The chemopreventive potential of sulforaphane and erucin

by

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A thesis submitted to the University of Surrey in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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University of Surrey

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**Abstract**

Administration of erucin (3 and 15 mg/kg) and sulforaphane (15 mg/kg) to rats for 10 days enhanced hepatic and pulmonary quinone reductase activity; sulforaphane also stimulated hepatic glutathione S-transferase, when monitored using 4-chloro-7-nitrobenzofurazan. Immunologically, both compounds increased the expression of hepatic and pulmonary glutathione S-transferases class α and μ, but not η, proteins. Neither compound influenced activity of selected cytochromes P450, although CYP1A and CYP1B1 protein levels rose markedly in both tissues. The lack of concordance between activity and protein expression is due to the fact that isothiocyanates are mechanism-based inhibitors. A similar pattern of cytochrome P450 and Phase II enzyme modulation was observed when precision-cut rat liver and lung slices were incubated with either isothiocyanate. Both compounds elevated total glutathione, quinone reductase and glutathione S-transferase in rat liver and lung slices. In limited studies conducted with human slices, erucin and sulforaphane, in contrast to rat, were at best weak inducers of quinone reductase raising the possibility of species differences in the inducibility of this enzyme by isothiocyanates.

Both isothiocyanates antagonised the benzo(a)pyrene-mediated induction of CYP1A in rat liver slices, and this may contribute to their chemopreventive activity.

Urinary excretion of indirect-acting mutagens (unchanged 2-amino-3-methylimidazo-[4,5-f]quinoline) was significantly suppressed in rats pre-treated with isothiocyanates for 11 days prior to exposure to a single dose of 2-amino-3-methylimidazo-[4,5-f]quinoline, but hepatic CYP1A and glutathione S-transferase activities were unaffected, indicating that they are not responsible for the increase in the metabolism of this carcinogen.
HPLC-MS methods were developed and validated for determining low sulforaphane levels in rat and human plasma. In rats, sulforaphane was rapidly absorbed, achieved high bioavailability but its pharmacokinetic behaviour was dose-dependent. Sulforaphane was also rapidly absorbed following intake of broccoli by human volunteers, achieving peak plasma levels within 1.5 hours. Repeated intake of broccoli did not alter its pharmacokinetic behaviour.
Acknowledgements

I would like to express my deep gratitude to my supervisors, Prof Costas Ioannides, Prof Maurice Sauer, Dr Nick Coldham and Dr Nikolai Kuhnert for all their support, guidance, generously shared knowledge and expertise throughout my studies. I gratefully acknowledge the financial support of the Association for International Cancer Research.

I would like to give my thanks to Dr Adriana Gielbert and Mr Laurence Howells of the Veterinary Laboratory Agency, Weybridge, for for their time and advice on the HPLC and LC/MS aspects of this project. I would also like to thank Eva Soltys, Amaka Okpara and Veronica Krizova for their contributions to the project. I am very grateful to Dr Shelagh Hampton, Dr John Wright, Dr Peter Kwasovski, Lora Tripkovic, Julia Darzi and Nicky Muirhead for their help with cannulation, vena puncture and collection of blood during the pharmacokinetic study in human volunteers. My thanks also go to all my colleagues and members of the technical staff at the University of Surrey for their assistance throughout this project, who are too numerous to name individually.

I am very grateful to all my friends for their encouragement and kind words.

Finally, I would like to thank my family for their remarkable patience, understanding and support.
Abbreviations

ACF  colon abberant crypt foci
AhR  aryl hydrocarbon receptor
AITC allyl isothiocyanate
API activator protein 1
ARE antioxidant response element
AUC area under the curve
B(a)P benzo(a)pyrene
BITC benzyl isothiocyanate
7-BQ 7-benenzyloxyquinoline
CDK cyclin dependent kinase
Clp plasma clearance
Cmax maximum plasma concentration
CO2 carbon dioxide
CPS counts per second
D dose
DBA dibenz[a,h]anthracene
DCNB 1,2-dichloro-4-nitrobenzene
DMBA 7,12-dimethylbenz[a]anthracene
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DTC dithiocarbamate
EBSS Earle’s balanced salt solution
ECL enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
EITC ethyl isothiocyanate
EROD ethoxyresorufin O-dealkylase
Erucin 4-methylthiobutyl isothiocyanate
F bioavailability
GIT gastro-intestinal tract
G-6-P glucose-6-phosphate
GST glutathione S-transferase
GSH reduced glutathione
GSSG oxidised glutathione
SFN-GS glutathione conjugate of sulforaphane
HA heterocyclic amines
HCl hydrochloric acid
HDAC histone deacetylase
7-HQ 7-hydroxyquinoline
ITC isothiocyanate
IQ 2-amino-3-methylimidazo-[4,5-f]quinoline
Kab absorption rate constant
KCI potassium chloride
KCN potassium cyanide
kel elimination rate constant
Keap1 Kelch-like ECH-associated protein 1
LC/MS coupled liquid chromatography-mass spectroscopy
LC/MS/MS coupled liquid chromatography-tandem mass spectroscopy
LDH lactate dehydrogenase
MAPK mitogen activated protein kinase

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1.1 Role of Diet in Carcinogenesis

The appearance of a mutated cell, a process called initiation, is directly or indirectly linked to multiple factors implicated in carcinogenesis such as DNA damage following exposure to ionising or UV radiation or chemical carcinogens, insertion of a gene sequence capable of causing transformations following infection with certain types of DNA or RNA viruses (HIV, HPV etc.) and hereditary predisposition (e.g. familial retinoblastoma, lack of expression of certain classes of detoxifying enzymes and DNA repair enzymes). However, the above factors are only responsible for the minority of human cancers. Chemical agents present in tobacco smoke, occurring naturally as food contaminants or formed during cooking or food processing are implicated in DNA damage and the appearance of aberrant cells (Farber et al., 1982 and Wogan et al., 2004). The majority of cancers, in theory, can be prevented by appropriate control of pollution, working conditions and especially changes in life style (e.g. cessation of smoking and reduced alcohol consumption) and change of diet (Doll and Peto, 1981).

The importance of diet in the incidence and prevalence of cancer has been widely recognised. Epidemiological evidence supports the relationship of excessive fat and red meat consumption, overeating and high alcohol intake with increased risk of colon, breast, ovary and prostate cancer. Even the method of cooking has been shown to be an important factor in carcinogenesis associated with diet as, for example, in the case of humans exposed to heterocyclic amines (HA) and polycyclic aromatic hydrocarbons (PAH) through smoking and/or consumption of well cooked, fried or barbecued meat and fish, who exhibit an increased risk of lung, colon, breast and prostate cancers (Ikeda et al., 1983).

In contrast, high consumption of fresh fruit, vegetables and whole grains has been strongly associated with a decreased risk of certain cancers (Steinmetz and Potter,
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1991, 1996; Rogers et al., 1993) indicating that, as well as being a source of certain toxins and carcinogens, human diet contains substances that can effectively prevent chemical-initiated carcinogenesis. The chemopreventive effect of fruit and vegetables is attributed either to individual or combined action of fibre, phytochemicals and micronutrients.

Chemoprevention is the ability of a substance to prevent, delay or reverse cancer development at non-toxic doses (Sporn and Suh, 2000). As the western population is ageing and life expectancy increases, the high incidence of cancer is becoming a major problem. Ongoing efforts to identify therapeutically useful chemopreventative agents have led to the assessment of various plant-derived compounds that could be responsible for low incidence of cancer in individuals consuming large quantities of fruit and vegetables. A number of naturally occurring non-toxic substances present in the diet have some degree of anticarcinogenic activity, some of which are very potent and could be developed for use as human chemopreventive agents.

Many naturally occurring compounds are direct antioxidants that act as free radical scavengers, for example, vitamin C, β-carotene and plant-derived polyphenols such as ellagic acid and α-tocopherol. Amongst other potential chemopreventive agents are plant-derived compounds such as coumarin (Maucher and von Angerer, 1994), diallyl disulphide (Dwivedi et al., 1992), flavonoids (Guthrie et al., 1998; Miyagi et al., 2000) and chlorphyllin (Smith et al., 2001).

1.2 Isothiocyanates and Cancer Prevention.

Consumption of a single group of vegetables, cruciferous vegetables, especially belonging to the genus Brassica (broccoli, Brussels sprouts, green cabbage) has been linked to reduced cancer incidence in many tissues in humans (Block et al., 1992;
Greenwald et al., 2001; Seow et al., 2002; Steinmetz and Potter, 1991 and Steinmetz and Potter, 1996). These plants contain a variety of substances including, carotenoids, vitamins, folic acid, selenium, dietary fibre, coumarins, flavonoids, fibre, chlorophyllin and glucosinolates that may contribute to the anticarcinogenic activity; amongst these, the glucosinolates are the most abundant class of phytochemicals.

The aglycone moiety of the glucosinolates is a precursor of a respective isothiocyanate (ITC). The ITCs are a class of compounds with diverse structures (Fig. 1.1), which are thought to be almost solely responsible for the chemopreventive activity associated with high intake of these vegetables (Verhoeven et al., 1996). Much attention has been devoted to the potential chemopreventive activity of several ITCs including allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), erucin and sulforaphane (SFN), which are believed to boost the cell’s capacity to detoxify reactive electrophiles by induction of phase II enzyme activity and increased cellular GSH levels (Fahey and Talalay, 1999) as well as reduced bioactivation of carcinogens associated with inhibition of a number of P450 enzymes.

\[
\begin{align*}
\text{AITC} & : & \quad & \begin{array}{c}
\text{N} \equiv \text{C} \equiv \text{S}
\end{array} \\
\text{BITC} & : & \quad & \begin{array}{c}
\text{N} \equiv \text{C} \equiv \text{S} \\
\text{C}_6\text{H}_5
\end{array} \\
\text{PEITC} & : & \quad & \begin{array}{c}
\text{N} \equiv \text{C} \equiv \text{S} \\
\text{C}_6\text{H}_5
\end{array} \\
\text{SFN} & : & \quad & \begin{array}{c}
\text{O} \\
\text{S} \equiv \text{N} \equiv \text{C} \equiv \text{S}
\end{array} \\
\text{ERUCIN} & : & \quad & \begin{array}{c}
\text{S} \equiv \text{N} \equiv \text{C} \equiv \text{S}
\end{array}
\end{align*}
\]

Figure 1.1: Structures of the major ITCs. Abbreviations: AITC – allyl isothiocyanate; BITC – benzyl isothiocyanate; PEITC – phenethyl isothiocyanate; SFN – sulforaphane
Purified and chemically synthesised ITCs such as AITC, BITC, PEITC and SFN effectively inhibited bioactivation and DNA damage inflicted by experimental carcinogens (IARC, 2004). This activity was consistent with the cancer chemopreventive activity in various organs in animal models (Hecht, 1996a, 1999b; Hetch et al., 1996b, 1999a; Conaway et al., 2000; Talalay and Fahey, 2001).

1.2.1 Formation of ITCs

Isothiocyanates (Fig. 1.2) occur naturally in Brassica and cruciferous vegetables as glucosinolate precursors. More than 100 naturally occurring glucosinolates have been identified so far. Glucosinolates are polar, non-volatile and heat-stable compounds that have no or little biological activity in the absence of exogenous myrosinase. In intact plants, the glucosinolates are physically segregated from the plant enzyme myrosinase, but are brought into contact on disruption of plant tissue during harvesting, chewing or food processing. Following cleavage of the glucose and sulphate, the unstable aglycone intermediates undergo spontaneous intramolecular rearrangements to form, depending on reaction conditions and type of myrosinase, a variety of degradation products including lipophilic, volatile and biologically active ITCs (Hecht, 2000 and Fahey et al., 2001).

![Figure 1.2: Formation of ITCs from their glucosinolate precursors. Adapted from Hecht (2000)](image)
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1.2.2 Chemopreventive activity of isothiocyanates

It is generally accepted that cancers induced by genotoxic chemicals begin with initiation, a process characterised by a DNA damage which remains unrepairedor misrepaired. Normally, most of such initiated cells are eliminated by apoptosis or by the cells of the immune system. Avoidance of apoptosis gives such initiated cells a growth advantage over the normal cells, which, as a result of clonal expansion, form foci of altered cells, a process called promotion. As the DNA of tumour cells is unstable and prone to further mutations, total loss of control over their survival, growth and proliferation will eventually result in malignancy.

The mechanism of anticarcinogenic activity of ITCs is multifactorial. It is believed that ITCs block emergence of carcinogen-induced aberrant cells as a result of their ability to modulate metabolism of carcinogenic compounds in favour of detoxification and excretion. Moreover, ITCs suppress tumour promotion and progression, operating mainly through a concerted modulation of multiple cellular signalling pathways involved in the regulation of apoptosis, cell growth, proliferation, differentiation and inflammation (Hecht et al., 2000; Myzak and Dashwood, 2006; Fimognari and Hrelia, 2007 and Juge et al., 2007).

1.2.3 ITC: Inhibition of tumour initiation

It is hypothesised that inhibition of cytochrome P450 and induction of phase II enzymes is one of the major mechanisms of tumour prevention by ITCs at the initiation stage (Thornalley, 2002; Hecht 1999). The notion that ITCs alter the balance between Phase I/Phase II metabolism to favour carcinogen deactivation has been strongly supported by experimental evidence. PEITC, consumed as a serving of watercress, enhanced urinary excretion of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in smokers (Hecht et al., 1999a). Cruciferous vegetables administered simultaneously with 2-amino-3-
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methylimidazol[4,5-f] quinoline (IQ) inhibited formation of colonic and hepatic preneoplastic lesions in the F344 rats (Knasmuller, 2003). ITCs such as BITC and PEITC administered to rodents prior to, or around the time of, treatment with high doses of carcinogens, such as azoxymethane, NNK, dibenz[a,h]anthracene (DBA), benzo(a)pyrene (B(a)P), 5-methylchrysene (5-MeC) and N-nitrosomethylbenzylamine (NMBA), have been shown to effectively inhibit tumorigenesis at different organ sites (Morse et al., 1991; Jiao et al., 1994; Chung et al., 1996; Hecht, 1999a and Hecht et al., 2002).

The chemopreventive activity of ITC appears to be tissue-specific, possibly reflecting the tissue-specific distribution and relative abundance of metabolizing enzymes. BITC administered to A/J mice concurrently with 5-MeC, DBA and B(a)P, potent animal lung carcinogens present in tobacco smoke, significantly reduced the incidence and multiplicity of the lung neoplastic lesions, while exerting no effect on stomach tumours in all PAH-treated groups (Wattenberg, 1987).

1.2.3.1 Modulation of Phase I enzyme activities by ITC

The majority of dietary, environmental and occupational carcinogens such as PAH, polycyclic aromatic amines (PAA), heterocyclic amines (HA) and nitrosamines are indirect acting carcinogens that require metabolic transformation by phase I enzymes to ultimate carcinogens, a process referred to as bioactivation. Such reactive intermediates are electrophilic in nature and covalently bind to critical cellular macromolecules such as DNA and proteins. Although the majority of DNA adducts are effectively removed by the cell defence systems, some can be misrepaired and, if this occurs at critical sites of critical genes such as pro-oncogenes and/or tumour-suppressing genes, can give rise to initiated cells.
Inhibition of Phase I enzymes and consequent interruption of bioactivation pathway(s) of parent carcinogens by phytochemicals such as ITCs is associated with decreased DNA adduct formation, mutation frequency, and, ultimately, inhibition or retardation of tumorigenesis. It is feasible to assess the effects of ITC on the extent of DNA/protein adduct formation both \textit{in vitro} and \textit{in vivo} and this can be used as a biomarker of the carcinogen bioactivation/deactivation and, ultimately, of ITC effects on carcinogen metabolism. Pre-treatment with PEITC decreased the unscheduled DNA synthesis in mucosal fragments of hamsters following exposure to NMBA (Soit et al., 2003) and formation of B(a)P-induced DNA and protein adducts in A/J mice (Sticha et al., 2002). Similarly, PEITC reduced the levels of PhlP-DNA and PhlP-protein adducts (Dingley et al., 2003). Exposure to high levels of carcinogen(s) may overwhelm the cellular defence systems (Knasmuller et al., 1996). A study utilising dietary relevant doses of SFN and PhlP provided strong evidence that both ITC concentrations and levels of carcinogen affect the chemopreventive efficacy of ITCs. The highest degree of protection against PhlP-mediated DNA damage afforded by SFN was observed at the lowest concentrations of carcinogen (Bacon et al., 2003), highlighting the importance of assessment of the chemopreventive potential of ITC at dietary relevant levels of carcinogens and ITC.

Alternatively, a direct assessment of enzyme level/activity can be used as an indicator of chemopreventive potential of ITC. Individual ITCs vary considerably in their potency and selectivity as CYP inhibitors (Jiao et al., 1996; Smith et al., 1993; Smith et al., 1996). It was suggested that the length of aliphatic carbon chain might influence the potency of cytochrome P450 inhibition (Hamilton et al., 1994). The data supporting this theory indicated that the potency of arylalkyl isothiocyanates to inhibit rat CYP1A1/2 and CYP2B1 increases if the length of the aliphatic carbon chain was increased up to C6 (Conaway et al., 1996). In contrast, BITC, which has a very short alkyl chain, showed a potent mechanism-based inhibition of rodent CYP1A1/2, CYP2B1, CYP2E1 and CYP3A2 as
well as human CYP2B6 and CYP2D6 (Goosen et al., 2001; Canistro et al., 2004) and rabbit CYP2E1 (Moreno et al., 1999). While many ITCs were effective inhibitors of most cytochrome P450 enzymes in vitro (Conaway et al., 1996; Goosen et al., 2001; Moreno et al., 1999 and Canistro et al., 2004), the in vivo effects are inconsistent. ITCs have been shown to either induce or inhibit P450 content and activity, depending on the specific ITC studied, experimental conditions, treatment protocol and tissue examined. For example, a short-term administration of PEITC to rodents resulted in inhibition of CYP2E1 and marked induction of CYP2B1 (Ishizaki et al., 1990). In contrast, hepatic and pulmonary CYP2E1 activity was elevated after chronic dietary administration of PEITC to mice (Smith et al., 1993). Dietary Brussels sprouts reduced IQ-initiated colon and liver aberrant foci in rats despite elevated hepatic CYP1A2 activity (Kassie et al., 2003a). CYP1A2 activity was also elevated in humans after consumption of Brassica vegetables (Lampe et al., 2000). Both P450 inhibition and induction were observed in rats fed broccoli samples from different cultivars and growth conditions indicating complex interactions of the individual ITC (Vang et al., 2001b).

The cytochrome P450 enzymes have remarkable substrate specificity. Suppression of bioactivation can be highly specific and depends on the structures of both the ITC and the carcinogen, reflecting ITC-induced disruption of the bioactivation pathway specific to the carcinogen in question. For example, BITC was shown to be a highly effective inhibitor of B(a)P-initiated lung tumorigenesis, while PEITC effectively suppressed formation of NNK-induced neoplasm lesions (Conaway et al., 2000; Hecht, 2000; Hecht et al., 2002) and inhibited human CYP1A2-mediated oxidative metabolism of NNK (Smith et al., 1996). Although intake of a mixture of ITCs theoretically may have a synergistic effect or/and protect against a wider range of carcinogens, a mixture of dietary BITC and PEITC (1 and 3 μmol/g diet), failed to reduce the level of B(a)P-
induced DNA and protein adducts, although it inhibited the formation of NNK-induced adducts (Boysen et al., 2003).

1.2.3.1.1 Molecular mechanisms of cytochrome P450 inhibition by ITC

Both mechanism-based and competitive inhibition have been described as mechanisms of Cytochrome P450 enzymes inactivation. PEITC competitively inhibited NNK bioactivation by mouse liver microsomes, but in vivo PEITC seems to act as a mechanism-based inhibitor of NNK metabolism (Smith et al., 1993). Mechanism-based inactivation is defined as loss of catalytic activity of the P450 enzyme as a result of irreversible adducts formation following bioactivation of the enzyme's substrate to a reactive intermediate. The mechanism-based inhibitors are selective for specific P450 enzymes and, unlike competitive inhibitors, do not readily dissociate. Benzyl isothiocyanate (BITC), for example, was a mechanism-based inactivator of rabbit CYP2E1 (Moreno et al., 1999), rat CYP1A1, CYP1A2 and CYP2B1 as well as human CYP2B6 and CYP2D6 (Goosen et al., 2001). BITC is metabolised to a reactive benzyl cyanate and benzylamine that covalently bind to and inactivate the enzyme. The covalent binding prevents further substrate binding and is thought to occur at the active site of the apoprotein (Goosen et al., 2001; Moreno et al., 1999). The inactivation of microsomal P450s caused by covalent modification or destruction of the haem moiety has not been observed experimentally (Goosen et al., 2001). Other enzymes, such as human CYP1A2 and CYP2C9, appear to be inhibited by PEITC competitively, whereas human CYP3A4 was inhibited both competitively and non-competitively (Nakajima et al., 2001).

1.2.3.1.2 Effect of thiol conjugates of ITC on cytochrome P450

Thiol conjugates of ITCs have also been shown to inhibit CYP enzymes, although they are less potent inhibitors than their parent compounds. Thiol conjugates of ITCs inhibited
CYP2E1, CYP1A1/2 and CYP2B1 (Jiao et al., 1996; Conaway et al., 1996). The inhibitory potency of individual thiol conjugates in aqueous solutions is determined by the rate of their deconjugation to release the parent ITC. Inhibition of CYP1A and CYP2B by aqueous dithiocarbamates (DTC) was greater with increased pre-incubation time and positively correlated with the extent of decomposition of the ITC conjugates, indicating that both the structure of parent ITC and the rate of the conjugate dissociation influence enzyme inhibition. However, in the presence of physiological concentrations of GSH, deconjugation of DTC is expected only to be marginal. If the rates of dissociation of thiol-ITCs in plasma and tissues are slow, as anticipated, then this would suggest that ITC-induced inhibition of P450 enzymes is unlikely to be a principal mechanism of their chemopreventive activity (Conaway, 2001).

1.2.3.2 ITC: effect of phase II metabolism

Reactive metabolites and ultimate carcinogens, generated as a result of bioactivation by P450s, may be detoxified by conjugation with glucuronic acid, GSH and sulphate, catalysed by phase II enzymes. The biological purpose of conjugation is to increase polarity and excretion of highly lipophilic compounds such as most carcinogens. The relative abundance of Phase I and II enzymes is critical and carefully regulated. ITCs such as PEITC have been shown to shift this balance in favour of conjugating enzymes.

1.2.3.2.1 Modulation of phase II enzymes in animals by ITCs

Feeding Brussels sprouts to rodents resulted in upregulation of hepatic UGT and decreased IQ-induced damage to hepatocytes (Humblot et al., 2004). PEITC induced activity/levels of GST, QR and UGT in the rodent liver, lung and gastric mucosa (Van Lieshout et al., 1998a, 1998b; Guo et al., 1992 and Kassle et al., 2003a). AITC significantly induced QR and GST in rat urinary bladder (Munday and Munday, 2002). The cysteine conjugates of BITC and PPITC similarly induced GST in many tissues of
female A/J mice (Zheng et al., 1992), indicating that certain ITC metabolites retain their bioactivity.

The induction of phase II enzymes by ITC appears to be tissue-specific. For example, AITC significantly induced QR and GST in rat urinary bladder. Moreover, while induction of QR reached a plateau after 2 weeks of treatment with AITC, the activity of bladder GST continued rising even after 21 days, suggesting that duration of treatment also influences the extent of Phase II enzyme induction (Munday and Munday, 2002). A single oral administration of PEITC to rats (1 mmol/kg bw) resulted in significant elevation of the liver QR (5-fold) and GST activities (1.5-fold) whereas activities of these enzymes were not significantly affected in the lung (Guo et al., 1992). GSTT1 was significantly elevated in gastric mucosa but not in the esophagus, colon, or liver of male W/A rats after dietary administration of PEITC (van Lleshout et al., 1998a; van Lleshout et al., 1998b).

1.2.3.2 Phase II enzyme modulation in humans by ITC

Dietary consumption of vegetables rich in glucosinolates by humans has also been associated with phase II enzyme induction, modulation of metabolism and, ultimately, excretion of carcinogens and hence decreased risk of cancer. Consumption of two ounces of watercress, which contains gluconasturtiil, the glucosinolate precursor of PEITC, increased the urinary excretion of NNAL and its glucuronide, the metabolites of the tobacco-specific nitrosamine NNK in smokers (Hecht et al., 1995). A recent epidemiological study in Chinese men has found a link between concentration of ITC metabolites in urine, GSTM1/T1 polymorphism and incidence of lung cancer. The GSTM1/T1-null smokers that had detectable levels of ITC metabolites in their urine were at the lowest risk of lung cancer, while GSTM1/T1-null smokers who had no detectable urinary ITC metabolites were at the greatest risk. This is the first epidemiological study
that established a direct link between ITCs and reduced risk of lung and colon cancer. Interestingly, individuals with the wild type of GSTM1/T1 genes had no appreciable protection from the consumption of Brassica vegetables. It has been hypothesised that the potent chemopreventive activity of ITCs observed in GSTM1/T1-deficient individuals was due to decreased excretion of ITCs so that their tissue concentrations would be considerably increased, and/or elevated global GST activity that might be sufficient to compensate for the function of missing enzymes encoded by silenced genes (Seow et al, 2002). Moreover, ITC-induced increase in plasma GSTA and peripheral lymphocyte GSTM was more pronounced in female than in male subjects (Thornalley, 2002). The major limitation of the above studies is lack of differentiation between individual glucosinolates and ITCs, because the cyclocondensation method often used to estimate ITC content does not allow differentiating between individual ITCs. The biochemical effects observed in human volunteers after consumption of Brassica vegetables are most likely to be a net result of bioactivity of the complex mixture of all plant glucosinolates. In other words, no individual glucosinolate can account for the cancer chemopreventive activity observed after consumption of Brassica vegetables (Vang et al., 2001a). For example, watercress contains at least three phase II enzyme inducers, two of which, 7-methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates, are 10-25 times more potent than PEITC (Rose et al., 2000).

1.2.3.2.3 Mechanism of phase II induction by ITC

*In vitro* studies have shown significant differences in the phase II enzyme inducing potential of individual ITCs which appears to correlate strongly with the intracellular accumulation of the ITCs. In turn, the extent of the intracellular accumulation of the ITC appears to be dependant on the ITC structure. The length of the side chain is of little importance while the oxidation state has a pronounced effect on the inducer activity as can be illustrated by comparing the inducing potency of erucin and SFN, the latter being
more than twice as potent as erucin at inducing OR and GST activities in the Murine Hepatoma Cells (Zhang et al., 1992).

ITCs are highly reactive towards GSH. Among ITCs, erucin and SFN have the highest rate of spontaneous, non-enzymatic reaction with GSH (Kolm et al., 1995). The intracellular accumulation of ITCs is accompanied by a rapid decline in the GSH levels and brings about oxidative stress. Experimentally induced depletion of GST in Hepa cells prior to treatment with ITCs enhanced their QR and GST inducing potency (Zhang and Talalay, 1998). Moreover, low lipophilicity and high reactivity towards GSH have been shown to increase inhibitory activity of ITC toward NNK-initiated lung tumorigenesis (Jiao et al., 1994), supporting the central role of GSH depletion and oxidative stress in induction of phase II enzymes by ITCs. Being highly reactive towards cellular GSH, the ITCs rapidly accumulate mostly as GSH conjugates, also known as dithiocarbamates (DTCs), and induce depletion of cellular GSH following transporter-mediated expulsion of DTCs out of the cell (Zhang, 2000). ITCs were characterised as monofunctional inducers that selectively upregulate Phase II enzymes without concurrent induction of Phase I enzymes (Miao et al., 2004). The 5'-flanking regulatory region of genes encoding phase II enzymes such as OR and GST contain Antioxidant Response Element (ARE), a binding site for the nuclear transcriptional factor Nrf2 (erythroid p45-related factor-2) activated by ITCs (Thornalley, 2002).

Figure 1.3: Activation by isothiocyanates of Nrf2 pathway regulating expression genes encoding phase II enzymes
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The Nrf2 normally resides in the cytosol bound to Kelch-like ECH-associated protein 1 (Keap1). In response to oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to ARE sequence in the upstream promoter of phase II genes (Fig. 1.3). The molecular mechanism of Nrf2-Keap1 complex dissociation in response to ITC-induced oxidative stress is not fully understood (Thornalley, 2002).

1.2.4 ITC-associated inhibition of tumour progression

In addition to the altered ratio of phase I/phase II enzymes that suppresses carcinogenesis at the initiation phase, ITCs inhibit experimentally induced tumorigenesis at the post-initiation and promotion phases. Dietary BITC and PEITC induced apoptosis in the lungs of rats following exposure to cigarette smoke for 28 consecutive days (D’Agostini et al., 2001). Furthermore, orally administered NAC conjugates of BITC and PEITC also decreased a multiplicity of (B(a)P)-initiated lung adenomas in A/J mice when administered using a post-initiation protocol. The inhibition of tumorigenesis was attributed to the induction of apoptosis (Yang et al., 2002). ITC-induced apoptosis and inhibition of cell proliferation have been reported in a number of studies (Huang et al., 1998; Chen et al., 1998; Yu et al., 1998; Gamet-Payrastre et al., 2000).

1.2.4.1 Pro-apoptotic activity of ITCs

Pro-apoptotic activity of ITC is another important aspect of the chemopreventive potential of ITCs as the majority of human cancers are resistant to apoptosis. There is some evidence that ITCs preferentially induce apoptosis in cells with malignant genotypes. For example, PEITC and AITC-induced apoptosis and growth inhibition was >10-fold greater in human leukaemia (HL60) cells compared with normal cells (Xu and Thornalley, 2000; Xu and Thornalley, 2001a).
Commitment of cells to apoptosis and inhibition of progression through the cell cycle appear to involve ITC-induced protein modification as a result of GSH depletion and oxidative stress (Xu and Thornalley, 2000; Payen et al., 2001). It has been suggested that ITC-associated activation of caspases and mitogen activated protein kinase (MAPK) family members occurs as a result of thiol modification and subsequent activation of protein tyrosine kinase and/or inhibition of protein tyrosine phosphatase activity and, ultimately, increased overall protein tyrosine phosphorylation (Xu and Thornalley, 2001b). Additionally, ITCs may alter expression of genes associated with apoptosis and regulation of cell progression through the cell cycle through change in the activity of stress-response transcription factors such as (activator protein) AP-1 and NF-κB, the downstream targets of MAPK, as well as transcription and/or activity of p53 protein (Yang et al., 2002 and Juge et al., 2007).

ITC-mediated apoptosis and inhibition of cell cycle progression associated with ITCs appears to involve induction of oxidative stress caused by GSH depletion following expulsion of the ITC-GS conjugates through the MRP protein channel (Xu and Thornalley, 2000; Payen et al., 2001). PEITC-induced apoptosis correlated with initial depletion of intracellular GSH and accumulation of the GSSG and GSH conjugates of PEITC, and was prevented by high concentrations of GSH (Xu and Thornalley, 2000; Xu and Thornalley, 2001a). The data on inhibitory activity of thiol-containing antioxidant N-acetyl cysteine on pro-apoptotic effects of ITCs are discordant: some studies reported inhibition (Chen et al., 1998), whereas others found that exposure to high concentrations of N-acetyl cysteine had no effect on the activation of MAPK pathway(s) (Xu and Thornalley, 2001b).

The mechanism of apoptosis induced by ITCs is not fully elucidated. ITCs have been shown to induce apoptosis by several distinct pathways, possibly activating more than
one pathway simultaneously. The MAPK pathway was one of the first pathways found to be activated by ITCs. Alternatively, ITC can induce apoptosis by activating the extrinsic or "death receptor" (DR) pathway or through activation of the mitochondrial pathway, involving release of the mitochondrial cytochrome C into the cytosol, and P53 pathway (Juge et al., 2006 and Fimognary and Hrelia, 2007).

1.2.4.2 Cell cycle arrest
Inhibition of progression through the cell cycle is associated with reduction in DNA synthesis and apoptosis. Progression through the cell cycle requires the presence of specific complexes of cyclin-dependent kinases (CDK) and their cyclins formed at specific stages of the cell cycle. The inhibition and activation of cyclins and CDK and, ultimately, the formation of the complexes are controlled by phosphorylation. ITCs induce cell cycle arrest that is characterised by the accumulation of cells at different stages of the cell cycle and sensitisation of cells to apoptosis, possibly as a result of ITC-induced suppression of transcription and/or function of cyclin-dependent kinases and up-regulated transcription of cyclins. The effects of ITCs on cell growth and cell cycle progression were initially studied in HeLa cells. The accumulation of cells in the G<sub>2</sub>/M phase interface and inhibition of cell growth was observed after treatment with AITC, BITC, or PEITC. These results suggest that ITC inhibit progression of cells through the cell cycle, inhibiting cell growth and predisposing cells to apoptosis (Hasegawa et al., 1993; Tang and Zhang, 2004).

1.2.5 ITC-induced oxidative stress
Oxidative stress is a condition arising in a cell from an imbalance between generation of free radicals and reactive oxygen species (ROS) and the ability of the cell antioxidant system to render them harmless. Free radicals and ROS are continuously produced as unavoidable by-products of cellular life processes such as substrate oxidation by P450
enzymes, mitochondria and peroxisomes. Mild and transient oxidative stress induced by low, dietary relevant doses of ITCs has been shown to alter cellular signalling pathways and transcription of genes encoding Phase II response proteins (conjugating, antioxidant and cellular thiol-synthesising enzymes). Such transient alterations in cellular homeostasis induced by ITCs at non-toxic levels boost cell antioxidant capacity and, consequently, diminish damage of tissues by chemical carcinogens and ROS (Fahey and Talalay, 1999 and Murata et al., 2000).

In contrast to the beneficial effects of mild changes in the cellular redox status in response to non-toxic concentrations of ITCs (in the lower μM range), toxic levels of ITCs are likely to result in excessive depletion of cellular GSH leading to extensive oxidative stress that exceeds cell antioxidant defences and leads to mutagenicity, induction of apoptosis and frank necrosis (Musk and Johnson, 1993). Treatment of cells with low, dietary achievable concentrations of ITCs induces only transient depletion of GSH levels followed by rebound elevation after 24h of exposure. While this is true for low concentrations of ITCs, exposure to high concentrations (50-500 μM) resulted in profound depletion of GSH levels and cytotoxicity (Zhang, 2000).

1.3 SFN as a Potential Chemopreventive Agent

SFN (Fig. 1.5) was first isolated from broccoli and identified as a potential chemopreventive agent in 1992 (Zhang et al., 1992). Glucoraphanin, the glucosinolate precursor of SFN, is the most abundant phytochemical found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage, and other cruciferous vegetables (Carlson et al., 1981; Zhang et al. 1992). A combination of factors such as prevalence of SFN in the Western diet, low toxicity and potent chemopreventive activity render SFN the most important ITC in relation to the human diet. Epidemiological data reveal an inverse relationship between high intake of cruciferous vegetables and risk of lung (Spitz et al.,
2000 and Zhao et al., 2001), colorectal, breast (Ambrosone et al., 2004), bladder (Zhao et al., 2007) and prostate cancer (Joseph et al., 2004). The experimental evidence supports a direct causal relationship between administration of SFN and suppression of chemically induced tumour development in animal models. SFN inhibited incidence and growth of mammary and colon tumours in rodents (Zhang et al., 1994; Chung et al., 2000, Shen et al., 2007). Treatment of rodents with SFN resulted in suppression of development of B(a)P-initiated forestomach (Fahey et al., 2002) and lung tumours (Hecht et al., 2002). SFN also suppressed formation of intestinal polyps in ApcMin/+ mice (Hu et al., 2006) and azoxymethane-induced ACF in mouse colon (Chung et al., 2000). Similarly, SFN protected against development of DMBA-induced mammary tumours (Zhang et al., 1994). Even topical application of SFN antagonised MBA-induced (Xu et al., 2006) and UV-light induced (Dinkova-Costova, 2006) skin tumourigenesis in rats.

Similarly to the other ITCs with chemopreventive activity, SFN evokes pleiotropic biological responses that reflect the chemopreventive activity of this phytochemical at different stages of carcinogenesis (Fig. 1.4). The mechanism responsible for the chemopreventive effects of SFN at the initiation stage appear to involve suppression of the bioactivation of carcinogens coupled with enhanced detoxification and elimination. At the promotion stage, SFN exerts chemoprotective activity through induction of apoptosis, cell cycle arrest (Juge et al., 2006 and Fimoganari and Hrelia, 2006) and permanent differentiation of tumour cells (Lee et al., 1999). Additional mechanisms include inhibition of histone deacetylase (HDAC) (Myzak et al., 2004, Myzak et al., 2006a, 2006b), anti-inflammatory activity (Wu et al., 2004, Ritz et al., 2006 and Talalay et al., 2007), antibacterial activity (Fahey et al., 2002) and upregulation of multidrug resistance-associated proteins (Payen et al., 2001). Finally, recent studies suggest that SFN also retards tumour progression as a result of inhibition of angiogenesis (Berti et
al., 2006 and Jackson et al., 2007) and suppression of activation of matrix metalloproteinases (Thejass and Kuttan, 2006).

![Diagram showing multiple targets for cancer chemoprevention for SFN](image)

**Figure 1.4: SFN: multiple targets for cancer chemoprevention**

### 1.3.1 Modulation of cytochrome P450 expression by SFN

Selective inhibition of specific P450s coupled with concomitant induction of Phase II enzymes is thought to be one of the most important mechanisms of the chemopreventive activity of SFN during the initiation stage of tumourigenesis. Several *in vitro* studies have shown selective inhibition of specific cytochrome P450 enzymes. SFN inhibited activities of CYP1A1/2 and CYP2B1/2 enzymes in rat hepatocytes and CYP3A4 in human hepatocytes (Maheo et al., 1997, Zhou et al., 2007). The activity of CYP1A2 and CYP3A4 enzymes was also inhibited by SFN in parenchymal liver cells (Jiao et al., 1996). Unlike BITC, which is a potent mechanism-based inactivator of CYP2E1, SFN was a weak competitive inhibitor of this enzyme (Barcelo et al., 1996). In human liver cells, SFN at low concentrations reflecting human intake inhibited CYP1A2 and CYP2E1, the major catalysts of bioactivation of IQ and NDMA respectively, as exemplified by inhibition of DNA damage (Barcelo et al., 1998). In a different study, however, even relatively high concentrations of SFN (40 μM) failed to alter the activity of human cytochrome P450s (Langouet et al., 2000).
SFN appears to be a rather weak inhibitor of cytochrome P450 enzymes. A 50% reduction in activity of rat CYP2E1 was observed at SFN concentration of 68 µM (Barcelo et al., 1996). At lower concentrations, (up to 40 µM), SFN had no effect on activity of either of the five major human cytochrome P450 studied, including CYP1A1, CYP1A2, CYP1B1, CYP2E1 and CYP3A4 (Langouet et al., 2000). At plasma levels achieved by dietary intake (2-7 µM), SFN failed to modulate expression of genes encoding for Phase I enzymes in human gastric mucosa (Gasper et al., 2007).

Contrary to the notion that SFN is a monofunctional inducer that selectively induces expression of Phase II enzymes without concurrent induction of Phase I enzymes (Zhang et al., 1992 and Miao et al., 2004), several cytochrome P450 enzymes were shown to be induced by SFN and its glucosinolate precursor glucoraphanin, suggesting the possibility of a cross-talk between the antioxidant response element (ARE) and xenobiotic response element (XRE) pathways. SFN induced CYP1A2 apoprotein levels (Yoxall et al., 2005) and up-regulated expression of genes encoding CYP1A, CYP2B and CYP3A in rat livers (Hu et al., 2004). Both, single and repeated oral administration of glucoraphanin to rats also resulted in upregulation of activity and apoprotein levels of hepatic CYP1A1/2, CYP3A2 and CYP2E1 (Perocco et al., 2006) and pulmonary CYP1A1/2, CYP2B1/2, CYP3A1/2 and CYP2C11 (Paolini et al., 2004). Collectively, these data suggest that inhibition of cytochrome P450 enzymes by dietary SFN plays, at best, a minor role in the chemoprevention afforded by this phytochemical.

1.3.2 Effect of SFN on Phase II enzymes

Phase II enzyme inducer activity appears to correlate well with the anticarcinogenic potential of ITCs (Talalay and Fahey, 2001) of which sulforaphane [(−)-1-isothiocyanate-4-(methylsulfinyl)butane] is the most potent inducer identified so far (Zhang et al., 1992; Zhang and Talalay 1998; Brooks et al., 2001 and Zhang and Callaway, 2002).
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SFN suppressed development of B(a)P-induced forestomach tumours in ICR mice with functional nrf2 gene, the product of which regulates transcription of phase II enzymes. Anticarcinogenic activity correlated with Phase II enzyme induction and was abrogated in mice lacking the Nrf2 gene (Fahey et al., 2002). Another study showed that Nrf2-null mice had a higher burden of B(a)P-induced tumours and lower basal GST activity, underscoring the role of inducible phase II enzymes in ITC-mediated chemopreventive effects (Ramos-Gomez et al., 2001).

QR protects the cell from quinone-semiquinone redox cycling and generation of ROS through obligatory two-electron reduction of exo- and endogenous quinones. QR is induced coordinately with other ARE-regulated phase II and anti-oxidant enzymes and may be used as a biomarker for global phase II enzyme induction (Jeong et al., 2006).

SFN is a potent inducer of phase II enzymes, such as QR, UGT and GSTs in primary rat and human liver hepatocytes, various cell lines and in a number of organs including skin, liver, lung, duodenum, forestomach, stomach, small intestine and urinary bladder following administration to animal models (Zhang et al., 1992; Zhang et al., 1994; Gerhauser et al., 1997; Fahey et al., 1997; Gao and Talalay, 2004; Maheo et al., 1997; Rose et al., 2000; Brooks et al., 2001; Misiewicz et al., 2004; Munday and Munday, 2004; Ritz et al., 2006 and Dinkova-Kostova et al., 2007) as well as in the skin of human volunteers (Xu et al., 2006 and Dinkova-Kostova et al., 2007).

Although after a single administration ITCs are rapidly conjugated and excreted almost completely during the initial 24h, their biological effects are prolonged because induced enzymes have a half-life of several days and can cope with a wide range of toxins. Significant elevation of QR activities in human adult retinal pigment epithelial cells, ARPE-19 cells, human HaCaT keratinocytes and L1210 mouse leukemia cells treated for
24 h with SFN was associated with markedly reduced cytotoxicity of the oxidative stressors (menadione, tert-butyl hydroperoxide, 4-hydroxynonenal, and peroxynitrite). The protection lasted several days after SFN removal until GSH levels and QR activity returned to control levels (Gao et al., 2001).

The induction of specific classes of GST appears to be tissue-specific. The SFN analogue compound-30 potently elevated levels of rodent GST\(\theta\) in the stomach and GSTA in the small intestine and liver while GSTM and GSTP were induced in the stomach and small intestine (van Lieshout et al., 1998a; van Lieshout et al., 1998b). The potency of SFN to upregulate the GST mRNAs also varied depending on the specific class of the enzyme. For example, SFN upregulated GSTA1/2 and GSTP1 mRNA levels, while transcription of GSTM1 remained unaltered (Maheo et al., 1997). The increased total expression/activity of GSTs may compensate for the lack of activity of a missing class of GST. GSTM1 and GSTT1 null polymorphism are present in at least 20% of individuals in most populations. Regular consumption of cruciferous vegetables by smokers, who are exposed to tobacco carcinogens such as PAHs, with one or both non-functioning genes encoding GSTM1 and GSTT1, is associated with a decreased risk of lung cancer (Sapone et al., 2007).

### 1.3.3 Modulation of cellular redox status by SFN

Mammalian cells generate ROS during normal aerobic metabolism such as cellular respiration and biotransformation of endogenous and exogenous chemicals. However, cells are equipped with complex mechanisms for neutralising ROS and free radicals including recycled ROS scavengers (cytoplasmic GSH and nuclear thioredoxin), antioxidant enzymes (superoxide dismutases, catalase, glutathione reductase, thioredoxin reductase and various types of peroxidases) and phase II enzymes (UGT, GSTs and QR). Plant-derived phytochemicals can enhance cell antioxidant capacity directly by scavenging ROS, or indirectly by modulating expression of genes coding for...
antioxidant and conjugating enzymes and cellular thiols. ITCs, including SFN, are indirect antioxidants that are not consumed themselves in redox reactions and exert their protective long-lasting effects even at low, physiologically achievable levels.

1.3.4 Antimutagenic effects of SFN

The level of formation of DNA adducts following exposure to genotoxic carcinogens is one of the biomarkers of their bioactivation. Although most DNA adducts are removed by the cell DNA repair systems, the level of the DNA adduct formation can be used to assess the overall bioactivation of carcinogens and their detoxication by chemopreventive agents. Even at low concentrations (up to 2 µM), SFN substantially suppressed formation of BaP- and 1,6-dinitropyrene-induced DNA adducts in the human mammary epithelial cell line MCF-10F and in cultured human epithelial cells BEAS-2B (Lee et al., 1999). The observed reduction in genotoxicity positively correlated with a potent induction of QR and GST expression (Singletary and MacDonald, 2000). SFN also afforded effective protection against formation of PhIP-induced DNA adducts in human hepatocytes. The decreased level of NDA adducts was accompanied by induction of GST1A1. Inhibition of genotoxicity of PhIP was ascribed to the increased detoxication of the carcinogen by induced conjugating enzymes, as SFN had no effect on CYP1A2 activity, an enzyme catalysing the first step in the bioactivation of the parent PhIP, and failed to reduce levels of PhIP-DNA adducts after binding had occurred (Bacon et al., 2003).

Inhibition of selective rat and human CYPs including CYP1A2 and CYP2E1 by SFN has also been associated with the antimutagenic effect of SFN. At low concentrations, reflecting human intake (0.1-10 µM), SFN inhibited IQ- and NDMA-induced DNA damage in human liver cells expressing human CYP1A2 and CYP2E1 respectively (Barcelo et al., 1998). Sulforaphene, a structural analogue of SFN, at concentrations ranging from 100
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to 500 nmol/plate inhibited the mutagenicity of a wide range of food-derived heterocyclic amines including IQ and PhIP in the Ames test in the presence of Aroclor 1254-induced rat liver S9, indicating inhibition of CYP1A2-mediated bioactivation of the heterocyclic amines (Shishu et al., 2002).

1.3.5 SFN-induced apoptosis and cell cycle arrest

SFN induced cell cycle arrest and/or apoptosis in mouse embryonic fibroblasts, non-transformed human T-lymphocytes, human medulloblastoma cells, human glioblastoma cells and in many cell lines (Gamet-Payrastre et al., 2000; Bonnesen et al., 2001; Chiao et al., 2002; Jackson and Singletary, 2004; Gingras et al., 2004; Fimognari et al., 2002; Misiewicz et al., 2004; Singh et al., 2004; Tang and Zhang, 2004; Choi and Singh, 2005; Karmakar et al., 2006).

1.3.5.1 SFN-induced oxidative stress and genotoxicity

Very high concentrations of SFN (>10 mmol) induced excessive oxidative stress and ROS-mediated mutagenicity in cultured yeast cells (Paolini et al., 2004). However, SFN failed to induce a mutagenic response when tested in the Salmonella assay (Moreno et al., 1999). At doses ranging from 0.1 to 10 μM, SFN was neither cytotoxic nor mutagenic to human liver cells (Barcelo et al., 1998).

1.3.6 Inhibition of tumour promotion and progression

Other putative mechanisms that might contribute to the chemopreventive activity of SFN include modulation of signalling pathways involved in cell differentiation and maintenance, antibacterial activity and suppression of angiogenesis.
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1.3.6.1 Induction of differentiation

SFN has been shown to induce terminal differentiation of HL-60 cells possibly as a result of inhibition of ornithine decarboxylase (ODC) activity in cultured mouse epidermal cells ME 308. ODC is a rate-limiting enzyme in the synthesis of polyamines that are involved in cell proliferation, differentiation and neoplastic transformation (Lee et al., 1999).

1.3.6.2 Antibacterial activity

SFN was bactericidal to intracellular *H. pylori* of human epithelial cell line HEp-2 and was a potent bacteriostatic agent against 3 reference strains and 45 clinical isolates of *H. pylori*, irrespective of their resistance to conventional antibiotics. The antibacterial activity of this phytochemical might contribute to protection against gastric cancer in humans (Fahey et al., 2002).

1.3.6.3 Induction of Phase III transporters

In addition to the modulation of Phase I/Phase II enzyme levels/activity, treatment with SFN upregulated expression of the multidrug resistance-associated protein (MRP2) in primary rat and human hepatocytes. MRP2 is a 190 kDa export pump integrated predominantly into the biliary domain of the plasma membrane of hepatocytes and is responsible for the efflux of reactive electrophilic carcinogens out of the cell before they attack cellular DNA. Increased levels of export pump protein MRP2 may contribute to the chemopreventive activity of SFN by facilitating biliary excretion of carcinogens. Additionally, induced expression of MRP may facilitate rapid expulsion of GS-SFN adducts and trigger apoptosis (Payen et al., 2001).

1.3.6.4 Modulation of transcriptional activity

In the nucleus, DNA is associated with histones. Acetylation/deacetylation of nuclear histones is an important mechanism of regulation of gene transcription and often
malfunctions in cancerous cells. Histone deacetylases (HDAC) catalyse acetylation of histones, a reaction that confers a negative charge on positively charged histones, breaking electrostatic interaction between histones and DNA, causing local relaxation of the chromatin and allowing the transcriptional factors and transcriptional machinery to access the DNA. Recently, SFN has been shown to inhibit HDAC activity both in vitro and in vivo (Myzak et al., 2004, 2006a and 2006b). HDAC inhibitors are thought to act through enhanced transcription of important tumour suppressing genes that might be silenced in cancer cells such as the cyclin-dependent kinase inhibitor p21^{Cip1/Waf1}, and genes promoting apoptosis, cell cycle arrest and terminal differentiation (Higdon et al., 2007).

1.3.6.5 Anti-inflammatory activity

Excessive generation of pro-inflammatory cytokines such as TNF-α and enzymes such as nitric oxide synthase, cyclooxygenase and prostaglandins is associated with suppressed immune response, inhibition of apoptosis, enhanced cell proliferation and increased invasiveness of cancer cells (Nuge et al., 2007). Pre-treatment with SFN suppressed pro-inflammatory effects of diesel extract in BEAS-2 cells (Ritz et al., 2006), protected human retina cells RPE against photooxidative damage (Gao and Talalay, 2004) and decreased oxidative stress and infiltration with macrophages in cardiovascular and kidney tissues of rats prone to hypertension and stroke (Wu et al., 2004). Topical application of a broccoli sprouts extract protected mouse and human skin from UV radiation damage (Talalay et al., 2007).

1.3.6.6 Suppression of angiogenesis

Growth of tumours requires development of a new internal capillary network to supply oxygen and nutrients. Inhibition of angiogenesis by SFN has been recently reported in immortalized human microvascular endothelial cells HMECC-1 (Bertl et al., 2006),
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human umbilical vein endothelial cells and mice bearing plugs impregnated with vascular endothelial growth factor (Jackson et al., 2007)

1.4 Dietary Intake of ITCs

Total human intake of glucosinolates from raw cruciferous vegetables varies between 8 and 300 mg per day per person in the UK, the Netherlands, Canada, the United States, and in Japan (Sones et al., 1984; Krul et al., 2002) and can be as high as 350 mg in Singapore (Zhao et al., 2001).

In Western countries, Brassica vegetables are consumed mostly cooked. Method of cooking can significantly influence glucosinolate intake. Almost 90% of all glucosinolates are lost due to leaching into boiling water. Other cooking methods such as microwaving, stir-frying or steaming, retain glucosinolates (Song and Thornalley, 2005). Excessive microwaving, however, can significantly diminish glucosinolate content. The estimated intake of SFN was 3-fold greater when the duration of broccoli microwaving was reduced from 5.5 to 2 min (Rungapamestry et al., 2007). Substantial quantities of glucosinolates are also lost during storage of cruciferous vegetables. For example, up to 26% of broccoli glucosinolate were lost after just 7 days of storage (Song and Thornalley, 2007). Moreover, since cooking deactivates plant myrosinase, only 10-20% of glucosinolates are expected to be converted to ITCs almost exclusively by gut bacteria (Conaway et al., 2000 and Shapiro et al., 2001).

Dietary intake of SFN can result in plasma concentrations of SFN in the lower μM range (Ye et al., 2002 and Song et al., 2005 and Gasper et al., 2005), although the intake of specific glucosinolates and of their corresponding ITC is difficult to calculate because of wide variations in the dietary habits among individuals in different geographical areas and vast variations in glucosinolate and ITC content. Different species and even different...
cultivars of the vegetable vastly differ in the type, proportion and level of glucosinolates. These are further influenced by the environmental and agricultural conditions during cultivation, duration and conditions of storage, age and part of the plant used. About the same amount of glucoraphanin can be provided by a 10g portion of young plants (young broccoli sprouts) containing 10-100 times higher levels of glucoraphanin than mature plants (Fahey et al., 1997; Howard et al., 1997).

1.5 Pharmacokinetics of SFN

1.5.1 Absorption

Little is known about the pharmacokinetics of SFN in humans. Previous studies have shown that SFN is relatively well absorbed from the GIT after hydrolysis by plant or bacterial myrosinase (Shapiro et al., 1998, Rouzaund et al., 2004 and Vermeulen et al., 2006). Intact glucosinolates are also absorbed from the gut. Following oral administration of glucoraphanin to F344 rats, a small fraction was detected in urine as intact glucoraphanin and in its reduced form glucoerucin, while 20% of the oral dose was hydrolysed by the gut microflora and recovered in urine as N-acetyl cysteine conjugates of SFN (SFN-NAC), free SFN and erucin (Bheemreddy and Jeffery, 2007). Since cooking deactivates plant myrosinase, degradation of glucosinolates to ITC in humans depends on activity of mammalian gut microflora with myrosinase activity. Rats and humans treated with broad-spectrum antibiotics had no urinary metabolites of ITC detected after consumption of cooked broccoli (Shapiro et al., 2001). It appears that the amount of ITC metabolites recovered in the urine over a 24 h period correlates with the estimated intake of ITC. This relationship can be used to indirectly assess the bioavailability of ITC from the plant matrix.
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The activity of plant myrosinase and, consequently, the bioavailability of ITCs from raw vegetables are considerably higher than the myrosinase activity of gut microflora and the bioavailability of cooked vegetable-derived ITCs. Consequently, the absorption profile would be very different between orally administered glucosinolates and ITC. Pre-formed ITC, being small lipophylic molecules, are expected to be rapidly absorbed throughout the gastro-intestinal tract (GIT) but mostly from the stomach and upper GIT, giving a steep rise in plasma concentration soon after their administration (Conaway et al., 1999). In contrast, absorption of ITCs following ingestion of plant glucosinolates is limited by their hydrolysis to ITC by gut microflora and takes place mainly in the lower intestines. Moreover, glucosinolates may be absorbed intact and undergo enterohepatic circulation (Bheemreddy and Jeffery, 2007). Therefore absorption of ITCs generated from their corresponding glucosinolate precursor is likely to be gradual and prolonged. It is unknown whether and to what extent such differences in absorption profile and site of absorption might influence biological effects of ITC (Johnson, 2002).

1.5.2 Metabolism

It is unknown whether administered and/or newly formed ITCs are subject to further metabolism by the gut microflora. ITCs are formed during hydrolysis at neutral pH. Nitriles, epithionitriles, thiocyanates or oxazolidine-2-thiones may also be produced at low pH and in the presence of metals such as Fe^{++} ions and chemicals that might alter myrosinase activity (Bones and Rossiter, 1996; Fenwick et al., 1983). Only marginal induction of phase II conjugating enzymes has been observed after feeding broccoli subjected to autolysis intended to facilitate generation of free ITCs (Keck et al., 2003), because autolysis favours formation of SFN-nitriles (Howard et al., 1997), which are weak inducers of conjugating enzymes (Matusheski and Jeffery, 2001).
The major elimination pathway of SFN and of its phase II metabolites is the mercapturic acid pathway (Fig. 1.6) (Kassahun et al., 1997; Rungapamestry et al., 2007; Janobi et al., 2006). Following absorption, SFN and other ITCs are metabolised to the corresponding mercapturic acids (N-acetyl-L-cysteine S-conjugates). All four mercapturates (glutathione, cysteineglycine, cysteine and N-acetyl cysteine conjugates of SFN) were detected in plasma of human volunteers after consumption of broccoli (Janobi et al., 2006). The major plasma metabolite was the cysteine conjugate of SFN, followed by the cysteineglycine conjugate. The GSH and N-acetyl cysteine conjugates of SFN were the least abundant plasma metabolites of SFN (Janobi et al., 2006). Following intraperitoneal administration of SFN in corn oil (50 mg/kg) to male Sprague-Dawley rats, GSH conjugate of a desaturated derivative of SFN, as well as GSH and NAC conjugates of SFN and erucin, the sulfide analogue of SFN, were detected in the bile.

![Diagram](image)

**Figure 1.5: The major pathway of ITC metabolism: mercapturic pathway.** Abbreviation: GST - Glutathione-S-Transferase; GTP - γ-Glutamyltranspeptidase; CGase - cysteinylglycinase; AT - Acetyltransferase (Shapiro et al., 1998)

Urinary metabolites were identified as the NAC conjugates of SFN and ERN, and respectively amounted to ~60% and ~12% of the SFN dose, indicating that oxidative
metabolism of ERN is favoured over reductive metabolism of SFN (Kassahun et al., 1997). The major plasma metabolite in the urine of human volunteers following ingestion of broccoli was also the N-acetyl cysteine conjugate of SFN. In contrast to rat, SFN-Cys was also present in human urine (Janobi et al., 2006). ITCs have also been shown to undergo oxidative metabolism by microsomal Cytochromes P450. In vivo, SFN appears to be metabolized by the phase I enzyme-mediated S-oxide reduction and dehydrogenation generating a desaturated derivative of SFN (Kassahun et al., 1997). BITC is metabolized by rat CYP1A1/2 and CYP2E1 to reactive intermediates such as benzyl cyanate and benzylamine that covalently bind to and inactivate the apoprotein (Goosen et al., 2000). No such intermediates were identified in vivo. In aqueous solutions, isocyanates are converted to corresponding amines, for example, PEITC is rapidly converted to phenethylamine in rats (Thorlalley, 2002).

1.5.3 Distribution
ITCs interact with sulfhydryl groups of cellular proteins to form reversible complexes, while interactions with amino groups are typically irreversible and give rise to thiourea derivatives (Thorlalley, 2002). Free ITCs diffuse freely through the cell plasma membrane into the cell where they become trapped after enzymatic and/or spontaneous conjugation with cellular thiols such as GSH. This conjugation is thought to be the major driving force responsible for the intracellular accumulation of SFN where it can reach concentrations a few orders of magnitude higher than extracellular levels. Almost 98% of SFN in Hepa 1c1c7 cells was in the form of GSH conjugates that were not metabolised further (Zhang, 2000). Exposure of Hepa 1c1c7 cells to SFN (5 µM) resulted in a very rapid cellular uptake and accumulation to 600 µM after 30 min of exposure (Zhang and Talalay, 1998). Rapid cellular accumulation is followed by a significant transport-mediated expulsion of DTCs (Zhang and Callaway, 2002 and Callaway et al., 2004).
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In plasma SFN exists predominantly as DTCs, which appear to dissociate, releasing parent SFN that diffuses through the plasma membrane into the cells (Ye et al., 2002). None or very little ITCs can enter the cell in conjugated form, rather 'free' ITC fractions appear to diffuse through the plasma membrane following reductive cleavage of GSH and other thiol conjugates. Free ITCs either re-enter the cells or are hydrolysed (under physiological conditions) to their inactive corresponding amines (Thornalley, 2002).

1.5.4 Excretion

Studies in animals and humans have shown that, similarly to other ITCs, the majority of the vegetable-derived, as well as synthesised SFN is rapidly excreted in the urine within 24 h mainly as mercapturates. About 60% of a single portion of broccoli sprouts containing 200 μmol of a mixture of SFN, erucin and iberin were recovered in urine as DTCs during the initial 8 h after consumption, indicating very short half-life of ITCs. The rate of urinary excretion of DTC following consumption of broccoli followed first-order kinetics (Shapiro et al., 1998) and was more than double that of the passive glomerular filtration rate, indicating net tubular secretion of DTC (Ye et al., 2002; Keck et al., 2003). Since the conjugation is reversible, accumulation of conjugates in the urinary bladder after administration of excessively high doses of ITC can result in toxicity and enhancement of tumourigenesis. For example, BITC orally administered at 50 mg/kg BW/day for 36 weeks to rats produced hyperplasia, dysplasia and carcinomas of the urinary bladder, while no other organs of the urinary system were affected. Such selective toxicity indicates non-genotoxic mechanisms of the cancer promoting activity of ITCs, involving chronic damage to the urinary bladder epithelium following spontaneous dissociation of the ITC conjugates (Okazaki et al., 2003). Metabolites of SFN emanating from the mercapturic acid pathway are excreted in the bile (Kassahun et al., 1997) and urine (Shapiro et al., 1998; Ye et al., 2002; Keck et al., 2003 and Janobi et al., 2006).
1.6 Chemopreventive Activity of Erucin

Erucin [1-isothiocyanato-4-(methylthio)butane], is another naturally occurring ITC, which differs from SFN only by the oxidation state of its sulphur atom (Fig.1.1) and, which is consequently relatively less polar. It occurs as the glucosinolate glucoerucin in rocket salad (Erucia sativa), where it is a major glucosinolate, and broccoli (Bennett et al., 2002 and Vaughn and Berhow, 2004). There is experimental evidence to suggest that glucoraphanin can be reduced to glucoerucin by mammalian enzymes (Bheemreddy and Jeffery, 2007). Glucoerucin is normally hydrolysed by plant myrosinase or gut bacteria to produce erucin. In broccoli, erucin comprises almost a quarter of all ITCs (Shapiro et al., 1998). It is also formed in vivo as a result of the oxidative metabolism of SFN (Kassahun et al., 1997).

In the Western diet, most cruciferous vegetables are usually cooked by boiling. During cooking, almost a third of all glucosinolates leach into the cooking water, although other cooking processes such as steaming and microwaving have minimal effects on glucosinolate loss (de Vos et al., 1988). More importantly, cooking of vegetables inactivates myrosinase, the enzyme that converts the glucosinolates to the active ITCs and, consequently, the bioavailability of ITC is very significantly reduced, with only 10-20% of all glucosinolates being converted to ITCs by mammalian gut bacteria (Conaway et al., 2000 and Shapiro et al., 2001). An advantage of rocket salad, the major source of the erucin glucosinolate, is that it is largely consumed raw and, consequently, neither glucosinolate levels nor myrosinase activity are adversely affected.

While SFN is arguably the most investigated ITC, the chemopreventive activity of erucin has been little studied. The experimental data suggest that erucin appears to share many of the biological activities of sulforaphane related to its chemopreventive activity, in some cases being far more potent.
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Erucin has been shown to modulate expression of Phase II enzymes both in vitro and in vivo. Mechanistic data show that the rate of spontaneous, non-enzymatic reaction of erucin with GSH is as high as that of SFN. Moreover, in contrast to SFN, which is a poor substrate for human GSTs, erucin is well metabolized by all major human GSTs, including GST1A1 GST1M1 GST1M4 and GSTP1 (Kolm et al., 1995). Since the inducing potential of individual ITCs appears to correlate well with the extent of their intracellular accumulation (Zhang and Talalay, 1998), the level of erucin accumulation within the cells is likely to match, or even surpass, that of SFN's, suggesting that the Phase II enzyme inducer potency of erucin is likely to be high. Both ITCs were equipotent inducers of GST and OR activities in the lung, liver, forestomach, glandular stomach and mucosa of proximal small intestine of mice as well as in the forestomach, duodenum and urinary bladder of female Spraque-Dawley rats following repeated oral administration (Zhang at al., 1992 and Munday and Munday, 2004). Erucin was also an effective inducer of QR and GST activity in murine hepatoma cells (Zhang at al., 1992), mRNA levels coding for QR and UGT1A1 in Caco-2 cells (Jacubikova et al., 2005) and mRNA levels of thioredoxin reductase (Wang et al., 2005).

Erucin profoundly decreased mitochondrial membrane potential, an early biomarker of apoptosis, disrupted progression of cells through the cycle, leading to accumulation of Caco-2 cells at the G2/M stage, a point where arrest of the cells cycle leads to loss of viability and commitment to apoptosis and enhanced expression of MRP2 in Caco-2 cells (Jacubikova et al., 2005). Upregulation of MRP2 transporter-related protein may facilitate efflux of carcinogens out of the cell, while rapid expulsion of GS-erucin adducts can trigger apoptosis.
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1.7 Project Objectives

1. To develop and validate an LC/MS method for detection of SFN in rat and human plasma following dietary levels of exposure.

2. To determine bioavailability and pharmacokinetic characteristics of SFN in rats.

3. To determine the pharmacokinetic parameters of SFN after single and repeated ingestion of broccoli by human volunteers.

4. To establish whether the major cytochromes P450 and Phase II conjugating enzymes are modulated by SFN and erucin in the rat liver and lung following exposure to low doses.

5. To evaluate ability of SFN and erucin to modulate cytochromes P450 and Phase II conjugation system in human and rat liver in comparative studies using precision-cut slices.

6. To investigate whether erucin, like SFN, can function as mechanism-based inhibitor of Cytochromes.

7. To determine whether exposure to erucin influences metabolism of the heterocyclic amine IQ.

8. To establish whether erucin, in comparison to SFN, modulates the B(α)P-induced upregulation of CYP1 as a possible mechanism of its anticarcinogenic activity.
Chapter 2

Materials and methods
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2.1. Chemicals and Apparatus

_Abcam, UK_
Human recombinant QR polyclonal antiserum raised in rabbit

_Alabama Research and Development Corporation, Munsford, Alabama, U.S.A_
Krumdieck Tissue Slicer

_Amersham Life Sciences_
ECL advance detection kit, blocking reagent, Hybond P PVDF membrane and mini camera

_BD Biosciences, Oxford, UK_
7-Benzylidyquinoline (7-BQ), 7-hydroxyquinoline (7-HQ), rat CYP1A1 goat polyclonal antiserum, rat CYP1B1 rabbit polyclonal antiserum, rat CYP2B1 goat polyclonal antiserum and rat CYP3A2 rabbit polyclonal antiserum

_BDH Chemicals Ltd, Poole, UK_
Ethylenediaminetetra-acetic acid disodium salt

_BIO-RAD Laboratories CA, U.S.A_
Bio-rad protein assay dye reagent, acrylamide (40%), bis-acrylamide (2%)

_Cambrex Bio Science Rockland, Rockland, US_
Low melting point agarose
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**Difco**

Bacto agar

**Direct Medical Supplies Ltd, UK**

IV Venfion cannulae (20G)

**Fisher Scientific UK Ltd, Loughborough, UK**

Acetonitrile, Tris base, dimethyl sulphoxide, potassium chloride and sodium chloride

**Fluka Buchs SG, Switzerland**

2, 5, 10 and 20 ml plastic syringes, formic acid, 4-chloro-7-nitrobenzofurazan (NBF-CI) and needle microlance 8G

**Gibco Life Sciences (Invitrogen), Paisley, UK**

Earle's balanced salt solution (EBSS), foetal bovine serum, gentamycin and RPMI 1640

**Helena Biosciences, Sunderland, UK**

12-Well Plates and 96-well plates

**Hawksley, Sussex, UK**

Cristaseal and heparinised capillary tubes (75mm in diameter)

**LKT Laboratories, Inc. Minnesota, US.**

Sulforaphane and erucin (99% pure)

**London Analytical bioscience Lab M**

Agar No.1
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**Melford Laboratories Ltd., Ipswich, UK**

β-nicotinamide adenine dinucleotide phosphate (NADP) and β-nicotinamide adenine dinucleotide phosphate reduced (NADPH)

**Sigma – Aldrich, Dorset, UK**

Ammonium persulphate, secondary anti-rabbit monoclonal antibody, secondary anti-goat monoclonal antibody, bovine serum albumin, 1-chloro-2,4-dinitro-benzene (CDNB), 3, 4-dichloronitro-benzene (DCNB), 5, 5' - dithio-bis(2-nitro)-benzoic acid (DTNB), 7-ethoxycoumarin, ethoxyresorufin, D-glucose, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione, 7-hydroxycoumarin, glycerol, β-glucuronidase, hydrocortisone-21-hemisuccinate, HRP-conjugated anti-rabbit and anti-goat secondary antibodies, insulin, β-mercaptoethanol, 7-methoxyresorufin, 2-methyl-1,4-naphthoquinone (menadione), 3-(N-morpholino)propanesulfonic acid (MOPS), tween 20, thiazoyl blue tetrazolium bromide (MTT), 7-pentoxyresorufin, polaroid film, pyronin Y, resorufin, D-saccharic acid, sodium dodecyl sulphate (SDS), sulphatase and N,N,N',N'-tetramethylethylenediamine (TEMED)

**Oxoid Ltd, Basingstoke, UK**

Oxoid nutrient broth No. 2

**Roche Diagnostics, Mannheim, Germany**

Cytotoxicity detection kit plus (LDH)

**RSCH Pharmacy, Guildford, UK**

Sterile IV sodium chloride 0.9% (500ml)
2.2 Methods

2.2.1 Animals

Male Wistar albino rats (200 - 250g) were purchased from Bantin and Kingman (B&K) Universal Ltd (Hull, UK). The animals were housed on sterilised sawdust in solid bottomed polypropylene cages, with 2-4 rats per cage except experiments involving urine collection where animals were housed singly in metabolic cages for no longer than 4 days. The ambient temperature was maintained at 22°C ± 3°C and humidity controlled in the range of 30-70%. Lighting was artificial, with a 12h light/12h dark cycle. Water and standard dry rodent food in the form of pellets was freely available at all times. The animals were allowed to acclimatise to the above conditions for at least 24h before sacrificing.

2.2.2 Preparation and maintenance of precision-cut tissue slices

Precision-cut tissue slices, of uniform thickness and diameter, were cut from human liver and rat liver and lung tissues and incubated under controlled conditions as described by Hashemi et al. (1999a).

2.2.2.1 Preparation of liver slices

Immediately after sacrifice of rats by cervical dislocation, livers were excised and placed in ice-cold EBSS enriched with D-glucose (25mM) and gassed with 95% oxygen/ 5% CO₂ for 1h. The liver lobes were cut free. With respect to the human liver, the tissue, delivered as 170-200g single piece, was cut into 1 cm thick sections using a scalpel. Tissue cylinders were cut with a hand-held coring tool (Vitron, Tuscan, US) to a diameter of 8 mm from the sections of human liver or whole lobes of rat livers. The tissue was kept submerged in ice-cold EBSS throughout the procedure. Liver slices (8mm in diameter) were cut to a thickness of 200-300µm from the tissue cylinder using a
Krumdieck tissue slicer (Alabama Research and Development corporation, Alabama, US) filled with chilled (4°C) oxygenated EBSS. The parts of the slicer that came into contact with the buffer and the tissue were wiped with 70% ethanol prior to use. The slicer was set to operate in the intermittent mode with a cycle speed of 40 cycles/min in order to minimise mechanical trauma to the tissue.

2.2.2.2 Preparation of lung slices

The process of preparation of lung slices is similar to that of the liver, but with a few differences. The first difference was that the lungs had to be stabilised with agarose. For this purpose, lungs were slowly infused with 0.75% solution of warm (37°C) low-melting point agarose (48ml/kg body weight) through a cannulated trachea (IV cannulae Venflon 20G). The trachea was clamped and the lungs were kept submerged in ice-cold (4°C) EBSS until the agarose solidified. At this point, the lobes of the lung were cut free and tissue cylinders were prepared in the same way as described for the liver. The second difference was that the thickness of the lung slices, set at 400-600 µm, was double that of the liver. The thickness of the lung slices, optimal for their viability during the maintenance in the culture medium, was determined experimentally (Umachandran and Ioannides, 2006).

2.2.2.3 Preparation of culture medium

Tissue slices were pre-incubated in the culture medium prepared as described by Lake et al. (1993), and consisted of RPMI 1640 supplemented with insulin (1µM), L-methionine (0.5mM), hydrocortisone-21-Hemisuccinate (0.1mM), foetal bovine serum (5% v/v) and gentamycin (50µg/mL). The incubation media contained, in addition, a series of concentrations of the test compound(s) prepared in DMSO. The final concentration of DMSO was equal in all treatment groups and did not exceed 0.5% (v/v).
2.2.2.4 Incubation of tissue slices

The tissue slices were transferred into 12-well culture plates (Dogterom, 1993), with each slice occupying a separate well containing the described above culture medium (1.5 ml), and were maintained at 37°C in an incubator equipped with an orbital shaker (Stuart orbital shaker, Barloword Scientific Ltd, Staffordshire, UK), in a humidified atmosphere containing 5% CO₂ (Galaxy B CO₂ incubator, Scientific Laboratory supplies Ltd, Nottingham, UK).

Tissue slices were initially pre-incubated under these conditions in order to allow the damaged cells to slough from the viable tissue. The pre-incubation period for liver slices was 0.5 h while the pre-incubation of the lung slices lasted 1 h in order to allow the agarose to melt. Tissue slices were then transferred to a fresh culture medium containing either test compound(s) or relevant solvent and were incubated further for up to 24 h. At the end of the incubation period, the slices were removed from the culture medium, briefly rinsed in KCL (0.154M) containing Tris (50mM, pH 7.4) and homogenised in the KCL-Tris buffer (12 slices in 1.2 ml).

2.2.3 LDH leakage

Lactate dehydrogenase (LDH) is an intracellular enzyme. LDH release from tissue slices into the incubation medium was used as an indicator of cytotoxicity. The LDH concentration was measured using a cytotoxicity detection kit plus (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. Three slices were used per each concentration of the test compound(s). On completion of incubation (24 h), the culture medium was aspirated and the remaining tissue slices were each homogenised in 1.5 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4. The media and homogenates were centrifuged at 2000 x g for 5 min at 4°C using a bench centrifuge. Duplicate aliquots (0.1 ml) of either the medium or homogenate
supernatants were mixed with 0.1 ml of freshly prepared reaction mixture (11.25 ml of dye were mixed with 0.25 ml of catalyst) using a 96-well plate. The plate was incubated at room temperature for 15 min. The reaction was terminated by addition of 0.05 ml of stop solution and absorbance was read at 492 nm. Leaked LDH was expressed as percent of total slice LDH released into the culture media (LDH medium + LDH slice homogenate = total LDH; LDH media/total LDH x 100 = % of total LDH released).

2.2.4 Metabolism of 7-ethoxycoumarin

The 7-hydroxylation of 7-ethoxycoumarin by tissue slices was determined using the method of van Iersel et al. (1994) and Steensma et al., (1994). Liver or lung slices were incubated with 7-ethoxycoumarin (50 μM) for various time periods up to 6 hours. At the end of each time period, the incubation medium was aspirated and divided into three equal aliquots. The slices were removed, briefly rinsed in 0.01 M PBS buffer, pH 7.4 collected into 1 ml of NaOH (0.5M) and sonicated (3X10 seconds bursts), allowing at least 1 minute time intervals between the bursts in order to prevent overheating. The protein content of the sonicated slices was determined as described in section 2.2.6.

Two of the aliquots of the aspirated incubation media, 100 μl in case of the liver and 400 μl in case of the lung slices, were incubated with ½ volumes of sodium acetate buffer (0.5M), pH 5.0 containing either β-glucuronidase (5000U/ml) or a combination of sulphatase (250U/ml) and D-saccharic acid 1,4-lactone (17mM), for 16h at 37°C in a shaking water-bath in a light-protected environment. The third aliquot was incubated under the same conditions but with ½ volume of the sodium acetate buffer (0.5M) only.

All three media aliquots and a series of known 7-hydroxycoumarin concentrations (0-5 μM) were diluted to 1 ml with KCL (0.154 M) containing Tris (50 mM) buffer, pH 7.4,
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acidified with 250 µl of HCl (4M), and extracted with 6 ml of chloroform for 30 minutes. At the end of the extraction process, an aliquot (5 ml) of the chloroform layer was transferred into clean test tubes and extracted with 3 ml of glycine-sodium hydroxide (0.5M), pH 10.5 for a further 30 minutes. The fluorescence of the aqueous layer was determined using an excitation wavelength of 380 nm and an emission wavelength of 452 nm. All samples and standards were analysed in triplicate replicates.

2.2.5 Preparation of subcellular fractions

Hepatic and pulmonary subcellular fractions were prepared as described by Ioannides and Parke (1975). The tissue was kept at 4°C throughout the procedure. On completion of the incubation, the slices were removed from the incubator, briefly rinsed in Tris (50mM) buffer containing KCl (0.154M), pH 7.4 and homogenised (12 slices per replicate in 1.2 ml of buffer) in Tris (50 mM) buffer containing KCl (0.154 M), pH 7.4. The homogenates prepared from the tissue slices were centrifuged at 9000 x g for 20 minutes at 4°C in eppendorf tubes using a bench micro-centrifuge (Eppendorf, model 5404). The whole livers, scissor-minced in three volumes of potassium chloride (1.15 % w/v) and lungs, scissor-minced in one volume of potassium chloride (1.15 % w/v), were homogenised in the same way as the tissue slices and homogenates were centrifuged at 9000 x g for 20 minutes at 4°C using a Beckman J2-21 floor centrifuge fitted with a JA-17 fixed angle rotor (Beckman Coulter Ltd, Bedfordshire, UK). The resulting supernatant (post-mitochondrial S9 fraction) was decanted, divided into aliquots and stored at -80°C prior to analysis.

The S9 aliquots were thawed at 4°C, and microsomal and cytosolic fractions were prepared by centrifugation at 105,000 x g for 45 min at 4°C using a Beckman L8-70M floor ultracentrifuge and a fixed angle 70 ITI type rotor (Beckman Coulter Ltd, Bedfordshire, UK). The supernatant (cytosolic fraction) was transferred to storage at -
80°C for subsequent analysis, whilst the microsomal pellet was immediately re-suspended in a volume of Tris (50 mM) buffer containing KCl (0.154 M), pH 7.4, equal to the volume of decanted cytosol.

### 2.2.6 Determination of protein content

Protein concentration was determined by the method of Bradford (1976). Aliquots (0.02 ml) of tissue sample were diluted with aqueous sodium hydroxide solution (0.5M) such that the protein concentration in the sample did not exceed 0.5mg/ml. The serial dilution of bovine serum albumin (0-0.5 mg/ml), prepared in sodium hydroxide solution (0.5M), was used to construct a calibration curve. Triplicate aliquots (10 µl) of either sample or standard were mixed with 0.2 ml of BioRad protein dye (diluted 5-fold with distilled water) using a 96-well plate. The samples were incubated at room temperature for 5 minutes and the absorbance was measured at 595 nm using an iEMS spectrometer plate reader. Results are expressed as milligrams of protein per millilitre of subcellular fraction preparation.

### 2.2.7 Determination of microsomal CYP1A and CYP2B activity

Microsomal CYP1A subfamily predominantly O-deethylates ethoxyresorufin (EROD) and O-demethylates methoxyresorufin (MROD), while the CYP2B subfamily predominantly O-depentylates pentoxyresorufin (PROD) to a fluorescent product resorufin. Ethoxyresorufin, methoxyresorufin and pentoxyresorufin were used as model substrates in simple, direct fluorimetric assay (Burke and Mayer, 1974, 1983; Lubet et al., 1985). The microsomes derived from the tissue slices had a low enzymatic activity and were pre-warmed to 37°C for 5min in water bath prior to adding to a cuvette. The following reagents were added in a fluorimetric cuvette:
Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th></th>
<th>Microsomes from tissue slices</th>
<th>Whole liver/lung microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris - HCL buffer (0.1M, pH 7.8)</td>
<td>1.80</td>
<td>2.00</td>
</tr>
<tr>
<td>Microsomal suspension*</td>
<td>0.25</td>
<td>0.05</td>
</tr>
<tr>
<td>EROD/MROD/PROD (0.5 mM)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*25% (w/v) for whole liver, 50% (w/v) for whole lung or 12 slices/ml

The reaction was initiated by addition of 0.01 ml of NADPH (50mM) and the rate of resorufin formation was monitored for up to 10 min at +37°C using a Varian fluorimeter controlled by Carry Eclipse software, at excitation and emission wavelengths set at 571 and 586 nm respectively; the slit widths were set at 5 mm. Enzyme activities were calculated using a standard curve constructed by plotting fluorescence against known concentrations of resorufin (0-250 pmole) and are expressed in units of pmol per minute per mg of microsomal protein.

2.2.7.1 Modulation of EROD activity by erucin or SFN in hepatic microsomes

These studies were carried out to determine whether erucin and SFN can function as competitive inhibitors of rat CYP1A1. The rate of the O-dealkylation of ethoxyresorufin was monitored by adapting the method described in section 2.2.7. EROD activity was measured in the presence of either erucin or SFN (10 and 25 μM) using microsomes prepared from the livers of rats induced with Aroclor 1254 as described in section 2.2.16.4. An aliquot (0.04 ml) of each of the stock solutions of the relevant test compound (0.5 and 1.25 mM), prepared in 50% ethanol, was added to the reaction mixture (2 ml) to achieve the required final concentration. Both test compounds at each concentration were tested against five concentrations of the substrate ethoxyresorufin.
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An aliquot of the relevant stock solution (10 μl) of the substrate (2, 10, 20, 100 and 200 μM) was added to the reaction mixture (2 ml) to achieve the required final substrate concentration (0.01, 0.05, 0.1, 0.5 and 1 μM respectively). Each activity was measured in triplicate. The following reagents were added in a fluorimetric cuvette:

<table>
<thead>
<tr>
<th>Tris - HCL buffer (0.1M, pH 7.8)</th>
<th>1.90 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal suspension (25% w/v)</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>EROD (2 – 200 μM)</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Test compound (0.5 or 1.25 mM)</td>
<td>0.04 ml</td>
</tr>
</tbody>
</table>

The reaction was initiated by addition of 0.01 ml of NADPH (50mM) and the rate of resorufin formation was monitored for up to 1 min at 37°C as described above.

2.2.7.2 Inhibition of EROD activity by erucin, SFN and their metabolites

These studies were undertaken to determine whether erucin and SFN are mechanism-based inhibitors of rat CYP1A1. EROD activity in presence of erucin or SFN (25 μM) was determined by adapting the method described in section 2.2.7. Each activity was measured in triplicate. The following reagents were added in a fluorimetric cuvette:

<table>
<thead>
<tr>
<th>Tris - HCL buffer (0.1M, pH 7.8)</th>
<th>1.90 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal suspension (25% w/v)*</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>NADPH (0.5 mM)</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Test compound (1.25 mM)</td>
<td>0.04 ml</td>
</tr>
</tbody>
</table>

*Microsomes were prepared from the livers of rats induced with Aroclor 1254 as described in section 2.2.16.4
Chapter 2: Materials and methods

All components of the assay except the substrate were pre-incubated for periods of time up to 1 h in a shaking water bath at 37°C to facilitate the metabolism of ITCs. The reaction was initiated by addition of an aliquot (10 μl) of ethoxyresorufin (0.5 mM) and the rate of resorufin formation was monitored for up to 1 min at 37°C as described above.

2.2.7.3 Effect of glutathione on EROD activity after pre-incubation with erucin/SFN

These studies were undertaken to determine whether oxidized or reduced glutathione could reverse erucin/SFN-associated inhibition of rat CYP1A1 activity. EROD was assessed adapting the method described in section 2.2.7, the only difference being that, once again, all components of the assay except 7-ethoxyresorufin were pre-incubated in the presence of erucin/SFN (25 μM) and reduced or oxidized glutathione (5-200 μM). Each determination was carried out in triplicate. The following reagents were added in a fluorimetric cuvette:

- Tris - HCL buffer (0.1M, pH 7.8) 1.90 ml
- Microsomal suspension (25% w/v)* 0.05 ml
- NADPH (0.5 mM) 0.01 ml
- Test compound (1.25 mM) 0.04 ml
- GSH or GS-SG (0.2-8 mM) 0.05 ml

*microsomes were prepared from the livers of rats induced with Aroclor 1254 as described in section 2.2.16.4

All components of the assay except the substrate were pre-incubated for 0.5 h in a shaking water bath at 37°C to facilitate the metabolism of ITCs. The reaction was
initiated by addition of an aliquot (10 µl) of ethoxyresorufin (0.5 mM) and the rate of resorufin formation was monitored for up to 1 min at 37°C as described above.

### 2.2.8 Determination of microsomal CYP3A activity

7-Benzylquinozoline (7-BQ) is demethylated, predominantly by CYP3A, to the fluorescent product 7-hydroxyquinoline (7-HQ). The assay was based on a method described by Stresser et al. (2002), and utilises 7-BQ as a marker substrate for the CYP3A subfamily of the cytochrome P450 mixed-function enzyme system. The following reagents were added to a fluorimetric cuvette:

<table>
<thead>
<tr>
<th></th>
<th>Microsomes from tissue slices</th>
<th>Whole liver/lung microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer (0.1M, pH 7.8)</td>
<td>1.75 ml</td>
<td>1.95 ml</td>
</tr>
<tr>
<td>Glucose-6-phosphate (G-6-P, 3.3 mM)</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>G-6-P dehydrogenase (0.4 U/ml)</td>
<td>0.04 ml</td>
<td>0.04 ml</td>
</tr>
<tr>
<td>Microsomal suspension</td>
<td>0.25 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>7-BQ (4 mM)</td>
<td>0.01 ml</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

The reaction was initiated by addition of 0.01 ml of NADP (1.3mM) and the contents of the cuvette were mixed by inversion. The rate of 7-HQ formation was monitored for up to 10 min at excitation and emission wavelengths set at 410 and 538 nm respectively, the slit widths being set at 5 mm. The enzyme activities were calculated using a standard curve constructed by plotting fluorescence against 7-HQ (0 to 0.5 nmole/ml).

### 2.2.9 Determination of NADPH-Cytochrome C reductase activity

This enzyme is localised in the microsomes and functions as a component of the mixed-function oxidase system. The activity is measured essentially by the method described by Williams and Kamin (1962). The activity of NADPH-cytochrome C reductase was
measured in the presence of either erucin or SFN using microsomes prepared from the livers of control rats. An aliquot (0.1 ml) of each of the stock solutions of the relevant test compound (0.03, 0.075, 0.15, 0.3, 0.75 and 1.5 mM), prepared in 50% ethanol, was added to the reaction mixture (3 ml) to achieve the required final concentration (1, 2.5, 5, 10, 25 and 50 μM respectively). The solvent vehicle (50% ethanol) was used as a control. The following reagents were added to both cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Test cuvette</th>
<th>Reference cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate buffer (50mM), pH 7.6 containing KCN (1 mM)</td>
<td>1.8 ml</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>Cytochrome C (0.1 mM)</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Test compound (0.03 – 1.5 mM) or solvent</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Microsomal suspension</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

An aliquot (0.1ml) of NADPH (30mM) was added to the test cuvette to initiate the reaction. The contents of the cuvettes were mixed by inversion. The increase in absorbance over time was followed at 550 nm for up to 2 min using a Kontron 860 spectrophotometer. Enzyme activities were calculated using a molar extinction coefficient of 18.5 mM⁻¹ cm⁻¹ and were expressed in units of nmol per minute per mg of microsomal protein.

### 2.2.10 Determination of total glutathione content

The total cytosolic glutathione content was measured essentially as described by Akerboom and Sies (1981). An aliquot (0.2 ml) of the cytosolic fraction prepared from whole livers or lungs (diluted 8 fold with KCl, 1.15%, w/v) or tissue slices (undiluted) was mixed with perchloric acid (2 M; 0.2 ml) containing EDTA (4 mM) to precipitate the proteins. The mixture was neutralised with potassium hydroxide (2 M; 0.2 ml)
containing MOPS (0.3 M). Standards of glutathione were taken through the same procedure. All standards and samples, analysed in duplicate, were centrifuged at 2,000 x g for 10 mins. The supernatant was decanted and used in the assay. The following reagents were added to a spectrophotometer cuvette:

Potassium phosphate buffer (0.1 M, pH 7) containing EDTA (2.5mM) 1.00 ml  
NADPH (1.5 mg/ml) 0.05 ml  
Glutathione reductase (6U/ml) 0.02 ml  
Sample or standard 0.10 ml  

The reaction was initiated by addition of 0.10 ml of 5,5'-dithio-bis(2-nitro)-benzoic acid (DTNB, 3.8mM). The contents of the cuvette were mixed by inversion and the reaction was followed at 412 nm for up to 2 min using a Kontron 932 spectrophotometer. Total glutathione concentration was calculated from a standard curve constructed by plotting glutathione reductase activity against glutathione concentrations.

2.2.11 Determination of quinone reductase activity

The assay was performed using the method described by Prohaska and Santamaria (1988), which is based on measuring an increase in absorbance due to reduction of a yellow dye 3-[4,5-dimethylthiazolyl-2-yl]-2,5diphenyl tetrazolium bromide (MTT) to a blue-coloured product, formazan, in the presence of the electron acceptor menadione.

The following reagents were added in both spectrophotometer cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Test cuvette</th>
<th>Reference cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer (25 mM, pH 7.4) containing tween 20 (0.083%, w/v)</td>
<td>1.00 ml</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>MTT (10 mM)</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>
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NADPH (50 mM)  0.01 ml  0.01 ml
Cytosol (diluted 10 to 50-fold)  0.05 ml  ---
Potassium chloride (1.15%, w/v)  ---  0.05 ml

The reaction was initiated by addition of 0.1 ml of menadione (10 mM) to both cuvettes and followed at room temperature at 610 nm for 1 min using a dual beam Contron Uvikon 860 spectrophotometer. Enzyme activities were calculated using a molar extinction coefficient of 11.3 mM$^{-1}$ cm$^{-1}$ and were expressed in units of nmol per minute per mg of total cytosolic protein.

2.2.12 Determination of glutathione-S-transferase activities

The glutathione-S-transferases (GSTs) are a family of cytosolic enzymes catalysing the conjugation of glutathione to electrophiles. The assay was carried out using 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB) or 4-chloro-7-nitrobenzofurazan (NBD-Cl) as accepting substrates using an adaptation of method described by Habig et al., (1974) for CDNB and DCNB and Ricci et al., (1994) for NBD-Cl. The change in absorbance over time due to the conjugation of substrate with glutathione was monitored at room temperature for 1 min in a dual beam Contron Uvikon 860 spectrophotometer at 340 nm for CDNB, 345 nm for DCNB and 419 nm for NBF. All samples were analysed in duplicate.

For measurement of GST activity using CDNB and DCNB as marker substrates, the following were added to both cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Reference cuvette</th>
<th>Test cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate buffer (0.1 M, pH 7.4)</td>
<td>1.00 ml</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>CDNB/DCNB (25 mM)</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>
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Reduced glutathione (25 mM)  0.30 ml  0.30 ml
Cytosol (10 to 50 fold diluted)  ---  0.05 ml
Potassium chloride (1.15%, w/v)  0.05 ml  ---

For measurement of GST activity using NBD-Cl as the accepting substrate the following were added to both cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Reference cuvette</th>
<th>Test cuvette</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate buffer (0.1 M, pH 5.0) containing reduced glutathione (0.5mM)</td>
<td>1.00 ml</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>NBD-Cl (4 mM)</td>
<td>0.05 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Cytosol (10 to 50 fold diluted)</td>
<td>---</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Potassium chloride (1.15%, w/v)</td>
<td>0.05 ml</td>
<td>---</td>
</tr>
</tbody>
</table>

Enzyme activities were calculated using the molar extinction coefficients of 8.5, 9.6 and 14.5 mM⁻¹ cm⁻¹ for CDNB, DCNB and NBD-Cl respectively, and were expressed in units of nmol per minute per mg of cytosolic protein.

2.2.13 Quantification of protein levels by Western blot analysis

Western blot is commonly used in the analysis of proteins in complex mixtures. Initially, proteins are separated by electrophoresis through a polyacrylamide gel using a Bio-Rad Mini Protean 3 kit (Bio-Rad, Hertfordshire, UK) according to the method devised by Laemmli (1970). The separated proteins are then transferred to a polyvinylidene fluoride (PVDF) membrane (pore size of 0.2 μm) as described by Towbin et al. (1979) where they are immobilised and the proteins of interest are visualised by a mean of antibody binding (Burnette, 1981). This technique is a useful tool in the qualitative and quantitative analysis of proteins of interest in complex mixtures.
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2.2.13.1 Discontinuous polyacrylamide gels

All Western blot equipment was cleaned with 70% methanol prior to use. Gels were prepared in glass cassettes, each assembled from a spacer plate with permanently bonded spacers (0.75mm) and a short plate aligned together and secured in a casting frame. The casting frame was then clamped upright onto the casting stand securing the gel cassette perpendicular to the bench surface and sealing it off by pushing down against the grey rubber gasket. The resolving gel monomer solution was prepared by combining 5.43 ml of water, 4.50 ml of resolving gel buffer (Tris-HCl (1.5M) containing SDS (0.4% w/v), pH 8.8), 5.62 ml of acrylamide (40% w/v, 2.95 ml of bis-acrylamide (2% w/v), 0.18 ml of ammonium persulphate (10% w/v, freshly prepared) and 0.018 ml of TEMED. The resolving gel monomer solution was poured up to a marked level (1cm below the teeth of the completely inserted comb), immediately overlayed with water (1ml) to avoid gel dehydration and allowed to polymerise for 30 min. The excess water was drained.

The stacking gel monomer solution consisted of 7.15 ml of water, 2.5 ml of stacking gel buffer (0.5M Tris containing SDS (0.4% w/v, pH 6.8), 0.7 ml of acrylamide (40% w/v), 0.4 ml of bis-acrylamide (2% w/v), 0.05 ml of freshly prepared ammonium persulphate (10% w/v),) and 0.01 ml of TEMED. The stacking gel monomer solution was poured between the glass plates above the resolving gel. The combs were inserted into the glass cassette and the gel was allowed to polymerise for 45 minutes to 1 hour, after which the combs were carefully removed. The gel cassette sandwiches, released from the casting frame, were mounted onto a clamping frame and inserted into the Electrode Assembly placed into the electrophoresis tank filled with running buffer diluted 5-fold. The 5x concentrated running buffer, pH 8.3, was prepared in advance by dissolving 15.15g Tris base, 72g glycine and 5g SDS in 1L of water.
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2.2.13.2 Sample preparation and loading

The samples with equal total protein concentration (1 to 2 mg/ml) were diluted 1:1 with sample loading buffer consisting of 4.80 ml of water, 1.20 ml of Tris buffer (0.5M), pH 6.8, 0.96 ml of glycerol, 0.92 ml of SDS (10% w/v), 0.48 ml of β-mercaptoethanol and 0.60 ml of pyronin Y (0.05% w/v). The samples in loading buffer were heated for 5 min at 95°C in a heating block, cooled and centrifuged at 3000g for 10 sec. Equal volumes of each sample (10-60 µl) were loaded into the corresponding wells using gel loading tips, reserving one well in each gel for a molecular marker (5 µl) to aid identification of sample and protein of interest. The molecular marker was also used as a quality control sample to ensure the quality of the running and transfer procedures. The gels were run for 75 to 90 min at a constant current of 40 mA until the band of pink dye reached the lower end of the gel. At this point the power pack was switched off and the electrode assembly was taken out of the buffer tank.

2.2.13.3 Transfer of polypeptides to PVDF membrane

The gel cassette sandwiches were released from the electrode assembly and the glass plates were separated with a gel releaser. The gels were floated off the glass plate in a tray filled with transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). The PVDF membrane (pre-wetted in 100% methanol), blotting paper, scotch brite and the gels were equilibrated in transfer buffer for 15 min. A PVDF membrane and the gel were sandwiched between two single pieces of blotting paper, cut to the size of the gel, and secured between two pieces of scotch brite in the transfer cassette. The gel was facing the black side (electronegative) of transfer cassette. The cassettes were inserted into the transfer module placed in the electrophoresis tank filled with transfer buffer. The proteins from the gel were transferred onto the membrane at a constant current of 100 mA for 15 h at room temperature.
2.2.13.4 Detection and visualisation of proteins

The blotting sandwich was disassembled upon completion of the transfer procedure, and the membrane was placed into disposable 50 ml plastic containers. The detection of proteins bound to the membrane was a three step process, each of which lasted 1 h and was performed at room temperature. Each membrane was first blocked to prevent non-specific binding of primary and secondary antibodies with ECL Advance blocking reagent (20 ml, 2% w/v) dissolved in a washing buffer (TBS, pH 7.6, containing 0.1% tween 20), then incubated with the primary antibody and, lastly, incubated with secondary antibody conjugated with horse radish peroxidase (HRP). The membrane was washed (3x10 min) in washing buffer at the end of each of these three procedures. The apoprotein bands were visualised using an ECL Advance detection kit and an ECL mini-camera. The development and exposure periods varied from 15 sec to 2 min depending on the intensity and clarity of the image on the photograph. The optical density of the protein bands was quantified using the GeneTool software (Syngene corporation, Cambridge, UK). The band representing the control was assigned a value of 100%, while the bands representing treatment(s) were expressed as percentage of the control.

2.2.14 The Ames mutagenicity test

The assay (Maron and Ames, 1983) was performed in a class II microbiological safety cabinet swabbed with 70% ethanol immediately prior to use. All solutions and equipment were autoclaved at 120°C for 15 minutes. Each assay incorporated positive and negative (spontaneous reversion rate) control and was performed in triplicate.

2.2.14.1 Minimal agar plates

A molten solution of bactoagar (1.5% w/v) was supplemented with 25 ml of D-glucose (40% w/v) and 10 ml of Vogel Bonner salts (1% magnesium phosphate, 10% citric acid,
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50% di-potassium hydrogen orthophosphate and 17.5% of sodium ammonium phosphate (w/v). A 30 ml aliquot of the bactoagar mix was then poured into a single petri dish and allowed to solidify. The minimal plates were prepared in advance and stored at 4°C up to one month before use.

2.2.14.2 Preparation of bacterial culture

The YG1024 bacteria used in the current studies were freshly grown from permanent copies, stored at -80°C, by incubating the inoculated nutrient broth containing ampicillin (0.025 mg/ml) at 37°C for 12h in a shaking water bath. The fresh cultures were either used immediately or after storage at 4°C for up to 72h. The permanent copies were prepared from fresh cultures, which were mixed with the cryopreservative dimethyl sulphoxide (DMSO, 9% v/v) and stored at -80°C.

2.2.14.3 Testing the quality of bacteria

The following tests were carried out in duplicate on the tester strain used in a mutagenicity assays to confirm that bacteria were viable and retained the required, genetically-engineered mutations.

2.2.14.3.1 Histidine auxotrophy

A fresh bacterial culture was streaked across the minimal agar plate supplemented with an aliquot (100 μl) of “high” histidine solution (0.1 M) and biotin (0.5 mM), smeared with a sterile plastic spreader. For comparison, a fresh bacterial culture was also streaked across the minimal agar plate without the “high” histidine solution. The plates were then left at +37°C for 24h in an Astell Hearson incubator. Growth only in the histidine-streaked plates indicates histidine requirement.
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2.2.14.3.2 R-factor plasmid

An aliquot of fresh culture (100μl) was plated with top agar (2 ml) supplemented (5% v/v) with the “high” histidine solution containing histidine (0.1 M) and biotin (0.5 mM). The top agar was allowed to set and a sterile filter disk, with 25 μl ampicillin (8mg/ml), was placed in the center of the plate which was then incubated at +37°C for 24h. The presence of the plasmid is indicated by the absence of bacterial growth around the ampicillin-impregnated disk.

2.2.14.3.3 Presence of rfa mutation

An aliquot of fresh culture (100μl) was plated with top agar (2 ml) supplemented (5% v/v) with the “high” histidine solution containing histidine (0.1 M) and biotin (0.5 mM). The top agar was allowed to set and a sterile filter disk, impregnated with 25 μl crystal violet solution (1mg/ml) was placed in the center of the plate. The plate was then incubated at +37°C for 24h. The presence of the rfa factor is indicated by the lack of bacterial growth around the crystal violet-impregnated disk.

2.2.14.4 Activation system

The activation system consisted of potassium phosphate buffer (0.2M, 70% v/v, pH 7.4) containing co-factors (NADP, 20 μmol/ml; glucose-6-phosphate, 25 μmol/ml), potassium chloride (0.33 M, 10% v/v), magnesium chloride (0.08 M, 10% v/v) and liver S9 (10% v/v). The S9 fraction was prepared from livers of W/A rats injected intraperitonealy with a single dose of Aroclor 1254 (0.5 g/kg of body weight) dissolved in corn oil (0.2 g/ml), and killed on the 5th day after dosing.

2.2.14.5 Plate incorporation test

Molten top agar (0.6% w/v) containing sodium chloride (0.5% w/v), histidine (0.05 mM) and biotin (0.05 mM), was dispensed as aliquots (2 ml) into sterile plastic tubes and
kept at 45°C in a water bath. An aliquot (0.1 ml) of filtered urine (Millipore filter, 0.2
μl), a solution of 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) in DMSO (0.5-10
μg/ml) or a relevant solvent (1.15% KCl and DMSO respectively), bacterial culture (0.1
ml) and activation system (0.5 ml) were added to each plastic tube containing top agar
in the order described. Immediately after addition of the activation system, the contents
of the tube were mixed and plated on a minimal agar plate. Once the top agar solidified,
the plates were inverted and incubated for 48h at 37°C for 24h in an Astell Hearson
incubator. The colonies were counted using a Gallenkamp Colony counter. Each assay
was carried out in triplicate.

2.2.15 Statistical evaluation

Results are expressed as mean ± standard deviation. The significance of the differences
between the means was analyzed using the unpaired Student’s t-test. The effects of a
second compound were analysed using two-way analysis (ANOVA) of variance. The
statistical significance is expressed as follows:

- P>0.05 not significant
- P<0.05 significant (*)
- P<0.01 very significant (**)
- P<0.001 extremely significant (***)
Chapter 3: Pharmacokinetics of SFN in rats

Chapter 3

Pharmacokinetics of SFN in rat
3.1 Introduction

Reactive ITCs can spontaneously and non-specifically bind to thiol groups of macromolecules, forming reversible thiocarbomoyl complexes with thiol groups of proteins (Thornalley, 2002). Moreover, the principal metabolic pathway of ITC in mammals involves enzymatic conjugation with cellular glutathione, the first step in the mercapturic acid pathway, followed by further metabolic transformation to generate sequentially the cysteinylglycine-, cysteine- and, finally, N-acetylcysteine conjugates (Kassahun et al., 1997; Conaway et al., 2002 and Bheemreddy and Jeffery, 2007). These reversible conjugates, known as dithiocarbamates (DTCs), are the predominant form of ITCs in plasma (Mennicke et al., 1983). Total ITC/DTC concentrations in vegetable extracts and biological fluids can be quantified using the cyclocondensation assay (Zhang et al., 1996 and Ye et al., 2002). The major limitation of this method is lack of differentiation between ITC and their DTC derivatives. Use of LC/MS instrumentation allows identifying and quantifying of individual ITCs in biological materials.

Many studies reporting the biological activity of SFN have been carried out in vitro (Conaway et al, 2002, Higdon et al., 2007), but in order to relate these data to the in vivo studies, it is essential that the pharmacokinetic behaviour of this phytochemical is established. In the present studies, an LC/MS method was developed, validated and employed for the specific, accurate and sensitive analysis of SFN in rat plasma following single oral administration of low doses. Moreover, intravenous administration of SFN was used to determine its absolute bioavailability at doses reflecting human intake.
Chapter 3: Pharmacokinetics of SFN in rats

3.2 Methodology

3.2.1 Animals

The conditions at which the animals were maintained during acclimatisation and study periods are described in section 2.2.1. Rats were allowed to acclimatise to the study conditions for 4 days.

3.2.1.1 Preliminary study

Three male Wistar albino rats (200g ± 10%) were treated with a single dose of 1.0, 5.0 mg/kg or 20 mg/kg, corresponding to 5.6, 28 and 112 μmol/kg, of SFN, dissolved in 1 ml of water. Blood samples (100 μl) were withdrawn from the tipped rat tail at regular time intervals for up to 8 hours, and placed into lithium-heparinised centrifuge microtubes. A sample was also obtained 24 h prior from untreated rats.

3.2.1.2 Main pharmacokinetic study

Sixteen male Wistar albino rats (200g ± 10%), randomly assigned to 4 groups of 4 animals each, were treated with SFN either as a single intravenous dose (0.5 mg/kg), in a volume of 100 μl, or single oral doses of 0.5, 1.0 and 5.0 mg/kg, corresponding to 2.8, 5.6 and 28 μmol/kg, dissolved in 1 ml of water. Blood samples (100 μl) were withdrawn from the rat tail at regular time intervals for 8 hours, and placed into lithium-heparinised centrifuge microtubes. A sample was also obtained 24 hours after SFN administration as well as from untreated rats.

3.2.2 Sample preparation

Aliquots of the plasma (40 μl) were made to 1 ml with 0.01 M phosphate-buffered saline (pH 7.3) and were subsequently extracted with chloroform (5 ml) twice for 30 minutes; the layers were separated by centrifugation at 510g for 10 minutes. The combined
chloroform extracts were evaporated under nitrogen using a N-EVAP model 111 (Organomation Assoc. Inc, USA) and reconstituted in 40 µl of the HPLC mobile phase (10 % acetonitrile in water containing 0.1% formic acid), and 20 µl was injected for analysis. Quality control and standard sulforaphane solutions of known concentrations (0.05, 0.5 and 1.8 µg/ml) were also carried through the same extraction procedure. Calibration curves were constructed by plotting a peak area of SFN standards versus their theoretical concentration (0.01- 2 µg/ml).

3.2.3 Determination of sulforaphane in rat plasma

Plasma levels of SFN in rat plasma were determined by LC-MS/MS. Separation of SFN from matrix constituents was achieved using an Ultimate 3000 (Dionex, Camberley, UK) HPLC employing a Synergi 4u Fusion-RP analytical column (80Å particle size, 150 x 1 mm) fitted with a KrudKatcher in-line filter, both supplied by Phenomenex (Macclesfield, UK). Mobile phase consisted of solvent A (10 % acetonitrile in water containing 0.1% formic acid) and solvent B (90 % acetonitrile in water containing 0.1% formic acid). The analyte was eluted with a linear gradient (0 to 20%) of organic solvent B over 22 min. The column was then washed with 80% of solvent B over 3 min and re-equilibrated for a further 10 min; the eluent flow rate was 0.05 ml/min. Under these conditions, the retention time of SFN was about 18 minutes in a total run time 35 minutes.

\[
\begin{align*}
\text{C} & \rightarrow \text{S} \\
178 & \rightarrow 114 \\
178 & \rightarrow 72
\end{align*}
\]

Figure 3.1: Structures of SFN transitions monitored in the MRM mode
Chapter 3: Pharmacokinetics of SFN in rats

SFN was detected on-line using an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) equipped with a turbo-ion electrospray probe, operating in the positive ionisation mode. The analyte was detected by monitoring the m/z 114 and 72 mass transitions (Fig. 3.1) in the multiple reaction monitoring (MRM) scan mode, under the following conditions: turbo ion-spray interface temperature, 350°C; collision-activated dissociation gas pressure and curtain gas pressure, 3 and 40 p.s.i. respectively; turbo ion-spray voltage, 5000 V; declustering, entrance and focusing potential, 11, 10 and 300 V respectively; collision energy 18 and 41 V for m/z 114 and 72 transitions respectively; collision cell exit potential, 15 V. Acquisition of data was achieved using the Analyst software (version 1.4; Applied Biosystems).

3.2.4 Pharmacokinetic analysis

Pharmacokinetic analysis was carried out using PK solutions™ software package (version 2.0, Summit Research Services, Ohio, USA). Because of the very long terminal phase (t_{1/2} = 65.6 hours after intravenous administration), pharmacokinetic parameters were calculated from the better defined AUC_{0-24}, rather than from AUC_{0-\infty}, to avoid introducing an inaccuracy in extrapolating the terminal phase to time infinity. AUC_{0-24} was calculated using the trapezoidal rule utilising the time data determined experimentally. Absolute bioavailability (F) was determined from the ratio of the oral to intravenous dose-normalised AUC_{0-24} values. Apparent volume of distribution (Vd) was calculated from the equation Vd = FD/ AUC_{0-24} k_{el}, where D is the dose and k_{el} is the elimination rate constant. Plasma clearance (Cl) was determined using the equation Cl = FD/ AUC_{0-24}. Finally, C_{max} and T_{max} were determined graphically from the plasma concentration versus time plots.
Chapter 3: Pharmacokinetics of SFN in rats

3.2.5 Statistical evaluation

Animal data, analysed individually, are presented as mean ± standard deviation, where n=4. The significance of the differences between the means was analyzed using the unpaired Student's t-test. The statistical significance is expressed as follows:

P>0.05 not significant
P<0.05 significant (*)
P<0.01 very significant (**)
P<0.001 extremely significant (***)

3.3 Results

3.3.1 Optimisation of chromatographic separation and sample preparation procedure

The Ultimate 3000 HPLC system was connected to a API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray source (TIS). The direct combination of LC with mass spectrometer reduces the stress on chromatographic separations because of the mass selectivity and distinctive fragmentation patterns, but a satisfactory chromatography is required to achieve good peak shapes. Sharp peak shape of SFN was achieved with a Synergi 4u Fusion-RP analytical column (80Å particle size, 150 x 1 mm), selected based on manufacturer's recommendation as the most suitable for small and weakly hydrophobic molecules and fitted with a KrudKatcher in-line filter. The best peak shape of SFN was achieved with a linear gradient (0 to 20%) of acetonitrile in water over 22 min, resulting in SFN elution time of about 18 minutes at flow rate of 0.05 ml/min.

Sample clean-up procedures, including protein precipitation with acids or methanol or acetonitrile, solid phase extraction (SPE) and liquid-liquid extraction with hexanol or
chloroform, were evaluated. Due to the small sample volume and low analyte concentrations, protein precipitation with acids resulted in an unacceptably high loss of sample (results not shown). Extraction of SFN using solid phase extraction (SPE) Oasis MCX (mixed ion exchange and hydrophobic interactions mode) cartridges resulted in highly inconsistent recoveries of SFN from plasma matrix, which varied up to 4-fold between replicates (results are not shown). Almost complete loss of SFN, which remained in aqueous phase, was observed when highly hydrophobic hexanol was used for extraction. Finally, extraction of SFN with the more polar chloroform, as detailed in section 3.3.3, resulted in high recoveries at low, medium and high concentrations.

3.3.2 Optimisation of MS/MS detection of SFN

The optimal source-dependent parameters recommended by the manufacturer for the API 2000 triple quadrupole mass spectrometer, equipped with a turbo-ion electrospray (TIS) probe, operating in the positive ionisation mode, at 50 μl/min LC flow rate were: TIS interface temperature, 350°C; collision-activated dissociation gas pressure and curtain gas pressure, 3 and 40 p.s.i. respectively; TIS voltage, 5000 V; including TIS sprayer.

The compound-dependent parameters of the instrument for the MRM scan were optimised using a direct infusion of SFN (5 μg/ml) dissolved in solvent comprising 1:1 water:acetonitrile and acidified with formic acid (0.1 % v/v). SFN spectra were acquired over the mass range m/z 100-300 using full-scan (Q1 scan) operating in the positive mode (Fig. 3.2). The protonated SFN ion [M + H]^+ of m/z 178 was selected for the product ion scan to establish the optimal pressure of the CAD gas (collision gas in the collision cell) and collision energy (CE) necessary to yield the characteristic transitions (product ions) of SFN (precursor ion) that can be monitored in the multiple reaction
monitoring (MRM) mode. The optimal CAD gas and CE for fragmentation of m/z 178 to m/z 114 and 72 transitions were 18 and 41 V respectively (Fig. 3.3).

Figure 3.2: Mass spectrum of SFN acquired in the full-scan mode
Chapter 3: Pharmacokinetics of SFN in rats

Continued

Figure 3.3: Collision-induced dissociation of SFN. Product ion spectra of the (M+H)+ ion of SFN (m/z 178) at CE set to 0 (A) and formation of m/z 178 → 114 and 178 → 72 transitions (product ions) at CE set at 18 (B) and 41 V (C) respectively. CAD was set to 3.
3.3.3 Validation LC/MS/MS method for determining SFN in rat plasma

At the retention time for SFN (18 min), there were no interfering peaks on the chromatograms obtained from blank plasma extracts (Fig. 3.4). The calibration curve for SFN (Fig. 3.5), constructed using the least square linear regression analysis of 14 data points, had excellent linearity over the tested range (0.01 to 2 µg/ml).

Figure 3.4: A representative chromatogram of extracted blank rat plasma. No significant matrix interferences were observed at the retention time for SFN (18 min).

Figure 3.5: Calibration curve for determination of SFN in rat plasma.
Chapter 3: Pharmacokinetics of SFN in rats

The limit of detection (LOD), defined as the lowest concentration of the analyte that generates signal to noise ratio of 3 was calculated to be 1 pmole/injection (5 ng/ml, following a 20 µl injection) (Fig. 3.6 A). The limit of quantification (LOQ), defined as the lowest concentration that produces an instrument response with a signal to noise ratio of 5, was 3 pmole/injection (15 ng/ml, following a 20 µl injection) (Fig. 3.6 B).

Figure 3.6: Representative LC/MS/MS chromatograms (MRM mode, 114 and 72 m/z transitions) of sulforaphane in spiked plasma extracts at the limit of detection (A) and quantification (B). The outer peak is the more abundant 114 transition, whereas the inner peak represents the less abundant 72 m/z transition.

Recovery for SFN, determined by comparing the instrument response obtained from spiked plasma samples taken through the extraction procedure and the analyte added
Chapter 3: Pharmacokinetics of SFN in rats

directly to the blank matrix extract and representing 100% at three different concentrations (0.05, 0.5 and 1.5 µg/ml, n=6), was 91, 91 and 88% respectively. At the same concentrations, inter-assay variation was 1.0, 2.2 and 3.2 % CV respectively, whereas intra-assay variation was for the same concentrations was 1.5, 6.5 and 5.0 % CV respectively (n=4).

3.3.4 Pharmacokinetics of SFN in rats

A preliminary dose-ranging study showed that SFN was easily detectable in plasma of rats treated orally with either of the three dose levels (1.0, 5.0 and 20 mg/kg) and that the lowest oral dose, compatible with the developed detection method, could be decreased to 0.5 mg/kg (results not shown).

In the main pharmacokinetic study, four groups of male Wistar albino rats (n=4), received either a single intravenous (0.5 mg/kg) or oral (0.5, 1.0 and 5.0 mg/kg) dose of SFN. Analysis of the time-course changes in the plasma levels of SFN, plotted using a semi logarithmic plot, following intravenous administration to rats showed that the plasma profile was best described by a two-compartment pharmacokinetic model (Fig. 3.7). Within two hours, plasma levels declined to about 10% of the 0.5 h level, they then remained fairly constant indicating a long terminal phase. Similarly, following oral administration of the same dose, a marked and rapid decline in plasma levels of SFN was evident between 1 hour after administration, when peak levels were achieved, and 2 hours after administration, followed by a prolonged terminal phase (Fig. 3.7). At the higher doses, however, the decline in plasma levels, after maxima have been attained, was more gradual. The pharmacokinetic parameters of SFN following oral and intravenous administration are shown in table 1. The $C_{\text{max}}$ and $\text{AUC}_{0-24}$ values in orally-treated rats increased with dose, but not proportionately; rise in $\text{AUC}_{0-24}$ and $C_{\text{max}}$ values was lower than would be anticipated. Comparison of $\text{AUT}_{0-24}$ values between the
intravenously- and orally-treated groups, at the 0.5 mg/kg dose, indicate an absolute bioavailability of 82% which, however, decreased at the higher doses. Finally, the rate of absorption constant $k_{ab}$, biological half-life $t_{1/2}$ and apparent volume of distribution decreased at the highest dose used.

A

**Intravenous dose (0.5mg/kg)**

![Graph A: Intravenous dose (0.5mg/kg)](image)

B

**Oral dose (0.5mg/kg)**

![Graph B: Oral dose (0.5mg/kg)](image)
Figure 3.7: Concentration of SFN in rat plasma versus time curves. Male Wistar albino rats were exposed to a single intravenous dose of 0.5 mg/kg (A) or single oral doses of 0.5, 1.0 and 5.0 mg/kg (B, C and D respectively) of SFN. Blood samples (100 μl) were withdrawn from the rat tail at time intervals for up to 24 hours after SFN administration. Values represent the mean ± SD, where n=4.
Chapter 3: Pharmacokinetics of SFN in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intravenous (0.5 mg/kg)</th>
<th>Oral (0.5 mg/kg)</th>
<th>Oral (1.0 mg/kg)</th>
<th>Oral (5.0 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>-</td>
<td>46.4 ± 19.4</td>
<td>84.0 ± 19.6*</td>
<td>211.3 ± 24.3***</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>-</td>
<td>0.5 ± 0.0</td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>$k_{\text{ab}}$ (h$^{-1}$)</td>
<td>-</td>
<td>5.78 ± 1.70</td>
<td>3.74 ± 0.44</td>
<td>2.57 ± 1.36*</td>
</tr>
<tr>
<td>$k_{\text{el}}$ (h$^{-1}$)</td>
<td>0.011 ± 0.003</td>
<td>0.012 ± 0.002</td>
<td>0.015 ± 0.005</td>
<td>0.028 ± 0.009*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>65.6 ± 16.3</td>
<td>62.2 ± 20.07</td>
<td>50.5 ± 16.0</td>
<td>27.3 ± 10.8**</td>
</tr>
<tr>
<td>$Cl_p$ (ml/h)</td>
<td>265 ± 28</td>
<td>264 ± 18</td>
<td>260 ± 28</td>
<td>263 ± 10</td>
</tr>
<tr>
<td>$V_d$ (l)</td>
<td>25.4 ± 8.3</td>
<td>23.8 ± 5.8</td>
<td>19.3 ± 7.7</td>
<td>10.4 ± 4.4*</td>
</tr>
<tr>
<td>AUC$_{0-24}$ (ng ml$^{-1}$ h)</td>
<td>476 ± 46</td>
<td>392 ± 26</td>
<td>493 ± 69*</td>
<td>1001 ± 37***</td>
</tr>
<tr>
<td>F %</td>
<td>100</td>
<td>82.4 ± 5.3</td>
<td>51.8 ± 7.2***</td>
<td>21.0 ± 0.8***</td>
</tr>
</tbody>
</table>

Table 3.1: Absolute bioavailability and pharmacokinetic parameters of sulforaphane following administration of low doses. Results are presented as mean ± SD for four animals. *P<0.05; **P<0.01; ***P<0.001, when compared to the 0.5 mg/kg oral dose.

3.4 Discussion

An analytical method, utilising LC-MS/MS, that allows the determination of SFN in small volumes of rat plasma following exposure to low dietary doses was developed, validated and employed to determine its absolute bioavailability and pharmacokinetic characteristics in the rat following intravenous and oral administration. An 150g serving of fresh broccoli is expected to release 56-112 mg of SFN (Howard et al., 1997) so that the intake for a 70 kg individual would be 0.8-1.6 mg/kg. The doses employed in this study of 0.5-5.0 mg/kg were chosen to represent similar levels of intake.

In the current studies the animals were not deprived of food prior to administration of SFN, as it could have an impact on its metabolic clearance. The principal pathway of metabolism of this compound involves conjugation with glutathione, followed by further processing of the conjugate to the mercapturate (Kassahun at al., 1997). Withdrawal of
Chapter 3: Pharmacokinetics of SFN in rats

food can result in a rapid decline in the cellular levels of glutathione (Pessayre et al., 1979).

Following oral administration, SFN peak plasma concentrations were attained at about one hour, indicating rapid absorption, compatible with its lipophilicity and small molecular size. Similarly, in studies conducted in human volunteers, isothiocyanates, measured as total dithiocarbamates, were rapidly absorbed reaching peak plasma levels one hour after ingestion of broccoli sprout preparations (Ye et al., 2002). At the highest dose only, the absorption rate constant decreased, and this may explain in part the fact that $C_{\text{max}}$ values did not rise proportionately to the dose. These observations raise the possibility that SFN may to some extent be absorbed by a carrier-mediated transport mechanism that is saturated at this dose level.

Oral absolute bioavailability of SFN in rats was over 80% at the lowest oral dose studied. It is likely that SFN is subjected to modest first-pass metabolism as glutathione and mercapturate metabolites of this isothiocyanate can be generated by intestinal as well as hepatic enzymes, or even possibly in the blood as it contains low levels of glutathione that can interact chemically with the isothiocyanate. Studies using human jejunum in situ have established that SFN is well absorbed by enterocytes where it is conjugated with glutathione during absorption and secreted back into the lumen (Petri et al., 2003).

In the present study, the oral bioavailability of SFN was dose-dependent, being only about 20% at the highest dose studied of 28 µmol/kg, i.e. a quarter of that observed at a dose of 2.8 µmol/kg. These observations imply that intake of SFN supplements may not be as effective as envisaged in achieving high plasma levels of the compound. Isothiocyanates display high protein binding (Ji et al., 2005 and Kassie et al., 2000), presumably because of their facile interaction with $-\text{SH}$ groups, and it is conceivable that
at the higher doses protein-binding sites are saturated so that SFN remains free and available for metabolism and excretion. It is relevant to point out that albumin contains a single residue of cysteine (Cys34). The observed dose-dependent decrease in biological half-life values concord with such a mechanism of action. Non-linear pharmacokinetics in rats have also been reported for PEITC, having an aromatic substituent (Ji et al., 2005), and collectively these observations indicate that the isothiocyanate group is more likely to be responsible for this effect rather than the substituent.

Following intravenous and oral dosing a rapid marked drop was observed in the plasma levels of SFN, and this most likely reflects cellular uptake. Isothiocyanates such as SFN, attain very high intracellular concentrations as a result of their interaction with glutathione (Zhang and Talalay, 1998, Ye and Zhang, 2001 and Zhang and Callaway, 2002). As the absorbed SFN is readily conjugated with glutathione and possibly other thiols, the concentration gradient drives the further cellular uptake of the isothiocyanate, which can achieve mM concentrations, and is accompanied by a marked drop in glutathione levels (Conaway et al., 2005). The glutathione and cysteinylglycine conjugates are exported through membrane transporters such as P-glycoprotein (Zhang, 2000 and Callaway et al., 2004). It has been demonstrated in in vitro studies that peak intracellular levels of isothiocyanates are attained within 3 hours of exposure, and intracellular concentration may be as much as 100-fold higher than extracellular concentration (Zhang and Talalay, 1998). Such extensive intracellular localisation would explain the large apparent volume of distribution. Elimination of SFN was characterised by a long terminal phase; in fact, no major difference was evident in plasma levels between 6 and 24 h following intravenous administration or oral administration at the lower doses. Most likely this was a consequence of protein binding, rendering the isothiocyanate unavailable for elimination through metabolism and excretion.
In summary, the present study demonstrates that in the rat, following oral administration of dietary doses, SFN was rapidly absorbed, achieving high absolute bioavailability at low doses. However, dose-dependent pharmacokinetics was evident, with bioavailability decreasing with increasing dose levels.
Chapter 4: Pharmacokinetics of SFN in humans

Pharmacokinetics of SFN in humans
4.1 Introduction

The possibility of SFN accumulation following repeated daily exposure was indicated by an observation that SFN plasma levels did not significantly differ between 6 and 24 h after a single administration to rats by oral gavage (Chapter 3), and following ingestion of a single portion of broccoli preparations by humans (Gasper et al., 2005 and Janobi et al., 2006). Moreover, in epidemiological studies the chemopreventive effect of Brassica vegetables was related to the level of consumption (reviewed by Conaway et al., 2002 and Higdon et al., 2007). Additionally, suppression of the genotoxic effects of experimental carcinogens by ITCs in animals was noted after their addition to the diet continuously for extended periods of time (Staretz et al., 1997; Sticha et al., 2002 and Dingley et al., 2003). Thus knowledge of the pharmacokinetic behaviour of SFN following repeated intake will enhance our understanding of its mechanism of action. Currently, it is unknown whether SFN accumulates in humans regularly consuming Brassica vegetables, since the previous studies (Conaway et al., 2005; Gasper et al., 2005; Song et al., 2005 and Janobi et al., 2006) focused only on its fate following ingestion of a single serving of broccoli. The present study aimed to evaluate the pharmacokinetic behaviour of SFN in human volunteers following single and repeated intake of commercially available broccoli purchased locally.

A nano-flow LC-tandem mass spectrometry (LC-MS/MS) method was developed and successfully applied to determine SFN concentration in human plasma. Among various tandem mass analysers, triple quadrupole has very good quantitative capabilities in the multiple reactions monitoring (MRM) mode, allowing monitoring of two or more characteristic transitions of each analyte, thus ensuring its unambiguous identification and accurate quantification at low levels in complex matrices (Willoughby et al., 2002).
Chapter 4: Pharmacokinetics of SFN in humans

4.2 Methodology

4.2.1 Broccoli preparation, storage and administration
Raw broccoli florets, purchased locally, were washed, liquidised using a Russell Hobbs blender, strained, divided into portions (300ml) and frozen at -20°C. Each portion of liquidised broccoli was defrosted at room temperature (18 – 22°C) prior to consumption.

4.2.2 Human volunteers
The study was ethically approved by the University of Surrey Ethics Committee (reference EC/2007/41/SBMS) and conducted in the Clinical Investigation Unit (CIU) of the University. All participants gave a written informed consent and their good health was confirmed by their doctors (general practitioners). Six volunteers (Table 4.1) were recruited according to the following criteria:

- Aged between 18-60 years old, male and female
- Non-smoker
- Healthy (no known medical complications)
- Not taking any medication or dietary supplements, with the exception of contraception.
- Must not have donated blood or participated in a trial involving blood collection in the past two months prior to the start of the study.
- Must not be pregnant

Volunteers were asked to attend the Clinical Investigation Unit (CIU) before the start of the study for collection of a blood sample (10 ml) for full blood screening to screen for possible blood disorders. The participants, whose full blood screening results confirmed their suitability for the study, were asked to avoid consumption of ITC-containing vegetables for at least one day before and throughout the study period. The participants were asked to consume 300 ml of liquidised broccoli for 10 consecutive
mornings and blood samples were withdrawn at regular time intervals over 24 h periods on two occasions: after intake of the first and the last portion of broccoli.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Body weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant 1</td>
<td>Female</td>
<td>27</td>
</tr>
<tr>
<td>Participant 2</td>
<td>Female</td>
<td>33</td>
</tr>
<tr>
<td>Participant 3</td>
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<td>46</td>
</tr>
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<td>26</td>
</tr>
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<td>Participant 5</td>
<td>Male</td>
<td>59</td>
</tr>
<tr>
<td>Participant 6</td>
<td>Female</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 4.1: Details of volunteers participating in the study

4.2.3 Blood collection

Blood samples were taken through a cannula inserted into a vein in the forearm on the first day of the study (visit 1). The first sample of blood (15 ml) was taken before the ingestion of broccoli to measure the baseline plasma concentration of SFN, and further 9 blood samples (15ml each) were collected following intake of broccoli at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 h post-dosing. On the second day of the study (visit 2), a single blood sample (24h post-treatment, 15 ml) was taken from a vein in the forearm through a vena puncture. Visits 3 and 4 were the same as visits 1 and 2 (Fig. 4.1).

Figure 4.1: Experimental design of the study into the pharmacokinetic fate of SFN in humans following single and repeated ingestion of broccoli. Healthy volunteers (n=6) consumed liquidised broccoli (300 ml) for 10 consecutive mornings and samples of their blood were collected for up to 24 h following ingestion of the first (visits 1 and 2) and the last (visits 3 and 4) portion. A sample of blood prior to ingestion of broccoli was also obtained.
Chapter 4: Pharmacokinetics of SFN in humans

4.2.4 Sample preparation

Human plasma samples were prepared for analysis by LC-MS/MS following a method described by Janobi et al. (2006). Aliquots of the plasma (0.5 ml) were defrosted on ice and mixed with ice-cold trifluoroacetic acid (10 % v/v) to precipitate plasma proteins. The samples were centrifuged at 10,000 x g for 10 min, the supernatant was decanted, filtered through a 0.22 μm syringe filter (Millex, Millipore corp.) and 1 μl was injected for analysis. Quality control (5.0 and 40.0 ng/ml) and standard (1.0-50.0 ng/ml) solutions of SFN in blank human plasma were also carried through the same sample clean-up procedure. Calibration curves were constructed by plotting the peak area of SFN standards versus their theoretical concentration.

SFN content in the consumed by the volunteers broccoli preparation was determined by adapting method described by Gasper et al. (2005). Aliquots of liquidised broccoli (1.0 ml) were sonicated for 5-7 sec to disrupt any remaining plant tissue, diluted 1000-fold with solvent (95 % water, 5 % acetonitrile and 0.1 % of formic acid), filtered through a 0.22 μm syringe filter (Millex, Millipore corp.), and 1 μl was injected for analysis. Standards (1.0-50.0 ng/ml) of SFN were prepared in the same solvent used for dilution of broccoli preparation.

4.2.5 Determination of SFN in human plasma by LC-MS/MS

Plasma levels of SFN were determined by high-performance liquid chromatography (HPLC)-Chip/MS system, featuring the HPLC-Chip/MS cube interface. Separation of SFN from matrix constituents was achieved using an Agilent 1200 (Agilent Technologies, Germany) LC system equipped with an in-line filter and employing a reusable custom-made chip, containing a pre-column and 150 mm x 75 μm ACE 5 AQ analytical column supplied by Agilent (Agilent Technologies, Germany). Mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (90 % acetonitrile in water
containing 0.1% formic acid). The sample was loaded onto the chip pre-column and then flushed (8 μl) under isocratic conditions (5% of organic solvent B) using a capillary pump operating at a flow rate of 4 μl/min. The analyte was eluted under isocratic conditions (65% of organic solvent B) using a nano pump operating at a flow rate of 0.6 μl/min. Under these conditions, the retention time of SFN was 1.2 minutes in a total run time of 3 minutes.

SFN was detected on-line using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Germany) equipped with an electrospray ionisation source sprayer, operating in the positive ionisation mode. The sprayer voltage was set to 1900 V, gas temperature and pressure were set at 300°C and 4 l/min respectively. The analyte was detected by monitoring the specific transitions in the positive MRM scan mode. The collision energy (CE) and fragmentor voltage (Table 4.2) necessary to maximise the yield of the characteristic transitions (product ions) of the protonated SFN [M + H]^+ precursor ions were optimised by direct infusion of SFN (0.2 μg/ml) dissolved in a solvent comprising 1:1 water:acetonitrile and acidified with formic acid (0.1 % v/v). Data were acquired and qualitatively analysed using the Agilent MassHunter Workstation and the Qualitative Software respectively. The acquired data were quantified using the Agilent MassHunter Quantitative Software (Agilent Technologies, Germany).

<table>
<thead>
<tr>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Fragmentor (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>178.1</td>
<td>119.1</td>
<td>110.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>114.1</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>72.1</td>
<td></td>
<td>21</td>
</tr>
</tbody>
</table>

Table 4.2: Optimal MS/MS parameters for detection of SFN in positive MRM mode


Chapter 4: Pharmacokinetics of SFN in humans

4.2.6 Data analysis

The pharmacokinetic parameters were calculated using a PK Solutions ™ software package (version 2.0, Summit research Services, Ohio, USA). Because of the long terminal phase (mean \( t_{1/2} > 33 \) h following single and repeated ingestion of broccoli), pharmacokinetic parameters of SFN were calculated from the better defined Area Under the Curve (AUC)\(_{0-24}\), rather than from AUC\(_{0-\infty}\) to avoid introducing an inaccuracy in extrapolating the terminal phase to time infinity. AUC\(_{0-24}\) was calculated using the trapezoidal rule utilising the data determined experimentally. Apparent volume of distribution (Vd) was calculated from the equation \( Vd = \frac{FD}{AUC_{0-24}} \), where \( k_{el} \) is the elimination rate constant, \( F \) is the bioavailability and \( D \) is the dose. Plasma clearance (Cl\(_p\)) was determined using the equation \( Cl_p = \frac{FD}{AUC_{0-24}} \). Since bioavailability of SFN in humans following ingestion of broccoli can not be calculated from the available data, it was assumed that absorption of SFN was rapid and complete, i.e. \( F=1 \). Such assumption is based on the previous findings that the bioavailability (\( F \)) of low doses of SFN (0.5 mg/kg) in rats was close to 1. Finally, \( C_{\text{max}} \) and \( T_{\text{max}} \) were determined graphically from the plasma concentration versus time plots.

4.2.7 Statistical evaluation of pharmacokinetic parameter

Data were analysed individually and statistically evaluated using the paired t-test. Comparisons were performed between the pharmacokinetic parameters calculated from the data obtained for all six volunteers after single and following repeated intake of broccoli. The statistical significance is expressed as follows:

\[
\begin{align*}
P &> 0.05 \quad \text{not significant} \\
P &< 0.05 \quad \text{significant (*)} \\
P &< 0.01 \quad \text{very significant (**)}
\end{align*}
\]
4.3 Results

4.3.1 Development and validation of LC-MS/MS method for SFN detection in human plasma

The compound-dependent parameters of an Agilent 6410 triple quadruple mass spectrometer for the MRM scan were optimised using direct infusion of SFN (0.2 µg/ml). SFN spectra (Q1 scan) in the positive mode were acquired over the mass range m/z 100-250 (Fig. 4.2). The protonated SFN ion [M + H]+ of m/z 178.1 (precursor ion) was fragmented to produce SFN-specific product ions of m/z 72, 114 and 119 for monitoring in the multiple reaction monitoring (MRM) mode using the product ion scan operating in the positive mode (Fig. 4.3). SFN was separated from the matrix constituents under isocratic conditions at a flow rate of 0.6 µl/min using the LC system equipped with an in-line filter and employing a reusable custom-made chip, combining a pre-column and an ACE 5 AQ analytical column designed to retain the hydrophilic and weakly hydrophobic molecules. SFN eluting under these conditions produced a good peak shape, high sensitivity and a short run time of 3 min. At the retention time for SFN (1.2 min), there were no interfering peaks on the chromatograms obtained from blank human plasma extracts (Fig. 4.4).

Figure 4.2: Mass spectrum of SFN acquired in the full-scan mode
Figure 4.3: Collision-induced dissociation of SFN. Fragmentation of the \((\text{M+H})^+\) ion of SFN (m/z 178.1) to SFN-specific product ions of m/z 178 → 114.1, 178 → 119.1 and 178 → 72 at collision energy set to 5 (A) and 10 V (B)
Figure 4.4: A representative chromatogram of extracted blank human plasma. No significant matrix interferences for SFN transitions (product ions) of m/z 178 → 72 (A), 178 → 114.1 (B) and 178 → 119.1 (C) were observed at the retention time for SFN (1.2 min).
The limit of quantification (LOQ), defined as the lowest concentration that produces an instrument response with a signal to noise ratio of 5, was 1 pg/injection (Fig. 4.5). The calibration curve for SFN (Fig. 4.6), constructed using the least square linear regression analysis of 7 data points, displayed very good linearity over the tested concentration range (1–50 ng/ml, using the injection volume of 1 μl). The inter-assay variation at two different concentrations (5 and 40 ng/ml) was 9.7 and 7.4 % CV respectively (n=4), whereas the intra-assay variation for the same concentrations was 6.3 and 2.5 % CV respectively (n=4).

Figure 4.5: Representative LC-MS/MS chromatograms of SFN in spiked human plasma at the limit of quantification. 1 μl of 1 pg/ml SFN standard in blank human plasma was injected for analysis. SFN transitions (product ions) of m/z 178 → 119.1 (A), 178 → 114.1 (B) and 178 → 72 were monitored in positive MRM mode.
4.3.2 Pharmacokinetics of SFN humans following single and repeated ingestion of broccoli

The SFN content of liquidised broccoli was 1.3 mg per 100 g (3.9 mg per consumed portion). Analysis of the time-course changes in the plasma levels of SFN in humans, plotted using a logarithmic plot (Fig. 4.7), following both single and repeated ingestion of broccoli, showed that the plasma profile fitted best a two-compartment pharmacokinetic model, as was the case with rats (Chapter 3). The maximum plasma concentration of SFN (Tmax) after ingestion of a single portion of liquidised broccoli was attained in 1.0 to 1.5 h in all six volunteers. Similarly, plasma SFN concentration peaked at 1.0 to 1.5 h following repeated ingestion of liquidised broccoli. After both single and repeated intake of broccoli, plasma levels rapidly declined reaching 50% at around 3 h and falling to about 10-15% from the peak levels by about 8 h, and remained fairly constant between 8 and 24 h (Fig. 4.7), indicating a relatively long terminal phase. However, despite the relatively long terminal phase, the maximum plasma levels of SFN following single or repeated ingestion of broccoli did not significantly differ (Table 4.3), suggesting lack of a cumulative effect.
Chapter 4: Pharmacokinetics of SFN in humans

Volunteer 1

Volunteer 2

Plasma SFN concentration (pg/mL)

Time (h)

---

Single intake

Repeated intake
Chapter 4: Pharmacokinetics of SFN in humans

Volunteer 3

Plasma SFN concentration (pg/ml)

Volunteer 4

Plasma SFN concentration (pg/ml)

Time (h)

Single intake

Repeated intake
Figure 4.7: SFN plasma concentrations in human volunteers following single and repeated ingestion of broccoli versus time curves. Healthy volunteers (n=6) consumed liquidised broccoli (300 ml) for 10 consecutive mornings and blood samples were collected for up to 24 h following ingestion of the first (single intake) and the last (repeated intake) portion. A sample of blood prior to ingestion of broccoli was also obtained. Results are mean ± SD of duplicate determinations.
### Single broccoli intake

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Volunteer 1 (ng/ml)</th>
<th>Volunteer 2 (ng/ml)</th>
<th>Volunteer 3 (ng/ml)</th>
<th>Volunteer 4 (ng/ml)</th>
<th>Volunteer 5 (ng/ml)</th>
<th>Volunteer 6 (ng/ml)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$</td>
<td>14.07</td>
<td>11.32</td>
<td>12.41</td>
<td>10.37</td>
<td>10.65</td>
<td>14.07</td>
<td>12.15 ± 1.65</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>0.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>$k_{\text{ae}}$ (h⁻¹)</td>
<td>2.3</td>
<td>2.3</td>
<td>1.7</td>
<td>2.4</td>
<td>2.1</td>
<td>1.9</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>$k_{\text{el}}$ (h⁻¹)</td>
<td>0.025</td>
<td>0.011</td>
<td>0.027</td>
<td>0.011</td>
<td>0.020</td>
<td>0.054</td>
<td>0.025 ± 0.016</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>27.4</td>
<td>62.9</td>
<td>25.3</td>
<td>62.3</td>
<td>34.4</td>
<td>12.8</td>
<td>37.5 ± 20.6</td>
</tr>
<tr>
<td>$Cl_p$ (ml/h)</td>
<td>55.4</td>
<td>54.4</td>
<td>58.5</td>
<td>65.8</td>
<td>80.1</td>
<td>52.0</td>
<td>61.0 ± 10.5</td>
</tr>
<tr>
<td>$V_d$ (l)</td>
<td>2.19</td>
<td>4.94</td>
<td>2.14</td>
<td>5.91</td>
<td>3.98</td>
<td>0.96</td>
<td>3.35 ± 1.90</td>
</tr>
<tr>
<td>AUC₀⁻₂₄ (ng ml⁻¹ h)</td>
<td>70.39</td>
<td>71.69</td>
<td>66.61</td>
<td>59.31</td>
<td>48.71</td>
<td>75.03</td>
<td>65.29 ± 9.74</td>
</tr>
</tbody>
</table>

### Repeated broccoli intake

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Volunteer 1 (ng/ml)</th>
<th>Volunteer 2 (ng/ml)</th>
<th>Volunteer 3 (ng/ml)</th>
<th>Volunteer 4 (ng/ml)</th>
<th>Volunteer 5 (ng/ml)</th>
<th>Volunteer 6 (ng/ml)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$</td>
<td>15.54</td>
<td>12.84</td>
<td>13.16</td>
<td>9.68</td>
<td>9.33</td>
<td>13.93</td>
<td>12.41 ± 2.44</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>$k_{\text{ae}}$ (h⁻¹)</td>
<td>3.7</td>
<td>4.6</td>
<td>1.7</td>
<td>2.2</td>
<td>3.0</td>
<td>2.0</td>
<td>2.9 ± 1.2</td>
</tr>
<tr>
<td>$k_{\text{el}}$ (h⁻¹)</td>
<td>0.041</td>
<td>0.016</td>
<td>0.027</td>
<td>0.010</td>
<td>0.032</td>
<td>0.030</td>
<td>0.026 ± 0.011</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>17.0</td>
<td>43.7</td>
<td>25.4</td>
<td>67.8</td>
<td>21.5</td>
<td>23.2</td>
<td>33.1 ± 19.3</td>
</tr>
<tr>
<td>$Cl_p$ (ml/h)</td>
<td>63.4</td>
<td>53.8</td>
<td>54.7</td>
<td>64.3</td>
<td>71.7</td>
<td>57.6</td>
<td>60.9 ± 6.8</td>
</tr>
<tr>
<td>$V_d$ (l)</td>
<td>1.56</td>
<td>3.42</td>
<td>2.00</td>
<td>6.26</td>
<td>2.22</td>
<td>1.93</td>
<td>2.90 ± 1.76</td>
</tr>
<tr>
<td>AUC₀⁻₂₄ (ng ml⁻¹ h)</td>
<td>61.56</td>
<td>72.00</td>
<td>71.25</td>
<td>60.97</td>
<td>54.36</td>
<td>67.73</td>
<td>64.65 ± 6.87</td>
</tr>
</tbody>
</table>

Table 4.3: Pharmacokinetic parameters of SFN in humans following single and repeated ingestion of liquidised broccoli. Healthy volunteers (n=6) consumed liquidised broccoli (300 ml) for 10 consecutive mornings and blood samples were collected for up to 24 h following ingestion of the (single intake) and the last (repeated intake) portion. A sample of blood prior to ingestion of broccoli was also obtained.
4.4 Discussion

An analytical LC-MS/MS method that allows determination of SFN present at low concentrations in human plasma was developed, validated and employed to determine its pharmacokinetic behaviour following single and repeated ingestion of commercially available broccoli.

In the current study SFN was rapidly absorbed attaining peak plasma concentration levels between 60 and 90 min following either single or repeated ingestion of broccoli. This concords with previous studies in humans, where plasma ITCs, measured as total dithiocarbamates (ITCs and their thiol conjugates) (Ye et al., 2002) or as SFN (Gasper et al., 2005 and Janobi et al., 2006) reached peak levels by 1.0 to 2.0 hour after ingestion of raw or lightly microwaved broccoli. However, the rate of absorption appears to vary considerably and can be significantly less rapid. ITCs, measured as total plasma dithiocarbamates, peaked between 2 to 6 h after ingestion of Broccoli sprouts (Conaway et al., 2000). The discrepancy in the absorption rate between the studies by Conaway et al. (2000) and Ye et al. (2002) can not be attributed to differences in methodology and study design because the amount of ITCs ingested by each volunteer (about 0.2 mmol) and the methodology (both studies used cyclocondensation method) for determining total plasma ITCs is sufficiently similar between the latter study and the study by Ye et al. (2002). The cyclocondensation method lacks discrimination between individual ITCs and their mercapturates (Conaway et al., 2000 and Ye et al., 2002). The increase in time it takes to reach the \( C_{\text{max}} \) may be due to inter-individual differences in the GST phenotype. Human GSTA1, GSTA2, and to a lesser extent GSTM, can bind to and sequester glutathione conjugates of ITCs (Meyer et al., 1995). Consequently, in GSTA and GSTM-positive individuals such sequestered GS-ITC conjugates could be released from the cells into plasma after a time lag.
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In the rat study (Chapter 3), the pharmacokinetic behaviour of SFN was dose-dependent (Chapter 3), with the decline in plasma levels becoming steeper at lower dose levels. The average intake of SFN in the current study was approximately 0.07 mg/kg, which is nearly an order of magnitude lower than the lowest (0.5 mg/kg) dose administered to rats; however, the decline in plasma SFN levels in humans was considerably less steep compared with the rat, raising several possibilities: 1) the intact glucosinolates were present in the broccoli preparation, 2) most of the absorbed SFN dose may be bound to plasma proteins, limiting its availability for metabolism, and/or a substantial part of the SFN dose may be absorbed from the gut after a time lag following dissociation of the reversible complexes with food constituents. As for the first possibility, the absorption of SFN, formed from its precursor glucoraphanin as a result of myrosinase activity of the colon bacteria (Rungapam et al., 2007) has been shown to be delayed by a few hours (Ye et al., 2002 and Rouzaud et al., 2004). Consequently, presence of intact glucosinolates in the broccoli preparation could distort the pharmacokinetic behaviour of SFN at the absorption/distribution phase. Glucoraphanin content of broccoli preparation remains to be determined in order to establish whether its was fully converted to SFN. However, the broccoli florets used in the current study were liquidised to maximise myrosinase-dependent formation of SFN from its glucosinolate precursor, so that most of SFN in such broccoli preparation had been formed and was available for immediate absorption (Rungapam et al., 2007). The second possibility is supported by the observation that the Vd of SFN in humans was significantly lower compared with rats, implying that most of the absorbed dose could be bound to plasma proteins. SFN, being highly reactive towards thiol groups of proteins, readily forms reversible dithiocarbamates (Thornalley, 2002; Kassie et al., 2000 and Zhang, 2001) and is likely to bind to plasma albumin. Thus the amount of “free” SFN capable of entering cells and available for metabolism would be limited to the rate of spontaneous dithiocarbamates dissociation. It should also be noted that the elimination of SFN may be enhanced by
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binding non-enzymatically to thiols such as homocysteine, cysteine, cysteineglycine and glutathione, which are present in plasma at micromolar levels. However, given that most plasma thiols exist either in the oxidised form or are bound to plasma proteins and are unavailable for binding (Mansoor et al., 1992, Andersson et al., 1993 and Di Giuseppe et al., 2004), the extent of such binding is unlikely to be high. Finally, ITCs readily form reversible complexes with thiol groups of macromolecules (Thornalley, 2002). Such non-specific binding to food constituents may limit immediate availability of ITCs for absorption. The bound fraction of ITC dose is likely to be absorbed after a time lag, following dissociation ITC-macromolecule complexes (Johnson, 2002).

Maximal concentration of SFN achieved in human plasma after either single or repeated ingestion of broccoli did not exceed 80 nmol/l. This level is considerably lower than was previously reported in humans after ingestion of various broccoli preparations (Gasper et al., 2005 and Janobi et al., 2006). The observed discrepancy is likely to be due to a number of factors influencing the glucosinolate content of broccoli. Up to a third of glucosinolates can be lost during storage (Song and Thornalley, 2007). Broccoli used in the current study was purchased from a supermarket and is likely to have been stored for some time prior to sale, whereas broccoli used in the previous human trials was freshly harvested or of a variety selected for its high glucosinolate content. Furthermore, a number of additional factors significantly influence the glucosinolate content and the extent of their conversion to isothiocyanates, such as the cultivar, age of the plant, weather, soil and processing conditions of broccoli (Howard et al., 1997; Song and Thornalley, 2007). Consequently, the glucosinolate and SFN content of broccoli vary widely. The SFN content of Broccoli used in the current study was 1.3 mg per 100 g, but could be as high as 49 mg/100 g (Howard et al., 1997). In the present study, broccoli was obtained from a local supermarket in order to simulate the levels of intake by the vast majority of consumers.
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There was no significant difference between the pharmacokinetic profiles of SFN following a single and repeated ingestion of broccoli. Although SFN plasma levels did not significantly decrease between 8 and 24 h, suggesting that it may accumulate after repeated intake, this phenomenon was not observed. Considering that the rate of either \textit{in vivo} or \textit{in vitro} dithiocarbamate dissociation to release "free" ITC that can enter the cell is slow (Ye et al., 2002), binding to plasma proteins such as albumin, which contains a single residue of free cysteine (Sugio et al., 1999), can be regarded as possible mechanism of ITC accumulation. Moreover, the low Vd of SFN (about 3 l) in humans was consistent with its plasma localisation as a result of extensive protein binding. It appears that the dose of SFN (0.07 mg/kg for a 70 kg individual) employed in the current study was insufficient for clear manifestation of the accumulation effect.

It is not clear whether the pharmacokinetic behaviour of SFN in humans may be dose-dependent, as was noted in rats, and/or species-specific. The apparent Vd of SFN in rat (Chapter 3) was clearly dose-dependent and large (25 l at 0.5 mg/kg dose level), whereas in humans following either single or repeated Broccoli intake it was significantly smaller (3 l). At higher dose levels achieved after consumption of Broccoli sprouts, the apparent Vd of total ITCs in human was about 60 l, indicating possible distribution throughout the total body water (Ye et al., 2002). However, the cyclocondensation method used by Ye et al., (2002) to determine plasma ITC levels does not allow the differentiation between individuals ITCs and their mercapturates; thus it is impossible to relate the apparent Vd of total dithiocarbamates to that of SFN. Subsequent studies on metabolic fate of SFN in humans after ingestion of various broccoli preparations used more sensitive LC/MS/MS methodology specific for SFN, but, unfortunately, no PK parameters were calculated (Gasper et al., 2005; Song at al., 2005 and Janobi at al., 2006), although the SFN plasma levels after ingestion of "standard" broccoli appear to decline slower compared to "high" glucosinolate broccoli (Gasper et al., 2005).
Chapter 4: Pharmacokinetics of SFN in humans

suggesting that the pharmacokinetic behaviour of SFN in humans may be dose-dependent. Additional studies are required to establish whether pharmacokinetic behaviour of SFN in humans is dose-dependent.

Biphasic elimination of SFN in humans following intake of broccoli, characterised by a long terminal phase, is most likely a consequence of protein binding, limiting its metabolism and excretion. Non-linear and dose-dependent (Ji and Morris, 2003 and Ji et al., 2005) pharmacokinetic behaviour in rats has also been reported for PEITC, which has an aromatic substituent, and collectively these observations indicate that the isothiocyanate group is responsible for this effect rather than the substituent. A recent study conducted in our laboratory (N. Konsue, personal communications) indicated that maximum PEITC plasma concentrations (Cmax) in rats were higher after repeated oral administration.

In summary, the results of the present study show that SFN does not appear to accumulate in humans after repeated consumption of Broccoli, most likely reflecting its low SFN content. The overall pharmacokinetic behaviour of SFN in humans, following either single or repeated ingestion of broccoli, and in rat appear to be similar, although, in view of significant differences in dose levels, it is not possible to reach a definite conclusion.
Chapter 5

In vivo modulation of rat hepatic and pulmonary xenobiotic-metabolising enzyme systems by erucin and SFN
Chapter 5: Modulation of xenobiotic-metabolising enzymes by erucin and SFN

5.1 Introduction

Previous studies have demonstrated that SFN is an effective suppressor of chemically-induced tumour development in rodents (Zhang et al., 1994; Chung et al., 2000; Fahey et al., 2002, Hecht et al., 2002; Changjiang et al., 2006; Dinkova-Costova, 2006 and Shen et al., 2007). Of the various chemopreventive mechanisms of ITCs operating at different stages of tumourigenesis, the ability of these phytochemicals to suppress generation and/or to enhance detoxification and elimination of mutagens is of paramount importance at the initiation stage. Both, inhibition of cytochromes P450 (Barcelo et al., 1996, Jiao et al., 1996, Maheo et al., 1997 and Zhou et al., 2006) and marked induction of Phase II enzymes such as QR and GST (Zhang et al., 1992; Zhang et al., 1994; Gerhauser et al., 1997; Fahey et al., 1997; Gao and Talalay, 2004; Maheo et al., 1997; Rose et al., 2000; Brooks et al., 2001; Misiewicz et al., 2004; Munday and Munday, 2004; Ritz et al., 2006 and Dinkova-Kostova et al., 2007) have been reported previously following exposure to SFN. Even when administered at dose levels that approximate human intake, sulforaphane augments activity of antioxidant enzymes such as QR (Yoxall et al., 2005) and GST (Zhang and Talalay, 1998).

While SFN is one of the most extensively researched ITCs, surprisingly few studies evaluated the effect of erucin on XMEs. It appears that both compounds are equipotent inducers of GST and OR activities when administered to rodents (Zhang et al., 1992 and Munday and Munday, 2004). Considering that in the Western diet, most cruciferous vegetables, the major source of the glucosinolate precursor of SFN, are usually consumed cooked, erucin has an important advantage over SFN in that rocket salad (Eruca sativa), the principal source of the glucosinolate precursor of erucin glucosinolate (Vaughn and Berhow, 2004), is ingested raw. Thermal processing of vegetables leads to a substantial loss of glucosinolates, but more importantly, inactivates plant myrosinase, the enzyme that converts the glucosinolates to the active ITCs and, consequently, ITC
bioavailability is substantially reduced, as only 10-20% of all glucosinolates are converted to ITCs by mammalian gut bacteria (Conaway et al., 2000 and Shapiro et al., 2001).

Since previous in vitro (Zhang at al., 1992 and Jakubikova et al., 2005) and in vivo (Zhang at al., 1992 and Munday and Munday, 2004) studies focussed only on modulation of Phase II enzyme systems, the aim of this study was to evaluate the potential of erucin to modulate cytochrome P450 enzymes as well as Phase II enzymes in the liver, the principal site of xenobiotic metabolism, and the lung, the target tissue in ITC-mediated chemoprevention (Higdon et al., 2007), when administered to rats at dietary relevant doses. Moreover, the effect of erucin on activity and expression of XME is compared to that of sulforaphane.

Moreover, as isothiocyanates, with either aliphatic or aromatic side chain, can be metabolically converted to intermediates that can inactivate the cytochrome P450 (Goosen et al., 2000, 2001; Moreno et al., 1999), the ability of erucin to act as a mechanism-based inhibitor was evaluated and compared with sulforaphane. Finally, the ability of erucin to modulate the CYP1A-associated metabolic activation of the heterocyclic amine IQ to mutagens was evaluated using the Ames mutagenicity assay.

5.2 Methods

5.2.1 Test compound formulation and storage

The control group of animals received tap water containing the solvent vehicle only (DMSO, 0.05% v/v). Stock solutions of erucin and sulforaphane were prepared in DMSO (300mg/ml w/v) and diluted with tap water to achieve final concentrations of 30mg/l and 150mg/l (v/v) for erucin, corresponding to approximate daily doses of 3 and 15
mg/kg respectively, and 150mg/l for sulforaphane, approximating daily dose of 15 mg/kg. All aqueous solutions of ITCs were prepared in advance and stored at – 20°C as aliquots that were defrosted as required at 4°C overnight prior to administration.

5.2.2 Animal receipt and study design

The commercial source and the conditions at which the animals were maintained during acclimatisation and study periods are described in section 2.2.1. Twenty male Wistar albino rats (about 180g) were randomly assigned to 4 groups of 5 animals as follows:

- **Group A:** Control, tap water and normal standard rodent diet
- **Group B:** Low dose of erucin – 30mg/litre in tap water and standard rodent diet
- **Group C:** High dose of erucin – 150mg/litre in tap water and normal rodent diet
- **Group D:** High dose of SFN – 150mg/litre of tap water and normal rodent diet

After the acclimatisation period of 4 days, the animals were maintained on the respective treatment regimes for 10 days. At the end of the study, animals were killed by cervical dislocation and their terminal body weights were recorded. Livers and lungs were excised and placed in ice-cold solution of KCl (1.15%, w/v). The tissues were processed and stored as described in section 2.2.6.

5.2.3 Determination of XME activities

The dealkylations of methoxy-, ethoxy-, pentoxy-resorufin and 7-benzyloxyquinoline were determined using the microsomal fraction as described in sections 2.2.7 and 2.2.8. The following assays were performed in the cytosol: QR using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as substrate, glutathione S-transferase activity using as accepting substrates CDNB, DCNB and NBD-Cl, and total glutathione (sections 2.2.10-2.2.12). Protein concentration in both fractions was
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determined as described in section 2.2.6. Finally, changes in enzyme protein expression
were identified and quantified by Western blot analysis (section 2.2.13).

5.2.4 Inhibition of CYP1A1 by erucin and SFN

The methodology of assessing whether erucin and SFN could function as competitive
and/or NADPH-dependent inhibitors of CYP1A1 in Aroclor-1254 induced rat hepatic
microsomes is described in Chapter 2 (sections 2.2.7.1 and 2.2.7.2). The ability of
 oxidised and reduced glutathione to prevent inhibitory effect of these compounds on rat
CYP1A1 activity was determined as detailed in section 2.2.7.2.

5.2.5 Ames mutagenicity studies

Mutagenic activity elicited by IQ was monitored using the Ames mutagenicity assay in
the presence of an activation system containing 10 % (v/v) hepatic S9 preparations
from control, erucin- and Aroclor 1254-treated rats, and employing Salmonella
typhimurium YG1024 as the indicator strain, as detailed in section 2.2.14. The Aroclor
1254-induced hepatic S9 fraction was prepared from the livers of W/A rats injected
intraperitonealy with a single dose of Aroclor 1254 (0.5 g/kg of body weight) dissolved in
corn oil (0.2 g/ml), and killed on the 5th day after dosing.

5.2.6 Inhibition of cytochrome P450 by erucin and SFN

The methodology for assessing whether erucin and SFN could function as competitive
and/or NADPH-dependent inhibitors of CYP1A1 in Aroclor-1254 induced rat hepatic
microsomes is described in Chapter 2 (sections 2.2.7.1 and 2.2.7.2). The ability of
oxidised and reduced glutathione to reverse inhibitory effect of erucin and SFN on rat
CYP1A1 activity was determined as detailed in section 2.2.7.2.
5.3 Results

5.3.1 Effects of erucin and sulforaphane on water-intake and body weight

The animals remained in good health throughout the duration of the study; no clinical or behavioural signs of systemic toxicity were apparent among the different treatment groups. The body weight and water consumption were recorded daily (on weekdays only). Addition of test compound to the drinking water did not deter the animals from drinking; consequently, although the water consumption fluctuates (Fig. 5.1 A), the fluctuation pattern does not significantly differ among the groups. All animals progressively gained weight (Fig. 5.1 B) at the expected rate, with no significant differences between the groups.

Figure 5.1: Body weight gain (A) and intake of water (B) supplemented with erucin or sulforaphane by rats. Male Wistar albino rats were administered erucin (30 or 150 mg/l), SFN (150 mg/l) or solvent vehicle DMSO, 0.05% v/v) in drinking water for 10 days. Results are presented as the means for five animals, but error bars have been omitted for clarity.
5.3.2 Modulation of rat hepatic XMEs activity and expression by dietary doses of erucin and sulforaphane

Treatment of rats with either compound failed to modulate the hepatic dealkylations of methoxy-, ethoxy- or pentoxy resorufin and of 7-benzoyloxyquinoline (Fig. 5.2). Immunoblot analysis employing antibodies to rat CYP1A1 recognised a single band, presumably CYP1A2, whose levels were modestly (30%) elevated by the highest

![Graphs showing effects of erucin treatment on EROD, MROD, PROD and 7-BQ activities.](image)

**Figure 5.2: Effect of treatment with erucin on activities of rat hepatic cytochrome P450 enzymes.** Male Wistar albino rats were administered erucin (30 or 150 mg/l), SFN (150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 10 days. EROD (A), MROD (B), PROD (C) and 7-BQ (D) activities were determined in microsomal fraction using ethoxy-, methoxy- or pentoxyresorufin and 7-benzyloxyquinoline as respective probes. Results are presented as mean ± SD, where n=5. *P<0.05.
dose of erucin. SFN also significantly elevated (100%) CYP1A2 expression at the highest dose level (Fig. 5.4 A). A single band was also recognised by the antibodies to CYP1B1, expression of which increased to a similar extent following treatment of the animals with the higher dose of erucin and SFN (Fig. 5.4 A). Treatment with erucin failed to modulate the levels of the CYP2B1 and CYP3A2 proteins, detected as single bands by the respective anti-rat serum (Fig. 5.4 A).

Figure 5.3: Effect of treatment with erucin on rat hepatic phase II enzyme activities.
Male Wistar albino rats were administered erucin (30 or 150 mg/l), SFN (150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 10 days. The activities of GSTs, analysed using CDNB (A), DCNB (B) or NBD-CI (C) as accepting substrates, QR (D) and total glutathione (E) were determined in the cytosolic fraction. Results are presented as mean ± SD, where n=5. *, P<0.05, **, P<0.01 and ***, P<0.001.
Figure 5.4: Modulation of rat hepatic cytochrome P450 (A) and Phase II (B) enzymes by treatment with ITCs. Rats were exposed to erucin or SFN for 10 days through their drinking water. Hepatic microsomal (A) and cytosolic (B) proteins were resolved by SDS-PAGE before being transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using antibodies to cytochrome P450 proteins, followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 5 (CYP2B1, CYP3A2, GST\(\alpha\) and GST\(\mu\)) or 40 \(\mu\)g (CYP1A, CYP1B1, GST\(\eta\) and QR) of total protein. Molecular markers were run concurrently to aid band identification. The values in italics show optical density percentage of each band relative to control.
Total glutathione levels and cytosolic glutathione S-transferase activity in the liver, determined using CDNB or DCNB as substrates, were unaffected by either, erucin or SFN treatment; however, when NBD-Cl was used as a substrate, activity was significantly enhanced by SFN treatment only. Both compounds augmented quinone reductase activity (Fig. 5.3). Immunoblot analysis indicated that erucin, at both dose levels, as well as SFN, up-regulated the levels of GSTα, GSTμ, and, in particular, QR, while GSTπ was unaffected (Fig. 5.4 B).

5.3.3 Modulation of pulmonary XME activities and expression in rats treated with dietary doses of erucin and sulforaphane

In the lung, neither isothiocyanate modulated the O-dealkylation of the alkoxyresorufins (Fig. 5.5); 7-benzyloxyquinoline demethylase was not detectable in this tissue. Immunoblot studies revealed that erucin, at both doses, as well as sulforaphane elevated pulmonary CYP1A1 apoprotein levels (Fig. 5.7 A). Similarly, the higher erucin dose and sulforaphane increased the band recognised by antibodies to CYP1B1 by 80 and 70% respectively. No changes were evident in the expression of CYP2B1 or CYP3A2 (Fig. 5.7 A).

Neither GST activity, monitored with CDNB or NBD-Cl, nor total glutathione levels were influenced by the isothiocyanate treatment. Quinone reductase activity, which was higher in the lung compared with the liver, was clearly elevated by the treatments with erucin or sulforaphane (Fig. 5.6). The increase in quinone reductase activity was paralleled by a similar rise in protein levels determined by immunoblot, which more than doubled in animals treated with the high erucin dose and sulforaphane respectively, in comparison with controls (Fig. 5.7 B). Erucin, at the higher dose, similar to sulforaphane, raised the levels of GSTα by 25-30% whereas GSTπ and GSTμ were unaffected (Fig. 5.7 B).
Figure 5.5: Effect of treatment with erucin and sulforaphane on rat pulmonary cytochrome P450 enzyme activities. Male Wistar albino rats were administered erucin (30 or 150 mg/l), SFN (150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 10 days. EROD (A) and PROD (B) activities were determined in microsomal fraction using ethoxy- or pentoxyresorufin as respective probes. Results are presented as mean ± SD for five rats. *P<0.05.

Figure 5.6: Effect of treatment with erucin and sulforaphane on rat pulmonary phase II enzyme activities. Male Wistar albino rats were administered erucin (30 or 150 mg/l), SFN (150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 10 days. The activities of GSTs, analysed using CDNB (A) or NBD-Cl (B) as accepting substrates, QR (C) and total glutathione levels (D) were determined in the cytosolic fraction. Results are presented as mean ± SD, where n=5. *, P<0.05 and **, P<0.01.
Figure 5.7: Modulation of rat pulmonary cytochrome P450 (A) and Phase II (B) enzymes by treatment with erucin and sulforaphane. Rats were exposed to erucin or sulforaphane through their drinking water for 10 days. Pooled pulmonary microsomal (A) and cytosolic (B) proteins were resolved by SDS-PAGE before being transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. Immunoblot analysis was carried out using antibodies to cytochrome P450 proteins, followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 5 (CYP2B1, GSTα and GSTµ), 20 (CYP3A2, GSTα and QR) or 40 µg (CYP1A and CYP1B1) of total protein. Molecular markers were run concurrently to aid band identification. The values in italics show optical density percentage of each band relative to control.
5.3.4 Inactivation of EROD by erucin and SFN

To investigate whether the observed decrease in B(a)P-mediated up-regulation of CYP1A1 by ITCs was associated with their potential to act as competitive and/or mechanism-based inhibitors of this enzyme, the inhibitory activity of erucin and SFN (25 and 50 μM) on CYP1A1-mediated O-deethylation of ethoxyresorufin (0-2 μM), was determined in vitro using hepatic microsomes from Aroclor-1254 induced rats. Michaelis-Menten constant ($K_m$) and $V_{max}$ values (Table 5.1), obtained from the linear Lineweaver-Burk plot (Fig 5.8), were not influenced by erucin, indicating lack of direct inhibitory effect of these phytochemicals on CYP1A1 enzyme activity.

The potential of erucin and SFN to function as mechanism-based inactivators of CYP1A1, was evaluated in hepatic microsomes prepared from Aroclor 1254-induced rats by monitoring the rate of CYP1A1-mediated O-deethylation of ethoxyresorufin following pre-incubation of ITCs in the presence of NADPH. Mechanism-based enzyme inactivation is characterised by a metabolic conversion of the inactivator to a reactive intermediate in the presence of NADPH. After 0.5 h pre-incubation of hepatic microsomes with erucin or SFN (0-100 μM), EROD activity declined in a concentration-dependent manner, with only 20 and 30% of residual EROD activity detected at 100 μM erucin and SFN respectively (Figs. 5.9 A and B). The ITC concentration required to achieve a 50 % inhibition of EROD activity was about 25 μM.

In further studies, hepatic microsomes, prepared from Aroclor 1254-induced rats, were pre-incubated for different time periods, up to 1 h, at 37 °C in the presence of erucin or SFN (0 or 25 μM) and NADPH, to determine whether NADPH-dependent EROD inhibition by ITCs is time-dependent. Although both erucin and SFN significantly inhibited EROD activity in a time-dependent manner (EROD activity was about 50 % at 30 min), erucin displayed a stronger inhibitory activity compared with SFN (Fig. 5.9 C).
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Figure 5.8: Lineweaver-Burk plot of EROD activity in hepatic microsomes in the presence of erucin (A) or SFN (B). The rate of ethoxyresorufin O-deethylation was monitored in Aroclor 1254-induced microsomes in the absence and presence of erucin (10 and 25 μM), employing multiple ethoxyresorufin concentrations (0-2 μM). Each point represents the mean ± SD of triplicate determinations.

The potential of reduced and oxidised glutathione to prevent ITC-mediated inhibition of O-deethylation of ethoxyresorufin was evaluated using Aroclor 1254-induced rat liver microsomes pre-incubated in the presence of erucin or SFN (25 μM), NADPH and
reduced or oxidised glutathione (0-200 µM) for 0.5 h. Reduced glutathione prevented inhibition of microsomal EROD by erucin and SFN in a concentration-dependent manner, restoring activity to control values at glutathione concentrations of 100 to 200 µM (Fig. 5.10 A). In contrast, oxidised glutathione failed to prevent ITC-mediated loss of EROD activity under similar experimental conditions (Fig. 5.10 B).

### Table 5.1: Effect of erucin (A) and SFN (B) on EROD kinetics in Aroclor 1254-induced rat hepatic microsomes.

The rate of ethoxyresorufin O-deethylation was monitored in the absence and presence of SFN (10 and 25 µM) over multiple substrate concentrations (0-2 µM). $K_m$ and $V_{max}$ values were determined by non-linear curve fitting. The values represent mean ± SD of triplicate determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3031 ± 173</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>10 µM erucin</td>
<td>2929 ± 165</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>25 µM erucin</td>
<td>2878 ± 169</td>
<td>0.28 ± 0.06</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3031 ± 173</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>10 µM SFN</td>
<td>2972 ± 227</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>25 µM SFN</td>
<td>3058 ± 183</td>
<td>0.32 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 5.9: Effect of pre-incubation on the inhibition of the hepatic O-deethylation of ethoxyresorufin by erucin and sulforaphane. Microsomes from Aroclor 1254-treated rats, supplemented with NADPH, were pre-incubated for 30 minutes in the presence of erucin (A) or sulforaphane (B) (0-100 μM), and reaction was initiated by addition of ethoxyresorufin. The 100% activity after a 30-minute pre-incubation in the two studies was 2.74 ± 0.20 and 3.18 ± 0.21 nmol/min per mg protein. C. Microsomes from Aroclor 1254-treated rats, fortified with NADPH, were pre-incubated for various periods of time (0-60 minutes) in the absence or presence of erucin or sulforaphane (25 μM), and reaction was initiated with the addition of the substrate. Results are presented as mean ± SD of triplicate determinations. *, P<0.05; **, P<0.01, ***, P<0.001.
Figure 5.10: Effect of glutathione on the erucin- and sulforaphane-mediated inhibition of the O-deethylation of ethoxyresorufin. Microsomes from Aroclor 1254-treated rats, fortified with NADPH, were pre-incubated for 30 minutes in the presence of erucin or sulforaphane (25 μM), and reduced (A) or oxidised (B) glutathione (0-200 μM); reaction was initiated with the addition of ethoxyresorufin. The 100% activity after a 30-minute pre-incubation in the two studies was 2.33 ± 0.08 and 3.25 ± 0.17 nmol/min per mg protein. Results are presented as mean ± SD of triplicate determinations. *, P<0.05; **, P<0.01, ***, P<0.001.
5.3.4 Effect of pre-treatment with erucin and SFN on mutagenicity of IQ

The bioactivation of the heterocyclic amine IQ to genotoxic intermediates in the Ames test was determined in the presence of hepatic post-mitochondrial preparations from control, Aroclor 1254-, erucin- and sulforaphane-pretreated rats. A concentration-dependent mutagenic response, induced by IQ, was evident in the presence of all activation systems, but the Aroclor 1254 system was clearly the most efficient; treatment with either isothiocyanate caused a modest decrease in mutagenic response (Fig. 5.11).

Figure 5.11: Effect of erucin treatment on the rat hepatic activation of IQ to mutagenic intermediates in the Ames test. Rats were exposed to erucin, at two dose levels, or SFN through their drinking water for 10 days. Mutagenic activity was determined in the presence of activation systems containing hepatic S9 (10%, v/v) derived from control, Aroclor 1254- (positive control) and erucin-treated rats, Salmonella typhimurium YG1024 and IQ (100-1000 ng/plate). Mutagenic activity of IQ (1000 ng/plate) was 7596 ± 1240 revertant colonies per plate in the presence of Aroclor 1254-induced activation system. The spontaneous reversion rate of 48 ± 8 has already been subtracted. Results are presented as mean ± SD for triplicate plates.
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5.4 Discussion

The aim of this study was to evaluate the potential of the aliphatic isothiocyanate erucin to modulate glutathione S-transferase and quinone reductase, two pivotal enzymes in the detoxication of reactive intermediates and prevention of oxidative stress, and cytochromes P450, the most important enzyme system in the generation of these intermediates. The choice of Phase I enzymes monitored in this project was based on the following considerations: the role in bioactivation of pro-carcinogens, the possibility of interactions with drugs and the impact on the biotransformation of xenobiotic compounds.

Activities and expression levels of CYP1A1, CYP1B1 and CYP2B1 enzymes were monitored in this project because of their role in bioactivation of pro-carcinogens. The CYP1 family is involved in bioactivation of polycyclic aromatic hydrocarbons, aromatic and heterocyclic amines to highly mutagenic chemical species (reviewed by Nebert et al., 2004 and Ma and Lu, 2007). CYP2B1 is involved in bioactivation of human environmental and dietary carcinogenic N-nitrosamines (Parkinson, 1996). Finally, since about 60% of all prescription drugs are hydroxylated by human CYP3A4 (Guengerich, 1999), effect of ITCs on rat CYP3A2 enzyme was determined to evaluate the potential of interactions with drugs in humans as a result of altered biotransformation of xenobiotics.

Since the liver is the principal site of metabolism and the lung is a target tissue in ITC-associated chemoprevention (Higdon et al., 2007), this project focussed on these two rodent tissues. The effects of erucin on hepatic and pulmonary enzymes, following exposure to dietary doses, were compared with those of sulforaphane. Previous studies in mice have indicated the potential of erucin and sulforaphane, at least at high doses administered by gavage, to stimulate quinone reductase and glutathione S-transferase
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activities in the liver and other tissues (Zhang et al., 1992), but this is the first study evaluating the potential of this isothiocyanate to modulate cytochrome P450 enzymes. Both isothiocyanates were given to rats in the drinking water at dose levels corresponding to the estimated human total ITC intake (Howard et al., 1997). Since the human consumption levels of erucin or glucoerucin have not been defined, the doses employed in these studies were based on the consumption of sulforaphane, a structurally similar isothiocyanate. A 250g serving of fresh broccoli will release 93-187 mg of sulforaphane (Howard et al., 1997), so that the intake for a 70 kg individual would be 1.3-2.7 mg/kg. The lower dose of erucin employed in the current study of approximately 3 mg/kg represents such dietary level of intake.

None of the cytochrome P450 activities studied was modulated by treatment with either ITC in the liver or lung. Hepatic and pulmonary CYP1A1 protein levels, determined immunologically, were, however, elevated by both compounds. CYP1B1 could not be monitored at the activity level as a selective substrate is not available, but immunoblot analysis indicated that treatment with SFN and erucin at the higher dose, elevated the levels of this enzyme in the liver. In the lung, both isothiocyanates led to an increase in the single band detected by the CYP1B1 antibodies. Neither compound had any effect on expression of either hepatic or pulmonary CYP2B or CYP3A2 protein. It is important to note that no statistical evaluation was performed on the western blots since only a single pooled sample was analysed per each group. Therefore, only variation in the immunologically detected amount of protein greater than 10-15% relative to control was interpreted as an indication of a likely treatment-induced change.

As isothiocyanates, with either aliphatic or aromatic side chain, can be metabolically converted to intermediates that cause cytochrome P450 inhibition, the ability of erucin to act as a mechanism-based inhibitor was evaluated and compared to sulforaphane.
The Km values for the O-deethylation of ethoxyresorufin in hepatic microsomes of Aroclor 1254-induced rats in the presence or absence of ITCs did not significantly differ, indicating lack of competitive inhibition of CYP1A1 enzyme by erucin or SFN, at least at concentrations up to 25 μM. Interestingly, SFN also failed to modulate CYP1A1 activity in microsomal preparations (Langouet et al., 2000), but not in rat hepatocytes (Jiao et al., 1996, Maheo et al., 1997 and Zhou et al., 2006), suggesting a possibility that SFN metabolite(s), generated in the whole cells, rather than parent compound itself, was the inhibitor of this enzyme. Mechanism-based inhibition is characterised by a time- and concentration-dependent inhibition that requires presence of NADPH. This type of inhibition is usually irreversible and, since inactivated enzyme has to be replaced by newly synthesised protein, has longer-lasting effects than competitive inhibition (Murray, 1997). Of the two isothiocyanates, erucin was marginally more potent at suppressing, in a time- and concentration-dependent manner, the O-deethylation of ethoxyresorufin. The fact that inhibition was prevented by reduced, but not oxidised, glutathione concords with the generation of an electrophilic metabolite(s). A more reactive isocyanate, derived from the cytochrome P450-mediated isothiocyanate oxidation has been postulated as the possible entity that interacts and inactivates cytochromes P450 (Lee, 1994). Such reactive metabolites were also identified in case of BITC, which inactivated rat CYP1A1 and CYP1A2 following metabolism to a reactive benzyl cyanate and benzylamine that covalently attached to and inactivated the enzymes (Goosen et al., 2001; Moreno et al., 1999). Moreover, α-naphthylisothiocyanate was converted by rat liver microsomes, in the presence of NADPH, to metabolites that bound irreversibly to microsomal proteins (El-Hawari and Plaa, 1977). Although both ITCs acted as mechanism-based inhibitors of CYP1A, they are unlikely to be metabolized by these enzymes. Aliphatic ITCs such as SFN and erucin are small molecules that are most likely to be the substrates for microsomal CYP2E1. However, neither the activity nor expression of this cytochrome P450 was influenced by SFN in preliminary studies and,
therefore, were not determined in the current studies (C. Ioannides, personal communications).

Erucin, at both dose levels, as well as SFN, significantly increased hepatic quinone reductase activity as previously described for sulforaphane (Yoxall et al., 2005). For both tissues, Western blot analysis indicated that this was due to increased cytosolic concentrations of quinone reductase protein. In accordance with the present observations, AITC also induced the activity of pulmonary QR in rat (Munday and Munday, 2002). On the other hand, exposure of rats to phenethyl isothiocyanate failed to enhance this activity in the lung (Guo et al., 1992), highlighting the importance of the side chain, since SFN and erucin, as well as AITC, have an aliphatic side chain whereas in the case of phenethyl isothiocyanate it is aromatic. Glutathione S-transferase activity was monitored using three substrates; CDNB is a substrate for a number of the cytosolic transferases (Habig et al., 1974). The much higher activity in the liver compared with the lung reflects the higher capacity of the former tissue to detoxicate electrophiles. Both compounds failed to influence activity in either liver or lung. DCNB is a substrate associated with the \( \mu \)-family, but no activity was detectable in lung as a result of poor expression in this tissue (Sherrat and Hayes, 2002). Hepatic activity, whatever the accepting substrate, was not influenced by erucin treatment although a modest rise in apoprotein levels was evident. Finally, NBD-CI has been reported to be selective for the \( \alpha \)-class (Ricci et al., 1994), and the very low activity in the lung demonstrates the poor expression of this class in this tissue (Sherratt and Hayes, 2002). A rise in GST activity was not seen with any of the three accepting substrates studied, whereas GST\( \alpha \) and GST\( \mu \) were up regulated at the protein level. Considering that ITCs have been previously shown to generate metabolites that are cytochrome P450 inactivators (Goosen et al., 2001 and Nakajima et al., 2001), it may be speculated that a hepatic metabolite(s) of erucin and SFN may be sufficiently stable to escape the endoplasmic reticulum and
inhibit cytosolic GST activity. In lung, similarly no change in activity was manifested by
treatment with either isothiocyanate, but GSTα levels increased, GSTπ was not
modulated by either isothiocyanate in either tissue. Thus, the effects of isothiocyanates
on the GST system are both compound and tissue specific. In terms of effectiveness as
Phase II enzyme inducers, erucin was as potent as SFN, which is not surprising since
erucin is extensively oxidised to form SFN and, likewise, sulforaphane is reduced to
erucin in rat (Kassahun et al., 1997).

In order to assess whether the erucin treatment had any impact on the ability of liver
microsomes to bioactivate chemical carcinogens, the metabolic conversion of the
heterocyclic amine 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) to mutagens was
investigated using the Ames mutagenicity assay. This compound was used as model
carcinogen as previous studies showed that sulforaphane inhibits the genotoxicity and
DNA-adduct formation of heterocyclic amines in cell cultures (Barcelo et al., 1998 and
Bacon et al., 2003). Erucin treatment caused a modest decrease in the bioactivation of
IQ but no clear concentration-dependent effect was evident. Sulforaphane similarly had a
modest effect in agreement with a previous in vivo study (Yoxall et al., 2005).

In summary, the present studies have demonstrated that following intake of erucin, at a
dose corresponding to human intake, the only enzyme modulated of those studied was
quinone reductase; an increase was seen in both liver and lung. No cytochrome P450
enzyme activity was perturbed by the erucin treatment implying that attenuated
generation of genotoxic intermediates is unlikely to be a chemopreventive mechanism at
dietary dose levels. These observations allow the conclusion that erucin may exert a
chemopreventive effect by affording protection against the carcinogenicity of quinones,
such as those formed by the oxidation of polycyclic aromatic hydrocarbons. At higher
doses, which may be achieved by the consumption of dietary supplements, the
expression of other enzymes was modulated, at least at the protein level. In general, no marked differences were observed between erucin and sulforaphane. However, as the major source of erucin is rocket salad which is largely consumed uncooked, it may prove a more effective chemopreventive agent than sulforaphane as it is likely to achieve a higher bioavailability, because the principal sources of glucosinolate precursor of SFN are vegetables which are commonly consumed cooked, resulting in loss of the glucosinolate and their lower conversion to the isothiocyanate. Finally, the ability of dietary isothiocyanates to up-regulate CYP1B1 expression in the liver and lung has been detected for the first time.
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Chapter 6

Tissue and species differences in the modulation of xenobiotic metabolising enzymes by erucin and sulforaphane
Chapter 6: Modulation of xenobiotic-metabolising enzymes by isothiocyanates

6.1 Introduction

Modulation of cytochrome P450 and Phase II detoxification enzymes expression is central to the chemopreventive effects of ITC during the initiation stage of carcinogenesis (Hecht, 2000 and Fimognari and Hrelia, 2007). Since down-regulation of cytochrome P450 enzymes leads to suppression of bioactivation, it is necessary to clarify whether erucin, like SFN, modulates expression of Phase I enzymes, as the focus of previous studies was the modulation of QR and GST (Zhang et al., 1992 and Jacubikova et al., 2005). Previous in vitro studies have investigated the effect of SFN on expression of cytochrome P450 enzymes in rat (Jiao et al., 1996; Maheo et al., 1997 and Zhou et al., 2007) and human liver parenchymal cells (Maheo et al., 1997) and microsomes (Langouet et al., 2000), as well as phase II enzymes in rat and human liver hepatocytes (Maheo et al., 1997), human retinal pigment epithelial cells (Gao and Talalay, 2004) and various cell lines (Zhang et al., 1992, Brooks et al., 2001, Basten et al., 2002, Bacon et al., 2003, Misiewicz et al., 2004 and Ritz et al., 2006). The present studies aim to compare modulation of xenobiotic-metabolising enzyme expression and activity by erucin and SFN in rat and human liver slices as well as in two rat tissues, lung and liver, following their incubation under identical conditions. Moreover, given that an increased global expression/activity of GSTs is thought to compensate for the lack of activity of a non-functioning class of GST in individuals with null polymorphism (Sapone et al., 2006), it is important to investigate the effect of these aliphatic ITCs on expression of distinct classes of GSTs since these data remain incomplete.

The use of precision-cut tissue slices generates metabolic and toxicological data that is indicative of in vivo responses (Berthou et al., 1989) and, therefore, is an ethical alternative to animals. The advantages of precision-cut tissue slices over isolated cells include preservation of normal tissue architecture, cell heterogeneity and cell-cell interactions within their original matrix. The technique, developed by Krumdieck,
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Brendel and Sipes (Krumdieck et al., 1980; Brendel et al., 1987; Sipes et al., 1987) can be employed in the preparation of precision-cut slices of uniform thickness and diameter from a number of tissues including liver, kidney, lung, prostate, spleen and heart (Azri et al., 1990; Stefaniak et al., 1992; Ball et al., 1996; Parrish et al., 1992, 1995, 2002), allowing one to investigate metabolic, toxicological and pharmacological profiles of test compounds in target tissues. More importantly, use of precision-cut tissue slices prepared from a variety of species such as laboratory animals (Sivapathasundaram et al., 1994; Price et al., 1995 and Hashemi et al., 1999) and humans (Renwick et al., 2000) facilitates cross species comparisons.

The current studies investigate the effects of different concentrations of erucin on expression of xenobiotic-metabolising enzymes (XME) in precision-cut slices generated from rat liver, the major site of xenobiotic metabolism, and lung, a target tissue for chemopreventive activity of ITC (Higdon et al., 2007). The potential of erucin to alter activity/levels of major enzyme systems, including cytochrome P450 enzymes, QR and GSTs is compared with that of SFN. Moreover, the present study, utilising precision-cut slices prepared from rat and human liver, explores whether the effect of erucin and SFN on XME expression and activity differs between these two species.

6.2 Methods

The methodology for preparation and incubation of precision-cut rat liver and lung slices is described in Chapter 2 (section 2.2.3). Liver and lung slices were incubated with sulforaphane or erucin (1-50 μM) for 24 h. Control slices were incubated with dimethyl sulphoxide (DMSO), the solvent vehicle (final concentration 0.1% v/v). At the end of the incubation period, tissue slices were homogenised and resolved into microsomal and cytosolic fractions used in the determination of Phase I and II enzyme activities, total protein and total glutathione levels as described in sections 2.2.6-2.2.14. Changes in
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enzyme protein expression were determined by Western blot analysis (section 2.2.13). The functional and metabolic viability of the tissue slices was monitored by measuring the extent of lactate dehydrogenase leakage in the incubation medium and the rate of biotransformation of the diagnostic substrate 7-ethoxycoumarin (7-EC) as described in sections 2.2.4 and 2.2.5 of the Chapter 2.

6.2.1 Human liver

Portions of human livers (about 200g each) from donors who suffered a lethal brain injury were obtained from the Human Tissue Bank, UK. The first liver (Donor 1) came from a Caucasian male, aged 26, smoker, who was maintained on ventilation for four days prior to death. The second liver (Donor 2) was obtained from a non-smoking Caucasian female, aged 54, who was maintained on artificial ventilation for two days prior to death. Both livers were HIV, hepatitis B and C-negative. The time from the moment of donors’ death to the beginning of the incubation of the liver slices did not exceed 12 h on both occasions.

6.3 Results

6.3.1 Assessment of metabolic tissue slice viability: metabolism of 7-EC

Rat liver and lung slices were incubated with 7-HC for up to 6 h. The slice protein content remained unchanged in both tissues throughout the incubation period. The

![Figure 6.1: Protein content of rat tissue slices. Rat liver and lung slices were incubated in RPMI 1640 containing 7-ethoxycoumarin (50 µM) for up to 6 h. The slices were individually homogenised in PBS. The results are expressed as means ± SD, where n=3, with each analysis carried out in duplicate. All slices were generated from a single rat.](image)
protein content of the lung slice homogenates was 60% of that of the liver slices. The protein concentration of the slice homogenates was expressed as mg/slice (Fig. 6.1). The concentrations of the glucuronide and sulphate conjugates of 7-HC in the culture medium increased in a time-dependant manner, while the levels of free 7-HC remained low but constant throughout the incubation period. The rate of the biotransformation of 7-HC by the liver slices was 3-fold greater than the metabolic activity of the lung slices (Fig. 6.2).

Figure 6.2: Cumulative metabolism of 7-ethoxycoumarin by rat lung (A) and liver (B) slices. The slices were incubated in modified RPMI 1640 with 7-ethoxycoumarin (50 μM) for up to 6 h. Metabolites of 7-ethoxycoumarin were determined in the media for each incubation period. Results are expressed as means ± SD of duplicate analysis of each of three slices per time point. All slices were generated from a single rat.
6.3.2 Effect of erucin on expression and activity of xenobiotic-metabolising enzymes in precision-cut rat liver slices

In liver slices, no significant differences in the activities of the microsomal MROD, PROD or 7-BQ demethylase were recorded following incubation with erucin (0–50 μM) for 24 h. Hepatic EROD, however, was significantly inhibited at the highest concentration (50μM) of erucin (Fig. 6.3). In contrast, Western Blot analysis revealed a modest elevation of hepatic CYP3A2 and pronounced concentration-dependent rise in CYP1A and CYP1B1 apoprotein levels, while the protein levels of CYP2B1 remained unaffected (Fig. 6.5).

Figure 6.3: Effect of erucin on activities of Phase I enzymes in cultured rat liver slices. Liver slices were incubated in modified RPMI 1640 culture medium containing erucin (0 to 50μM) for 24 h. EROD (A), MROD (B), PROD (C) and 7-BQ (D) activities were determined in microsomal fraction using ethoxyresorufin, methoxyresorufin, pentoxyresorufin and 7-benzoxyquinoline as respective probes. Values represent the means ± SD where n=3. *, P<0.05.
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QR activity in rat liver slices was induced in concentration-dependent fashion, with a 74\% increase achieved at 25 \( \mu \text{M} \); at the highest concentration of 50 \( \mu \text{M} \), activity decreased to control levels (Fig. 6.4). Immunologically, there was a pronounced concentration-dependent increase in QR protein level, reaching a maximum of 3.5-fold at the 10 \( \mu \text{M} \) concentration. The magnitude of the induction diminished at the highest erucin concentrations (Fig. 6.5).
Figure 6.5: Modulation of hepatic cytochrome P450 enzymes (A) and phase II enzymes (B) by treatment with erucin. Liver slices were incubated in modified RPMI 1640 culture medium containing erucin (0 to 50 µM) for 24h. Hepatic microsomal (A) and cytosolic proteins (B) were resolved by 10 % (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using either anti-rat serum to cytochrome P450 (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (CYP2B1, CYP3A2, GSTα and GSTµ) or 40 µg (CYP1A1, CYP1B1, GSTn and QR) of total protein. Molecular markers were run concurrently to aid band identification. The values in italic show optical density percentage of each band relative to control.
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Of the GST enzymes, global hepatic GST activity, determined with the broad-spectrum diagnostic substrate CDNB, was modestly elevated (about 20%) but the statistical significance was achieved only at concentration of 2.5 μM. The activity of GSTα, determined using the marker substrate NBD-CI, increased by 40%, while GSTμ activity, determined using DCNB as the marker substrate, was without change (Fig. 6.4). At the protein level, GSTα and GSTμ levels were modestly elevated, while GSTn protein did not change (Fig. 6.5).

Total cytosolic GSH content was analysed using GSH reductase-coupled 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. Treatment with erucin significantly elevated total glutathione level in a concentration-dependent fashion. However, the glutathione levels started to fall at 25 μM and were halved at the highest concentration (Fig. 6.4).

### 6.3.3 Effect of erucin on expression and activity of xenobiotic-metabolising enzymes in precision-cut rat lung slices

Following incubation of rat lung slices with a range of erucin concentrations (0–50 μM), there was no significant change in the activities of monitored cytochrome P450 enzymes, except at the highest concentration (50μM), at which PROD activity was inhibited by almost 50 % (Fig. 6.6). CYP3A2 activity was not detectable when 7-benxyloxyquinoline was used as a probe. Western blot analysis showed a modest decrease in pulmonary CYP2B1 protein levels, which diminished by 23 % at 50 μM (Fig. 6.8). CYP1A1 apoprotein levels were elevated by erucin in a concentration-dependent manner (up to 2-fold) before returning to control values at 50 μM. Similarly, treatment with erucin caused a marked and concentration-dependent increase in pulmonary CYP1B1 apoprotein. There was no major change in CYP3A2 apoprotein levels (Fig. 6.8).
Figure 6.6: Effect of erucin on activities of Phase I enzymes in cultured rat lung slices.

Lung slices were incubated in modified RPMI 1640 culture medium containing erucin (0 to 50µM) for 24 h. EROD (A) and PROD (B) activities were determined in microsomal fraction using ethoxy- and pentoxyresorufin as respective probes. Values represent the means ± SD where n=3, each replicate comprised up to 12 slices. *, P<0.05, **, P<0.01.

Figure 6.7: Effect of erucin on activities of Phase II enzymes in cultured rat lung slices. Lung slices were incubated in modified RPMI 1640 culture medium containing erucin (0 to 50µM) for 24 h. The activities of GST, analysed using CDNB as substrate (A), QR (B) and total glutathione (C) were determined in the cytosolic fraction. Values represent the means ± SD, where n=3; each replicate comprised up to 12 slices. *, P<0.05, **, P<0.01 and ***, P<0.001.
Figure 6.8: Modulation of pulmonary cytochrome P450 enzymes (A) and phase II enzymes (B) by treatment with erucin. The slices were cut from rat lungs stabilised with agarose (0.75 % w/v) and incubated in the modified RPMI 1640 culture supplemented with erucin (0 to 50μM) for 24 h. Pooled pulmonary microsomal (A) and cytosolic proteins (B) were resolved by 10 % (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. Immunoblot analysis was carried out using either anti-rat serum to cytochrome P450 (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (CYP2B1, CYP3A2, GSTn), 20 (GSTα, GSTμ, and QR) or 40 μg (CYP1A and CYP1B1) of total protein. Molecular markers were run concurrently to aid band identification. The values in italic show optical density percentage of each band relative to control. The values in italics show percentage of optical density of each band relative to control.
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The activities of pulmonary QR and GST, when CDNB was used as an accepting substrate, were significantly elevated (Fig. 6.7). GST activity was not detectable when either DCNB or NBD-Cl served as accepting substrate. Immunologically, QR and GST protein levels increased in a concentration-dependent manner, declined at the highest erucin concentration. Treatment with erucin had no effect on GST \( \mu \) and GST \( \eta \) protein expression (Fig. 6.8).

The total cytosolic glutathione level in lung slices was 10% of hepatic levels. There was a concentration-dependent increase in the pulmonary glutathione levels, but at the highest dose level concentration of glutathione fell to 60% of the control values (Fig. 6.7).

6.3.4 Effect of SFN on expression and activity of xenobiotic-metabolising enzymes in precision-cut rat liver slices

When rat liver slices were incubated with SFN (0–50 \( \mu \)M) for 24 h, the only change in microsomal EROD, MROD, PROD and 7-BQ demethylase activities was observed at the highest concentration, at which the activities of all monitored cytochromes P450 diminished, but statistical significance was attained only for MROD and PROD (Fig. 6.9). In contrast, treatment with SFN induced expression of hepatic CYP1A1, CYP1B1 and, to a lesser extent, CYP3A2 protein in a concentration-dependent manner, as analysed by Western Blot. However, effect of induction declined at the highest concentrations of SFN. Treatment with SFN had no effect on expression of hepatic CYP2B1 (Fig. 6.11).

SFN markedly induced hepatic QR, both at the activity (Fig. 6.10) and, in particular, at the protein level (Fig. 6.11). Maximal induction of QR was observed at 5 and 10 \( \mu \)M, but further increase in SFN concentration diminished magnitude of induction. Global GST activity rose in a concentration-dependent manner, but the statistical significance was
achieved only at 10 and 25 µM SFN concentrations. GSTo and GSTµ activities were induced by SFN by approximately 40 and 75 % respectively (Fig. 6.10). The effect of SFN on the expression of hepatic GSTs varied depending on the GST class. GSTo and GSTµ protein levels were elevated, while GSTn levels did not appear to alter (Fig. 6.11). Following 24 h incubation of rat liver slices with SFN, the total cytosolic glutathione levels were elevated in a concentration-dependent manner, but dropped to 59 % of the control value at 50 µM (Fig. 6.10).

Figure 6.9: Effect of sulforaphane on rat hepatic cytochrome P450 enzyme activities. Rat liver slices were maintained on sulforaphane-supplemented RPMI 1640 culture media for 24 h. Activities of EROD (A), MROD (B), PROD (C) and 7-BQ demethylase were determined in microsomal fraction. Values are presented as means ± SD, where n=3; each replicate comprised of up to 12 slices. *, P<0.05 versus control. **P<0.05.
Figure 6.10: Effect of sulforaphane on activities of Phase II enzymes in cultured rat liver slices. Liver slices were incubated in modified RPMI 1640 culture medium containing erucin (0 to 50 μM) for 24 h. The activities of GSTs, analysed using CDNB (A), CNBODI (B) and DCNB (C) as substrates, QR (D) and total glutathione (E) were determined in the cytosolic fraction. Values represent the means ± SD where n=3. *, P<0.05, **, P<0.01 and ***, P<0.001.
Figure 6.11: Modulation of hepatic cytochrome P450 enzymes (A) and phase II enzymes (B) by exposure to sulforaphane. Liver slices were incubated in modified RPMI 1640 culture medium containing sulforaphane (0 to 50 \( \mu \text{M} \)) for 24h. Hepatic microsomal (A) and cytosolic proteins (B) were resolved by 10 % (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. Immunoblot analysis was carried out using either anti-rat serum to cytochrome P450 (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 \( \mu \text{g} \) (CYP2B1, CYP3A2, GST\( \alpha \) and GST\( \mu \)) or 40 \( \mu \text{g} \) (CYP1A, CYP1B1, GST\( \gamma \) and QR) of total protein. Molecular markers were run concurrently to aid band identification. The values in italic show optical density percentage of each band relative to control. Molecular markers were run concurrently to aid band identification. The values in italic show percentage of optical density of each band relative to control.
6.3.5 Effect of SFN on expression and activity of xenobiotic-metabolising enzymes in precision-cut rat lung slices

In the lung slices incubated with SFN for 24 h, EROD activity was inhibited by 33 and 44% at the highest concentration levels. Activity of pulmonary PROD was without change (Fig. 6.12). At the apoprotein level, SFN induced expression of the microsomal CYP1A1, CYP1B1 and CYP3A2 levels, the effect being more marked in the case of CYP1A1. At the two highest concentrations (25 and 50 μM), CYP1B1 protein levels were markedly decreased. Modulation of pulmonary CYP2B1 by SFN was not observed (Fig. 6.14).

![Figure 6.12: Effect of sulforaphane on activities of Phase I enzymes in cultured rat lung slices.](image)

Similar to erucin, SFN significantly increased QR and, to a lesser extent, total GST activities in lung slices (Fig. 6.13). Immunologically, there was a marked and concentration-dependent increase in QR and a less pronounced rise in GSTα protein, whereas GSTn and GSTμ were unaffected by this treatment (Fig. 6.14).
Figure 6.13: Effect of sulforaphane on rat pulmonary Phase II enzyme activities and total glutathione levels. Lung slices were incubated in modified RPMI 1640 culture medium containing SFN (0 to 50 µM) for 24 h. The activities of GST, analysed using CDNB as an accepting substrate (A), QR (B) and total glutathione (C) were determined in cytosolic fraction. Values represent the means ± SD, where n=3; each replicate comprised 12 slices. *, P<0.05, **, P<0.01 and ***, P<0.001.

Total GSH levels in lung slices after exposure to SFN (0-50 µM) for 24h were moderately elevated in a concentration-dependent fashion. GSH concentrations fell to control levels in lung slices exposed to the highest SFN concentrations (25 and 50 µM).
Figure 6.14: Modulation of pulmonary cytochrome P450 enzymes (A) and phase II enzymes (B) by sulforaphane. The slices were cut from rat lungs stabilised with agarose (0.75 % w/v) and incubated in the modified RPMI 1640 culture supplemented with SFN (0 to 50μM) for 24 h. Pooled pulmonary microsomal (A) and cytosolic proteins (B) were resolved by 10% (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. Immunoblot analysis was carried out using either anti-rat serum to cytochrome P450 (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (CYP2B1, CYP3A2, GSTn), 20 (GSTa, GSTμ and QR) or 40 μg (CYP1A and CYP1B1) of total protein. Molecular markers were run concurrently to aid band identification. The values in italic show optical density percentage of each band relative to control. The numbers in italics show percentage of optical density of each band relative to control.
6.3.6 Effect of erucin on expression and activity of xenobiotic-metabolising enzymes in precision-cut human liver slices

When slices prepared from the 1st human liver were incubated with erucin (0-50 µM) for 24 h, no significant changes in activities of hepatic EROD, MROD or 7-BQ demethylase were observed. At the highest concentration, however, activity of hepatic MROD was significantly reduced (Fig. 6.15). Activity of human PROD was not determined due to limited amounts of available human liver. The expression of human CYP1A, CYP2B1 and CYP3A4 was suppressed only at the highest concentration of erucin (50 µM), while hepatic CYP1B1 apoprotein levels remained unchanged (Fig. 6.17).

In slices prepared from a second human liver, incubation with erucin similarly led to a significant decrease in the activities of both microsomal enzymes studied, EROD and 7-
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BQ demethylase, at the highest concentration (50µM) only (Fig. 6.15). Modulation of MROD and PROD activities by erucin in the second human liver was not evaluated due to limited amounts of this tissue. The Western Blot analysis revealed reduction in microsomal CYP2B1 and CYP3A4 protein levels (40% and 70% respectively) following treatment with the highest concentration of erucin. In contrast, CYP1A1 and CYP1B1 expression in liver of the second donor was modestly up-regulated (up to 60%) by erucin (Fig. 6.17).

Figure 6.16: Effect of erucin on activities of human Phase II enzymes (Donor 1 and 2). Slices were incubated in modified RPMI 1640 culture medium containing erucin (0-50µM) for 24 h. The activities of GST, monitored using CDNB (A) or NBD-Cl as substrate, QR and total glutathione levels were determined in the cytosolic fraction. Results are presented as mean ± SD, where n=3; each replicate comprised up to 12 slices. *, P<0.05.
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Figure 6.17: Modulation of hepatic cytochrome P450 enzymes (A) and phase II enzymes (B) by exposure to erucin (Donors 1 and 2). Slices from two human livers were incubated in the modified RPMI 1640 culture medium containing erucin (0 to 50 µM) for 24h. Hepatic microsomal (A) and cytosolic proteins (B) were resolved by 10% (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using either rat antibodies to cytochrome P450 (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 10 (GSTs), 20 (CYP1A and CYP3A4) or 40 µg (CYP1A, CYP1B1 and QR) of total protein. Molecular markers were run concurrently to aid band identification. The values in italics show optical density percentage of each band relative to control. The values in italics show percentage of optical density of each band relative to control.
In the 1st human liver, treatment with erucin had no effect on activity of QR and GST, the latter being monitored using either CDNB or NBD-Cl as a marker substrate (Fig. 6.16). GST activity was not detectable in either of human livers when DCNB was used as a probe. Western blot analysis of the phase II enzymes expression revealed that erucin, unexpectedly, failed to up-regulate expression of QR or GST. Once again, the highest concentrations of erucin were associated with suppressed expression of QR, GSTn and GST\(\mu\), whereas GST\(\alpha\) protein levels were not affected (Fig. 6.17).

In contrast, in slices generated from the second human liver, erucin enhanced activities of GST, determined using either broad-spectrum CDNB or GST\(\alpha\)-specific NBD-Cl as marker substrates, with the most marked increase observed at 5 and 10 \(\mu\)M. Further increase in erucin concentration, however, was associated with reduction in GST activity (Fig. 6.16). Neither QR nor GST\(\mu\) activities were detectable. Similarly, erucin modestly up-regulated expression of QR and all GST isoforms except GST \(n\), although GST\(\alpha\) protein levels fell below control values at the highest erucin concentration (Fig. 6.17).

Total glutathione levels in slices prepared from the 1st liver were unaffected by treatment with erucin (Fig. 6.16). In the liver of the 2nd donor, however, a concentration-dependent increase in total glutathione concentration was evident after 24 h incubation with erucin. Marked glutathione depletion at the highest concentration of erucin was observed in both human livers (Fig. 6.16).

3.3.7 Effect of sulforaphane on expression and activity of xenobiotic-metabolising enzymes in precision-cut human liver slices

SFN had no effect on activity of cytochromes P450 monitored in either human liver following 24 h incubation, except at the highest concentration (50 \(\mu\)M), which was associated with a significant decrease in activities of most phase I enzymes (Fig. 6.18). Effect of SFN on MROD activity in the 1st human liver, as well as modulation of PROD in
either human livers, was not determined due to shortage of this tissue. At the protein level, SFN had no effect on expression of cytochrome P450 enzymes in the 1st human liver. In the 2nd human liver, however, CYP1A1 and CYP1B1 expression was modestly up-regulated (up to 40%) by treatment with SFN, while CYP2B1 and CYP3A4 apoprotein levels remained mostly unaffected (Fig. 6.20). Protein levels of all four monitored cytochromes P450 fell in both human livers in response to treatment with the highest SFN concentration, as in rat (Fig. 6.20).

![Figure 6.18](image1.png)

**Figure 6.18:** Effect of sulforaphane on activities of human Phase I enzymes (Donor 1 and 2). Slices were generated from livers of two donor and incubated in modified RPMI 1640 culture medium containing sulforaphane (0 to 50 μM) for 24 h. The activities of EROD, MROD and 7-BQ demethylase were determined in the microsomal fraction using ethoxyresorufin (A), methoxyresorufin (B) and 7-benzyloxyquinoline (C) as substrates in microsomal fraction. Values are means ± SD, where n=3; each replicate comprised 12 slices. *, P<0.05 and **, P<0.01.

None of the phase II enzymes studied was influenced by SFN at the activity level at concentrations less than 50 μM (Fig. 6.19). In contract, in the 2nd human liver, global hepatic GST and GSTa activities almost doubled at 10 μM. (Fig. 6. 19). After 24 h incubation with SFN, activities of QR in the 1st liver and GSTμ in both human livers were undetectable. Immunologicaly, SFN, like erucin, had no effect of expression of either
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Phase II enzyme in the 1st human liver, but, in the 2nd human liver, this ITC modestly up-regulated expression of GSTα, GSTμ and QR enzymes, with the highest increase achieved at 5 and 10 μM. No concentration-dependent change in human GSTn expression was detected in the 2nd liver (Fig. 6.20). Use of the highest concentration of SFN (50 μM) resulted in declining protein levels and activity of phase II enzymes (Figures 6.19 and 6.20).

Figure 6.19: Effect of sulforaphane on activities of human Phase II enzymes (Donor 1 and 2). Slices were incubated in modified RPMI 1640 culture medium containing erucin or sulforaphane (0 to 50μM) for 24 h. The activities of GST, with CDNB or CNBOD as substrate), QR and total glutathione were determined in cytosolic fraction. Values represent the means ± SD, where n=3; each replicate comprised 10 slices. *, P<0.05 and **, P<0.01.
Figure 3.20: Modulation of hepatic cytochrome P450 enzymes (A) and phase II enzymes (B) by exposure to sulforaphane (Donor 1 and 2). Slices from the liver of a male donor were incubated in modified RPMI 1640 culture medium containing sulforaphane (0 to 50 μM) for 24h. Each lane was loaded with 10 (GSTs), 20 (CYP1A and CYP3A4) or 40 μg (CYP1A, CYP1B1 and QR) of total protein. Hepatic microsomal (A) and cytosolic proteins (B) were resolved by 10 % (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membranes. Immunoblot analysis was carried out using either anti-rat antibodies to cytochrome P450 proteins (A) or to phase II enzymes (B), followed by the appropriate peroxidase-labelled secondary antibody. Molecular markers were run concurrently to aid band identification. The values in italics show percentage of optical density of each band relative to control.
Total glutathione levels in the 1st human liver remained mainly unaltered, whereas in the 2nd human liver, treatment with SFN increase total glutathione concentration in a concentration dependent manner (Fig. 6.19). At the highest concentration, SFN markedly decreased glutathione levels in both human livers (Fig. 6.19).

3.3.8 ITC toxicity to tissue slices

Initially, slices were exposed for 24 h to ITC concentrations ranging from 0 to 100 μM. However, the necrotic macroscopic appearance and significant loss of the metabolic activity in slices suggested that the concentrations of ITC exceeding 50 μM could be toxic. To investigate whether these phytochemicals are toxic to the cultured tissues, extent of the lactate dehydrogenase (LDH) leakage into the incubation medium was measured after 24 h incubation with either SFN or erucin. The results of the latter experiment confirmed that both SFN and erucin were marginally toxic to rat liver tissue at concentration of 50 μM and highly toxic at concentration of 100 μM. The lung slices tended to leak less intracellular LDH into the medium after 24 h incubation but, compared with liver slices, appeared to be more susceptible to ITC-induced toxicity; mild toxicity was apparent at concentrations of 25 μM SFN (Fig. 6.21).
Figure 6.21: Toxicity of erucin (A) and sulforaphane (B) to rat liver slices. LDH leakage was measured in the liver and lung slices incubated in modified RPMI 1640 containing a range of concentrations (0 to 100 μM) of erucin or sulforaphane for 24 h. LDH release is expressed as percentage of total LDH. Results are presented as mean ± SD of duplicate measurements from each of three slices per concentration. *, P<0.05; **P<0.01; ***P<0.001.
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6.4 Discussion

A major mechanism underlying ITC-associated chemopreventive activity is alteration of the carcinogen metabolism in favour of detoxification and elimination, achieved by suppressing their generation, through impairment of cytochrome P450-mediated activation, and by enhancing their detoxication by up-regulating protective enzyme systems such as quinone reductase and the glutathione S-transferases (reviewed by Hecht, 2000 and Fimognari and Hrelia, 2007). It is assumed that similar mechanisms operate in humans exposed to these compounds through diet. The use of precision-cut tissue slices allows, for the first time, an investigation of the effects of erucin and SFN on carcinogen-metabolising enzyme systems in human tissue, in comparison with rat following exposure to these compounds under identical conditions. The obvious advantage of this approach is a direct assessment of outcome from exposure of the human tissue(s) to the test compounds that eliminates the necessity to extrapolate experimental observations from animals to humans.

Other important advantages of this experimental technique include preservation of the tissue architecture and cell heterogeneity, both are important factors when studying modulation of the XMEs in vitro. In control adult rat most hepatic cytochromes P450, including CYP1A2, CYP2B1 and CYP3A2, are distributed non-uniformly, being localised predominantly in the centrilobular hepatocytes, whereas CYP1A1 shows a significantly less zone-specific pattern of expression and is heterogeneously expressed by hepatocytes throughout the parenchyma (Murray and Burke, 1995). CYP2B is also abundant in the midzonal hepatocytes (Heinonen et al., 1996). The predominant classes of cytosolic GSTs in adult rat liver are alpha and mu, both being uniformly expressed by hepatocytes (Tee et al., 1992). The rat bile duct epithelial cells express GST class mu and pi. In the rat lung, CYP2B and CYP3A are expressed by Clara cells of the bronchial epithelium and type II alveolar epithelial cells, whereas CYP1A is localised in Clara cells.
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of the bronchial epithelium (Murray and Burke, 1995). All three major classes of the rat cytosolic pulmonary GSTa are localised in Clara cells and ciliated cells of the bronchial epithelium (Murray and Burke, 1995).

One of the major disadvantages of the tissue slice system is the short duration of their maintenance in culture media, thus limiting the timescale of in vitro studies to a maximum of 72 h (Hashemi et al., 1999; Hashemi et al., 2000 and Umachandran and Ioannides, 2006). However, the duration of exposure, known to influence the inducing potential of ITCs on phase II enzymes (Munday and Munday, 2002), is an important factor to be considered when extrapolating the in vitro findings to an in vivo situation. Tissue slices provide valuable data on biological and toxicological effects of test compounds in target organs, however, it is essential to ensure that they remain viable during incubation in a culture medium.

6.4.1 Biotransformation of 7-ethoxycoumarin

Viability of cultured tissue can be monitored using a variety of biochemical markers, including intracellular ATP content, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction, K⁺ retention, enzyme leakage (De Kanter et al., 1999; Monteil et al., 1999; Price et al., 1995) and a biotransformation of a marker substrate such as 7-ethoxycoumarin (Steensma et al., 1994).

Biotransformation of 7-ethoxycoumarin (7-EC) is a sensitive indicator of the metabolic capability of slices as both Phase I and II enzymes activities are monitored during incubation. 7-Ethoxycoumarin is metabolised by the microsomal Phase I enzymes to 7-hydroxycoumarin (7-HC), which is subsequently enzymatically conjugated with sulphate and glucuronide (Barr et al., 1991a, 1991b and Steensma et al., 1994). The rate of metabolism of the diagnostic substrate 7-hydroxycoumarin (7-HC) was determined to
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ensure the quality and validity of the subsequent experiments involving the use of precision-cut liver and lung slices as an *in vitro* model employed to study the effect of erucin and SFN on activity and expression of xenobiotic-metabolising enzymes (XMEs). The formation of the hydroxylated product 7-HC and its sulphate and glucuronide conjugates from a parent compound 7-EC during incubation for up to 6 h approximated the levels previously reported for this model substrate for rat liver (Price et al., 1995, Hashemi and Ioannides, 1999, 2000 and De Kanter et al., 1999) and lung slices (Price et al., 1995 and Umachandran et al., 2004), allowing the conclusion that the tissue slices were metabolically viable under the experimental conditions of this and subsequent studies.

### 6.4.2 Species and tissue differences in the modulation XME enzyme activities and expression by erucin and SFN

Slices of both rat tissues and human liver were incubated with the isothiocyanates for 24 h, the time period required to achieve maximal induction (Pushparajah et al., 2007). Neither compound modulated the activity of any cytochrome P450 studied in rat lung and liver, as well as in both human livers, except at the highest concentrations used (50 μM), in which case the activities of most human and rat cytochromes P450 significantly fell. Such effect is most likely to be a manifestation of cytotoxicity caused by exposure to high concentrations of ITCs as will be discussed later in this chapter. Western blot analysis, on the other hand, revealed a pronounced and concentration-dependent rise in CYP1A1 and, to a lesser extent, CYP1B1 apoprotein in rat liver and lung. In the liver of Donor 2, CYP1A1 and CYP1B1 apoprotein was modestly elevated, but no similar changes in expression of these enzymes were evident in slices from the liver of Donor 2. Thus there are marked inter-individual differences in modulation of CYP1A1 and CYP1B1 expression by both compounds.
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The CYP1A subfamily consists of two members: CYP1A1 and CYP1A2, the activity of which is commonly determined using ethoxy- and methoxyresorufin as respective marker substrates. However, in hepatic microsomes of control rats, ethoxyresorufin is primarily deethylated by CYP2C6 (Burke et al., 1994) and CYP2C11 (Nakajima et al., 1990). EROD and MROD activities in both rat tissues were without change at non-toxic concentrations of ITCs, although inhibition of EROD by SFN was previously detected at ≥ 5 μM in primary rat hepatocytes (Maheo et al., 1997). In contrast, the expression of rat pulmonary and hepatic CYP1A1 was markedly up-regulated in the concentration-dependent manner by both compounds. Considering that CYP1A1, unlike CYP1A2, is not constitutively expressed in rat and human liver (Casarett et al., 2001), the protein recognised by the polyclonal anti-rat CYP1A1/2 serum in control rat and human liver is most likely to be CYP1A2. CYP1A2, on the other hand, is not expressed in extrahepatic tissues of most mammals, thus pulmonary protein detected by the polyclonal anti-rat CYP1A1/2 serum is CYP1A1. Although the polyclonal rat CYP1A1/2 antibodies, used in the western blot analysis, have the same affinity for both rat enzymes, only a single band was detected because separation of rat CYP1A1 and CYP1A2, which have molecular weights of 59 and 58 kDa respectively, is difficult to achieve. A lack of change in the rat EROD activity despite a marked increase in CYP1A1 protein suggests loss of catalytic activity due to the mechanism-based inhibition of this enzyme by both compounds as has previously been discussed in chapter 5. The activities of human EROD in both livers and MROD in the liver of donor 1 were not influenced by treatment with non-toxic concentrations of either erucin or SFN. Interestingly, the basal EROD activities in the liver of Donor 1, a smoker, did not differ from that of Donor 2, a non-smoker. The lack of inhibiting activity on CYP1A has previously been demonstrated with SFN using bacteria expressing human enzymes (Langouet et al., 2000). Immunoblot using the polyclonal anti-rat CYP1A1/2 serum that cross-reacts with human CYP1A protein, revealed a modest elevation of CYP1A1/2 protein in the liver of the 2nd donor. CYP1A1/2,
visualised as a single band of approximately 58 kDa, was not induced by either ITC in the liver of the 1st donor, indicating genetic variability.

Due to lack of CYP1B1-selective diagnostic substrate, the activity of this enzyme was not monitored. Both ITCs markedly induced expression of this enzyme in rat liver and, to a lesser extent, in lung. In contrast, human CYP1B1 was marginally induced only in the liver of the 2nd donor, clearly demonstrating species differences. The overall pattern and magnitude of rat CYP1B1 expression following treatment with erucin and SFN resembles that of CYP1A1. The similarities are not surprising since expression of mammalian CYP1A1 and CYP1B1 is regulated by a common ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR) (reviewed by Nebert et al., 2004). Although SFN is thought to act as a monofunctional inducer that selectively induces Phase II enzymes without concurrent induction of AhR-dependant Cytochrome P450 enzymes (Miao et al., 2004 and Higdon et al., 2007), current results indicate that there is a cross-talk between ARE-regulated and AhR-dependent gene regulation pathways. Indeed, SFN up-regulated rat CYP1A1 apoprotein (Yoxall et al., 2005) in vivo.

Activities of CYP2B1 and CYP3A were determined using pentoxyresorufin and 7-benzyloxyquinoline as respective diagnostic substrates. Both compound failed to alter either activity or expression of CYP2B1, rat CYP3A2 and human CYP3A4 in any of the tissues studied at non-toxic concentrations. In contrast, SFN inhibited hepatic PROD in rat primary hepatocytes (Maheo et al., 1997) and CYP3A4 catalytic activity and mRNA levels in human liver parenchymal cells (Maheo et al., 1997 and Zhou et al., 2007), but was without effect on the human enzymes expressed by bacteria (Langouet et al., 2000), the conflicting outcomes probably arising due to differences in experimental protocols. The anti-rat CYP3A2 serum, used in the western blot analysis, recognises and
strongly binds to rat CYP3A2 and human CYP3A4, but cross-reacts weakly with CYP2B and CYP2C, which appeared as an additional faint band on some blots.

NADPH:quinone oxidoreductase-1 (QR) is a Phase II enzyme that protects the cell from quinone-semiquinone redox cycling and generation of ROS through obligatory two-electron reduction of exogenous compounds, such as those generated by the oxidation of polycyclic aromatic hydrocarbons and endogenous quinones (Brunmark and Cadenas, 1989). In the studies with the rat tissue slices, both compounds up-regulated activity and expression of pulmonary and hepatic QR, in accordance with observations made previously both, in vitro (Zhang at al., 1992, Gerhauser et al., 1997 and Ritz et al., 2006) and in vivo (Zhang at al., 1992, Yoxall et al., 2005 and Chapter 5). The inducing effect of erucin and SFN on rat hepatic and pulmonary QR activities and expression was concentration-dependent. As a QR inducer, SFN was only marginally more potent than erucin, indicating that the oxidation state of sulphur has little impact on the ability of these isothiocyanates to modulate Phase II enzymes. However, when human liver slices from two different donors were used, only a weak rise in protein levels was noted in only one of the two human livers studied, in marked contrast to the observations made in rat. These findings suggest that human QR may be resistant to induction by these aliphatic isothiocyanates. However, the aromatic isothiocyanate, phenethyl isothiocyanate, also failed to induce this enzyme or increase protein levels in three human livers, whereas increases were observed in rat slices incubated under identical conditions (N Konsue, personal communication). Collectively, these data illustrate an important species difference between rat and human with implications for the chemopreventive activity of these isothiocyanates, as it would imply that chemopreventive potential might be less pronounced in human compared with rat. The lack of induction of human QR by erucin and SFN observed in current studies OR is surprising given that QR mRNA was induced in human colonic adenocarcinoma cells (Jacubikova et al., 2005) by erucin while SFN
similarly up-regulated expression of QR in human retinal pigment epithelial cells (Gao and Talalay, 2004) and in various human cell lines (Brooks et al., 2001 and Misiewicz et al., 2004). The differences may be consequence of the use of tissue slices as an in vitro model, resembling the in vivo environment more closely than immortalised cell lines or isolated cells, or/and human polymorphism. The non-functioning QR gene found in 20% of caucasians and almost 50% of other ethnic groups (Gaedigk et al., 1998). Furthermore, human Nrf2, the transcriptional factor involved in activation of ARE-controlled genes encoding phase II enzymes, is also polymorphic. This could influence its expression and function (Marzec et al., 2007) and may influence QR inducibility. A more definitive conclusion would require the analysis of more human liver samples.

Conjugation with polar glutathione moieties is a major pathway of detoxification and excretion of mutagens. Glutathione S-transferase (GST) activity was monitored using three substrates. CDNB is a substrate for several cytosolic transferases, whereas DCNB is a substrate predominantly metabolised by the \( \mu \)-family and NBD-CI has been reported to be selective for the \( \alpha \)-class (Ricci et al., 1994 and Sherrat and Hayes, 2002). Both compounds stimulated (GST) activity in rat liver, with all three substrates, with DCNB being the most sensitive, followed by NBD-CI, implying a rise in the \( \mu \)- and \( \alpha \)-class enzymes, and the immunological studies revealed modest increase in the protein levels of these classes, but not in the \( \pi \)-class. Of the pulmonary GST enzymes, rat GST activity, measured with the CDNB, was modestly elevated by both ITCs to the same degree, however, neither phytochemical influenced expression of the pulmonary GST\( \mu \), indicating that in rat, the effect of ITCs on GST is tissue and class-specific. When human liver slices were investigated, glutathione S-transferase activity was not detectable when DCNB was used as substrate; even in rat slices the lowest activity was obtained with this substrate. Incubation of liver slices for 24 hours leads to loss of glutathione S-transferase activity, particularly when assessed using DCNB (Hashemi et al., 1999), which may fall below the
levels of detection. The response seen in the two human livers following incubation with the two isothiocyanates differed. Neither compound modulated glutathione S-transferase activity, whatever the substrate used, or protein expression following Western blot analysis when studies were performed with liver slices from Donor 1. In the liver from Donor 2, however, both isothiocyanates caused moderate elevation of glutathione S-transferase activity, accompanied by a similar rise in the levels of immunologically determined GSTα and GSTμ with no concentration-dependent changes in GSTπ, similar to what was noted in rat liver slices. Enhanced expression of the human GST GSTA1/2 and GSTM1 \textit{in vitro} has been reported previously for SFN (Maheo et al., 1997, Bacon et al., 2003 and Ritz et al., 2006). The varied response obtained in the two human livers may be attributed to either genetic differences or to factors linked to the time of liver removal relative to the time of death and/or transport of the tissue. The latter is unlikely as, in fact, the basal hepatic glutathione S-transferase activity in Donor 1 was much higher than that of Donor 2. Clearly, more human livers need to be analysed for a clearer picture to emerge. Similarly, both compounds increased total glutathione levels in rat liver but only in human liver from Donor 2. The ability of isothiocyanates to enhance glutathione synthesis in animals has already been documented with sulforaphane (Zhang, 1992), but this is the first time that this has been demonstrated with erucin.

6.4.3 Cytotoxicity of erucin and SFN and effect on total GSH levels

The activities of most cytochromes P450 decreased in both rat tissues and human liver at the highest concentrations of erucin and SFN (25 and 50 μM). Moreover, the increases in activity of the various enzymes observed in rat and human slices at lower concentrations were no longer evident at the highest isothiocyanate concentration used. Such a pattern of XME modulation by ITCs is consistent with cytotoxicity as supported by the observations that these and higher concentrations increased leakage of lactic
dehydrogenase. Furthermore, the highest concentrations of ITCs depleted tissue glutathione.

Cytotoxic effects of erucin and SFN on liver and lung slices were evaluated using a simple colorimetric assay based on quantifying lactate dehydrogenase (LDH) activity released from damaged cells into the medium. The extent of LDH leakage, expressed as percentage of total liver slice LDH content, is a commonly used marker of parenchymal cell membrane integrity, however, no clear relationship between the degree of LDH leakage and slice viability has been established (Barr et al., 1991a and 1991b and Elkins et al., 1996). Although the LDH leakage from control slices was quite high (up to 40%) by 24 h of incubation, the slices retained their metabolic activity.

Both compounds were toxic to cultured rat and lung slices at 50 μM, and highly cytotoxic at 100 μM. Although the LDH leakage was not monitored in human liver slices, it is reasonable to expect manifestation of toxicity at high concentrations of ITCs, such as falling activities and/or cessation of induction of some enzyme, as indeed was the case with both human livers.

GSH is the major cellular scavenger of free radicals and electrophiles. At 50 μM, both ITCs profoundly decreased total cellular glutathione levels in the human liver as well as in both rat tissues. Rapid intracellular accumulation has been reported previously with SFN in Hepa 1c1c7 cells (Zhang and Talalay, 1998). Since ITCs are highly reactive towards glutathione, 95-98% of intracellular SFN exists as glutathione conjugates (Zhang, 2000) that are then rapidly exported out of the cells (Zhang and Callaway, 2002 and Callaway et al. 2004). Therefore it is not surprising that exposure of rat and human tissue slices to excessively high concentrations of SFN (≥ 25 μM) results in profound glutathione depletion and associated cytotoxicity. Erucin appears to sequester cellular
glutathione to the same degree as SFN. In contrast, after 24 h incubation of rat liver and lung slices with lower concentrations of erucin or SFN, glutathione levels were increased in a concentration-dependent manner. SFN substantially increased GSH levels previously in lymphoblastoid (Misiewicz et al., 2004) and in HepG2-C8 (Kim et al., 2003) cells. Such rebound up-regulation of glutathione synthesis in response to treatment with non-toxic concentrations of ITCs was only apparent in the liver from second donor. In contrast, glutathione levels in the human liver of the 1st donor did not recover by 24 h, indicating inter-individual differences in response to glutathione depletion among humans.

6.4.4 Conclusion

Neither erucin nor SFN modulated the activities of cytochromes P450, suggesting that suppression of bioactivation of human mutagens by dietary levels of erucin and SFN is unlikely to be a relevant mechanism of their anticarcinogenic activity. On the other hand, the likelihood of food-drug interactions that can lead to undesired side effects in humans taking medications may also be significantly decreased. Erucin and sulforaphane elevated glutathione S-transferase expression in isoform-specific manner in both rat and human liver. However, quinone reductase, either at the activity or protein level, was inducible by these compounds only in rat, and very poorly, or not at all, in human liver.
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7.1 Introduction

Humans are exposed repeatedly throughout the course of life to low levels of carcinogenic nitrosamines, heterocyclic amines (HA) and polycyclic aromatic hydrocarbons (PAH) present in foods such as smoked, grilled, fried or barbecued meat and fish, tobacco smoke and environment. Such carcinogens require cytochrome P450-mediated metabolic bioactivation to form highly reactive chemical species that covalently bind to the cellular DNA. Chemical mutagens and carcinogens are highly hydrophobic in nature and require metabolic modification to a more polar derivative that can be safely eliminated from the body. The first step of their metabolism involves hydroxylation by cytochrome P450 enzymes. Depending on the specific cytochrome P450 involved, the hydroxylation reaction is the first metabolic step leading to either detoxification or bioactivation. A large number of human carcinogens, including polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HA), are bioactivated by the CYP1, a highly inducible cytochrome P450 family (Ma and Lu, 2007). CYP1 expression is regulated by a ligand-activated transcriptional factor, the aryl hydrocarbon receptor (AhR). Many AhR ligands are CYP1 substrates, some of which, such as the classical PAH B(a)P, potently induce expression of this enzyme (McFadyen et al., 2003 and Nebert et al., 2004). CYP1 family consists of two subfamilies, CYP1A and CYP1B. The latter, composed of a single enzyme CYP1B1, is poorly expressed in the liver, but is present in extrahepatic tissues. The former subfamily consists of two enzymes, CYP1A1 and CYP1A2. CYP1A2 is liver-specific, whereas CYP1A1 is poorly expressed in the liver but occurs in extrahepatic tissues (Nebert et al., 2004). CYP1A1 induction has been associated with increased risk of cancer in animals (Gelboin, 1980) and humans (Proctor, 2001).

One of the putative mechanisms of suppressed tumour initiation in experimental animals by dietary erucin and SFN is acknowledged to be inhibition of the CYP1A-associated
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metabolic bioactivation of chemical carcinogens (Juge et al., 2007). However, SFN upregulated CYP1A1 apoprotein (Yoxall et al., 2005). Moreover, both erucin and SFN markedly induced CYP1A1 apoprotein in the in vivo and in vitro studies described in Chapters 5 and 6 respectively, indicating that these ITCs may modulate cellular signalling pathways involved in the regulation of CYP1A enzyme expression. It was, therefore, hypothesised that these phytochemicals may interfere with B(a)P-induced upregulation of CYP1A1 enzyme by B(a)P by possibly antagonising its binding to the AhR. The purpose of the present studies was to investigate whether concomitant treatment with ITC and B(a)P may antagonise B(a)P-mediated CYP1A1 induction and, consequently, the extent of bioactivation of carcinogens.

7.2 Methods

7.2.1 Effect of concomitant treatment with ITCs and B(a)P-mediated upregulation of CYP1A1 expression

The possibility of ITCs interacting with B(a)P was evaluated in precision-cut rat liver slices prepared and maintained as described in sections 2.2.2.1, 2.2.2.3 and 2.2.2.4. Liver slices were incubated in modified RPMI 1640 containing either a mixture of BaP (1 or 5 μM) with erucin/SFN (5 - 50 μM) or the ITCs alone for 24 h. The appropriate controls were incubated with the solvent vehicle (0.1%, v/v), dimethyl sulfoxide (DMSO). The toxicity of the ITC/B(a)P mixtures to liver slices was assessed by monitoring the LDH leakage (section 2.2.3). Tissue slices were homogenised and resolved into microsomal and cytosolic fractions (section 2.2.5), and the former was used in the determination of CYP1A1 activity (section 2.2.7) and apoprotein levels (section 2.2.13). Total protein was determined as described in section 2.2.6.
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7.2.2 Effect of pre-treatment with ITCs on mutagenicity of urinary IQ

The antimutagenic potential of each of the two test compounds, SFN and erucin, was assessed in rats maintained on water containing the test compounds or solvent vehicle for "long" (10 days) or "short" (1 day) period of time prior to exposure to a single dose of IQ. Body weight was recorded at the beginning (day 1) and at the end of the study (terminal body weight). At the end of the study, the animals were killed by cervical dislocation; the livers were removed, snap-frozen in liquid nitrogen and subsequently stored at -80°C until processed for analysis as described in section 2.2.5. The urine, collected prior to and post IQ administration, was analysed using the Ames mutagenicity test carried out in the presence of an activation system (10% of Aroclor 1254-induced S9) and employing Salmonella typhimurium strain YG1024, characterised by over-expression of O-acetylation as described in section 2.2.14.

7.2.2.1 Test compound formulation and storage

Stock solutions of erucin and sulforaphane were prepared in DMSO (300 mg/ml) and diluted with tap water to achieve final concentrations of 30mg/l and 150mg/l (v/v), corresponding to approximate daily doses of 3 and 15 mg/kg for each of the ITCs. The tap water for control animals was supplemented with the solvent vehicle only (DMSO, 0.05% v/v). All aqueous solutions of test compounds and solvent vehicle were prepared in advance and stored at -20°C as aliquots that were defrosted as required at 4°C overnight prior to administration. The heterocyclic amine 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) was dissolved in corn oil at a concentration of 1.25mg/ml and stored at 4°C.

7.2.2.2 Animal maintenance

The commercial source and conditions at which the animals were maintained during acclimatisation and study periods are described in section 2.2.1.
7.2.2.3 Design of study into the effects of "long"-term pre-treatment with ITCs on the IQ-mediated urinary excretion of mutagens

The experimental design of the study is summarised in Figure 7.1. Twelve male W/A rats were examined and randomly assigned to 3 groups of 4 animals each. Following an acclimatisation period of three days, the animals were maintained on water containing test compounds or solvent vehicle for 14 days as detailed below:

- **Group A**  Control, DMSO (0.05%, v/v) in tap water
- **Group B**  Low dose (30mg/l) of erucin or SFN in drinking water containing DMSO (0.05%, v/v)
- **Group C**  High dose (150mg/l) of erucin or SFN in drinking water in water containing DMSO (0.05%, v/v)

![Experimental design of the 14 day (long-term) study into the effects of erucin or sulforaphane on the IQ-mediated excretion of mutagens in urine.](image)

Figure 7.1: Experimental design of the 14 day (long-term) study into the effects of erucin or sulforaphane on the IQ-mediated excretion of mutagens in urine. Male Wistar albino rats (n=4) were maintained on either erucin (30 and 150 mg/l) or SFN (30 and 150 mg/l)-supplemented water for 14 days; controls received water containing solvent vehicle (DMSO, 0.5% v/v). Eleven days after the treatment commenced, a single dose of IQ (5 mg/kg of body weight) was administered by oral gavage to all animals. Urine was collected daily, starting 24h before and finishing 72 h after the IQ administration and urinary excretion of mutagens determined using the Ames mutagenicity assay.

On the 10th day of treatment (24h prior to the IQ administration), all animals were transferred into metabolic cages where they were housed singly for the last 4 days of
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On the 11th day of the treatment, all animals received a single dose of IQ (5 mg/kg body weight) administered by oral gavage. Urine was collected on a daily basis starting 24h before and finishing 72h after IQ administration.

### 7.2.2.4 Design of study into the effects of "short"-term pre-treatment with ITCs on IQ-mediated urinary excretion of mutagens

The experimental design of this short-term study is outlined in figure 8.2. Twelve male W/A rats were randomly assigned to 3 groups of 4 animals each. Following an acclimatisation period of three days, all animals were transferred into metabolic cages where they were housed singly throughout the study (4 days), and received the following treatments:

- **Group A**  
  Control, DMSO (0.05%, v/v) in tap water

- **Group B**  
  Erucin (150mg/l) in drinking water containing DMSO (0.05%, v/v)

- **Group C**  
  SFN (150mg/l) in drinking water in water containing DMSO (0.05%, v/v)

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![Figure 7.2: Experimental design of the "short"-term study into the effects of ITCs on the IQ-mediated urinary excretion of mutagens. Male Wistar albino rats (n=4) were maintained on erucin (150 mg/l) or SFN (150 mg/l)-supplemented water for 4 days. Twenty four h after the treatment commenced, a single dose of IQ (5 mg/kg of body weight) was administered by oral gavage to all animals. Urine was collected daily and excretion of mutagens determined using the Ames mutagenicity assay.](image-url)
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After a 24 pre-treatment with the ITCs, all animals received a single dose of IQ (5 mg/kg body weight), administered by oral gavage. Urine was collected on a daily basis starting 24h before and finishing 72 h after the IQ administration.

7.2.2.5 Determination of XME activities

Rate of ethoxy- and methoxyresorufin dealkylation was determined in hepatic microsomal fraction (section 2.2.7). Activity of cytosolic glutathione S-transferase was monitored using CDNB as the accepting substrate (section 2.2.12). Protein concentration in both fractions was determined as described in section 2.2.6.

7.3 Results

7.3.1 Effect of ITC/B(a)P mixture on LDH leakage

Lactate dehydrogenase (LDH) leakage into the incubation medium after a 24 h incubation of liver slices with erucin/SFN and B(a)P alone, as well as with their mixtures, was monitored as an indicator of cytotoxicity (the data on toxicity of individual ITCs is presented in Chapter 6, section 6.3.2). There was no change in the extent of LDH leakage from liver slices incubated with B(a)P alone (0-100 μM) for 24 h (Fig. 7.3). Following 24 h treatment of liver slices with mixtures of erucin (0-50 μM) and B(a)P (1 and 5 μM), a weak, but statistically significant toxicity was observed at 50 μM erucin combined with either 1 or 5 μM B(a)P (Fig. 7.4 A). Similarly, LDH leakage in hepatic slices treated with mixtures of SFN (0-50 μM) and B(a)P (1 and 5 μM) was only increased at the highest SFN level irrespective of B(a)P concentration (Fig. 7.4 B).
**Figure 7.3:** Leakage of LDH from rat liver slices incubated with BaP. LDH leakage was measured in liver slices incubated in modified RPMI 1640 supplemented with BaP (0-100 μM) for 24 h. LDH release is expressed as percentage of total LDH. Values are presented as means ± SD of duplicate determinations of triplicate slices per concentration.
Continued

![Graph showing LDH leakage from rat liver slices incubated concurrently with B(a)P and erucin (A) or SFN (B). LDH leakage was measured in liver slices incubated in modified RPMI 1640 containing a mixture of B(a)P (1 and 5 μM) and either erucin or SFN (5 - 50 μM) for 24 h. LDH release is expressed as percentage of total LDH. Values are presented as means ± SD of duplicate determinations of triplicate slices per concentration. *, P<0.05.](image)

**Figure 7.4: Leakage of LDH from rat liver slices incubated concurrently with B(a)P and erucin (A) or SFN (B).** LDH leakage was measured in liver slices incubated in modified RPMI 1640 containing a mixture of B(a)P (1 and 5 μM) and either erucin or SFN (5 - 50 μM) for 24 h. LDH release is expressed as percentage of total LDH. Values are presented as means ± SD of duplicate determinations of triplicate slices per concentration. *, P<0.05.

### 7.3.2 Effect of erucin and SFN on B(a)P-mediated upregulation of CYP1A1 expression in liver slices

Initially, rat liver slices were incubated for 24 h with B(a)P (0-20 μM) alone to establish the concentration levels optimal for CYP1A1 induction. Hepatic CYP1A1 activity, determined using ethoxyresorufin as a diagnostic substrate, was induced by B(a)P in a concentration-dependent manner, increasing almost 6- and 16-fold at 1 and 5 μM respectively. The B(a)P-mediated CYP1A1 induction was linear up to B(a)P concentration of 5 μM. Therefore, B(a)P concentration of 1 μM was selected for detecting a possible synergistic or additive effect of ITC on B(a)P-mediated EROD induction, whereas 5 μM was optimal for evaluating the possibility of an antagonistic effect (Fig. 7.5).
When liver slices were incubated with either ITC alone (0-50 μM) for 24 h, hepatic EROD activity was significantly inhibited by both compounds only at the highest concentration (50μM) (Fig. 7.6), while Western blot analysis revealed a concentration-dependent increase in CYP1A1 apoprotein levels (Fig. 7.7). In contrast, concurrent treatment of rat liver slices with erucin or SFN (0-50 μM) and BaP (1 and 5 μM) diminished the inducing potential of the latter (Fig. 7.6). The antagonistic effect of both ITC was clearly concentration-dependent (Figures 7.6). Both ITCs caused a similar concentration-dependent decrease in the B(a)P-mediated increase in CYP1A1 protein expression (Fig 7.7 A and B). The change in expression of CYP1A1 was plotted taking into account amounts of loaded protein (Fig 7.7 C), since a linearity has already been established between protein concentration and optical density of the protein band (Pushparajah et al., 2007).
Figure 7.6: Effect of erucin (A) and SFN (B) on BaP-mediated increase in EROD activity. Slices from the liver of control rats were incubated in modified RPMI 1640 containing either mixtures of BaP (1 and 5 μM) and erucin (5-50 μM) or the test compounds alone for 24 h. Hepatic EROD activity was determined in microsomal preparation. Results are presented as mean ± SD of triplicate determinations. Each replicate comprised a pool of 6-10 slices. *, P<0.05; **, P<0.01. P<0.001.
Figure 7.7: Effect of erucin (A) or SFN (B) on B(a)P-mediated increase in hepatic CYP1A1 apoprotein. Slices from livers of control rats were incubated in modified RPMI 1640 containing either a mixture of B(a)P (5 μM) and ITC (0 - 50 μM) or ITC alone for 24 h. Microsomal proteins were resolved by 10 % (w/v) SDS-PAGE and then transferred electrophoretically to the Hybond-P polyvinylidene difluoride membrane. Immunoblot analysis was carried out using rat CYP1A1 antibodies followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 5 (B(a)P alone and B(a)P-erucin groups) or 40 μg (control and erucin only groups) of total protein. Molecular markers were run concurrently to aid band identification. The values in italics above the immunoblots show optical density (percentage) of each band relative to respective control (solvent vehicle-treated slices). C. Graphical presentation of quantitative changes in the apoprotein levels taking into account protein levels loaded.
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7.3.3 Effect of a “long”-term treatment with erucin or SFN on urinary excretion of mutagens in rats exposed to IQ

The animals remained in good health throughout the duration of the study; no clinical or behavioural signs of systemic toxicity were apparent among the different treatment groups. Since a single 250 g rat consumed at least 25 ml of water daily, ITC intake was calculated to be 0.75 and 3.75 mg per rat or 3 and 15 mg per kg for the low and higher dose levels respectively. Neither compound had any significant impact on water consumption, body weight gain or relative liver weight (Table 7.1).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg/day)</th>
<th>Average daily water intake (ml/day)</th>
<th>Average daily body weight gain (g)</th>
<th>Relative liver weight (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>---</td>
<td>27.4 ± 1.9</td>
<td>4.1 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>Low erucin</td>
<td>3</td>
<td>30.3 ± 6.9</td>
<td>4.2 ± 0.3</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>High erucin</td>
<td>15</td>
<td>25.7 ± 4.1</td>
<td>4.2 ± 0.7</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg/day)</th>
<th>Average daily water intake (ml/day)</th>
<th>Average daily body weight gain (g)</th>
<th>Relative liver weight (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>---</td>
<td>27.4 ± 0.3</td>
<td>4.4 ± 2.9</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Low SFN</td>
<td>3</td>
<td>27.8 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>High SFN</td>
<td>15</td>
<td>31.5 ± 3.1</td>
<td>4.2 ± 0.7</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

Table 7.1: Daily water consumption, body weight gain and relative liver weight of rats pre-treated with erucin (A) or SFN in water and exposed to IQ. Male Wistar albino rats were treated with ITC (30 and 150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 14 days. Eleven days after the treatment commenced, a single dose of IQ (5 mg/kg) was administered by oral gavage to all animals. Results are presented as mean ± SD, where n=4.
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Effect of pre-treatment of rats with ITCs (low and high dose) for 11 days prior to exposure to IQ (5 mg/kg) on excretion of the indirect-acting urinary mutagens was determined using the Ames mutagenicity test in the presence of an Aroclor 1254-induced activation system. Both ITCs suppressed the indirect-acting mutagenicity of urine, but a clear concentration-dependent effect was evident only in the case of erucin (Fig. 7.8 A). Excretion of the indirectly-acting mutagens in urine in the first 24 h after IQ administration was significantly suppressed in animals maintained on the higher dose of either compound, whereas at lower dose statistical significance was attained only for erucin (Fig. 7.8 A). Treatment with lower dose of SFN decreased the indirect-acting mutagenicity of urine, but statistical significance was not achieved as a result of wide variations and small size (n=4) of groups (Fig. 7.8 B). Significant suppressing effect of erucin persisted up to 72 h after IQ administration (Fig. 7.8 A). Suppressing effect of SFN on excretion of indirect-acting mutagens was evident only in the first 24 h after administration of IQ (Fig. 7.8 B). No direct-acting mutagenicity was detected, i.e. in the absence of the activation system (results are not shown).

To investigate whether the observed ITC-induced decrease in urinary mutagenicity following exposure to IQ was associated with a change in CYP1-mediated metabolism of this model HA, the rate of hepatic O-deethylation (EROD) and O-demethylation (MROD) of ethoxy- and methoxyresorufin, catalysed by CYP1A1 and CYP1A2 enzymes respectively, were determined. Neither ITC modulated microsomal EROD or MROD (Fig. 7.12 A and B), suggesting that other mechanisms may be involved. Activity of GST, determined using CDNB as a marker substrate, was marginally elevated by the higher doses of both compounds, but statistical significance was not reached due to small size and wide variations between the groups (Fig. 7.12 C).
Figure 7.8: Effect of 11 day pre-treatment with erucin (A) and SFN (B) on urinary excretion of mutagens in rats following IQ administration. Male Wistar albino rats (n=4) were treated with ITC (30 and 150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 14 days. Eleven days after the treatment commenced, a single dose of IQ (5 mg/kg of body weight) was administered by oral gavage to all animals. Urine was collected daily, starting 24h before and finishing 72 h after the IQ administration. Urinary mutagenic activity was determined by the Ames mutagenicity test employing S. typhimurium strain YG1024 in the presence of Aroclor 1254-induced activation system. Results are presented as mean ± SD of triplicate determinations. The spontaneous reversion rates of 20 ± 3 and 38 ± 6 for erucin and SFN respectively have already been subtracted. *, P<0.05; **, P<0.01.
Figure 7.9: Effect of erucin and SFN on rat hepatic EROD, MROD and GST. Male Wistar albino rats were treated with ITC (30 and 150 mg/l) or solvent vehicle (DMSO, 0.05% v/v) in drinking water for 14 days. Eleven days after the treatment commenced, a single dose of IQ (5 mg/kg) was administered by oral gavage to all animals. Hepatic EROD (A) and MROD (B) were determined in the microsomal fraction, whereas GST (C) activity was determined in the cytosolic fraction using CDNB as an accepting substrate. Results are presented as mean ± SD, where n=4, each sample assayed in duplicate. *, P<0.05; **, P<0.01.
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7.3.4 Effect of short-term pre-treatment with erucin or SFN on urinary excretion of mutagens in rats treated with IQ

To investigate whether a duration of ITC pre-treatment prior to IQ exposure can influence urinary mutagenicity, rats were pre-treated with ITC for one day prior to IQ administration. The animals remained in good health; no clinical or behavioural signs of systemic toxicity were apparent among the different treatment groups. Neither compound had any significant impact on water consumption, body weight gain or relative liver weight (Table 7.2).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg/day)</th>
<th>Daily water intake (ml/day)</th>
<th>Daily body weight gain (g)</th>
<th>Relative liver weight (% of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>---</td>
<td>23.9 ± 0.9</td>
<td>4.9 ± 0.7</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>High erucin</td>
<td>15</td>
<td>20.9 ± 3.6</td>
<td>5.5 ± 0.9</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>High SFN</td>
<td>15</td>
<td>22.5 ± 6.0</td>
<td>5.1 ± 0.6</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

Table 7.2: Daily water consumption, body weight gain and relative liver weight of rats pre-treated for 24h with erucin (A) or SFN prior to IQ administration. Male Wistar albino rats were maintained on erucin (150 mg/l), SFN (150 mg/l) or solvent vehicle (DMSO, 0.5 % v/v)-supplemented water for 4 days. Twenty four h after the treatment commenced, a single dose of IQ (5 mg/kg) was administered by oral gavage. Results are presented as mean ± SD, where n=4.

Analysis of urine in the presence of an Aroclor 1254-induced activation system revealed that neither erucin nor SFN influenced excretion of the indirect-acting mutagens (Fig. 7.13), whereas no mutagenicity was detected in the absence of the activation system (results not shown).
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7.4 Discussion

7.4.1 Interactions of ITCs with B(a)P

Inhibition of Phase I enzymes and, consequently, interruption of the bioactivation pathway(s) of parent carcinogens by dietary ITCs has been associated with decreased DNA damage, mutation frequency and, ultimately, inhibition or retardation of tumourigenesis (Juge et al., 2007). The reverse is also true, especially in case of the CYP1A1, induction of which has been associated with increased risk of cancer in animals (Gelboin, 1980) and humans (Proctor, 2001).

Neither CYP1A1 apoprotein nor activity were down-regulated by the individual ITCs. In contrast, both ITCs antagonised in a concentration-dependent manner the AhR-mediated...
Induction of CYP1A1 enzyme activity and expression by B(a)P in liver slices following their concurrent incubation for 24 h. B(a)P is a potent inducer of its own metabolism and bioactivation to genotoxic metabolites mostly by CYP1A1, induction of which has been linked to an increased risk of cancer in animals (Gelboin, 1980) and humans (Proctor, 2001). Curbing its up-regulation by inducers such as B(a)P and, consequently, bioactivation of human carcinogens such as PAHs and HAs, therefore represents a novel mechanism that may contribute to the pleiotropic anticarcinogenic effects of erucin, SFN and, possibly other ITCs. Given that induction of CYP1A1 by B(a)P is AhR-controlled, it is possible that ITCs disrupt activation by B(a)P and/or interfere with downstream events involving formation of active transcription factor and binding to the promoter of XRE region of AhR-controlled battery of genes. Recently SFN has been shown to bind to the steroid and xenobiotic receptors (SXR) directly and disrupt the SXR-coactivator interactions necessary for the activation of the gene transcription in primary human hepatocytes (Zhou et al., 2007).

Alternatively, the inhibiting effect of ITCs on B(a)P-mediated up-regulation of CYP1A1 could be explained by direct mechanism-based inactivation of this enzyme as described in Chapter 5. However, the latter mechanism does not explain a decrease in CYP1A1 apoprotein levels. Moreover, ITC-induced loss of CYP1A1 activity, prevented even by relatively low levels of reduced GSH (0.2 mM) is unlikely to take place in hepatocytes where physiological GSH concentration is significantly higher (Chapter 5).

7.4.2 Effect of "long-" and "short"-term pre-treatment with ITCs on excretion of urinary mutagens in IQ-treated rats

Heterocyclic amines are another important class of CYP1A-bioactivated chemicals, associated with increased risk of human cancers (Ikeda et al., 1983, Robbana-Barnat et al., 1996, De Stephani et al., 1997 and Sinha et al., 2000) and established carcinogens
Chapter 7: Effect of isothiocyanates on metabolism of carcinogens

in animals (Adamson et al., 1994), which are formed in protein-rich food such as red meat, poultry and fish during heat processing, especially grilling, broiling or frying. The mutagenic activity of the heterocyclic amines, such as 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) (Fig. 7.11), arises as a consequence of metabolic activation of the parent compounds, which are oxidised by microsomal CYP1A2, with minor contribution from CYP1A1 to form the N-hydroxyamino derivatives that are subsequently conjugated with sulphate and acetate to generate esters that spontaneously break-down to form the nitrenium ion, the presumed ultimate carcinogen that interacts with DNA (Davis et al., 1993). IQ was chosen as a model mutagen for several reasons: 1) it is a food mutagen and thus likely to be consumed concurrently with ITCs formed in cruciferous vegetables, 2) previous studies have shown a SFN-mediated decrease in IQ-induced genotoxicity and/or mutagenicity (Barcelo et al., 1998; Bacon et al., 2003 and Yoxall et al., 2005) and 3) it is highly mutagenic in the Ames mutagenicity assay thus enabling the determination of IQ-mediated mutagenicity in rat urine.

Since the mechanism of ITC-mediated chemoprevention is partially based on impairment of the metabolic pathways of chemical carcinogens that produce genotoxic metabolites and enhanced detoxification of their reactive intermediates (Fahey and Talalay, 1999), the aim of the current studies was to investigate whether erucin can modulate the metabolism of IQ as exemplified by urinary excretion of pro-mutagens. Mutagenic activity was determined using the Ames assay employing Salmonella typhimurium bacteria strain YG1024, genetically modified to over-express O-acetyl transferase, the enzyme involved in the bioactivation of IQ (McArdle et al., 1999). The effects of long- and short-term pre-treatment of rats with erucin or SFN prior to IQ administration on excretion of urinary mutagens was evaluated at the dose levels reflecting human intakes (Howard et al., 1997). Direct-acting urinary mutagenic activity, i.e. in the absence of the activation system, was not detected in either long- or short-term study. Treatment of
Chapter 7: Effect of isothiocyanates on metabolism of carcinogens

urine with aryl sulphatase or β-glucuronidase failed to increase the IQ-induced mutagenic response in previous studies (McArdle et al., 1999), therefore this methodology was not applied to the current studies.

"Long-term" pre-treatment of rats with low and high dose of erucin prior to IQ administration resulted in a significant and dose-dependent reduction in excretion of indirect-acting mutagens in urine collected during the first 24h after IQ administration. Marked suppressing effect of erucin persisted up to 72 h after IQ administration, but only at the high dose group. SFN was a less potent suppressant of the indirect-acting mutagenicity, decreasing it only in the first 24 h after IQ administration and only at the high dose. Indirect mutagenic activity reflects the levels of unchanged IQ that is converted by the CYP1A, present in the activation system, to the hydroxylamine, the proximate mutagen responsible for the mutagenic effect in the Ames assay. The only other metabolites that may contribute to indirect mutagenic activity are N-acetyl IQ and demethylated IQ, but both are minor metabolites (Barnes and Weisburger, 1985). In contrast, the ring hydroxylated metabolites of amino-compounds can not be activated despite having an intact exocyclic amino group (Tong et al., 1986 and Ioannides et al., 1989) and the N-conjugates of IQ such as the sulphamate lack mutagenic activity (Turesky et al., 1986). Since the indirect-acting urinary mutagens most likely represents the unchanged parent compound, the decrease in their excretion can be caused by induction of liver CYP1A1 and, more importantly CYP1A2, the latter being the principal enzyme involved in cytochrome P450-mediated detoxification, as well as bioactivation pathways of IQ metabolism (McArdle et al., 1999 and Yoxall et al., 2004). However, activities of either EROD or MROD in the current studies did not differ between the treated and control animals. IQ itself is a very weak inducer of the CYP1 family (Rodrigues et al., 1989) and it is extremely unlikely to influence this enzyme systems following administration of a single oral dose. The CYP1A enzyme activities were only
determined in the liver, the principal site of IQ metabolism, the contribution of other organs to the metabolism of IQ, which could be conceivably differentially modulated by isothiocyanates, has not been accounted for. Another feasible explanation is that a change in the activity of other enzyme systems, such as sulfotransferases or UGTs can shift the IQ metabolism so as to favour the competing detoxification pathways. This mechanism is supported by the observation that the extent of Phase II enzyme induction in rats can be enhanced by increased duration of ITC treatment (Munday and Munday, 2002). Furthermore, the protective effect of ITC in the current studies was only apparent after "long"-term, but not 24 h pre-treatment with ITC prior to IQ administration. Activity of sulfotransferases and UGTs were not determined and it remains to be established whether direct conjugation with sulphur and/or glucuronide of the exocyclic amino group (Turesky et al., 1986 and Armbrecht et al., 2007), which are likely to promote its detoxification, may be enhanced. Glucuronide and sulphur conjugates (Fig. 7.11) of the exocyclic amino group are expected to increase the direct mutagenicity

![Figure 7.11: Structures of IQ and its major metabolites. Adapted from Armbrecht et al. (2007)](image-url)
following treatment of urine with β-glucuronidases or aryl sulphatases respectively, which was not the case (McArden et al., 1999), suggesting that these metabolites are not good substrates and/or may be resistant to the activity of these enzymes (Turesky et al., 1986). Ring hydroxylation by cytochrome P450 enzymes followed by glucuronide and sulphur conjugation (Fig. 7.11) are also among the important detoxification pathways of IQ (Armbrecht et al., 2007) that may be enhanced by ITCs. It is conceivable that ITCs excreted in urine act as mechanism-based inactivators of CYP1A in S9 preparations used in the activation system. The latter mechanism is unlikely for at least two reasons. Firstly, the urinary concentrations of ITC even at the high dose level does not exceed 2 μM (Chapter 3) and was further reduced 20-fold when urine was incorporated into the top agar for plating, whereas effective CYP1A inhibition (50 %) can be achieved in vitro at concentrations of erucin and SFN of 25 μM. Secondly, the chemopreventive effect of ITCs against IQ-associated mutagenicity was evident only after "long"-term administration, but not after 1-day pre-treatment. Considering that most of ITC dose is excreted within first 24 h after administration, duration of ITC pretreatment should not affect CYP1A inhibition if such mechanism was operative. To gain a better understanding of the mechanism of ITC-mediated attenuation of IQ mutagenicity, it is necessary to investigate the metabolic profile of IQ before and after ITC administration. Finally, it can not be ruled out that the observed decrease in the urinary excretion of indirect-acting following long-term pre-treatment of rats with ITC prior to exposure to IQ is attributed to the enhancement of the metabolic pathway(s) leading to bioactivation of this mutagen and formation of DNA and/or protein adducts and thus promoting mutagenic effects of IQ. Further studies are required to ascertain whether ITCs are capable of promoting IQ-induced DNA damage.
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7.4.3 Conclusion

Both ITCs, erucin and SFN, attenuated B(a)P-mediated induction of CYP1A, possibly as a result of mechanism-based inactivation of this enzyme by ITC and/or antagonism of B(a)P binding to the AhR. Moreover, the current studies demonstrate that "long"-term, but not short-term administration of ITCs diminishes urinary excretion of indirect-acting mutagens in rats exposed to a single dose of IQ, although the mechanism of the modulating effects remains to be elucidated.
Final discussion
8.1 Effects of erucin and SFN on phase II enzymes and glutathione levels

Diets rich in Brassica vegetables have been linked to reduced incidence of various human cancers (Higdon et al., 2007 and Sapone et al., 2007). The observed beneficial health effects of these plants are ascribed to ITCs, the most abundant class of phytochemicals present in these vegetables. They occur in the form of glucosinolate precursors that are converted to their respective ITCs by the endogenous plant enzyme myrosinase following disruption of the plant material (Talalay and Fahey, 2001). Numerous in vitro and animal studies have confirmed the beneficial chemopreventive activity of these phytochemicals.

The most extensively investigated ITCs include AITC, BITC, PEITC and SFN, most of which are inducers of phase II enzymes such as QR, GST and UGTs, and inhibitors of cytochromes P450 (Hecht, 2000). Among these, SFN is arguably the most extensively investigated ITC, whereas erucin has received much less attention. One of the postulated mechanisms of the anticarcinogenic activity of ITCs involves impairment of the metabolic activation of chemical carcinogens and enhanced detoxification of their reactive metabolites. Consequently, the carcinogens are bio-transformed through metabolic pathways that lead to inactive products that the body can readily eliminate (Thornalley, 2002 and Juge et al., 2007). At the dietary relevant doses (Howard et al., 1997), which were the focus of the current in vivo studies, only hepatic and pulmonary QR activity was elevated by erucin. When rats were treated with low doses of erucin, no detectable change in GST activity was achieved, although the GSTα and GSTμ protein levels were marginally up-regulated. Immunoblots can be more sensitive than biochemical assays and can identify changes in the protein expression that are too small to be detected at the catalytic activity level. However, the sensitivity of immunoassays depends on the source and quality of antibodies and reagents. Alternatively, the GST isoenzymes may be catalytically inactive; such possibility is supported by previous...
Chapter 8: Final discussion

studies reporting ITC-induced inactivation of GSTs following irreversible binding of substrate, rendering the enzyme catalytically inactive (Zhang et al., 1995). The elevation of rat GST apoprotein was isoenzyme-specific, as neither compound modulated GST expression (Chapter 5), the latter being in agreement with the current studies in precision-cut slices (Chapter 6).

8.1.1 Are dietary achievable plasma levels of SFN sufficient for its in vitro biological activity

*In vivo* exposure to SFN modestly enhanced GST activity when assessed by NBD-Cl, but the effect was only evident at a dose-level of 15 mg/kg, which is higher than human dietary exposure. Following oral administration of SFN to rats at the dietary dose of 0.5 mg/kg, the peak plasma concentration achieved was <0.3 μM (Chapter 3), which is below the lowest dose (3 mg/kg) employed in the present study (Chapter 5). The minimal concentration of erucin and SFN required to achieve a clear induction of both rat and human phase II enzymes in liver slices was 2.5 μM (Chapter 3), which can be attained in humans following Broccoli intake (Ye et al., 2002 and Gasper et al., 2005). However, it should be emphasised that the broccoli used in previous studies was selected for high glucosinolate concentration and/or was grown for the purpose of the study. Concentration of glucosinolates and consequently ITCs in commercially supplied Brassica vegetables can vary up to 10 fold at each step of the food production chain (Dekker et al., 2000). The broccoli used in the study described in Chapter 4 was purchased from a local supermarket to match the levels of SFN that can be reached by the general population. However, even after repeated consumption of large quantities of raw broccoli, the maximal plasma concentration of SFN (80 nM) was below the levels compatible with a measurable change in biological activity in rat and human tissue slices (Chapter 6). Moreover, the intake of ITCs, including SFN, by the general population may be further reduced substantially as a result of storage and cooking (Song and
Thornalley, 2007 and Rungapamestry et al., 2007). In this respect erucin, the principal source of which, the rocket salad, is consumed raw, has an important advantage over SFN. Currently it is not known whether plasma levels of erucin compatible with its biological activity can be attained by consumption of Brassica vegetables, because their glucoerucin content and consequently human intake have not yet been defined.

8.1.2 Role of GSTs in tumourigenesis

Since induction of GSTs by ITCs is one of the putative mechanisms of their anticarcinogenic activity, it is important to consider the complexity of the role of GSTs in tumourigenesis.

GSTs are unusual in that they are present at very high intracellular concentrations and are characterised by surprisingly low catalytic efficiency (Meyer et al., 1995). Most abundant mammalian cytosolic GSTs belong to class α, μ and η. Their expression is regulated by complex mechanisms through multiple pathways including the ARE, XRE, GST P enhancer I glucocorticosteroid-responsive element and AhR and, therefore is species-, age- and sex-specific (Hayes and Pulford, 1995). The biological role of GST is not confined to metabolic conjugation and subsequent clearance of xenobiotic substrates. The human GSTμ and GSTη are involved in cellular signalling regulating cell survival and death presumably by means of direct interaction with proteins at the site independent of their catalytic site, binding to and sequestering signalling proteins. Although induction of GSTs is thought to be beneficial to health, elevated levels of GSTs can lead to resistance to apoptosis initiated by a variety of stimuli (Townsend and Tew, 2003).
8.1.3 Erucin and SFN as substrates for GSTs

The major elimination pathway of SFN both in rodents (Kassahun et al., 1997 and Bheemreddy and Jeffery, 2007) and in humans (Janobi et al., 2006) is the mercapturic acid pathway, the first step of which entails GST-catalysed conjugation with glutathione. Moreover, the enzymatic rate of ITC conjugation with glutathione is high (Zhang et al., 1995; Kolm et al., 1995 and Meyer et al., 1995). It is therefore necessary to consider whether ITCs can enhance their own metabolism through induction of GST activity, increasing the rate of conjugation with glutathione and consequently elimination.

The induction of GSTs by erucin and SFN in both rat and human tissue slices was isoenzyme-specific, with GST class α and μ, but not η being up-regulated (Chapter 6). Human GSTM1 and GSTP1 are the most efficient catalysts of GSH conjugation with ITCs, whereas the GSTA1, GSTM2 and GSTM4 are significantly less efficient (Zhang et al., 1995 and Kolm et al., 1995). However, GSTA1 and, to a lesser extent, GSTA2 and GSTM favour direct binding to GS-ITC conjugates over binding of GSH and ITC alone, sequestering the conjugates and thus promoting the conjugation of ITCs with GSH by other classes of GSTs (Meyer et al., 1995). Expression of rat and human GSTμ was modestly up-regulated (Chapter 6) and, since GSTM1 appears to be the main contributor to ITC metabolism in humans due to high catalytic efficiency towards these compounds and high hepatic concentration of this isoenzyme, it may be speculated that erucin and SFN have the potential to induce their own metabolism; however, paradoxically, GSTM-null subjects excreted SFN metabolites at a faster rate than their GSTM-positive counterparts (Gasper et al., 2005), although it was hypothesised that diminished risk of cancer in GSTM1 and GSTT1-null individuals is associated with slower rate of ITC metabolism and clearance (Higdon et al., 2007). In view of significant polymorphism in GST expression in humans, the overall ITC metabolism is likely to depend on the individual’s phenotype. Marked inter-individual variations in response to treatment with
erucin and SFN were evident following incubation with human liver slices, expression of GSTs being modestly elevated in only one of the two donors (Chapter 6).

8.1.4 Effect of erucin and SFN on glutathione levels

GSH is the major cellular thiol and is regarded as the first line of cellular defence against genotoxic chemical carcinogens and ROS. Therefore, a rise in GSH levels will boost cell antioxidant capacity, diminishing damage that could be inflicted by electrophiles to cellular macromolecules such as DNA, proteins and lipids (Fahey and Talaley, 1999 and Murata et al., 2000). Both SFN and erucin elevated GSH levels following incubation with rat liver and lung slices (Chapter 6), as has been shown for SFN in previous in vitro studies (Brooks et al., 2001; Ye and Zhang, 2001; Kim et al., 2003 and Misiewicz et al., 2004). Although the extent of the ITC-induced increase in total GSH levels was similar in rat liver and lung slices, this effect is probably of greater importance in the lung, where basal levels of glutathione are much lower (Chapter 6). Such up-regulation of glutathione synthesis may be an adaptive response to a temporary decline in cellular GSH following exposure to non-toxic (up to 25 μM) concentrations of ITCs (Chapter 6). Owing to high enzymatic and non-enzymatic rate of reaction with GSH, ITCs utilise cellular GSH to form mercapturates (Zhang, 2000 and Zhang, 2001), their principal metabolites both in rodents (Kassahun et al., 1997 and Rungapamestry et al., 2006) and in humans (Janobi et al., 2006). In contrast, concentrations of erucin and SFN exceeding 25 μM were cytotoxic and seemed to evoke more profound depletion of cellular glutathione levels which did not rebound by 24 h of incubation (Chapter 6).

Of the two human livers, modest elevation of total GSH levels in response to non-toxic concentrations of either ITC was only apparent in the liver from the second donor, indicating inter-individual differences in response to glutathione depletion among humans. GSH levels in the second human liver were induced by ITCs in parallel with the
other phase II enzymes, presumably because the genes encoding glutathione synthesis enzymes such as \( \gamma \)-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis, are also ARE-responsive and are therefore co-coordinately up-regulated with other phase II enzymes (Zhang et al. 1992; Bonnesen et al., 2001; Brooks et al., 2001 and Morimitsu et al., 2001).

In contrast to the \textit{in vitro} outcome, neither compound influenced hepatic or pulmonary GSH concentrations when administered to animals at low dose levels (Chapter 5). The lack of detectable change in GSH levels \textit{in vivo} most likely reflects insufficient concentration of ITCs in rat plasma (Chapter 3) as already discussed in section 8.1.1.

\textbf{8.1.5 Modulation of QR activity by erucin and SFN}

Both compounds markedly up-regulated expression and activity of rat pulmonary and hepatic QR both \textit{in vitro} and, more importantly, \textit{in vivo} (Chapters 5 and 6, Zhang et al., 1992; Matusheski and Jeffery, 2001; Munday and Munday, 2004 and Yoxall et al., 2005), an enzyme that may stabilize p53 protein, resulting in increased apoptosis in cancerous cells (Asher et al., 2001), and which also facilitates the detoxication of quinones (Dinkova-Kostova and Talalay, 2000). Since this enzyme system is responsible for the detoxification of quinones, it may be inferred that erucin and sulforaphane have the potential to antagonise the carcinogenicity of polycyclic aromatic hydrocarbons in rat tissues through such a mechanism. Intriguingly, when human liver slices from two different donors were used, neither ITC influenced expression and/or activity of QR in the first liver, whereas only a weak rise in protein levels was noted in the second, in marked contrast to the observations made in rat. The possible mechanism(s) and implication of the observed differences in the inducibility of rat and human QR are discussed in section 8.6.
8.2 Effect of erucin and SFN on activity and expression of phase I enzymes

Inhibition of phase I enzymes, particularly of the CYP1 family is associated with suppressed bioactivation of carcinogens to genotoxic metabolites and is viewed as one of the mechanisms underlying anticarcinogenic activity of ITCs (Fimognari and Hrelia, 2007 and Juge et al., 2007), although the suppression of CYP1A-mediated deactivation pathways is frequently overlooked. Modulation of cytochromes P450 activity by erucin and SFN was not observed either in vivo, after oral administration to rats for two weeks at low dose levels, or in vitro, following exposure of rat and human tissue slices for 24 h to non-toxic concentrations (Chapter 5 and 6). However, the expression of CYP1A and CYP1B1 proteins was up-regulated both in vivo and in vitro, indicating catalytic incompetence of these enzymes. In concord with a previous study (Yoxall et al., 2005), SFN was a mechanism-based inhibitor of rat hepatic EROD, as was erucin, but the ITC-mediated loss of EROD activity was completely prevented by reduced GSH even at concentration (0.2 mM), i.e. below the physiological levels (about 5 mM) found in rat hepatocytes (Chapter 5). Moreover, the inhibiting effect was only apparent at relatively high ITC concentrations (≥ 25 μM) that are significantly higher than dietary achievable levels in humans as has already been discussed above. Consequently, it is unlikely that this mechanism is operative under conditions that prevail in vivo.

The observation that CYP1A apoprotein, but not the activity, was induced by ITCs is consistent with mechanism-based inactivation of this enzyme (Chapters 5 and 6). Contrary to the notion that SFN is a monofunctional inducer that selectively up-regulates expression of phase II enzymes through the ARE pathway without concurrent induction of AhR-regulated CYP1A family of enzymes (Zhang et al., 1992 and Miao et al., 1994), both compounds enhanced CYP1A and CYP1B1 protein levels (Chapter 6), indicating a possibility of cross-talk between the AhR- and ARE-regulated pathways. The up-regulation of the CYP1 family by erucin and sulforaphane was an unexpected and
surprising observation, as these are small molecular weight aliphatic compounds. Extensive studies have shown that the inducers of the CYP1 family are essentially aromatic planar compounds with a large area/depth\(^2\) ratio (Lewis et al., 1986; Lewis et al., 1987). Amongst the most avid ligands to the AhR receptor are the toxin TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and large hydrophobic planar aromatic compounds such as the polycyclic aromatic hydrocarbons (Denison et al., 2002; Denison and Nagy, 2003 and Pushparajah et al., 2008), although compounds comprising a single substituted aromatic ring, such as diaminotoluenes, can also serve as agonists (Cheung et al., 1996). Erucin and sulforaphane, both small molecular weight aliphatic compounds, are clearly strong inducers of the CYP1 family despite their structural unsuitability to function as Ah receptor agonists. However, up-regulation of the CYP1 family, independent of binding to the Ah receptor, has been reported (Ayalogu et al, 1995; Boyd et al, 1995; Lesca et al., 1995).

The inducing effect of erucin and sulforaphane on CYP1 protein expression was clearly much less pronounced in human liver slices compared with the observations made in rat slices, incubated with these compounds under identical conditions. Neither ITC influenced CYP1A and CYP1B1 apoprotein levels in the liver from Donor 1, whereas both compounds modestly elevated expression of these enzymes the liver of Donor 2, but much less effectively compared with rat. These observations point to a possible species difference in the up-regulation of CYP1 by these isothiocyanates. Alternatively, polymorphism in CYP1 enzymes (Nagata, K. and Yamazoe, Y., 2002), AhR (Denison and Nagy, 2003) or co-activator and related regulating proteins may influence AhR affinity to agonists and, consequently, the CYP1 inducibility in humans, although the impact of these polymorphic variations on the induction of CYP1 proteins is not entirely clear (Harper et al., 2002). It is imperative that additional human samples are investigated for an unequivocal conclusion to be drawn.
8.2.1 Effect of erucin and SFN on B(a)P-mediated induction of CYP1A

Both ITCs, erucin and SFN, attenuated B(a)P-mediated induction of CYP1A. The antagonistic effect of erucin and SFN on B(a)P-mediated up-regulation of CYP1A is unlikely to be explained entirely by mechanism-based inactivation of this enzyme by ITC because, firstly, physiological GSH concentrations are sufficiently high to prevent the inhibiting activity of ITCs (Chapter 5) and, secondly, both compounds antagonised the effect of B(a)P not only at the activity, but also at the apoprotein level (Chapter 7). However, mechanism-based inhibitors, also known as suicidal substrates, can destroy cytochromes (Murray, 2007). In order to confirm that the observed decrease in CYP1A protein level is due to ITC-induced down-regulation of its expression and not the consequence of protein degradation, expression of this enzyme needs to be evaluated at the mRNA level.

ITCs may antagonise the inducing effect of B(a)P on AhR-controlled CYP1 expression by virtue of direct binding to the receptor and disruption of co-activator recruitment and preventing the formation of the complexes necessary for the initiation of gene transcription. Direct interaction of SFN with the steroid and xenobiotic receptors (SXR) has been reported in primary human hepatocytes (Zhou et al., 2006). In view of the major involvement of AhR receptor in carcinogenesis, the potential of ITCs to modulate its function deserves further investigation. Furthermore, the biological function of the AhR is not limited to the regulation of XME expression. The AhR is implicated in cellular processes such as endocrine homeostasis, cell proliferation, apoptosis and terminal differentiation (Puga et al., 2002, Pocar et al, 2005 and Barouki et al., 2007). Further studies into the effect of ITCs on the AhR receptor binding are required for better understanding of this aspect of their biological activity. However, clear antagonism of B(a)P-mediated CYP1A1 up-regulation was achieved at relatively high ITC concentrations of ≥ 10 μM, but such effects, once again, are unlikely to persist in vivo following
exposure to the dietary relevant doses of erucin and SFN, because of the low plasma levels.

8.3 Effect of erucin and SFN on mutagenicity of IQ

Both ITCs suppressed the excretion of indirect-acting mutagens in rats exposed to a single dose of IQ, when mutagenicity was assessed in the presence of an activation system, indicating the potential to influence the metabolic fate of this heterocyclic amine, but only after repeated administration of ITC at approximate daily dose levels of 93.2 μmol/kg for erucin, and 84.7 μmol/kg for SFN. Erucin antagonised direct-acting mutagenicity of IQ even at lower, dietary achievable (Howard et al., 1997) dose level of 18.6 μmol/kg (Chapter 7). The indirect-acting mutagenicity in the urine of rats exposed to IQ mainly reflects levels of unchanged IQ (Barnes and Weisburger, 1985), since the ring-hydroxylated metabolites (Tong et al., 1986 and Ioannides et al., 1989) and the N-conjugates of IQ such as the sulphamate (Turesky et al., 1986) lack mutagenic activity and, furthermore, can not be metabolically converted to mutagens. Heterocyclic amines, such as IQ, are bioactivated through N-oxidation, catalysed by the CYP1 family and, in particular CYP1A2 and, to a lesser extent, CYP1A1 and CYP1B1 (Kim and Guengerich, 2005 and Turesky, 2005). Therefore, a decrease in the urinary indirect-acting mutagenicity indicates enhanced metabolism of IQ. However, neither erucin nor SFN elevated the hepatic CYP1A2 and CYP1A1 activities, indicating that the enhanced metabolism of IQ can not be attributed to up-regulation of these enzymes. Furthermore, the observed favourable change in IQ metabolism can not be explained by augmented reduction of the electrophilic hydroxylamine metabolite to the parent compound (Turesky, 2005), because neither ITCs induced activity of GST when a broad-spectrum substrate CDNB was used. The lack of modulating effect of low doses of ITCs on activities of CYP1A and GST is consistent with observations made in the in vivo study (Chapter 5). A feasible mechanism of the decreased mutagenic activity is enhancement
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by ITCs of other important detoxication pathways of IQ, including N-glucuronidation and sulphation (Turesky, 2005 and Armbrecht et al., 2007). PEITC enhanced UGP glucuronyl transferase (UGT) activity but decreased sulphotransferase in rats (Dingley et al., 2003). At the mRNA and protein levels, SFN up-regulated UGT1A1 in HepG2 cells but not in human hepatocytes (Basten et al., 2002 and Bacon et al., 2003). The effect of erucin on these enzyme systems in the liver has not been investigated. Since activities of sulfotransferases and UGTs were not determined, it remains to be established whether direct conjugation with sulphate and/or glucuronide of the exocyclic amino group pathways can be modulated by ITCs. In contrast, the decrease in urinary excretion of the heterocyclic amines PhIP and 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) in humans after ingestion of Brussels sprouts and broccoli (Murray et al 2001) appears to be due to enhanced CYP1A2 activity and increased metabolism of the amines (Walters et al 2004). Whether this effect can be attributed to the ITC content of the vegetables or other constituents also significantly contribute to and/or synergistically enhance the anticarcinogenic effects of ITCs remains to be elucidated.

8.4 Relative potencies of SFN and erucin to modulate xenobiotic metabolising enzymes expression and mutagenicity of IQ

Overall, both compounds were equipotent modulators of xenobiotic metabolising enzymes expression in rat lung and rat and human liver. However, erucin was marginally more potent as mechanism-based inhibitor of hepatic EROD activity (Chapter 5). Erucin also more effectively suppressed urinary mutagenicity of IQ when administered to rats at low doses (Chapter 7). In the in vivo study, unlike erucin, SFN significantly induced hepatic GST when analysed using NBD-Cl as a marker substrate (Chapter 5). Interestingly, human GSTs significantly vary in their catalytic efficiency towards different ITCs, with erucin being one of the best substrates for GSTs, in contrast to SFN, the poorest aliphatic substrate for all four human GSTs, despite their structural
similarity. Due its sulphur oxidation state, SFN is relatively polar and, in contrast to erucin, does not bind readily to the hydrophobic substrate-binding site of GSTs (Zhang et al., 1994 and Kolm et al., 1995), suggesting that SFN is likely to have the lowest clearance rate and, therefore, the highest GST inducing potency. However, in the in vivo studies in rat (Chapter 5), and in incubated rat and human tissue slices (Chapter 6), both compounds were essentially equipotent inducers of GSTs, possibly as a result of in vivo metabolic reduction of SFN to erucin, one of its major metabolites in rats (Kassahun et al., 1997; Bheemreddy and Jeffery, 2007).

8.5 Liver and lung as target tissues for ITCs

The overall pattern of ITC-induced modulation of phase I and phase II modulation was very similar in both tissues. However, the effect of both compounds on the xenobiotic metabolising enzymes expression in vivo was more pronounced in liver compared with lung, possibly reflecting differences in the anatomical location and metabolic capacity of these organs. Up-regulation of the rat pulmonary QR by the aliphatic ITC SFN was also reported previously in mice (Zhang at al., 1992). In contrast, aromatic PEITC failed to enhance QR activity in the lung (Guo et al., 1992 and Konsue and Ioannides, 2008), suggesting that effect of ITCs on this enzyme could be compound-specific and be influenced by the structure of the side-chain. Up-regulation of QR and GST enzyme systems by ITCs appears to be of greater importance in the lung. Lung is a major target tissue for many tobacco carcinogens such as PAH and heterocyclic amines, which can damage this tissue following their in situ cytochromes P450-mediated metabolic bioactivation to genotoxic metabolites (Ioannides and Lewis 2004). Moreover, experimental evidence supports the view that reactive intermediates generated in the liver, the principal site of bioactivation, may be transported to extrahepatic tissues, although the underlying mechanism(s) has not been elucidated (Wall et al 1991; Kaderlik et al 1994). Since the extent of DNA-binding intermediates would dependent on
both rate of their P450-catalysed formation and phase II-mediated detoxification, up-regulation of enzyme systems such as QR and GSTs by both ITCs may be responsible, at least partially, for the observed chemopreventive activity of ITCs in this tissue following exposure of animals to polycyclic aromatic hydrocarbons (Conaway et al., 2002 and Conaway et al., 2005). More importantly, both compounds enhanced pulmonary QR expression and activity in vivo, following repeated administration to rats at dietary relevant doses (Chapter 5).

8.6 Rat as surrogate for human in studies relating to ITCs

Rat may not be a good surrogate for human when studying potential of ITCs to modulate expression of xenobiotic metabolising enzymes. Expression of QR was very modestly elevated in only one of the two human livers studied, in marked contrast to the observations made in rat (Chapter 6). This could reflect a compound-specific failure of these aliphatic ITCs to induce human QR. However, the aromatic PEITC also failed to up-regulate activity and protein levels of this enzyme in three human livers, whereas increases were observed in rat slices incubated under identical conditions (N. Konsue, personal communication). These data could also indicate a possible species differences between rat and human with implications for the chemopreventive activity of these ITCs, as it would imply that chemopreventive potential might be less pronounced in human compared with rat. The failure of ITCs to induce human QR (Chapter 6) is surprising given that erucin up-regulated QR mRNA in human colonic adenocarcinoma cells (Jacubikova et al., 2005), whereas SFN potently augmented expression of QR in the skin of human volunteers following topical application of broccoli preparations (Dinkova-Kostova et al., 2007), in human retinal pigment epithelial cells (Gao and Talalay, 2004) and various human cell lines (Brooks et al., 2001 and Misiewicz et al., 2004). The observed difference could be attributed to human polymorphism. A non-functioning QR gene has been found in 20% of caucascians and almost 50% of other ethnic proup
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(Gaedigk et al., 1998 and Begleiter et al., 2006). Furthermore, genetic polymorphism in human Nrf2, the transcriptional factor involved in activation of ARE-controlled genes encoding phase II enzymes, could influence its expression and function (Marzec et al., 2007) and may lead to inter-individual differences in QR inducibility. A more definitive conclusion would require the analysis of additional human liver samples. Nevertheless, these studies highlight the need for caution when extrapolating from animals to humans.

In contrast, rat appeared to be an acceptable surrogate for human when studying the pharmacokinetic (PK) fate of SFN (Chapters 3 and 4). Although the calculated PK parameters in rat and human were broadly similar, several important points need to be considered when comparing the results of these two studies. The eating behaviour and diet composition of the two species are very different. Rats are nocturnal animals and eat mostly during the night time. They were dosed with SFN (Chapter 3) early in the morning, when their stomachs were likely to be full. In contrast, humans ingested broccoli (Chapter 4) after overnight fasting and had their breakfast and subsequent meals during the day time. It is pertinent to note that reactive ITCs can form covalent bonds with thiol, hydroxyl and amino groups of macromolecules (Keck et al., 2003). It remains to be established whether such spontaneous and non-specific binding to food constituents could limit bioavailability of orally ingested ITCs, especially when consumed concurrently with protein-rich food (meat, fish, milk etc.). Reversible binding of ITCs to food thiols is likely to delay absorption. Moreover, the propensity of ITC to bind to plasma proteins is likely to limit their cellular uptake. Thus, following administration at dietary relevant dose levels, concentrations of SFN in tissues could be lower than in plasma and below the levels compatible with its biological activity (i.e. about 2.5 µM). Finally, maximum PEITC plasma concentrations in rats were higher after repeated oral administration (N. Konsue, personal communications). In contrast, repeated consumption of broccoli by humans was not associated with a rise in SFN Cmax.
However, the dose of SFN ingested by humans was extremely low (Chapter 4), making direct comparison difficult. A definite conclusion as to whether rat is a good surrogate for human as far as ITCs are concerned would require additional studies using similar dose levels and allometric scaling.

8.7 Chemopreventive effectiveness of erucin and SFN

The most marked effect manifested at low dose levels by both compounds in vivo was up-regulation of QR. This mechanism could be partially responsible for the documented chemopreventive effect of ITCs following exposure of animals to chemical carcinogens that are metabolised to quinones (reviewed by Hecht, 2000; Conaway et al., 2002 and Conaway et al., 2005). However, additional mechanisms, including induction of apoptosis, suppression of cell proliferation and inflammation are likely to contribute to the observed chemopreventive activity of these phytochemicals. These mechanisms appear to function at the in vivo ITC concentrations similar to those required for up-regulation of Phase II enzymes. SFN-induced cell cycle arrest was observed in vitro at concentrations as low as 2.5μM (Miziewicz et al., 2004) in lymphoblastoid cells, 3μM in human T-cell leukaemia cells (Fimognari et al., 2002) and 4.3μM in human leukaemia 60 cells (Zhang et al., 2003). SFN induced apoptosis at 2.5μM in human lymphoblastoid cells (Miziewicz et al., 2004) and at ≥ 5μM in medulloblastoma cells (Gingras et al., 2004), but in most studies higher concentrations (10-40 μM) were required (Cho et al., 2005; Choi et al., 2007 and Karmakar, 2006). Erucin also inhibited cell proliferation in breast cancer MCF7 and Norman human mammary epithelial cells at 28 and 46μM respectively (Azarenko et al., 2006). At 5μM, SFN suppressed inflammatory response in airway epithelial cells stimulated with diesel extract (Ritz et al., 2006). Collectively, these data demonstrate that minimal ITC concentrations necessary for their biological activity could be achieved in vivo. Moreover, at these concentrations, ITCs could activate
multiple chemopreventive mechanisms simultaneously. However, such concentrations can only be achieved in humans following ingestion of broccoli with high glucosinolate content, i.e. broccoli sprouts, as discussed in section 8.1.1. Alternatively, ITCs may be ingested as dietary supplements.

To achieve ITC plasma concentrations (around 2.5-5 μM) sufficient for their chemopreventive activity in vivo, a 70 kg person would have to ingest at least 560 mg of SFN (8 mg/kg) of ITCs, assuming that 100% of the ingested dose is absorbed. This is equivalent to 43 kg of common broccoli per day. Therefore for chemopreventive purpose, ITCs would have to be used as dietary supplements rather than to be obtained from broccoli. However, repeated intake of excessively high doses (>25 mg/kg) of ITCs was associated with mutagenicity in laboratory animals (Dannick et al., 1982; Rao et al., 1995 and Kassie et al., 2003b). Therefore, if ITCs are to be taken repeatedly for long periods of time in the form of dietary supplements, it is essential that their daily dose be optimised to avoid overdose-induced toxicity.

8.8 Future work

Of more than 100 naturally occurring glucosinolates, only very few (Hecht, 2000 and Conaway et al., 2002) have attracted attention. The chemopreventive potential of the remaining ITCs deserves evaluation.

Another point that should be considered is that Brassica vegetables contain a mixture of glucosinolates, and it is conceivable that the combined biological effect of their respective ITCs could result in either synergy or antagonism. Clinically important synergism among chemoprotective agents has already been reported (Brenner, 2000 and Torrance et al., 2000). On the other hand, both P450 inhibition and induction were reported in rats fed different varieties of broccoli (Vang et al., 2001b), presumably as a
consequence of change in the glucosinolate ratio (Fahey et al., 1997 and Howard et al., 1997).

The current studies demonstrated that long-term pre-treatment with dietary relevant doses of ITCs was effective at suppressing mutagenicity of IQ, presumably as a result of altered metabolism. It would be informative to establish whether the enhanced conjugation of IQ with sulphate and glucuronide could be the underlying mechanism.

The important role of antiproliferative, anti-inflammatory and pro-apoptotic activity of ITCs in inhibition of the post-initiation stages of carcinogenesis has been increasingly recognised (Conaway et al., 2002; Myzak and Dashwood, 2006c and Juge et al., 2007). However, additional studies are needed to establish whether such mechanisms take place in vivo following administration of low doses of ITCs.

Finally, additional human livers are required to establish whether the observed differences in modulation of the QR and CYP1 expression by ITCs are due to inter-species differences or human polymorphism. Moreover, it is important to evaluate the ability of both compounds to antagonise B(a)P-mediated up-regulation of CYP1A in human liver, elucidate the mechanistic basis for this interaction and evaluate whether it can be achieved in vivo following oral administration of dietary relevant doses of both ITC and carcinogen.

8.9 Conclusion

To summarise, the maximal plasma concentrations of SFN in humans, following ingestion of Broccoli, or in rats, after oral administration of low doses approximating human intake, were well below those required to achieve XEM modulation, antagonise B(a)P-mediated induction of CYP1A or suppress mutagenic activity of IQ. Moreover, the
observed peak in plasma SFN concentration was transient. However, up-regulated expression of QR and GSTs in humans can be achieved following ingestion of Broccoli with high glucosinolate content. i.e. Broccoli sprouts. Moreover, other Brassica constituents may contribute to and/or synergistically enhance the anticarcinogenic effects attributed to these phytochemicals.
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