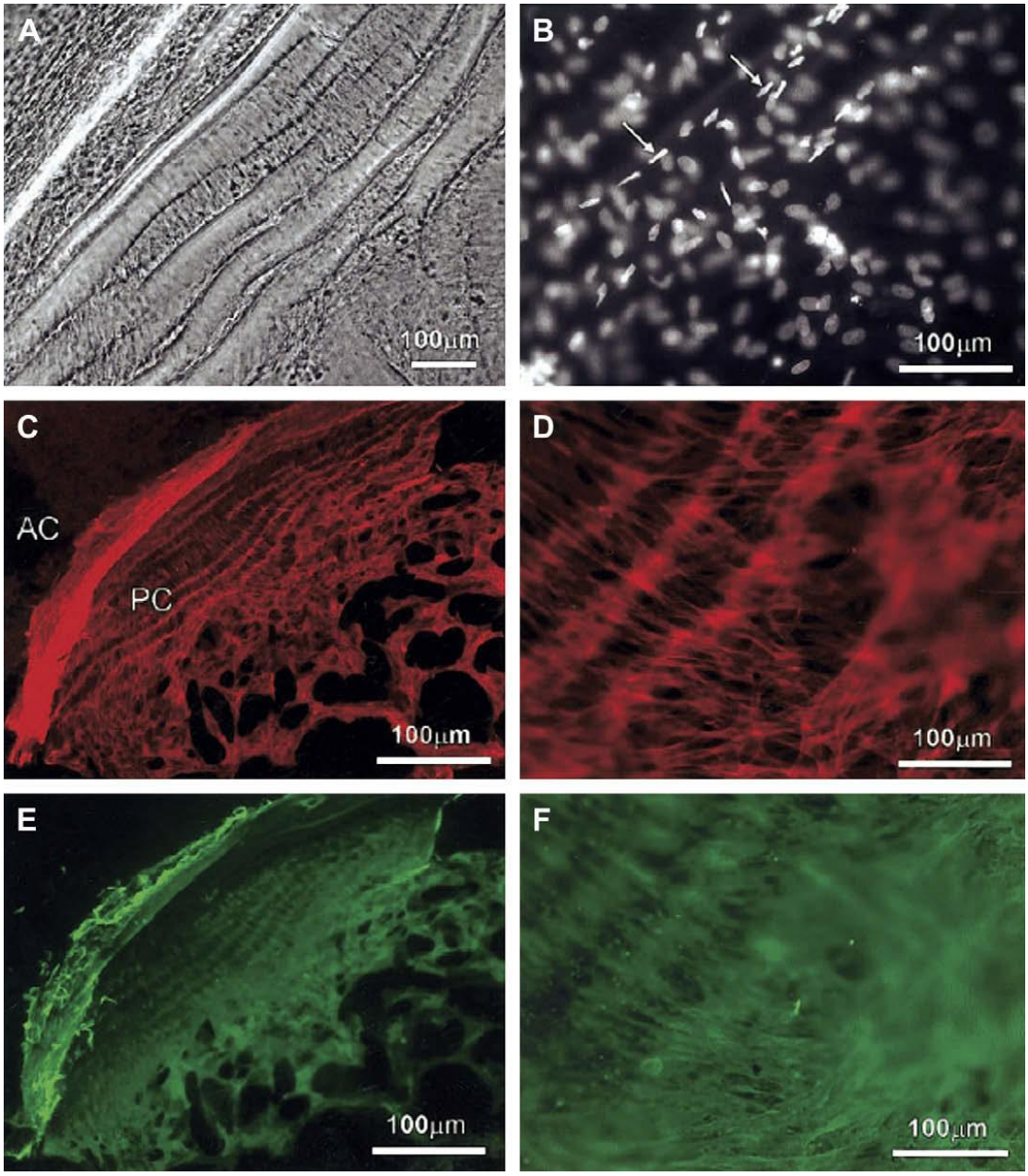

Figure 1.9 Examination of a capsular bag removed from a donor eye that had undergone cataract surgery 32 days before the time of death. (A) A phase-contrast micrograph shows wrinkling of the posterior capsule and the associated cellular morphology. (B) Fluorescence micrograph illustrating several layers of nuclei, some of which exhibit a spindle shape (arrows) and are oriented along capsular wrinkles. (C) Fluorescence micrograph showing F-actin distribution in cells growing across the posterior capsule (PC) and also those growing on the outer surface of the anterior capsule (AC). (D) A higher-magnification fluorescent micrograph demonstrating the F-actin organization of cells residing on the posterior capsule in association with matrix contraction. (E) Fluorescence micrograph showing a-SMA distribution in cells growing across the posterior capsule and also those growing on the outer surface of the anterior capsule (AC). (F) A higher-magnification fluorescence micrograph demonstrating the a-SMA organization of cells residing on the posterior capsule in association with matrix contraction (Wormstone et al., 2002)

1.5.2 Experimental models for PCO

PCO is a major clinical problem and several models have been developed to study this fibrotic disease. These include *in vivo* animal studies (Behar-Cohen et al. 1995, Cobo et al. 1984); capsular bag models (Davidson et al. 2000) (Liu et al. 1996, Wormstone 2002); cell culture studies (Nishi et al. 1996, Wormstone et al. 2004) (Kurosaka et al. 1995); *in vivo* observations (Hollick et al. 1999, Ursell et al. 1998) and analysis of post-mortem material (Saika et al. 2000, Wormstone et al. 2001).

The most commonly used model for *in vivo* animal studies is the rabbit. Apart from ethical considerations, the main drawback with this approach is that cell growth in non-primates may be different to that in primates. In primates, PCO is solely caused by lens cell growth, whereas in animals, cells from surrounding tissues like iris can migrate on to the posterior capsule (Behar-Cohen et al. 1995, Cobo et al. 1984). Furthermore, different species generate different inflammatory responses compared to primates, following trauma to the eye (Bito 1984). It is also difficult *in vivo* to establish data relating to the progression of PCO in animal systems and much of the information obtained is from detailed end-point examinations.

Capsular bag models have emerged in recent years and are based on sham cataract operations (Davidson et al. 1998, Liu et al. 1996, Nagamoto and Bissen-Miyajima 1994, Saxby et al. 1998). A capsular bag is produced that is identical to that generated *in vivo*, this system has the correct spatial organization of cells, which is the same as *in vivo* and the cells grow on their natural matrix. At the onset of experiments, the central posterior capsule is free of cells, but with time, cells encroach on this space

and in some cases can modify the matrix in such a way that wrinkles are formed in the capsule (Liu et al. 1996, Wormstone 2002). The factors that control these functional events can be investigated and a match paired experimental approach is often employed (Liu et al. 1996, Maidment et al. 2004, Wormstone 2002) (Duncan et al. 1997, Wormstone et al. 2001). The capsular bag model has also been developed and applied in bovine (Saxby et al. 1998) and canines (Davidson et al. 1998). A critical aspect of the capsular bag model is the maintenance of the capsular bag shape. In order to achieve this some groups have employed capsular rings (Nagamoto and Bissen-Miyajima 1994, Saxby et al. 1998), while others have used fine pins to maintain the circular shape (Figure 1.10) (Davidson et al. 1998, Liu. et al. 1996). Furthermore, some of these systems permit the insertion of an IOL and therefore the role it plays can also be investigated on a day to day basis (Figure 1.9). the capsular bag model also allows for different surgical procedures like phacoemulsification, to be compared in a controlled way (Quinlan et al. 1997). Although, the capsular bag model is an excellent system to study lens cell growth and behaviour, the model can not identify the pothtial side feects to other ocular tissues, nor address the physical roles that other tissues may play. In addition, this system of investigation is dependent on the availability of human donor material which is relatively scarce.

Cell lines are a valuable experimental tool for investigating PCO when the availability of human donor material is limited. Cell lines have advantages as they provide a homogenous population of cells, which should respond in a similar manner, which enables a contimuous series of experiments to be carried out. In recent years human lens cell lines have been generated and are now commonly used.

The FHL 124 cell line has many advantages, the cells express phenotypic lens epithelial markers αA crystallin, Pax6 and FOXE3 and have 99.5% similarity of gene expression with the native lens epithelium (Wormstone et al. 2004). The cell line can be grown on a foreign matrix and cells still maintain a similar gene expression profile of phenotypic lens makers compared to the native tissue (Wormstone et al. 2004).

Improvements in technology have enabled *in vivo* observations regarding PCO progression to be made. Camera systems have been developed that can detail changes to the capsular bag within the visual axis (Hollick et al. 1999, Ursell et al. 1998). This allows for patient assessment at various time points and changes to the capsular bag can be observed over time. While this technique can not provide information at the single cell level it does provide an excellent insigt into the rates of PCO progression.

The analysis of post-mortem material provides an important understanding of which matrix components, cellular markers or growth factors are present within the capsular bag as PCO progresses. Electron microscopy (Marcantonio et al. 2000), immunocytochemistry (Marcantonio et al. 2000) (Saika et al. 2000), RT-PCR (Wormstone et al. 2001) and ELISA (Wormstone et al. 2001), (Wormstone et al. 2000) have been used to analyse post-mortem material. Although this type of investigation provides " scene of the crime" information it does not provide definitive proof that various molecular suspects are responsible for PCO devepment.

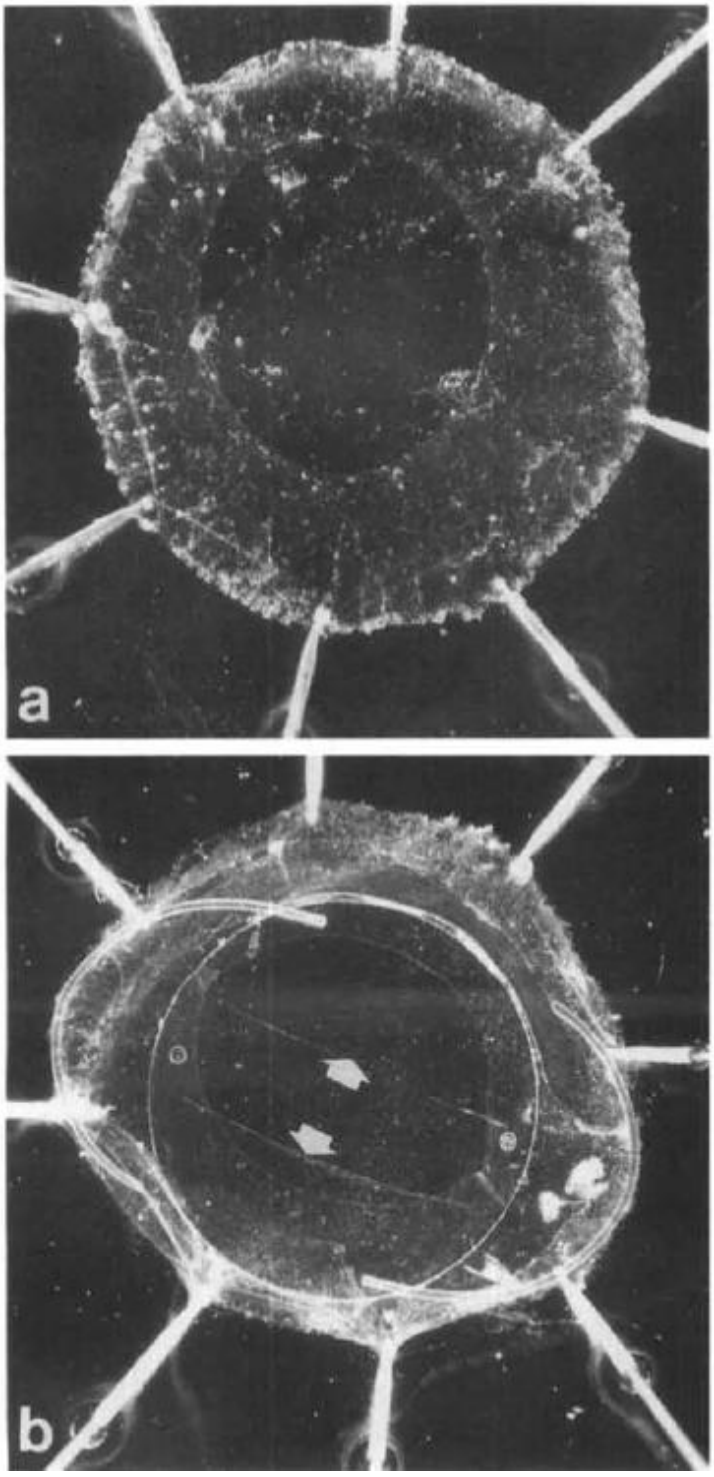

Figure 1.10 Low power dark-field views of capsular bag preparations pinned out and immersed in culture medium, in the absence of an IOL (a) the disc shape opening in the anterior capsule reveals the posterior beneath. When an IOL is present (b) the supporting loops (haptics) distend the capsular bag and creases can be seen on the posterior capsule (arrows). The micrograph represents a field of view of 1.8×1.25 cm (Liu et al. 1996)

1.6 Dietary antioxidants and cataract prevention

Different types of diet have been associated with an increased or decreased risk of cataract. Epidemiological studes have shown that dietary intakes rich in fruit and vegetables are linked to a reduced risk of cataract. An Italian case-control study investigated the effects of dietary factors on the risk of cataract (Chiu and Taylor 2007). Significant inverse relationships were seen between spinach, cruciferous vegetables, tomatoes, peppers, citrus fruits, and melon.

The effects of vitamins on cataract using multivitamin supplements have been investigated in several recent studies. An important decrease in risk for nuclear cataract was seen in the prospective Longitudinal Study for Cataract (33%) (Leske et al. 1997). A modest decrease in cataract progression was also found in the Rocha European American Cataract Trial (REACT) (Chylack et al. 2002). However, in the prospective Nurses' Health study, there was no association between multivitamin supplement consumption and cataract (Hankinson et al. 1992). Another antioxidant formulation, containing only three vitamins (A, C and E), has been used in the Age- Related Eye Disease study (AREDS) (AREDS group, 2001) and the Women's Health study (Christen et al. 2004). These two randomized placebo controlled studies did not show any significant effect of the supplementation of antioxidants on the development or progression of age-related lens opacities.

The relationship between vitamin C intake and risk for cataract has been investigated in several human studies, in the Nurses' Health study, a decreased prevalence of lens opacities has been found in women using vitamin C supplements (Hankinson et al.

1992). Like for vitamin C the results of studies investigating the effects of vitamin E on cataract are not consistent. Positive effects of vitamin E on cataract were observed in some studies (Leske et al. 1991, Rouhiainen et al. 1996, Vitale et al. 1993), but not in others (Lyle et al. 1999, Mohan and Muralidharan 1989). All these studies considered the vitamin E serum level except The Beaver Dam Eye study (Lyle et al. 1999) which measured the total dietary intake. Vitamin E serum levels were inversely associated with risk of nuclear cataract in the Lens Opacities case control study (Leske et al. 1991) and the prospective Baltimore Longitudinal study on Aging (Vitale et al. 1993).

A prospective study of the effect of vitamin A, or β-carotene, on the risk of cataract formation was conducted in the United States (Chasan-Taber et al. 1999). The results show a lower risk of developing cataract for women consuming high amounts of vitamin A, comparing to those consuming low amounts of vitamin A. three previous prospective studies did not obtain the same results. None of them found a significant association between vitamin A and cataract. Because lutein and zeaxanthin, two other carotenes, have been found in human cataractous lenses after extraction (Yeum et al. 1999), they have been well studied for their possible effect on cataract. Studies investigating the effect of carotenes on risk of cataract found an inversely related risk of cataract with high lutein and zeaxanthin dietary intake (Chasan-Taber et al. 1999, Lyle et al. 1999).

Another class of molecule investigated for their effects on cataract are flavonoids. Among these phenolic compounds, procyanidins, catechins and quercetin are chiefly studied. Several studies have investigated the effects of procynidin extracts on cataract

formation in rats. Procyanidins, or proanthocyanidins, are part of the flavanol group, which is a subclass of flavnoids. Procyanidins and their antioxidative metabolites were found to supress cataract formation (Durukan et al. 2006), to prevent cataract formation or its progression (Osakabe et al. 2004, Yamakoshi et al. 2002) and to postpone it (Nguyen et al. 1999). It is proposed that daily consumption of procyanidins in food may offer a prophylactic solution against onset and progression of cataract.

In addition to epidemiology studies, information has also been gleaned from experimental approaches in the laboratory. Numerous oxidants, including hydrogen peroxide, have been found to induce opacity in lenses from a number of species (Spector et al. 1995, Truscott 2005). A variety of antioxidants have been found to protect lens cells and prevent opacification (Sanderson et al. 1999).

Quercetin is a dietary bioflavonoid which has been shown to have protective effects against lens opacification in *in vivo* and in vitro models of cataract (Beyer-Mears and Farnsworth 1979, Cornish et al. 2002, Lija et al. 2006, Sanderson et al. 1999). Quercetin effectively inhibited H_2O_2-induced lens opacification in a lens organ culture with hydrogen peroxide (LOCH) model of cataract (Sanderson et al. 1999). A recent study looking at quercetin and its interaction with the hypoxia-inducible factor-1 (HIF-1) pathway raises important questions to be considered in relation to the lens. Triantafyllou used HeLa cells to investigate the effects of flavonoids on HIF-1a (Triantafyllou et al. 2008). It was found that as well as an increase in HIF-1a levels, that quercetin (100 mM) also inhibited translocation of HIF-1a to the nucleus via the p44/42 MAPK pathway. This inhibition became more apparent when the HIF-1 pathway was stimulated either

by iron chelation or hypoxia (1% O_2). In human lens epithelial cells, it was found that 65% of the genes with 10 mM quercetin increased expression were regulated by the HIF-1 pathway (Radreau et al. 2009). The quercetin-induced increase and nuclear translocation of HIF-1α was reversed by addition of excess iron (100 mM) (Radreau et al. 2009). These results demonstrate that quercetin activates the HIF-1 signaling pathway in human lens epithelial cells.

1.7 Isothiocyanates (ITCs)

Numerous epidemiological studies have shown that consumption of large quantities of fruits and vegetables, especially cruciferous vegetables (e.g. Broccoli and Brussels sprouts), can protect against carcinogenesis, mutagenesis, drug toxicities, and other chronic diseases (Conaway et al. 2002, Lam et al. 2009).

1.7.1 Chemical structure and classification of Isothiocyanates

All ITCs are characterized by the presence of an N=C=S functional group which is found in a variety of cruciferous vegetables. ITC compounds include Ally isothiocyanate (AITC) from cabbage, mustard, and horseradish; benzyl isothiocyanate (BITC) from lepidium cress; phenethyl isothiocyanate (PEITC) from watercress and garden cress; and SFN (SFN) from broccoli, cauliflower, brassicas, and kale (Verhoeven et al. 1997).

The chemopreventative effect of cruciferous vegetables is thought to be partially due to their relatively high content of glucosinolates (β-thioglucoside N-hydroxysulfates),

which distinguishes them from other vegetables (Zhang 2000, 2012). Glucosinolates all contain two parts: a common glycone moiety and a variable aglycone side chain derived from amino acids. Although over 120 different side chain structures have been described, relatively few occur in dietary crucifers (Table 1) (Figure 1.11). They are hydrolyzed to an aglycone R-C (-SH) =N-O-SO_3^- by the enzyme myrosinase (thioglucoside glycohydrolase, EC 3.2.3.1).

Table 1 Source of Isothiocyanates from Dietary Vegetables (Cheung and Kong 2010b)

Structure	Chemical name	Food
CH3-S-CH2-CH2-CH2-N=C=S	3-Methylthiopropyl	Cabbages
CH3-S-CH2-CH2-CH2-CH2-N=C=S	4-Methylthiobutyl	Arugula
CH3-S(=O)-CH2-CH2-CH2-N=C=S	3-Methylsulfinylpropyl (iberin)	Broccoli, Brussels sprouts, cabbages
CH3-S(=O)-CH2-CH2-CH2-CH2-N=C=S	4-Methylsulfinylbutyl (SFN)	Broccoli, Cauliflower
CH2=CH-CH2-N=C=S	2-Propenyl (AITC)	Mustards, cabbages, Brussels sprouts, Cauliflower
 -CH2-N=C=S	Benzyl (BITC)	Lepidium cress
 -CH2-CH2-N=C=S	2-Phenethyl (PEITC)	Watercress, radishes, turnips

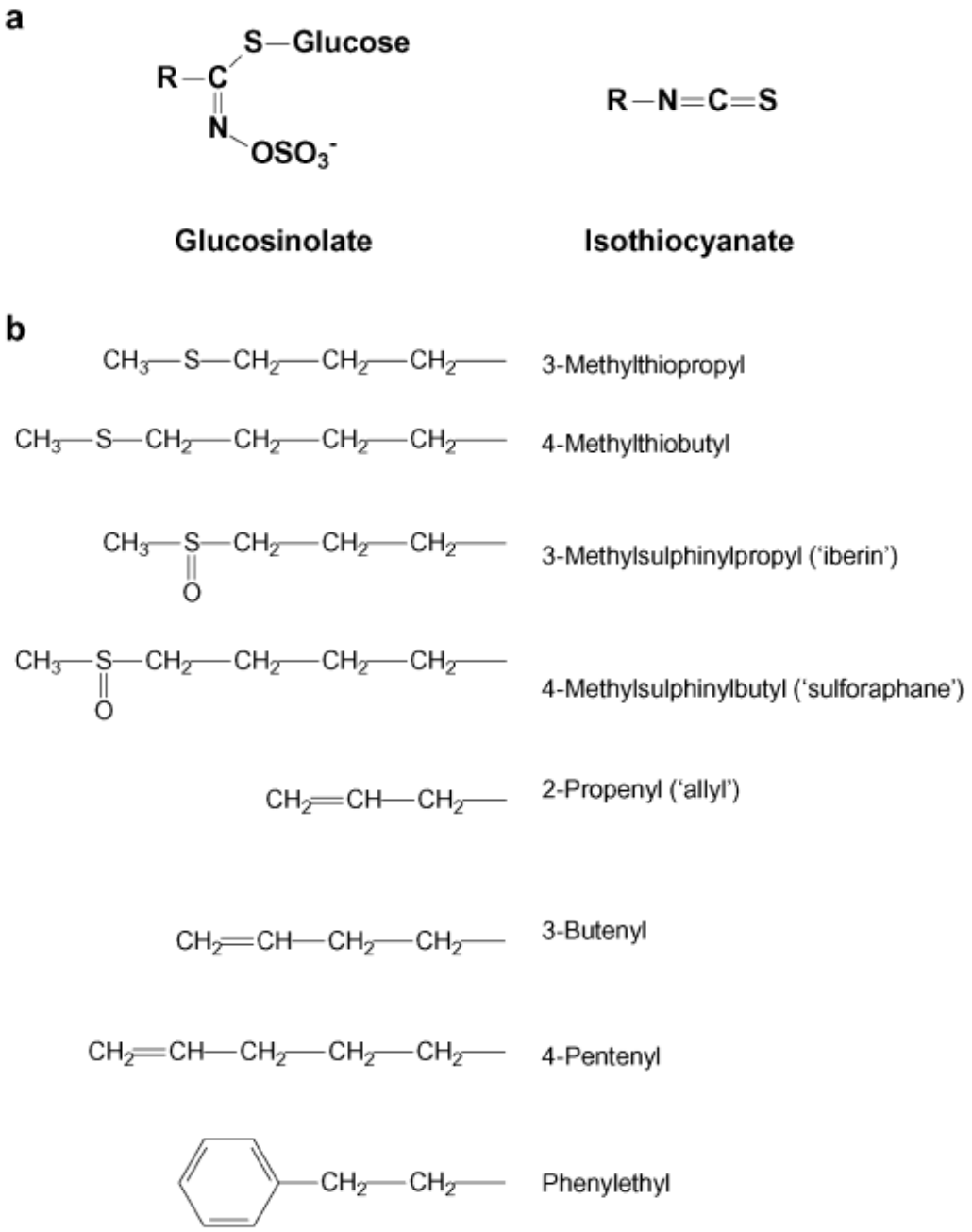

Figure 1.11 (A) The general chemical structure of glucosinolates and isothiocyanates, where R represents the variable side chain. (B) Examples of side chain structures (R) of glucosinolates and isothiocyanates (Juge, et al 2007).

S-(N-aralkylthiocarbamoyl)cysteine derivatives are formed from the reaction with cysteinyl thiols. Nucleophilic attack of isothiocyanates by amino groups forms thiourea derivatives with the structure RNH-C(=S)-NHR′. Nucleophilic attack of isothiocyanates by hydroxide ion forms monothiocarbamate derivatives of the form RNH-C(=S)-O–. These compounds eliminate carbonyl sulfide COS, which produces the corresponding amine derivative RNH2. These two sequential reactions constitute the irreversible hydrolysis of isothiocyanates that is associated with the loss of pharmacological activity. These are spontaneous reactions that occur under physiological conditions. They initially deliver a RNH-C(=S)-group to the nucleophile; hence, the reaction is called thiocarbamoylation (Figure 1.12).

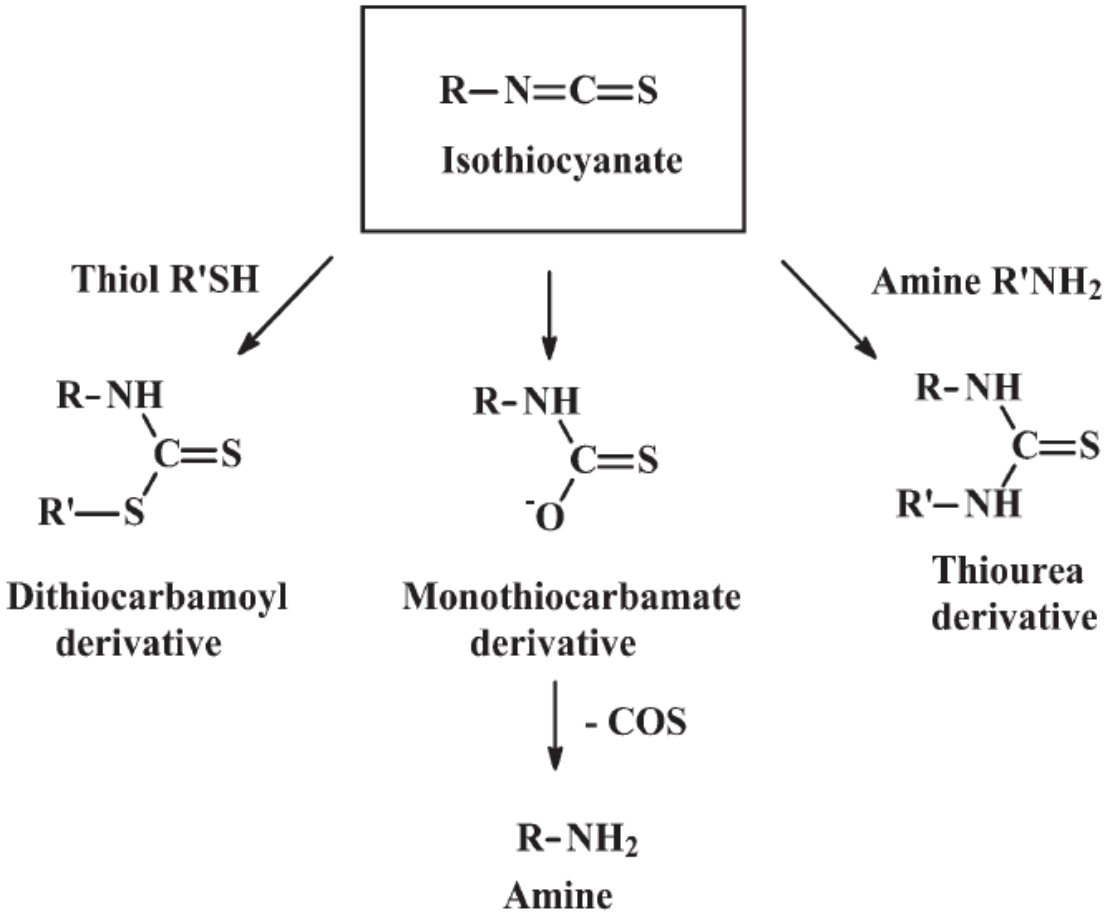

Figure 1.12 Thiocarbamoylation of isothiocyanates (Wu et al. 2009)**.**

Significant losses of isothiocyanates are expected during Figure 1.12. Hydrolysis of glucosinolates by myrosinase and formation of isothiocyanates food processing. The thioglycosidic bond is hydrolyzed, glucose is released and an unstable aglycone is formed (Figure 1.13). The aglycone fragments eliminate sulfate SO_4^{2-} and form isothiocyanate R-N=C=S. Glucosinolates are normally located in the cytoplasm of plant tissue (Rask et al. 2000). Myrosinase is expressed on the external surface of the plant cell wall, so it cannot access the glucosinolates (Rask et al. 2000). When the tissue is ruptured by chewing, preparation for cooking, heating, or insect attack, myrosinase interacts with the released glucosinolates and hydrolysis products are formed.

Some glucosinolates give rise to other products such as epithionitrile and oxazolidine derivatives, which depends on the glucosinolate, temperature, pH and presence of reducing agents. Some isothiocyanates are volatile and will be lost to the atmosphere by vaporization at the boiling point and evaporation at temperatures below the boiling point; for example, loss of allyl isothiocyanate occurs at a boiling point of 88 ℃. Isothiocyanates also are hydrolyzed at physiological temperatures, a process that becomes more rapid at higher cooking temperatures (Shapiro et al. 1998). This is probably due to isothiocyanate volatility and susceptibility to hydrolysis, and it explains why ingested cooked vegetable material often has a very low content of isothiocyanate and a much higher (>100 fold) content of glucosinolates (Shapiro et al. 1998). The myrosinase acting on the glucosinolates may also originate from other sources within the gastrointestinal tract (Zhang 2012, Zhang and Callaway 2002).

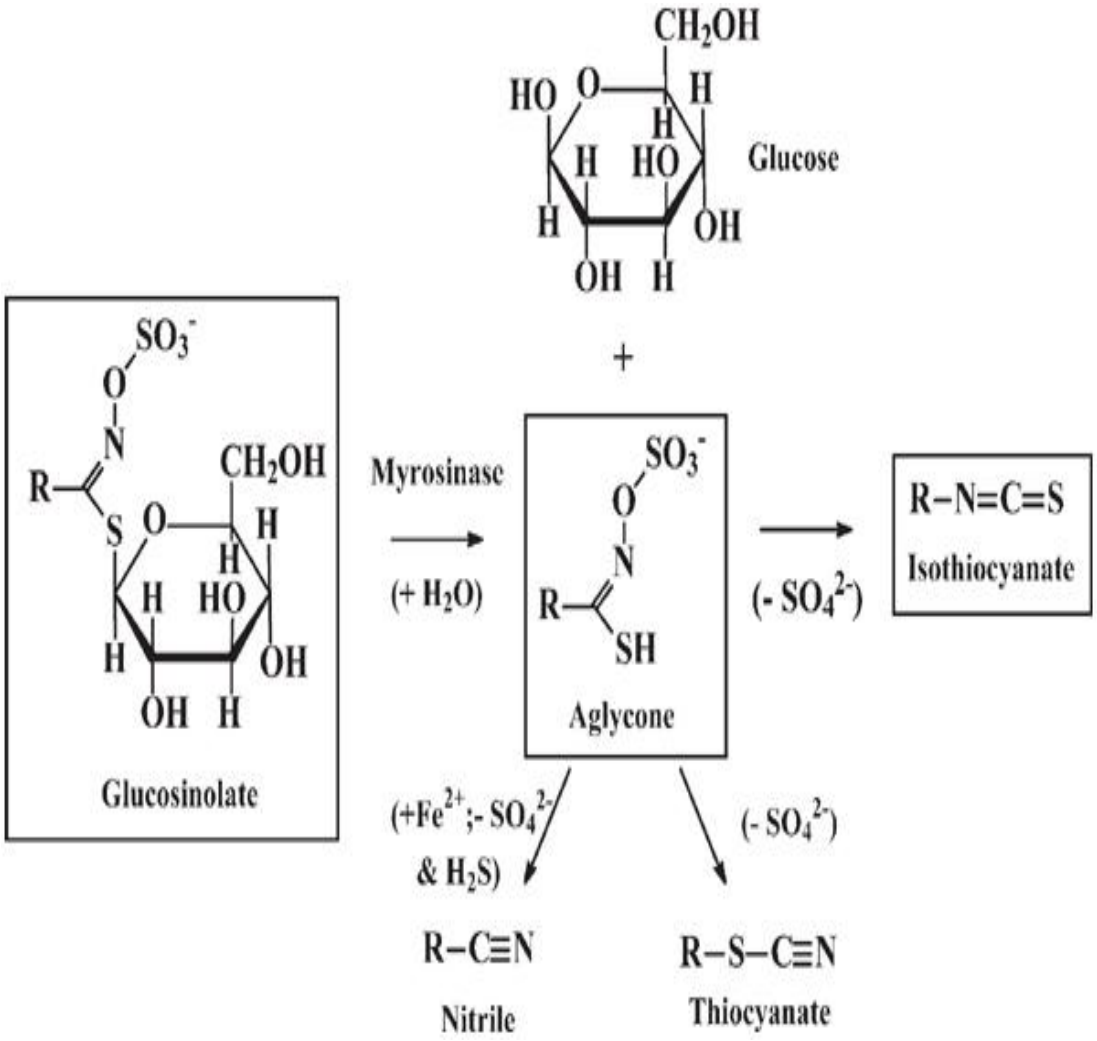

Figure 1.13 Hydrolysis of glucosinolates by myrosinase and formation of isothiocyanates (Wu et al. 2009)

1.7.2 Isothiocyanates metabolism and cellular uptake

After absorption through daily diet, ITCs are usually metabolised via the mercapturic acid pathway (Figure 1.14) (Egner et al. 2008). In addition, the central carbon of the –

N=C=S group reacts with the sulfhydryl group of glutathione S-transferases (GSTs). ITCs cross the gastrointestinal epithelium and the capillary endothelium by passive diffusion after being ingested in the lumen of the gastrointestinal tract. They then cross the plasma membrane into cells of tissues by binding to thiols of plasma protein and react with glutathione to form the glutathione conjugate, S-(N-alkyl/arylthiocarbamoyl) glutathione, which is promoted by GSTs. The GS conjugate is expelled from cells by transporter proteins, known as multidrug resistant proteins (MRPs). In the extracellular medium, they undergo successive enzymatic modifications, first by γ-glutamyl transferase (γ-GT) and then dipeptidase, forming a cysteine conjugates which is transported to the liver. Here, the cysteine conjugate is acetylated to Nα-acetyl derivative or mercapturic acid and transported to the kidney and excreted in the urine (Zhang 2001, 2012) (Figure 1.13).

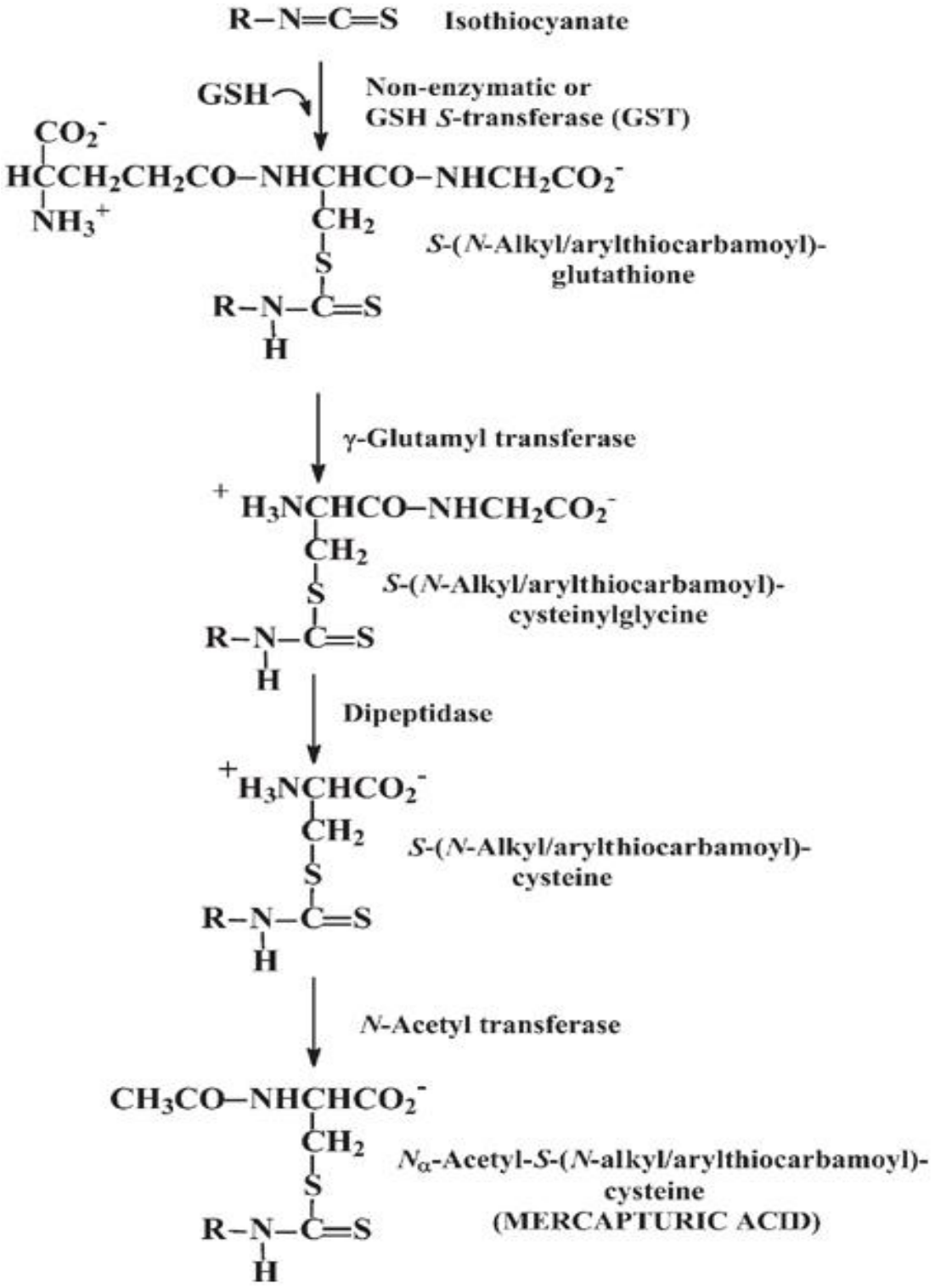

Figure 1.14 Metabolism of isothiocyanates by the mercapturic acid pathway. R is the aliphatic or aromatic substituent of the isothiocyanate (Zhang et al. 2012).

Mercapturic acid derivatives of dietary ITCs have been detected as major urinary metabolites in rats (approximately 72% of a single oral dose of SFN) (Kassahun et al. 1997) and human subjects (nearly 60% of a single oral dose of broccoli Sprouts) (Ye et al. 2002b) after consumption of glucosinolates.In addition, in a rat model, 24 hours after a single oral dose of ITCs, 72% of SFN was recovered in urine; only 1% was detected in the second 24 hours urine sample (Kassahun et al. 1997). The results show that the bioavailability of ITCs is high. Moreover, even after repeated SFN dosing (oral broccoli sprout extracts containing 25 μM ITCs at 8 hour intervals for 7 days) (Shapiro et al. 2006), ITCs also can be disposed of in urine extremely fast, and this is closely related to its rapid absorption (Petri et al. 2003a). The one critical factor which influences the absorption and bioavailability of ITCs is myrosinase activity. Myrosinase activity influences the hydrolysis process of ITCs from glucoraphanin. Mammalian cells have no myrosinase activity. The myrosinase enzyme only exists in cruciferous plant cell walls and gut microbial flora (Kassahun et al. 1997). In cruciferous plants, physical disruption like chopping, cutting and chewing can release the enzyme. However, the myrosinase enzyme is heat-labile during the cooking process, and the enzyme activity can be significantly reduced by up to 3-fold (Conaway et al. 2000). Another source of myrosinase activity is the microbial flora in the gut system. The evidence from an experiment compared isolated human fecal bacteria (Shapiro et al. 1998b) and F344 rates (Bheemreddy and Jeffery 2007) dosed with glucoraphanin. The results indicate that colonic microbial flora can catalyse glucoraphanin to ITCs.

Many studies showed that ITCs is taken up by cells predominantly, if not entirely, through GSH conjugation reactions in cells, and that cellular GST promotes uptake of

ITCs by enhancing the conjugation reaction. ITCs penetrate cells by diffusion, but once inside the cells, they are rapidly conjugated via their –N=C=S group with cysteine sulfhydryl groups of glutathione (GSH) and of proteins (Figure 1.15) (Zhang 2000). ITCs is rapidly accumulated in human and animal cells, with the peak intracellular ITCs accumulation reached within 0.5–3 hours of exposure and up to 100- to 200-fold over the extracellular ITCs concentration or up to millimolar levels (Zhang and Talalay 1998, Zhang and Callaway 2002). In addition, lipophilicity of ITCs does not seem to influence uptake of ITCs by cells (Zhang 2012).

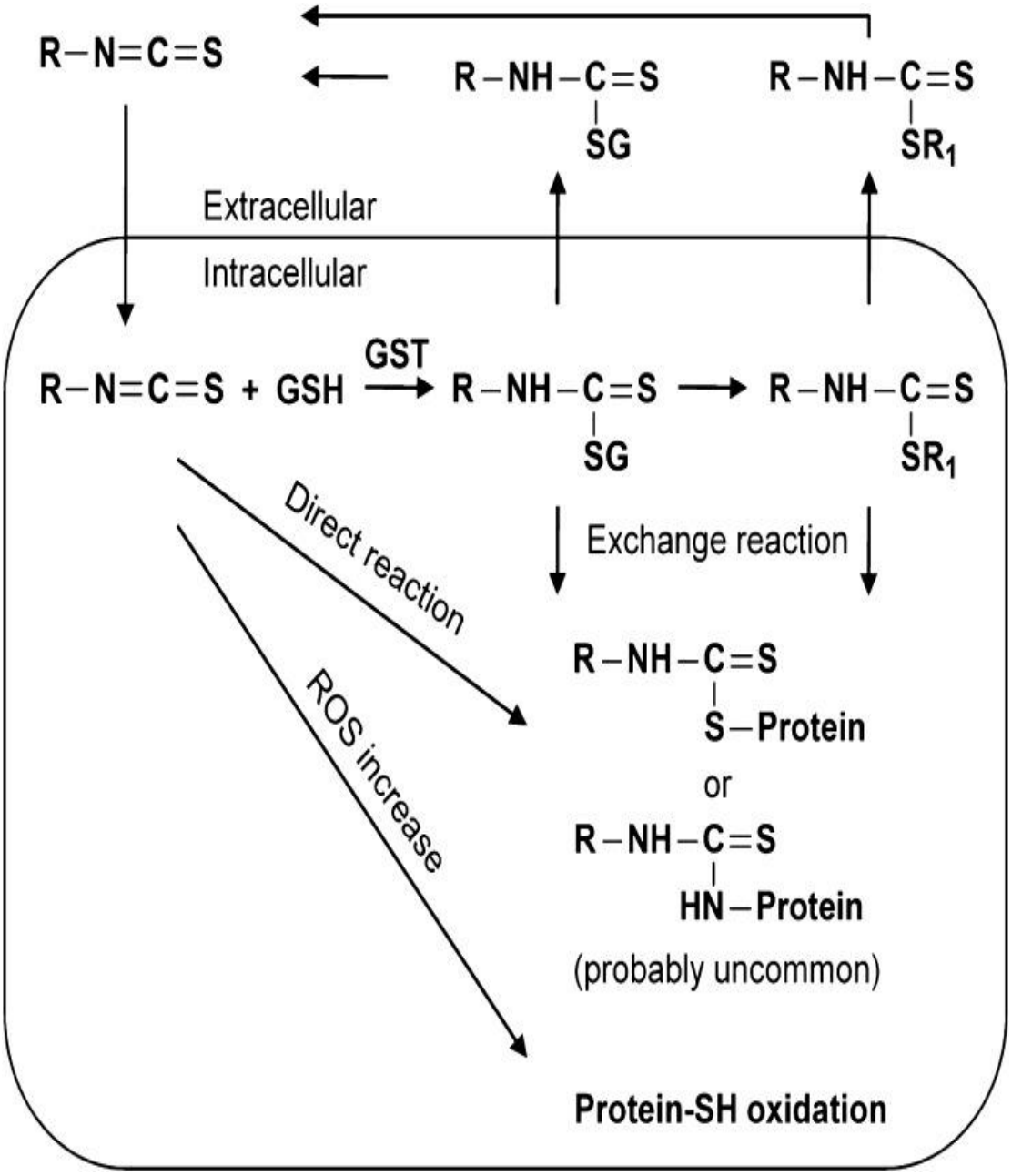

Figure 1.15 Cellular uptake and elimination of ITCs and intracellular protein modification. ITCs (R-N=C=S) are believed to enter a cell by diffusion, but once in the cell are rapidly accumulated via conjugation with intracellular thiols, primarily GSH but also proteins. The GSH conjugates may be further metabolized via the mercapturic acid pathway ('R-NH-C (=S)-SR1' stands for these metabolites), which are expelled from the cell via membrane transporters. The conjugates may modify cellular proteins via exchange reactions with cysteine sulfhydryl groups. ITCs and their thiol conjugates may also bind to certain proteins via reaction with amino groups and may also cause protein thiol oxidation by stimulating cellular reactive oxygen species production (Zhang et al. 2012).

1.8 Sulforaphane

Sulforaphane (SFN, R-1-isothiocyanato-4-methylsulfinylbutane), an ITC obtained in the diet through the consumption of broccoliis an isothiocyanate (Figure 1.15) (Hintze et al. 2003). Most cultivars of broccoli accumulate between 2 and 10 mmol/ g of 4 methylsulfinyl glucosinolate in their florets. Higher levels on a dry weight basis may sometimes be found within broccoli seedlings (sprouts) a few days after germination, although these rapidly decline as the seedlings age. A high glucosinolate variety of broccoli has, however, been specially bred to accumulate about threefold higher levels of glucosinolates in its florets. The most abundant glucosinolate in broccoli is glucoraphanin, which upon hydrolysis by myrosinase or intestinal flora yields the isothiocyanat, SFN, as shown in Figure 1.16 .

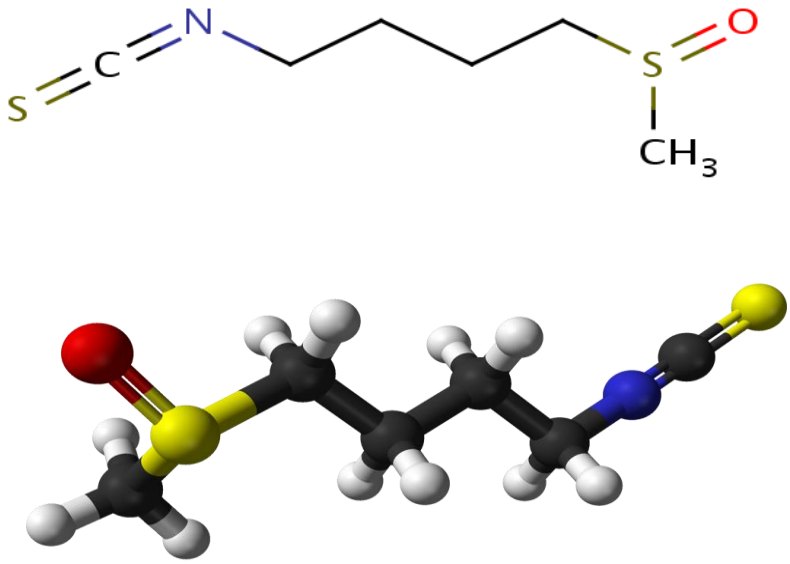

Figure 1.16 General chemical structure of SFN

(http://www.hmdb.ca/metabolites/HMDB05792)

When raw florets or sprouts are macerated or eaten, between 60 and 80% of the glucosinolate is converted to the SFN-nitrile, as opposed to the ITC, due to the combined effects of myrosinase and a non-catalytic protein cofactor (ESP-like). However, mild cooking can preserve myrosinase activity while denaturing ESP, resulting in almost 100% conversion to SFN. Further cooking denatures myrosinase, and intact glucosinolates are ingested. However, these can be converted to SFN in the colon by microbial thioglucosidase activity.

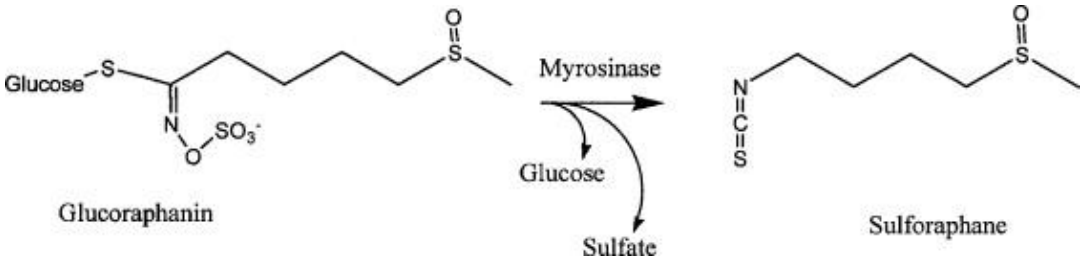

Figure 1.17 Hydrolysis of glucoraphanin to SFN by myrosinase (Cwik et al. 2010)

1.8.1 SFN metabolism

After absorption through daily diet, SFN is usually metabolised via the mercapturic acid pathway (Egner et al. 2008). SFN reacts with glutathione to give rise to a glutathione-SFN conjugate, which is catalyzed by Glutathione-S-Transferases (GSTs). After that, step-wise cleavage of glutamine and glycine first by the enzymes γ-glutamyl transpeptidase (GTP) and then by cysteinylglycinase (CGase) yields a L-cycteine conjugate. Then N-acetyltransferase (NAT) acetylates the L-cysteine conjugate to produce an N-acetyl-L-cysteine conjugate (mercapturic acid derivative). This final product is then removed from the body through urine (Figure 1.17).

Mercapturic acid derivative, present in urine, reflects the uptake of SFN and the intake of glucoraphanin from cruciferous vegetables (Fahey et al. 1997). Higher amounts of SFN conjugates in the blood and SFN-derived mercapturic acid metabolites in urine were found when broccoli was eaten raw (bioavailability 37%) versus cooked (bioavailability 3.4%) (Vermeulen et al. 2008).

In addition, in a rat model, 24 hour after a single oral dose of SFN, 72% of SFN was recovered in urine; only 1% was detected in the second 24 hour urine sample (Kassahun et al. 1997). The results show that the bioavailability of SFN is high. Moreover, even after repeated SFN dosing (oral broccoli sprout extracts containing 25 μmol ITC at 8 hour intervals for 7 days) (Shapiro et al. 2006). SFN also can be disposed of in urine extremely fast, and this is closely related to its rapid absorption (Petri et al. 2003).

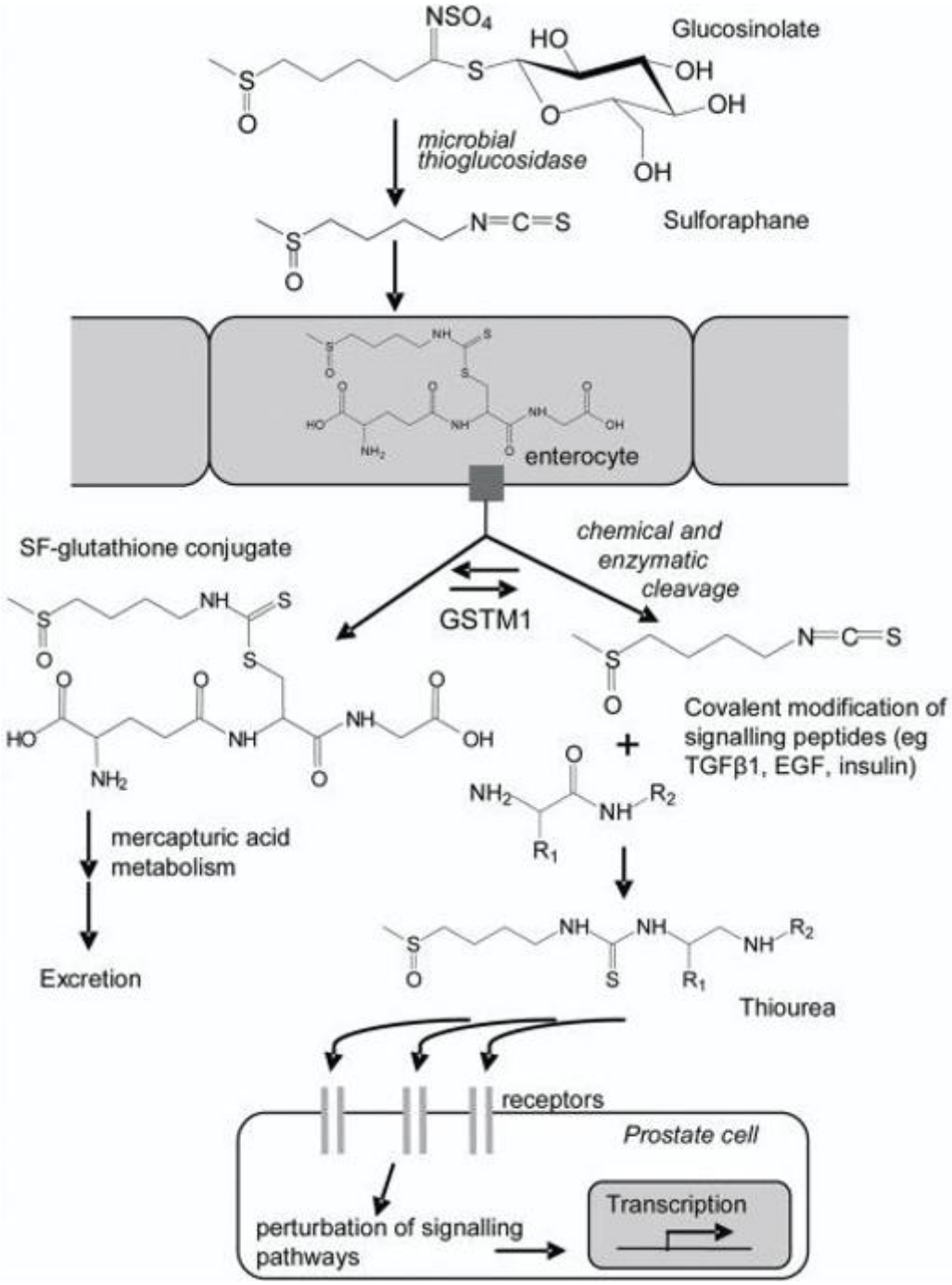

Figure 1.18 Metabolism of 4-methylsulphinylbutyl glucosinolate and SFN. Upon entry into enterocytes SFN is rapidly conjugated to glutathione, exported into the systemic circulation and metabolized through the mercapturic acid pathway. Within the low glutathione environment of the plasma the SF-glutathione conjugate may be cleaved, possibly mediated by GSTM1, leading to circulation of free SFN in the plasma. This free SFN can modify plasma proteins including signalling molecules, such as TGFβ, EGF and insulin (Traka et al. 2008).

1.8.2 Functional roles of SFN

SFN has been found to be a very potent chemopreventive agent in numerous animal carcinogenesis models, as well as cell culture models, exerting its chemopreventive effects through regulation of diverse molecular mechanisms (Cheung and Kong 2010). Several mechanisms have been proposed for the chemopreventive effect of SFN. These includes an early stage of research focused on "the blocking activity" of SFN through induction of phase II detoxification proteins (Juge et al. 2007). Further studies have recognized inhibition of phase I enzymes as also involved in activation of procarcinogens (Juge et al. 2007). Moreover, SFN may also influence or stop cancer development though several other mechanisms which are involved in controlling cell proliferation (Juge et al. 2007). These mechanisms include induction of apoptosis, induction of cell cycle arrest, and anti-inflammatory effects (Juge et al. 2007). Most likely, these factors interact together to reduce the risk of carcinogenesis. Understanding the different responses of cells and tissue to SFN is of great therapeutic interest.

1.8.2.1 Cytoprotection and "blocking" mechanisms

Much of our understanding of the ability of SFN to protect cells has come from chemoprevention studies. Drug metabolism generally plays a key role in the "blocking" effect of SFN. Drug metabolism takes place by two consecutive processes, phase I and phase II metabolism (Woolf and Jordan, 1987; Woolf, 1999). Phase I (Cytochrome P450 (CYP)) drug-metabolising enzymes (DMEs) are usually involved in oxidation, reduction, or hydrolysis of chemicals including carcinogens and these chemical reactions have been implicated in the bioactivation of carcinogens

(conversion of procarcinogens to carcinogens) (Wogan et al. 2004). These reactive metabolites can form adducts with endogenous biomolecules such as DNA, RNA, and proteins, and play a key step in initiating cellular damage and cancers. Therefore, modulation of CYP activity to decrease the activation of procarcinogens could be a plausible target for chemoprevention through preventing cancer initiation (Patel et al. 2007) . It has been reported that isothiocyanates could impair CYP activity by acting as competitive inhibitors as well as mechanism-based inhibitors, but the mechanism has not been extensively studied Human CYP isoforms that are involved in drug metabolism include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A (Elbarbry et al. 2007, Shimada et al. 1994). These reactive metabolites can form adducts with endogenous biomolecules such as DNA, RNA, and proteins, and play a key step in initiating cellular damage and cancers. Therefore, modulation of the CYP activity to decrease the activation of procarcinogens could be a plausible target for chemoprevention through preventing cancer initiation (Patel et al. 2007) .

SFN was shown to inhibit the activity of CYP1A1 and CYP1A2 in the human hepatoma cell line HepG2 (Skupinska et al. 2009a, Skupinska et al. 2009b). Also in rat models, SFN has a dose-dependent inhibition effect on the bioactivities of CYP1A1 and CYP2B1/2 (Maheo et al. 1997). The key step in the carcinogenesis of polycyclic aromatic hydrocarbons (PAH) is their biotransformation to oxyderivatives by CYP1A1 and CYP1A2. Therefore, inhibition of these CYP enzymes by SFN may explain its chemopreventive effect in rats against the carcinogen, aflatoxin B1 (Maheo et al. 1997). In further research, using the p-nitro-phenol hydroxylase assay, SFN was shown to have an anti-mutagenic effect against N-nitrosodimethylamine (NDMA) in

acetone-induced rat liver microsomes (Puccini et al. 1989). The procarcinogen is known to be activated by CYP2E1 to cause DNA damage, and this was inhibited by SFN in a dose-dependent manner (Barcelo et al. 1996). This confirmed that SFN is a potent competitive inhibitor for CYP2E1. On the other hand, SFN had no effect on the direct-acting, sodium azide (Paolini et al. 1997). This suggests that SFN may provide protection against carcinogens which are substrates for CYP2E1. Although, the mechanism by which SFN inhibits CYP activity has not been extensively studied, it has been reported that isothiocyanates impair CYP activity by acting as competitive inhibitors as well as mechanism-based inhibitors (Goosen et al. 2001, Nakajima et al. 2001)

The next process in drug metabolism involves phase II enzymes. Phase I enzymes produce hydroxylated intermediates. These intermediates are highly reactive and so have to be further metabolised by the phase II enzyme system. Phase II enzymes are detoxifying and excretory enzymes. Phase II reactions involved the metabolites produced during phase I metabolism, conjugating them with molecules such as uridine 5'diphosphate-glucouronic acid, glutathione or sulfate to produce less toxic and more readily eliminated metabolites. Also Phase II reactions have been extended to include proteins that catalyze reactions that lead to comprehensive cytoprotection against electrophiles and reactive oxygen species (Talalay 2000). Knockout of one or more of these phase II proteins, which include NADPH: quinine oxidoreductase (QR), glutathione S-Transferases (GST), UDP glucuronosyltransferases (UGT), glutathione reductase (GR), and heme oxygenase-1 (HO-1), in animals caused a significant increase in carcinogen induction and spontaneous tumorogenesis (Henderson et al. 1998b, Long et al. 2001, Long et al. 2000b). Many researchers have documented that

SFN is the most potent naturally occurring phase II detoxification enzyme inducer in both animals and humans (Fahey et al. 2002, Talalay 2000). Several in vitro studies have shown the effectiveness of SFN in inducing the activity of phase II proteins. SFN was found to potently induce the activity of QR, GST-α and γ- glutamylcysteine synthetase, and increase intracellular glutathione synthesis in human prostate cancer lines (Brooks et al. 2001). SFN and its glutathione conjugate significantly induced both UGT1A1 and GSTA1 mRNA and protein levels in human hepatoma HepG2 cells and colorectal adenocarcinoma HT29 cells (Basten et al. 2002). Similarly, in *in vivo* studies, rats fed for 14 weeks with 200 mg/day of dried broccoli sprouts containing glucoraphanin, the precursor for SFN, had significantly decreased oxidized GSH and protein nitrosylation, as well as increased glutathione (GSH) content, GSH reductase and GSH peroxidase activities in cardiovascular and kidney tissues (Wu et al. 2004).

There are three cellular components that regulate the gene expression of phase II proteins. These are Kelch-like ECH-associated protein 1 (Keap 1); Nuclear factor (erythroid-derived 2) - like 2 (Nrf2); and the antioxidant response element (ARE). Disruption of Nrf2-Keap1 interactions is one of the mechanisms to explain how SFN influences Phase II enzymes. In the absence of SFN and other inducers, Nrf2 is sequestered in the cytoplasm by Keap 1. Upon exposure to SFN, SFN can react with the thiol group of Keap1 and release the binding Nrf2 causing dissociation from Keap1. Nrf2 undergoes nuclear translocation and binds to the ARE and activates the transcription of phase II genes (Keum et al. 2006, Yu et al. 1999). Nrf2 knockout mice have been used to evaluate the importance of Nrf2. Feeding mice with broccoli seed as a source of SFN for 7 days, resulted in an increase in protein expression of NQO-1, GSTA1/2, GSTA3, GSTM1/2 in the stomach, small intestine and liver of wild-type

mice but not in Nrf2 knockout mice (McWalter et al. 2004). It shows that the ability of SFN to induce the expression of phase II proteins is mediated through Nrf2 as shown in Figure 1.19.

Collectively, the cytoprotective effect of SFN could be attributed to its effect on the balance between procarcinogen activation (by inhibiting phase I enzymes) and carcinogen detoxification (by enhancing phase II enzymes) (Figure 1.19) (Elbarbry and Elrody 2011).

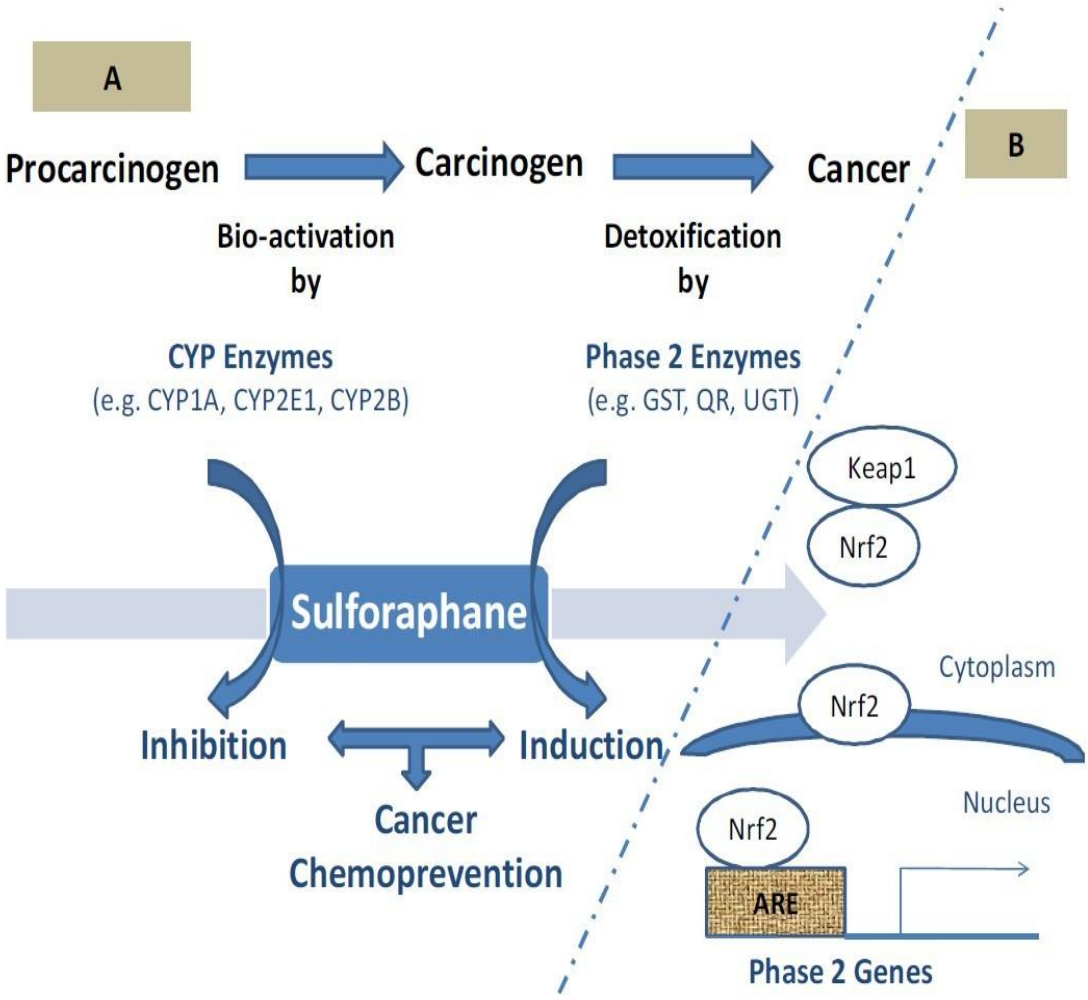

Figure 1.19 Cytoprotection by sulforaphane via modulation of drug metabolizing enzymes (DME). A: SFN interferes with cancer initiation through inhibition of CYP enzymes that are involved in bioactivation of procarcinogens and/or induction of phase II detoxification proteins. B: Proposed mechanism for the induction of phase II enzymes by SFN. SFN causes Nrf2 to dissociate of from its inhibitor Keap1 and translocate to the nucleus where it binds to the ARE and activates phase II genes (Elbarbry et al. 2011).

1.8.2.2 Cytostatic, anti-proliferative and cell death mechanisms

Recent studies showed that SFN may be involved in other anti-proliferation mechanisms including inhibition of cell growth by activation of apoptosis, cell cycle arrest, opromotion of ER stress and disruption of normal tubulin polymerization (Juge et al. 2007).

Apoptosis, or programmed cell death, is a highly regulated process that occurs under a range of physiological and pathological conditions as part of the cellular mechanism. Apoptosis is important in the development and maintenance of homeostasis and in the elimination of damaged cells. Inappropriate regulation of apoptosis leads to serious disorders such as neural degeneration, autoimmune diseases and even cancers (Elbarbry and Elrody 2011). The morphological features of apoptosis include cell shrinkage, chromatin condensation and fragmentation of the cell into compact membrane enclosed structures. Apoptosis can be induced by genotoxic compounds and various environmental stresses.

Induction of apoptosis is hypothesized to be through intracellular activation of caspases, a family of cysteine proteases, which are responsible for initiation and execution of apoptosis (Figure 1.20). Also caspase-independent pathways mediate induction of apoptosis, such as release of the mitochondrial protein apoptosis inducing factor (AIF) into the cytosol, activation of a family of Ca^{+2}-activated cytosolic proteases called calpains (Juge et al. 2007), or modulating the activation of transcription factors such as NF-kB and AP1 family members which are involved in

induction of cell survival genes (Mi et al. 2007). The first evidence for a cytostatic effect of SFN was reported in human colon cancer cells, where decreased viability in HT29 and Caco-2 cells was observed as a result of treatment with 15 and 50 mM, respectively (Gamet-Payrastre et al. 1998). In in vitro studies，colon cancer cell lines treated by SFN have been used to study features of apoptosis (Gamet-Payrastre et al. 2000a). It also it has been demonstrated in ovary (Bryant et al. 2010), prostate (Shankar et al. 2008) (Chiao et al. 2002b), bladder (Shan et al. 2006) and brain cells (Jiang et al. 2010). Several mechanisms, including both caspase-dependent and independent, have been proposed to explain the induction of apoptosis by SFN and have been reviewed (Juge et al. 2007a). The first report on the involvement of caspases in SFN-mediated apoptosis was by Chiao and colleagues in prostate LNCaP cells (Chiao et al. 2002a). Subsequently, SFN was shown to activate the mitochondrial/ intrinsic apoptotic pathway. This involves release of cytochrome c from the mitochondria into the cytosol, which in turn binds to the apoptosis protease activation factor-1 (Apaf-1) and leads to activation of the “initiator” caspase-9 (Choi and Singh 2005, Karmakar et al. 2006). Moreover, SFN was also shown to activate the death receptor/extrinsic pathway of apoptosis in prostate cancer cells. This pathway is initiated by “death ligands” such as Fas-L, TRAIL or tumor necrosis factor-a (TNF-a) and involves induction of caspase-8 and subsequent activation of “effector” caspases (Choi and Singh 2005, Singh et al. 2004). Additionally, SFN has been shown to activate caspase-12 following damage of the endoplasmic reticulum (Karmakar et al. 2006).

It was demonstrated that the initial signal for SFN-induced apoptosis is possibly from ROS (Myzak et al. 2007). ROS may play a key role in SFN- induced apoptosis.

Research using PC3 prostate cancer cells has shown that ROS are accompanied by a disruption of mitochondrial membrane potential, cytosolic release of cytochrome C, and apoptosis. The possible mechanism is that SFN treatment increases mitochondrial ROS production and induces apoptosis as indicated by the release of cytochrome C via both deathreceptor and mitochondrial caspase cascades. High doses of SFN are a necessary condition for inducing ROS production. When HT-29 colon cancer cells were treated with 50 μM SFN, the cell cycle arrest response was blocked by the addition of antioxidants N-acetyl-cysteine (NAC) or GSH, indicating that generation of ROS was indispensable for growth arrest under the assay conditions. However, in the DU145 cell line, only 10 μM SFN was enough to raise the ROS. In PC3 and LnCap human prostate cells less than 40 μM SFN treatment showed an induced effect of the formation of acidic vesicular organelles and autophagy; at the same time mitochondrial cytochrome C release and apoptosis were inhibited (Herman-Antosiewicz et al. 2006). Obviously, ROS production after SFN exposure has the ability to influence cell death in prostate and colon cancer cells, although the mechanisms have not been fully investigated.

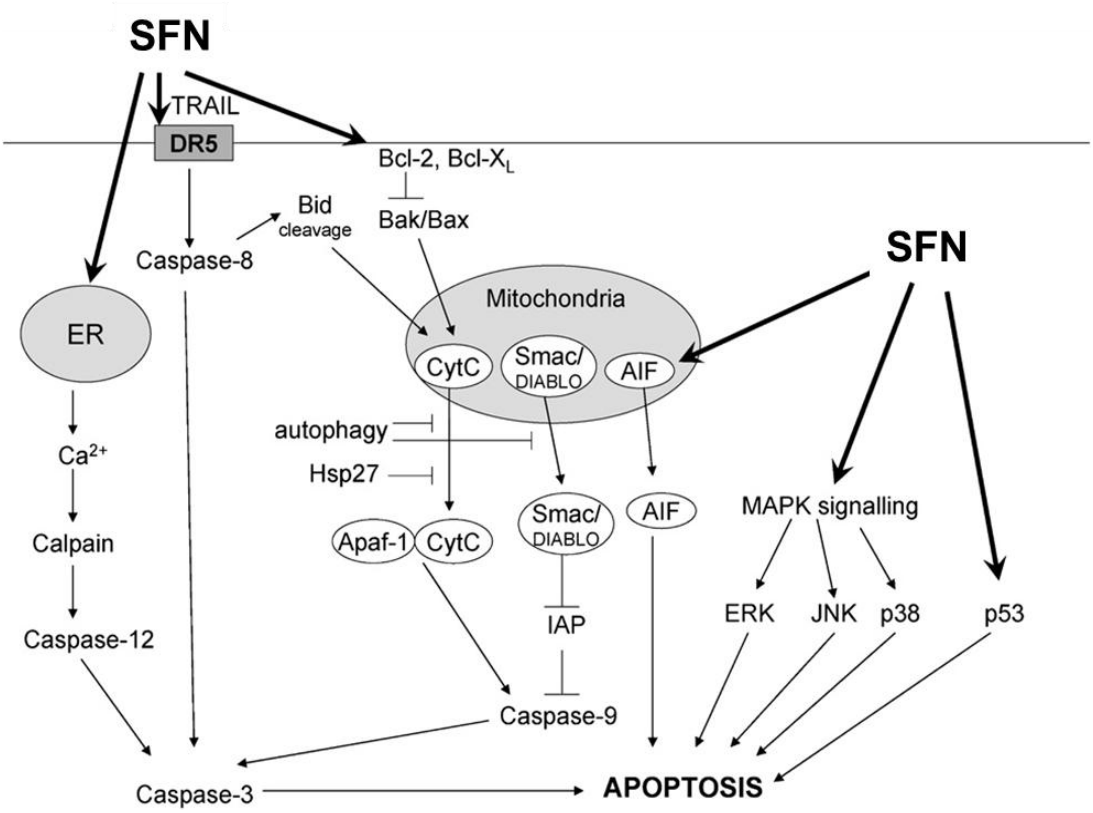

Figure 1.20 SFN induces apoptosis by affecting multiple targets of the mitochondrial/intrinsic and the receptor/extrinsic apoptotic pathways as well as caspase-independent pathways. All the apoptotic components shown in this figure have documented altered expression or (in) activation in response to SFN treatment (modified from Juge, 2007).

The other anti-proliferation mechanisms of SFN involve cell cycle arrest (Juge et al. 2007). The progression of cell cycle through the four stages, G1, S, G2 and M, is highly regulated by a number of mechanisms or checkpoint proteins (Figure 1.21). These cell cycle regulators are essential in cytoprotective responses to stress, including DNA damage and abnormal mitogenic signals (Juge et al. 2007). The phases of the cell cycle are regulated by cyclin-dependent kinase (CDK) molecules and cyclins, which drive the cell from one phase to the next and in turn are regulated by inhibitors. This process offers many potential targets for chemopreventive agents such as SFN. The key regulators of cell cycle progression are the cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors (CDKIs). Normal cell growth is maintained through a balance between the cyclin/CDK complexes that promote cell growth and CDKIs that promote cell cycle arrest.

There are three possible mechanisms of SFN-mediated cell cycle arrest (Figure 1.21). First is through inhibition of cyclins which, together with CDK, drive the cell cycle from one phase to the next (Chiao et al. 2002b). In both prostate and colon cancer cells, SFN inhibited the expression of cyclin D1 and DNA synthesis along with a G1 phase cell cycle block (Chiao et al. 2002). Another mechanism by which SFN affects the cell cycle is through up-regulation of CDKIs, which bind and inhibit the activity of cyclin/CDK complexes and regulate cell cycle progression through the four phases (Shan et al. 2006). Treatment of epithelial ovarian cancer cells with SFN resulted in dose-dependent decrease in expression of G1 phase cyclins while increasing the expression of CDKIs (Bryant et al. 2010). A third possible mechanism for the arrest in cell cycle progression by SFN was described by Jackson and colleagues and involved disruption of microtubules by inhibition of tubulin polymerization (Jackson

and Singletary 2004a). Tubulin plays a pivotal role in cell division, motility and intracellular trafficking in all eukaryotes (Nogales 2000). Drugs that inhibit its polymerization and interfere with mitosis have gained much attention in cancer drug discovery. The first evidence that SFN inhibits tubulin polymerization was observed in the mouse mammary carcinoma cell line F3II (Jackson and Singletary 2004a). In this case, low concentrations of SFN (15 mM) caused mitotic cells to display aberrant and mildly depolymerized spindles, whereas high doses of SFN (100 – 300 mM) inhibited tubulin polymerization. Similarly, the inhibition of tubulin polymerization was specifically attributed the SFN molecule which was also shown in bovine endothelial cells (Jackson and Singletary 2004a) and human breast adenocarcinoma cells (Jackson and Singletary 2004b).

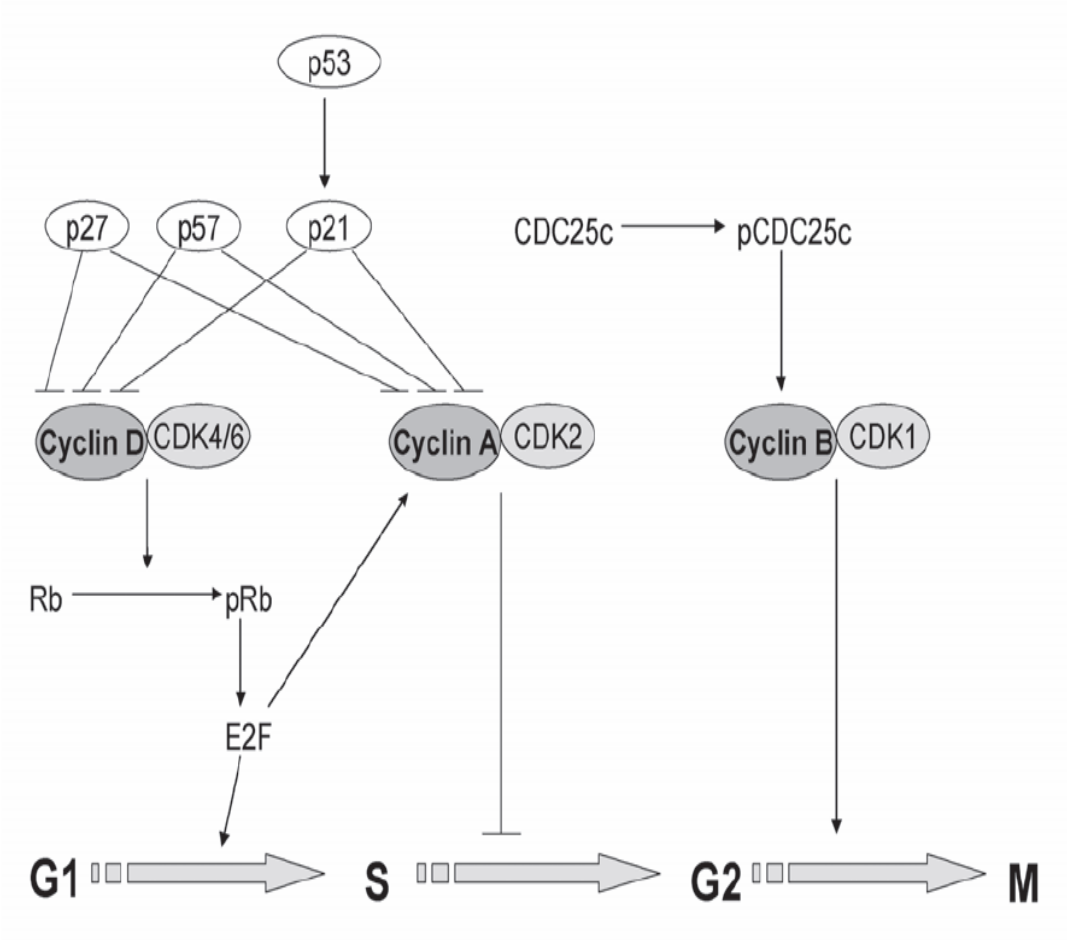

Figure 1.21 SFN arrests the cell cycle by modulating different stages of cell cycle progression. Only members of the cell cycle machinery that have been documented to alter expression or activation status in response to SFN treatment are shown (Jugea, 2007).

The endoplasmic reticulum (ER) is the site of protein synthesis, where folding and trafficking are initiated, and also the mediator of internal and external stresses (Figure 1.22) (Boyce and Yuan 2006). ER stress can arise because unfolded or mis-folded proteins are produced within the ER. Normal levels of unfolded proteins can be counteracted by a number of ER chaperone proteins, but if the level continues to increase, for example through prolonged oxidative or osmotic stress, then it is detected by the molecule Bip which in turn activates one or more of 3 stress pathway initiators (PERK, IRE1 and ATF6).

ER stress can also be seen as a depletion of ER calcium with a concomitant rise in cytosolic calcium. Once a prolonged stress is sensed then a range of external pathways are initiated. For example, when EIF2α is phosphorylated, then translation of certain proteins is inhibited. This apparently is a protective pathway, which presumably results from an inhibition of the synthesis of unfolded proteins. Interestingly, expression of Ca-ATPase, whose function is inhibited by thapsigargin (Tg) is in fact up-regulated after Tg exposure presumably via a feedback mechanism (Liu et al. 2002).

Many of the other pathways lead to cell death through apoptosis, for example calpain activation, which is calcium dependent, is particularly harmful to the lens and has been implicated in cataract formation (Sanderson et al. 2000). ER stress has been implicated in oxidative stress mediated lens opacity and lens cell death (Wang et al. 2012). In cases where ER stress is activated by Tg or arsenic trioxide lens cell death has resulted. There is also a limited body of information that suggests SFN can

promote ER stress in non-ocular cells (Deng et al. 2012, Doudican et al. 2012b). If SFN can induce an ER stress in lens cells it is likely cell viability will be compromised.

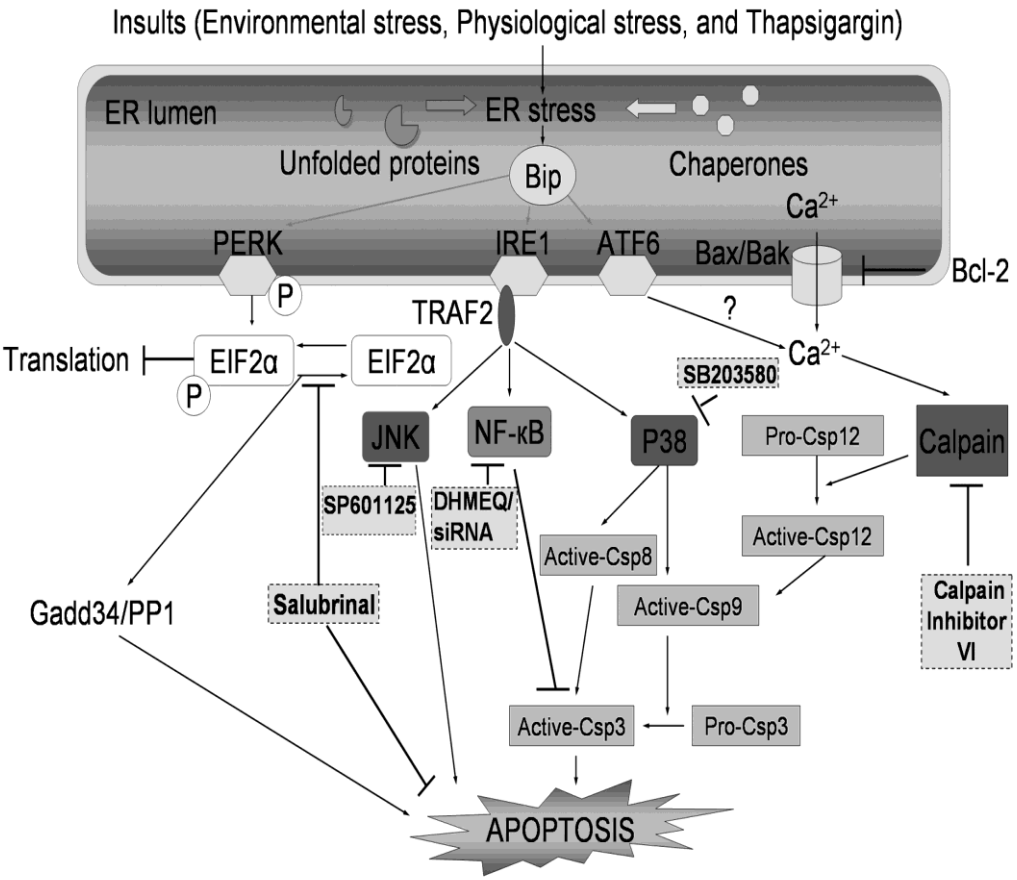

Figure 1.22 Diagram showing the different stress-sensing receptors both within the ER (eg Bip and Calcium) and on the ER membrane (PERK, ATF6 etc) and downstream mechanisms leading to apoptosis. (Boyce and Yuan 2006, Wu and Kaufman 2006)

Aims

Cataract and PCO are important medical problems affecting millions worldwide. Reducing the incidence of these conditions would have a marked effect on the personal well being of millions of people. SFN is reported to be both protective and destructive to cells in a dose-dependent manner in many cells, tissues and organs. The current study therefore tested the hypothesis that SFN could provide putative therapeutic benefit to patients against cataract and PCO formation. To test this hypothesis the following aims were tested:

1) To determine if the isothiocyanate, SFN could yield protection to lens cells against oxidative stress.
2) To identify the mechanisms by which SFN can elicit cytoprotection to lens cells against oxidative stress.
3) To establish the ability of SFN to initiate death of lens cells and prevent PCO formation.

CHAPTER 2
MATERIALS AND METHODS

All reagents were purchased form Sigma (Poole, Dorset) unless otherwise stated.

2.1 Cell culture

FHL 124 is a non-virally transformed cell line generated from human capsule-epithelial explants (Reddan et al. 1999), showing a 99.5% homology (in transcript profile) with the native lens epithelium (Wormstone et al. 2004). FHL 124 cells were routinely cultured in 25 cm^2 tissue culture flasks and maintained in Eagle's Minimal Essential Medium (EMEM) (Gibco, Paisley, UK) supplemented with 5% Foetal Calf Serum (Gibco, Paisley, UK) with 50 μg/ml gentamicin antibiotic (Sigma-Aldrich, MO, USA). Then FHL 124 cells were confluent, medium was aspirated from the 25 cm^2 tissue culture flasks and the cell layer was washed with 3 ml of Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich, MO, USA) for 1 minute. The wash solution was then aspirated from the flask and 3 ml of trypsin-EDTA (0.05% and 0.2% respectively) was added and maintained at room temperature for 1-2 minutes until the cells were detached from the base of the flask. At this point the trypsin was neutralized with 7 ml of EMEM supplemented with 5% FCS and a cell suspension formed through several pipetting cycles. The cell suspension was transferred to a 25 ml sterile universal tube and cells were counted using a hemocytometer. The cell suspension was centrifuged for 7 minutes at 800 rpm, which resulted in the formation of a cell-pellet. The supernatant was carefully removed from the universal tube and discarded. The remaining cell-pellet was then re-suspended in an appropriate volume of 5% FCS-EMEM and either: transferred to a new flask (passaged); seeded on

coverslip (10,000 per coverslip for immunocytochemistry); seeded on 35 mm tissue culture dishes (30,000 per dish qRT-PCR; 30,000 per dish for Western blot; 30,000 per dish for microarrays; 35,000 per dish for alkaline comet assay) or seeded on to 96 well plates (5000 per well for ApoTox-Glo Assay; 10,000 per well for LDH Assay; 5000 per well for MTS; Promega, Southampton, UK). All preparations were maintained at 35 ℃ in a humidified atmosphere of 95% air and 5% CO_2 incubator.

2.2 Cell viability and death assays

2.2.1 ApoTox-Glo Triplex Assay

FHL 124 cells were seeded on 96 well plates at a density of 5000 cells per well. 24 hours prior to addition of experimental conditions, culture medium was replaced with serum-free EMEM. The medium was removed from wells and replaced with freshly prepared medium and placed in experimental conditions. Plates were incubated at 35 ℃, 5% CO_2 for the experimental duration (up to 72 hours). The ApoTox-Glo Triplex Assay (Promega, Southampton, UK) was used to measure FHL 124 cell viability, cytotoxicity and apoptosis following manufacturer's instructions. Briefly, viability and cytotoxicity are measured by fluorescent signals produced when either live-cell or dead-cell proteases cleave added substrates GF-AFC (viability) and bis-AAF-R110 (cytotoxicity). Fluorescence of the cleaved products is proportional to either viability or cytotoxicity. GF-AFC can enter cells and is therefore only cleavable by live-cell proteases, which incidentally becomes inactive when cell membrane integrity is lost; bis-AAF-R110 cannot enter the cell, and is cleaved only by dead-cell protease leaked from cells lacking membrane integrity. Both cleaved substrates have

different excitation and emission spectra. Apoptosis is measured by the addition of a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7) which is cleaved in apoptotic cells to produce a luminescent signal. Fluorescence was measured at $380_{Ex}/510_{Em}$ (viability), $485_{Ex}/520_{Em}$ (cytotoxicity) and luminescence (apoptosis) with a FLUOstar Omega plate reader (BMG LabTech, Aylesbury, UK).

2.2.2 MTS assay

A cell proliferation assay (CellTiter 96 AQueous; Promega, Southampton, UK) was used in accordance with the manufacturer's instructions to assess the viability of the cells. This assay is a colorimetric method for determining the number of viable cells undergoing proliferation. The assay is based on the cellular conversion of a tetrazolium salt (MTS) into a formazan product. The resultant absorbance is directly proportional to the number of living cells in culture. In brief, 5000 cells were seeded in 96-well plates for 24 hours then serum-starved for 24 hours before exposure to SFN or vehicle control for a further 24 hours before placing in experimental conditions for 72 hours, as previously described. Then 25 µl of reagents (CellTiter 96 AQueous One Solution; Promega) was added directly to the culture wells and incubated for 1 hour, and absorbance was measured at 490 nm with a FLUOstar Omega plate reader (BMG LabTech, Aylesbury, UK). Cell viability was expressed as a percentage, with 100% representing the signal from untreated cells and 0% representing the background signal from empty wells.

2.2.3 Lactose Dehydrogenase (LDH) Assay

LDH is a soluble cytosolic enzyme that is released into the culture medium when the integrity of the cell membrane is lost (Bonfoco et al. 1995a, Bonfoco et al. 1995b).

Therefore, a high LDH assay reading would indicate cytotoxicity. The LDH assay reaction consists of two steps. Firstly, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. Secondly, $NADH/H^+$ reduces the tetrazolium salt Iodotetrazolium chloride (INT) to form a formazan product (Callewaert et al. 1983, Korzeniewski and Callewaert 1983). Following exposure to experimental conditions, the culture medium for the LDH assay was harvested. Total LDH was measured by permeabilising the remaining cells by addition of an equal volume of 2% Triton-X100 dissolved in cell culture medium. Samples were centrifuged for 5 minutes at 13000 rpm to obtain a cell-free supernatant and 100 µl was dispensed into wells of a 96-well plate. 100 µl LDH substrate (Diaphorase/NAD^+ mixture with stabilized INT and sodium lactate was then added, prepared according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). Plates were incubated for 10 minutes at room temperature in the dark before measuring absorbance at 490 nm with a FLUO star Omega plate reader (BMG LabTech). Appropriate background readings were made and subtracted from corresponding absorbance values. Results were expressed as relative fluorescent units (RFU) or as a percentage total releasable LDH using the following formula:

$$\%\ \text{Total Releasable LDH} = \frac{(\text{Sample absorbance} - \text{medium control absorbance})}{((\text{TritonX100 positive control absorbance} - \text{TritonX100 control absorbance}) + (\text{sample absorbance} - \text{medium control absorbance})) * 100}$$

2.3 Alkaline Comet Assay

The alkaline comet assay, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analysing DNA strand breaks in individual cells. The experimental protocol is summarised in Figure 2.1.

FHL 124 cells were seeded onto 35mm plastic culture dishes at a density of 35,000 cells per dish and grown until approximately 70% confluency. At this time point the medium was removed from each dish and replaced with 1.5ml serum-free EMEM for 24 hours before placing the cells in experimental conditions for a further 24 hours. Cells were pre-treated with 1 µM SFN for 24 hours prior to exposure to 30 µM H_2O_2 and incubated at 35 ℃, 5% CO_2. The cells were washed with ice cold phosphate buffered saline (PBS), harvested, counted, re-suspended in PBS containing 10% DMSO and frozen at -80 ℃ until the alkaline comet assay was performed. Samples were defrosted and approximately 25,000 cells per sample centrifuged at 3000 rpm for 5 minutes at 4 ℃. Pellets were re-suspended in 0.6% low melting point agarose, dispensed in duplicate onto glass microscope slides (pre-coated in 1% normal melting point agarose) and allowed to set on ice, under a glass coverslip. Once set, the coverslips were removed and slides transferred into ice-cold lysis buffer (100mM disodium EDTA (Fisher Scientific, Loughborough, UK), 2.5 M NaCl (Fisher Scientific, Loughborough, UK), 10 mM Tris-HCl (Formedium, Fisher Scientific, Loughborough, UK), pH 10.0 with 1% triton-X-100 added immediately prior to use) for 1 hour. Slides were washed twice with ice-cold dH_2O for 10 minutes, transferred to a flatbed electrophoresis tank (Perceptive Instruments, Bury St Edmunds, UK) and incubated in freshly prepared ice-cold electrophoresis buffer (300 mM NaOH, 1 mM disodium EDTA, pH 13) for 30 minutes, followed by electrophoresis in the same buffer at 21V (1V/cm) for 30 minutes. Procedures were performed protected from

direct light. Slides were drained of electrophoresis buffer and flooded with neutralisation buffer (0.4 M Tris-HCl, pH 7.5) for 30 minutes, washed twice in dH_2O for 10 minutes and dried at 37 ℃. Slides were stained with SYBR Green I nucleic acid stain diluted from a 10,000 X stock in 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 5 minutes protected from light at room temperature, drained and dried at room temperature prior to visualisation. 100 comets were randomly analysed per sample (50 per gel), with images captured by a Zeiss Axiovert fluorescence microscope (Zeiss, Cambridge, UK) and comets scored using Comet Assay IV Lite analysis software (Perceptive Instruments, Bury St Edmunds, UK).

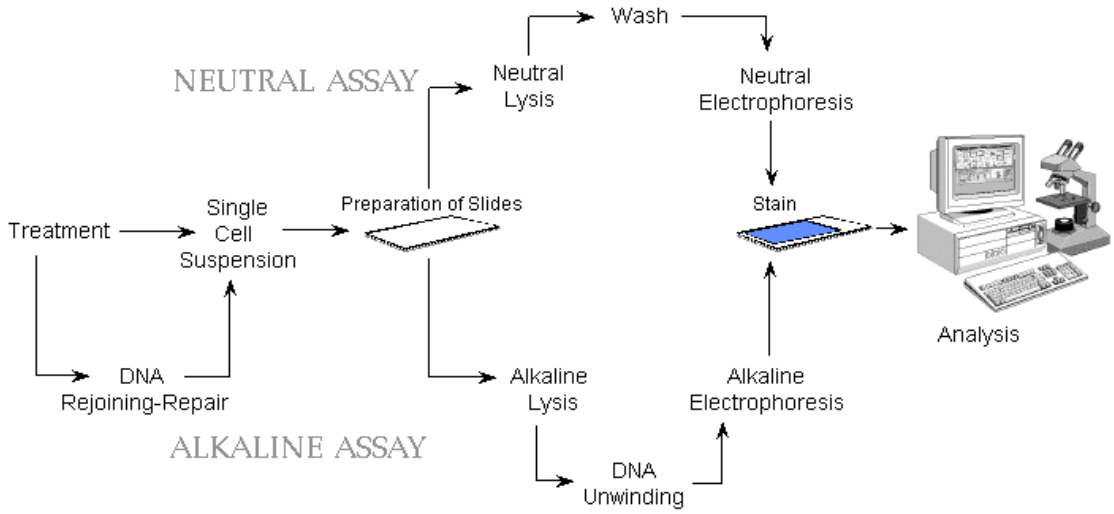

Figure 2.1 Manual comet assay system

(http://www.loats.com/docs/HALOcomet/HALOcomet.htm)

2.4 Analysis of gene expression by quantitative Real- Time Polymerase Chain Reaction (qRT-PCR)

2.4.1 Cell culture

Cells were seeded onto 35mm culture dishes (150,000 cells in 1.5ml) and grown to 90% confluency. Cells were treated with 0, 10, 30μM SFN for 24 hours.

2.4.2 Total RNA Extraction

RNA was extracted from FHL 124 cells using an RNeasy® mini kit (Qiagen Ltd, Crawley UK). The monolayer of cells was lysed by adding 350μl of buffer RLT containing 2- β-mercaptoethanol, to each culture dish. The cell lysate was collected using a cell scraper. The cell lysate was transferred into a sterile eppendorf tube and was passed through a 20-gauge needle (0.9nm diameter) fitted to an RNase-free syringe three times. This process homogenizes the cells allowing the RNA to be released from all FHL 124 cells. 350μl of 70% ethanol was added to the homogenized lysate, and was mixed via pipetting. The ethanol was added to the lysate to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. 700 μl of the solution was transferred to an RNeasy mini column placed in a 2 ml collection tube. The columns were centrifuged for 30 seconds at 8000 rpm and the flow-through was discarded. A series of washes using different buffers were carried out in order to produce the pure RNA. 700 μl of RW1 buffer was added to the RNeasy mini column, centrifuged for 30 seconds and removal of flow-through was carried out. The RNeasy mini column was fitted to 1 new collection tube and two washes with 500 μl of RPE buffer. The initial centrifugation was for 15 seconds and the second centrifugation for 2 minutes, which gave rise to a dry RNase silica-gel membrane containing the RNA. Using 50μl of RNase free water the RNA was eluted from the

column into a sterile 1.5ml eppendorf and centrifuged for 1 minute.

2.4.3 Total RNA quality assessment

The NanoDrop ND-1000 spectrophotometer (NanoDrop Technolgies, Delaware, USA) was calibrated using a blank sample of 1µl of sterile double distilled water. The absorbance (nm) and RNA concentration (µg/µl) were recorded for each sample. RNase-free filter pipettes were used in all procedures involving the RNA to prevent contamination of the sample.

2.4.4 First strand cDNA synthesis

For generation of cDNA by reverse transcription, 750 ng (FHL 124 cells) of total RNA was applied to 0.5 ml RNase-free thin walled Eppendorf tubes and samples diluted with appropriate volumes of double distilled water to give a final concentration of 75 ng/µl. Equal volumes of random primers (Promega, Southampton, UK) and 10mM dNTP (Bioline, London, UK) were mixed together and 2µl of this mix was added to each of the diluted RNA samples, before brief vortex mixing and centrifuging for 30 seconds at 13,000 rpm. Samples were placed in a Peltier Thermal Cycler-DNA Engine (MJ Research Inc, Reno, NV) and incubated at 65℃ for 5 minutes, followed by 5 minutes incubation on ice and a brief 15 seconds centrifuge at 13,000 rpm. A mixture containing: 40 µl of RNase out Recombinant Ribonuclease inhibitior; 100 mM DTT and 5X first strand buffer (Invitrogen, Paisley UK) were prepared in the ratio of 1: 2: 4 respectively. 7 µl of this mixture was then added to each sample. Samples were centrifuged for 30 seconds at 13,000 rpm before incubation at 25 ℃ for 10 minutes in the Peltier Thermal Cycler. This was followed

by a 42 ℃ incubation step for 2 minutes. Samples were removed from the Thermal Cycler and 1 µl of Superscript II™ (Invitrogen Ltd, Paisley UK) was pipetted into each sample. Samples were placed back into the Thermal Cycler (without centrifugation) and reverse transcription was performed with incubation at 42 ℃ for 50 minutes followed by a 70 ℃ incubation for 14 minutes. The generated cDNA samples were diluted with sterile double distilled water to a final concentration of 7 ng/ µl.

2.4.5 TaqMan qRT-PCR

QRT-PCR reactions were performed using an ABI prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) under the following conditions: 50 ℃ for 2 minutes, 95 ℃ for 10 minutes, and then 40 cycles, each consisting of 15 seconds at 95 ℃ and 1 minute at 60 ℃. Each reaction was performed in 25 µl and contained reverse transcribed RNA, primers and probes (sequences for primers and probes are given in Table 2) and TaqMan PCR Master Mix (Applied Biosystems). Primers and probes (Figure 2.2) were bought as predesigned TaqMan probe and primer sets provided by Applied Biosystems. The threshold cycle (Ct) values, defined as the point at which the fluorescent signal is recorded as statistically above background, were obtained with the 7500 Fast system software 2.0.5 (Applied Biosystems).

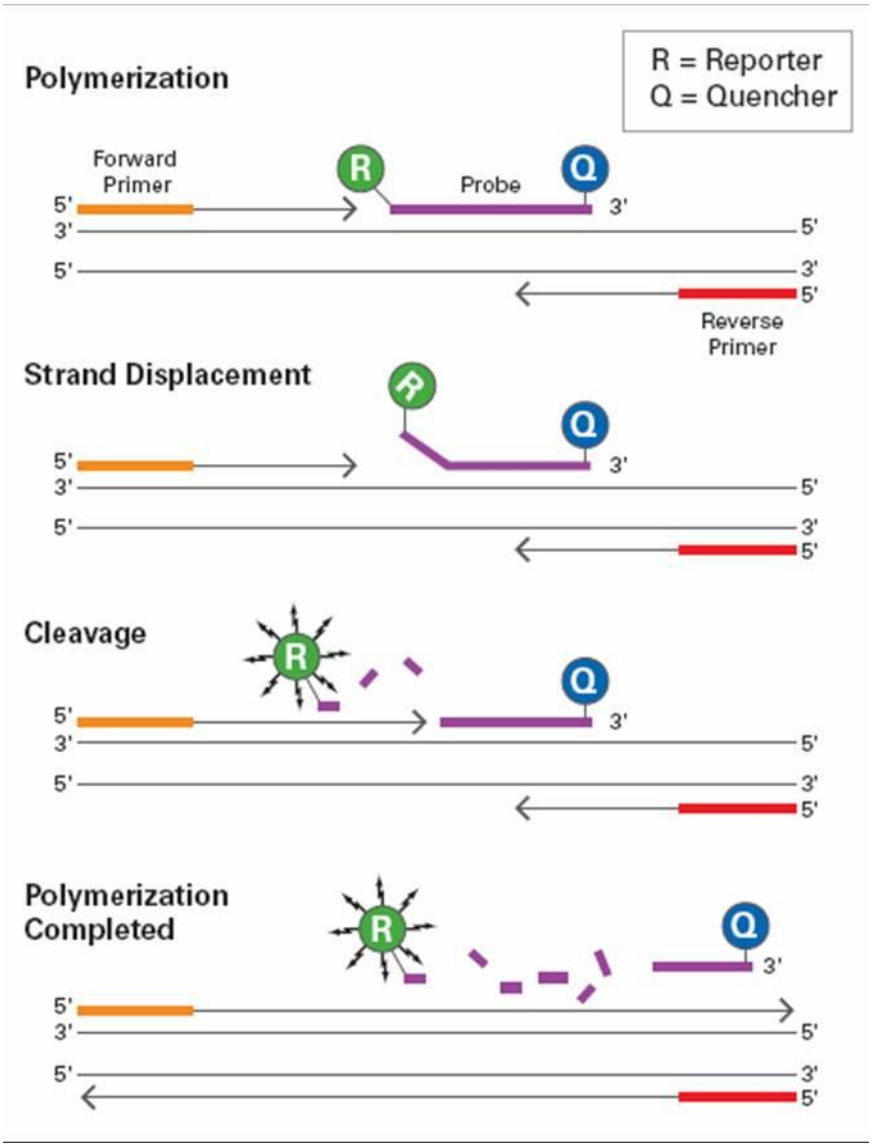

Figure 2.2 TaqMan probe chemistry mechanism

http://www.asuragen.com/Services/services/gene_expression/ab_taqman.aspx

Table 2. Pre-designed Taqman probe/primer sets for genes of interest

Gene name	Protein encoded	RefSeq	TaqMan primer probe set
ATF6	ATF6	NM_007348	Hs00232586_m1
ERN1	IRE1	NM_001433	Hs00176385_m1
EIF2AK3	EIF2α	NM_003836.3	Hs00178128_m1
HSPA5	BiP	NM_004836.3	Hs99999174_m1

2.5 Analysis of gene expression by Illumina gene microarray

RNA was extracted from FHL 124 cells as described in the previous section using an RNeasy® mini kit (Qiagen Ltd, Crawley UK). The monolayer of cells was lysed by adding 350 µl of buffer RLT containing 2- β-mercaptoethanol, to each culture dish. The cell lysate was collected using a cell scraper. The cell lysate was transferred into a sterile Eppendorf tube and was passed through a 20-gauge needle (0.9 nm diameter) fitted to an RNase-free syringe three times. This process homogenizes the cells allowing the RNA to be released from all FHL 124 cells. To the solution 350 µl of 70% ethanol was added to the homogenized lysate, and was mixed via pipetting. The ethanol was added to the lysate to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. 700 µl of the solution was transferred to an RNeasy mini column placed in a 2ml collection tube. The columns were centrifuged for 30 seconds at 8000 rpm and the flow-through was discarded. A series of washes using different buffers were carried out in order to produce the pure RNA. 700 µl of RW1 buffer was added to the RNeasy mini column, centrifuged for 30 seconds and removal of flow-through was carried out. The RNeasy mini column was fitted to 1 new collection tube and two washes with 500 µl of RPE buffer with an initial centrifugation was for 15 seconds, the second centrifugation for 2 minutes gave rise to a dry RNeasy silica-gel membrane containing the RNA. Using 50 µl of RNeasy free water the RNA was eluted form the column into a sterile 1.5 ml Eppendorf and centrifuged for 1 minute. RNA samples were shipped on dry ice to a commercial microarray facility. RNA quantity and quality was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technolgies, Delaware, USA) and an Agilent RNA pico labchip (Wokingham, UK); only samples with an RNA integrity number

(RIN) ≥ 9 were used in the study. Samples were normalised to 100 ng and were processed according to the Illumina Whole-Genome Gene Expression Direct Hybridisation Assay Guide, using the Ambion Kit: Illumina® TotalPrep™-96 RNA Amplification Kit. Qualitative and quantitative quality control was performed on the labelled cRNA and 1.5 μg of labelled cRNA was hybridised to a HumanHT-12.v4 Beadchip and scanned by the Illumina BeadArray reader.

Illumina microarray (BeadArray) unnormalised probe profile data were analysed using the Bioconductor package (http://www.bioconductor.org) in R (http://www.r-project.org). Firstly, the data from different chips were loaded into R to be background corrected, quantile normalised and variance stabilised (Lim et al. 2008). The normalised data from all the arrays have been deposited in the Array Express database with an accession number (to be confirmed). Lists of differentially expressed genes were computed by using the eBAYES statistic (Smyth 2004) to compute a p-value. In contrast to a basic t-test, the eBAYES t-test we perform is relative to a FC threshold and this allows us to formally test the hypothesis that a gene is more differentially expressed than a given FC that is the ratio of average expression level between two groups. Differentially expressed genes were determined by using a combination of a log fold change (lfc) and a p-value threshold criteria since that has been found to find genes more likely to play a physiological role (McCarthy and Smyth 2009). We identified differentially expressed genes that had a fold change threshold of more than 1.3, that is, 0.3786 log fold change and a p value of <0.05. In addition, we used the Benjamini and Hochberg method to compute adjusted P-values (q values) and control the FDR rate. We considered any gene with an FDR q-value of 0.25 to be significant.

2.6 Western blot analysis

2.6.1 Protein extraction

FHL 124 cells were seeded onto 35 mm culture dishes at 150,000 cells in 1.5 ml 5% FCS-EMEM and were maintained until 90% confluency. The medium was replaced with non-supplemented EMEM and cells were cultured for a further 24 hours before exposure to experimental conditions. FHL 124 cells were washed with ice cold PBS, then lysed using Daub's lysis buffer, 150 mM NaCl, 1% Triton X-100, 1MM EDTA, 10% (w/v) glycerol (BDH Laboratory, Poole, UK), 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 250 ml distilled water (dH2O) supplemented with protease inhibitors phenylmethylsulfonylfluoride (PMSF) (1 mm) and aprotinin (10 µg/ml). Cultured cells were scraped using a cell scraper, transferred to Eppendorf tubes and left on ice for 20 minutes. Whole cell lysates were centrifuged at 13000 rpm at 4 ℃ for 10 minutes. The supernatant was then collected and the pellet of cell debris discarded.

2.6.2 Protein quantification

The protein concentration of each lysate was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). A range of protein standards (0-1000 µg/ml) was obtained by dilution of bovine serum albumin (BSA) in Daub's lysis buffer. A constant volume (10 µl) of protein standard and "undetermined" lysate samples was introduced into separate wells of a 96 well plate. All standards were tested in triplicate, and all samples in duplicate. Each well received 40 µl of dH_2O followed by 200 µl of the two supplied BCA reagents in a 50:1 ratio. The plate was covered and placed on a shaker for 1 minute prior to 1 hour incubation at 37 ℃. The

absorbance at 550 nm was then measured using BMG LabTech plate reader. A protein standard concentration curve was generated allowing the determination of the concentration of the samples.

2.6.3 Sample preparation

Samples were made to a constant protein concentration by dilution with dH_2O. Each sample received 20% (v/v) loading buffer (160 mM Tris, 4% (w/v) Sodium dodecyl suphate (SDS) (both Melford Laboratories, Ipswich, UK), 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 12% (v/v) β-mercaptoethanol, and pH 6.8). Protein samples with loading buffer were heated at 85℃ for 5 minutes to ensure complete protein denaturation and cooled down on ice for 5 minutes. Finally, samples were spun at 13000 rpm for 30 seconds.

2.6.4 SDS-PAGE gel electrophoresis

Protein samples together with ECL DualVue Western blotting maker (GE Healthcare, Little Chalfont, UK) were loaded onto SDS-PAGE gels for electrophoresis. The running gel layer was prepared at 8% acrylamide by mixing dH_2O, 40% acrylamide (Bio-Rad Laboratories, Hempstead, UK), 4x lower gel buffer (1.5 MTris and 0.4% (w/v) SDS, pH 8.8), ammonium persulphate (600 μg/ml) and TEMED (1.2 μl/ml). The stacking gel, which allows the proteins to stack together when they enter the gel, was made at 5% acrylamide. It consisted of dH_2O, 40% acrylamide, 4x upper gel buffer (0.5 M Tris and 0.4% (w/v) SDS, pH 6.8), and ammonium persulphate and TEMED as previously described. The gel was run at 4℃ at a constant current of

0.03A until the dye front reached the bottom of the gel.

2.6.5 Protein transfer

The SDS-PAGE gel together with thick blotting paper and a polyvinylidene fluoride (PVDF) membrane (Perkin Elmer Life Sciences, Boston, MA, USA), previously activated by a 10 second immersion in 100% (v/v) methanol (Fisher Scientific, Loughborough, UK), were incubated in transfer buffer solution (48 mM Tris, 39 mM glycine (Fisher Scientific, Loughborough, UK), 4% (v/v) methanol and 0.0375% (w/v) SDS, pH 8.3) at room temperature for 20 minutes. The gel and membrane were held between the two pieces of thick blotting paper. This "sandwich" was placed on a semi-dry transfer blotter (Trans-Blot, Bio-Rad laboratories, Hercules, CA, USA). Proteins were transferred from the gel to the membrane, at 0.3 mA per gel and 15 V for 35 minutes. Following transfer, molecular weight marker could be seen on the membranes, indicating protein transfer was successful.

2.6.6 Immunoblotting and development

Transferred proteins were blocked in 0.1% (v/v) Tween-20 in PBS (PBST) with 5% (w/v) fat-reduced milk powder (Marvel) for 1 hour at room temperature with gentle agitation. The PBST blocking solution was then removed and the blots were placed overnight at 4℃ with 5% (w/v) Marvel PBST containing the primary antibody (Anti-NQO1, 1:500; Anti-TXNRD1, 1:2000; Anti-β actin, 1:1000). Membranes were washed 4 times in 1% (w/v) Marvel PBST at 10 minute intervals with gentle agitation prior to 1 hour incubation at room temperature with secondary antibody (Anti-mouse IgG 1:1000) (Anti-rabbit IgG 1:1000) diluted in 1% (w/v) Marvel PBST. Membranes

were washed 5 times in PBST at 10 minute intervals, followed by a 10 minute wash in PBS. Proteins were detected using the chemiluminescent ECL plus Western Blotting Detection System (GE Healthcare, Little Chalfont, UK). The volume of ECL Plus reagent to be used was determined by the surface area of the membranes (100 μl per cm^2 of membrane). The ELC Plus reagent was left on the membranes for 5 minutes in the dark at room temperature. This reagent was then drained away and the membranes were placed between two layers of cling film inside a Hypercassette (GE Healthcare, Little Chalfont, UK). In a dark room, the membranes were exposed to high performance chemiluminescence film (GE Healthcare, Little Chalfont, UK), which is then placed into developing (Kodak GBX developer), stop (Photosol SB80, Basildon, UK) and fixative (Ilford Imaging, Mobberley, UK) solutions to reveal protein bands. The photographic films were scanned using an HP Scanjet 5470c (Hewlett Packard Development Company, USA). Band intensity was determined with Kodak 1D 3.5 software (Kodak Scientific Imaging Systems, Rochester, NY, USA). β-actin was used as a loading control and for band intensity normalisation.

Table 3. Antibodies used for Western blot experiment

Target	Host	Dilution	Supplier
Primary Antibodies			
Anti-NQO1	mouse	1:500	Abcam, Cambridge, UK
Anti-TXNRD1	mouse	1:2000	Abcam, Cambridge, UK
Anti-β actin	rabbit	1:1000	Cell signalling, Boston, USA
Secondary Antibodies			
Anti-mouse IgG HRP conjugate	sheep	1:1000	GE Healthcare, Little Chalfont, UK
Anti-rabbit IgG HRP conjugate	donkey	1:1000	GE Healthcare, Little Chalfont, UK

2.7 Immunofluorescence

Preparations were rinsed three times with PBS followed by a 10-30 minutes fixation in 4% formaldehyde in PBS, and then rinsed in 0.02% BSA, 0.05% IGEPAL in PBS three times. Preparations were permeabilised with PBS containing 0.5% Triton X-100 for 30 minutes. Three washes were made (0.02% w/v and 0.05% v/v respectively). Non-specific sites were blocked with either normal goat or donkey serum (1:50 in 1% w/v BSA in PBS) for 60 minutes. Primary antibody (anti NQO1 and anti TXNRD1 mouse monoclonal antibodies (Abcam, Cambridge, UK); anti-Nrf2 rabbit polyclonal (Abcam, Cambridge, UK) and anti-vimentin mouse monolclonal (Sigma. Poole, Dorset) was diluted 1:100 in 1% BSA in PBS and applied overnight at 4℃ followed by washing 3 times for 5 minutes with shaking with 0.02% BSA, 0.05% IGEPAL in PBS. ALEXA 488-conjugated goat anti-mouse or donkey anti-rabbit secondary antibody (used at 1:200; Molecular Probes, Leiden, NL) was applied for 60 minutes at 37℃ in a moist atmosphere. The stained preparations were again washed extensively, floated onto microscope slides, and mounted with Hydromount mounting medium (National Diagnostics, Hull, UK). Images were viewed using fluorescence microscopy (Zeiss) and captured using a digital camera and Zeiss Axiovision software. Where applicable images were quantified using ImageJ.

2.8 Whole pig lens culture

Fresh porcine eyes were obtained from a local slaughterhouse. The tissue collection conformed to the ARVO statement of the Use of Animals in Ophthalmic and Vision Research. Eyes were placed in sterile containers and covered with Eagle's minimum essential medium (EMEM) containing 200 U/ml penicillin and 200 µg/ml streptomycin. They were stored at 4℃ before dissection. Within 24 hours post-mortem, lenses were dissected by anterior approach following cornea removal and incubated in bicarbonate-CO_2-buffered EMEM (pH 7.4), containing 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, and 50 µg/ml gentamicin at 35℃. After a pre-culture period of 24 to 72 hours to ensure no damage had arisen from the isolation procedure, lenses were exposed to 2µM SFN for 24 hours prior to adding 2 mM H_2O_2. During the experimental period, lens images were taken at the starting point (T= 0), at 24 hours and at 4 days using a charge coupled device (CCD) camera (UVP, Cambridge, UK) with Synoptics software (Synoptics, Cambridge, UK). At the end of 24 hours culture in the presence of experimental conditions, the medium was collected for LDH assay. Dark field images of lenses were taken using a CCD camera. Grey scale values of the central for each lens was measured using Image J 1.45s analysis software and expressed as arbitrary units.

2.9 Dissection and Culture of human capsular bags

A sham cataract operation was performed on the donor eyes under a dissecting microscope (Figure 2.3). Using an insulin needle, the anterior capsule was breached about 3 mm from the equator, and an incision was made from that point to the centre of the capsule. By tugging the flap, created by this incision, with surgical forceps a continuous curvilinear capsule rhexis was created, such that a disc of anterior capsule was removed, leaving an opening approximately 5 mm in diameter. The resultant window enabled the lens fibres mass to be removed by hydroexpression. Residual fibres were removed by joint irrigation with Hartmann's solution and aspiration. At this point, an IOL can be implanted through the anterior opening if required. The resultant capsular bag was then dissected free of the zonules and secured on a sterile 35 mm Polymenthymethacrylate (PMMA) Petri-dish. Eight entomological pins (Watkins and Doncaster, Cranbrook, UK) were inserted through the edge of the capsule to retain its circular shape. Capsular bags were maintained in EMEM and incubated at 35℃ in a 5% CO_2 atmosphere. The medium was replaced every 2 days and on-going observations of cell growth were performed with a Nikon phase-contrast microscope (Nikon, Tokyo, Japan). Images were acquired with a digital camera (Coolpix 950; Nikon, Tokyo, Japan).

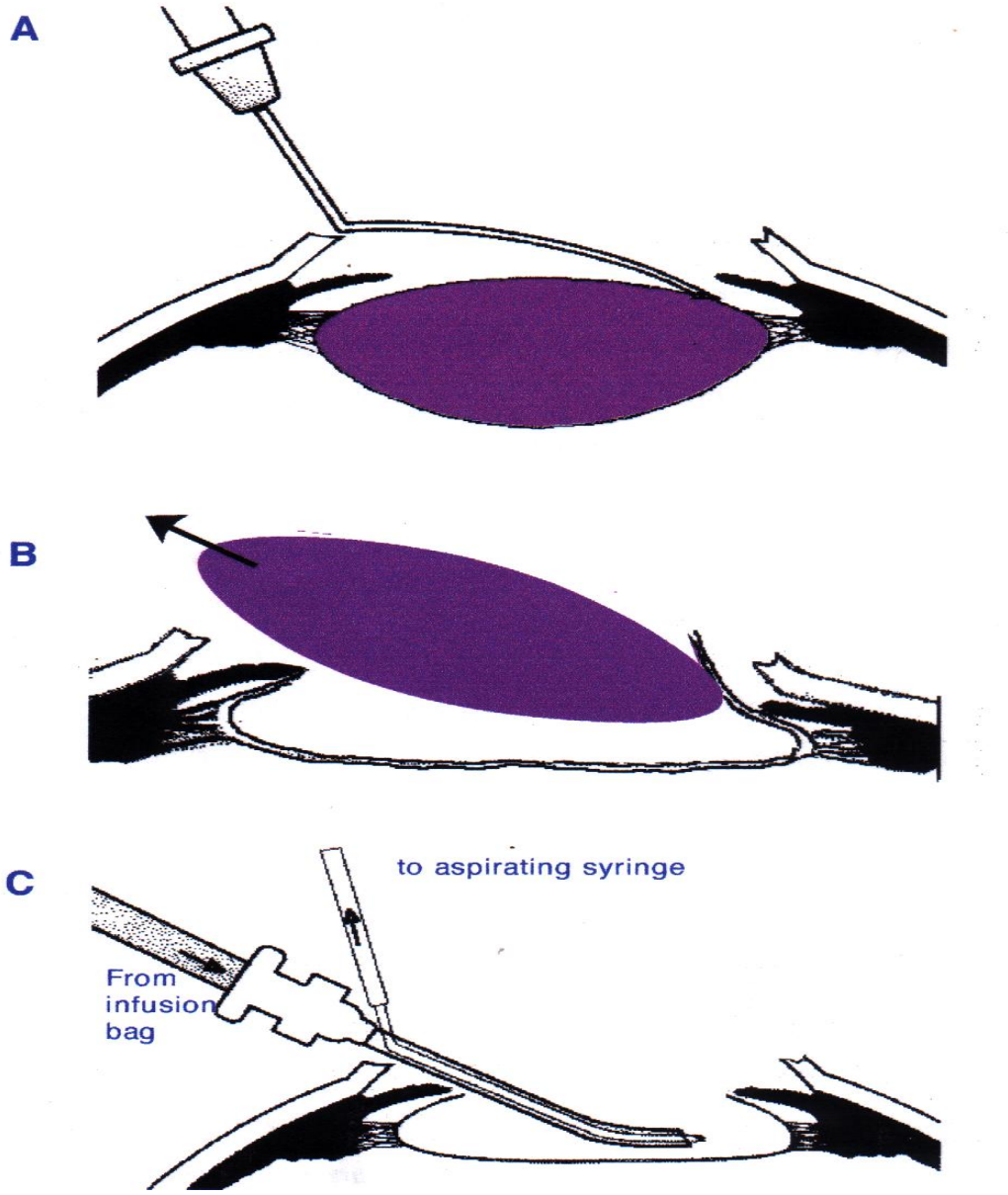

Figure 2.3 The major stages of a sham cataract operation (A) A small incision is made in the anterior capsule (B) the lens fibre mass is removed (C) and an aspirating syringe is used to remove any remaining cortical matter (modified from Sandford et al. 1994).

2.10 Statistical analysis

Student’s t-test analysis was performed using Excel software to determine any statistical difference between two groups (Excel; Microsoft, Redmond, WA). When the effect that one factor have on one dependent variable is studied, one-way analysis of variance (ANOVA) is used to compare the means of several different groups. It is a generalization of Student's t-test which compares means of two groups. The null-hypotheses that is tested with an ANOVA is that there is no difference between the group means, and a low p-value indicates that the nullhypothesis should be rejected. The ANOVA will only tell you whether there is a significant difference of means between the groups, but not which of the groups that differ from each other. If the ANOVA results in a p-value below the threshold value (e.g. <0.05), you can do a post hoc test to see if there is a significant difference between pairs of groups. There are three different post hoc tests: Tukey-Kramer's, Bonferroni's, and Dunnett's test. One ANOVA with Dunnett’s or Tukey’s post-hoc analysis using SPSS18.0 for Windows (SPSS Inc, Illinois) were performed to determine any statistical difference between multiple groups, significance was determined using a p value of $\leqslant 0.05$. One way ANOVA with Tukey’s post hoc analysis was employed to assess multiple groups when all or many pairwise comparisons are of interest. One way ANOVA with Dunnett's post hoc analysis was employed to assess all groups compared against one control group. A 95% confidence interval was used to assess significance.

CHAPTER 3
SULFORAPHANE CAN PROTECT HUMAN LENS CELLS AGAINST OXIDATIVE STRESS

3.1 Introduction

Cataract renders millions in the world blind and is notably a disease that largely afflicts the elderly (Javitt et al. 1996). Its management places a significant strain on healthcare budgets (Taylor 1993). At present, the only means to treat cataract is by surgical intervention (Wormstone et al. 2009) and it is predicted that 32 million operations will be performed annually by the year 2020. Delaying the onset of cataract is therefore a major healthcare priority across the globe. Oxidation plays a key role in the formation of cataract (Spector 1995). Oxidative stress – a cellular imbalance between production and elimination of ROS, such as superoxide, hydrogen peroxide and peroxynitrite – is considered to be of major pathophysiological relevance for a variety of pathological processes. Thus, it is valuable to identify agents, which might enhance the cellular antioxidant defence systems within the lens, such as compounds that antagonize the deleterious action of ROS on biomolecules. The mode of action of these compounds could be either to scavenge ROS directly or to trigger protective mechanisms inside the cell, thereby resulting in improved defense against ROS. Dietary intake is one possible means of enhancing protective systems and is an area worthy of study.

ITCs, which are derived from glucosinolates found in cruciferous vegetables are characterized by sulfur containing N=C=S functional groups. ITCs can inhibit many types of tumour formation in animal models and their consumption is inversely

correlated with the risk of cancer in human (Lee and Cho 2008, Trachootham et al. 2008). Protective mechanisms of ITCs have been proposed including the induction of phase II detoxification enzymes and inhibition of phase I carcinogen-activating enzymes. The isothiocyante SFN is an interesting candidate that to date has not been studied extensively in the lens.

SFN, which is found in broccoli and other brassicas, is a product of hydrolytic conversion of 4-methysulphinylbutyl glucosinolate (glucoraphanin) by an endogenous myrosinase (Hintze et al. 2003a) and has been identified as a very potent chemopreventive agent in numerous animal carcinogenesis models as well as cell culture models, exerting its chemopreventive effects through regulation of diverse molecular mechanisms (Tanito et al. 2005).

The most studied role of SFN in chemoprevention is its ability to induce phase II detoxification enzymes, cell cycle arrest and apoptosis. Induction of phase II enzymes is one means by which SFN enhances the cellular antioxidant capacity. Enzymes induced by SFN include Nrf2-regulated enzymes such as GSTs and NQO1 which can function as protectors against oxidative stress (Prawan et al. 2005, Yanaka et al. 2005). SFN is also a very potent inducer of HO-1 that catalyzes the conversion of heme to biliverdin which in turn is reduced enzymatically to bilirubin (Jeong et al. 2005, Keum et al. 2006, Prestera and Talalay 1995). Among the various genes encoding proteins that possess antioxidant characteristics, HO-1 has attracted particular interest as it is finely upregulated by stress conditions and generates products that might have important biological activities. HO-1 displays antioxidant, antiapoptotic, and anti-inflammatory

effects and appears to have a complex role in angiogenesis (Prawan et al. 2005, Ryter et al. 2006). Experimental evidence suggests that SFN activates NF-E2 p45-related factor-2 transcription factor in binding antioxidant response elements in the promoter regions of target genes, thereby increasing cellular defences against oxidative stress (Tanito et al. 2005, Wagner et al. 2010). Intake of 200 μmol broccoli isothiocyantates (mainly SFN) in human has been reported to result in SFN plasma levels in the low micromolar range (Ye et al. 2002a).

3.2 Aims

Protecting the lens against oxidative stress is of great importance in delaying the onset of cataract. An important aim was therefore, to determine if the SFN could yield protection to lens cells against oxidative stress. This was achieved through the use of cell viability/ cell death assays using a human lens cell line (FHL 124) and whole porcine lens culture.

3.3 Results

3.3.1 Effects of SFN on cell viability, cytotoxicity and apoptotic cell death

Cell viability relative to untreated control group was not significantly affected by SFN exposure of 5 μM and below for 24 hours and 72 hours (Figure 3.1A and D). However, at SFN concentrations of 10 μM and above a significant reduction in cell viability was observed; this effect became more pronounced with increasing concentrations of SFN (Figure 3.1A and D). A significant increase in cytotoxicity was also seen with SFN exposure between 10-100 μM (Figure 3.1B and E). Apoptosis was detected with the ApoToxGlo triplex assay using CaspaseGlo to detect Caspase3/7 activity and, consistent with the other measurements, a significant increase was identified following 10-100 μM SFN exposure (Figure 3.1C and F).This suggests that, at these doses, SFN (≤ 5 μM) is not toxic to FHL 124 cells, and can therefore be studied for cytoprotective effects against H_2O_2.

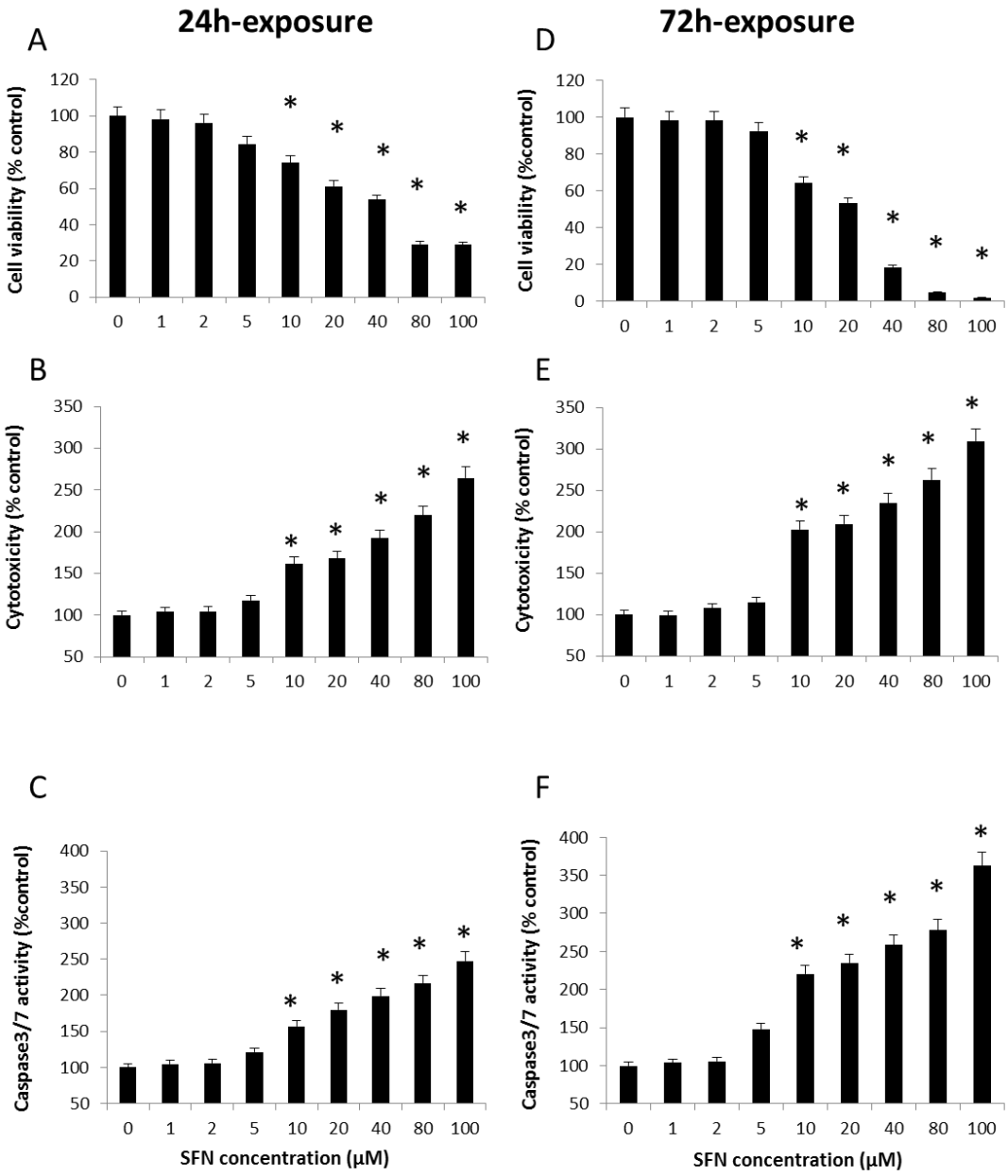

Figure 3.1 Concentration-dependent effects of sulforaphane on FHL 124 cell viability (A & D), cytotoxicity (B & E) and apoptosis (C & F) detected by the ApoToxGlo triplex assay following a 24 (A-C) and 72 hour (D-F) culture period with SFN. The data are presented as mean ± SEM (n=4). * indicates a significant difference between the treated group and untreated controls ($p \leq 0.05$; ANOVA with Dunnett's post hoc test).

3.3.2 Effects of SFN on protection of lens cells against oxidative stress

3.3.2.1 Effects of hydrogen peroxide on FHL 124 cell viability detected by the MTS assay

An MTS assay was used to quantify cellular viability of FHL 124 cells in response to increasing concentrations of hydrogen peroxide (H_2O_2). Figure 3.2 show that the application of 1-10 μM H_2O_2 did not significantly affect cell viability following a 24 hours culture period. However, exposure to 30 – 300 μM H_2O_2 over this experimental period did result in a significant change. The consequence of 30 μM H_2O_2 treatment was a significant reduction of cellular viability to 59 ± 5% of the control levels. The addition of 100 μM H_2O_2 caused a significant 90 ± 6% decrease in the survival of FHL 124 cells compared to control levels.

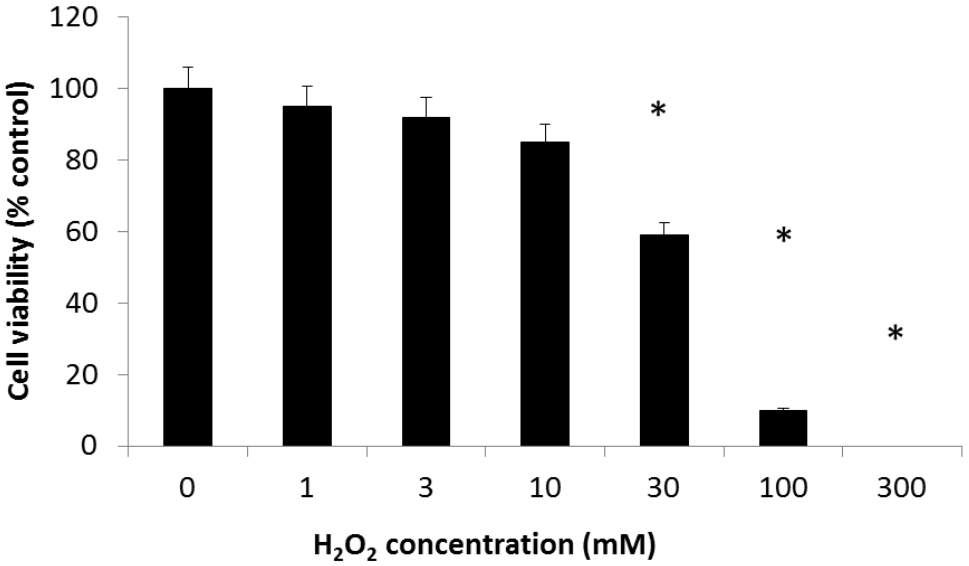

Figure 3.2 Effects of H_2O_2on FHL 124 cell viability. The cell viability of FHL 124 cells after exposure to H2O2 (0-300 μM) conditions over a 24 hour period was assessed using the MTS assay. The data are expressed as % cell survival in comparison to the untreated control. Each column represents the mean ± SEM of 4 independent experiments. * Represents a significant difference between untreated control and treatment group (by one way ANOVA with Dunnett's post hoc test; $p<0.05$).

3.3.2.2 Effects of SFN co-treatment against hydrogen peroxide-induced FHL 124 cell death detected by the MTS assay

To investigate whether SFN had direct antioxidant properties, FHL 124 cells were co-treated with 1 µM SFN and 30 µM H_2O_2 for 24 hours (Figure 3.3). Cells viability was tested by MTS assay. 30 µM H_2O_2 reduced FHL 124 cell viability to 55% of the control population. 1 µM SFN alone had no effect on cell viability (Figure 3.3). Cells co-treated with 1 µM SFN and 30 µM H_2O_2 exhibited a reduction in the viable cell population, which was significantly different to the control group, but did not significantly differ from the cells treated with 30 µM H_2O_2 alone (Figure 3.3), indicating no significant protection of the cells by SFN against H_2O_2 toxicity using this approach.

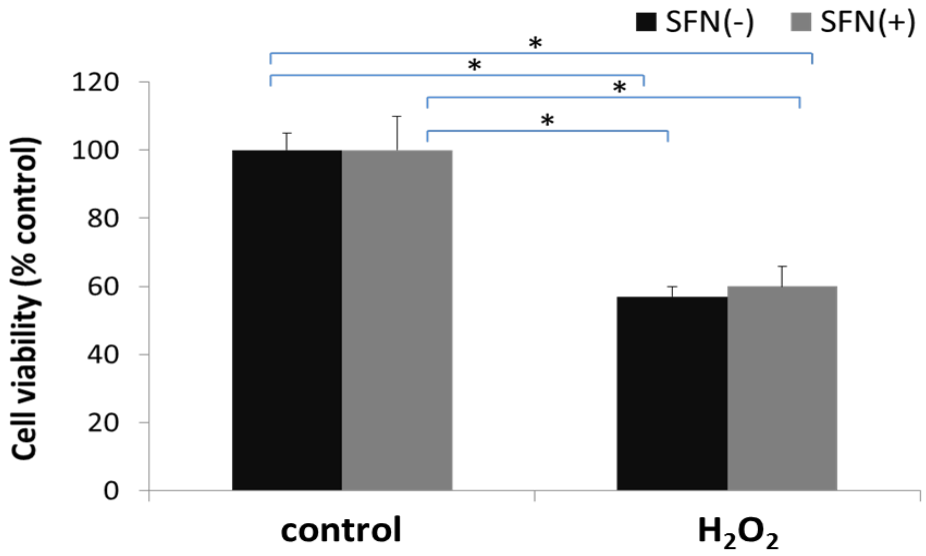

Figure 3.3 The viability of FHL 124 cells following exposure to 30 µM H_2O_2 together with 1µM SFN over a 24 hour period assessed using the MTS assay. The data is expressed as % cell survival in comparison to control, represented as 100%. Each column represents the mean ± SEM of 4 independent experiments. * Represents a significant difference between untreated control and treatment group (by one way ANOVA with Tukey's post hoc test; $p<0.05$).

3.3.2.3 Effects of SFN pre-treatment against hydrogen peroxide-induced FHL 124 cell death detected by the MTS assay

To investigate whether SFN has indirect antioxidant properties, FHL 124 cells were incubated with 1 μM SFN for 24 hours prior to exposure to 30 μM H_2O_2. SFN was then removed (Figure 3.4 A) or retained (Figure 3.4 B) before adding H_2O_2. Figure 3.4 B shows that addition of 30 μM H_2O_2 significantly reduced the viable cell population, (again detected using the MTS assay), within 24 hours such that levels were 56 ± 4% compared to the untreated control. FHL 124 cells pre-treated with 1 μM SFN, prior to addition of 30 μM H_2O_2, and retained for the experimental duration demonstrated a significant increase (~80% protection) in the viable population that was comparable to control populations. When FHL 124 cells were incubated with SFN for 24 hours, then removed prior to H_2O_2 addition marked cytoprotection (~80%) was still observed (Figure 3.4 A).

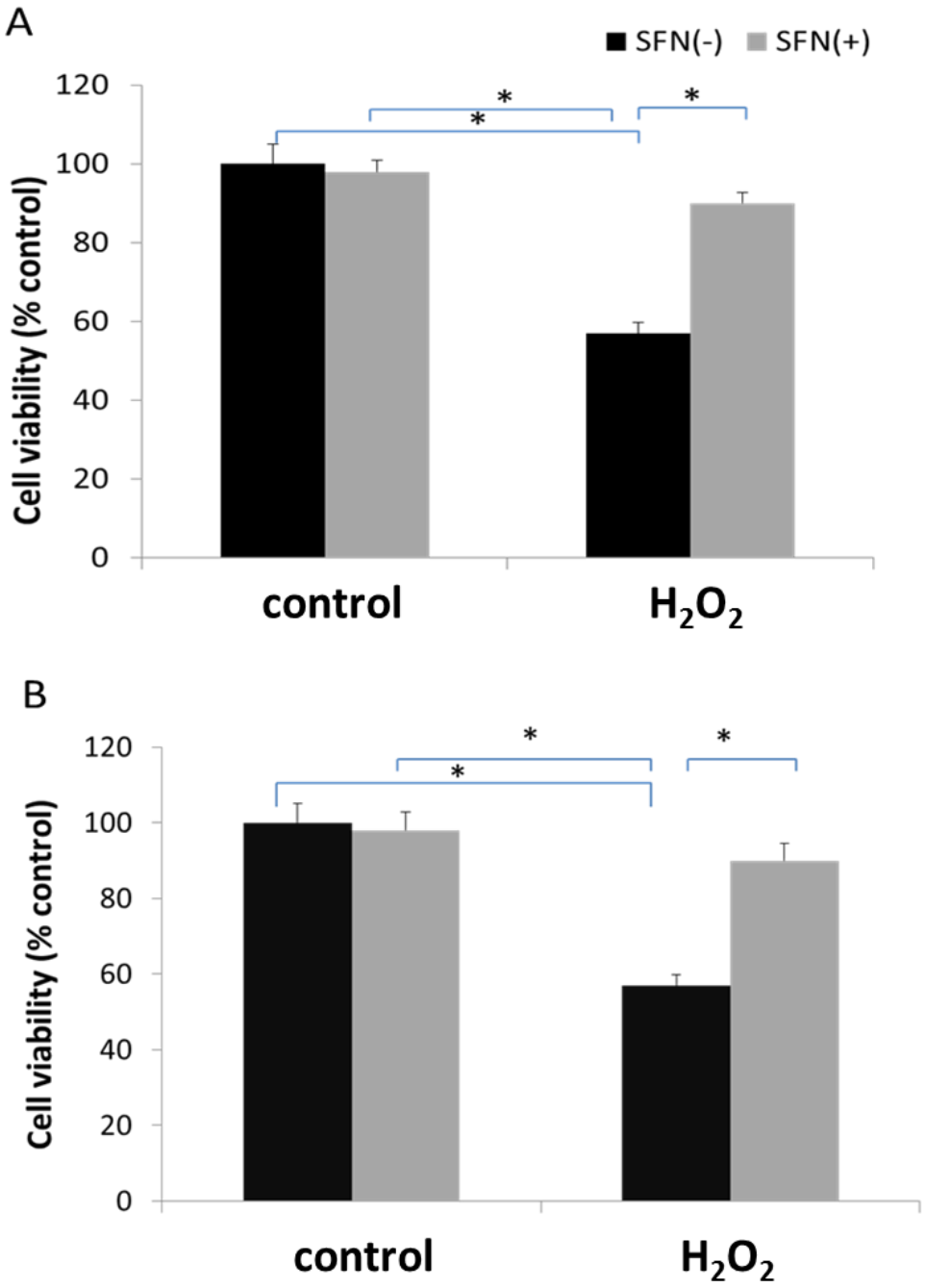

Figure 3.4 The viability of FHL 124 cells exposure to 1µM SFN for 24 hours, followed by 30µM H_2O_2 in the absence of SFN (A) and in the presence of SFN (B) for a further 24 hours using MTS assay. The data is expressed as % cell survival in comparison to control, represented as 100%. Each column represents the mean ± SEM of 4 independent experiments. * Represents a significant difference between untreated control and treatment group (by one way ANOVA with Tukey's post hoc test; $p<0.05$).

3.3.2.4 SFN protection of lens cells against oxidative stress detected by the ApoToxGlo Triplex Assay

Due to the results of the MTS assay performed previously, the ApoTox-Glo Triplex Assay was used in order to have an independent means of verifying the results. Exposure of FHL 124 cells to 30 µM H_2O_2 for a 24 hours period resulted in a significant decrease in cell viability and a significant increase in cytotoxicity and apoptosis (Figure 3.5 B and C). In agreement with the previous experiments, addition of 1 µM SFN to the cells had no discernible effect on cell viability, cytotoxicity or apoptosis relative to serum-free maintained control cells (Figure 3.5). However, pre-treatment of cells with 1 µM SFN significantly inhibited H_2O_2 induced effects, such that cell viability, cytotoxicity and apoptosis did not significantly differ from serum-free or SFN (alone) maintained cells (Figure 3.5).

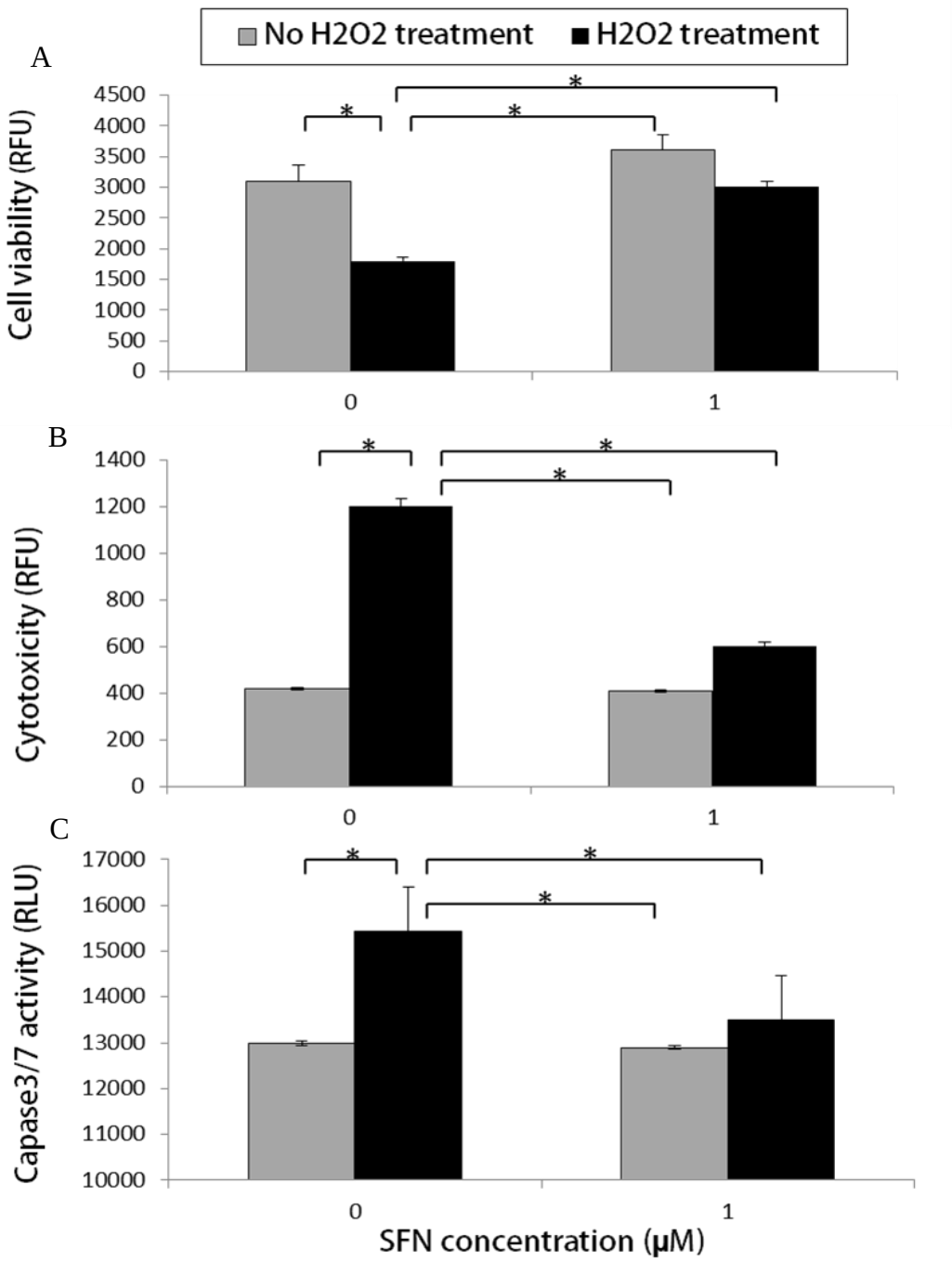

Figure 3.5 SFN protection against oxidative stress induced loss of cell viability (A), cytotoxicity (B) and apoptosis (C), following a 24 hour experimental period, determined using the ApoTox-Glo assay. Cells were pre-treated with 1 µM SFN for 24 hours prior to exposure to 30 µM H_2O_2. Data are presented as mean ± SEM (n=4). * indicates a significant difference between treated the indicated groups (by one way ANOVA with Tukey's post hoc test; $p<0.05$).

3.3.2.5 SFN protection of lens cells against oxidative stress determined by the LDH Assay

To support the ApoToxGlo Triplex Assay data, the LDH assay was employed to assess cell damage/death. Treatment with 30 µM H_2O_2 invoked a significant increase in LDH release into the medium (Figure 3.6). This effect was inhibited by pre-treatment of the cells with 1 µM SFN. No difference in LDH levels was observed between the SFN/ H_2O_2 treated cells and the SFN alone group or serum-free controls (Figure 3.6).

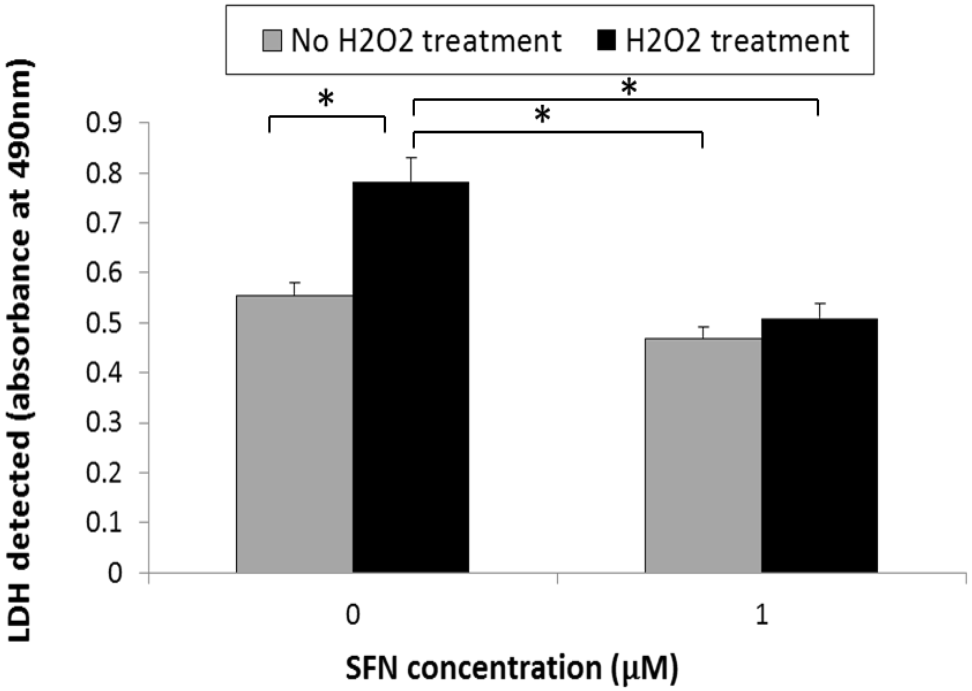

Figure 3.6 SFN protection against oxidative stress induced cell damage/death, following a 24 hours experimental period, determined using the LDH assay. Cells were pre-treated with 1 µM SFN for 24 hours prior to exposure to 30 µM H_2O_2. Data is expressed as mean ± SEM (n=4). * Represents a significant difference between the indicated groups (by one way ANOVA with Tukey's post hoc test; $p<0.05$).

3.3.2.6 Effects of SFN pre-treatment against hydrogen peroxide-induced FHL 124 cell DNA damage determined by the Alkaline Comet Assay

A number of studies have identified that oxidative stress induces DNA strand breaks in human cells (Jantzen et al. 2012, Liu J. et al. 2012). To investigate such effects in this experimental system, the alkaline comet assay was used to investigate DNA damage (and its repair) induced by oxidative stress in FHL 124 cells over time. Exposure to 30 μM H_2O_2 resulted in greatest levels of DNA strand breaks in cells harvested at the 30 minutes time point, which demonstrated a mean value for DNA in the tail of 52.7% (Figure 3.7 B). This declined with time, but remained significantly elevated during the following 2 hours. There were significantly lower levels of DNA breaks when cells were pre-treated with 1 μM SFN (Figure 3.7 B), indicating an enhanced antioxidant defense. Treatment with 1 μM SFN alone for this time period did not significantly change levels of DNA strand breaks when compared to untreated cells.

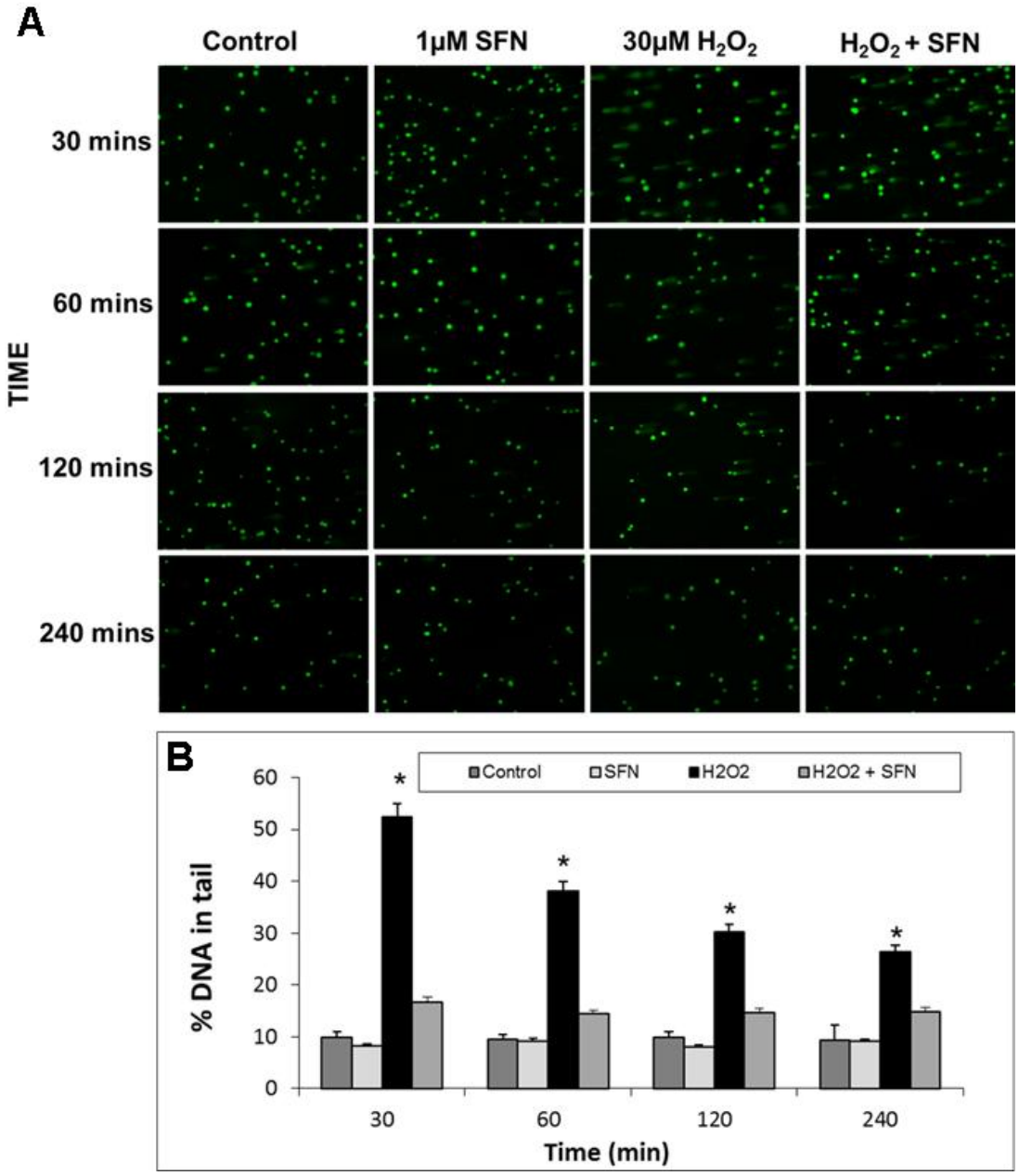

Figure 3.7 SFN suppression of DNA damage of FHL 124 cells induced by oxidative stress detected using the Comet assay following a 30, 60, 120 or 240 minute experimental period. Cells were pre-treated with 1 µM SFN for 24 hours prior to exposure to 30 µM H_2O_2. DNA strand breaks were measured by the alkaline comet assay; tails were measured for at least 100 comets/sample. (A) Representative fluorescent micrographs for each experimental group. (B) Pooled quantitative data from 4 individual experiments. Data are presented as mean ± SEM. * Represents a significant difference between the indicated group and all other treatments (by one way ANOVA with Tukey's post hoc test; $p<0.05$).

3.3.2.7 SFN protection of lens cells against oxidative stress assessed in a whole lens culture system

To further test the protective nature of SFN against oxidative stress we employed a porcine whole lens culture system (Tamiya et al. 2000). Following dissection, whole porcine lenses did not demonstrate any notable opacity. Lenses maintained in serum-free medium remained transparent over the four day culture period. This can be clearly seen in Figure 3.8 A as the grid placed beneath the lens is clearly visible. Similarly, with the dark-field image presented in Figure 3.8 B, minimal white light scattering regions were observed. Addition of 2 μM SFN to the cultures did not affect transparency, such that lenses appeared similar to the serum-free (SF) control group. Exposure to 2 mM H_2O_2 induced a marked change in transparency that appeared as a cloudiness in the peripheral cortex that progressed over time, such that the majority of the cortical region was affected (Figure 3.8 A and B). At day 1, the peripheral lens had begun to opacify, such that the grid could not be seen clearly through these regions and was associated with light scatter (Figure 3.8 B). When 2 mM H_2O_2 was added to lenses pre-incubated in SFN then maintained in 2 μM SFN, opacity was still observed but this was less marked than the H_2O_2 only treated group. Quantification of these images also demonstrated a significant decrease in opacification levels, such that with 2 μM SFN, a 40% decreased opacification relative to H_2O_2 alone treated lenses was observed at day 4 (Figure 3.8 D). At end point (day 4), the culture medium was analyzed for LDH. Serum-free and SFN only treated lenses had no discernible levels of LDH in the culture medium (Figure 3.8 C). Addition of hydrogen peroxide induced a dramatic increase in LDH levels, which was suppressed in the presence of SFN.

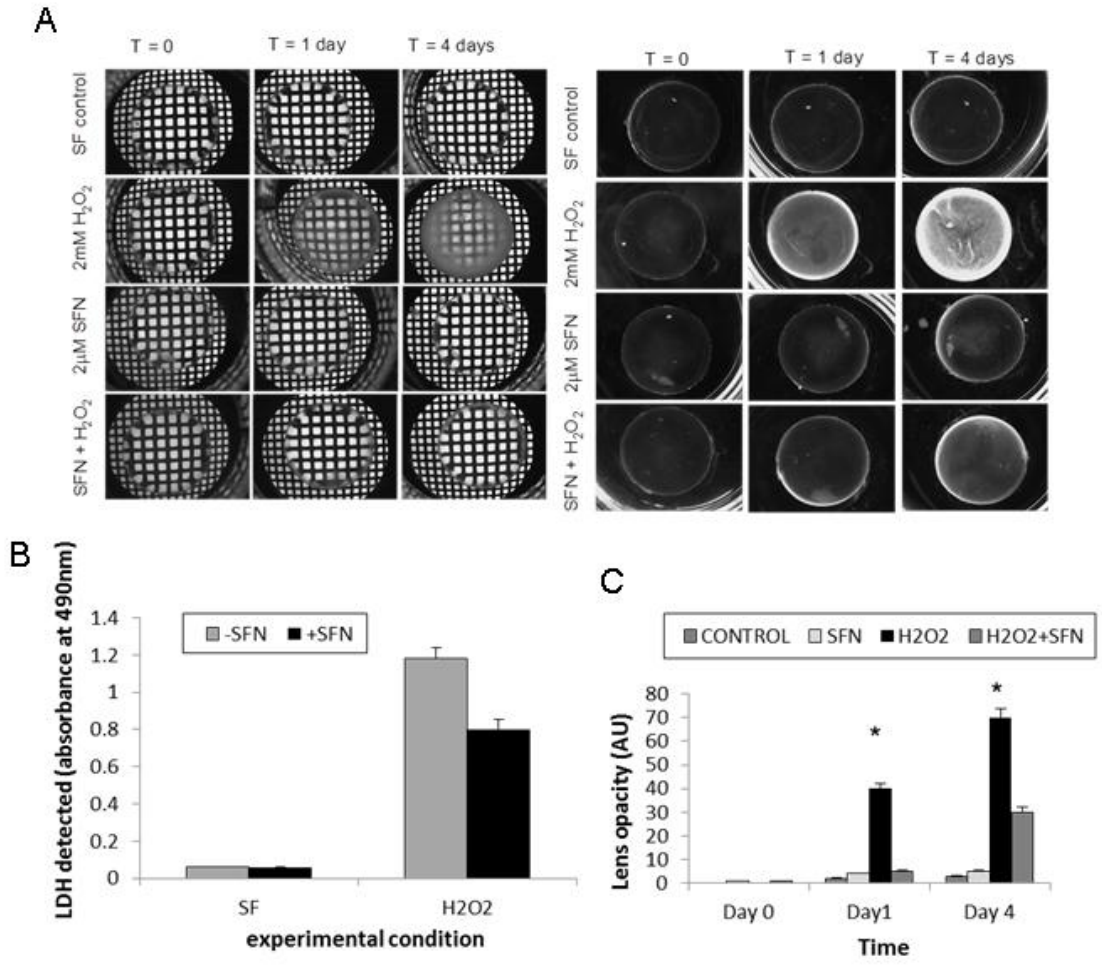

Figure 3.8 Pre-incubation (24 hrs) with SFN reduces hydrogen peroxide induced lens opacity. (A) Representative bright-field and (B) dark-field images of whole pig lens organ cultures over time. (C) Pooled data showing LDH levels within the culture medium at end-point; data are presented as mean ± SEM (n=4). (D) Quantification of lens opacity over time (n= 4); data are presented as mean ± SEM (n=4). SFN was applied at 2 µM and H_2O_2 at 2 mM. * Represents a significant difference between the indicated groups ($p \leq 0.05$; ANOVA with Tukey's post-hoc test).

3.4 Discussion

Cataract is the major cause of blindness worldwide and cataract surgery is the most common surgical procedure performed on the elderly (Wormstone et al. 2009). The large cost of this operation and possible complications associated with it favour long-term goals of avoiding cataract formation, or significantly retarding onset of the disease. In relation to cataract, it has long been recognised that oxidative stress has a significant role to play in the pathophysiology of the disease (Spector et al. 1995, Truscott 2005) and recent experiments clearly show increased protein oxidation in human lenses with cataract compared to age-matched controls (Hains and Truscott, 2008). Furthermore, diets rich in antioxidants have been linked with a reduced risk for cataract in several epidemiological studies (Jacques and Chylack 1991, Tavani et al. 1996). Whilst much research relating to protection against cataract by antioxidants has focussed on the antioxidant vitamins, although the mechanisms of protection are not known (Cumming et al. 2000).

Oxidative stress can be involved in the development of cataract (Spector et al. 1995, Truscott 2005). It was shown that the concentration of H_2O_2 in the aqueous humour increases dramatically in cataractous eyes compared to normal (Spector A. and Garner 1981). The presence of H_2O_2 or other oxidants may lead to damage to the lens such as formation of high molecular weight protein aggregates and oxidation of preoteins and membrane lipids (Bhuyan et al. 1986, Garner and Spector 1980, Spector 1984). These may contribute to the loss of transparency and therefore to the formation of cataract. In this chapter, the effects of H_2O_2 on human lens epithelial FHL 124 cells were investigated.

In the FHL 124 cell line, a dose-dependent reduction in cell viability was observed following 24 hour H_2O_2 treatment. For these cells, the IC50 was approximately 30 Mm H_2O_2. This is comparable with results in lens epithelial cells of human (HLE B3) (Seomun et al. 2005, Xing and Lou 2002, Yao et al. 2008) and rabbit (TOTL-86) origin (Ohguro et al. 1999). In addition, loss of transparency has been observed in ex *vivo* lenes of several origins treated with H_2O_2 (Petersen et al. 2007, Sanderson et al. 1999, Wang et al. 1997).

SFN has been identified in numerous cell and animal carcinogenesis models to be an effective chemopreventive agent which utilises a diverse range of molecular mechanisms to achieve this (Juge et al. 2007a). In this chapter, the effects of SFN against H_2O_2-induced FHL 124 cell death was investigated. SFN showed cytoprotective properties against H_2O_2. The reduction in cell viability observed with 30 μM H_2O_2 was prevented in the presence of SFN (1 and 2 μM). SFN has potent antioxidant properties and these could therefore be responsible for the protective effect cells have against oxidative against oxidative stress.

The current work demonstrates that pre-treatment of a lens cell line or whole lens cultures with SFN yielded protection for lens epithelial cells against hydrogen peroxide induced DNA damage, cell death and transparency loss. It is therefore of interest to consider the mechanisms by which SFN provides this protection (Cheung and Kong 2010b). Putative mechanisms include a direct interaction with the oxidative stressor (H_2O_2), inhibition of apoptotic signals or via modification of DNA repair

systems enhancing activity of anti-oxidant defence enzymes (Astley et al. 2004, Azevedo et al. 2011).

From the experimental data it is unlikely that SFN causes its effect by direct anti-oxidant actions because when co-treated no significant protection was observed; to achieve protection pre-treatment was required. ITCs could be rapidly accumulated in human and animal cells, with the peak intracellular ITCs accumulation reached within 0.5–3 hours of exposure and up to 100- to 200-fold over the extracellular ITC concentration or up to millimolar levels (Zhang 2001, 2012). This suggests modifications to the cell are made to enhance protection or that transport of SFN to key areas within the cells takes time. DNA damage detected using the alkaline comet assay was reduced by SFN pre-treatment. This may be a result of upstream effects of SFN giving rise to less damage or because of the increased efficiency of mechanisms that repair DNA strand breaks, such as non-homologous end joining (NHEJ) (Sekine-Suzuki et al. 2008, Yu et al. 2009). This can be tested in the future by the use of siRNA knockdowns of the repair systems. If upstream factors reduce DNA damage then no difference will be observed by reduction of DNA repair capacity.

Enhancing the antioxidant defence systems within the lens is a worthwhile aim and dietary supplements provide a logical means to achieve this (Chiu and Taylor 2007). Several intervention studies have been carried out, which have not demonstrated clinical benefit. A number of factors have contributed to these outcomes, including the period of observation and the point of intervention. However, it has been suggested that the use of diet enrichment before the age of 50 is likely to yield long-term benefit

(Chiu and Taylor 2007). There is also evidence available that demonstrates high consumption of brassicas can reduce the risk of cataracts in both animals and humans (Moise et al. 2012, Vibin et al. 2010). It may therefore be feasible that SFN can be used in conjunction with other antioxidants to further enhance the defence systems of the lens against persistent oxidative stress and potentially delay cataract formation.

The work presented in the current study clearly shows, for the first time, that SFN can protect human lens cells and the lens itself from significant oxidative stress. Thus enrichment of the diet with SFN has the potential to provide benefit to individuals in their defence against cataract formation. From the findings presented it is highly likely that SFN is acting as an indirect antioxidant and thus identification of these indirect pathways in SFN protection will be of value and relevance to cataract research. The data also suggest that exposure to elevated levels of SFN can have an adverse effect on lens cell survival and indeed promote cell death by apoptosis. This later trait could also be exploited in the treatment of posterior capsule opacification.

CHAPTER 4
IDENTIFICATION OF PROTECTIVE MECHANISMS OF SULFORAPHANE IN LENS CELLS

4.1 Introduction

The data presented in the previous chapter convincingly shows that SFN can provide protection to lens cells against oxidative stress. It was therefore logical to try to elucidate the cellular mechanisms of this action. A general screening was employed to determine if multiple pathways were involved. The majority of the literature, investigating multiple cell types, has suggested two major protective mechanisms of ITCs. These are the induction of phase II detoxification enzymes and the inhibition of phase I carcinogen-activating enzymes (Zhang 2012) (Figure 1.18).

Phase I drug-metabolizing enzymes (DMEs) are principally mediated by cytochrome P450 (CYP) enzymes that are mainly expressed in the liver (Shimada et al. 1994). They are usually involved in oxidation, reduction, or hydrolysis of chemicals including carcinogens and these chemical reactions have been implicated in the bioactivation of carcinogens (conversion of procarcinogens to carcinogens) (Wogan et al. 2004). Human CYP isoforms that are involved in drug metabolism include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, CYP3A4/5 and CYP4A (Elbarbry et al. 2007, Shimada et al. 1994). Reactive metabolites can form adducts with endogenous biomolecules such as DNA, RNA, and proteins, and play a key step in initiating cellular damage and cancers. Therefore, modulation of the CYP activity to decrease the

activation of procarcinogens could be a plausible target for chemoprevention through preventing cancer initiation (Patel et al. 2007) .

SFN was shown to inhibit the activity of CYP1A1 and CYP1A2 in the human hepatoma cell line HepG2 (Skupinska et al. 2009a, Skupinska et al. 2009b). Also in rat models, SFN has a dose-dependent inhibition effect on the bioactivities of cyp1A1 and cyp2B1/2 (Maheo et al. 1997). The key step in the carcinogenesis of polycyclic aromatic hydrocarbons (PAH) is their biotransformation to oxyderivatives by CYP1A1 and CYP1A2. Therefore, inhibition of these CYP enzymes by SFN may explain its chemopreventive effect in rats against the carcinogen, aflatoxin B1 (Maheo et al. 1997). In further research, using the p-nitro-phenol hydroxylase assay, SFN was shown to have an anti-mutagenic effect against N-nitrosodimethylamine (NDMA) in acetone-induced rat liver microsomes (Puccini et al. 1989). The procarcinogen is known to be activated by CYP2E1 to cause DNA damage, and this was inhibited by SFN in a dose-dependent manner (Barcelo et al. 1996). This confirmed that SFN is a potent competitive inhibitor for CYP2E1. On the other hand, SFN had no effect on the direct-acting, sodium azide (Paolini et al. 1997). This suggests that SFN may provide protection against carcinogens, which are substrates for CYP2E1. Although, the mechanism by which SFN inhibits CYP activity has not been extensively studied, it has been reported that isothiocyanates impair CYP activity by acting as competitive inhibitors (Goosen et al. 2001, Nakajima et al. 2001).

Phase II reactions mediate the metabolites produced during phase I metabolism with molecules such as uridine 5’diphosphate-glucouronic acid, glutathione or sulfate to

produce less toxic and readily eliminated metabolites. Also, Phase II reactions have been extended to include proteins that catalyze reactions which lead to comprehensive cytoprotection against electrophiles and reactive oxygen species (Talalay 2000).

Phase II enzymes include glutathione S-transferases (GSTs), which catalyze the nucleophilic addition of glutathione (GSH) to electrophilic groups of a broad spectrum of xenobiotics (Pool-Zobel et al. 2005); NAD(P)H:quinone oxidoreductase (QR-1), which catalyzes the two-electron reduction of quinones to hydroquinones (Cullen et al. 2003), preventing the one-electron reduction of quinones by other quinone reductases that would result in the production of radical species (Vasiliou et al. 2006); and UDP-glucuronosyltransferases (UGTs), which transfer glucuronic acid to hydrophobic chemicals (Saracino and Lampe 2007). Knockout of one or more of phase II proteins in animals led to a significant increase in carcinogen-induced and spontaneous tumorogenesis (Henderson et al. 1998, Long et al. 2000). Therefore, modulation of this cellular metabolism by inducing phase II enzymes could block or reduce the process of cancer initiation.

Many researchers have documented that SFN is the most potent naturally occurring phase II detoxification enzyme inducer in both animals and humans (Fahey et al. 2002, Talalay 2000). Several *in vitro* studies have shown the effectiveness of SFN in inducing the activity of phase II proteins. SFN was found to potently induce the activity of QR, GST-α and γ- glutamylcysteine synthetase, and increase intracellular glutathione synthesis in human prostate cancer lines (Brooks et al. 2001). SFN and its glutathione conjugate significantly induced both UGT1A1 and GSTA1 mRNA and protein levels in

human hepatoma HepG2 cells and colorectal adenocarcinoma HT29 cells (Basten et al. 2002). Similarly, in *in vivo* studies, rats fed for 14 weeks with 200 mg/day of dried broccoli sprouts containing glucoraphanin, the precursor for SFN, had significantly decreased oxidized GSH and protein nitrosylation, as well as increased glutathione (GSH) content, GSH reductase and GSH peroxidase activities in cardiovascular and kidney tissues (Wu et al. 2004).

There are three cellular components that regulate the gene expression of phase II proteins. These are Keap 1, Nrf2, and the ARE. In the absence of SFN and other inducers, Nrf2 is sequestered in cytoplasm by Keap 1. Upon exposure to SFN, SFN can react with the thiol group of Keap1 and release the binding Nrf2 causing dissociation from Keap1. Nrf2 undergoes nuclear translocation and binds to ARE and activates the transcription of phase II genes (Keum et al. 2006b, Yu et al. 1999). Nrf2 knockout mice have been used to evaluate the importance of Nrf2. Feeding mice with broccoli seed as a source of SFN for 7 days, resulted in an increase in protein expression of NQO-1, GSTA1/2, GSTA3, GSTM1/2 in the stomach, small intestine and liver of wild-type mice but not in Nrf2 knockout mice (McWalter et al. 2004). It shows that the ability of SFN to induce the expression of phase II proteins is mediated through Nrf2 as shown in Figure 4.1. Collectively, the chemopreventive effect of SFN could be attributed to its effect on the balance between procarinogen activation (by CYP enzymes) and carcinogen detoxification (by phase II enzymes). The aforementioned *in vitro* and *in vivo* studies may indicate that protection against carcinogenesis by SFN is attributed to its ability to inhibit the activity of CYP enzymes involved in procarcinogen bioactivation and/or its ability to enhance the capacity for detoxification of electrophiles and oxidants (Elbarbry and Elrody 2011).

4.2 Aims

Having determined that SFN could elicit protection to lens cells against oxidative stress it was then important to identify the mechanisms by which SFN can elicit cytoprotection to lens cells against oxidative stress. To achieve this goal an illumina gene microarray was carried out to assess SFN regulation of gene expression. At the protein level SFN induced changes were assessed using western blot and immunocytochemistry. Moreover, distribution of Nrf2 in response to SFN was assessed using immunofluorescence.

4.3 Results

4.3.1 Effects of SFN on gene expression FHL 124 cells detected by Illumina gene microarray

As an initial screen to identify gene changes induced by SFN, an Illumina gene microarray was performed. The data revealed that 9 genes were significantly up-regulated following a 24 hour exposure to 1 μM SFN (Table 4) and 9 genes were also up-regulated by 2 μM SFN (Table 5). There were 8 genes up-regulated in both 1 and 2 μM SFN exposures (Figure 4.1). SFN treatment resulted in an increased expression of several Nrf2 regulated genes. However, the elevated expression of the target genes was only demonstrated at the mRNA level. It was therefore of interest to look at proteins levels and see whether their expression was modulated by SFN or not.

Table 4 Increased gene expression in FHL 124 cells in response to 24 hour exposure to 1 μM SFN, determined using Illumina gene microarrays. All genes presented were increased ≥ 1.3 fold in the SFN treated group relative to non-treated control and were statistically different ($p \leq 0.05$; Student's t-test and $q \leq 0.25$; Benjamin Hochberg analysis)

Official Symbol	Official Full Name	Location	Summary	Fold change	p value	q value
NQO1	NAD(P)H dehydrogenase, quinone 1	16q22.1	This protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species.	2.04735	0.0000151	0.087413526
OKL38	Oxidative stress induced growth inhibitor	16q23.3	Encodes an oxidative stress response protein that regulates cell death	1.96899	0.000000304	0.010537
LOC392437	unknown	unknown	unknown	1.63879	0.000023	0.113791741
TXNRD1	thioredoxin reductase 1provided	12q23-q24.1	This gene encodes a member of the family of pyridine nucleotide oxidoreductases and plays a role in selenium metabolism and protection against oxidative stress.	1.60769	0.0000141	0.087413526
PIR	pirin (iron-binding nuclear protein)	XP22.2	The encoded protein is an Fe(II)-containing nuclear protein expressed Act as a transcriptional cofactor Involved in the regulation of DNA transcription and replication.	1.41567	0.00000459	0.053056757
G6PD	glucose-6-phosphate dehydrogenaseprovided	xq28	This gene encodes glucose-6-phosphate dehydrogenase whose main function is to produce NADPH	1.39306	0.0000028	0.048589621
EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	1q42.1	Epoxide hydrolase is a critical biotransformation enzyme., which functions in both the activation and detoxification of epoxides.	1.37074	0.0000117	0.087413526
FTL	ferritin, light polypeptide	19q13.33	FTL encodes the light subunit of the ferritin protein. affect the rates of iron uptake and release in different tissues	1.35383	0.0000539	0.169818382
PANX2	pannexin 2provided	22Q13.33	This protein and pannexin 1 are abundantly expressed in central nervous system (CNS) and are coexpressed in various neuronal populations	1.32656	0.0000269	0.116426773

Table 5 Increased gene expression in FHL 124 cells in response to 24 hour exposure to 2 μM SFN, determined using Illumina gene microarrays. All genes presented were increased ≥ 1.3 fold in the SFN treated group relative to non-treated control and were statistically different ($p \leq 0.05$; Student's t-test and $q \leq 0.25$; Benjamin Hochberg analysis)

Official Symbol	Official Full Name	Location	Summary	Fold change	p value	q value
OKL38	Oxidative stress induced growth inhibitor	16q23.3	Encodes an oxidative stress response protein that regulates cell death	2.18195	0.0000000551	0.001911616
NQO1	NAD(P)H dehydrogenase, quinone 1	16q22.1	This protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species.	2.09728	0.0000106	0.046724545
LOC392437	unknown	unknown	unknown	1.74835	0.00000619	0.046724545
TXNRD1	thioredoxin reductase 1provided	12q23-q24.1	This gene encodes a member of the family of pyridine nucleotide oxidoreductases and plays a role in selenium metabolism and protection against oxidative stress.	1.72358	0.00000318	0.036786402
PIR	pirin (iron-binding nuclear protein)	XP22.2	The encoded protein is an Fe(II)-containing nuclear protein expressed Act as a transcriptional cofactor Involved in the regulation of DNA transcription and replication.	1.56149	0.000000263	0.004559489
GCLM	glutamate-cysteine ligase, modifier subunit	1p22.1	Glutamate-cysteine ligase is the first rate limiting enzyme of glutathione synthesis.	1.53588	0.0000597	0.159341311
FTL	ferritin, light polypeptide	19q13.33	FTL encodes the light subunit of the ferritin protein. affect the rates of iron uptake and release in different tissues.	1.42567	0.0000106	0.046724545
EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	1q42.1	Epoxide hydrolase is a critical biotransformation enzyme., which functions in both the activation and detoxification of epoxides.	1.37391	0.0000108	0.046724545
G6PD	glucose-6-phosphate dehydrogenaseprovided	xq28	This gene encodes glucose-6-phosphate dehydrogenase whose main function is to produce NADPH	1.34738	0.000009	0.046724545

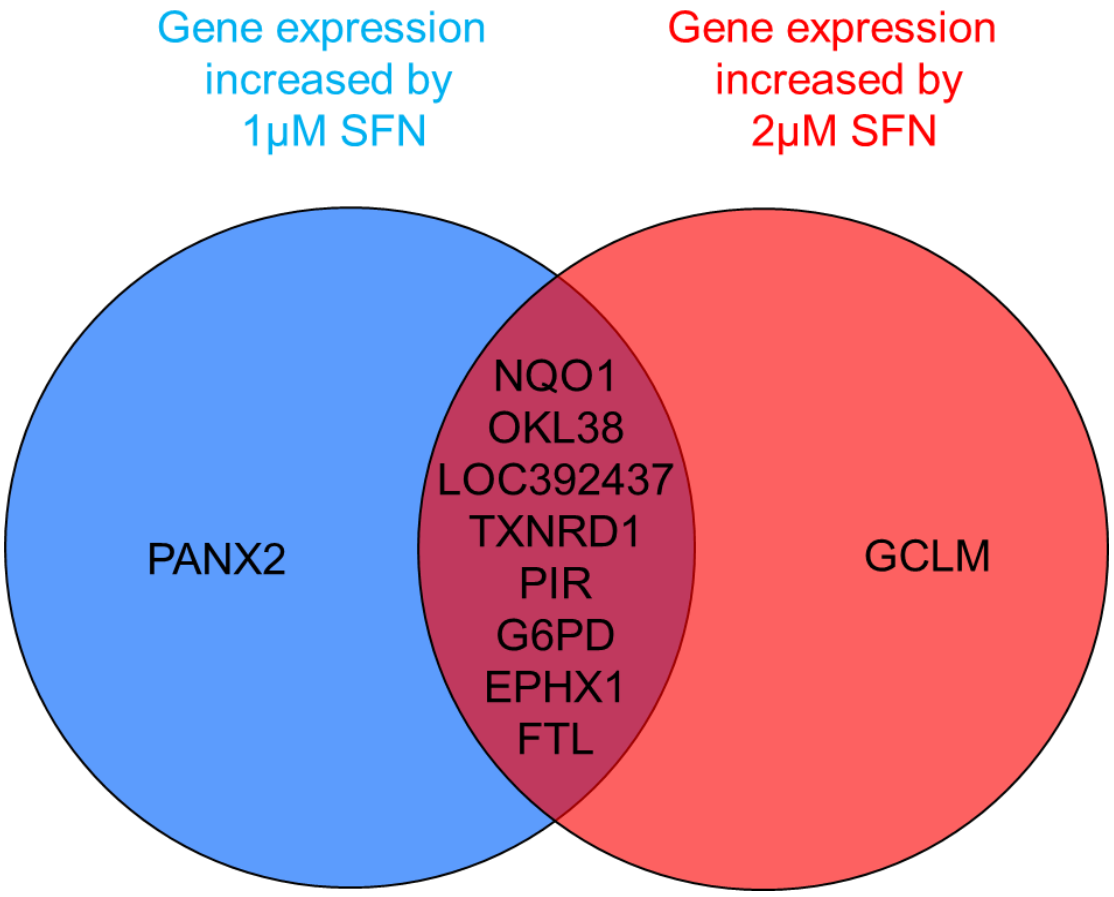

Figure 4.1 A Venn diagram showing the effect of 24 hour exposure of 1 and 2 μM SFN on increased gene expression of FHL 124 cells identified by Illumina gene microarray.

4.3.2 Effects of SFN on protein levels of Nrf2 regulated genes in FHL 124 cells detected by Western blot methods

NQO1 and TXNRD1 had been identified as being significantly increased in expression by SFN from the microarray study. They are classic phase II enzymes and have been reported to show increased expression in a number of cells and tissues treated with SFN (Matusheski and Jeffery 2001). To further investigate the effects of SFN on protein levels of these two genes, we measured protein expression of NQO1 and TXNRD1 in FHL 124 cells following SFN treatment using western blotting. A concentration dependent increase in NQO1 and TXNRD1 protein level was observed using Western blot methods (Figure 4.2). Significant elevation was observed at SFN concentrations $\geq$ 0.5 μM, with a peak observed with 2 μM SFN. This was a 2.5-fold elevation relative to untreated control cells for TXNRD1 (Figure 4.2 B). Similarly, NQO1 protein levels were increased by 2.3-fold compared to untreated control cells after 24 hour incubation with 2 μM SFN (Figure 4.2B). Protein expression was further evaluated using immunocytochemistry (Figure 4.3). Using this approach the distribution of both NQO1 and TXNRD1 was seen to be largely cytoplasmic (Figure 4.3 A). Quantification of these images also demonstrated a significant increase in protein levels, such that with 2 μM SFN, an 8-fold and 13-fold increase relative to untreated control cells was observed for NQO1 and TXNRD1 respectively (Figure 4.3 B).

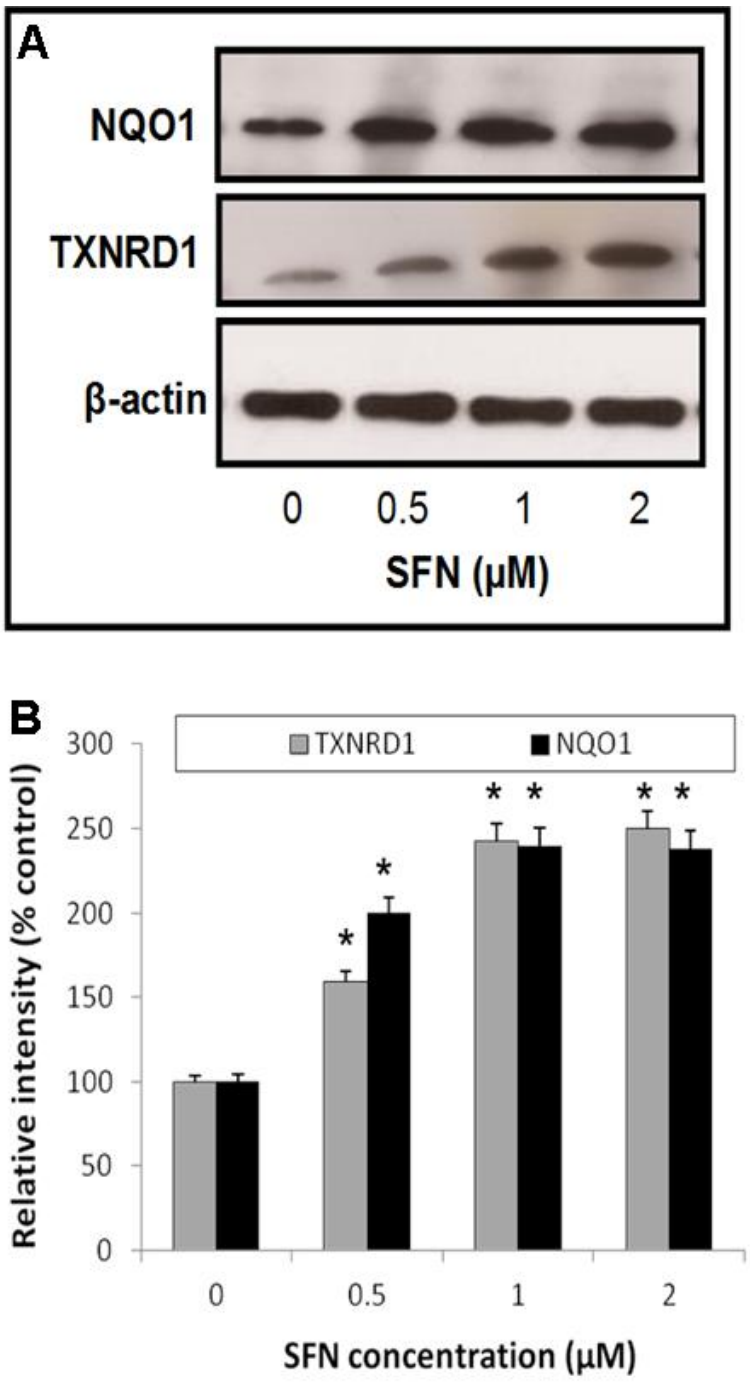

Figure 4.2 SFN induced NQO1 and TXNRD1 protein increase in FHL 124 cells, detected using western blot. (A) Representative blots showing NQO1, TXNRD1 and β–actin levels within FHL 124 cells. (B) Quantitative data derived from band intensities; the protein band intensities for NQO1 and TXNRD1 were normalized to β-actin. Data are presented as mean ± SEM (n=4). *Indicates a significant difference between treated and the non-treated control group ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

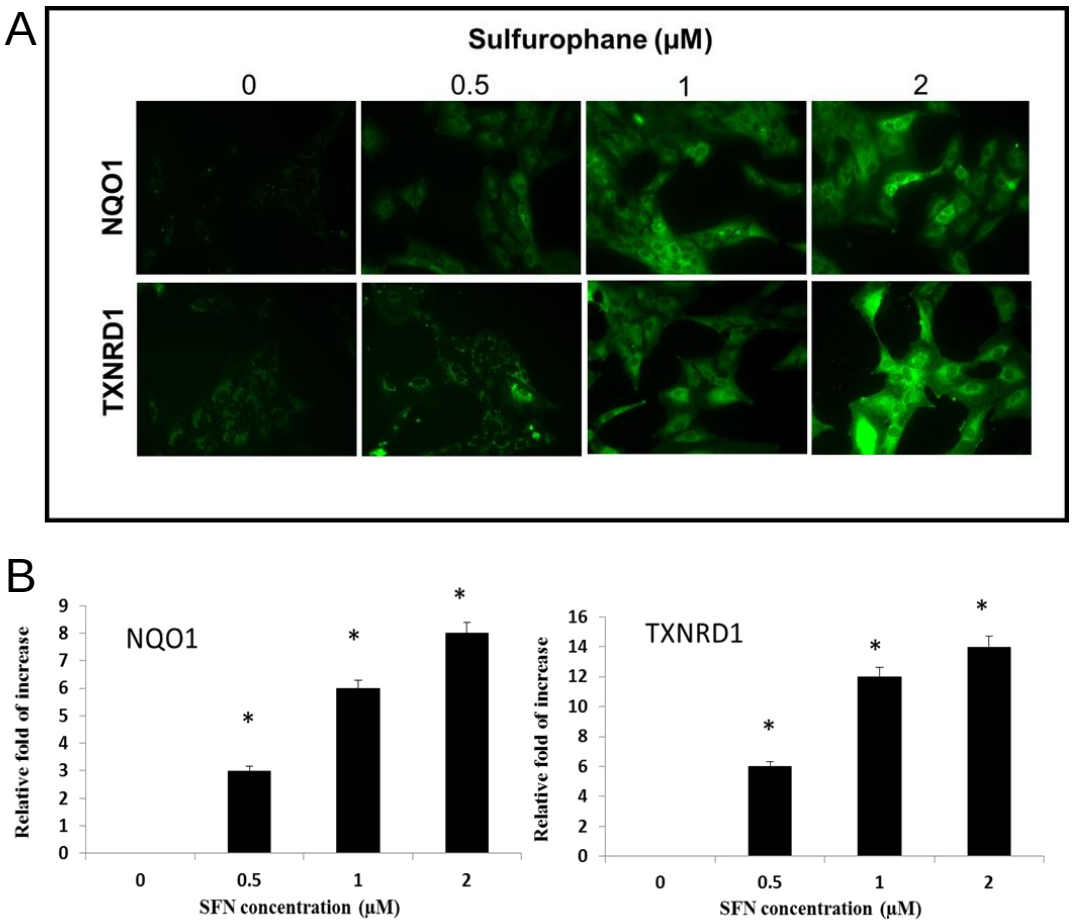

Figure 4.3 Immunofluorescence analyses of NQO1 and TXNRD1 distribution and expression within FHL 124 cells following exposure to SFN for a 24 hour period. (A) Representative images showing the organisation of NQO1 and TXNRD1 within FHL 124 cells. (B) Quantitative data derived from fluorescence micrographs showing changes in protein level in response to SFN. Data are presented as mean ± SEM (n=4). *Indicates a significant difference between treated and control groups ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

4.3.3 Effects of SFN on activation of transcription Factor Nrf2 in FHL 124 cells detected by Immunocytochemistry

Interestingly, it was observed that all 9 of the genes (LOC392437 unknown) (Figure 4.1) detected using the gene microarray that showed increased expression were reported in the literature to be regulated by the Nrf2 pathway. As Nrf2 is therefore a likely candidate in the regulation of SFN protection, further investigation was carried out to determine whether SFN could induce Nrf2 nuclear translocation in human lens epithelial cells. The accumulation and translocation of Nrf2 to the nucleus would indicate activation of Nrf2 and the induction of the Keap1-Nrf2- ARE pathway (Shelton and Jaiswal 2013). Immunocytochemistry experiments were carried out to look at the location of Nrf2 proteins within FHL 124 cells following SFN treatment for 4 hours. As shown in Figure 4.4 A, only cytoplasmic labelling of Nrf2 with no distinct nuclear staining was observed in the non-stimulated cells, whereas an intense nuclear labelling was observed in the SFN-stimulated cells. In addition, the SFN treatment of the cells led to a dose-dependent increase in the Nrf2 levels (Figure 4.4 B).These data clearly show that Nrf2 is translocated to the nucleus indicating Keap1-Nrf2-ARE pathway activation in response to SFN exposure.

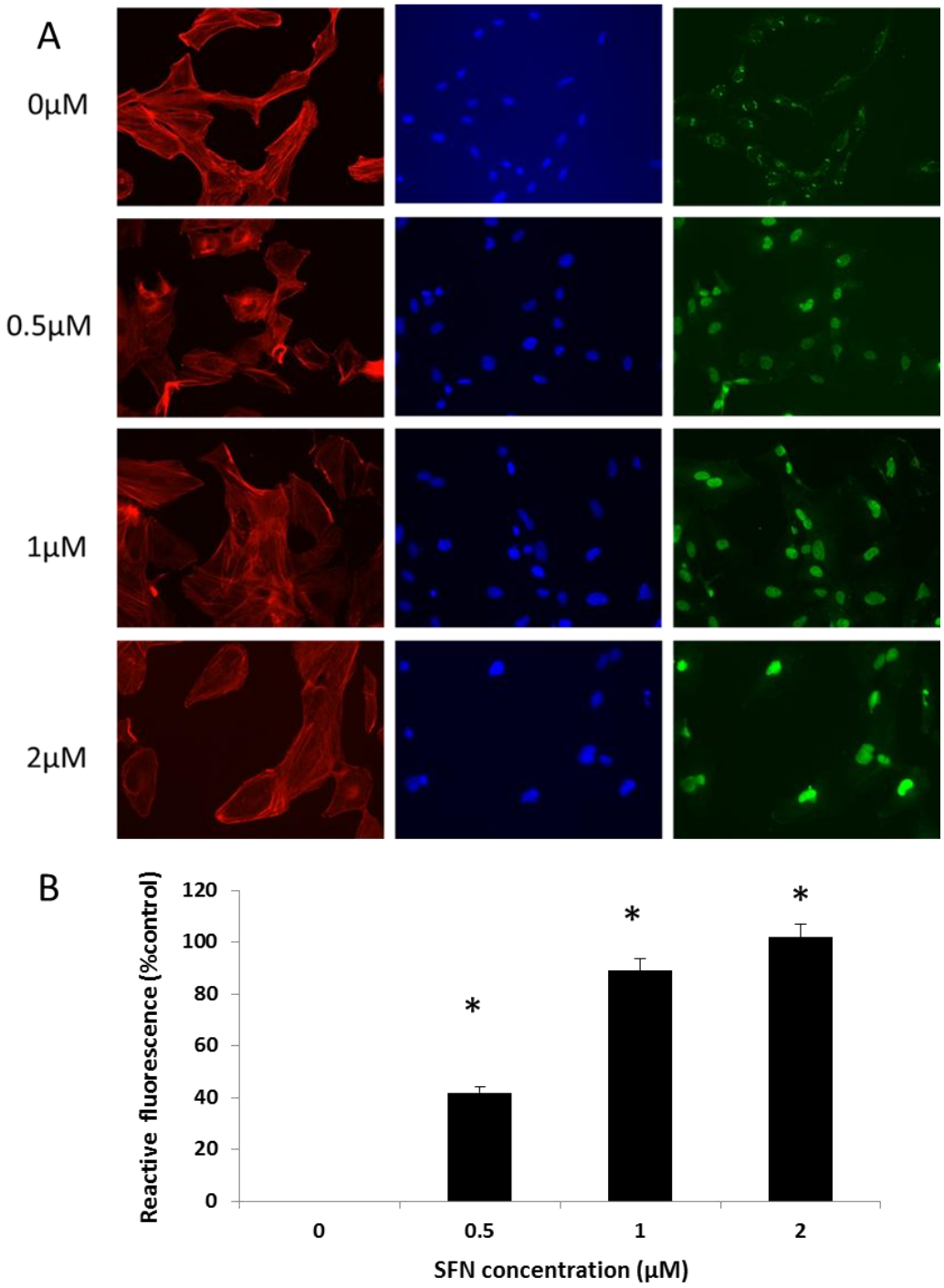

Figure 4.4 Immunocytochemistry of FHL 124 showing induction of nuclear translocation of Nrf2 in response to SFN treatment. (A) Representative fluorescent micrographs showing Nrf2 distribution following 4 hour exposure to 0, 0.5, 1, 2 µM SFN treatments. Nuclei and actin filaments are labelled using DAPI (blue) and Texas red X-Phalloidin (red) respectively. (B) Quantitative data derived from fluorescence micrographs showing changes in Nrf2 protein level in response to SFN. Data are presented as mean ± SEM (n=4). *Indicates a significant difference between treated and control groups ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

4.4 Discussion

The widespread belief that oxidative damage plays a major role in age related cataract and in a number of chronic diseases has focused scientific and public attention on the possibility that antioxidants could prevent or at least retard these processes (Lou 2003, Williams 2006). Antioxidants are of two types: direct and indirect. Direct antioxidants for example glutathione (GSH), tocopherols, ascorbic acid and carotenoids are substances that can inactivate free radicals or that prevent free-radical initiated chemical reactions (Lou 2003). In contrast, indirect antioxidants are not able to participate in radical or redox reactions as such, but they enhance the antioxidant capacity of cells by a variety of mechanisms (Lou, 2003).

This chapter has demonstrated that SFN can elicit protective benefit to lens cells. SFN is unlikely to be a direct-acting antioxidant in our system, since it is very unlikely that the isothiocyanate group can participate in oxidation or reduction reactions under physiological conditions (Barton and Ollis, 1979). Data from chapter 3 showed that protection against oxidative stress occurred in lenses that had been pre-treated with SFN for 24 hours, but protection was absent when SFN was added at the same time as the oxidative insult. This suggests that SFN is able to induce protective mechanisms within the cells rather than having direct antioxidant effects.

In considering SFN as an agent that could reduce the risk of cataract formation, it is plausible that SFN could act via multiple pathways. Oxidative stress is a recognised factor in the formation of cataract(Spector 1995, Taylor et al. 1995, Truscott 2005)

and SFN, as a potent antioxidant, could indirectly inhibit oxidative damage. One possibility is that SFN could raise tissue GSH levels and thus enhance a non- enzyme antioxidant defence capacity (Zhang and Talalay 1994). The GCLM gene encodes glutamate-cysteine ligase, which is the first rate limiting enzyme in glutathione synthesis. A significant upregulation of this gene was observed using microarray analysis with 2 μM SFN treatment and may suggest that this defence mechanism is positively regulated by SFN or by stimulating the ARE as other inducers of Phase II enzymes.

Modification of enzymatic antioxidant defence systems is also likely to play a key a role in resistance of oxidative stress and the data presented support this notion. Dietary isothiocyanates are breakdown products of glucosinolates from cruciferous vegetables, for example broccoli (Wu et al. 2009). There are many studies that demonstrate ITCs are potent inducers of chemopreventive enzymes including antioxidant enzymes (Keum et al. 2005, Thornalley 2002). The current study has addressed this issue by an Illumina gene array to determine the effects of SFN on gene expression. The data revealed that 8 genes were significantly up-regulated following 24 hours exposure to both 1 and 2 μM SFN, of which a number are associated with anti-oxidant defence. Of the genes that were up-regulated, all are reported in the literature to be controlled by the transcription factor Nrf2. TXNRD1 and NQO1 are classic phase II enzymes and have been reported to show increased expression in a number of cells and tissues (Matusheski and Jeffery 2001). G6PD encodes glucose-6-phosphate dehydrogenase which is a cytosolic enzyme whose primary function is to produce NADPH; this gene is also reported to be regulated by Nrf2 signalling (Shelton and Jaiswal 2013). Moreover, NADPH is utilised by NQO1

as a hybride donor in the conversion of quinones to hydroquinones. Therefore upregulation of G6PD is likely to facilitate the antioxidant activity of NQO1. Nrf2 signalling has also been implicated in the expression of pirin in small airway epithelium (Hubner et al. 2009). In smokers, Nrf2 signalling was more active and this was associated with increased expression of pirin relative to non-smokers (Hubner et al. 2009). Moreover, it was identified that the promoter region of the pirin gene contains functional antioxidant response elements (Hubner et al. 2009). In addition, EPHX1 and FTL, which encode epoxide hydrolase 1 and ferritin light chain respectively are also shown using a mouse model to be induced by 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), which is a highly potent chemopreventive agent. In Nrf2 knockout animals, the induction of EPHX1 was suppressed indicating an important role of Nrf2 signalling in its regulation (Malhotra et al. 2010). In the case of FTL, the CCDO-Im induced expression was ablated in the absence of Nrf2 (Liby et al. 2005). OKL38, which encodes oxidative stress induced growth inhibitor 1, is reported to show induction following oxidative stress (Li et al. 2007). In cancer cells it is believed that following DNA strand breaks, OKL38 interacts with p53 and relocates to the mitochondrion to initiate cytochrome c release and apoptosis; OKL38 is therefore defined as a tumour suppressor (Hu et al. 2012). In the current system, OKL38 is up-regulated in response to SFN, but under these conditions cells continue to survive and grow. In addition, cytotoxicity/apoptosis does not differ from controls. Oxidative stress induced DNA damage is suppressed by SFN and perhaps without this cue, OKL38 does not interact with p53 and induce apoptosis. The expression of OKL38 remains curious and further inhibition studies will be required to elucidate its putative role in SFN protection in the lens.

The common factor linking all the genes shown to be up-regulated is Nrf2/keap1 signaling (Li et al. 2007b). It was therefore of importance to establish whether Nrf2 signaling can take place in lens cells in response to SFN. The data confirm that Nrf2 translocation to the nucleus does occur and thus it is reasonable to hypothesise that the protection observed with SFN against oxidative stress is largely mediated by Nrf2 regulation. SFN is known to influence this pathway through interaction with the thiol group on keap1, which liberates Nrf2 from the complex. Nrf2 then translocates to the nucleus and initiates transcription (Juge et al. 2007a). As a result of the experiments presented here, it will therefore be of great interest in the future to determine more of the role of Nrf2 in expression of the genes identified from the microarray data as this could also contribute to any potential anti-cataract activity. Such work will involve establishing the kinetics of Nrf2 nuclear translocation in response to SFN using immunocytochemistry and GFP tags, Nrf2/ARE reporter assays and siRNA approaches to assess functional involvement of the individual genes and Nrf2 signaling.

It will also be of key importance to investigate the effects of SFN on Nrf2 pathway under conditions of reduced oxygen in order to specifically relate to the conditions experienced by the lens in vivo. In addition, the bioavailability of SFN to the lens requires consideration. Peak plasma concentrations of total SFN (largely as SFN metabolites) are reported in the low micromolar range in bioavailability studies following intake of a SFN-rich food. Whether SFN reaches the lens and in what form is an important question for future research.

In summary, pre-treatment of lens cells with SFN can modify anti-oxidant defence mechanisms by induction of Keap1-Nrf2-ARE pathway activation, thus rendering lens cells more capable of suppressing the daily insult of oxidative stress. Improved intake through an SFN-rich diet or use of supplements could provide a novel approach to retard the onset of cataract formation in the human lens.

CHAPTER 5
THE PUTATIVE THERAPEUTIC BENEFITS OF SULFORAPHANE APPLICATION IN PREVENTING POSTERIOR CAPSULE OPACIFICATION

5.1 Introduction

PCO is the most common complication of cataract surgery and likely the most common cause of non-refractive decreased postoperative vision (Schaumberg et al. 1998, Apple et al. 1992). At present the only means of treating cataract is by surgical intervention. PCO reflects the wound-healing process of the LECs that remain in the capsular bag after cataract surgery. Residual LECs within the capsular bag (formed at cataract surgery to hold the IOL) rapidly grow and proliferate across the posterior lens capsule and migrate over the anterior surface of the IOL thus causing a secondary reduction in vision quality (Wormstone 2002). The most common changes with regard to the all important visual axis result from fibrosis; this is generally associated with wrinkling/contraction of the posterior capsule and increased cell aggregation. In addition, formation of swollen globular cells known as Elschnig's pearl often occur (Kappelhof et al. 1987). In the peripheral capsular bag, lens fibre differentiation occurs, frequently leading to the appearance of Soemmerring's ring (Kappelhof et al. 1987). Additional features can also contribute to visual deterioration, including anterior fibrosis and anterior capsular phimosis (Sciscio and Liu 1999, Waheed et al. 2001).

In the present study we investigated the potential benefits of SFN in the prevention of PCO. At low concentrations SFN can provide benefit to lens cells, but at higher concentrations it is reported to act in a manner that can disrupt the function of cells in a detrimental way, such as a reduction in proliferation by cell cycle arrest (Jackson and Singletary 2004a, b) and induction of cell death by apoptosis.

There are three possible mechanisms of SFN-mediated cell cycle arrest. First is through inhibition of cyclins which, together with CDK, drive the cell cycle from one phase to the next (Chiao et al. 2002). A second mechanism affects the cell cycle is through up-regulation of CDKIs, which bind and inhibit the activity of cyclin/CDK complexes and regulate cell cycle progression through the four phases (Shan et al. 2006). A third possible mechanism for the arrest in cell cycle progression involves disruption of microtubules by inhibition of tubulin polymerization (Jackson and Singletary 2004a, b).

Induction of apoptosis is hypothesized to be through intracellular activation of caspases, a family of cysteine proteases, which are responsible for initiation and execution of apoptosis. Also caspase-independent pathways mediate induction of apoptosis, such as release of the mitochondrial protein apoptosis inducing factor (AIF) into the cytosol, activation of a family of Ca^{+2}-activated cytosolic proteases called calpains (Juge et al. 2007), or modulating the activation of transcription factors such as NF-kB and AP1 family members which are involved in induction of cell survival genes (Mi et al. 2007). Apoptosis in response to SFN is known to occur through these mechanisms in various cells and tissues (Gamet-Payrastre et al. 2000). It also it has been demonstrated in ovary (Bryant et al. 2010, Chiao et al. 2002, Jiang et al. 2010,

Shan et al. 2006, Shankar et al. 2008). It has also been proposed that oxidative stress can mediate cell death by apoptosis through induction of ER stress (Wang et al 2012).

As SFN is also reported to promote ER stress in other systems establishing a possible whether ER stress plays an important role in SFN mediated cell death is of interest To date no information is available on SFN regulated cell cycle arrest, apoptosis or ER stress in the lens.

5.2 Aims

Prevention of PCO is an important are of lens biology. The aim of this chapter was therefore to establish the ability of SFN to initiate death of lens cells and prevent PCO formation.To achieve this, the efficacy of SFN was assessed using a human lens cell line and capsular bag system. Using these models, cell viability, cell death and ER stress were evaluated .

5.3 Results

5.3.1 Effects of SFN on FHL 124 cell viability by MTS assay

FHL 124 cell survival was decreased by SFN in a dose dependent manner (Figure 5.1). Cells were exposed to a range of SFN concentrations (0-300 μM**)** for 2 minutes, 30 minutes, 1 hour and 24 hours prior to end point at 24 hours**.** When continually exposed to SFN for the 24 hours experimental period, evidence of impaired growth/survival was first observed at 10 μM. A similar pattern was observed when cells were exposed to SFN for 1 hour. With 30 minutes exposure to SFN, a significant reduction in cell number was observed with a treatment of 30 μM SFN and above. With a 2 minute exposure period, 100μM SFN was required to observe a significant reduction in cell number/viability.

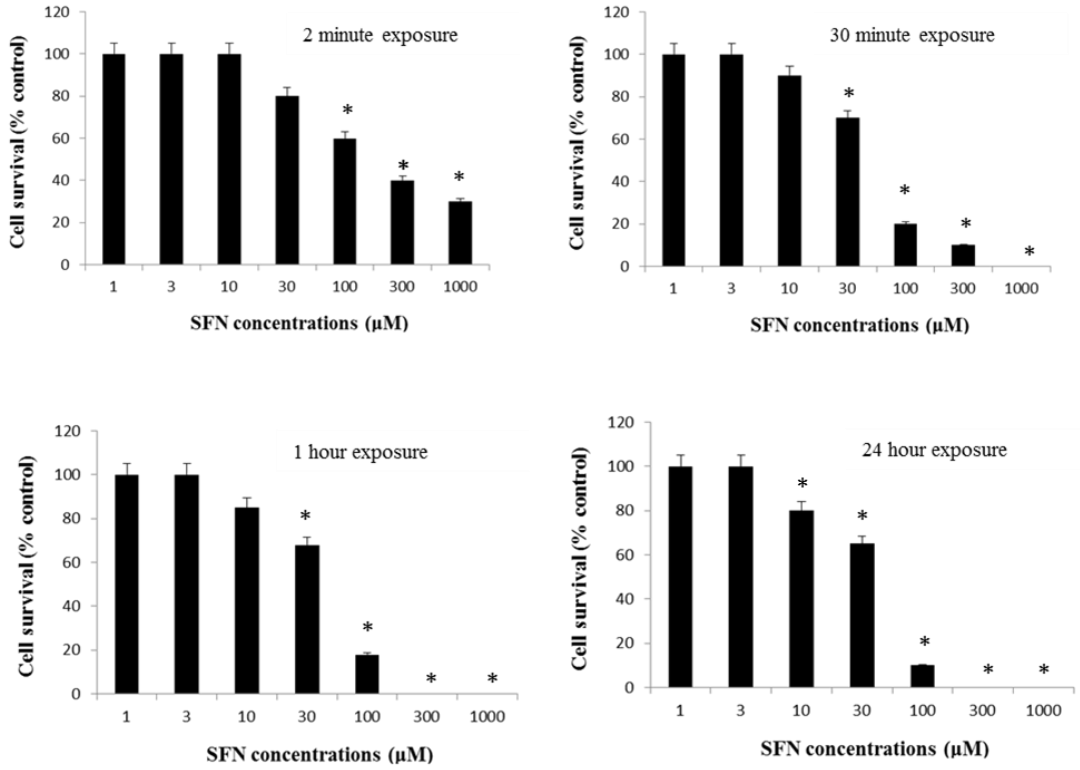

Figure 5.1 Dose-dependent effects of SFN on FHL 124 cell survival. Cell survival was evaluated following a 24 hour experimental culture period. Cells were exposed to SFN for the first 2 minutes, 30 minutes, 1 hour or continuously exposed for 24 hours. The Data are expressed as mean± SEM (n=4). * indicates a significant difference between the treated group and untreated controls ($p \leq 0.05$; ANOVA with Dunnett's post hoc test).

5.3.2 Effect of SFN on human capsular bags

A sham cataract operation was performed on human donor lenses which were then cultured in serum free medium in the presence of SFN for 30 days. Capsular bags maintained in standard culture conditions demonstrated progressive cell growth across denuded regions of the anterior capsule; the outer anterior capsule and importantly the previously cell-free posterior capsule. At day 8 cells could be clearly seen on the central posterior capsule. The level of growth in capsular bag preparations treated with 1mM SFN was similar to control untreated preparations (Figure 5.2). Cells were also observed on the central posterior capsule with 10 μM SFN, but growth appeared retarded. No cells were observed on the central posterior capsule with 100 μM SFN and indeed the cells on the anterior capsule appeared distressed (Figure 5.2). Following 30 days of culture (end-point), control capsular bags exhibited complete coverage of the posterior capsule (Figure 5.3 A). Exposure to either 0 or 1 μM SFN for the first 24 hours of culture had negligible effect on cell coverage by day 30. The capsular bag that was exposed to 10 μM SFN demonstrated a marked reduction in cells growing (Figure 5.3) and a 24 hour treatment with 100 μM SFN lead to widespread cell death and completely inhibited growth onto the posterior capsule (Figure 5.4). To further validate this observation, capsular bags treated with SFN were examined by immunocytochemistry. This clearly confirmed that an abundant population of lens cells resided on the posterior capsule when treated with 10 μM SFN, but there were no cell present in the 100 μM treated group (Figure 5.5).

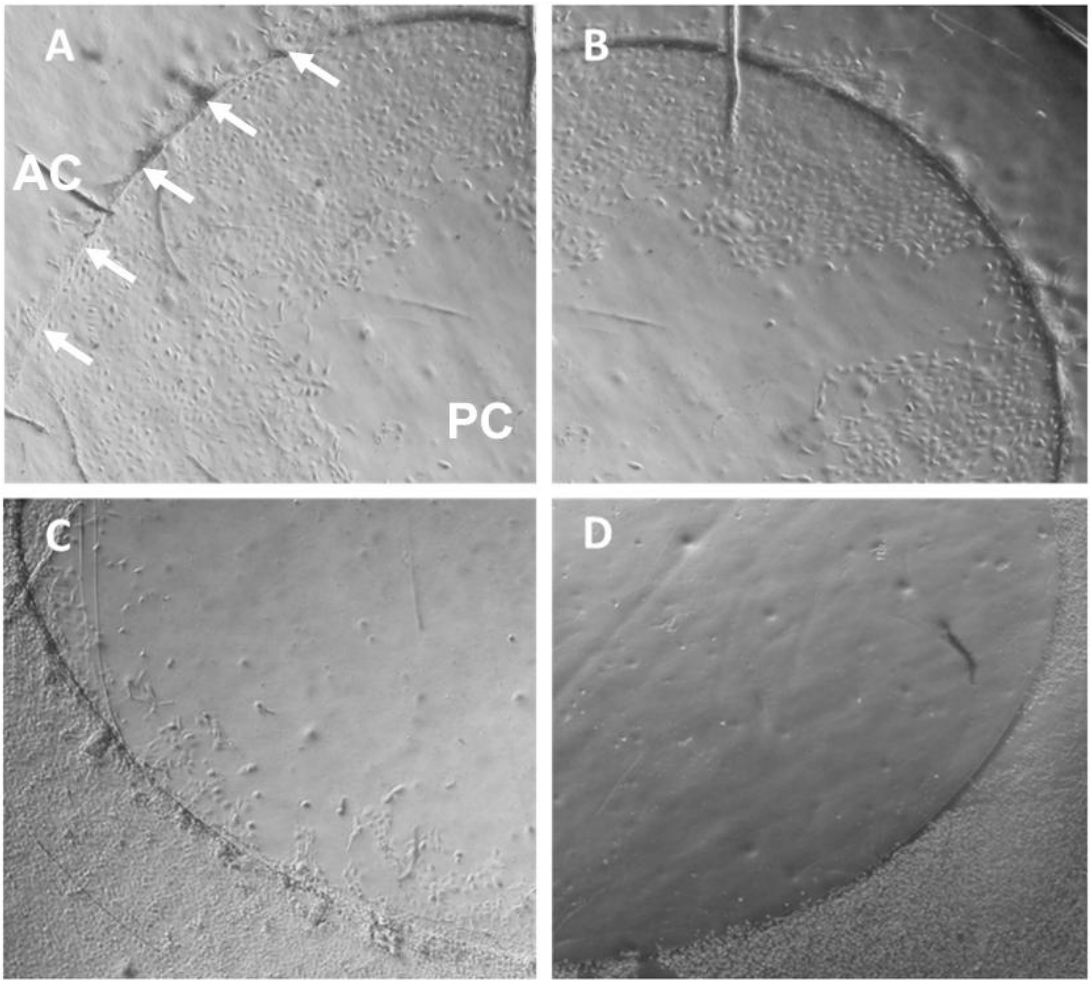

Figure 5.2 Dark-field images of four capsular bag quarters showing the posterior capsule (PC), capsulorhexis (arrowed) and outer anterior capsule (AC) captured after 8 days. Capsular bags were treated with medium alone (A); 1 μM SFN (B); 10 μM SFN (C) or 100 μM SFN (D).

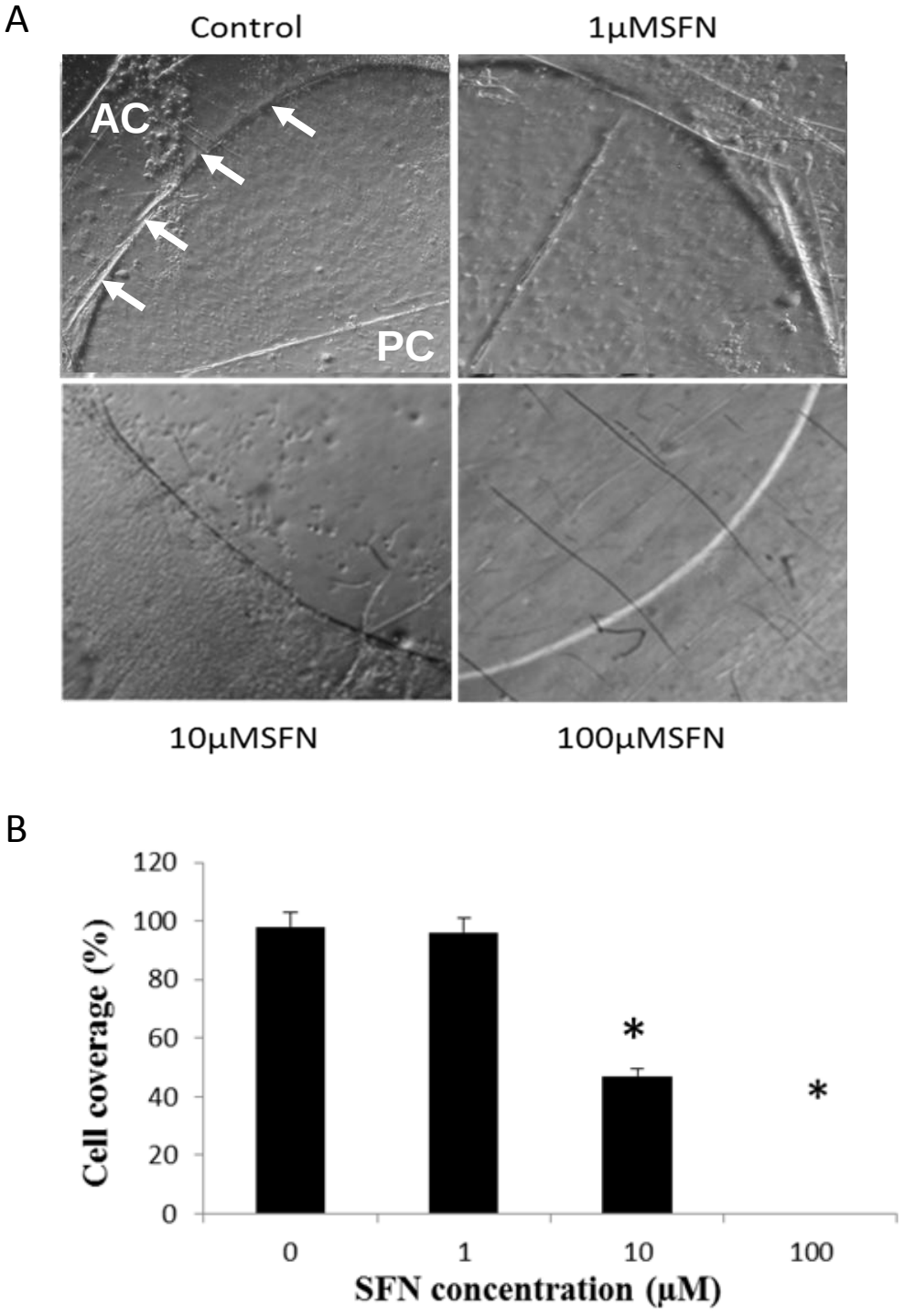

Figure 5.3 (A) Modified dark-field images of four capsular bag quarters showing the posterior capsule (PC), capsulorhexis (arrowed) and outer anterior capsule (AC) captured after 30 days. Capsular bags were treated with medium alone; 1 µM SFN; 10 µM SFN or 100 µM SFN. (B) Quantitative data derived from modified dark-field images showing changes in cell coverage on the posterior capsule in response to SFN. Data are presented as mean ± SEM (n=4). *Indicates a significant difference between treated and control groups ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

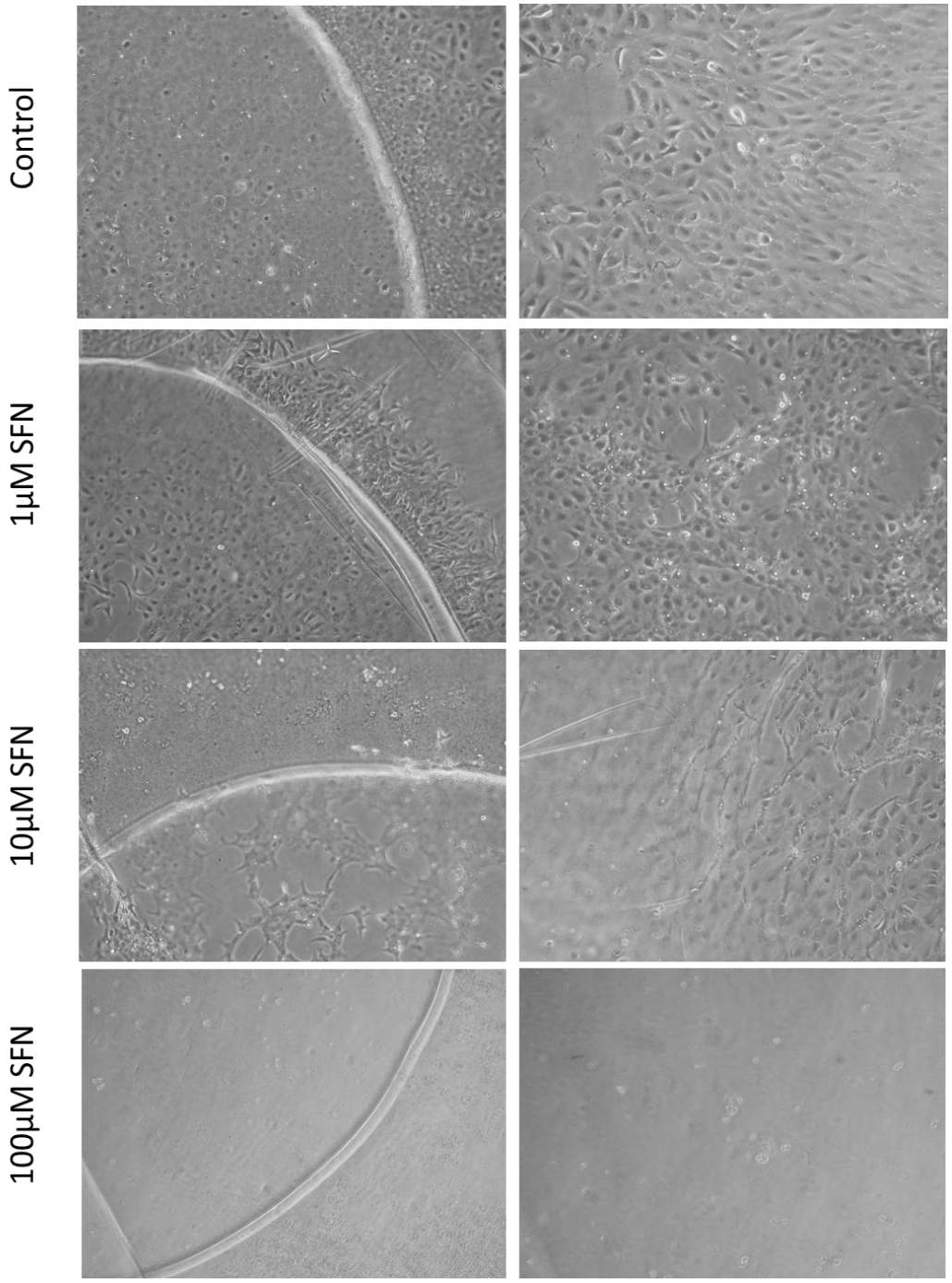

Figure 5.4 Low and high power phase-contrast images of cells growing in different regions of human lens capsular bag preparations following 30 days in the presence or absence of SFN. The field of view of all micrographs in the left hand column represent 2000 ×1427μm and in the right column represent 800 × 571 μm.

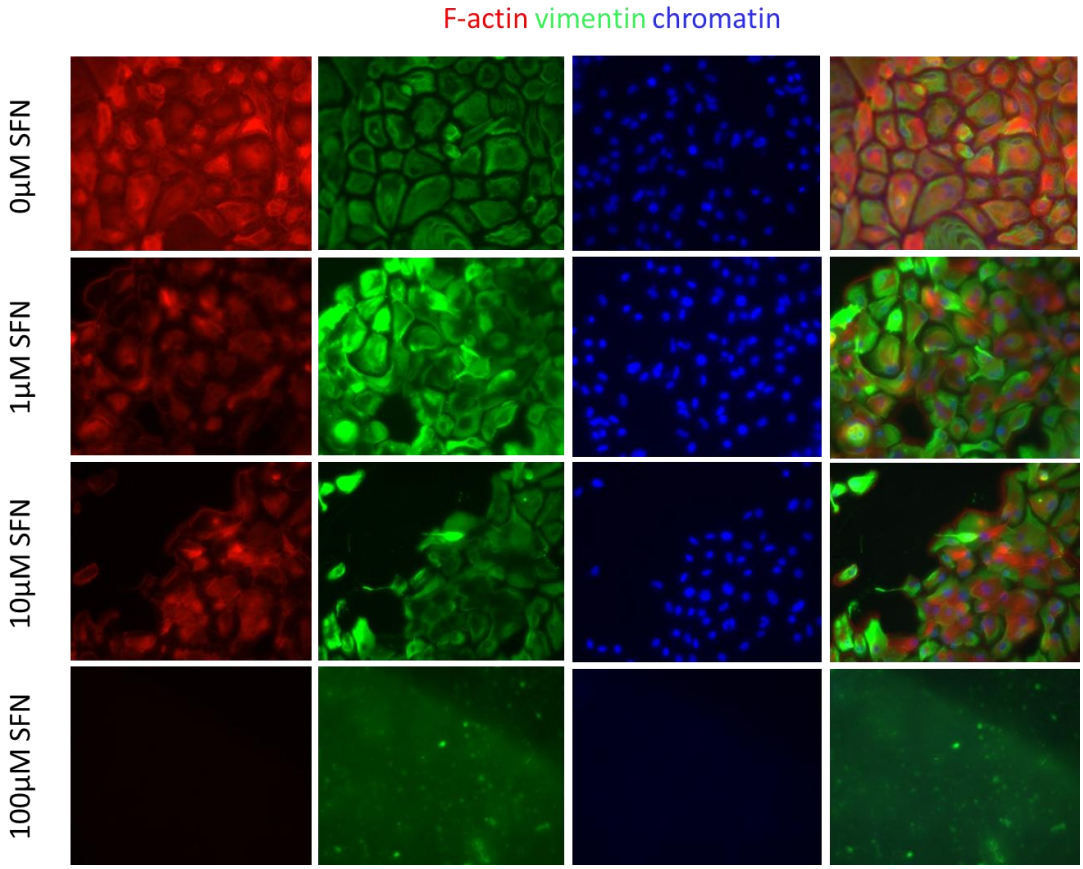

Figure 5.5 Fluorescent micrographs of lens cells growing on the central posterior capsule of capsular bags treated with 0, 1, 10 or 100uM SFN. Preparations had been cultured for 30 days. A population of cells on the posterior capsule of capsular bags treated for 24 hours with 1or 10 µM SFN can clearly be seen, whereas treatment with 100 µM totally ablated the cell population. The field of view of all micrographs is 448 × 342 µm.

5.3.3 Effect of SFN on ER stress in FHL 124 cells

In order to investigate the putative involvement of endoplasmic reticulum (ER) stress in SFN mediated events, Real-time PCR and Western blot analysis were employed. FHL 124 cells were treated with 10, 30 and 100 μM SFN for 24 hours. Real-time PCR revealed increases in all four ER stress gene products tested in FHL 124 cells after exposure to SFN for 24 hours (Figure 5.6). The levels of BiP, IRE1 and EIF2α in lens cells were significantly increased within 24 hours of exposure to 10 μM SFN (Figure 5.6). All genes were significantly elevated at the 30 and 100 μM SFN concentrations. At the protein level, BiP, ATF6, EIF2α and IRE1 were significantly up-regulated in response to a 24 hours 100 μM SFN treatment. When cells were exposed to 30 μM SFN, there were significant increases in BIP, IRE1 and EIF2α expression (Figure 5.7).

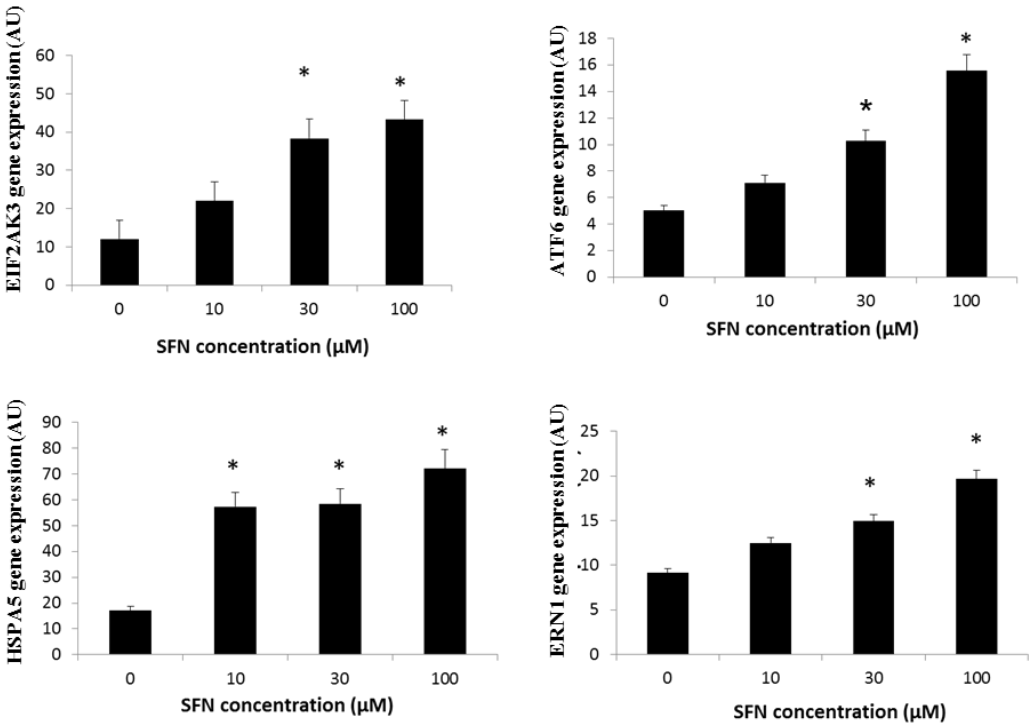

Figure 5.6 Real-time PCR analysis of SFN effects on ER stress gene expression. Cells were exposed to 0, 10, 30, 100μM SFN and changes in (A) EIF2AK3, (B) ATF6, (C) HSPA5 and (D) ERN1 gene expression were detected. Values were normalized to 18S gene expression. The data represent mean ± SEM (n= 4). *Represents a significant difference between untreated control and treated groups ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

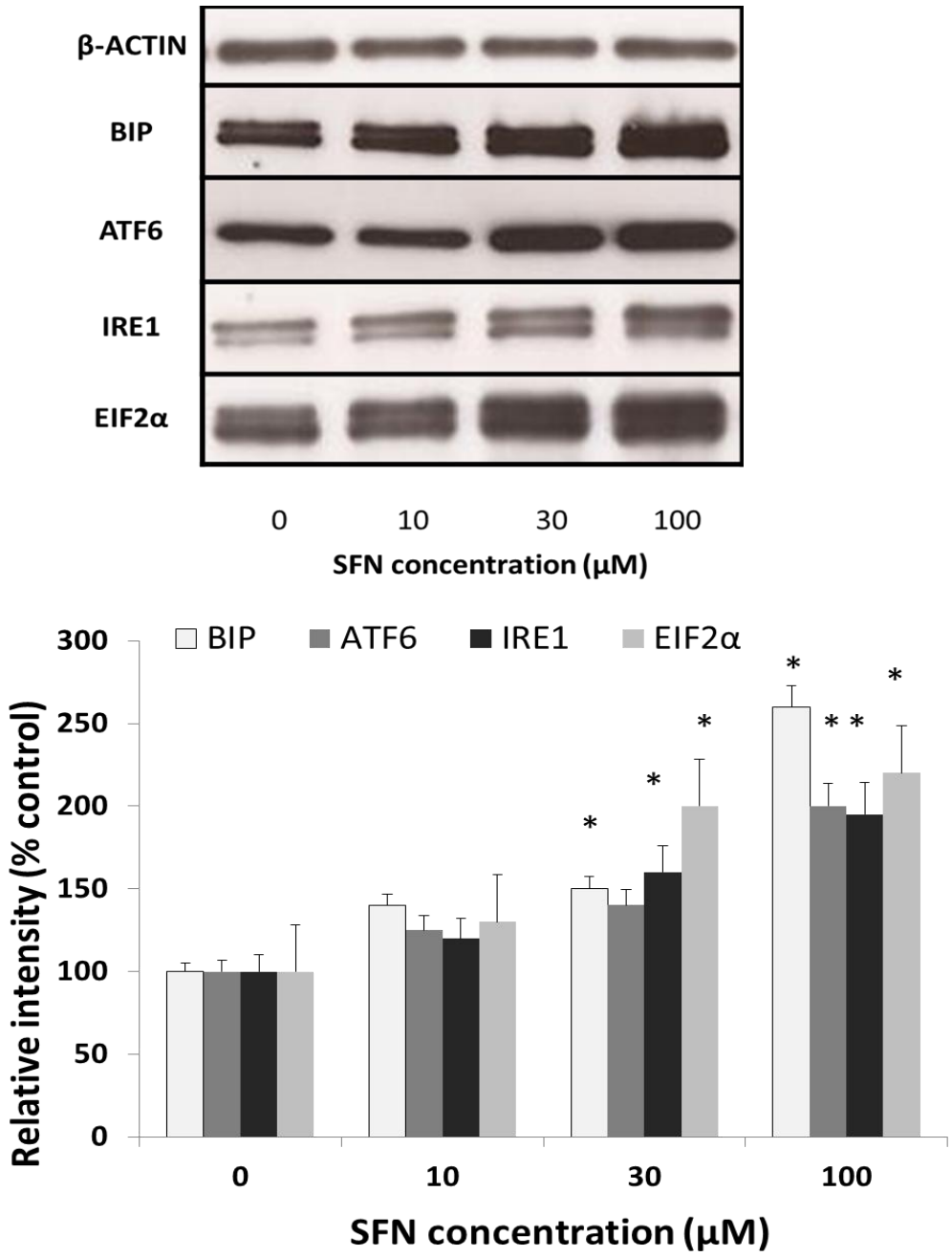

Figure 5.7 SFN induced BIP, ATF6, IRE1, and EIF2α protein increase in FHL 124 cells, detected using western blot. (A) Representative blots showing BIP, ATF6, IRE1, and EIF2α and β–actin levels within FHL 124 cells. (B) Quantitative data derived from band intensities; the protein band intensities for BIP, ATF6, IRE1, and EIF2α were normalized to β-actin. Data are presented as mean ± SEM (n=4). *Indicates a significant difference between treated and the non-treated control group ($p \leq 0.05$; ANOVA with Dunnett's post-hoc test).

5.4 Discussion

PCO is the most common complication of cataract surgery (Apple et al. 1992a). At present the only means of treating cataract is by surgical intervention, and this initially restores high visual quality (Allen and Vasavada 2006). Unfortunately, PCO develops in a significant proportion of patients to such an extent that a secondary loss of vision occurs (Wormstone et al. 2009). It has been reported that there are various ideas and methods to prevent (or at least decrease the incidence of) proliferation of the lens epithelium. However, the existing possibilities to prevent PCO formation are still not satisfactory. Two means of PCO prevention need to be discussed. Firstly, there is the mechanically induced, local restriction of proliferating cells (contact inhibition). Secondly, the inhibition of PCO formation by cytotoxic drugs or gene therapy which is perhaps even more important (Duncan et al. 1997a, Fernandez et al. 2004, Jordan et al. 2001)

Although SFN has been studied extensively in recent years, this work has largely focused on cancer (Jackson and Singletary 2004b, Keum et al. 2005). However, the functional role of SFN in the prevention of PCO, which affects a large proportion of cataract patients, has not been investigated. In the present study, the hypothesis was tested that SFN can inhibit the development of PCO. In the present study, application of SFN leads to widespread cell death using both a human lens cell line and human capsular bag model. High doses of SFN are a necessary condition for inducing ROS production. The concentration required to successfully kill cells in the capsular bag was significantly greater than that needed to in the cell line. This difference can be

explained by the presence of residual lens fibres in the capsular bag. These fibre cells can protect the underlying epithelial cells (particularly those located in the equatorial region) by acting as a physical barrier and providing many additional SH sites for SFN to act upon. Therefore greater concentrations are required to reach the cells in the equatorial region.

With regard to the relevance of cytotoxic drugs and thereby the possible induction of apoptosis in FHL 124 cells, one first needs to focus on the characterization of the pathways leading to cell death. A clear and detailed understanding of extracellular and intracellular mechanisms of the underlying molecular steps leading to the death of the cell is crucial for the understanding and the distinct pharmacological triggering of cell-specific, proapoptotic cascades. One possible problem for PCO prevention using a toxic compound will be the healthy, anatomically narrow and physiologically fragile surroundings of the capsular bag. Already a Perfect Capsule device recently developed by Maloof and colleagues (Maloof et al. 2003) applied to the intraocular lenses did show that the strictly local and defined application of any pharmaceutical seems to be absolutely necessary to prevent intraocular side effects. . This method has shown that there was no leakage to surrounding tissues, and the population of epithelial cells remaining in the bags after surgery was reduced (Maloof et al. 2003).

The present study demonstrates that SFN can induce ER stress, which was demonstrated by ER stress gene and protein induction. Previous work has shown that thapsigargin, a calcium ATPase inhibitor and arsenic trioxide can also induce ER stress in lens cells. An important question to answer is whether the short-term

exposure to SFN will cause progressive death long-term as is observed with thapsigargin (Duncan et al. 1997b, Wang et al. 2005). It will therefore be of interest to extend the culture period in the cell line MTS experiments to see if death at high concentrations does become greater over time. This data could then be fed into the capsular bag work and a 2 minute exposure using the perfect capsule system could be applied and assessed.

Combinatorial treatment with SFN and Arsenic trioxide (As_2O_3) might provide a promising therapeutic approach for a variety of diseases such as multiple myeloma (MM) (Doudican et al. 2012) and acute promyelocytic leukemia (APL) (Doudican et al. 2010). SFN enhances the cytotoxic effects of As_2O_3 through a ROS-dependent mechanism. Cellular exposure to SFN in combination with As_2O_3 resulted in a dramatic increase in levels of ROS compared to treatment with either agent alone. SFN, alone or with As_2O_3, decreased intracellular glutathione (GSH) content. Furthermore, addition of the free radical scavenger N-acetyl-l-cysteine (NAC) rescued cells from As_2O_3/isothiocyanate-mediated cytotoxicity. As As_2O_3 has been associated with ER stress in the lens and investigated for its potential benefits in the prevention of PCO (Zhang et al. 2007), this suggests that SFN deserves further investigation in combination with As_2O_3 in the treatment of PCO.

In conclusion, SFN is capable of killing lens epithellial cells by apoptosis. SFN is also capable of promoting ER stress, which may mediate SFN induced apoptosis. Application of SFN using a controlled druge delivery device at the time of surgery is a novel therapeutic approach for the prevention of posterior capsule opacification.

CHAPTER 6
GENERAL DISCUSSION

Several epidemiological studies have shown that consumption of large quantities of cruciferous vegetables such as broccoli and brussels sprouts, can protect against carcinogenesis, mutagenesis, drug toxicities, and some chronic diseases (Conaway et al. 2002). Much of this chemopreventive effect has been attributed to the physiological effect of the isothiocyanates, especially SFN. SFN was first identified as a potential chemopreventive agent in 1992 and since much research has focused on it. (Zhang et al. 1992), much research has focused on it. The ultimate chemopreventive effects of ITCs probably involve multiple mechanisms. However, a considerable portion of their effects is attributed to the enhanced detoxification of carcinogens (Phase II enzyme activation) as well as blocking carcinogen activation (Phase I enzyme inhibition) (Zhang 2012). On the other hand, SFN could also play a cytostatic role (Zhang 2012). Recent studies showed that mechanisms include the activation of apoptosis and inhibition of cell growth by cell cycle arrest. SFN induced apoptotic cell death can occur via the caspase pathway (Kim et al. 2003, Yu et al. 1998) or the p53-dependent pathway (Huang et al., 1998). Furthermore, SFN can also inhibit the IKK-IκBα-NF-κB signaling pathway (Xu et al. 2005a, Xu et al. 2005b), and induce cell cycle arrest (Gamet-Payrastre et al. 2000b) and/or potentially induce cell death genes leading to apoptosis. Other possible mechanisms such as disruption of normal tubulin polymerization, inhibition of cytochrome P450 enzymes and anti-inflammatory effect may also lead to cytotoxicity (Zhang 2012). These protective and cytotoxic actions of SFN are concentration dependent and cell/tissue dependent. If managed appropriately SFN application can be used to prevent stress induced damage,

for example in the case of cataract, or if required initiate cell loss to eliminate unwanted cells as is the case in PCO.

Several studies have suggested that excessive exposure of the lens to high levels of oxygen was a risk factor for nuclear cataract and oxidative damage is strongly implicated in cataract formation (Giblin et al. 1995, Palmquist et al. 1984). The mechanism involves disruption of the redox system, membrane damage, proteolysis and protein aggregation. The current work supports this concept as application of hydrogen peroxide, acting as an oxidative stress inducer caused cell death of human lens epithelial cells. SFN (1 µM) was shown to confer full cytoprotection against H_2O_2-induced FHL 124 cell death. Protection was observed when cells were pre-treated with SFN for 24 hours, but no protective effects were shown without pre-treatment. It is therefore important to consider the mechanisms by which SFN provides this protection, and such mechanisms could arise from a number of different angles. Possibilities include a direct interaction with the oxidative stressor (H_2O_2), enhancing activity of anti-oxidant defence enzymes, inhibition of apoptotic signals or via modification of DNA repair systems. From the experimental data it is unlikely that SFN causes its effect by direct anti-oxidant action because when co-treated no protection was observed; to achieve this pre-treatment was required, and also SFN is unlikely to be a direct-acting antioxidant in our system, as the isothio-cyanate group can participate in oxidation or reduction reactions under physiological conditions (Barton and Ollis, 1979). The cytoprotective properties of SFN pre-treatment in FHL 124 cells suggested that SFN could induce changes in gene expression, leading to cytoprotective against H_2O_2. Changes at mRNA levels were investigated by an Illumina gene microarray in SFN-treated FHL 124 cells and all the 8 up-regulated

genes were known to be controlled by the transcription factor Nrf2 in the literature.

Nrf2 is a critical transcription factor mediating amplification of the mammalian defense system against environmental stresses. It has been reported that Nrf2 translocates into the nucleus and binds to the ARE in conjunction with small Maf proteins after activation by chemopreventive agents as well as other redox/oxidative stresses, leading to transcriptional activation of downstream genes encoding GSTs (Chanas et al., 2002; Hayes et al., 2000; Ishii et al., 2000; Nguyen et al., 2000), γ-GCS (Wild et al., 1999), HO-1 (Ishii et al., 2000; Alam et al., 1999), NQO1 (Lee et al., 2001a,b) and thioredoxin reductase (Kimet al., 2001). The function of Nrf 2 and its downstream proteins has been shown to be important for protection against oxidative stressors and chemical-induced cellular damage in liver (Enomoto et al., 2001; Chan et al., 2001) and lung (Chan and Kan, 1999), for prevention of cancer formation in the gastrointestinal tract (Ramos-Gomez et al., 2001; Fahey et al., 2002), as well as promotion of the wound-healing process (Braun et al., 2002).

NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) and Thioredoxin reductase 1(TXNRD1) classic phase II enzymes, are highly-inducible enzymes that are regulated by the Keap1/Nrf2/ARE pathway. They are important in detoxifying quinones and maintaining the cellular redox balance. Evidence for the importance of the antioxidant functions of TXNRD1 and NQO1in combating oxidative stress is provided by demonstrations that induction of TXNRD1 and NQO1 levels or their depletion (knockout, or knockdown) are associated with decreased and increased susceptibilities to oxidative stress, respectively (Matusheski and Jeffery 2001). One

common feature of these proteins is that they use NADPH as an electron donor. So, for efficient detoxification and maintenance of cellular redox status, it would be beneficial to up-regulate these proteins together with the appropriate reducing potential (NADPH) to support enzymatic reactions. Glucose-6-phosphate dehydrogenase (G6PD) enzyme can directly generate NADPH so G6PD is likely to facilitate the antioxidant activity of NQO1 by producing NADPH. Pirin (PIR, iron-binding nuclear protein) could active Nrf2 signalling has been implicated in small airway epithelium by comparing smokers and non-smokers (Hubner Mol Med 2006). Ferritin (FTL), an antioxidant, is known to possess an ARE, requires Nrf2 for basal expression (Kato and Niitsu 2002). In addition, proper management of free iron is also important for minimizing oxidative stress, and this can be best achieved by ferritin. Ferritin converts Fe^{2+} to Fe^{3+} (ferroxidase activity) and sequesters it, thereby preventing Fe^{2+} from participating in the Fenton reaction. Thus, up-regulation of HO-1 together with ferritin is a way to increase antioxidant potential while minimizing hydroxyl radical formation. In Nrf2 knockout animals, the induction of EPHX1 was suppressed indicating an important role of Nrf2 signalling in its regulation. OKL38, which encodes oxidative stress induced growth inhibitor 1, is reported to show induction following oxidative stress (Li et al. 2007). In cancer cells it is believed that, following DNA strand breaks, interacts with p53 and relocates to the mitochondrion to initiate cytochrome c release and apoptosis; OKL38 is therefore defined as a tumour suppressor (Hu et al. 2012). In our system, OKL38 is upregulated in response to SFN, but under these conditions cells continue to survive and grow. In addition, cytotoxicity/apoptosis does not differ from controls. Oxidative stress induced DNA damage is suppressed by SFN and perhaps without this cue, OKL38 does not interact with p53 and induce cell damage. The expression of OKL38 remains curious and

further inhibition studies will be required to elucidate its putative role in SFN protection in the lens.

As there is a strong implication that Nrf2 signalling is playing a role in SFN protection of lens cells. Future studies should explore the role of Nrf2. Such investigations should build on the current body of data to determine the kinetics of Nrf2 translocation. To achieve this, immunocytochemistry could again be employed, but other techniques such as GFP tagged Nrf2 or ARE reporter assays could be used to establish time and concentration dependent effects on Nrf2 distribution and activity. In addition, it will be important to test the hypotheis that Nrf2 is critical in SFN protection of the lens. SiRNA targeted against Nrf2 could be employed to disable Nrf2 signalling. In the absence or at reduced levels of Nrf2 the ability of SFN to protect lens cells can be assessed using the ApoToxGlo Triplex assay. The LDH assay could be used to measure this effect in whole pig lens cultures. If Nrf2 is critical for protection SFN will not demonstrate any benefit in the absence of Nrf2. Similarly, gene expression can be assessed in Nrf2 siRNA treated cells to determine if the genes upregulated on the gene array are indeed under Nrf2 regulation as predicted.

Lens epithelial cell function and survival are critical to prevent cataract formation. However, in the case of PCO an uncontrolled growth of cells over the posterior capsule of the lens occurs. The remaining lens epithelial cells after surgical intervention rapidly grow and proliferate across the posterior lens capsule. The LECs can also migrate over the anterior surface of the intraocular lens. Cells ultimately encroach on the visual axis and induce light scattering changes due to matrix

contraction and matrix deposition. One approach to address this problem is to kill the cell population that remains following surgery. As higher concentrations of SFN can induce cell death it seemed to be a reasonable candidate. SFN was found to induce cell death by apoptosis and to induce ER stress. Previous work has shown that ER stressors such as thapsigargin can reduce protein synthesis and proliferation (Thomas et al. 1999, Wang et al. 2005). Interestingly, thapsigargin does not lead to immediate cell death; it appears that the downregulation in protein synthesis prevents vital proteins being synthesized to replace those that are naturally broken down over time for example growth factor receptors (Wang et al. 2005). As a consequence survival signals are lost and cell death by apoptosis results. As SFN has been identified as an ER stressor it will be interesting to see if a similar mode of action is employed in lens cells. To test this FHL 124 cells exposed to SFN for 2 minutes could remain in culture for extended periods for example 24, 48 and 72 hours. If SFN acts in the same way as thapsigrgin the level of cell death at active concentrations will progressively increase. This has important implications to PCO as application of any drug is likely to occur at the time of surgery and thus if a 2 minutes exposure can have long-term consequences it would be a valuable tool in PCO prevention (Duncan et al. 2007).

In summary, at low concentrations SFN can yield protection to lens cells against oxidative stress and appears to achieve this, at least in part, through upregulation of the Nrf2 signalling pathway leading to increase expression of antioxidants within the cell. At high concentrations cell cycle arrest and apoptosis can be induced. Moreover, SFN also induces ER stress in lens cells that is also likely to initiate cell death by apoptosis. In conclusion, the present body of work has developed our understanding of SFN in the lens and its putative uses in the prevention of cataract and PCO

formation.

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APPENDIX I – RNA QUALITY CONTROL FOR GENE MICROARRAY EXPERIMENTS

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QC REPORT

Michael Wormstone Project ID: S016317 Date: 20/06/11

METHODOLOGY:

1st QC – Validation of total RNA

RNA LabChip® Analysis

RNA integrity will be assessed by running a small aliquot of samples on the Agilent Bioanalyzer 2100 using RNA 6000 Nano or Pico Assay. This analysis results in both gel-like image as well as electrophoretic data. Indications of RNA degradation are:

- decreasing ratio of ribosomal bands;
- additional peaks below the ribosomal bands;
- decrease in overall RNA signal;
- shift towards shorter fragments.

Genomic contamination can also be seen on these images, indications are:

- a 'hump' or a smear seen after the 28s ribosomal peak

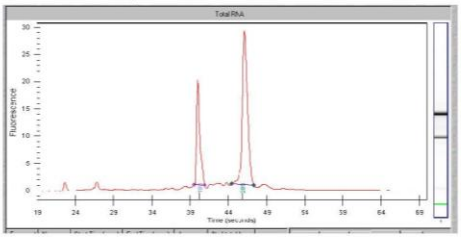

Figure 1: Intact total RNA as represented by two strong ribosomal peaks with a 28S/18S ratio close to 2.0.

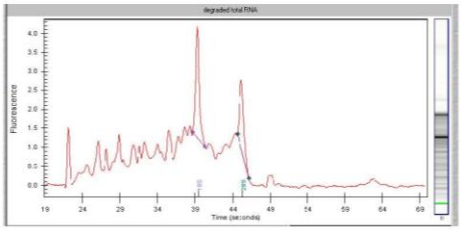

Figure 2: Degraded total RNA as represented by raised baseline.

The RNA integrity number (RIN) is a value provided by the Agilent Bioanalyser software to estimate the integrity of total RNA samples. This looks at the entire electrophoretic trace of the RNA sample including the presence or absence of degradation products. A RIN number of 10 indicates 'perfect' RNA, a RIN number of 5 partially degraded RNA and a RIN of 1 totally degraded RNA.

RESULTS:

Key:

green	passes quality control
orange	questionable quality – only to be continued with caution
red	fails quality control

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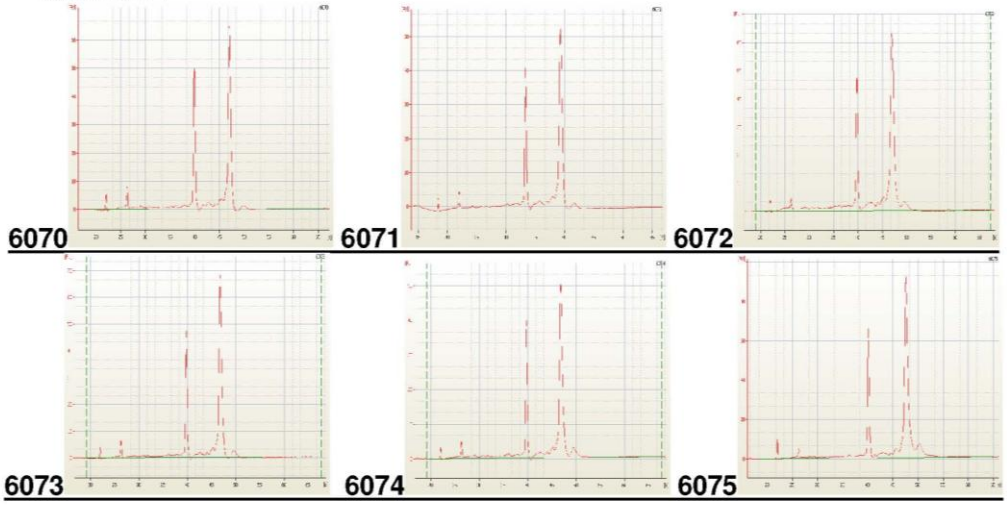
SAMPLE INFORMATION

Internal ID	Tube_ID
6070	A-0
6071	A-1
6072	A-2
6073	B-0
6074	B-1
6075	B-2
6076	C-0
6077	C-1
6078	C-2
6079	D-0
6080	D-1
6081	D-2

RESULTS:

Electropherograms - Agilent RNA pico labchip#

- **6070-6071**



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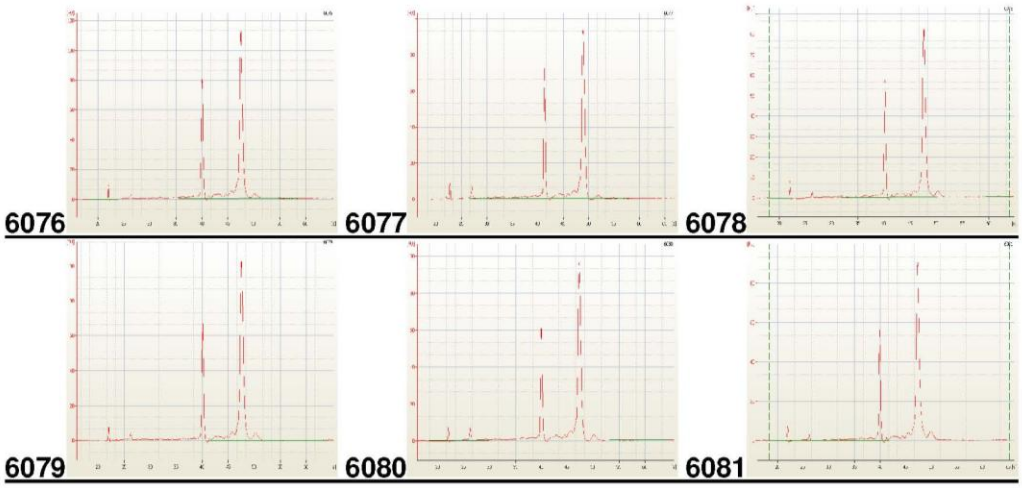

Result interpretation - Agilent RNA *pico* labchip

Internal ID	Tube ID	RIN Number	Pass / Fail / Questionable
6070	A-0	9.6	Pass
6071	A-1		Pass
6072	A-2	9.3	Pass
6073	B-0	9.7	Pass
6074	B-1	9.7	Pass
6075	B-2	10	Pass
6076	C-0	10	Pass
6077	C-1	9.7	Pass
6078	C-2	10	Pass
6079	D-0	10	Pass
6080	D-1	9.8	Pass
6081	D-2	10	Pass

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Result interpretation – Nanodrop

Internal ID	Tube ID	260/280	ng/µl	yield (ng)
6070	A-0	2.00	135.48	1490.28
6071	A-1	1.99	149.36	1642.96
6072	A-2	2.00	140.92	1550.12
6073	B-0	1.98	78.08	858.88
6074	B-1	1.99	86.40	950.4
6075	B-2	2.00	89.32	982.52
6076	C-0	1.98	80.88	889.68
6077	C-1	1.97	79.08	869.88
6078	C-2	1.93	74.20	816.2
6079	D-0	1.89	25.08	275.88
6080	D-1	1.96	49.72	546.92
6081	D-2	2.00	50.04	550.44

Results Summary

Internal ID	Sample ID	Agilent	Nanodrop
6070	A-0	Pass	Pass
6071	A-1	Pass	Pass
6072	A-2	Pass	Pass
6073	B-0	Pass	Pass
6074	B-1	Pass	Pass
6075	B-2	Pass	Pass
6076	C-0	Pass	Pass
6077	C-1	Pass	Pass
6078	C-2	Pass	Pass
6079	D-0	Pass	Pass
6080	D-1	Pass	Pass
6081	D-2	Pass	Pass