# SYNTHESIS OF SULFORAPHANE AND INHIBITION OF CYTOCHROME P450 ENZYMES AS A BASIS FOR ANTIGENOTOXICITY

SÍLVIA BARCELÓ BATLLORI

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

JULY 1997

THE UNIVERSITY OF ASTON IN BIRMINGHAM

# SYNTHESIS OF SULFORAPHANE AND INHIBITION OF CYTOCHROME P450 ENZYMES AS A BASIS FOR ANTIGENOTOXICITY

A thesis submitted by Sílvia Barceló Batllori
for the degree of Doctor of Philosophy
1997

## SUMMARY

Many dietary factors have been associated with a decreased risk of developing cancer. One potential mechanism by which these factors, chemopreventors, protect against cancer may be via alteration of carcinogen metabolism. The broccoli constituent sulforaphane (1-isothiocyanate-4-methylsulfinylbutane) ($CH_3$-SO-$(CH_2)_4$-NCS) has been isolated as a potential inducer of phase II detoxification enzymes and also protects rodents against 9,10-dimethyl-1,2-benz*[a]*anthracene-induced mammary tumours. The ability of sulforaphane to also modulate phase I activation enzymes (cytochrome P450) (CYP450) was studied here. Sulforaphane was synthesised with an overall yield of 15 %, essentially via 1-methylsulfinylphthalimidobutane, which was oxidised to the sulfoxide moiety. Deprotective removal of phthalimide yielded the amine, which was converted into sulforaphane by reaction with *N,N'*-thionocarbonyldiimidazole. Purity (95 %) was checked by $^{1}$H-NMR, $^{13}$C-NMR and infrared and mass spectrometry. Sulforaphane was a competitive inhibitor of CYP2E1 in acetone-induced Sprague-Dawley rat microsomes ($K_i$ 37.9 ± 4.5 μM), as measured by the p-nitrophenol hydroxylase assay. Ethoxyresorufin deethylase activity (EROD), a measurement of CYP1A activity, was also inhibited by sulforaphane (100 μM) but was not competitive, and a preincubation time-dependence was observed. In view of these results, the capacity of sulforaphane to inhibit *N*-nitrosodimethylamine (NDMA)-induced genotoxicity (CYP2E1-mediated) was studied using mouse liver activation systems. Sulforaphane (>0.8 μM) inhibited the mutagenicity of NDMA (4.4 mg/plate) in *Salmonella typhimurium* strain TA100 after pre-incubation for 45 min with acetone-induced liver 9000 g supernatants from Balb/c mice. Unscheduled DNA synthesis induced by NDMA (33.5 μM) in mouse hepatocytes was also reduced by sulforaphane in a concentration-dependent manner (0.064-20 μM). Sulforaphane was not genotoxic itself in any of these systems and cytotoxic only at high concentrations (>0.5 mM and > 40 μM respectively). The ability of sulforaphane to modulate the orthologous human enzymes was studied using a human epithelial liver cell line (THLE) expressing individual human CYP450 isoenzymes. Using the Comet assay (a measurement of DNA strand breakage under alkaline conditions), NDMA (0.01-1 μg/ml) and IQ (0.1-10 μg/ml) were used to produce strand breaks in T5-2E1 cells (expressing human CYP2E1) and T5-1A2 cells (expressing human CYP1A2) respectively, however no response was observed in T5-neo cells (without CYP450 cDNA transfection). Sulforaphane inhibited both NDMA and IQ-induced DNA strand breakage in a concentration-dependent manner (0.1-10 μM). The inhibition of metabolic activation as a basis for the antigenotoxic action of sulforaphane in these systems (bacteria, rodent hepatocytes and human cells) is further supported by the lack of this chemopreventor to influence $NaN_3$ mutagenicity in *S. typhimurium* and $H_2O_2$-induced DNA strand breakage in T5-neo cells. These findings suggest that inhibition of CYP2E1 and CYP1A by sulforaphane may contribute to its chemoprotective potential.

**Key words**: sulforaphane/chemoprevention/cytochrome P450/*N*-nitrosodimethylamine/ 2-amino-3-methylimidazo[4,5-f]quinoline

*Si vols una bona vida*
*no et planyis mai del passat:*
*comença sempre a refer-te*
*com si tot just fossis nat.*
*Ton dever de cada dia*
*el dia ja te'l dirà:*
*complau-te en allò que facis*
*I estima allò que l'altre fa.*
[Goethe]

**ALS MEUS PARES,**
**GRACIES PEL VOSTRE AMOR**
**I TOT EL QUE M'HEU ENSENYAT.**

***ACKNOWLEDGMENTS***

Firstly, I would like to express my gratitude to Dr. Kevin Chipman (University of Birmingham, Biochemistry) for his supervision, patience, encouragement and help. Without him, most of this work would had not been accomplished.

I would like to thank Dr. Mike Coleman for all his help and proof reading and Dr. Andy Gescher for supervision during my first year. Special thanks go to Dr. John Gardiner and all the members of the medicinal chemistry lab for their help in the organic synthesis. I am also grateful to Nuala Helsby and Martin Hargeaves for their help in the kinetic studies.

I also would like to acknowledge Katherine Macé, Elizabeth Offord and Andrea Pfeifer from Nestlé Research Center, Lausanne (Switzerland) for supplying the cell lines used in this project.

I am indebted to all the present and past members of the 4th floor of Biochemistry, University of Birmingham, for making lab work and coffee breaks more bearable. Special thanks to all the members of Dr. Chipman group and especially to Jane Davies, for her helpful technical support and proof reading, Clement Musonda for teaching me cell culture techniques and Fiona Elcock for her support. I would also like to express my gratitude to Hillary Cross for scientific and technical suggestions and encouragement.

I am also incredibly grateful to a number of people outside academia. Thanks to Jim, Alan, Phil and Willy for the use of their computers and help. My thanks also go to my flatmate Helen for proof reading, support and friendship. Also special thanks to Pili and Mireille for their friendship and help, and to Ian for friendship and constant support.

Finally, I will be eternally indebted to my parents Joan Mª and Assumpta, and brothers Joan, Lluis and David, for their love, encouragement, education and also economic support throughout this thesis. Words can not express my gratitude towards them.

***CONTENTS*** **Page**

## LIST OF TABLES

### Chapter 1



### Chapter 2



### Chapter4

**Chapter 5**

## LIST OF FIGURES

### Chapter 1



### Chapter 2

**Chapter 3**



**Chapter 4**

## Chapter 5

## ABREVIATIONS

| | |
|---|---|
| 2-AAF | 2-Acetylaminofluorene |
| 4-NC | 4-Nitrocatechol |
| $AFB_1$ | Aflatoxin $B_1$ |
| ARE | Antioxidant response element |
| B[*a*]P | benzo[*a*]pyrene |
| BAA | Bicyclic aromatic amines |
| BHA | Tert-butyl-4-hydroxyanisole |
| BHQ | Tert-butylhydroquinone |
| BITC | Benzylisothiocyanate |
| *m*-CPBA | m-Chloroperoxybenzoic acid |
| BSA | Bovine serum albumin |
| CYP | Cytochrome P450 isoform |
| CYP450 | Cytochrome P450 |
| DAS | Diallylsulfide |
| DASO | Diallylsulfoxide |
| $DASO_2$ | Diallylsulfone |
| DMBA | 7,12-Dimethylbenz[*a*]anthracene |
| DMEM | Dulbeco's modified Eagle's Medium |
| EROD | Ethoxyresorufin deethylase |
| GCIJ | Gap junction intercellular communications |
| Glu P-1 | 2-Amino-6-methyldipyrido[1,2,-*a*:3',2'-*d*]imidazole |
| GP | Glutathione peroxidase |
| GR | Glutathione reductase |
| GRE | Glucocorticoid response element |
| GSH | Glutathione |
| GST | Glutathione-*S*-transferase |
| HAA | Heterocyclic aromatic amines |
| HBSS | Hank's balanced salt solution |
| HMPA | Hexamethylphosphoroustriamine |
| IQ | 2-Amino-3-methylimidazo[4,5-*f*]quinoline |
| LDA | lithium diisopropylamide |

| | |
|---|---|
| LDH | Lactate dehydrogenase |
| LHMDS | Lithium hexamethyldisilazide |
| MeIQ | 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline |
| MeIQx | 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline |
| NAT | *N*-Acetyltransferase |
| NBMA | *N*-Nitrosobenzylamine |
| NDEA | *N*-Nitrosodiethylamine |
| NDMA | *N*-nitrosodimethylamine |
| ß-NF | ß-Naphthoflavone |
| NNG | Net nuclear grain |
| NNK | 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| PAH | Polycyclic aromatic hydrocarbon |
| PBS | Phosphate buffer saline |
| PEITC | Phenetylisothyocyanate |
| PhIP | 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine |
| PHITC | Phenylhexylisotiocyanate |
| PITC | Phenylisothiocyanate |
| PNP | *p*-Nitrophenol |
| PPAR | Peroxisome proliferator activated receptor |
| QR | Quinone reductase |
| ROS | Reactive oxygen species |
| SCGE | Single cell gel electrophoresis |
| SOD | Sodium superoxide dismutase |
| TCDD | 2,3,7,8-Tetrachlorodibenzo-p-dioxin |
| TDI | N,N'-thionocarbonyldiimidazole |
| THF | Tetrahydrofuran |
| THLE | Human epithelial liver cells |
| TPA | tetradecanoyl-phorbaol-13-acetate |
| Trp P-1 | 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole |
| UDS | Unscheduled DNA synthesis |
| UGT | UDP-glucurosyltransferases |
| XRE | Xenobiotic response element |

# *CHAPTER 1*

# *General Introduction*

Cancer is currently one of the major causes of death in developed countries, and it is estimated that at least one person in four will contract cancer during his/her lifetime. The involvement of genetic factors and also exogenous factors in the aetiology of cancer has been demonstrated (Doll and Peto, 1981). Exogenous factors include mainly radiation, viruses, environmental factors and diet. In addition to early cancer diagnosis and therapy, cancer prevention has become a priority issue during the last decades (Weinstein, 1991). The strategies for prevention include the elucidation of mechanisms of carcinogenesis and the identification of carcinogenic and "anticarcinogenic" factors. This project is concerned with mechanisms of cancer prevention, in particular the modulation of metabolic activation of carcinogens by a potential anticarcinogenic dietary factor, sulforaphane.

## *1.1. Chemical carcinogenesis*

Cancer is a process in which a population of cells escape normal growth control to become a malignant tumour (Vile and Morris, 1992). A malignant tumour is a group of fully transformed cells with abnormal proliferating capacity and which can invade adjacent tissues and spread (metastasis) to other sites in the body forming secondary tumours. Normal cells become malignant when they are no longer able to complete the differentiation process and grow continuously expressing the transformed phenotype (Vile and Morris, 1992, Trosko, 1989). The nature of the primary events leading to the production of abnormally proliferating cells has been associated with various genetic changes.

### 1.1.1. Mutation and cancer

The "Mutational Theory of Cancer" was first introduced by Boveri in 1911 (Strauss, 1981) and implies that there is an association between malignant transformation and genetic changes, i.e. mutations occurring in somatic cells. Several lines of evidence support this theory. Firstly, the majority of human and animal tumours have a clonal origin, i.e. derive from divisions from a single transformed cell (Strauss, 1981). Secondly, a large number of carcinogens are also mutagens. Further evidence comes from cancer-prone patients with hereditary DNA repair deficiency, such as Xeroderma Pigmentosum (Setlow, 1978). The recent identification of cellular (proto)-oncogenes and tumour suppressor genes provides

additional support. Proto-oncogenes encode proteins involved in signal transduction pathways regulating cell growth, differentiation and division, and are only expressed during restricted periods of differentiation and organ development (Yamamoto, 1993). More than 50 families have been identified, of which *c-Ha-ras*, *c-myc*, *neu* and *nef* have been widely studied (Stanley, 1995). Activated proto-oncogenes have been found in many human and animal tumours (Stanley, 1995; Fearon and Vogelstein, 1991). On the other hand tumour suppressor genes have been found to be inactivated or lost in several types of cancers (Weinstein, 1991; Levine *et al*, 1991). It is important to note in this context that activation of proto-oncogenes and inactivation of tumour suppressor genes can result from chromosomal rearrangements, insertion of foreign DNA sequences, deletions or point mutations.

It thus appears that carcinogenesis is a complex process, where mutation is probably a necessary initiating step but not sufficient, requiring further events which will ensure clonal expansion and metastasis (Pitot and Dragan,1994).

### 1.1.2. Multistage carcinogenesis

The transformation of a normal cell to a malignant cancer, carcinogenesis, is a multistep process. Animal and human cancer studies have provided evidence that cancer results from a sequential accumulation of genetic alterations in somatic cells. For example, in mouse skin tumour development, the transformation of normal cells to spindle cell carcinomas occurs after three different stages (*H-ras* mutation, *H-ras* amplification and loss, and p53 inactivation; Quintanilla *et al*, 1986; Ruggeri *et al*, 1991). In humans, studies of colorectal cancer have demonstrated that six mutation events are necessary to transform normal epithelium into adenomas, carcinomas and finally metastasis (Fearon and Vogelstein, 1990). These mutations include mutations of oncogenes (*FAP*, and *K-ras*), DNA-hypomethylation, loss of tumour suppressor genes (*DCC* and *p53*) and other undetermined alterations (Fearon and Vogelstein, 1990). A simplified model of multistage carcinogenesis has been proposed. Three main phases have been considered: initiation, promotion and progression (Figure 1.a.).

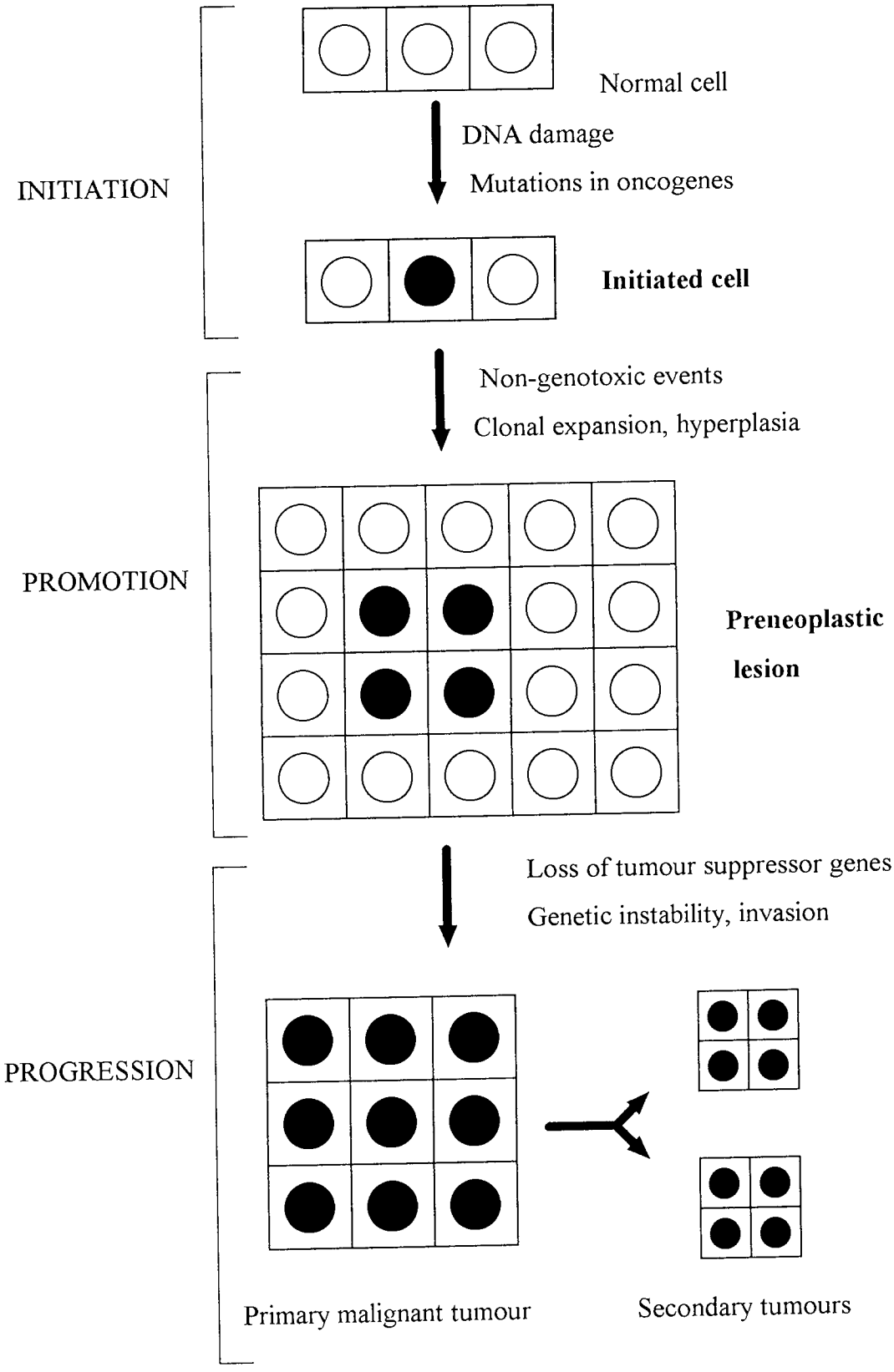



Figure 1.a. Schematic representation of multistage carcinogenesis

The **initiation** stage is an irreversible step which involves molecular changes in the structure of DNA (mutations) in a normal stem cell. At least one cell division will be necessary to achieve genetic fixation of the DNA damage and expression of the genotypic change (Pitot and Dragan, 1994). These mutations usually produce changes in proto-oncogenes (and sometimes tumour suppressor genes) which play a major role in growth control and the cell cycle (see above), thus allowing the clonal expansion of the mutated cells in a later stage. The mutations that activate these genes can be induced by a chemical (initiator), radiation, viruses or may occur spontaneously possibly mediated by oxidative damage (Ames and Gold, 1991).

The **promotion** step is considered to consist of clonal expansion of the initiated cells (Melnick, 1992). It is a reversible process which does not involve direct structural genetic changes, but may involve alterations in gene expression through perturbation of signal transduction (Melnick, 1992). The promoting agents are believed to act via a variety of pathways which often result in increased cellular proliferation (Schulte-Hermann *et al*, 1990; see section 1.1.3.1.). The promotion stage is believed to be the rate-limiting step of the carcinogenesis process (Pitot and Dragan, 1994; Pascale *et al*, 1993).

The **progression** stage is an irreversible stage during which cells with the malignant phenotype are formed. Progression is characterised by independent events such as invasion, metastatic growth, anaplasia and increased rate of growth (Pitot and Dragan, 1994). This stage is characterised by karyotype instability. Progressor agents can induce genomic alterations such as deletions, translocations and insertions in specific genes (Pitot and Dragan, 1994; Pascale *et al*, 1993) and also inhibit DNA repair and topoisomerase activity (Cortés *et al*, 1993). Tumour suppressor genes and particularly the *p53* gene, are lost or inactivated during this stage (Levine *et al*, 1990; Stanley, 1995). As for the initiation stage, progression can occur spontaneously possibly mediated by oxidative damage (Ames and Gold, 1991).

It thus appears that carcinogenesis is a complex process requiring several successive events. The initiation stage, where a DNA mutation is provoked by a chemical (or other agents) is a necessary event, but not sufficient. The mutated cell needs to be clonally expanded

(promotion) and finally transformed into a malignant neoplasm with metastasic capacity (progression).

### 1.1.3. Classification of chemical carcinogens

In view of the multistage nature of carcinogenesis, chemical carcinogens can be classified as initiator, promoter or progressor, depending on the stage induced by the carcinogen. An alternative classification divides carcinogens in complete and incomplete. A complete carcinogen is able to induce all the stages of carcinogenesis, i.e induces cancer from normal cells. An incomplete carcinogen may only induce initiation (Pitot and Dragan, 1994). Nevertheless, the classification of chemical carcinogens as genotoxic or non-genotoxic (epigenic) is ubiquitously used and accepted.

Genotoxic carcinogens are, by definition, chemicals that produce direct DNA damage leading to carcinogenesis, whereas non-genotoxic carcinogens do not produce DNA damage as a primary biological activity. A classification of genotoxic and non-genotoxic carcinogens according to their mode of action is shown in Table 1.a.. Genotoxic carcinogens will be described in detail in section 1.1.3.2.

#### 1.1.3.1. Non-genotoxic carcinogens

Non-genotoxic carcinogens are non-mutagenic and do not react with DNA. Because of this they generally are considered unable to possess initiating activity. Their mechanisms of action are complex and numerous, but generally they are mitogenic and able to induce cell proliferation (Green, 1991; Purchase, 1994), and might inhibit apoptosis (Schulte-Hermann *et al*, 1990), which would result in a selective process, allowing the survival and growth of initiated cells. These features suggest a role in the promotion stage, such as tetradecanoyl-phorbol-13 acetate (TPA) and phenobarbitone ((Schulte-Hermann *et al*, 1990). Nevertheless, a number of non-genotoxic chemicals do not possess promoting activity (Tennant, 1993), such as benzene. On the other hand some non-genotoxic carcinogens, e.g. peroxisome proliferators may be complete carcinogens by enhancing the occurrence of spontaneous mutations or may select cells with spontaneous tumours (Ames and Gold,

1991; Berry, 1993). Common characteristics of non-genotoxic carcinogens are the organ and species specificity, lack of common factors in their chemical structure and a threshold dose pattern (Berry, 1993).

The most common mechanisms by which non-genotoxic carcinogens act are briefly described in Table 1.a. One of the major mechanisms of action of non-genotoxic carcinogens appears to be related with the induction of cell proliferation (Purchase, 1994). This can be achieved by regulation of the expression of growth control genes (Green, 1991), by alteration of hormonal homeostasis (Purchase, 1994) or by inhibition of apoptosis (Schulte-Hermann *et al*, 1991). Non-genotoxic carcinogens have also shown to be able to increase expression of some oncogenes or inhibit expression of tumour suppressor genes (Green, 1991). Possible events leading to these alterations are enhancement of signal transduction, activation of nuclear receptors, or cytotoxic effects leading to regenerative hyperplasia (Green, 1991; Purchase, 1994). Regulation of gap junction intercellular communication (GJIC) is another possible mechanism of non-genotoxic carcinogens (Yamasaki *et al*, 1995). It is recognised that gap junctional intercellular communications (GJIC) may have tumour suppressor activity and thus may regulate cell proliferation and the clonal expansion of initiated cells by holding them in a quiescent state; this protection can be lost if a chemical is able to inhibit this function (Chipman, 1995). Several non-genotoxic carcinogens have been found to generate reactive oxygen species, which in turn, can lead to oxidative DNA damage, which has been correlated with both tumour initiation and to tumour promotion (Clayson, 1994, Oberley, 1995).

### 1.1.3.2. Genotoxic carcinogens

Genotoxic carcinogens are chemicals which primary activity is the alteration of the information encoded in the DNA, either directly or through the formation of a reactive metabolite (Purchase, 1994). Because of their genotoxic activity they are usually responsible for the initiation stage of carcinogenesis and some of them for the progression stage, during which mutations have been implicated. Genotoxic chemicals can be classified into three broad categories (Table 1.a.): direct acting agents, which react directly with DNA, procarcinogens, which interfere with DNA after metabolic activation and inorganic

chemicals, which interfere with DNA replication. The unifying characteristic of direct agents and procarcinogens is their electrophilicity (electron-deficiency), which allows them to react with nucleophilic sites in the DNA. Procarcinogens are not electrophilic *per se*, thus require metabolic activation (possibly via an intermediate, proximate carcinogens) to a highly reactive electrophilic form, the ultimate carcinogen. The majority of carcinogens belong to the procarcinogens class. Figure 1.b. shows typical examples of direct agents and procarcinogens, proximate and ultimate carcinogens. The transformation of a procarcinogen to ultimate carcinogen often involves oxidation of the substrate (Figure 1.b.) (Phase I reactions), but other type of reactions (Phase II reactions) can also occur. These will be discussed in detail in the following section (1.2.).

The interaction of genotoxic carcinogens with DNA can lead to several lesions. Many ultimate carcinogens bind nucleophilic sites in DNA to produce DNA adducts. One of the most nucleophilic sites is the $N^7$ position of guanine and many carcinogens form DNA adducts at this position (for example aflatoxin $B_1$, 2-acetylaminofluorene (Bolt, 1988). Other common lesions are the attack of alkylating agents (such as *N*-nitrosodimethylamine (NDMA)) to nucleophilic oxygen in DNA (Essigman and Wood, 1993). Free radical intermediates, which are formed sometimes during the metabolism of chemical carcinogens, can also induce DNA lesions, such as 8-hydroxyguanine, either directly or via the formation of reactive oxygen species (ROS) (Floyd, 1990). DNA adducts can lead to base mispairing (Singer, 1986) and bulky adducts can also block DNA synthesis resulting in a non-coding lesion (Friedberg, 1992). Different adducts will lead to different types of mutation (Essigmann and Wood, 1993). The interaction of carcinogens and DNA can also lead to other structural changes, such as single and double DNA strand breaks (Sina *et al*, 1983), pyrimidine dimmers or DNA strand crosslinks.

The cell can respond to these changes in three different ways; Firstly, the tolerance mechanism, where the damage is circumvented without being repaired (Friedberg, 1994) and is error-prone, or secondly, DNA repair mechanisms can be activated. DNA lesions can be reversed directly by removal of damaged bases by alkyltransferases (Pegg and Byers, 1992) or can be restored by excision repair. Base excision repair involves removal of the damaged or mispaired base, cleavage of the damaged DNA strand break, removal of several

neighbouring bases with subsequent action of DNA polymerases and lygases to yield a complete double stranded DNA without damaged bases (Regan and Setlow, 1974). The action of DNA polymerases introduces a potential for a mutation to occur in the form of a mispaired base (Cheng and Loeb, 1997). This possibility of mutation is much higher in nucleotide excision repair, where a longer base sequence is removed and repaired (Hoeijmarkers and Bootsma, 1994). A third response to DNA damage can be the deliberate removal of the cell by apoptosis. Extensive DNA damage can activate p53 to prime cells for apoptosis should repair not be possible (Tlsty, 1997).

Thus, genotoxic carcinogens can induce DNA structural changes as a result of the failure of DNA repair systems. These changes include point mutations, frameshift mutations, insertions, deletions or changes in chromosome structure or number (Pitot and Dragan, 1996). Consequently DNA adducts, DNA strand breaks, mutations, DNA binding and repair and chromosome aberrations have proved useful as endpoints in the identification of genotoxic carcinogens. Short-term genotoxicity tests include the *Salmonella typhimurium* reverse mutation assay, micronucleus test, unscheduled DNA synthesis and the single cell electrophoresis assay, which will be discussed later (chapters 4 and 5).

The formation of DNA adducts, as well as mutations, depends on the reactivity of the ultimate carcinogen and the overall metabolism of the chemical agent (see following section 1.2.) and DNA repair systems. Once a mutation has appeared during the synthesis of a new DNA strand from a damaged template, cell replication will play a primordial role to allow transformation of the mutated cell into a malignant tumour (see 1.1.2.). When these mutations occur in proto-oncogenes and tumour suppressor genes, which play an important role in the control of cell growth and differentiation (Pitot and Dragan, 1994; Stanley, 1995) (see section 1.1.2.), persistence of this mutation is ensured.

Table 1.a. Classification of chemical carcinogens based on their mode of action

| CLASSIFICATION | MODE OF ACTION | EXAMPLES |
|---|---|---|
| **I. Genotoxic** | Agents which interact with DNA as primary function | |
| 1. Direct acting | Organic electrophiles; direct alteration of DNA by covalent binding | Alkylating agents, lactones, imines |
| 2. Procarcinogen | Require conversion to direct acting electrophilic species through metabolic activation | Aromatics, polycyclic hydrocarbons, nitrosamines, halogenated hydrocarbons |
| 3. Inorganic carcinogen | Interference with DNA replication | Nickel, cadmium |
| **II. Epigenic or non-genotoxic** | Agents for which there is no evidence of interaction with DNA as primary function | |
| 4. Cytotoxin | Cytolethal; induction of regenerative cell proliferation; mutations may occur secondarily through several mechanisms (generation of reactive oxygen species); preferential growth of preneoplastic cells may be caused by selective killing of normal cells or expression of growth genes | nitrilo triacetic acid, chloroform |
| 5. Mitogen | Stimulation of mitogenic cell proliferation directly or via a cellular receptor; mutations may occur secondarily; preferential growth of preneoplatic cells may be caused by alterations of rates of cell birth or death. | phenobarbital, hexachlorocyclohexane |

Cont'd....

| | | |
|---|---|---|
| 6. Peroxisome proliferator | Generation of reactive oxygen radicals through lipid peroxidation; inhibition of apoptosis; growth control genes may be activated directly or via cellular receptors. | fenofibrate, diethylhexylphthalate |
| 7. Immunosuppressor | Enhancement of development of virally induced neoplasms through impairment or loss of tumour resistance. | azathioprine, cyclosporine |
| 8. Hormones and Hormonal-Altering Agents | Chronic stimulation of cell growth via activation of regulatory genes; alteration of hormonal homeostasis; apoptosis; generation of reactive oxygen species. | diethylstilbestrol, estrogens, synthetic androgens |
| 9. Solid-State Carcinogen | Unknown, although type of crystals and size seems to be a major factor; only mesenchymal cells are affected. | asbestos, fiberglass, metal foils |
| 10. Cocarcinogen | Enhances the carcinogenic process when administered simultaneously with a genotoxic carcinogen possibly by altering the metabolism of a procarcinogen or its absorption / elimination. | phorphol esters, ethanol |
| 11. Promoter | Promotes tumour formation by clonal expansion of a genotoxic-induced preneoplastic lesion. | phorbol esters, TPA, saccharin |
| 12. Progressor | Increased growth of initiated/promoted cells; induction of karyotypic changes to chromosomes; metastases. | arsenic salts, benzene, hydroxyurea |

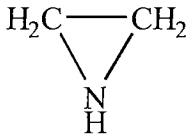
Adapted from Derelanko, (1995).

## DIRECT ACTING CARCINOGENS

Bis(chloromethyl)ether

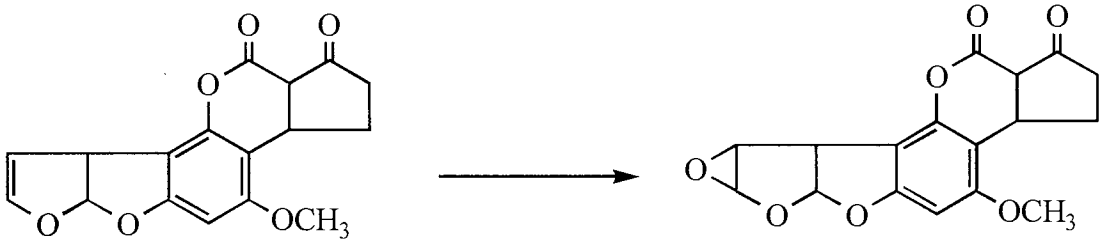


Beta-propiolactone



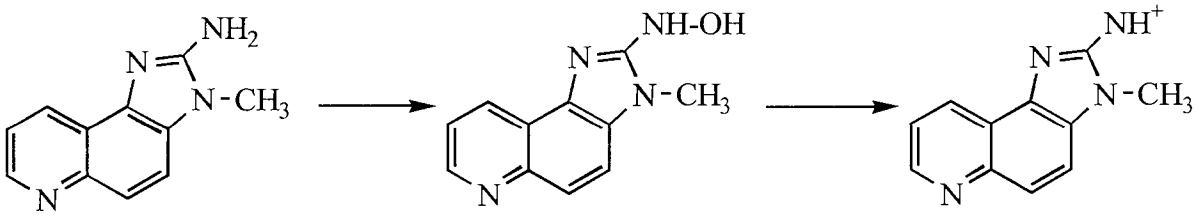
Ethylenimine

## PROCARCINOGENS, PROXIMATE AND ULTIMATE CARCINOGENS



Aflatoxin $B_1$
(Procarcinogen)

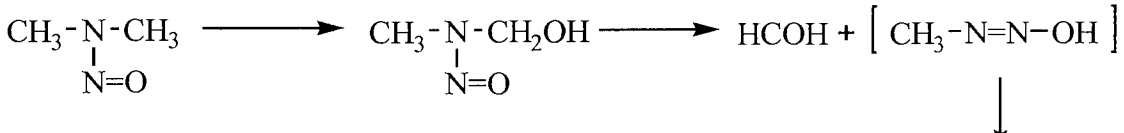
Aflatoxin $B_1$,2,3, epoxide
(Ultimate carcinogen)



2-Amino-3-methylimidazo
[4,5-*f*]quinoline (IQ)
(Procarcinogen)

N-hydroxy-IQ
(Proximate carcinogen)

Nitrenium ion
(Ultimate carcinogen)



*N*-nitrosodimethylamine
(Procarcinogen)

Hydroxomethyl, methy
nitrosoamine
(Proximate carcinogen)

$CH_3^+ + N_2 + OH^-$

Methyl carbonium ion
(Ultimate carcinogen)

Figure 1.b. Chemical structures of direct and indirect genotoxic carcinogens.

## *1.2. Metabolic activation and detoxification of carcinogens*

The ability of carcinogens to induce DNA mutations depends mainly on the reactivity of the parent compound or intermediate metabolites and on its overall metabolism. Carcinogens entering a biological system undergo absorption, disposition (tissue distribution), enzymatic and/or non enzymatic transformations and finally excretion. Although all these processes contribute to some extent to the observed amount and/or reactivity of the ultimate carcinogen, the biotransformation of the chemical is possibly the most important and determining factor.

The biotransformation of xenobiotics is a mechanism to eliminate lipophilic xenobiotics by transforming them into hydrophilic species easily excreted. Without biotransformation, lipophilic xenobiotics would be poorly excreted, accumulating in the body and eventually killing the organism (Parkinson, 1996). Although biotransformation is primarily a detoxification and protective mechanism, for certain xenobiotics, namely procarcinogens, biotransformation results in the formation of a metabolite more toxic than the parent compound. The extent of reactive metabolite formation and its degree of toxicity is dictated by the rates of the competing activation and deactivation pathways (Ioannides *et al*, 1995). This balance between activation and detoxification can be perturbed by several factors (saturation of deactivation pathways, selective induction of activating enzymes,...), thus increasing the production and accumulation of toxic and potential carcinogenic intermediates (Ioanides *et al*, 1995). These factors affecting the metabolism of carcinogens will be discussed later.

The reactions involved in the biotransformation of carcinogens and other xenobiotics are generally divided into Phase I and Phase II reactions (Parkinson, 1996). Phase I reactions generally introduce small functional groups (e.g. -OH, $-NH_2$, -SH or -COOH) into the carcinogen resulting in a small increase in hydrophilicity. Phase II reactions introduce a polar endogenous group (glucuronic acid, sulfate, acetyl, methyl, glutathione or amino-acids) resulting in a large increase in xenobiotic hydrophilicity, thus, promoting excretion. Substrates of Phase II reactions are generally xenobiotics or products from Phase I reactions (Parkinson, 1996) and Phase I reactions usually precede Phase II reactions.

Table 1.b. shows the most common Phase I and II enzymes involved in the biotransformation of carcinogens. Only those enzymes relevant to this project are described in detail below.

### 1.2.1. Cytochrome P450

Cytochrome P450 enzymes (CYP450) are the most important Phase I enzymes, due to the number of carcinogens (and other xenobiotics) that they metabolise and due to their catalytic versatility. They are found mainly in liver as well as in almost all tissues and they are usually located in the endoplasmic reticulum, although some mitochondrial forms have also been found (Guengerich, 1992). The basic and most common reaction catalysed by CYP450 is monooxygenation. The catalytic cycle of CYP450 will be described in more detail in the next section (1.3.1.). To date there have been identified more than 300 different forms of CYP450 and 50 different families (see section 1.3.2.) but in mammals only 4 families are involved in the metabolism of carcinogens (Nelson *et al*, 1993; 1995). The different isoenzymes have different catalytic activities and different substrate specificities, which sometimes overlap (see section 1.3.3.). The oxidation reactions that they catalyse are: hydroxylation of aliphatic or aromatic carbons, epoxidation of double bonds, heteroatom oxygenations, *N*-hydroxylations, heteroatom dealkylations, oxidative group transfer, clearance of esters and dehydrogenation. Some typical examples of carcinogen activation are the epoxidation of aflatoxin $B_1$ or benzo[*a*]pyrene, *N*-oxidation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-hydroxylation of 2-acetylaminofluorene or 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), and *N*-demethylation of *N*-nitrosodimethylamine (NDMA) (Guengerich and Shimada, 1991). CYP450 also can catalyse azo- and nitro-reductions and reductive dehalogenations. Different factors (genetic, environmental) may affect CYP450 activity and therefore affect the metabolic activation of carcinogens (see sections 1.3.4. and 1.3.5).

### 1.2.2. UDP-glucuronosyltransferases

UDP-glucuronosyltransferases (UGT) catalyse the conjugation of substrates with glucuronic acid which results in an increase of lipophilicity and recognition by the biliary

and renal transport systems, thus facilitating excretion. UGTs are localised in the endoplasmic reticulum of various tissues. Substrates for glucuronidation contain functional groups such as aliphatic alcohols and phenols, carboxylic acids, and primary and secondary aromatic and aliphatic amines (Miners and Mackenzie, 1992). Glucuronidation results in detoxification of most carcinogens, but in some cases, such as 2-aminonaphthalene, has the opposite effect resulting in activation. Two gene families have been found, UGT1 and UGT2 (with subfamilies A and B). In humans, the multiple forms are products of either a single UGT1 gene locus or multiple UGT2B genes (Bierly and Burchell, 1993). Some forms of UGTs can be induced by compounds such as polycyclic aromatic hydrocarbons (PAH), which are also inducers of the *Ah* receptor (see section 1.3.5) or by monofunctional inducers of phase II enzymes (see section 1.4.4).

### 1.2.3. N-Acetyltransferases

*N*-Acetylation is a major route of metabolism for aromatic amines and hydrazines resulting in transformation into less water soluble metabolites (amides and hydrazides respectively) (Evans, 1992). Aromatic amines can be both activated or deactivated, depending on their structure and whether they have previously been *N*-hydroxylated by CYP450 (generally CYP1A2). *N*-Acetylation generally results in detoxification of aromatic amines, whereas, *O*-acetylation (after P450 *N*-hydroxylation) results in activation of hydroxylamines to reactive products. *N*-Acetyltransferases (NAT) are cytosolic enzymes found in liver and other tissues, and two different forms have been found in humans, NAT1, expressed in most tissues, and NAT2, only expressed in liver (Vatsis *et al*, 1995). The two forms have different but overlapping substrate specificities. For example 2-aminofluorene is metabolised both by NAT1and NAT2, whereas IQ is preferentially metabolised by NAT2 (Kadlubar, 1994). Bicyclic and heterocyclic aromatic amines (BAA and HAA respectively), carcinogens in bladder and colon (Kadlubar, 1994), can be both activated (the *N*-hydroxyl form) or detoxified by NAT1 and NAT2 (BAA). On the other hand HAA (the *N*-hydroxyl form) can be activated by NAT2 but not detoxified by any of the two enzymes (Kadlubar, 1994). Genetic polymorphisms for NAT2 have been reported and individuals can be classifed as slow or fast acetylators (Grant *et al*, 1992). These polymorphisms can be an important risk factor in cancer susceptibility (Kadlubar, 1994) to

BAA and HAA due to their metabolic differences (see above). A polymorphism for NAT1 has recently been described and may be associated with increased colon cancer risk.

### 1.2.4. Glutathione-*S*-transferases

Glutathione-*S*-tranferases (GST) catalyse the conjugation of numerous electrophilic xenobiotics and also endogenous compounds with glutathione (GSH). GSTs are present in most tissues and are mainly cytosolic enzymes, although they may also be present in microsomal fractions (Ketterer, 1988; Pickett and Lu, 1989). Substrates for GST are hydrophobic, containing an electrophilic atom, and in some cases they react non-enzymatically with GSH to some extent. Apart from the metabolic function of GSTs, they can also have a role as a transport system for its conjugates (Baillie and Slatter, 1991). Substrates of GST can be conjugated directly to GSH (for example ß-propiolactone, 1,2-dichloro-4-nitrobenzene) or transformed to an electrophilic intermediate (usually by cytochrome P450) prior to GSH conjugation. Conjugation with GSH constitutes an important detoxification pathway for most of the electrophilic substrates, which otherwise would bind to DNA or other macromolecules (e.g. benzo[a]pyrene-4,5-oxide, aflatoxin $B_1$-8,9-oxide) (Ketterer, 1988). However, compounds such as dichloromethane or dibromoethane are converted to reactive metabolites by GSH conjugation (Guengerich, 1994) and for bromobenzene, GSH conjugation after oxidation by CYP450 leads to the generation of ROS.

GSTs can exist as homodimers or heterodimers and they are divided into four gene families $\alpha$, $\mu$, $\pi$ and $\theta$. In humans the alpha class contains three different genes, hGSTA1, A2 and A3; five genes have been identified in the mu class, hGSTM1 (with three alleles A, B and 0), M2, M3 (with two alleles A and B), M4 and M5; one gene in the pi class, hGSTP1 (with three alleles A, B and C) and the theta class contains two genes, hGSTT1 (with two alleles A and 0) and T2 (Mannervick *et al,* 1992). Some compounds can be substrates of several classes, for example 1-chloro-2,4-dinitrobenzene (A,M and P), whereas other chemicals may be preferentially conjugated with only one class (Ketterer, 1988). For instance, alkene oxides are substrates of the mu class, and in rodents, aflatoxin $B_1$ 8,9-oxide is conjugated only with the Yc subunits of the alpha class. Thus, aflatoxin $B_1$

causes tumours in rat (low levels of GST Yc) but is much less potent in mice, which have high levels of GST Yc (Eaton and Gallagher, 1994). A genetic polymorphism for hGSTM1 has been described, and individuals with the homozygote null allele seem to be at risk for cigarette smoking induced lung cancer (Hayashi *et al*, 1992) and also for adenocarcinoma and urothelial cancer (Lear *et al*, 1996; Katoh *et al*, 1995). Polymorphisms of hGSTM3, T1 and P1 have also been identified, and their link with various types of cancer is under study (Warwick *et al*, 1994; Lear *et al*, 1996).

The α and μ class are inducible by several agents including 3-methylcholanthrene, phenobarbitone, antioxidants and Michael acceptors in rodents (Nguyen *et al*, 1994) and also by monofunctional and bifunctional inducers (Prochaska and Talalay, 1988) (see section 1.4.4.). The induction is a result of increased levels of mRNA due to transcriptional activation (Nguyen *et al*, 1994). This can be mediated by different response elements in the enhancer regions of the GSTs genes (xenobiotic response element (XRE), Barbie box, antioxidant response element (ARE) and/or glucocorticoid response element (GRE)) (see section 1.4.4.).

### 1.2.5. Quinone reductases

Quinone reduction can be mediated by two different mechanisms. The one electron reduction, catalysed by NADPH-cytochrome P450 reductase usually is accompanied by formation of ROS (Parkinson, 1996). The two electron reduction, catalysed by DT-diaphorase generally promotes deactivation of quinones, preventing their participation in oxidative cycling and depletion of intracellular GSH (Atallah *et al*, 1987). Several forms of the enzyme have been found in rodents and humans. In humans, two different forms have been identified: an inducible form, $NQO_1$ (NADPH-quinone oxidoreductase, or also named quinone reductase (QR)), which accounts for the majority of the activity and a non inducible form, $NQO_2$. Polymorphims for both isoforms have been described (Rosvold *et al*, 1995; Jaiswal *et al*, 1990). QR has been found to be induced by phenolic antioxidants, planar aromatic compounds and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Favreau and Pickett, 1995). The mechanisms of induction of QR will be discussed in detail in section 1.4.4 (monofunctional and bifunctional inducers).

### 1.2.6. Metabolic activation systems in testing for carcinogens

The majority of short-term assays used in the testing of potential carcinogens, are carried out in bacteria or mammalian cell cultures (see section 1.1.3.1.), which lack the metabolic capacity of intact mammals. Metabolic activation must therefore be provided to these systems. The liver is the first organ to encounter ingested nutrients, drugs and enviromental toxicants and functions as first-pass clearance of toxins and carcinogens (Molsen, 1996). It is therefore the organ where most carcinogens will be metabolised, and contains high levels of phase I and II enzymes, hence its widely used as a metabolic activation system. Both post-mitochondrial supernatant (S9) and microsomes from rodent liver are commonly used (Maron and Ames, 1983). Other tissues have also been used for the testing of carcinogens, for example lung homogenates may be used for the testing of cigarrette smoking derived aromatic amines (Shimada *et al*, 1992a, b).

The addition of exogenous metabolic activation systems is not always necessary; such is the case of intact hepatocytes, which retain cytochrome P450 and phase II enzyme activities (Madle *et al*, 1994) in the short term. Alternative metabolic systems have recently been developed by the construction of cell lines that express cDNAs encoding cytochrome P450s (see section 5.1.1 and 5.1.2.). Despite the usefulness of the above mentioned *in vitro* metabolic activating systems, they do not mimic the mammalian *in vivo* situation and the final balance between activation and detoxification may be different *in vivo* than *in vitro*.

Table 1.b. Phase I and Phase II enzymes involved in biotransformation of carcinogens

| REACTION | ENZYME | LOCALIZATION | SUBSTRATES |
|---|---|---|---|
| | | Phase I | |
| Oxidations | Cytochromes P450 | all tissues (microsomes) | nitrosamines, PAH, arylamines |
| | Flavin-monooxygenases | liver, kidney, lung (microsomes) | acethylhydrazine, thiobenzamide |
| | Prostaglandin-H-synthase | extrahepatic tissues (microsomes) | 5-nitrofurans |
| | Alcohol dehydrogenase | liver, kidney, lung, gastric mucosa (cytosol) | ethanol |
| | Aldehyde dehydrogenase | stomach and extrahepatic tissues (cytosol) | acetaldehyde |
| Hydrolysis | Cytochromes P450 | all tissues (microsomes) | |
| | Carboxylesterases | liver and extrahepatic tissues (microsomes and cytosol) | vinylacetate, 1,3-dimethyl-3-phenyl-1-nitrosourea |
| | epoxide hydrolases | liver and extrahepatic tissues (microsomes and cytosol) | benzo[a]pyrene, styrene-7,8-epoxide |
| Reductions | Cytochromes P450 | all tissues (microsomes) | carbon tetrachloride, halothane |
| | Azo, nitro-reductase | intestinal microflora | 2,6-dinitrotoluene |
| | NADPH-cytochrome P450 reductase | all tissues (microsomes) | menadione |

Cont'd...

| Phase II | | | |
|---|---|---|---|
| Glucuronidation | UDP-glucuronosyl-transferases | liver and extrahepatic tissues (microsomes) | aromatic and aliphatic amines, alcohols, 2-aminophthalene |
| Sulfatation | Sulfotransferases | liver, kidney, intestinal tract (cytosol) | hydroxymethyl-PAH, phenols, safrole |
| Methylation | Methyltransferases | extrahepatic tissues, (cytosol) | amines, sulfhydryl-containing xenobiotics |
| Acetylation | N-Acetyl-transferases | liver and extrahepatic tissues (cytosol, mitochondria) | bicyclic and heterocyclic aromatic amines, IQ |
| Amino acid conjugation | N-Acyltransferases Aminoacyl-tRNA-synthetase | liver (mitochondria, cytosol) | carboxylic acids, hydroxylamines |
| Glutathione conjugation | Glutathione-S-transferases | liver and extrahepatic tissues (cytosol) | propiolactone, benzo[a]pyrene-4,5-oxide, aflatoxin $B_1$ |
| Quinone reduction | Quinone reductase* (or DT-diaphorase) | liver and extrahepatic tissues (cytosol) | menadione |

Adapted from: Parkinson, 1996.

* Quinone reductase is not a classic Phase II enzyme, but its regulation resembles that one of these enzymes.

## *1.3. The cytochrome P450 superfamily.*

The most important enzymes involved in phase I reactions and metabolism of carcinogens are the cytochrome P450 system (see section 1.2.1.). The cytochrome P450 enzymes (CYP450) are a superfamily of heme-containing monooxygenases with a unique spectral absorption at 450 nm corresponding to the $Fe^{2+}$-CO complex and due to the presence of a cysteinyl thiolate fifth ligand to the iron (Ortiz de Montellano, 1989). Cytochrome P450 enzymes are monomers of about 48 to 60 kDa and the amino acid sequence that contains the cysteine-thiolate fifth ligand is highly conserved throughout all CYP450 forms. (Gonzalez and Gelboin, 1992). To date, over 300 CYP450 genes, belonging to prokaryotes and eukaryotes, including mammals and plants have been recognised (Nelson *et al*, 1993, Nelson, 1995). They are expressed virtually in all mammalian tissues with the exception of erythrocytes and striated muscle, with the highest activity in the liver. Although CYP450s have been found in several organelles, the microsomal fraction (endoplasmic reticulum) shows the highest activity towards xenobiotics (in the text CYP450 will imply microsomal enzyme unless otherwise stated).

CYP450s are believed to have existed for over one billion years and their main function is considered to have been the metabolism of steroids to maintain membrane integrity. CYP450s involved in xenobiotic metabolism appear to have evolved as a defense mechanism against toxic substances in the food chain (mainly plants) (Gonzalez and Gelboin, 1992). Thus, the diversity of CYP450 forms as well as species differences and polymorphisms seems to be the result of diet changes through the species evolution (Gonzalez and Gelboin, 1994).

### 1.3.1. Mechanisms of catalysis by cytochrome P450

The cytochrome P450 system consists of two hemoproteins, cytochrome P450 and cytochrome $b_5$, two flavoproteins, NADPH cytochrome P450 reductase and NADH cytochrome $b_5$ reductase and employs two pyridine nucleotides, NADPH and NADH. The enzymes are embedded in the phospholipid bilayer which facilitates their interaction. The flavoproteins function is that of transferring electrons form NAD(P)H to the cytochrome

P450. In general NADPH cytochrome P450 reductase donates the first electron required for catalysis and either NADPH cytochrome P450 reductase or cytochrome $b_5$ can supply the second electron to cytochrome P450 (Sevrioukova and Peterson, 1995). In contrast to the multiple forms of P450s, only one form of NADPH-cytochrome P450 reductase and cytochrome $b_5$ have been found, and they are present in a much lower ratio than cytochrome P450. The reason of this seems to be that the flavoproteins can transfer electrons much faster than cytochrome P450 can use them (Sevrioukova and Peterson, 1995).

The most common reactions catalysed by CYP450 were described in section 1.2.1. The catalytic cycle of CYP450 has been described and reviewed by several authors (Guengerich 1995; Ortiz de Montenallo, 1995; Groves and Hang, 1995). Briefly, the substrate binds to the heme iron which is then reduced from ferric ($Fe^{3+}$) to ferrous ($Fe^{2+}$) with an electron from NADPH-cytochrome P450 reductase. Then, molecular oxygen binds to the iron to give a ferrous cytochrome P450-dioxygen complex which is further reduced by a second electron to give a peroxoiron (III) complex and which becomes FeOOH after the addition of two protons. Finally a molecule of water is released from this complex and the oxygen atom is transferred to the substrate, which then dissociates from the heme in its ferric ($Fe^{3+}$) state. This is appropiate for most of the P450 reactions, although there are some exceptions (Guengerich *et al,* 1995).

### 1.3.2. Multiple forms of cytochrome P450

The cytochrome P450 superfamily can be classified into three different general categories (Gonzalez and Gelboin, 1992): i) soluble forms found in bacteria and with high substrate specificity, ii) forms involved in highly specific steroid oxidations, some of which are mitochrondrial, and iii) forms that are bound to the endoplasmic reticulum of the cell, with broad substrate specifity. The cytochrome P450 isoenzymes have been classified into families (more than 40 % amino acid sequence identity) (families 1, 2, 3, etc), and subfamilies (at least 55% amino acid sequence identity) (2A, 2B, 2C, ..) (Nelson *et al,* 1993). Aproximately 350 cytochrome P450 genes have been sequenced, belonging to 50 families and 82 subfamilies, found in vertebrates (mammals, fish and birds), invertebrates,

plants, and bacteria (Nelson, 1995). In mammals 12 families comprising 22 subfamilies have been identified. Table 1.c. lists most of the cytochrome P450 genes identified.

In humans (and rodents), the major gene families are 1, 2, 3, 4, 7, 11, 17, 19 and 21. Families 7, 11, 17, 19, 21 and 27 are found extrahepatically and are involved in the metabolism and biosynthesis of steroid hormones and other endogenous substrates (Guengueric h, 1995). Families 1, 2, 3 and 4 (hepatic and extrahepatic) are involved in Phase I reactions. The distribution of the different isoenzymes in the human liver, the major organ involved in carcinogen metaboism (see section 1.2.6.) is shown in figure 1.c. However these levels can vary considerably due to several factors (see sections 1.3.4. and 1.3.5.). The different cytochrome P450 isoforms involved in carcinogen metabolism are described briefly in the following section.

Table 1.c. Cytochrome P450s.

| GENE SYMBOL | SPECIES |
|---|---|
| CYP1A1 | Human, rat, mouse, rabbit, hamster, trout |
| CYP1A2 | Human, rat, mouse, rabbit, hamster, chicken, Trout |
| CYP1B1 | Human, mouse |
| CYP2A1, 2A2, 2A3 | Rat |
| *cyp2a4, 2a5* | Mouse |
| CYP2A6, 2A7 | Human |
| CYP2B1, 2B2, 2B3, 2B8 | Rat |
| CYP2B6 | Human |
| *cyp2b9, 2b10* | Mouse |
| CYP2C6, 2C7, 2C11, 2C12, 2C13 | Rat |
| CYP2C8, 2C9, 2C10, 2C18, 2C19 | Human |
| CYP2D1, 2D2, 2D3, 2D4, 2D5 | Rat |
| CYP2D6 | Human |
| *cyp2d9, 2d10, 2d11, 2d12, 2d13* | Mouse |
| CYP2E1 | Human, rat, mouse, rabbit, monkey |
| CYP2E2 | Rabbit |
| CYP2F1 | Human |
| CYP3A1, 3A2 | Rat |
| CYP3A3, 3A4, 3A5, 3A7 | Human |
| CYP3A6 | Rabbit |
| CYP3A14, 3A15 | Guinea pig |
| CYP3A9 | Rat |
| *cyp3a11, 3a16* | Mouse |

Cont'd...

| | |
|---|---|
| CYP4A1, 4A2, 4A3 | Rat |
| CYP4A4, 4A5, 4A6, 4A7, 4A8 | Rabbit |
| CYP4A9, 4A11 | Human |
| *cyp4a10, 4a12* | Mouse |
| CYP4B1 | Human |
| CYP4F3 | Human |
| CYP5A1 | Human, rat |
| CYP7 | Human, rat, rabbit, bovine |
| CYP8 | Bovine |
| CYP11A1 | Human, rat, rabbit, chicken, bovine, pig |
| CYP11B1, 11B2 | Human, rat, mouse, bovine |
| CYP17 | Human, rat, mouse, pig, chicken |
| CYP19 | Human, rat, mouse, pig, chicken |
| CYP21A2 | Human |
| CYP21 | Sheep, Pig |
| CYP24 | Human |
| CYP27 | Human, rat, rabbit |
| CYP51-CYP69 | Lower eukariotes |
| CYP71-CYP99 | Plants |
| CYP101-CYP116 | Bacteria |

Adapted form Nelson (1995).

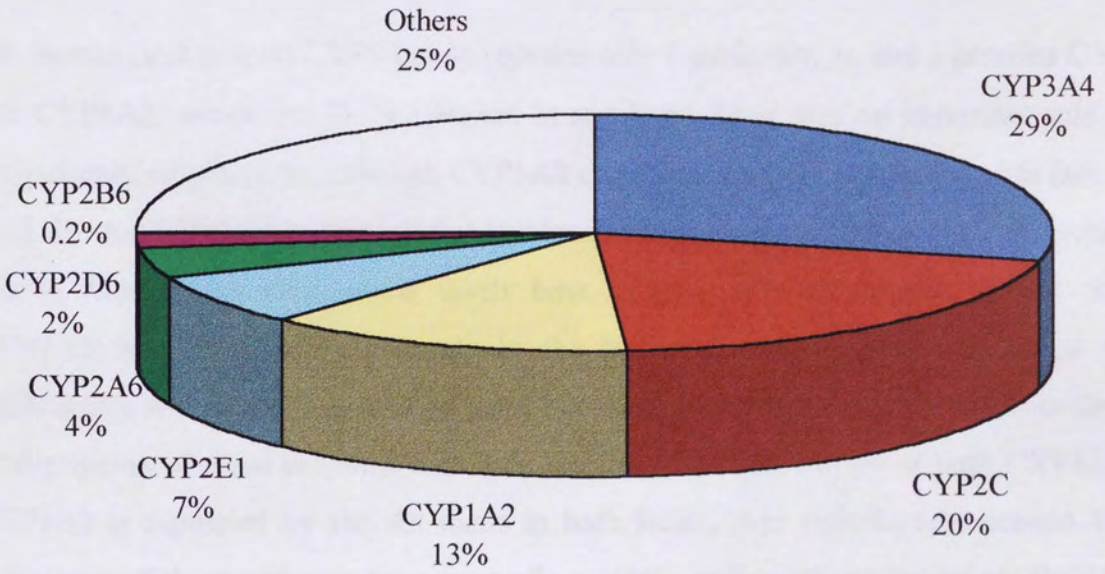



Figure 1.c. Distribution of cytochrome P450 enzymes in the human liver.

Adapted from Guengerich (1995).

## 1.3.3. P450s involved in the metabolism of carcinogens and their substrate specificity

The main features and substrate specificty of cytochrome P450 families involved in the metabolism of carcinogens are described below and were extracted form several reviews (Gonzalez and Gelboin, 1994; Guenguerich and Shimada, 1991; Ioannides *et al*, 1995; Wrighton and Stevens, 1992 and Guengerich, 1995).

### 1.3.3.1. Cytochrome P450 family 1 (CYP1)

The human (and rodent) CYP1 family contains only 1 subfamily, A, and 2 proteins CYP1A1 and CYP1A2, which are 70 % identical in sequence. They play an important role in the activation of carcinogens, although CYP1A2 can also inactivate some substrates (see Table 1.c.). Human CYP1A1 is expressed at low levels in extrahepatic tissues (lung, placenta), but can be induced to considerable levels both in liver and extrahepatic tissues. Human CYP1A2 is expressed constitutively in the liver (13 %, see figure 1.c.) but not in extrahepatic tissues and it is also an inducible enzyme. Interspecies differences on the level of expression of these enzymes exist (see section 1.3.6.). Induction of both CYP1A1 and CYP1A2 is regulated by the Ah locus in both human and rodents (see section 1.3.4.). Substrates of these enzymes have generally a planar molecular configuration. CYP1A1 is responsible for the oxidation of polycyclic aromatic hydrocarbons (PAH) whereas CYP1A2 catalyses the *N*-hydroxylation of arylamines and heterocyclic amines (see table 1.d.).

### 1.3.3.2. Cytochrome P450 family 2 (CYP2)

Although this is one of the largest families, comprising a number of subfamilies, there is only one isoenzyme with a major role in carcinogen metabolism, the CYP2E1. Nevertheless, there are few other subfamilies which also have a minor role. CYP2A6 has been found to activate several carcinogens (see table 1.d.), but its only present at less than 1 % in human liver. In rodents it has also been found in extrahepatic tissues. The human isoenzyme CYP2D6 has been found to be associated with lung cancer and other illness (see section 1.3.5.). However its role in cancer aethiology is not fully understood, since it only activates one carcinogen (NNK) which is also activated efficiently by other CYP450s (see table 1.d.).

The CYP2E1 isoenzyme plays a major role in the bioactivation of many small molecular weight and generally hydrophilic carcinogens and other toxins, although it can also inactivate certain compounds (see table 1.d.). It is responsible for the metabolism of some nitrosamines (see 1.3 6. for species differences in their metabolism), carbon tetrachloride, azoxymethane, vinyl chloride, and organic solvents such as acetone, dimethylsulphoxide, benzene and alcohol and some drugs like paracetamol. CYP2E1 is constitutively expressed in liver and several extahepatic tissues (lung) and is inducible by ethanol and isoniazid in humans and also by acetone and certain physiological states in rodents (see section 1.3.4.1.2.).

### 1.3.3.3. Cytochrome P450 family 3 (CYP3)

The human CYP3A subfamily contains 4 different genes, CYP3A3, 3A4, 3A5 and 3A7, of which CYP3A4 is expressed to a greater degree than the others and also constitutes the major fraction of the total cytochrome P450 level in human liver (see figure 1.c.). However, in the rat two isoforms have been found: CYP3A1 and 3A2. CYP3A4 is also expressed in extrahepatic tissues (lung, intestine). CYP3A4 can activate a broad variety of carcinogens such as aflatoxin $B_1$, 6-aminochrysene, and some polycyclic aromatic hydrocarbons to their ultimate carcinogens (see table 1.d.). Deactivation of carcinogens has also been associated with the human CYP3 family, as for aflatoxin $B_1$ and 1,6-dinitropyrene. Whereas CYP1A and CYP2E1 substrates are quite specific (i.e. planar compounds for CYP1A and small compounds for CYP2E1), CYP3A metabolises mainly large compounds but lacks substrate specificity. The human enzyme is induced by barbiturates, dexamethasone and troleandomycin.

### 1.3.3.4. Cytochrome P450 family 4 (CYP4)

The principal function of CYP4 is the metabolism of endogenous substrates such as fatty acids and prostaglandins. CYP4B1, expressed in rodent lung, can activate a number of aromatic amines, but the human protein was unable to catalyze these reactions. There is a close link of this family with peroxisomal proliferators (non-genotoxic carcinogens in

rodents (see section 1.1) which have shown to induce rat CYP4A1, 4A2 and 4A3 and rabbit CYP4A6.

### 1.3.4. Regulation of enzyme activity by induction and inhibition.

Cytochrome P450 levels and activity in the liver can vary considerably among different individuals. These variations can be attributed to several factors. The first is human genetic polymorphisms. These are heritable DNA changes that can lead to the synthesis of a form of CYP450 with deficient catalytic activity or lack of inducibility. The most common human polymorphisms will be mentioned in section 1.3.5. The second and third mechanisms are enzyme induction and inhibition, described here.

#### 1.3.4.1. Induction of cytochrome P450 enzymes that metabolise carcinogens.

There are a few different mechanisms by which CYP450s can be induced. Inducers of CYP450s are often substrates themselves for the induced enzyme, so that the enzyme activity is increased as needed. In general, the induction of cytochrome P450 enzymes is a protective mechanism, since detoxification is the main function of these enzymes (see section 1.2.). Nevertheless, the induction of these enzymes in certain conditions might be a disadvantage and may enhance chemical toxicity (Ioannides and Parke, 1993). Cytochrome P450 can activate numerous procarcinogens (section 1.3.3), therefore its induction may result in an increase of ultimate carcinogen which can result in an increased chance of mutations and carcinogenesis (see section 1.1.3.1).

Inducers of cytochrome P450s can be classified into five different classes (Porter and Coon, 1991) represented by the following inducers: 3-methylcholanthrene (induces CYP1A1, CYP1A2), phenobarbitone (rat CYP2B1 and CYP2B2), pregnenolone-16α-carbonitrile (CYP3A1, CYP3A2), isozianid (CYP2E1) and clofibric acid (rat CYP4A1, CYP4A2, CYP4A3). Table 1.e. summarises the mode of action of these inducers. Because of their involvement in carcinogen activation and relevance to this project, CYP1A and CYP2E1 induction is described in detail bellow.

#### 1.3.4.1.1. CYP1A1 and CYP1A2 induction.

3-Methylcholanthracene, polycyclic aromatic hydrocarbons (PAHs), flavones, TCDD and some drugs and naturally occurring compounds induce rat CYP1A1 and CYP1A2 (cigarette smoking has shown to induce the human enzymes). The mechanism of induction has been widely studied in experimental animals and several reviews have been published (Whitlock and Denison, 1995; Okey *et al,* 1994). The induction of CYP1A1 involves activation of transcription of the *CYP1A1* gene and message stabilization, which results in an increase of levels of mRNA and newly synthesised protein. When the inducers are lacking, transcription of *CYP1A1* is suppressed by a repressor protein, thus explaining the low constitutive levels of CYP1A1 enzyme (Parkinson, 1996). The induction of CYP1A1 is mediated through the "Ah receptor" (named from the fact that it binds and mediates the response to aromatic hydrocarbons (Ah)). The induction of CYP1A1 transcription requires a second protein in addition to Ah, Arnt, which is involved in the binding of the ligand-receptor to DNA (Whitlock and Denison, 1995). The free Ah receptor appears to form an heteromer with two heat-shock proteins (hsp90) in the cytosol. When the ligand binds to the Ah receptor, the two hsp90 proteins dissociate from the receptor, enabling phosphorylation of Ah (via tyrosine kinases), which then can enter into the nucleus and bind the Arnt protein. Finally the phosphorylated complex ligand-Ah receptor-Arnt binds to regulatory sequences of DNA enhancing transcription of the *CYP1A1* gene (and other genes; Okey *et al,* 1994). These regulatory sequences, located in the enhancer region of the gene are known as xenobiotic responsive elements (XRE) and are also present in the upstream enhancer region of CYP1A2, GST, QR, UGT and aldehyde dehydrogenase (Parkinson, 1996). Thus, inducers of the Ah receptor and CYP1A1, will also induce CYP1A2, GST, QR, UGT and aldehyde dehydrogenase. The implications of this induction will be of great importance, since all these enzymes are involved in the metabolism of carcinogens (see section 1.2.) and will be discussed in following sections (1.4.3. and 1.4.4.).

The Ah receptor has been purified and cloned in C57BL/6 mouse (Burbach *et al,* 1992). Four different allelic variants of the Ah receptor have been identified, one low afinity, *Ah d* , which is responsible for the lack of Ah induction of certain mouse strains, and three high

affinity forms, *Ah b* (Poland *et al,* 1994). The human Ah receptor is similar to the rodents receptor, but in general has less affinity for halogenated aromatic hydrocarbons (Okey *et al,* 1994). The Ah receptor may also mediate the expression of genes that regulate cell growh and differentiation, which play a key role in the carcinogenesis process (see section 1.2.) (Okey *et al*, 1994).

#### 1.3.4.1.2. CYP2E1 induction

Rodent CYP2E1 can be induced by isoniazid, ethanol, acetone, pyrazole, fasting and diabetes (Koop and Tiernay,1990). In general CYP2E1 inducers also can inhibit CYP2E1 or be metabolised by CYP2E1 and/or can increase serum ketone bodies. CYP2E1 is induced by several mechanisms depending on the type of inducer. For example, compounds such as acetone, isoniazid or ethanol act by stabilization of the protein without mRNA increase. The CYP2E1 protein is stabilised by decreasing protein degradation (probably by blocking its c-AMP-dependent phosphorylation) (Song *et al*, 1989). On the other hand fasting increases gene transcription and diabetes induces CYP2E1 by mRNA stabilization (Whitlock and Denison, 1995). Human CYP2E1 has been shown to be induced in patients treated with ethanol or isoniazid (Guenguerich, 1995).

Table 1.d. Human CYP450s involved in the metabolism of procarcinogens.

| CYP450 | CARCINOGEN ACTIVATION | CARCINOGEN INACTIVATION |
|---|---|---|
| CYP1A1 | Benzo[a]pyrene, dimethylbenz[a]anthracene | |
| CYP1A2 | 2-aminofluorene, 2AAF, Aflatoxin $B_1$, 2-aminoanthracene, IQ, MeIQ, MeIQx, Glu P-1, Trp P-1, PhIP, NNK | Aflatoxin $B_1$, 1,3-dinitropyrene, 2-furylfuramide |
| CYP2A6 | Aflatoxin $B_1$, NNK, NDEA | |
| CYP2D6 | NNK | |
| CYP2E1 | Carbon tetrachloride, chloroform, methylene chloride, styrene, vinyl chloride, acrilonitrile, NDMA, NDEA, NNK, *N*-nitroso-*N*-methylbenylamine | trichloroethylene, ethylene dibromide, 1,1,1-tricholoethane |
| CYP3A4 | Aflatoxin $B_1$, aflatoxin $G_1$, 6-aminocrysene, 1-nitropyrene, 7,8-dihydroxy-7,8-dihydrobenzo[a]-pyrene, tris-(2,3-dibromopropyl) phosphate, senecionine | Aflatoxin $B_1$, senecionine, 1,6-dinitropyrene |

Adapted form Guengerich and Shimada (1991), Gonzalez and Gelboin (1994) and Guengerich (1995).
Abreviations: 2-AAF: 2-acetylaminofluorene, IQ: 2-amino-3-methylimidazo{4,5-*f*] quinoline, MeIQ: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, MeIQx: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline, Glu P-1: 2-amino-6-methyldipyrido[1,2,-*a*:3',2'-*d*]imidazole, Trp P-1: 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NDEA: *N*-nitrosodiethylamine, NDMA: *N*-nitrosodimethylamine.

Table 1.e. Inducers of cytochrome P450s involved in xenobiotic metabolism

| INDUCER | CYP450 INDUCED | MECHANISM |
|---|---|---|
| 3-methylcholanthrene, PAH*, TCDD, flavones, smoking*, cruciferous vegetables | rat and human CYP1A1 and CYP1A2 | Transcriptional activation and message stabilization Ah receptor-XRE domain |
| phenobarbitone*, phenytoin, butylated hydroxyanisole | rat CYP2B1, 2B2, 2A1, 2C6 and 3A2 human CYP3A4 | Transcriptional activation and message stabilization Barbie box |
| pregnenolone-16α-carbonitrile, steroids | CYP3A1, CYP3A2 | Transcriptional activation Protein stabilization |
| isoniazid*, acetone, ethanol*, pyrazone, benzene, fasting, diabetes | rat and human CYP2E1 | Transcriptional activation Protein stabilization mRNA stabilization Increased mRNA translation |
| clofibric acid, nafenopin, trichloroacetic acid | CYP4A1, CYP4A2, CYP4A3 | Transcriptional activation PPAR |

Based on Whitlock (1995) and Parkinson (1996).
Abreviations: PAH: polyaromatic hydrocarbons, TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, XRE: xenobiotic responsive element, PPAR: peroxisome proliferator activated receptor.
* Inducers of the human isoenzyme

### 1.3.4.2. Inhibition of cytochrome P450s

Cytochrome P450 inhibitors can be divided into three main categories, depending on their mechanism of action: reversible inhibition, metabolite intermediate complexation (quasi-irreversible) and autocatalytic inactivation (irreversible) (Murray and Reidy, 1990).

Reversible inhibitors can either bind to the ferric heme, the ferrous heme or might bind to lipophilic regions of the protein and simultaneously to the prosthetic heme iron (Ortiz de Montellano and Correira, 1995). The normal function of the CYP450 enzyme is restored once the inhibitor is eliminated from the organism. The second category includes chemicals, which are not inhibitors *per se*, and require metabolism by CYP450 to an intermediate capable of inhibiting P450 isoenzymes, which sequesters the enzyme in catallitically non-functional state (Murray and Reidy, 1990). The inhibitor forms a stable complex with CYP450 (metabolite-intermediate (MI)-complexation), which can be displaced resulting in reactivation of CYP450. The third class of inhibitors include compounds that are converted by CYP450 to radical intermediates which bind the enzyme and inactivate it. This inactivation can occur by covalent binding to the protein, covalent binding to the prosthetic heme group (leading to loss of heme) or by covalent binding of the modified heme (due to interaction with the inhibitor) to the protein (Ortiz de Montellano and Correira, 1995; De Matteis, 1978). Inhibitors that bind irreversibly to CYP450 are also named suicide inhibitors (Murray and Reidy, 1990). Restoration of CYP450 activity can only be achieved by *de novo* synthesis of the enzymes. Inhibitors that are catalysis-dependent (this includes second and third class) are also known as mechanism-based inhibitors.

Other mechanisms of inhibition have been described, such as depletion of cofactor supply, xenobiotic down regulation of CYP450s (e.g. diallylsulfide (Kwak *et al*, 1994)) or alteration of the biosynthesis of heme or apoprotein (Murray and Reidy, 1990).

Cytochrome P450 inhibitors can also be classified as non-selective inhibitors and selective inhibitors. Non-selective inhibitors generally exert their action to most of the P450 isoenzymes. For example, carbon monoxide inhibits all CYP450 isoforms by ferrous-heme complexation, SFK525A inhibits several isoforms by binding to the enzyme and

allylisopropylacetamine is a mechanism based inhibitor of several CYP450 forms (Correira, 1995). Selective-inhibitors inhibit selectively only a small number of isoforms. Examples of selective inhibitors are shown in table 1.f. Because of the involvement of CYP450s in carcinogen activation, the selective inhibition of cytochrome P450 isoenzymes is thought to have the potential to confer anticancer properties (see section 1.4.2.). However, adverse reactions may occur since CYP450s also detoxify certain carcinogens (see table 1.d.). The inhibition of hepatic carcinogen metabolism can also result in reduced first-pass clearance of many carcinogens, which may result in greater exposure to carcinogens in extrahepatic tissues.

Table 1.f. Selective inhibitors of human and rat liver P450s

| CYP450 | INHIBITOR | MECHANISM |
|---|---|---|
| 1A1 | 7,8-benzoflavone<br>*α-naphthoflavone* | reversible (competitve)<br>*reversible* |
| 1A2 | fluroxamine<br>furafylline (rat and human)<br>*α-naphthoflavone* | reversible (competitive)<br>irreversible (mechanism-based)<br>*reversible* |
| 2A6 | diethyldithioncarbamate* | irreversible |
| 2C9 | sulfaphenazole (also rat 2C6) | reversible (competitive) |
| 2D6 | quinidine<br>*ajamacicine (rat 2D)* | reversible (heme complexation)<br>*reversible* |
| 2E1 | 4-methylpyrazole<br>*disulfiram, diallylsulfide* | reversible (heme complexation)<br>*irreversible (mechanism-based)* |
| 3A4 | troleandomycin (also rat 3A1/2)<br>gestodene (also rat 3A1/2)<br>erythromycin | quasi-irreversible<br>irreversible (mechanism-based)<br>MI-complexation |

Adapted from Murray and Reidy (1990) and Correira (1995). Inhibitors in italics represent inhibitors of rat CYP450s.

* Diethyldithionocarbamate is not a totally selective inhibitor of CYP2A6; it has been shown to inhibit several CYP450 isoforms including 2E1.

## 1.3.5. Polymorphisms linked to carcinogen metabolising cytochrome P450 enzymes

As seen in section 1.3.4. the metabolic activation of many carcinogens can be modulated by induction and/or inhibtion of the CYPs involved in their metabolism. Genetic factors have also been found to play a role in CYPs expression, and therefore may contribute to carcinogen metabolism. Several cytochrome P450s, and also Phase II enzymes express genetic polymorphisms in the population. The most common polymorphisms found to date regarding drug metabolism enzymes are the CYP1A1, CYP2D6, CYP2E1, GSTM1 and NAT2. GSTM1 and NAT2 plymorphisms have already been described (section 1.2.).

A polymorphism of the MspI restriction site in CYP1A1 gene has been linked with CYP1A1 inducibility in some studies and with a high risk to lung cancer in Japanese populations (Kawajiri, 1990). MspI polymorphism consists of three genotypes: a predominant homozygous m1 allele (genotype A), the heterozygote (genotype B) and a homozygous rare m2 allele (genotype C). A significant correlation in a Japanese population between susceptibility to lung cancer and m2/m2 homozygosity was found (Kawajiri, 1990). However this association in Caucasian populations (10-fold lower frequency of m2 homozygous) is controversial (Rannung *et al*, 1995). A polymorphism resulting in aminoacid substitution (Ile-Val) has also been associated with lung cancer (Val/Val genotype).

The levels of CYP1A2 mRNA and protein have been shown to differ substantially among individuals and they have been classified as fast and slow CYP1A2 mediated caffeine metabolisers. However, a genetic polymorphism associated with this variability has not yet been identified (Daly *et al*,1994; Raunio and Pelkonen, 1995; Catteau *et al*, 1995).

The CYP2D6 (debrisoquine hydroxylase) polymorphism is the most studied human enzyme deficiency. The polymorphism is characterised by the inability of a subset of the population to metabolise debrisoquine, thus making possible a classification of individuals as poor and extensive metabolisers (PMs and EMs respectively) (Daly *et al*, 1994). The human CYP2D consists of two genes, CYP2D6 and CYP2D7 and a pseudogene, CYP2D8P. Three most

common defective alleles are CYP2D6A (a deletion of A2637 in exon 5), 2D6B (a transition of G1934 to A), and 2D6D (deletion of the entire gene) (Rannug *et al*, 1995). There is evidence in favor of a correlation between the EM phenotype and an increased risk of lung cancer (Raunnio and Pelkonen, 1995) and gastric cancer (Poulsen *et al*, 1993), although a negative correlation has also been reported (Rannug *et al*, 1995). The association between CYP2D6 polymorphism and lung and gastric cancer risk is not understood, mainly due to poor catalytic activity of CYP2D6 towards carcinogens (it is known only to activate NNK, see table 1.d.), and because CYP2D6 is mainly expressed in liver. Several authors speculate the possibility that other factors (oncogenes or tumour suppresor genes) may be linked to the CYP2D6 polymorphism (Poulsen *et al*, 1993).

Genetic polymorphims of CYP2E1 have been identified using the restriction enzymes DraI, TaqI, and RsaI and in Japanese populations have been associated with lung cancer risk (Raunio *et al*, 1995). However, no correlation was found in other populations, with less frequency of the polymorphic alleles (Raunio and Pelkonen, 1995). Another polymorphism of CYP2E1 has been found in the 5'-flanking region, using PstI and RsaI (Rannug *et al*, 1995). Althougth there was no association with lung cancer risk, interethnic differences in distribution of the rare allele RsaI- PstI+ have been observed (Raunio *et al*, 1995, Rannug *et al*, 1995). Recently, another polymorphism in the 3'-non coding region and the poly A tail has been found, but no associations with cancer risk have been described yet (Seree *et al*, 1995).

In summary, evidence indicates that a correlation exists between genetic polymorphisms and cancer risk, but this may vary in different ethnic groups. It is likely that individuals with genotypes coding for active P450 enzymes and inactive phase II enzymes might be at higher risk to develop cancer than individuals with the opposite genotype (Rannug *et al*, 1995). As a matter of fact correlations between certain cancer types and combined polymorphisms have been reported. For example CYP1A1 polymorphism combined with the GSTM1 null genotype have been associated with lung cancer risk (Hayashi, 1992; Nakachi, 1993), urothelial cancer risk (Katoh *et al*, 1995) and with basal cell carcinoma risk (Lear *et al*, 1996). Fast caffeine metabolisers (CYP1A2) and rapid acetylators (NAT2) were more prevalent among colorectal/polyp cases than among controls (Kadlubar *et al*, 1992).

However, more studies are needed to be able to predict an individual's suceptibility to chemical carcinogenesis.

### 1.3.6. Differences between orthologous human and rodent cytochrome P450 enzymes.

Evidence indicates that orthologous human and rodent, in particular rat, CYP450 forms catalyse the activation (and detoxification to a lesser extent, see section 1.3.3.) of carcinogens in a highly similar manner. Nevertheless, differences in expression, catalytic activities and substrate specificity between rodent and human CYP450s have been observed. In table 1.c. it can be seen that species specific CYP450 forms are found. For example, different isoforms of the subfamilies CYP2A, CYP2B, CYP2C, CYP2D, CYP3A and CYP4A are found in different species and in mice CYP2B and CYP2C isoforms are lacking. Also human polymorphisms are numerous (see section 1.3.5.) but rare in rodent species. These differences are relevant to cancer and may reflect interspecies variability in the susceptibility to chemically induced carcinogenesis.

The distribution of CYP450s in tissues, their expression and inducibility have been found to vary between species. For example CYP1A1 in rodents is constitutively expressed at low levels in liver and extrahepatic tissues, but in humans it is essentially an extrahepatic enzyme (Shimada *et al*, 1992b). It is inducible in both humans and rodents. Another important difference is that CYP3A4 is the major P450 in human liver, whereas in rats it is CYP2C11 (Paine, 1995).

Differences in substrate specificity between rodent and human CYP450s have also been found. Human CYP3A4 has a large spectrum of carcinogen substrates (see table 1.3.3), some of these carcinogens are metabolised by non-homologous forms in rat, such as aflatoxins, metabolised by CYP2C isoenzymes or benzo[*a*]pyrene, mainly catalysed by CYP1A1 in rat and mouse (Nedelcheva and Gut, 1994). Another example is the different isoforms involved in the metabolism of nitrosodialkylamines in humans and rats. Whereas NDEA in rats is mainly metabolised by CYP2E1, in humans it is metabolised by various isoforms, including CYP2A6, CYP2C, CYP2E1 and CYP3A4 (Bellec *et al*, 1996). Some carcinogens are catalysed by the same CYP450 forms in human and rat, but by a different

order of magnitude (for example IQ is activated more efficiently by human CYP1A2 than the rat homologue (Shimada and Nakamura, 1987). Inhibitors of CYP450 have also been found to have different specificity and potency in different species (for a review see Nedelcheva and Gut, 1994).

These interspecies variations in the metabolism of carcinogens (either due to different substrate specificities or different level of expression of different CYP450 isoforms) are thus, important and relevant to the response to carcinogens. Therefore, the extrapolation of animal data to human should be cautious, and for some cases may not be possible. Experimental approaches that might overcome these limitations include the use of human epidemiological data, human tissues, or human CYP450-expression systems (see chapter 5.1.).

### *1.4. Chemoprevention of cancer*

Cancer is currently the second leading cause of death worldwide, accounting for over seven million deaths per year, and it is estimated that it might become the leading cause by the year 2000 (Weinstein, 1991). The advances made in the treatment of cancer have resulted in a reduction in mortality in individuals less than 65 years old, however cancer incidence has increased substantially for all age groups (Doll, 1992, Weinstein, 1991). New perspectives and directions of research in cancer are therefore necessary.

Evidence indicates that 50 - 80% of human cancers are potentially preventable (Weinstein, 1991). This is possibly due to the fact that the causes of cancer are mainly exogenous factors, such as cigarette smoking, dietary factors (10-70 % of cancers are associated with diet), occupational and environmental chemicals, viruses and socioeconomic factors (Weistein, 1991, Doll and Peto, 1981). One logical approach to cancer prevention is to avoid exposure to these exogenous causes. However, this might not be always possible or desired (e.g. occupational and environmental factors, socioeconomic factors, cigarette smoking).

An alternative (and/or complementary) approach to the prevention of cancer is the potentiation of the host defence anticancer mechanisms by the intake of one or several compounds (De Flora *et al*, 1995; Wattenberg, 1992). This approach is referred as chemoprevention and was firstly introduced by Sporn when studing the chemopreventive properties of retinoids (Sporn et al, 1976). These compounds, chemopreventors, include naturally occurring chemicals in the diet and synthetic chemicals (often analogs of dietary compounds).

Chemopreventors are also called anticarcinogens and are often antimutagens, because of the close correlation between mutations and carcinogenesis (see section 1.1.), and because they often inhibit mutations induced by carcinogens. The endpoints and methods used for assessing carcinogens, can therefore also be used for the assessment of corresponding chemopreventors.

### 1.4.1. Chemoprevention and diet

Epidemiological data have indicated that consumption of diets rich in vegetables and fruits is associated with a low risk of certain cancers. For example an inverse relationship has been found between diets rich in fish, vegetables and β-carotene- and vitamin A-containing foods and risk of skin cancer in a case control study in Australia (Kune *et al*, 1992). An inverse association between vegetable and fruit consumption and lung cancer risk was found in the Iowa women's Health study (Steinmetz *et al*, 1993). Similar associations were found in a follow-up study in the United States for second primary oral and pharyngeal cancers (Day *et al*, 1994). The risk of developing second primary cancers was 40-60 % lower among individuals with high intake of vegetables, including dark yellow, cruciferous and green leafy vegetables (Day *et al*, 1994). A decreased colon cancer risk has been associated with frequent ingestion of cruciferous vegetables (Steinmetz *et al*, 1993 and Kune *et al*, 1987). As a consequence of the epidemiological evidence, international dietary guidelines have suggested increasing the consumption of fruits, vegetables and fiber and decreasing fat intake (Kritchersky, 1993).

The protective effect of vegetables and fruits has been attributed, at least in part, to micronutrients (or non-nutrients, i.e. compounds without nutritional value; Wattenberg 1983, 1992, 1996). These chemopreventors include compounds with very different chemical structures, such as phenols, indoles, aromatic isothiocyanates, methylated flavones, coumarins, selenium salts, protease inhibitors, ascorbic acid, tocophenols, retinol and carotenes (Wattenberg, 1992). They are found in a wide variety of foods, including allium vegetables, whole grains, cruciferous vegetables, leafy vegetables, coffee and tea, and citrus fruits (Dragsted *et al,* 1993).

It should be realised that diet may also be a source of many carcinogens, such as nitrosamines, polycyclic aromatic hydrocarbons or heterocyclic amines (Lindsay, 1996). However, the overall contribution of vegetables and fruits to cancer seems to be beneficial. The identification of chemopreventors in the diet and elucidation of their mechanisms of action are thus necessary for a better understanding of their impact in cancer prevention.

### 1.4.2. Mechanisms of action and classification of chemopreventors

In view of the multistage nature of carcinogenesis (section 1.1.2.), it can be anticipated that the mechanisms of chemoprevention will be numerous, depending on the stage at which they inhibit the carcinogenic process, i.e. initiation, promotion or progression. The different mechanisms of chemoprevention have been studied and reviewed by several authors (Wattenberg, 1983, 1992, Stavric, 1994, De Flora and Ramel, 1988).

Several classifications of chemopreventors have been proposed, according to the mechanisms of action. De Flora and Ramel (1988) classified anticarcinogens and antimutagens in three broad categories, 1. those working by extracellular mechanisms, 2. those working by cellular mechanisms before cancer initiation, and 3. those working by modulation of the promotion and progression stages (Table 1.g.). Chemopreventors can also be classified according to Wattenberg (1982, 1992) into compounds that prevent the formation of carcinogens from their precursors, blocking agents, which prevent carcinogens from reaching and reacting with critical target sites in the tissues, and suppressor agents, which suppress the expression of neoplasia in cells previously exposed to carcinogen .

The possible mechanisms of action of chemopreventors are summarised in Table 1.g.. The inhibition by extracellular mechanisms can be represented by vegetable fibres, which have shown to inhibit several types of cancers by absorbing the carcinogen in the gut (Dragsted *et al,* 1993; Ferguson, 1994) and by ascorbic acid, which prevents formation of endogenous nitrosamines by scavenging nitrite in the gut (Dragsted *et al,* 1993).

The mechanisms of inhibition of the initiation stage of carcinogenesis within the cell are various (Table 1.g.). The inhibition of cell replication is a possible mechanism, since cell division is important for the fixation of DNA damage (see section 1.1.3.). Several protease inhibitors have been found to act through this mechanism (Dragsted *et al,* 1993). Another mechanism is the trapping of activated and direct carcinogens, for example chlorophyllin inhibits mutagenesis of $AFB_1$, B[a]P and various HAAs (Ferguson, 1994), although it also modulates metabolism of carcinogens (Yun *et al,* 1995). The scavenging of free radicals and ROS is also potentially protective, since these species are genotoxic and induce carcinogenesis (see section 1.1.3.). Many antioxidants, such as flavonoids, β-carotene, vitamin A fall into this category (Stravic, 1994; Ferguson, 1994). Antioxidants can also inhibit tumour promotion and progression, as will be discussed later. Another possible mechanism of anticarcinogenesis is the modulation of DNA repair systems. Most mutations arise as a result of error-prone DNA repair (see section 1.1.3.2.), therefore the increase of fidelity of DNA replication, inhibition of error-prone repair pathways or inhibition of DNA repair leading to death of damaged cells would inhibit the formation of critical mutations (Ferguson, 1994). Compounds that act on repair and replication processes, such as vanillin, have been named "biomutagens" or "true mutagens" (Ferguson, 1994; Ohta, 1993). The protection of nucleophillic sites in DNA can prevent the reaction of the carcinogen with DNA, and thus inhibit the formation of mutations; ellagic acid and tannins have been found to act by this mechanism (Dragsted *et al,* 1993). Finally, the modulation of carcinogen metabolism can also inhibit carcinogenesis and mutagenesis, and will be discussed in detail in the following section.

Table 1.g. Mechanisms of inhibition of carcinogenesis and mutagenesis.

1. Inhibition of mutagens/carcinogens by extracellular mechanisms
    1.1. Inhibition of uptake
    1.2. Inhibition of endogenous formation
    1.3. Complexation, dilution and/or deactivation
2. Inhibition of mutation and cancer initiation by cellular mechanisms
    2.1. Inhibition of cell replication
    2.2. Stimulation of trapping and detoxification in non-target cells
    2.3. Modification of transmembrane transport
    2.4. Modulation of metabolism
    2.5. Blocking or competition with reactive molecules
    2.6. Modulation of DNA repair and control gene expression
3. Inhibition of tumour promotion and progression
    3.1. Scavenging of free radicals
    3.2. Inhibition of proteases
    3.3. Inhibition of cell proliferation
    3.4. Induction of cell differention
    3.5. Modulation of signal transduction
    3.6. Protection of intercellular communications
    3.7. Effects on growth factors, hormones, and/or immune system
    3.8. Inhibition of neovascularisation
    3.9. Physical,chemical or biological antineoplastic activity

Modified from De Flora and Ramel (1988) and De Flora *et al* (1995).

The promotion and progression stages of carcinogenesis are complex and many events are involved (see section 1.1.2.), therefore the mechanisms of inhibition of these stages will be numerous, and complex and are not fully understood yet (see Table 1.g.). Several antioxidants, such as retinoids and carotenoids have been found to inhibit tumour promotion and progression. Some of their mechanisms of action include increase of cell differentiation, increase of intercellular communication, and suppression of tumour cell growth (Schwartz, 1996). Ascorbic acid has also been found to inhibit growth of leukemia and lymphoma cell lines (Kao *et al*, 1993).

Some chemopreventors may act through various of these mechanisms at the same time, such as antioxidants (scavenging of genotoxic ROS, inhibition of promotion and progression; Rice-Evans and Miller, 1996), chlorophyllin or ascorbic acid (see above). Another example is that of *N*-acetylcysteine, which chemopreventor action includes extracellular mechanisms, modulation of carcinogen metabolism, stimulation of DNA repair and scavenging of ROS (Izzoti *et al*, 1994, De Flora *et al*, 1995).

### 1.4.3. Mechanisms of anticarcinogenesis by modulation of carcinogen activation

In sections 1.1. and 1.2. it was pointed out that the ability of chemical carcinogens to induce mutations was dependent on the reactivity of the ultimate carcinogen and its overall metabolism, which is dictated by the balance between activation and detoxification pathways. Thus, events enhancing the detoxification pathways will increase the elimination of carcinogen, resulting in a protective effect against carcinogenesis. Both the procarcinogen and ultimate carcinogen can be subject to detoxification, as well as direct acting carcinogens. An alternative mechanism of protection would occur by inhibition of the activation of procarcinogens leading to the formation of ultimate reactive carcinogenic metabolites (Wattenberg, 1983, 1992) (De Flora and Ramel, 1988). The different mechanisms of anticarcinogenesis by modulation of carcinogen metabolism are shown in figure 1.d..

Chemopreventors that modulate carcinogen metabolism (or "blocking agents") can then be classified in three groups (Wattenberg, 1983): 1. they inhibit the activation of

procarcinogens to their ultimate carcinogenic forms, 2. they induce carcinogen-detoxification systems, and 3. they act by scavenging the reactive forms of carcinogens. This last group include compounds such as chlorophyllin (section 1.4.2.) and nucleophiles such as GSH, and compounds that increase levels of GSH (De Flora and Ramel, 1988; Ketterer, 1988; Meister, 1994).

Since activation of carcinogens is generally mediated by cytochrome P450 enzymes and detoxification is catalysed by phase II enzymes (see section 1.2), it could be said that the inhibition of cytochrome P450s and the induction of phase II enzymes are two major mechanisms of chemoprevention. This is, however, a generalization, since some carcinogens are actually detoxified by cytochrome P450 (section 1.3.3.) and others are activated by phase II enzymes. The mechanisms of inhibiton of cytochrome P450 have already been described (see section 1.3.4.2) and it has been suggested that inhibition of P450 enzymes could be used as a target for chemoprevention of chemical carcinogens (Yang *et al,* 1994). Examples of inhibitors of CYP450 that have been shown to inhibit chemical carcinogenesis are diallylsulfide, flavonoids, and isothiocyanates (Yang *et al,* 1994).

The induction of detoxification enzymes, i.e phase II enzymes such as glutathione-*S*-tranferases (GST), UDP-glucuronosyltransferase (UGT) and quinone reductase (QR) can occur coordinated with induction of microsomal monooxygenase activity (i.e. CYP450) (bifunctional inducers) or without induction of the latter (monofunctional inducers). Examples of bifunctional inducers are β-naphthoflavone (β-NF), polycyclic aromatic hydrocarbons, certain flavonoids and azo-dyes and some monofunctional inducers are phenolic antioxidants such as tert-butyl-4-hydroxyanisole (BHA), isothiocyanates and coumarins (Talalay, 1989). There is controversy about the beneficial action of bifunctional inducers (Wattenberg, 1983, 1992). Althougth they seem to inhibit chemical carcinogenesis in experimental animals, the fact that they induce CYP450 (which catalyse the activation of many carcinogens (see section 1.3.3)) makes it possible that under certain conditions enhancement of carcinogenesis might occur. In fact, the induction of CYP1A has been considered as an indicator of potential carcinogenesis (Ioannides and Parke, 1993).

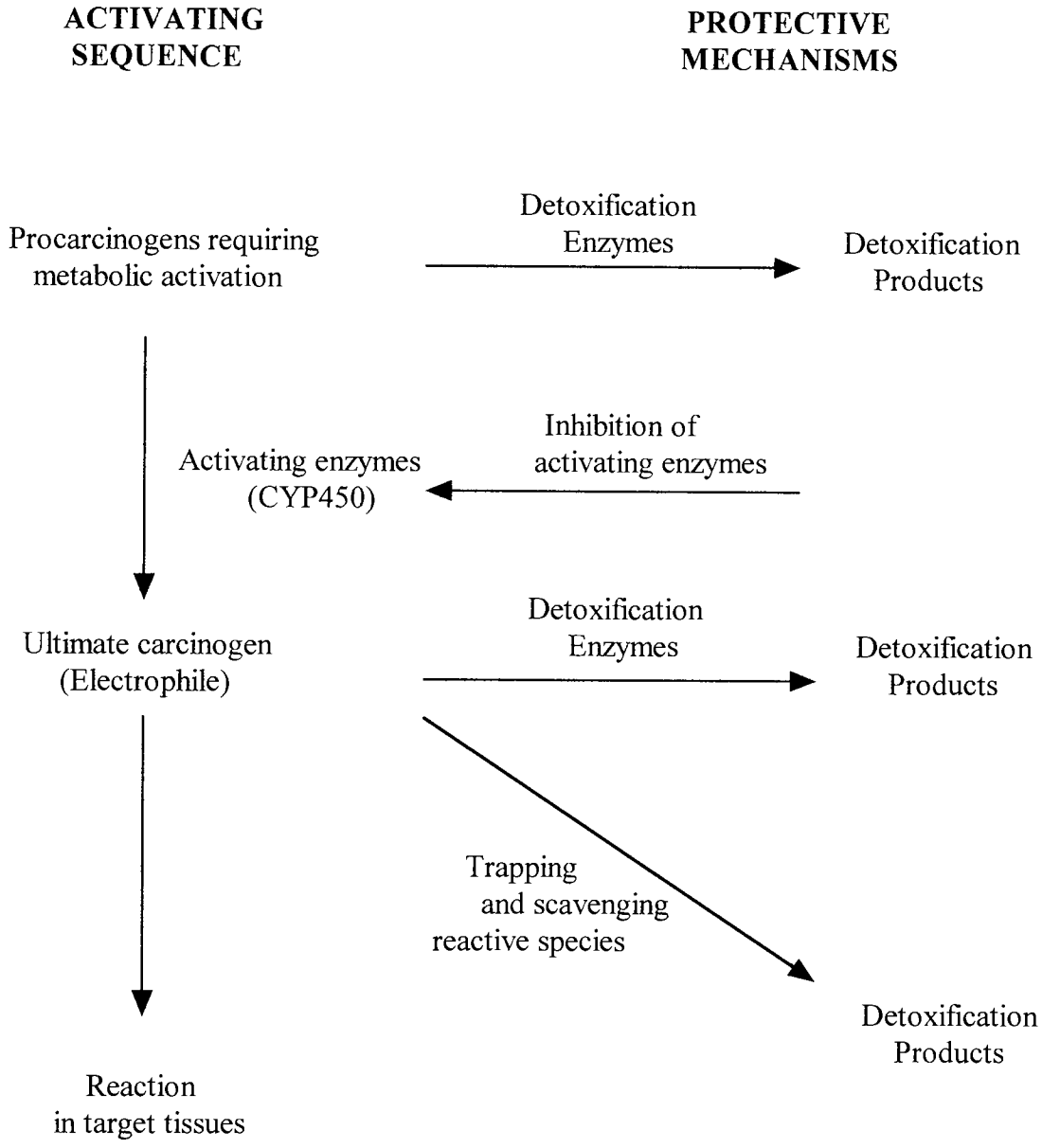



Figure 1.d. Mechanisms of chemoprevention by modulation of carcinogen metabolism.

Adapted from Wattenberg (1983).

### 1.4.4. Regulatory mechanisms of monofunctional and bifunctional inducers

The mechanisms of induction of cytochrome P450 activity and phase II enzymes (mainly GST and QR) have been studied by Prochaska and Talalay (1988). By using two experimental systems (Hepa 1c1c7 murine hepatoma cells and their mutants defective in Ah receptor function, and livers of mouse strains with high and low affinity Ah receptors), it was found that the induction of quinone reductase (and GST in mice) and CYP450 activity by bifunctional inducers was mediated througth the Ah receptor (Prochaska and Talalay, 1988) (see section 1.3.4.1.). Moreover, bifunctional inducers were incapable of inducing either quinone reductase or AHH activity in the absence of a functional Ah receptor. On the other hand monofuctional inducers did not require the Ah receptor to induce QR and GST activities (Prochaska and Talalay, 1988; Talalay, 1989). The mechanisms of induction of CYP1A1 and CYP1A2 by the Ah receptor were described in section 1.3.4.1. and it was shown that the induction of transcription was largely regulated by the upstream enhancer XRE, also present in Phase II enzymes.

The mechanisms of induction of Phase II enzymes by monofunctional inducers are not fully understood yet, but have been clarified more recently by the identification of other response elements in the upstream regions of several phase II enzymes.

The Antioxidant Response Element, ARE, has been identified recently in the 5'flanking upstream region of rat QR (Favreau and Picket, 1995). Compounds such has β-NF, tert-butylhydroquinone (BHQ) have been found to activate gene expression through the ARE. It is believed that this element is responsible for both basal and inducible transcription of QR (Favreau and Picket, 1995). Other enzymes have been found to contain ARE: rat GST Ya (contains ARE and XRE), mouse GST Ya has an EpRE (electrophile response element, similar to ARE only with two nucletides difference) and rat GST P, which contains a GPEI (GST P enhancer I, similar to rat QR ARE) and rat and mouse GST Ya ARE (Parkinson, 1996). In human QR, ARE contains TRE sequences (phorbol 12-tetradecanoate 13-acetate (TPA)-responsive elements) within the ARE, and has been found to bind AP-1 and Fos/Jun proteins ((Li and Jaiswal, 1992). However, rat QR ARE does not bind to c-Fos and c-Jun.

Recent studies have indicated that monofuctional inducers increase transcription of QR and GST *via* the EpRE/ARE consensus, but not via the TRE (Prestera and Talalay, 1995). The identity of the EpRE/ARE enhancer-binding proteins is controversial. Nevertheless, recent findings indicate the existence of a EpRE/ARE-specific protein, yet to be identified (Favreau and Pickett, 1995), althougth the EpRE/ARE could be induced both by the specific protein and AP-1 protein (Prestera and Talalay, 1995). However, the response to monofunctional inducers seems to be mediated by this specific protein rather than AP-1 (Prestera and Talalay, 1995).

### *1.5. Chemopreventor factors in cruciferous vegetables*

Cruciferous vegetables are one of the major groups of vegetables associated with a decreased risk of different types of cancer in humans (see section 1.4.1), such as colon, rectum and gastric cancer (Graham, 1983, Kune *et al*, 1987, Steinmetz and Potter, 1991). Experimental data from animals also indicate a positive relationship between cruciferous vegetables and a decrease in cancer development. Feeding rodents with Brussels sprouts, cauliflower, cabbage decreased the tumorigenicity effect of carcinogens such as polybrominated biphenols and aflatoxin $B_1$ in the liver, and 7,12-dimethylbenz[a]anthracene (DMBA) in rat breast (Stoewsand, 1995; Boyd *et al*, 1982; Dragsted *et al*, 1993). It is thought that this protective action is exerted by chemopreventor agents (non-nutrients) present in these vegetables (Wattenberg, 1983; Dragsted *et al*, 1993). Several chemopreventors have been isolated and identified from cruciferous and other vegetables. Some examples of chemopreventors in cruciferous vegetables and their anticarcinogenic effect are shown in table 1.h. The mechanisms by which these chemopreventors exert their action are various. For example, oltipraz chemoprevention has been associated with the induction of GST (phase II enzyme) and the inhibition of various cytochrome P450 isoenzymes (Langouët *et al*, 1995 and 1996, Primiano *et al*, 1995). Penethylisothiocyanate (PEITC) seems to protect against carcinogens by the inhibition of cytochrome P450 2E1 (Ishizaki *et al*, 1990; Guo *et al*, 1992). Benzyliosthiocyanate (BITC) chemopreventive action has been associated with its blocking and suppressing properties towards several carcinogens (i.e. inhibits initiation of carcinogenesis by modulation of carcinogen metabolism and also inhibits the promotion stage; Wattenberg, 1987 and 1992).

Table 1.h. Examples of chemopreventors in cruciferous vegetables

| CHEMOPREVENTOR | TUMOUR-INDUCING CARCINOGEN | SPECIES AND TARGET ORGAN |
|---|---|---|
| Benzylisothiocyanate | DMBA, B[a]P, NNK, diethylnitrosamine | rat lung, forestomach and mammary gland |
| Phenetylisothiocyanate | NBMA, NNK, DMBA | rat forestomach, lung (and mouse) and esophagus |
| Phenylhexylisothiocyanate | NNK | mouse lung |
| B-Sitosterol | N-methyl-N-nitrosourea | rat colon |
| Indole-3-carbinol | N-nitrosodiethylamine | rat liver |
| Oltipraz | Aflatoxin $B_1$, azoxymethane | rat liver and colon/small intestine |

Adapted form Dragsted *et al,* 1993 and Wattenberg, 1992.

NBMA: *N*-Nitrosobenzylmethylamine

### 1.5.1. Identification of a major inducer of phase II enzymes in broccoli: Sulforaphane

Induction of phase II enzymes has been used to detect the presence of blocking agents in complex natural products (Wattenberg, 1983 and 1992). A method based on the induction of QR in Hepa c1 c7 murine hepatoma cells has been developed recently (Prochaska *et al*, 1992) and allows the identification of monofunctional phase II enzymes inducers. By using this technique, sulforaphane [(-) 1-isothiocyanate-4(*R*)-methylsulfinylbutane] was identified as the most potent inducer of phase II detoxication enzymes in broccoli (Zhang *et al*, 1992). Sulforaphane at a dose of 15 μmol/mouse/day for 5 days induced mouse liver GST 2-fold and QR 2.5-fold (Zhang *et al*, 1992). Levels of GST and QR were also induced in forestomach, glandular stomach, proximal intestine and lung (Zhang *et al*, 1992). Because of the association between induction of phase II enzymes and chemoprevention (see sections 1.4.3 and 1.4.4.), and the potent induction of QR and GST by sulforaphane, this agent has been proposed as a potential chemopreventor. During the course of this study, Zhang *et al*, (1994) have demonstrated the ability of sulforaphane to inhibit DMBA-tumourigenicicty in rats.

### 1.5.2. Occurrence of isothiocyanates and sulforaphane

Isothiocyanates are found in cruciferous vegetables as their glucosinolate precursors. When the plant tissue is disrupted during processing, glucosinolates are decomposed by the enzyme myrosinase (or alternatively by microflora in the gastrointestinal tract) to glucose, sulfate, and unstable aglycones (sulfur-nitrogen containing compounds) (Fenwich *et al*, 1982). The formation of isothiocyanates or nitriles depends mainly on the type of substrate and the hydrolysis conditions, specially the pH (Stoewsand, 1995). At neutral pH, the formation of isothiocyanates is favoured, whereas under acidic conditions nitriles are formed (Figure 1.e.). Some unstable isothiocyanates, such as indole isothiocyanates, decompose spontaneously to give carbinols (McDanell *et al*, 1988).

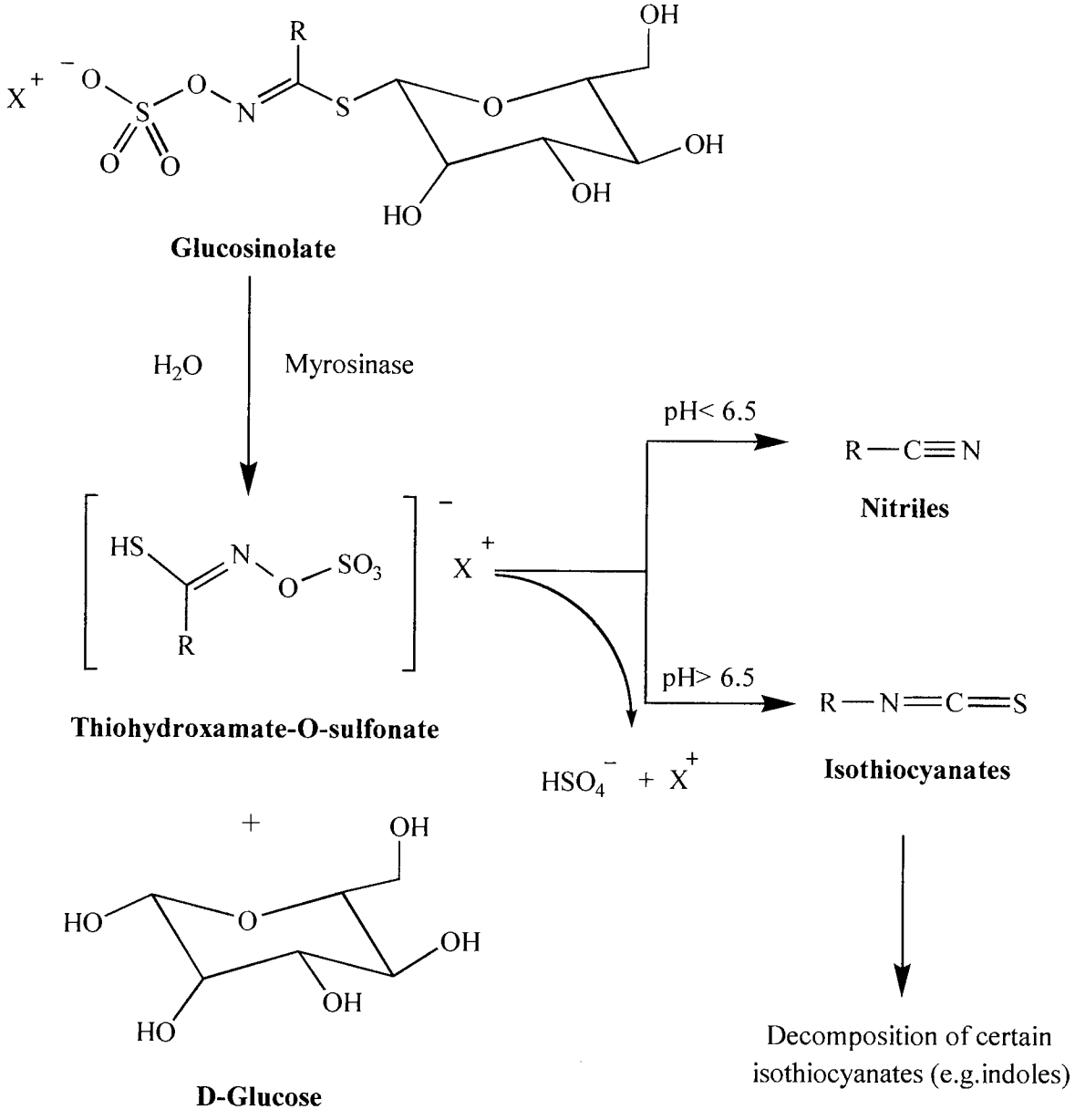



Figure 1.e. Decomposition of glucosinolates into isothiocyanates.

Glucosinates are present in seeds, roots, stems and leafs of several plants of which cruciferous vegetables (*Brassica oleracea*) are the most important. This family includes cabbage, Brussels sprouts, broccoli, cauliflower, kale, and turnip. Levels of glucosinolates in various cruciferous have been estimated. Loft *et al,* (1992) found a glucosinate content of 5-35 μmol/g dry weight of broccoli, of which indole glucosinates represent 60 % and glucoiberin and glucoraphanin representing a 10% of total glucosinolates (155 μmol/g powder). Glucoiberin and glucoraphanin are the precursors of 1-isothiocyanate-4-methylsulfinylpropane and sulforaphane (1-isothiocyanate-4-methylsulfinylbutane). Sulforaphane has been extracted from SAGA broccoli (8.9 mg of sulforaphane from 640 g of fresh broccoli, 0.08 μmol/g fresh weight) (Zhang *et al,* 1992), and also from hoary cress and other plants (Prochazka, 1959, Kjaer and Christensen, 1958).

### 1.5.3. Biochemical and biological properties of isothiocyanates and sulforaphane

The ability of isothiocynates and related compounds to induce phase II enzymes and/or inhibit cytochrome P450, as potential mechanisms of chemoprevention, was already discussed in sections 1.4. and 1.5.1..

Some cruciferous vegetables have been shown to induce cytochrome P450 enzymes (Bradfield *et al,* 1985; Wortelboer *et al,* 1992), and certain products of glucosinolate degradation appear to be responsible, at least in part, for this effect (Loft *et al,* 1992). The possible adverse consequences of this induction have already been described (section 1.4.2.).

The major metabolic pathway of isothiocyanates seems to be the reversible conjugation with glutathione, which can occur spontaneouly or mediated by GSTs (Baillie and Slatter, 1991). This conjugation also appears to allow the transport of conjugated isothiocyanates through the body membranes, followed by release of the original isothiocyanate in other sites (Baillie and Slatter, 1991).

Isothiocyanates have been known for many years for their harmful effect in animals fed large amounts of Brassica vegetables (Stoewsand, 1995). Isothiocyanates such as

allylisothiocyanates, benzylisothiocyanate and phenethylisothiocyanate have been shown to be cytotoxic in several cell types (Bruggeman *et al*, 1986; Babich *et al*, 1993). The two latter isothiocyanates have also been found to induce chromosome damage in the SVM cell line (Musk and Johnson, 1993). Some studies have indicated that allylisothiocyanate and phenylhexylisothiocyanate might be mutagenic and carcinogenic (Neudecker and Henschler, 1985, Rao *et al*, 1995). In reference to sulforaphane, there is no information regarding its metabolism or cytotoxicity. It thus appears that the final chemopreventive properties of certain isothiocyanates might be the result of the balance between beneficial (anticarcinogenic) and harmful (toxicity) effects.

### 1.5.4. Aims

Several isothiocyanates have been found to inhibit the tumourigenicity of several carcinogens in rodents (see table 1.h.), and one of their possible mechanisms of chemoprevention was that of modulation of carcinogen metabolism by inhibition of the activation pathways, i.e. cytochrome P450 and also by induction of detoxification pathways. It is conceivable that sulforaphane mediates chemoprotection via more than one mechanism. Its effect on phase I enzymes involved in the activation of carcinogens has so far been given scarce attention. The main purpose of this project was thus to study the ability of sulforaphane to inhibit carcinogen activation and genotoxicity by inhibition of cytochrome P450 enzymes.

The first objective of this project was to synthesise sulforaphane, since it was commercially unavailable. Because related isothiocyanates inhibited particularly CYP2E1 and CYP1A (Yang *et al*, 1994) and also the tumourigenesis of certain CYP2E1 and CYP1A substrates (see table 1.h.). The effect of sulforaphane on CYP2E1 and CYP1A was investigated. CYP2E1 and CYP1A are abundantly expressed in human liver and responsible for the metabolic activation of a range of many carcinogens, such as NDMA and IQ respectively. The influence of sulforaphane on the genotoxicity *in vitro* of these carcinogens was also assessed, in three different systems: bacterial (*Salmonella typhimurium*) using rodent enzymes for metabolic activation, rodent hepatocytes and human cell lines expressing human P450s.

# *CHAPTER 2*

## *Synthesis of sulforaphane*

## *2.1. Introduction*

### 2.1.1. Previous reported synthesis

The synthesis of sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane) has been described by Schmid and Karrer, (1948). The synthesis of sulforaphane was achieved starting from 1-bromo-4-phthalimidobutane (**I**) by conversion to 1-methylthio-4-phthalimidobutane (**II**), and subsequent oxidation, amine deprotection and finally conversion to the isothiocyanate product, 1-isothiocyanato-4-(methylsulfinyl)butane (**V**) (Schmid and Karrer, 1948) (Figure 2.a.). Specifically, the reaction between 1-bromo-3-phthalimidobutane (**I**) with gaseous thiomethanol gave 1-methylthio-4-phthalimidobutane (**II**), which was then oxidised to 1-methylsulfinyl-4-phthalimidobutane (**III**) by reaction with $H_2O_2$. Deprotection of the amine group (**IV**) was achieved by reaction with hydrazine hydrate and the isothiocyanate (**V**) was obtained *via* the dithiocarbamate by reaction with carbon disulfide and iodine (reported yield 58 %).

Zhang *et al,* (1992) prepared sulforaphane according to the method of Schmid and Karrer, (1948) except that gaseous thiomethanol was replaced by sodium thiomethoxide. An analogue of sulforaphane, sulforaphene (*trans*-4-methylsulphinyl-3-butenyl isothiocyanate) was synthesized *via* phthalimidobutyraldehyde by addition of thiomethanol and the amine group was transformed to the isothiocyanate also by reaction with carbon disulfide and iodine (Balenovic *et al*, 1966).

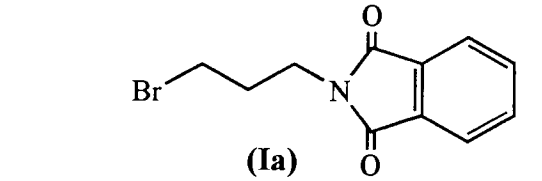
### 2.1.2. Alternative synthesis proposed

Alternative approaches to the synthesis of sulforaphane were proposed here (Figure 2.b.). For the first of these, 1-bromo-3-phthalimidopropane (**Ia**) would be used as starting material (in place of 1-bromo-4-phthalimidobutane) to be converted directly to 1-methylsulfinyl-4-phthalimidobutane (**III**) by nucleophilic substitution reaction. Although this synthetic route had not been described, the formation and reaction of methylsulfinyl carbanion, the required nucleophile for this approach, with other alkyl halides (conversion of **Ia** to **III** in figure 2.b.) had been described (Corey and Chaykovsky, 1965).

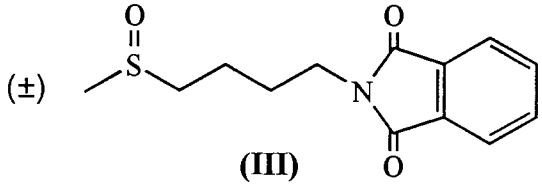
Aston University

**Content has been removed for copyright reasons**

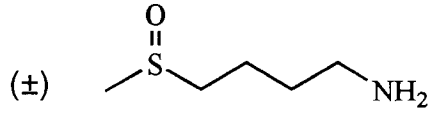
Figure 2.a. Synthesis of sulforaphane (Schmid and Karrer, 1948)



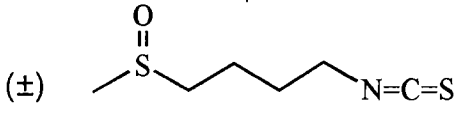

(Ia)




(III)




(IV)




(V)

**SULFORAPHANE**

Figure 2.b. Initial approach for the synthesis of sulforaphane.

1-Bromo-3-phthalimidopropane (**Ia**) would be converted directly to 1-methylsulfinyl-4-phthalimidobutane (**III**).

The main advantage of this proposed route was that it would involve one step less than the literature method, which theoretically would improve the overall yield and reduce time and expense. However, the route proposed by Schimd and Karrer (1948) introduces the possibility of choosing the chirality of the sulfoxide group of sulforaphane (by enantioselective sulfide oxidation), whereas the introduction of this fuctionality from DMSO does not (since the two methyls are non-distinguishable in DMSO). This could be significant, because isolated sulforaphane from plants has been identified as (*R*)-sulforaphane (Figure 2.c.). However, the inducer potency (induction of phase II enzymes) of isolated (*R*)-sulforaphane and synthetic (*R,S*)-sulforaphane were found to be closely similar (Zhang *et al*, 1992).

This first approach to the synthesis of (*R,S*)-sulforaphane *via* methylsulfinyl carbanion (Figure 2.b.) ultimately proved unsuccesful.

The second approach proposed here was based on the method by Schmid and Karrer (1948) and Zhang *et al.* (1992) (Figure 2.a.).


(*R*)-sulforaphane (*S*)-sulforaphane

Figure 2.c. Stereoisomers (enantiomers) of sulforaphane

## *2.2. Methods*

### 2.2.1. Chemicals and instruments

Standard chemicals and solvents were purchased from Aldrich Chemical Company Ltd., Lancaster Chemicals Company Ltd. or Sigma Chemicals Company Ltd. (UK). The following solvents were dried by heating under reflux over the appropiate drying reagent followed by distillation: dichloromethane ($P_2O_5$), methanol ($Mg(OMe)_2$), THF (Na, benzophenone), toluene (Na, benzophenone), acetone ($MgSO_4$) and acetonitrile ($CaH_2$). Flash column chromatography (Still *et al*, 1978) was performed using Sorbsil C60 silica gel. TLC was performed using Kieselgel 60 silica gel plates containing a fluorescent indicator. Spots were visualised under 254 nm UV light or with the aid of iodine or potassium permanganate.

$^1H$ and $^{13}C$-NMR spectra were recorded on a Brucker AC 250 ($^1H$ at 250MHz, $^{13}C$ at 62.5 MHz, both in $CDCl_3$, δ in ppm, tetramethylsilane as the reference). Infrared spectra were recorded on a Nicolet STIR spectrometer (KBr disk) and mass spectra were run by EPSRC Mass Spectrometry Service Centre, Chemistry Department, University College of Swansea, using a V.G. 7070E instrument. Gas chromatography was carried out on a PYE UNICAM-Series 204 with a SE-°30 column.

### 2.2.2. Preparation of 1-bromo-3-phthalimidopropane.(Ia)

3-Amino-1-bromopropane (5 g, 22.8 mmol) was dissolved in toluene (50 ml) and phthalic anhydride (4 g, 27.4 mmol) and triethylamine (2.3 g, 22.8 mmol) were added. The reaction mixture was heated under reflux in a flask fitted with a water separator for 4 hours and then cooled to room temperature, dried over $MgSO_4$ and evaporated. The crude product was purified by flash chromatography (hexane : ethylether 3 : 1) and obtained as white crystals (5.47 g, 90 %)

**$^1H$-NMR**: δ 7.82 (m, 2H, aromatic), 7.70 (m, 2H, aromatic), 3.81 (t, 2H, $J$ = 6.8 Hz, N-$CH_2$-), 3.39 (t, 2H, $J$ = 6.7 Hz, Br-$CH_2$-), 2.24 (m, 2H, Br-$CH_2$-$CH_2$-).

**$^{13}$C-NMR**: δ 168.6 (carboxyl), 134.4 aromatic), 132.3 (aromatic), 123.7 (aromatic), 37.0 (N-*C*$H_2$-), 31.9 (Br-*C*$H_2$-), 30.2 (Br-$CH_2$-*C*$H_2$-).

## 2.2.3. Preparation of 1-iodo-3-phthalimidopropane (Ib)

1-Bromo-3-phthalimidopropane (1 g, 3.7 mmol) was dissolved in dried acetone (30 ml) and to this sodium iodide (586 mg, 3.9 mmol) was added. The reaction was left stirring overnight at room temperature and covered with foil paper to prevent decomposition of iodide by light. $^1$H-NMR showed that some starting material (23 %) was still present (TLC gave the same $R_f$ for reactant and product). More NaI (277 mg, 1.85 mmol) was added and the reaction mixture was heated at 40 °C for one hour. The solution was then evaporated, filtered through a short silica column (hexane : ethylether 1:1) and washed twice with a saturated solution of $Na_2S_2O_3$ to eliminate the excess iodide. The solution was dried over $MgSO_4$ and evaporated to give the product as white crystals (0.84 g, 83 %).

**$^1$H-NMR**: δ 7.84 (m, 2H, aromatic), 7.71 (m, 2H, aromatic), 3.76 (t, 2H, $J$ = 6.8 Hz, N-C*H*$_2$-), 3.15 (t, 2H, $J$ = 2.1 Hz, I-C*H*$_2$-).), 2.23 (m, 2H, I-$CH_2$-C*H*$_2$-).

## 2.2.4. Synthesis of 1-methylthio-4-phthalimidobutane (II)

1-Bromo-4-phthalimidobutane (6 g, 21.2 mmol) was dissolved in dried methanol (50 ml) by heating under argon at 60°C, and then $CH_3SNa$ (3 g, 42.8 mmol) was added. The reaction mixture was heated under reflux for 3 h and solvents removed *in vacuo*. The crude product dissolved in $CH_2Cl_2$: MeOH 5:1 was chromatographed on flash silica gel eluting with $CH_2Cl_2$. The solution was dried ($MgSO_4$) and evaporated to yield a pale yellow oil (3.61 g, 68%) that crystallized standing at room temperature.

**$^1$H-NMR**: δ 7.82 (m, 2H, aromatic), 7.69 (m, 2H, aromatic), 3.69 (t, 2H, $J$ = 7.0 Hz, N-C*H*$_2$), 2.51 (t, 2H, $J$ = 7.1 Hz, S-C*H*$_2$), 2.06 (s, 3H, S-C*H*$_3$), 1.78 (m, 2H, S-$CH_2$-C*H*$_2$), 1.62 (m, 2H, N-$CH_2$-C*H*$_2$).

**$^{13}$C-NMR**: δ 168.1 (carbonyl), 133.8, 131.9, 123.0 (aromatics), 37.3 (N-*C*$H_2$), 33.4 (S-*C*$H_2$), 27.5 (S-$CH_2$-*C*$H_2$), 26.2 (N-$CH_2$-*C*$H_2$), 15.3 (S-$CH_3$).

## 2.2.5. Oxidation of 1-methylthio-4-phthalimidobutane (Figure 2.h.)

1-Methylthio-4-phthalimidobutane (0.53 g, 2.1 mmol) was dissolved in dried $CH_2Cl_2$ (20 ml) and cooled under argon at 0 °C. Meta-chloroperoxybenzoic acid (*m*-CPBA) hydrate (0.6 g, 1.1-1.6 mmol) was dissolved in dry dichloromethane (10 ml), dried over molecular sieves (to remove water from the *m*-CPBA), cooled to 0 °C and added to the reaction mixture. After 30 min of reaction at 0 °C, TLC showed no presence of starting material. The solution was partially evaporated and the product was purified by flash chromatography (500 ml $CH_2Cl_2$, $CH_2Cl_2$ : MeOH 10:1). The product obtained (0.506 g, 89 %) was a mixture of sulfoxide and sulfone (2:1).

**$^1$H-NMR** (sulfone (**IIIa**)): δ 7.83(m, 2H, aromatic), 7.72 (m, 2H, aromatic), 3.73 (t, 2H, $J$ = 6.3 Hz, N-C$H_2$-), 3.09 (t, 2H, $J$ = 7.5 Hz, $SO_2$-C$H_2$-), 2.9 (s, 3H, $SO_2$-C$H_3$), 1.87 (m, 4H, N-$CH_2$-(C$H_2$)$_2$-).

## 2.2.6. Preparation of 1-methylsulfinyl-4-phthalimidobutane (III)

The generation of sulfoxide from the sulfide was followed by gas chromatography. (GC). An SE-°30 column was placed in a Pye Unicam-Series 204 gas chromatograph. $H_2$ and air were used as carrier gasses and fixed at 1.0 and 0.5 kg/cm$^2$. $N_2$ was fixed at 0.8 kg/cm$^2$ and was the detecting gas. Temperatures of the column, detector, and injector were set at 210, 300 and 250 °C respectively. Retention times of sulfide, sulfoxide and sulfone were 8, 2 and 20 min respectively. 1-Methylthio-4-phthalimidobutane (4.165 g, 16.7 mmol) was dissolved in dry $CH_2Cl_2$ (50 ml) and cooled under argon at 0 °C. Meta-chloroperoxybenzoic acid dissolved in dichloromethane (0 °C) was gradually added in portions to the reaction mixture and left to react. A 1 ml sample was taken after each addition and diluted (1 µl, 7.5 µM) and analyzed by GC until the reaction was finished. The product was purified by flash chromatography (700 ml $CH_2Cl_2$, $CH_2Cl_2$ : MeOH 10:1) and obtained as a white solid (3.13g, 71%).

**$^1$H-NMR**: δ 7.82 (m, 2H, aromatic), 7.62 (m, 2H, aromatic), 3.73 (t, 2H, $J$ = 6.6 Hz, N-C$H_2$-), 2.74 (t, 2H, $J$ = 7.0 Hz, SO-C$H_2$-), 2.56 (s, 1H, SO-C$H_3$), 1.83 (m, 4H, N-$CH_2$-($CH_2$)$_2$-).

**$^{13}$C-NMR**: δ 168.3 (carbonyl), 134.0, 131.9, 123.2 (aromatics), 53.7 (SO-$CH_2$-), 38.7 (SO-$CH_3$), 37.0 (N-$CH_2$-), 27.6 (N-$CH_2$-$CH_2$-), 19.7 (SO-$CH_2$-$CH_2$-).

**IR**: Strong peaks at 1000 cm$^{-1}$ (SO) and 1700 cm$^{-1}$ (CO-N-CO).

## 2.2.7 Synthesis of 1-methylsulfinylbutanamine (IV)

The protected amine (2.97 g, 11.2 mmol) was dissolved in benzene (40 ml) under argon and heated to reflux. Hydrazine hydrate (6 ml, 12.4 mmol) was added *via* syringe and the reaction was left to reflux for 30 min. The diamine appeared as a compact white precipitate. The precipitate was filtered off and washed with $CHCl_3$. The extracts were dried over $MgSO_4$, filtered and evaporated to give a yellow oil (1.51 g, 75%).

**$^1$H-NMR**: δ 2.68 (m, 4H, N-C$H_2$- and SO-C$H_2$-), 2.53 (s, 3H, SO-C$H_3$), 1.77 (m, 2H, SO-$CH_2$-C$H_2$-), 1.56 (m, 2H, N-$CH_2$-C$H_2$-).

**$^{13}$C-NMR**: δ 54.3 (SO-$CH_2$-), 41.4 (N-$CH_2$-), 38.4 (SO-$CH_3$), 32.4 (N-$CH_2$-$CH_2$-), 19.9 (SO-$CH_2$-$CH_2$-).

**IR**: Strong peaks at 1000 cm$^{-1}$ (SO) and 1560 cm$^{-1}$ ($NH_2$).

## 2.2.8. Reaction of 1-methylsulfinylbutanamine (IV) with carbon disulphide.

a) Triethylamine. The amine (IV) (97 mg, 0.7 mmol) was dissolved in a methanolic solution of triethylamine (1.6ml, 2.75 M, 3.6 mmol), cooled to - 5 °C and a molar ethanolic solution of carbon disulphide (0.7 ml) was added. After standing at 0 °C for five hours the mixture was stirred for one hour at room temperature and then methanolic iodide (28 ml, 0.5 M, 14 mmol) was added dropwise under continuous stirring during one hour at room temperature. The reaction mixture was cooled at - 5 °C, the precipitate filtered off, and after evaporation, the residue was extracted with ether : chloroform 4 : 1. The extracts were washed several times with a $Na_2S_2O_3$/NaCl saturated solution, dried over $MgSO_4$ and evaporated to give a dark oil (86 mg) containing triethylamine, iodide and sulforaphane.

b) Dimethylethylamine. The amine (174 mg, 2 mmol), a methanolic solution of dimethylethylamine (3.7 ml, 10 mmol), a molar ethanolic solution of carbon disulfide (2 ml) and methanolic iodide (8 ml, 4 mmol) were mixed as described in a). The residue (100 mg) was chromatographed using $CH_2Cl_2$ : MeOH (0 %, 5 %, 10 %) as eluent system. The product (20 mg, 6 %) was obtained as an impure dark oil containing sulforaphane.

## 2.2.9. Synthesis of isothiocyanate-4-methylsulfinyl-butane; sulforaphane (V)

4-Methylsulfinylbutanamine (847 mg, 6.3 mmol) was dissolved in bromobenzene (30 ml), *N,N'*- thiocarbonyldiimidazole (1.36 g, 6.9 mol) was added and the mixture was heated at 140°C for one h. Analyses of a 5 ml sample ($^1$H- and $^{13}$C-NMR) indicated presence of final product and an intermediate (30%). Hydrochloric acid (3 ml, 3.2 mmol) was added dropwise and left reacting for 30 min until formation of a precipitate. After drying over $MgSO_4$, filtration and removal of solvent *in vacuo*, a pale brown oil (464 mg, 42%) was obtained, containing 10% of an intermediary. The crude product dissolved in $CHCl_3$: MeOH (5%) was chromatographed on flash silica gel eluting with $CHCl_3$: MeOH (0-5%). Two fractions were separated, the pure product as a yellow oil (131.5 mg, 28%) and a second fraction of product containing 10% of intermediate (136.4 mg).

**$^1$H-NMR**: δ 3.59 (t, 2H, *J*=6.0 Hz, SCN-C*H*$_2$-), 2.72 (m, 2H, SO-C*H*$_2$-), 2.59 (s, 3H, SO-C*H*$_3$), 1.90 (m, 4H, SCN-CH$_2$-(C*H*$_2$)$_2$-).

**$^{13}$C-NMR**: δ 53.3 (SO-*C*H$_2$-), 44.5 (SCN-*C*H$_2$-), 38.6 (SO-*C*H$_3$). 28.9 (SCN-CH$_2$-*C*H$_2$-), 20.0 (SO-CH$_2$-*C*H$_2$-).

**IR**: Strong peaks at 1000 cm$^{-1}$ (SO) and 2100 and 2180 cm$^{-1}$ (N=C=S).

**HRMS**: calculated for $C_6H_{11}NOS_2$ ($M^+$+H) 178.041, found 178.036.

**$^1$H-NMR** (intermediate (**Va**)): δ 8.50, 7.76, 7.03 (3s, 3H, imidazole), 3.81 (t, 2H, *J* = 6.35 Hz, N-C*H*$_2$-), 2.75 (m, 2H, SO-C*H*$_2$-), 2.60 (s, 3H, SO-C*H*$_3$), 1.92 (m, 4H, N-CH$_2$-(C*H*$_2$)$_2$-).

**$^{13}$C-NMR** (intermediate (**Va**)): δ 137.5, 129.4, 118.1 (imidazole), 53.3 (SO-*C*H$_2$-), 45.2 (N-*C*H$_2$-), 38.3 (SO-*C*H$_3$), 26.5 (N-CH$_2$-*C*H$_2$-), 20.0 (SO-CH$_2$-*C*H$_2$-).

## *2.3. Results and discussion*

### 2.3.1. Synthesis of 1-bromo-3-phthalimidopropane (Ia)

The first step in the alternative approach (Figure 2.b.), involves synthesis of 1-bromo-3-phthalimidopropane (**Ia**) from 3-amino-1-bromopropane and phthalic anhydride. 3-Amino-1-bromo-propane was chosen as starting material since bromide is a good leaving group for subsequent displacement by sulfur. 1-Bromo-4-phthalimidopropane (**Ia**) was thus prepared according to Bose *et al,* (1958). The amino group was protected by reaction with one equivalent of phthalic anhydride to give 1-bromo-3-phthalimidopropane (Figure 2.d.). This compound was clearly identified by its $^1$H NMR spectrum. This showed key aromatic peaks at 7.82 ppm (2H) and 7.70 ppm (2H), a 2H triplet ($J$ = 6.8 Hz) at 3.81 ppm for the methylene next to the phthalimide N, and a 2H triplet at 3.39 ppm ($J$ = 6.7 Hz) for the bromomethylene.

### 2.3.2. Attempted synthesis of 1-methylsulphinyl-4-phthalimidobutane (III) *via* the methylsulphinyl carbanion.

2.2.2.(a). Corey and Chaykovsky (1965) reported the formation of methylsulphinyl carbanion using sodium hydride (NaH), a strong base, to deprotonate dimethylsulfoxide (DMSO). Methylsulphinyl carbanion reacted with several aromatic compounds (benzophenone, benzaldehyde) to give the appropiate sulfoxides in good yields. However, our attempts to react the methylsulphinyl carbanion with 1-bromo-4-phthalimidopropane were unsuccessful (Figure 2.e.).

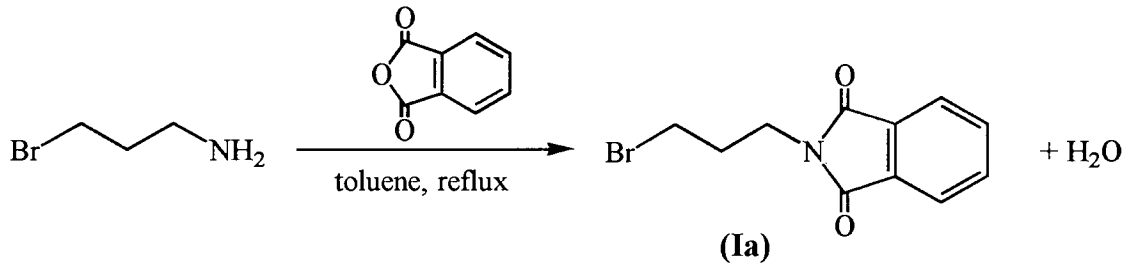



Figure 2.d. Synthesis of 1-bromo-3-phthalimidopropane (**Ia**).

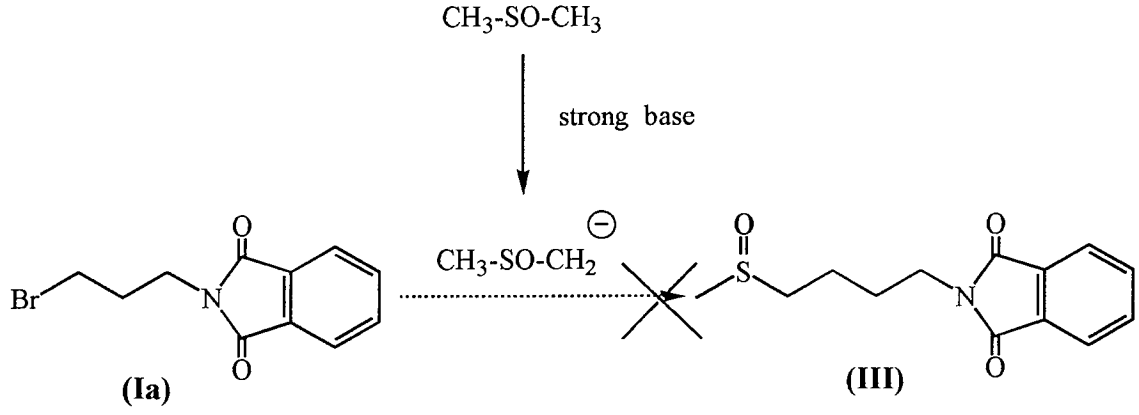



Figure 2.e. The attempted synthesis of 1-methylsulphinyl-4-phthalimidobutane (**III**) using the methylsulphinyl carbanion

The reaction between NaH and DMSO and 1-bromo-3-phthalimidopropane failed both in DMSO and tetrahydrofuran (THF) as solvents. Hexamethylphosphoroustriamine ($[(CH_3)_2N]_3PO$) (HMPA) (0.5-1 equivalent), a hydrocarbon ionisation promoter (through ion solvation stabilization), was added to the reaction mixture. However, the product could be neither isolated or identified, although TLC showed the formation of a material that was not starting material.

This led to investigation of a range of strong bases for the formation of methylsulphinyl carbanion using different solvents and temperatures, but none of these attempts were successful. (Table 2.a. summarises the different conditions used).

2.2.2.(b). A possible explanation for the failure of the reaction described in Figure 2.e. could be that the bromide of the group Br-$CH_2$- might not be displaced by the methylsulphinyl carbanion. The iodide derivate is potentially a more reactive group, since I is bigger and more electropositive than Br, and is therefore a better leaving group and might facilitate the reaction.

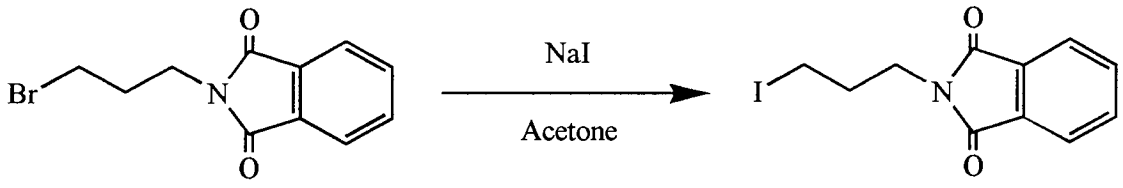
Thus, to prepare the desired iodo derivative, 1-bromo-3-phthalimidopropane was reacted with an excess of sodium iodide in dry acetone (Figure 2.f.). The product, 1-iodo-3-phthalimidopropane (**Ib**) was purified by flash chromatography using hexane : ethylether (1 : 1) as eluent and washed with a saturated solution of $Na_2S_2O_3$ to eliminate the excess iodine (83 % yield). The product was identified by $^1$H-NMR, since bromine is more electronegative than I, Br-$CH_2$- resonates at higher field (3.39 ppm, triplet, 2H) than I-$CH_2$- (3.15 ppm). The methylene group next to the phthalimide N (triplet, 2H) for the iodine derivative resonates at 3.76 ppm ( as compared with the bromide analogue at 3.81 ppm).

1-Iodo-3-phthalimidopropane was reacted with methylsulfinyl carbanion generated from DMSO under the same conditions described previously (section 2.2.2a. and table 2.a.), but no reaction occurred.

Table 2.a. Different conditions used for the reaction between DMSO and 1-bromo-3-phthalimidopropane.

| BASE | SOLVENT | $T_1$[a] | $T_2$[b] |
|---|---|---|---|
| NaH | DMSO | 70-75 °C | 50-60 °C |
| | THF | 70-75 °C | room temp.<br>reflux |
| | THF + HMPA | 70-75 °C | room temp.<br>reflux |
| | MeOH | 70-75 °C | reflux |
| LDA, LHMDS | THF + HMPA | -78 °C<br>0 °C<br>0 °C | 0 °C<br>room temp.<br>reflux |
| n-BuLi | THF | -78 °C | 0 °C |

[a] $T_1$ : temperature for the formation of methylsulphinyl carbanion
[b] $T_2$ : temperature of addition of 1-bromo-3-phthalimidopropane.
LDA: lithium diisopropylamide.
LHMDS: lithium hexamethyldisilazide.




**(Ia)** **(Ib)**

Figure 2.f. Preparation of 1-iodo-3-phthalimidopropane (**Ib**).

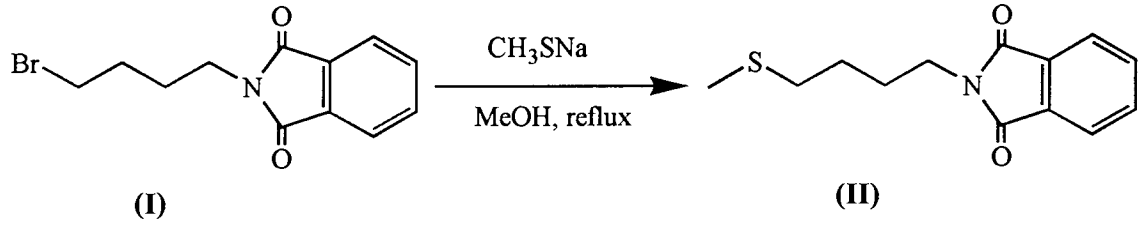
2.2.2.(c) In view of the failure to obtain 1-methylsulphinyl-4-phthalimidobutane (**III**) from either 1-bromo-3-phthalimidopropane (**Ia**) or 1-iodo-3-phthalimidopropane by reaction with methylsulfinyl carbanion, a second approach based on the methods of Schmid and Karrer (1948) and Zhang *et al*, (1992) was attempted. Thus, 1-bromo-4-phthalimidobutane (**I**) would be transformed into 1-methylthio-4-phthalimidobutane (**II**), oxidised and then converted to the free amine (**IV**), which was further transformed to the isothiocyanate, sulforaphane (**V**) (Figure 2.a.).

## 2.3.3. Synthesis of 1-methylthio-4-phthalimidobutane (II)

1-Methylthio-4-phthalimidobutane was synthesised according to the method of Zhang *et al,* (1992) by the route shown in Figure 2.g.. One equivalent of 1-bromo-4-phthalimidobutane was reacted with an excess of sodium thiomethoxide in methanol under reflux to yield the product (90 %), which was purified by flash column chromatography (Still *et al,* 1978). This compound was clearly identified by its $^{1}$H-NMR, showing a singlet at 2.06 (3 H) for the methyl next to the sulfide group (S-C$H_3$) and a 2 H triplet at 2.51 ($J$ = 7.12 Hz) for the methylene next to the sulfide (S-C$H_2$-). $^{13}$C-NMR showed the methyl group at 15.3 ppm and the methylene group (S-C$H_2$-) at 33.4 ppm.

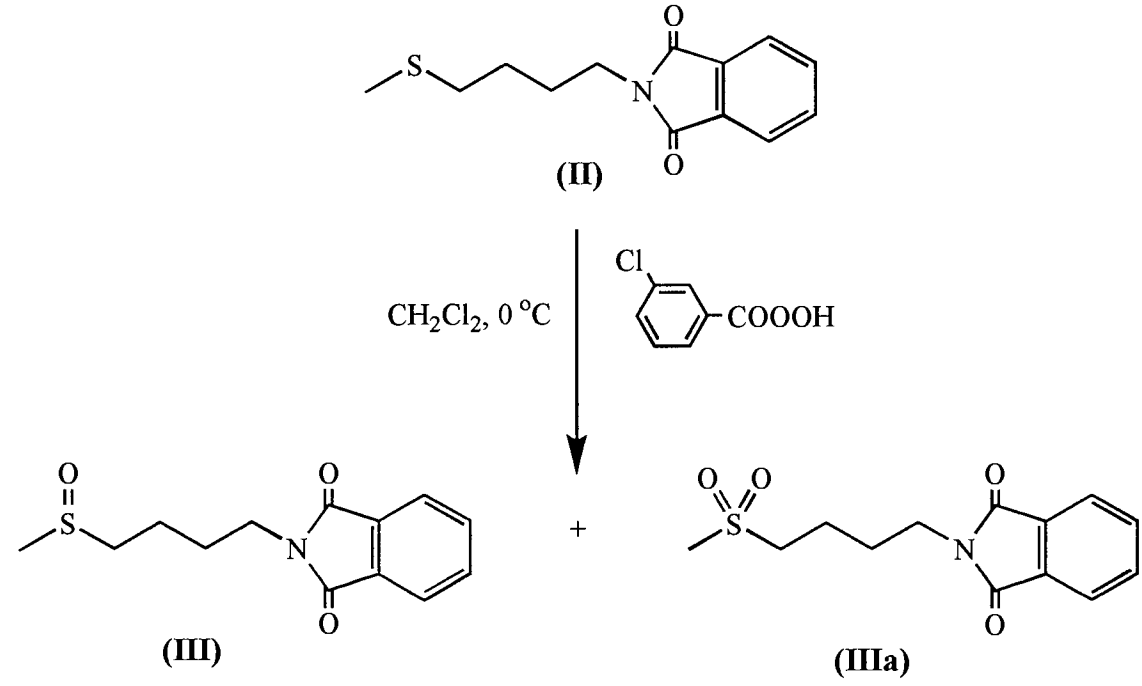
## 2.3.4. Oxidation of 1-methylthio-4-phthalimidobutane

1-Methylthio-4-phthalimidobutane (**II**) was oxidised to 1-methylsulfinyl-4-phthalimidobutane (**III**) by *m*-chloroperoxybenzoic acid (*m*-CPBA) at 0 °C in dry dichloromethane. Commercial *m*-CPBA contains from 10 % to 30 % of water. The addition of one equivalent of commercial *m*-CPBA to the sulfide gives as a main product the sulfoxide, but containing up to 20 % of the original sulfide, which would be difficult to separate due to the similarity of the structures. The addition of an excess of *m*-CPBA (1.5 equivalents of commercial acid) gave a mixture of sulfoxide (1-methylsulfinyl-4-phthalimidobutane) (**III**) and sulfone (1-methylsulfonyl-4-phthalimidobutane) (**IIIa**) as a result of over-oxidation (Figure 2.h.).




(I) (II)

Figure 2.g. Synthesis of 1-methylthio-4-phthalimidobutane (II)




(II) (III) (IIIa)

Figure 2.h. Oxidation of 1-methylthio-4-phthalimidobutane

The sulfone was identified by $^1$H-NMR since the hydrogens of the methyl group ($CH_3$-$SO_2$-) and the methylene group (-$SO_2$-$CH_2$-) gave resonance at higher frequency (2.9 and 3.09 respectively) than the sulfide analogues (2.56 and 2.74 respectively).

In order to avoid formation of sulfone (**IIIa**), the reaction was followed by gas chromatography using a SE-°30 column placed in a Pye Unicam-Series 204 gas chromatograph. The reaction was controlled qualitatively by following the decrease of sulfide (Figure 2.i.). Retention times of sulfide, sulfoxide and sulfone were 8, 2 and 20 min respectively. *m*-CPBA (0 °C) was gradually added in portions to the reaction mixture and left to react for 30 min-1 h. The sulfoxide (**III**) was separated from the excess *m*-CPBA by flash chromatography and obtained as a pure white solid in good yields (70-85 %).

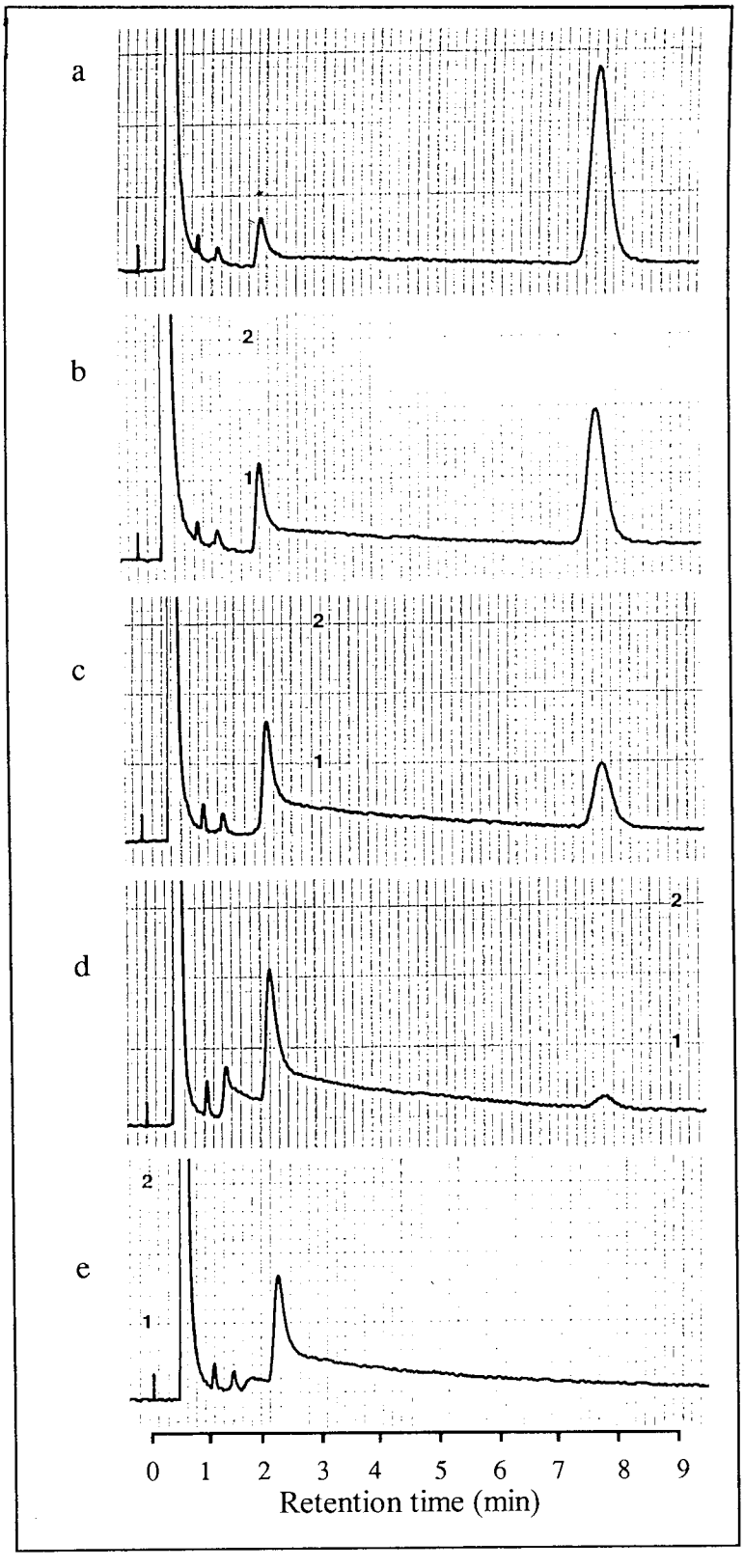



Figure 2.i. Oxidation of 1-methylthio-4-phthalimidobutane (**II**) followed by gas chromatography

Retention times are 8 min for the sulfide (**II**) and 2 min for the sulfoxide (**III**). Samples were analysed after 30 min-1 h reaction of the sulfide (II) with a) 0.5 equivalent *m*-CPBA, b) 0.3 eq., c) 0.2 eq., d) 0.1 eq. and e) 0.05 eq.

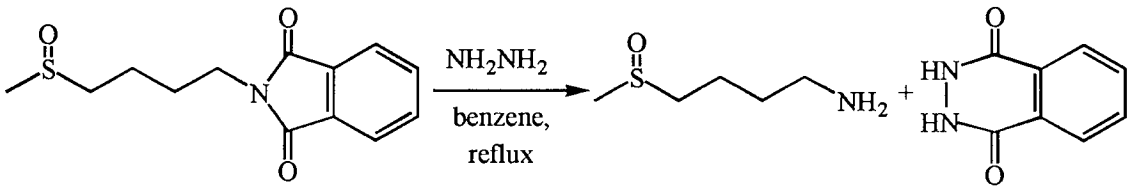
## 2.3.5. Synthesis of 1-methylsulfinylbutylamine

The amine (**IV**) 1-methylsulfinylbutylamine was obtained by deprotection of the phthalimide derivate (**III**) (Figure 2.j.). The method was based on that of Nicolaou *et al*, (1992) but hydrazine hydrate was used instead of methylhydrazine. One equivalent of (1-methylsulfinyl-4-phthalimidobutane) (**III**) was reacted with one equivalent of hydrazine hydrate for 30 min in benzene under reflux to give the free amine and phthaloyl hydrazide (white precipitate). 1-Methylsulfinylbutylamine (**IV**) was obtained in good yields (75-92%) after extraction from the reaction mixture in chloroform. The product was identified easily by $^{1}$H-NMR (lack of aromatic hydrogens and carbons) and by IR (1560 $cm^{-1}$ for the free amine compared to 1700 $cm^{-1}$ for the protected amine (CO-N-CO)). Key $^{1}$H-NMR data included a singlet at 2.53 for the methyl group (S-C$H_3$), and peaks at 2.68 for the $CH_2$ next to the sulfoxide and next to the amino group. $^{13}$C-NMR showed the *S*-methyl at 38.4 ppm, and IR showed both SO (1000 $cm^{-1}$) and $NH_2$ (1560 $cm^{-1}$) stretches.

## 2.3.6. Attempted synthesis of sulforaphane by reaction of the amine with carbon disulfide and iodine.

Numerous methods for obtaining isothiocyanates have been described and reported since the early fourties (Patai, 1977). One of the most popular methods is the reaction of an amine with carbon disulfide and iodine, via formation of a thiocarbamate intermediate.

Schmid and Karrer (1948) synthesised sulforaphane in 58 % yield from the amine (IV) by reaction with carbon disulfide for 3 days following addition of iodine and sodium methylate. Balenovic *et al.* (1966) used carbon disulfide, iodine and triethylamine ($Et_3N$) to obtain 4,4'-dimethylsulphinylbutylisothiocyanate from the appropiate amine in 68 % yield.




(III) (IV)

Figure 2.j. Synthesis of amine 1-methylsulfinylbutylamine (**IV**)

**DPT** **TDI**

Figure 2.k. Structures of 2-pyridylthionocarbonate (DPT) and *N, N'*-thiocarbonyldiimidazole (TDI).

The method of Balenovic *et al*, (1966) was employed in an attempt to synthesise sulforaphane. One equivalent of carbon disulfide was added to the amine (**IV**) dissolved in a methanolic solution of ($Et_3N$) at -5 °C and left to stand for 5 h at 0 °C and then stirred for one hour at room temperature. Iodine was added gradually over one hour at room temperature. A small amount of sulforaphane was extracted, but this contained $Et_3N$ and iodine which were the main components of the product obtained.

The same reaction was repeated using dimethylethylamine ($Me_2EtN$) instead of $Et_3N$, since it has a lower boiling point and would be easier to remove (by evaporation). Sulforaphane was obtained as an impure dark oil in 6 % yield.

## 2.3.7. Synthesis of sulforaphane.

Isothiocyanates have been obtained in good yields by reacting an amine precursor with di-2-pyridylthionocarbonate (DPT) (Kim and Yi, 1985) or *N*, *N'*-thiocarbonyldiimidazole (TDI) (Staab and Walker, 1962) (Figure 2.k.). Both reagents are commercially available, TDI being considerably cheaper than DPT.

4-Methylsulphinylbutylamine (**IV**) was reacted with one equivalent of TDI for one hour at room temperature. A sample of the reaction mixture was analysed by NMR prior to purification, showing formation of the isothiocyanate (**V**) sulforaphane, but also that an intermediate (**Va**) was present as the main product (60-70 %). Figure 2.l. shows the mechanism of reaction between the amine and TDI and formation of the intermediate which is transformed in a reversible reaction into sulforaphane. $^1$H-NMR spectra of the intermediate (**Va**) and of sulforaphane (**V**) are shown in figure 2.m. Key peaks for the intermediate include imidazole singlet peaks at 8.50 (1H), 7.76 (1H), and 7.03 (1H) (absent in the spectrum of sulforaphane) and a 2H triplet ($J$ = 6.35 Hz) at 3.81 for the methylene next to the imidazole N, whereas for sulforaphane the methylene next to the isothiocyanate N appears at 3.59 (triplet, $J$ = 6.0 Hz).

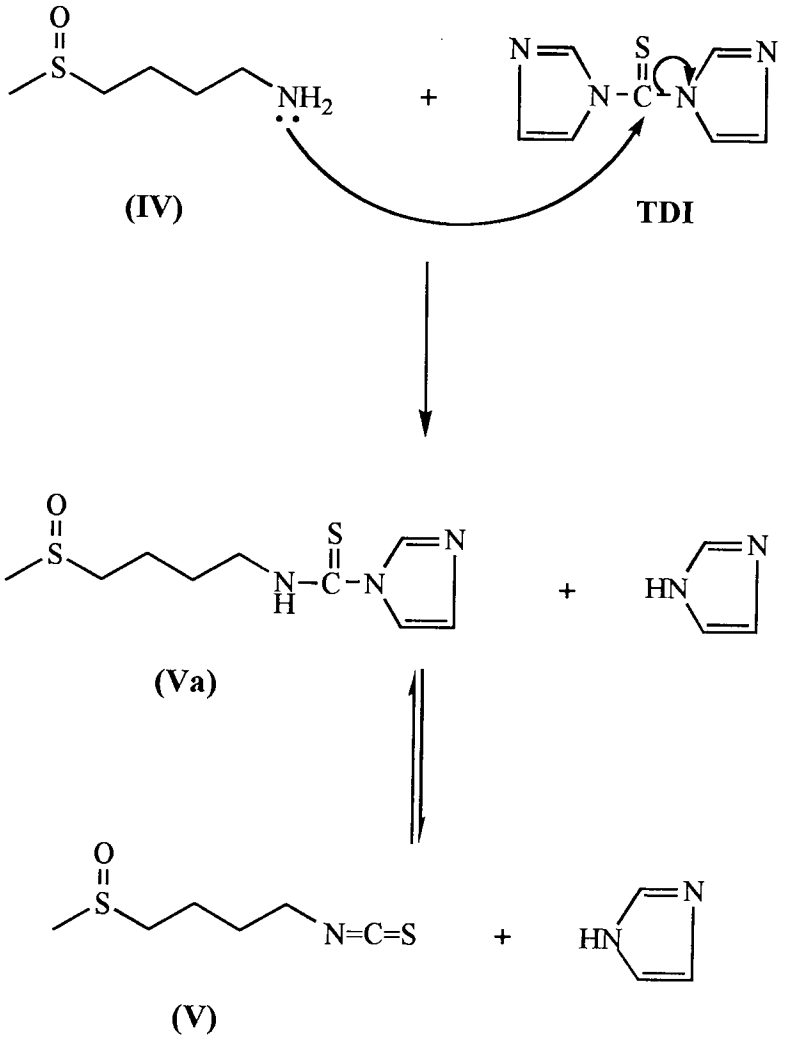



Figure 2.1. Mechanism of reaction between 4-methylsulphinylbutylamine (**IV**) and TDI.

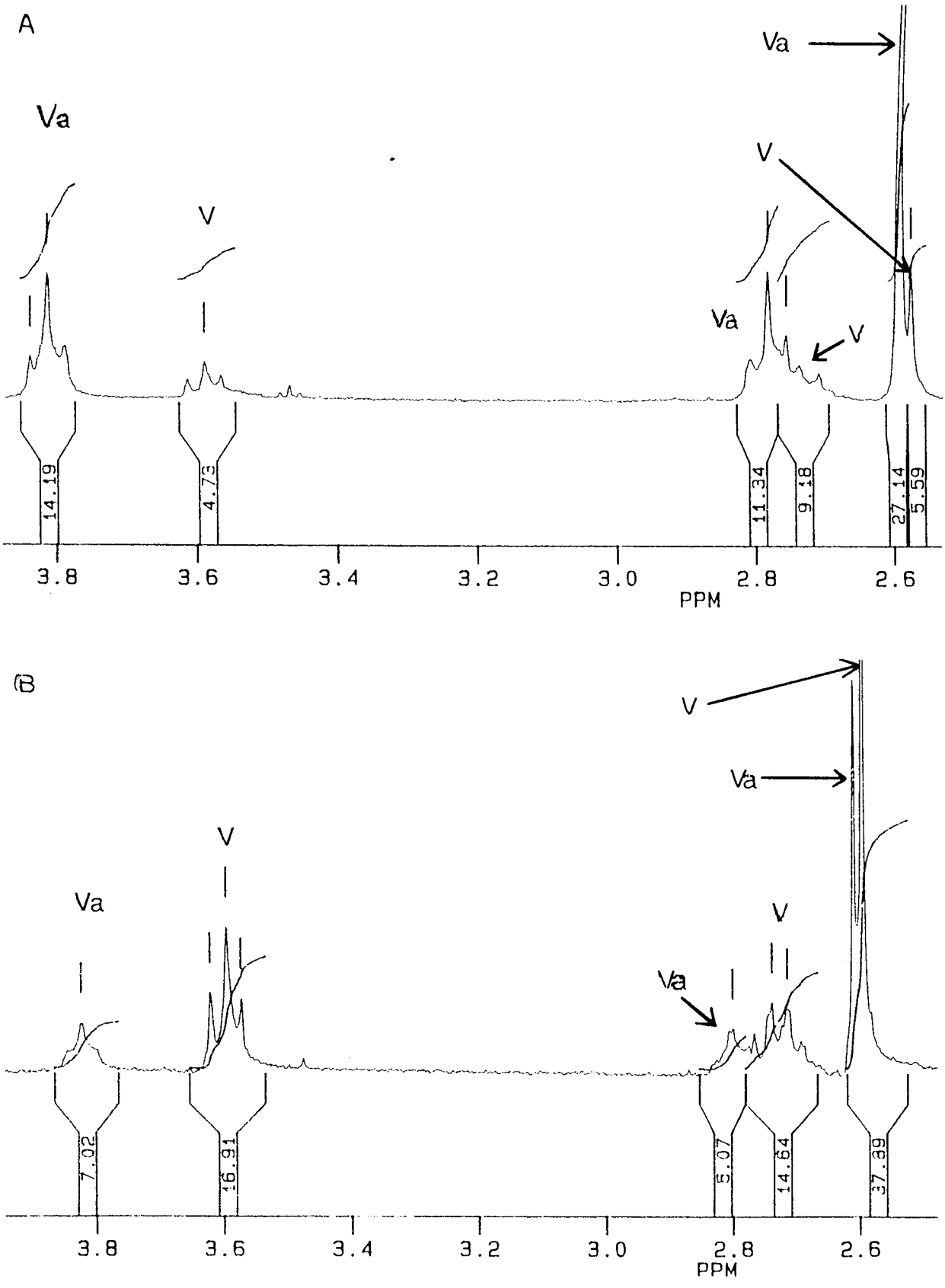



Figure 2.m. $^{1}$H-NMR spectra of sulforaphane (**V**) and intermediate (**Va**)

A) 30 % of sulforaphane was present when the reaction was carried out at room temperature. B) 70 % of sulforaphane was produced when the reaction was carried out at 140 °C.

In order to displace the reaction towards the formation of sulforaphane the reaction was attempted at higher temperatures (140 °C) and different solvents were also used (toluene and acetonitrile), but with little effect. Up to 70 % of sulforaphane was obtained by reaction under reflux in bromobenzene (compared to Baxter *et al*, 1956) (see Figure 2.m.).

The decomposition of the thiourea intermediate (**Va**) proceeded to near completion (90-95 % of product) by addition of HCl as acidic catalyst (Patai, 1977). The reaction starts by protonation (from HCl) of the imidazole nitrogen, followed by two electron migration from nitrogen ($(CH_2)_4$-N) to form a double bond between N and C=S, leading to elimination of imidazole, which is a stable leaving group due to its protonation. The displaced imidazole is then protonated by HCl to form its hydrochloride.(Figure 2.n.). The mechanism thus explains why the conversion of intermediate Va to sulforaphane is facilitated by acid conditions.

Sulforaphane was obtained after evaporation of solvent as a pale brown oil in 42 % yield, along with 10 % of the intermediate. Pure sulforaphane (> 95 %)was obtained after purification by flash chromatography using $CHCl_3$ : MeOH (0-5 %) as eluent. The overall yield of the synthesis was 15 %.

Sulforaphane was identified by NMR (see above for key peaks), IR (strong peaks at 2100 and 2180 $cm^{-1}$ corresponding to the isothiocyanate moiety) and mass spectrosocopy. The values agreed with those reported by Zhang *et al.* (1992).

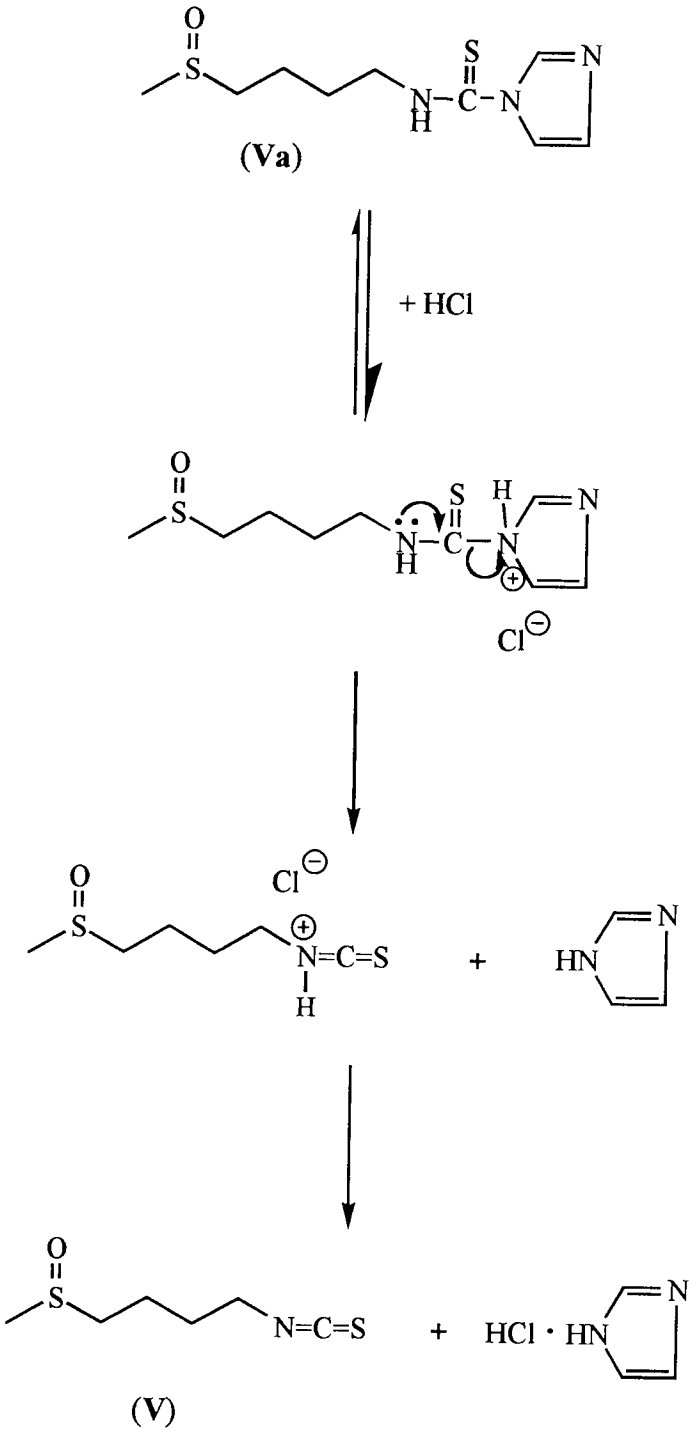



Figure 2.n. Conversion of thiourea intermediate (**Va**) to sulforaphane (**V**) by addition of HCl.

## *2.4. Conclusions*

Sulforaphane was synthesised (Figure 2.o.) in an overall yield of 15 %. Alkylation of 1-bromo-4-phthalimidobutane (**I**) with sodium thiomethoxide yielded 1-methylthio-4-phthalimidobutane (**II**). Oxidation of the sulfide group to sulfoxide (**III**) was achieved by reaction with *m*-CPBA at 0 °C in dry $CH_2Cl_2$ and was monitored by GC in order to avoid over-oxidation to the sulfone analogue. Deprotection of 1-methylsulfinyl-4-phthalimidobutane (**III**) to the amine 1-methylsulfinylbutylamine (**IV**) was accomplished by the use of hydrazine hydrate. Finally, conversion of the amine group to the isothiocyanate sulforaphane (**V**) was achieved by reaction of the amine (**IV**) with thiocarbonyldiimidazole (TDI) in a HCl catalysed mechanism. $^1$H-NMR of sulforaphane (**V**) and the different intermediates (**II** - **IV**) used for its synthesis are shown in **Figure 2.p**. Sulforaphane was identified by NMR, IR and mass spectrometry,and the values were in agrement with those reporeted previously (Zhang *et al*, 1992) (see 2.2.9. for actual values).

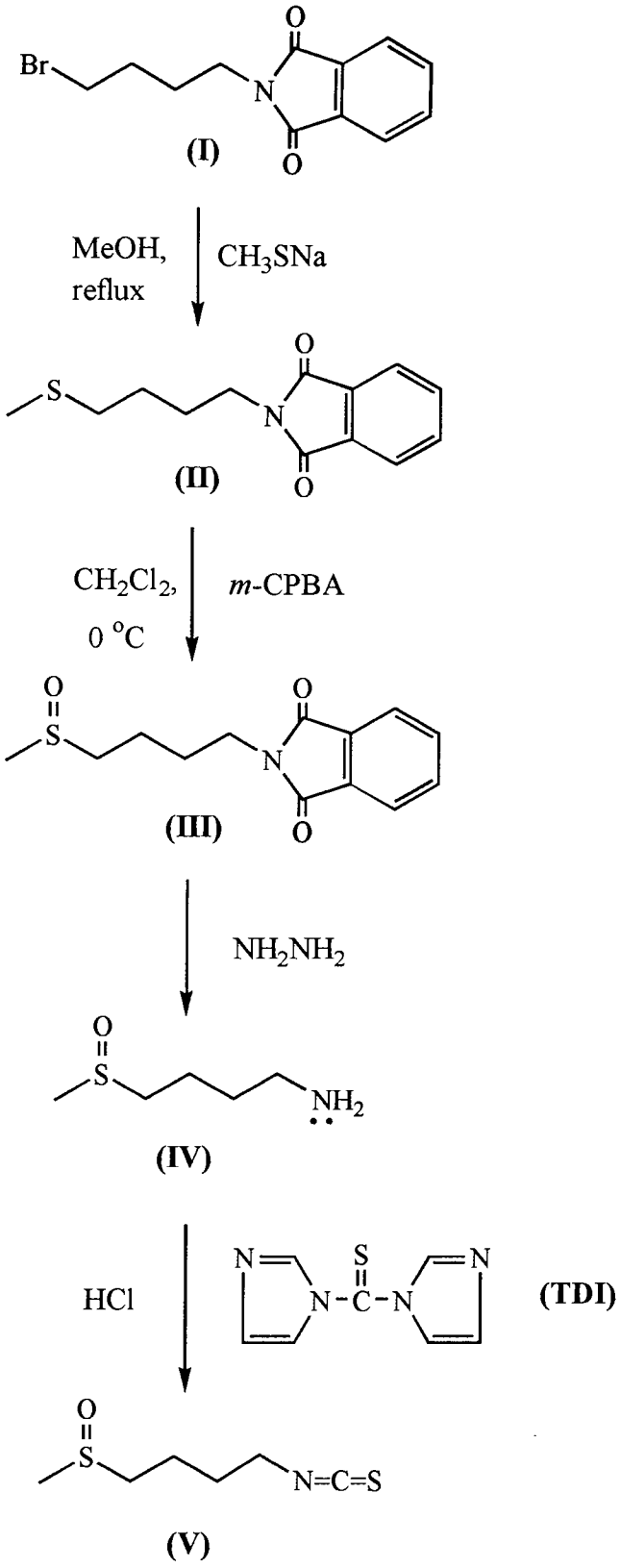
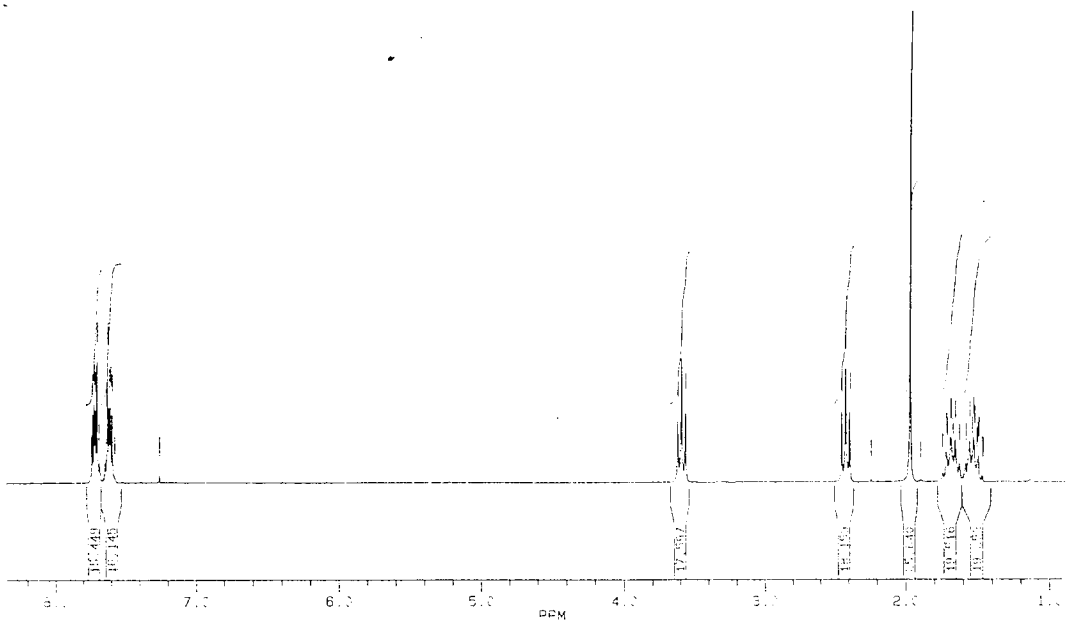
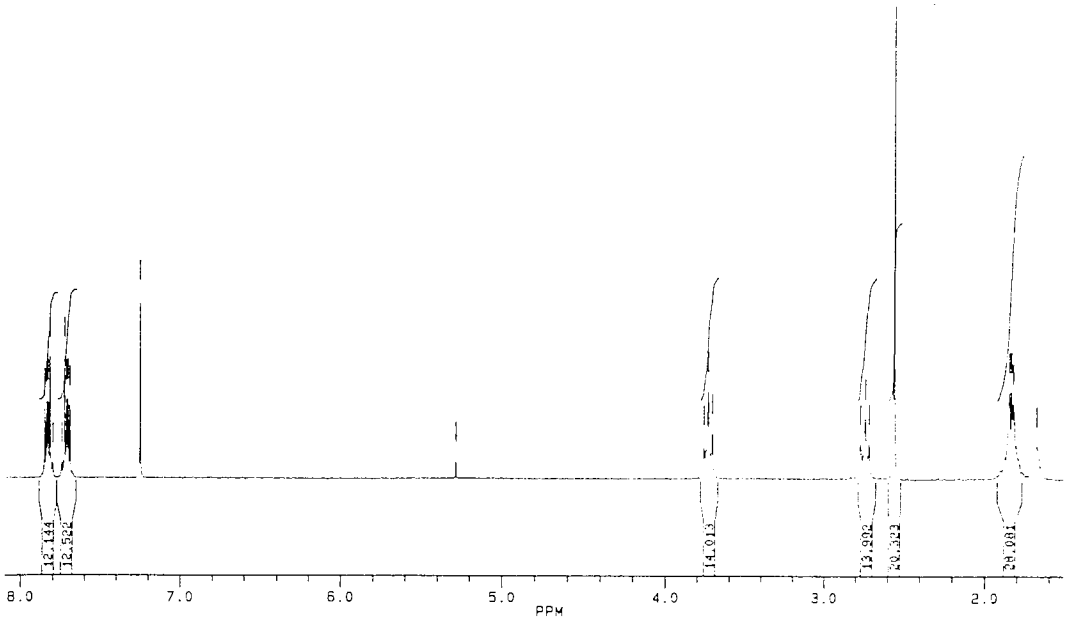



Figure 2.o. Overall synthesis of sulforaphane.

II

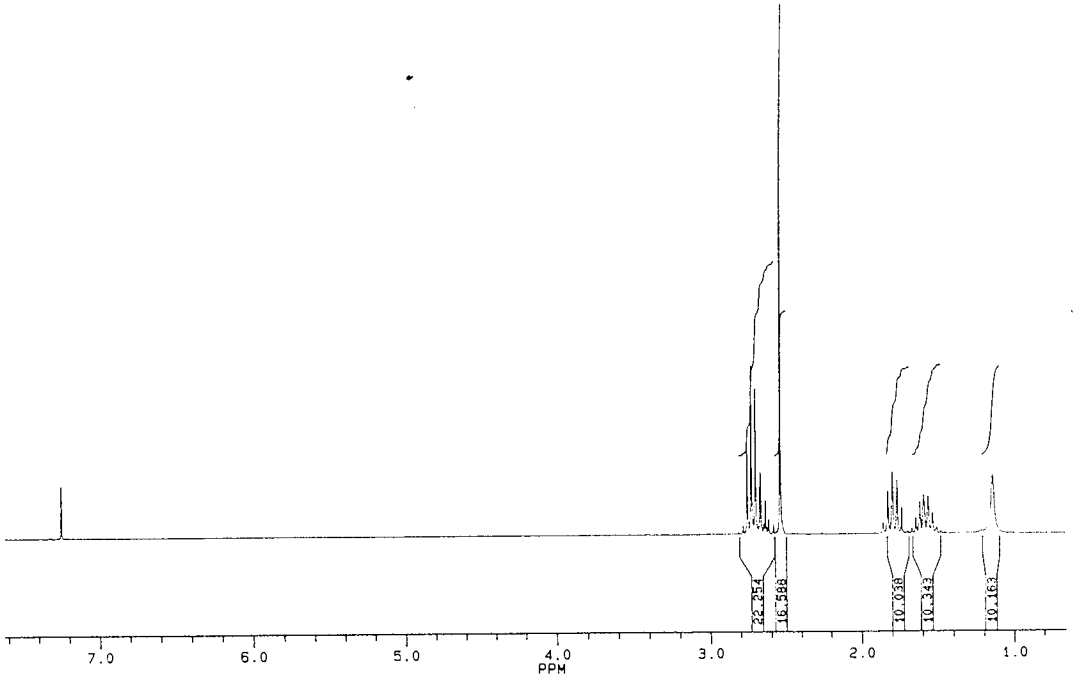



III




Cont'd.....

IV




V

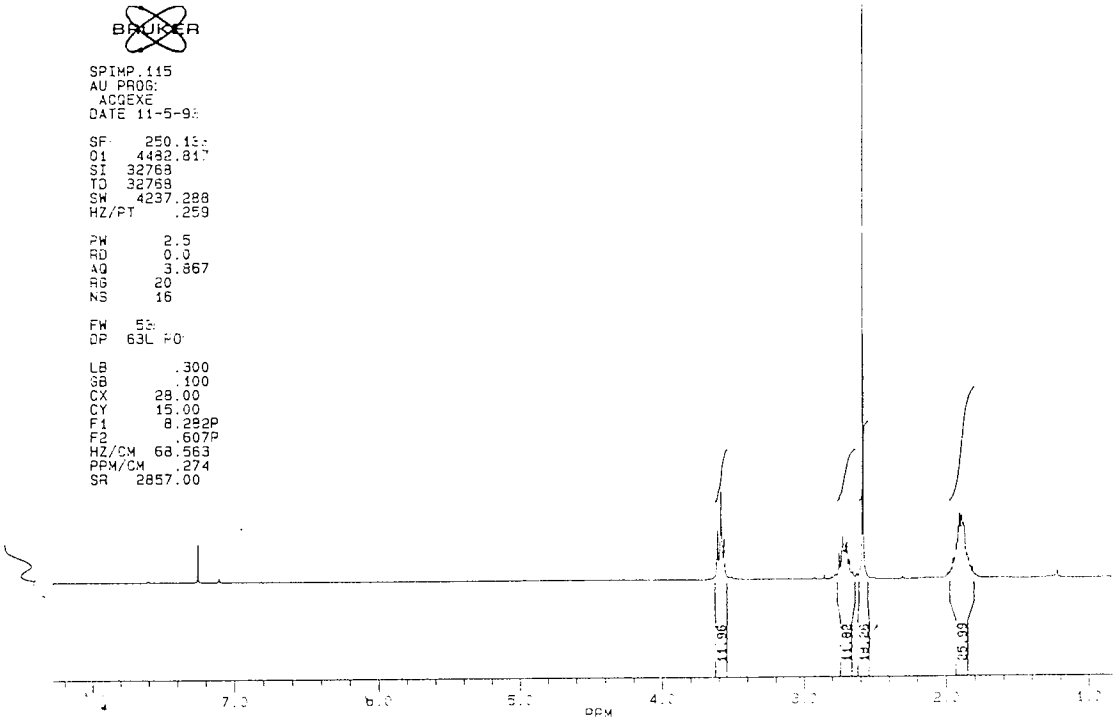



Figure 2.p. $^{1}$H-NMR spectra of yielded 1-methylthio-4-phthalimidobutane (**II**), 1-methylsulfinyl-4-phthalimidobutane (**III**), 1-methylsulfinylbutylamine (**IV**) and sulforaphane (**V**)

# *CHAPTER 3*

## *Inhibition by sulforaphane of rat CYP2E1 and CYP1A*

## *3.1. Introduction*

One of the possible mechanisms to prevent chemical carcinogenesis is by modulation of the metabolic activation of carcinogens (Wattenberg, 1983, 1992: De Flora and Ramel, 1988; and also see general introduction 1.4.3.). This may be achieved by inducing detoxification pathways or by inhibiting activation pathways. The broccoli constituent sulforaphane has been shown to induce phase II enzymes GST and QR (Zhang *et al,* 1992), and recently has also been shown to protect rats against DMBA-induced tumourigenesis (Zhang *et al,* 1994). Nevertheless, the potential of sulforaphane to inhibit the activation of carcinogens is not known.

Since cytochrome P450 enzymes play key roles in the activation of carcinogens (see section 1.3.3.), it has been suggested that their inhibition might be an indicative of potential chemoprevention (Yang *et al,* 1994). Several potential chemopreventors from brassica vegetables are thought to modulate carcinogen activation by inhibition of CYP450s. For example, the related isothiocyanate PEITC inhibits rat liver microsomal CYP2E1 (Ishizaki *et al,* 1991) and rat CYP1A2 (Guo *et al,* 1992). Other organosulfur compounds, such as diallylsulfide (DAS), diallylsulfoxide (DASO) and diallylsulfone ($DASO_2$) have also shown to inhibit rat hepatic CYP2E1 (Brady *et al,* 1991a). The ability of sulforaphane to inhibit CYP2E1 and CYP1A2 was thus studied in rat liver microsomes.

## *3.2. Methods*

Chemicals

All chemicals used were purchased from Sigma-Aldrich, unless otherwise stated.

### 3.2.1. Animal husbandry and treatments

#### 3.2.1.1. Acetone treatments

Male Sprague-Dawley rats (100 - 200 g) were obtained from Batin and Kingman Ltd. (Hull, UK), kept in 12 hour light/dark cycles and fed with Heygates Rat and Mouse Breeding Diet and water *ab libitum* (Pharmaceutical Sciences, Aston University). Treated animals were given drinking water containing acetone (1% v/v) for two weeks. Control animals were given drinking water.

#### 3.2.1.2. β-Naphthoflavone treatments

Male Sprague-Dawley rats were obtained from Harlan Ltd. (Heathfield, Sussex), kept in 12 hour light/dark cycles and fed with a Rat and Mouse Maintenance Diet (Special Diet Service, UK) and water ab libidum (BMSU, University of Birmingham). Treated animals were administered β-naphthoflavone in corn oil (80 mg/kg body weight) and control animals received corn oil.

### 3.2.2. Preparation of microsomes

Treated and control animals were killed by cervical dislocation and the livers were removed, washed in Tris buffer (50 mM, pH 7.4) KCl solution (0.154 M) and wet weighed. Livers were then cut into small pieces and homogenised in Tris buffer-KCl solution to give a 25 % homogenate. The homogenate was then centrifuged at 9000g for 20 min at 4 °C and the postmitochondrial supernatant was further centrifuged at $10^5$ g for 1 h at 4 °C in a Beckman L8-60M ultracentrifuge. The microsomal pellet was suspended in Tris buffer-KCl solution, recentrifuged at $10^5$ g for 1 h and resuspended in phosphate buffer (50 mM, pH 7.4) to give

a suspension equivalent to 0.5 g original liver wet weight/ml buffer. Microsomes were stored at -70 °C and an aliquot (1 ml) was used for protein determination.

### 3.2.3. Protein determination

Microsomal protein content was determined according to the method of Lowry *et al*, (1951).

The solutions required were:

- solution A: $Na_2CO_3$ (2 % w/v) in NaOH (0.1 M)
- solution $B_1$: $CuSO_4.5\ H_2O$ (1 % w/v)
- solution $B_2$: KNa tartrate (2 % w/v)
- solution C: this solution was prepared by addition of solution $B_1$ (1 % v/v) and solution $B_2$ (1 % v/v) in solution A (100 ml)

Microsomes were diluted 1:80 in NaOH (0.05 M). Bovine serum albumin standard curve (0 - 200 μg/ml) was prepared by diluting a stock solution (1 mg/ml) into NaOH (0.05 M). Solution C (5 ml) was added to the diluted microsomes (1 ml) and albumin standards (1 ml), mixed, and left standing at room temperature for 10 min. Folins reagent diluted in distilled water (1:1) (0.5 ml) was added to the mixture, and left for 30 min. Absorbance was measured at 750 nm.

### 3.2.4. *p*-Nitrophenol (PNP) hydroxylation assay

The *p*-nitrophenol (PNP) hydroxylation assay was based on Koop *et al*, (1989). All incubations were carried out, with duplicate samples, in 2 ml Ependorfs with shaking at 37°C. Incubations contained either control or induced rat liver microsomal preparations (200 μl), freshly prepared NADPH (1 mM) (100 μl, 10 mM), ascorbic acid (1mM) (100 μl, 10 mM), phosphate buffer (400-500 μl, 50 mM, pH 6.8), substrate PNP (0-500 μM) (100 μl, 0.5 - 5 mM), and (when required) inhibitors sulforaphane (25-250 μM) (100 μl, 0.25 - 2.5 mM), or PEITC (10 - 100 μM) (100 μl, 0.1 - 1 mM). Incubations were prewarmed at 37°C before addition of NADPH. The reaction was terminated after 30 min by addition of $HClO_4$ (0.5 ml, 0.7 M). In a preliminary study linearity of generation of 4-nitrocatechol

with time during the incubation period was established. The samples were ultracentrifuged at 13000 rpm for 5 min and then NaOH (100 µl, 10 M) was added to 1 ml of the samples. Absorbance was measured at 546 nm. A standard curve was prepared with 4-nitrocatechol (5 - 100 µM) (5 - 100 µl, 1 mM stock solution). Enzyme kinetic parameters were estimated using a enzyme kinetics computer package (Enzfit). Inhibition constant, $K_i$ (mean ± SD, n=3) was calculated from a Dixon plot (1/v vs. concentration of inhibitor).

### 3.2.5. Ethoxyresorufin deethylase (EROD) assay

The measurement of ethoxyresorufin deethylase (EROD) activity was based on the methods of Pohl and Fouts, (1980) and Burke *et al,* (1985) with several modifications. Preliminary studies were performed to determine optimal assay conditions (microsomal protein concentration and incubation time). All incubations were carried out in triplicate in centrifuge tubes wrapped in tin foil to avoid decomposition of ethoxyresorufin and resorufin by light. Microsomes (25 µg) (12.5 µl, 2 mg/ml), ethoxyresorufin (0-5 µM) (0-500 µl, 0.01 mM), magnesium chloride (12.5 mM) (12.5 µl, 1M), sulforaphane when required (100 µM) (50 µl, 2 mM) and Tris buffer (0.05 M, pH: 7.4) were preincubated for 2 min. Freshly prepared NADPH (1 mM) (10 µl, 10 mM) was added and then mixtures were incubated for 5 min at 37 °C in a shaking water bath. The reaction was stopped by addition of cold methanol (1 ml) and the tubes were left on ice for 10 min to allow protein precipitation. Samples were centrifuged for 10 min at 2000 rpm and fluorescence of supernatants was measured at 530 nm excitation / 585 nm emission in a Perkin Elmer Luminescence Spectrometer LS50B. A standard curve was prepared with resorufin (0 - 0.6 µM) (0 -120 µl, 5 µM). Enzyme kinetic parameters were estimated using a enzyme kinetics computer package (Grafit).

### 3.2.6. Spectral determination of cytochrome P450

Spectral determination of cytochrome P450 was based on the method of Omura and Sato (1964). Control rat liver microsomes (100 µl, 4 mg protein/ml), suforaphane (100 µM) (50 µl, 2 mM) and NADPH (1 mM) (10 µl, 10 mM) when required were incubated at 37 °C for 5 min in 1 ml of Tris buffer (0.1 M, pH 7.4) containing 20 % (v/v) glycerol and then

transferred to glass cuvettes. A reference sample was prepared containing microsomes in buffer. A baseline was recorded between 400 and 500 nm in a split-beam spectrometer (Uvikon 992 Spectrophotometer, Kontron Instruments). A few grains of sodium dithionite were added to both reference and sample cuvettes. Carbon monoxide freshly made by addition of approximately 10 ml of sulfuric acid onto sodium formate was gently bubbled into the sample cuvette for 1 min. The spectrum was then re-scanned from 400 to 500 nm.

## *3.3. Results*

### 3.3.1. Induction by acetone of PNP hydroxylase activity

The induction of PNP hydroxylase activity was studied in rat liver microsomes from rats treated with acetone (see methods 3.2.1.1.). Protein content of microsomes was determined by the Lowry method (section 3.2.3.) from a Bovine serum albumin (BSA) standard curve ($r = 0.996$) (Figure 3.a.). Microsomal protein content was 6.28 and 6.41 mg/ml for control and acetone-induced microsomes respectively.

The standard curve of 4-nitrocatechol (4-NC) (5-100 μM) is shown in Figure 3.b.. Data correlated well with a linear regression ($r = 0.997$). The effect of acetone pretreatment on 4-nitrocatechol production from PNP in rat hepatic microsomes is shown in Figure 3.c. Acetone pretreatment clearly induced PNP hydroxylase activity as shown by an increase of activity (4-NC production) in acetone-induced microsomes compared to control microsomes (Figure 3.c.). PNP hydroxylase activity in control liver microsomes was characterised by a $K_M$ of 69.52 ± 7.92 μM and a Vmax of 0.53 ± 0.02 nmol/mg/min. PNP hydroxylase activity in acetone-induced microsomes was characterised by a $K_M$ of 119.24 ± 7.61 μM and a Vmax of 1.96 ± 0.05 nmol/mg/min. Thus, acetone pretreatment resulted in 3.7-fold increase of Vmax in rat liver microsomes.

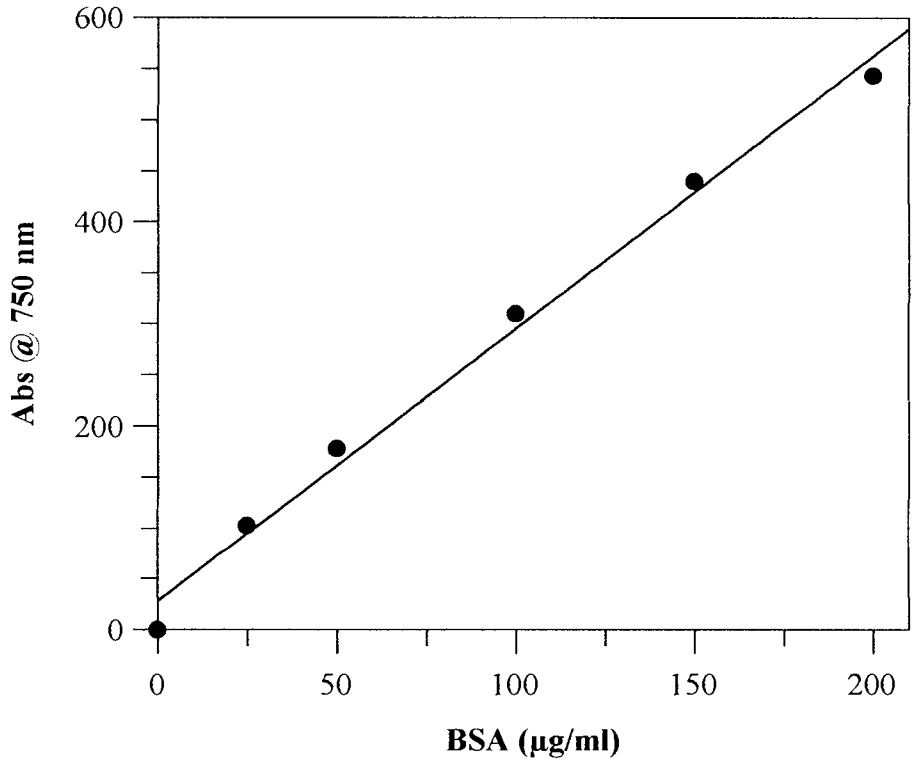



Figure 3.a. Bovine serum albumin (BSA) standard curve

Protein was determined by the Lowry method (section 3.2.3.). Values are duplicates.

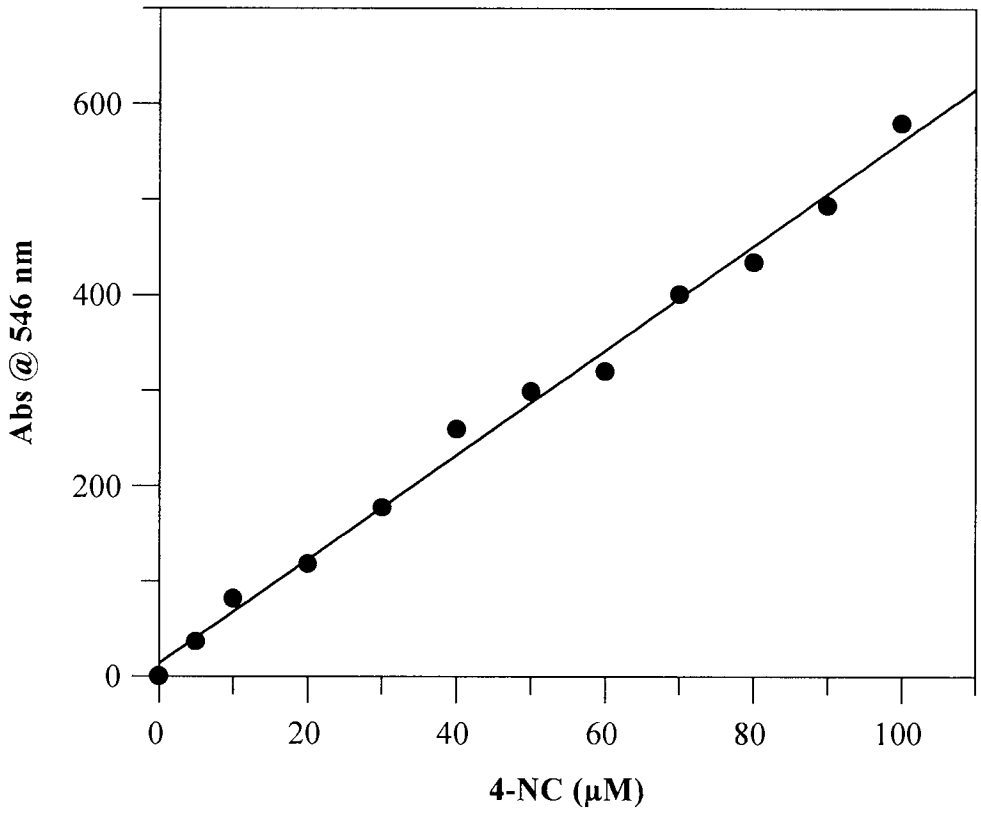



Figure 3.b. 4-Nitrocatechol (4-NC) standard curve.

Perchloric acid (0.5 ml) and NaOH (100 μl) were added to the 4-NC standards (1 ml), mixed and absorbance read at 545 nm. Values are duplicates.

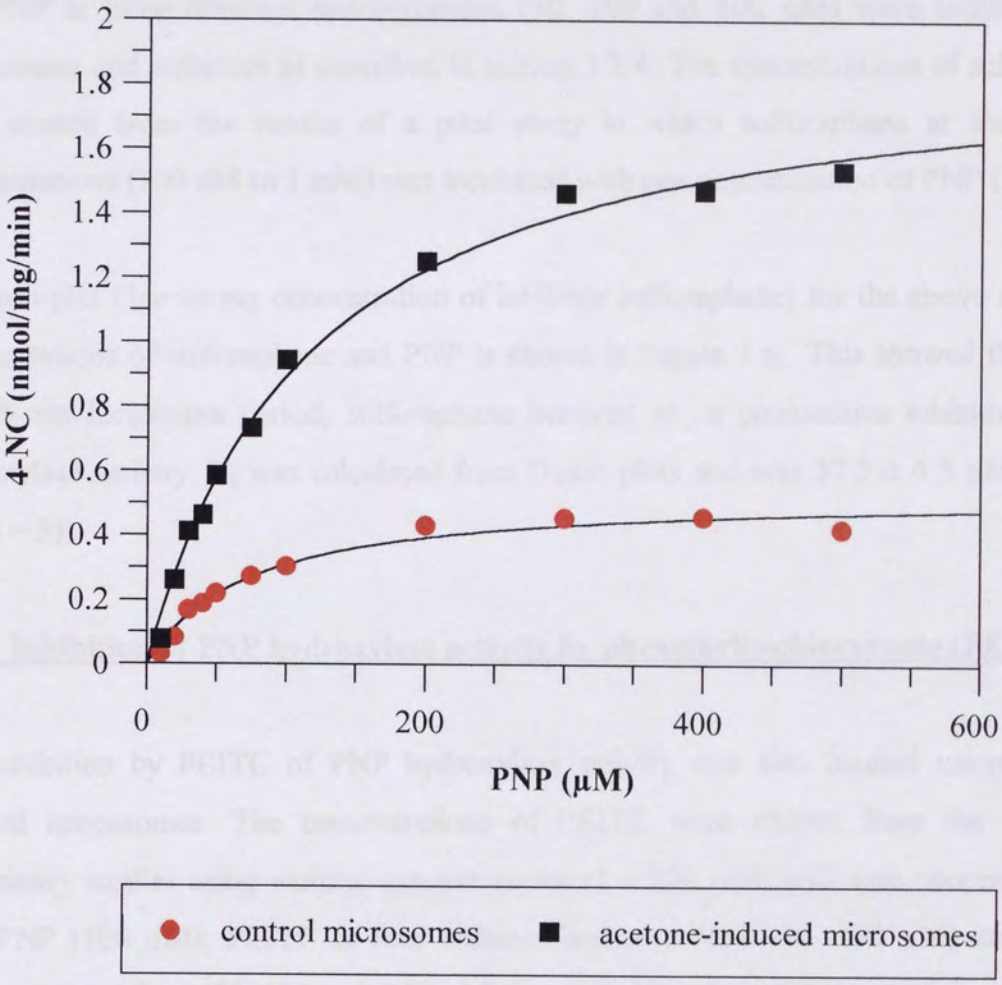



Figure 3.c. Induction by acetone pretreatment of PNP hydroxylase activity in rat liver microsomes.

Incubations were carried out at 37 °C for 30 min and the reaction was stopped by addition of perchloric acid. Absorbance was measured at 546 nm after addition of NaOH (section 3.2.4.). Values are the mean of duplicates. Acetone-induced and control microsomes were prepared from acetone treated and control animals respectively (3.2.1. and 3.2.2.)

### 3.3.2. Inhibition of PNP hydroxylase activity by sulforaphane

The inhibition by sulforaphane of PNP hydroxylase activity was studied using acetone-induced rat liver microsomes. Sulforaphane at four different concentrations (25 - 250 μM) and PNP at three different concentrations (50, 100 and 500 μM) were incubated with microsomes and cofactors as described in section 3.2.4. The concentrations of sulforaphane were chosen from the results of a pilot study in which sulforaphane at six different concentrations (100 nM to 1 mM) was incubated with one concentration of PNP (100 μM).

A Dixon plot (1/v versus concentration of inhibitor sulforaphane) for the above mentioned concentrations of sulforaphane and PNP is shown in Figure 3.d.. This showed that during the 30-min incubation period, sulforaphane behaved as a competitive inhibitor of PNP hydroxylase activity. $K_i$ was calculated from Dixon plots and was 37.5 ± 4.5 μM (mean ± SD, n = 3).

### 3.3.3. Inhibition of PNP hydroxylase activity by phenethylisothiocyanate (PEITC)

The inhibition by PEITC of PNP hydroxylase activity was also studied using acetone-induced microsomes. The concentrations of PEITC were chosen from the results of preliminary studies using various concentrations (1 - 100 μM) with one concentration of with PNP (100 μM). PEITC at four different concentrations (10 -100 μM) and PNP at three concentrations (50, 100 and 250 μM) were used for the inhibition studies.

A Dixon plot for PEITC and PNP is shown in Figure 3.e.. PEITC was a competitive inhibitor of PNP hydroxylase activity during the 30-min incubation period and a $K_i$ of 13.1 ± 1.1 μM (mean ± SD, n = 3) was obtained from Dixon plots.

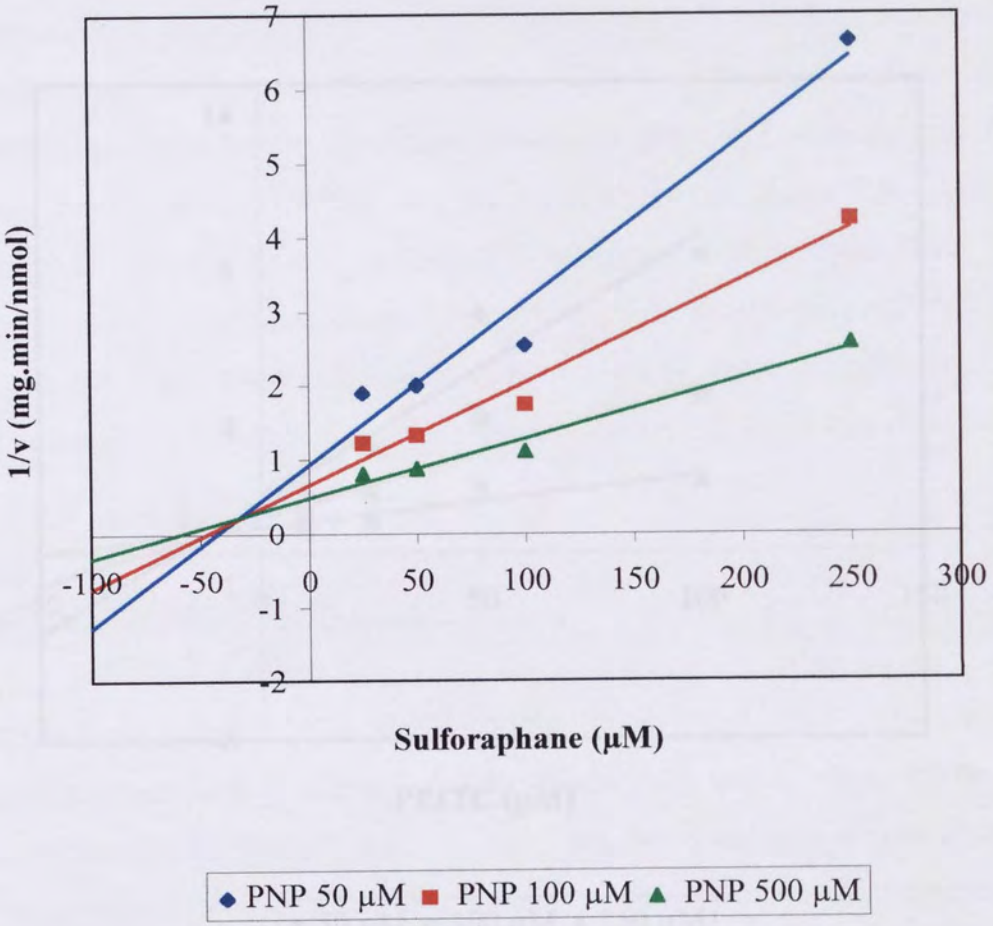



Figure 3.d. Inhibition by sulforaphane of PNP hydroxylase activity in acetone-induced rat liver microsomes (Dixon plot).

Sulforaphane (50-250 µM), PNP (50-500 µM), acetone-induced microsomes (200 µl) and cofactors were incubated for 30 min at 37 °C (see methods 3.2.4.). Absorbance was measured at 546 nm after protein precipitation and addition of NaOH. Values are means from duplicate analyses. $K_i$ was calculated as mean ± SD, n=3.

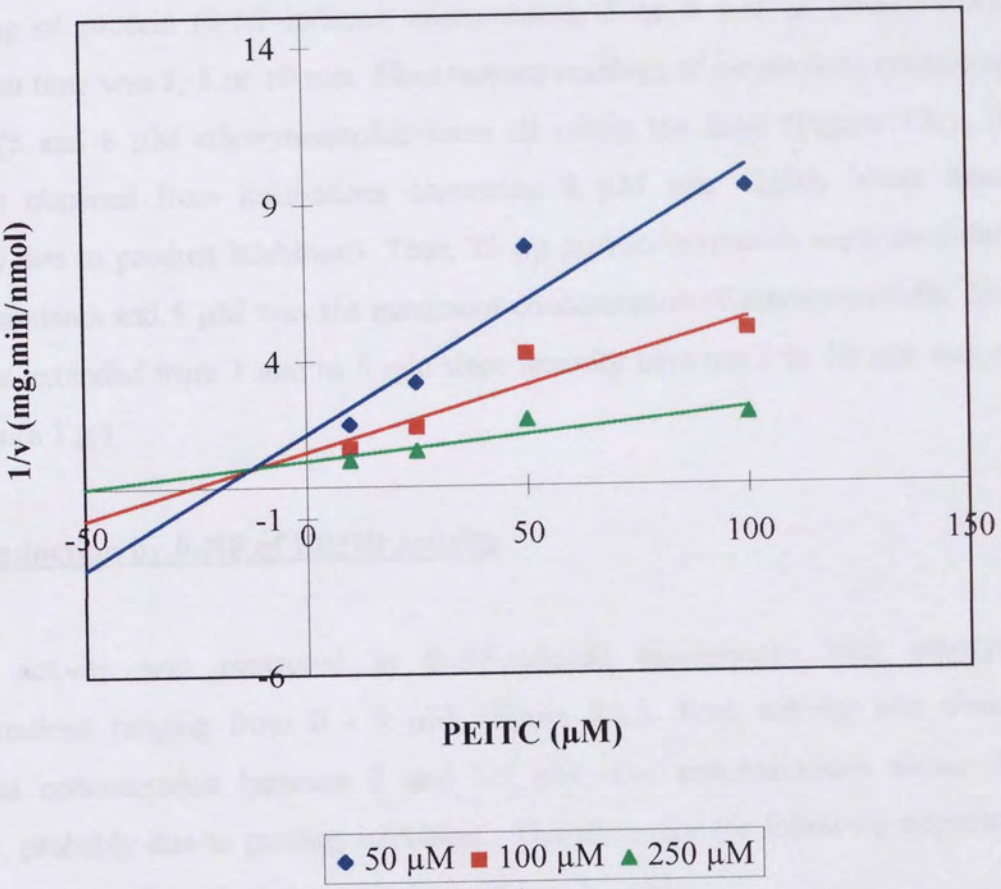



Figure 3.e. Inhibition by PEITC of PNP hydroxylase activity in acetone-induced rat liver microsomes (Dixon plot)

Phenethylisothiocyanate (PEITC) (10 - 100 µM), PNP (50 -250 µM), acetone-induced microsomes (200 µl) and cofactors were incubated for 30 min at 37 °C (see methods 3.2.4.). Values are means from duplicate analyses and $K_i$ was calculated as mean ± SD, n=3.

### 3.3.4. Determination of EROD activity assay conditions.

EROD activity was calculated from a resorufin standard curve (Figure 3.f.) which correlated well with a linear fit (r = 0.999). Microsomal protein used in the incubations containing control microsomes (0.1 mg/incubation) was too high when ß-NF-induced microsomes were used. EROD activity was measured in several incubations containing 25 or 50 µg of protein (ß-NF-induced microsomes), 5 or 8 µM of ethoxyresorufin and incubation time was 3, 5 or 10 min. Fluorescence readings of incubations containing 25 µg protein (5 and 8 µM ethoxyresorufin) were all within the scale (Figure 3.h.). However resorufin obtained from incubations containing 8 µM was slightly lower than 5 µM (possibly due to product inhibition). Thus, 25 µg protein/incubation were used throughout the experiments and 5 µM was the maximum concentration of ethoxyresorufin. Incubation time was extended from 3 min to 5 min since linearity between 3 to 10 min was observed (see Figure 3.g.)

### 3.3.5. Induction by ß-NF of EROD activity

EROD activity was measured in β-NF-induced microsomes with ethoxyresorufin concentrations ranging from 0 - 5 µM (Figure 3.h.). Peak activity was observed for substrate concentration between 2 and 2.5 µM, and concentrations above decreased activity, probably due to product inhibition. Therefore, for the following experiments, the highest concentration of ethoxyresorufin used was 2.5 µM.

The effect of ß-NF pretreatment on resorufin production in rat hepatic microsomes is shown in Figure 3.h. An induction of EROD activity was observed for ß-NF-induced microsomes compared to control microsomes. EROD activity in control liver microsomes was characterised by a $K_M$ of 0.23 ± 0.06 µM and a Vmax of 0.35 ± 0.03 nmol/mg/min, whereas ß-NF-induced microsomes gave a $K_M$ of 0.81 ± 0.14 µM and a Vmax of 6.8 ± 0.5 nmol/mg/min (ethoxyresorufin 0-2.5 µM). Thus, pretreatment of rats with ß-NF resulted in 19.4-fold increase of Vmax in liver microsomes.

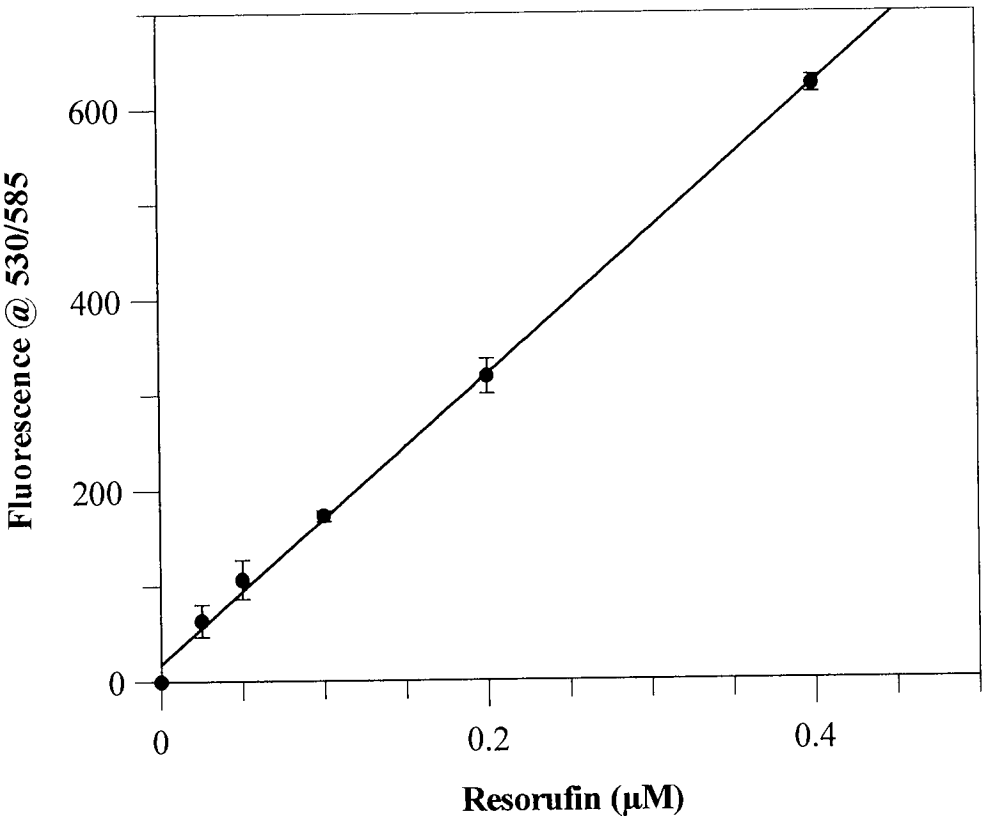



Figure 3.f. Resorufin standard curve

Ethanol (1 ml) was added to resorufin standards (1 ml), left standing on ice for 10 min, and fluorescence was measured at 530 nm excitation / 585 nm emission. Values are the mean ± SD, n=3.

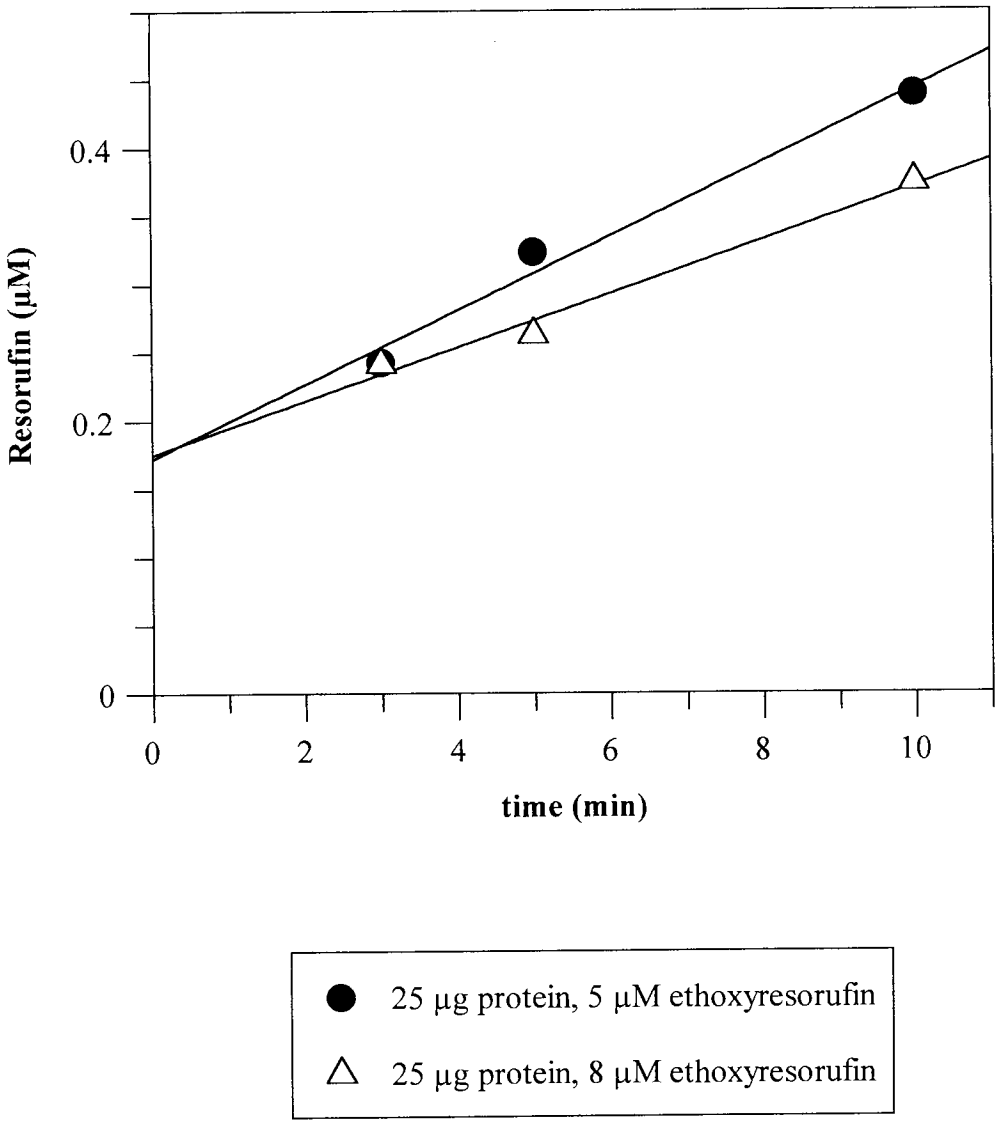



Figure 3.g. Effect of protein concentration, time and ethoxyresorufin concentration on EROD activity in ß-NF-induced rat liver microsomes.

Ethoxyresorufin (5 and 8 µM), ß-NF-induced rat liver microsomes (25 and 50 µg protein) and cofactors were incubated for 3, 5, and 10 min at 37 °C. After protein precipitation (see methods 3.2.5.), fluorescence was measured at 530 / 585 nm. Values are from duplicate analyses.

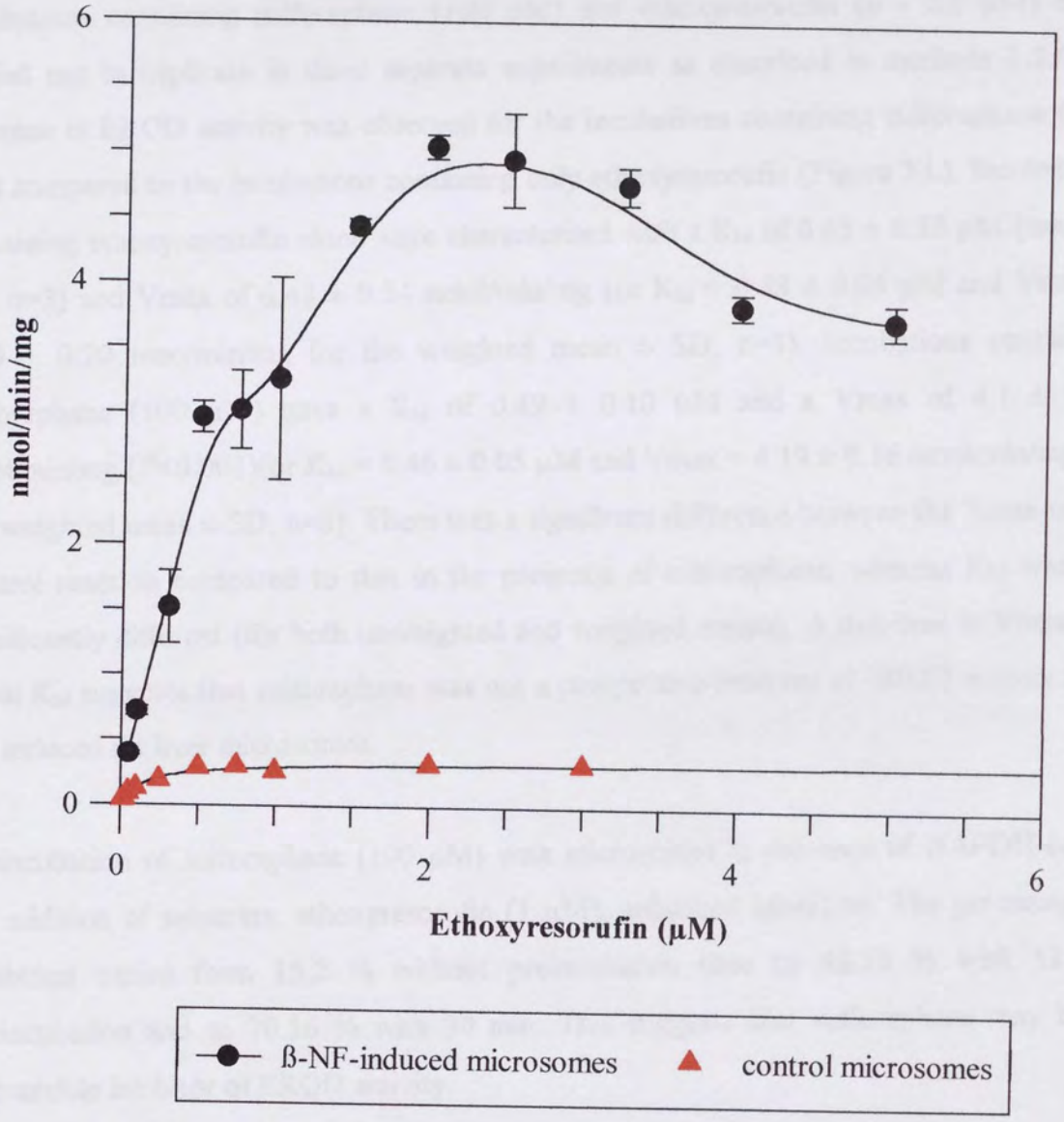



Figure 3.h. EROD activity in ß-NF-induced and control rat liver microsomes

Incubations containing 25 µg or 0.1 mg of protein for ß-NF-induced or control microsomes respectively, were carried out at 37 °C for 5 min and the reaction was stopped by addition of methanol. Fluorescence was measured at 530/585 nm. Values are the mean ± SD, n=3 for ß-NF microsomes and the mean of n=2 for control incubations. Km for ß-NF-induced microsomes was calculated excluding ethoxyresorufin concentrations of 3, 4 and 5 µM.

### 3.3.6. Inhibition of EROD activity by sulforaphane

The inhibition of EROD activity by sulforaphane was studied in ß-NF-induced microsomes. Incubations containing sulforaphane (100 µM) and ethoxyresorufin (0 - 2.5 µM) were carried out in triplicate in three separate experiments as described in methods 3.2.5. A decrease in EROD activity was observed for the incubations containing sulforaphane (100 µM) compared to the incubations containing only ethoxyresorufin (Figure 3.i.). Incubations containing ethoxyresorufin alone were characterised with a $K_M$ of 0.65 ± 0.32 µM (mean ± SD, n=3) and Vmax of 6.48 ± 0.54 nmol/min/mg (or $K_M$ = 0.48 ± 0.04 µM and Vmax = 6.40 ± 0.20 nmo/min/mg for the weighted mean ± SD, n=3). Incubations containing sulforaphane (100 µM) gave a $K_M$ of 0.49 ± 0.10 µM and a Vmax of 4.1 ± 0.56 nmol/min/mg ($P$<0.001)(or $K_M$ = 0.46 ± 0.05 µM and Vmax = 4.19 ± 0.16 nmol/min/mg for the weighted mean ± SD, n=3). There was a significant difference between the Vmax of the control reaction compared to that in the presence of sulforaphane, whereas $K_M$ was not significantly different (for both unweighted and weighted means). A decrease in Vmax and equal $K_M$ suggests that sulforaphane was not a competitive inhibitor of EROD activity in ß-NF induced rat liver microsomes.

Preincubation of sulforaphane (100 µM) with microsomes in presence of NAPDH before the addition of substrate, ethoxyresorufin (1 µM), enhanced inhibition. The percentage of inhibition varied form 15.2 % without preincubation time to 42.75 % with 12 min preincubation and to 70.36 % with 30 min. This suggests that sulforaphane may be an irreversible inhibitor of EROD activity.

### 3.3.7. Effect of sulforaphane on cytochrome P450 spectra

The ability of sulforaphane to destroy the heme iron atom was assessed by measurement of cytochrome P450 spectra. The presence of sulforaphane (with or without NADPH) in the incubations did not change the spectra from that of control rat liver microsomes (Figure 3.j.). Absorbance at 450 nm (representative of functional CYP450) and absorbance at 420 nm (representative of inactive CYP450) did not vary for incubations containing sulforaphane compared to the control incubations.

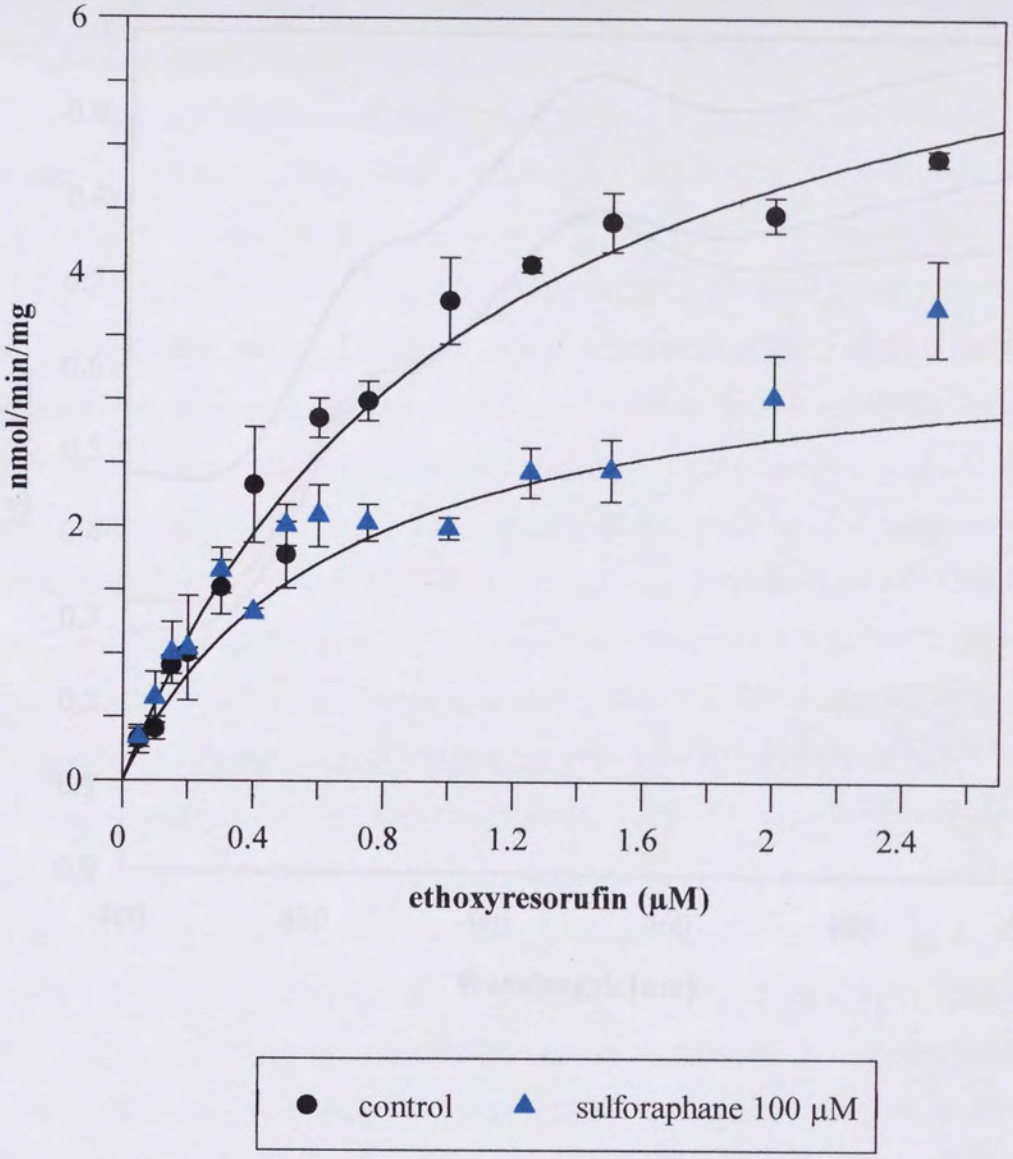



Figure 3.i. Inhibition of EROD activity by sulforaphane in ß-NF-induced rat liver microsomes

ß-NF-induced microsomes with or without sulforaphane (100 µM) were incubated for 5 min at 37 °C and EROD activity was measured as described in methods 3.2.5. Values are the mean ± SD, n=3. Km and Vmax were calculated as the mean ± SD of three separate experiments.

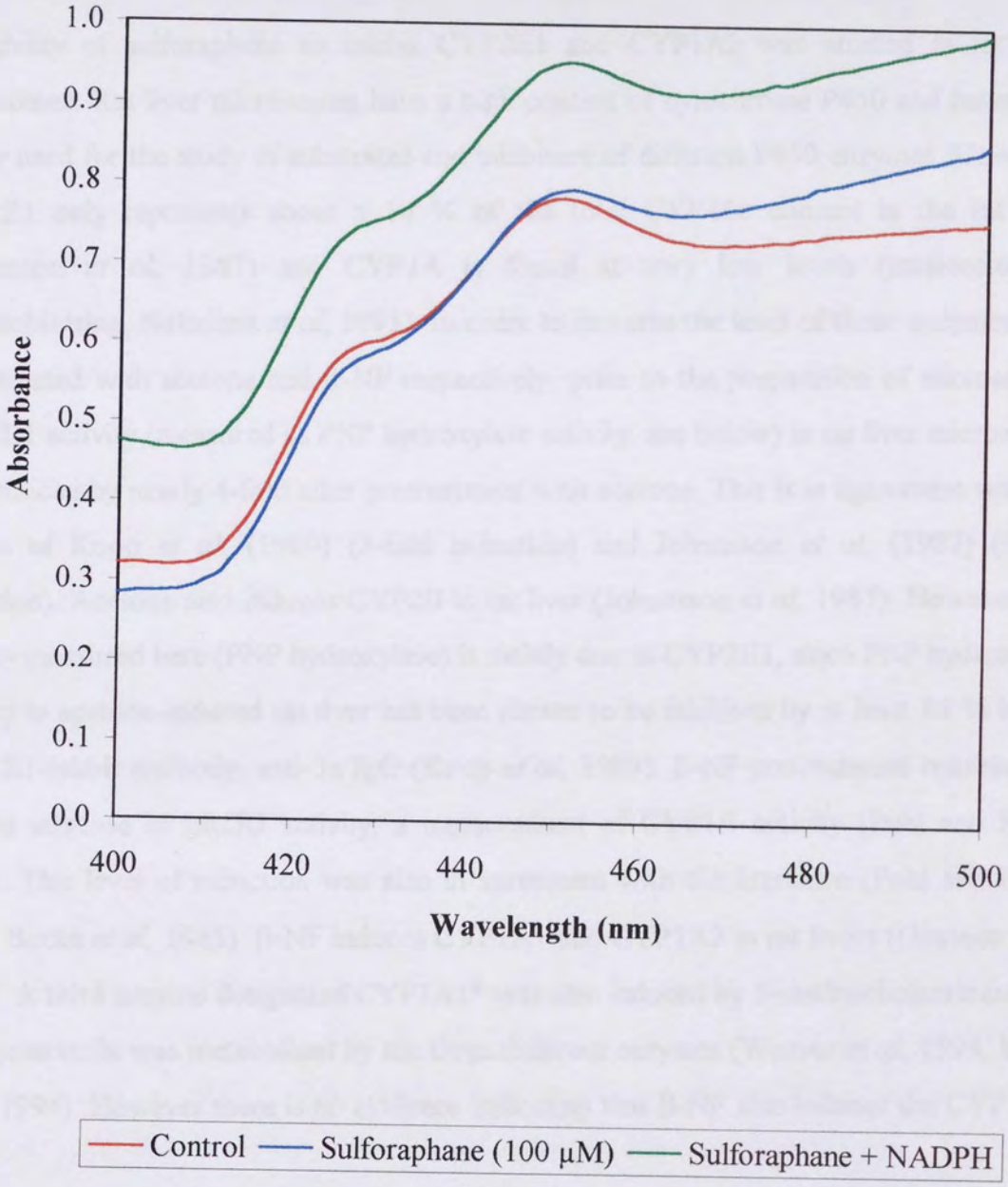



Figure 3.j. Cytochrome P450 spectrum in control rat liver microsomes with or without sulforaphane

Microsomes were incubated with or without sulforaphane (100 μM) and NADPH at 37 °C for 5 min. Spectrophotometric determination of cytochrome P450 was as described in methods 3.2.6.

## *3.1. Discussion*

The ability of sulforaphane to inhibit CYP2E1 and CYP1A2 was studied in rat liver microsomes. Rat liver microsomes have a high content of cytochrome P450 and have been widely used for the study of substrates and inhibitors of different P450 enzymes. However, CYP2E1 only represents about a 10 % of the total CYP450 content in the rat liver (Johansson *et al*, 1987) and CYP1A is found at very low levels (undetected by immunoblotting, Nakajima *et al*, 1993). In order to increase the level of these enzymes, rats were treated with acetone and β-NF respectively, prior to the preparation of microsomes. CYP2E1 activity (measured as PNP hydroxylase activity, see below) in rat liver microsomes was induced by nearly 4-fold after pretreatment with acetone. This is in agreement with the studies of Koop *et al*, (1989) (3-fold induction) and Johansson *et al*, (1987) (9-fold induction). Acetone also induces CYP2B in rat liver (Johansson *et al*, 1987). However, the activity measured here (PNP hydroxylase) is mainly due to CYP2E1, since PNP hydroxylase activity in acetone-induced rat liver has been shown to be inhibited by at least 85 % by the CYP2E1-rabbit antibody, anti-3a IgG (Koop *et al*, 1989). β-NF pretreatment resulted in a 19-fold increase in EROD activity, a measurement of CYP1A activity (Pohl and Fouts, 1980). This level of induction was also in agreement with the literature (Pohl and Fouts, 1980, Burke *et al*, 1985). β-NF induces CYP1A1 and CYP1A2 in rat livers (Oinonen *et al*, 1994). A third enzyme designated CYP1A1* was also induced by 3-methylcholanthrene and ethoxyresorufin was metabolised by the three different enzymes (Weaver *et al*, 1994, Burke *et al*, 1994). However there is no evidence indicating that β-NF also induces the CYP1A1* form.

The different extent of enzyme induction by acetone and β-NF (4- and 19-fold respectively) might be explained in terms of the mechanisms of induction. Acetone has shown to induce CYP2E1 enzyme mainly due to a decrease in protein degradation rate (Song *et al*, 1989). On the other hand, the induction of CYP1A1 and CYP1A2 by β-NF is a result of an increase of mRNA and protein levels (Oinonen *et al*, 1994), and is accompanied by an induction of QR. β-NF binds the Ah receptor via XRE (see section 1.3.4.1); CYP1A1 contains many XRE sites in the upstream region, thus explaining the high levels of induction by this agent.

Sulforaphane inhibited both PNP hydroxylase and EROD activity (indicators of CYP2E1 and CYP1A activities respectively (see above)) in acetone- and β-NF-induced microsomes respectively. Sulforaphane was a competitive inhibitor of CYP2E1, whereas for CYP1A a competitive inhibition was not observed. CYP1A activity also decreased markedly with increasing preincubation time, which indicates that sulforaphane is an irreversible inhibitor. For comparison, the inhibitory potential of PEITC towards CYP2E1 was also studied. PEITC was also a competitive inhibitor of PNP hydroxylase activity, but more potent than sulforaphane ($K_i$ 3-fold lower). The inhibitory potential of PEITC correlated with previous studies, although smaller values for the $K_i$ were reported, which may be attributed to the measurement of different indicators of CYP2E1 activity (NDMA-demethylase activity) (Ishizaki *et al*, 1990). The moderate inhibitory effect of sulforaphane compared to that of PEITC is in agreement with the recent work of Jiao *et al*, (1996). A comparison of the inhibitory potential of sulforaphane between these studies is difficult, since the latter authors calculated the $IC_{50}$ values (approximately 100 μM). Furthermore, CYP2E1 activity was measured as *N*-nitrosodimethylamine demethylation activity in liver microsomes from untreated rats, and incubation times were also different. Another important point is that the sulforaphane used in this work was synthesised as the racemic mixture (*R, S*) whereas sulforaphane used by Jiao *et al*, (1996) was the naturally occurring *R* stereoisoform. It is known that stereoisomery can be an important factor in metabolism ( Caldwell, 1996), and thus the racemic mixture may have a different inhibitory effect than the *R* isoform. The lower inhibitory potential of *R*-sulforaphane compared to arylalkylisothiocyanates observed by Jiao *et al*, (1996) might be related to the presence of the arylgroup. PEITC and related isothiocyanates also decreased EROD activity in rat and mouse liver and lung (Guo *et al*, 1992, 1993). This inhibition was mixed, with a competitive and a non-competitive component. Recently, PEITC was also reported to inhibit human CYP1A2 in a competitive and suicide (irreversible) manner (Smith *et al*, 1996). Sulforaphane also showed to inhibit irreversible CYP1A (possibly both CYP1A1 and CYP1A2 since the EROD assay measures the activity of both enzymes, see above), although the competitive component was not seen here. CYP2E1 metabolises chemicals of small molecular weight (Yang *et al*, 1990). This supports the competitive effect observed for sulforaphane, a relatively small molecule. On the other hand, CYP1A is known to metabolise planar molecules of relatively high

molecular weight (Lewis *et al,* 1993, 1994). Considering sulforaphane structure, it is therefore unlikely that it would be a metabolite of CYP1A, thus supporting the non-competitive inhibition observed here.

Sulfur containing compounds are known for their capacity to inhibit CYP450 (De Matteis, 1978, Murray and Reidy, 1990). Compounds such as carbon disulfide, the garlic component DAS, disulfiram, diethyldithiocarbamate and isothiocyanates such as PEITC have been shown to inhibit particularly CYP2E1 and some of them also inhibited other isoforms such as CYP1A2. Most of these compounds appear to exert an irreversible inhibition, including PEITC (Ishizaki *et al,* 1990, Smith *et al,* 1996) or oltipraz, also a sulfur compound which appears to be a competitive and irreversible inhibitor of CYP1A2 and CYP3A4 (Langouët *et al,* 1995). The inactivation of CYP450 by organosulfurs can occur by several mechanisms: loss of heme moiety, covalent binding to the apoprotein, or a combination of both (De Matteis, 1978). It has been suggested that the inactivation by organosulfurs is probably due to binding with the apoprotein and possibly after they are oxidatively activated by the enzyme (Ishizaki *et al,* 1990, Jiao *et al,* 1994). It is thus likely that the competitive inhibition of sulforaphane towards CYP2E1 might be accompanied by an irreversible component, as it was observed for CYP1A inhibition. CYP450 contains many residues with groups such as amino, hydroxy or thiol (cysteine) (see Tan *et al.,* 1997 for CYP2E1), it is likely that sulforaphane might react with some of these groups. The inability of sulforaphane to reduce heme protein in rat liver microsomes (Figure 3.j.) supports the theory of apoprotein binding. Other possible mechanisms of irreversible inhibition involve depletion of cofactor supply, xenobiotic-down regulation of CYP450 or inhibition of heme or apoprotein synthesis (Murray and Reidy, 1990). Whether sulforaphane also interacts with cytochrome P450:NADPH reductase or cytochrome $b_5$ or regulates protein synthesis is not known, although this would not be a basis in microsomal incubations. However no evidence of this exists for other isothiocyanates.

The possibility of covalent binding between sulforaphane and residues in CYP450 could either result form the reaction of the isothiocyanate group or the sulfoxide group with these residues. Furthermore, the covalent interaction could occur via a metabolite of sulforaphane, since this has shown to be competitive for the active site of CYP2E1.

Arylalkylisothiocyanates have shown to be suicide inhibitors (inactivation is metabolism-dependent) of CYP2E1 (Ishizaki *et al*, 1990) and CYP1A2 (Smith *et al*, 1992). This has been attibuted, at least in part, to the isothiocyanate moiety. It is therefore likely that sulforaphane, which bears an isothiocyanate group, will also inactivate CYP2E1 and CYP1A2. The inactivation of CYP2E1 by DAS, DASO and $DASO_2$ has been attributed to the sulfone group (DAS and DASO are metabolised to the sulfone) (Brady *et al*, 1991; Reicks and Crankshaw, 1996; Kwak *et al*, 1994). Two metabolic options are open for sulfoxide groups, reduction to sulfide or oxidation to sulfone (Damai, 1987). For example the antirheumatic drug sulindac undergoes sulfoxide reduction (Damai, 1987). Nevertheless DAS and DASO are transformed to sulfones (see above). Evidence exists indicating that sulforaphane is metabolised to its sulfide and sulfone analogues in rats (Baillie, personal communication). It is therefore likely that the sulfone group might be partially responsible for the inactivation, as for the allyl organosulfurs.

Sulforaphane has recently been shown to inhibit the tumourigenic effects of DMBA in rats (Zhang *et al*, 1994). DMBA is activated mainly by CYP1A, although CYP2B and CYP3A may also contribute in rat microsomes (Lambard *et al*, 1991). Sulforaphane was a potent inducer of GST and QR in several tissues of mouse (Zhang *et al*, 1992), thus might activate detoxification pathways of many carcinogens. The question that arises is whether the inhibition observed here *in vitro* in rat liver microsomes will also be observed *in vivo*, and whether the inhibition of DMBA might be due to the induction of detoxification enzymes, or the inhibition of CYP450, or possibly a combination of both. DMBA induced mammary tumours in female rats, however the ability of sulforaphane to modulate CYPs in this tissue is not known.

The inhibition of CYP450 has been proposed as a mechanism of inhibition of carcinogenesis and mutagenesis (Wattenberg, 1989; De Flora and Rumey, 1988) and several agents that do so, have been proposed as chemopreventors (see general discussion, chapter 6). Rat CYP1A and CYP2E1 have been shown to activate numerous carcinogens to their reactive ultimate species. CYP1A1 is responsible for the activation of benzo[*a*]pyrene, and CYP1A2 for carcinogens such as 2-aminofluorene, and IQ (see Nedelcheva and Gut, 1994). CYP2E1 metabolises the potent carcinogen NDMA, and chlorinated compounds such as chloroform

and carbon tetrachloride (Yang *et al*, 1990). The inhibition of these enzymes may thus result in inhibition of these carcinogens activation. Since sulforaphane inhibits these enzymes, it is possible that it might inhibit the activation of some of these carcinogens, and therefore their carcinogenic effects. The ability of sulforaphane to modulate the genotoxic effect of some of these carcinogens (NDMA and IQ) was therefore studied (see chapter 4 and 5).

# *CHAPTER 4*

## *Inhibition by sulforaphane of N-nitrosodimethylamine (NDMA)-genotoxicity mediated by rodent CYP450*

## *4.1. Introduction*

### 4.1.1. Genotoxicity tests as predictors of carcinogenesis

It is widely accepted that mutation is a necessary event in the carcinogenesis process (see section 1.1.1 of general introduction) and consequently, mutagenicity and genotoxicity have been used as endpoints for the detection and screening of genotoxic carcinogens. Numerous short-term genotoxicity tests are currently used which allow the measurement of the end points such as induction of gene mutations, chromosomal aberrations, DNA damage and repair, mitotic recombinations and sister chromatid exchange (Venitt and Parry, 1984, Carere *et al*, 1995). The test systems used in these assays include bacteria, plants, insects and cultured mammalian cells and also laboratory animals for *in vivo* assessments. Some of the main advantages of these tests are rapidity, simplicity, reproducibility and sensitivity. However, they also present some limitations, such as (for some assays) a lack of metabolic activating enzymes, thus requiring the addition of an external metabolic system such as mammalian liver homogenates. there is also a possibility for obtaining false positives or false negatives. It should also be recognised that non-genotoxic carcinogens are not detected by these tests. Nevertheless, short-term tests (often used in a battery) have shown a good correlation with rodent carcinogenicity data (Ames *et al*, 1976; De Serres and Shelby, 1979) and also are a good prediction of human carcinogenicity, 84 % sensitivity according to Wilbourn et al, 1986, although differences between rodent and human carcinogenicity also exist (67 % sensitivity according to Brockman and De Marini, 1988; Bridges, 1988).

#### 4.1.1.1. Mutagenicity: *Salmonella typhimurium* assay

The *Salmonella typhimurium* preincubation test was developed by B. Ames (Maron and Ames, 1983) and is one of the most commonly used short-term tests. The assay can detect reverse mutation from amino acid auxotrophy to prototrophy. The bacterial strains used in this assay carry base substitutions or frameshift mutations in operons coding for the histidine. The assay determines whether the test chemical can reverse this mutation by introducing a second mutation, and thus allowing bacteria to grow to form colonies in a histidine-deficient medium. All bacterial strains contain GC base pairs at the histidine

mutation (except strain TA102, see table 4.a.), so they are selective for agents which react with these bases (Venitt *et al*, 1984).

In addition to the histidine mutation, bacterial tester strains contain a number of mutations that increase their ability to detect mutagens (see table 4.a.). One mutation, rfa, increases permeability of the cell wall to large molecules, the uvrB mutation results in deficiency in excision repair, thus increasing the sensitivity to mutagenic and lethal effects of mutagens, since the bacterial strains cannot excise DNA adducts (Maron and Ames, 1983). The plasmid pKM101, present in some of the strains (see table 4.a.), carries a gene (*muc*$^+$) which participates in "SOS" DNA repair, a repair pathway induced by DNA damage which confers increased resistance to the lethal effects of several mutagens at the expense of an increased rate of mutability (Venitt *et al*, 1983, 1984).

One of the major limitations of these bacterial strains is that they lack metabolic activation enzymes, so that direct carcinogens can be detected but not pre-carcinogens. This is overcome by the addition of an external metabolic system, which generally is rodent liver homogenates (S9 fraction), which have a high content of both phase I and phase II enzymes (Maron and Ames, 1983). To increase the sensitivity of certain mutagens, animals can be treated animals with several agents (ß-NF, Arocolor 1254) which will increase cytochrome P450 content of S9. A considerable number of mutagens first detected by this assay have been shown to be carcinogenic in experimental animals and approximately 60 % of known carcinogens are mutagenic in this test (Harper *et al*, 1983).

#### 4.1.1.2. DNA repair: Unscheduled DNA synthesis

One of the mechanisms by which a cell can respond to DNA changes and damage is excision repair (see general introduction 1.1.3.1.), which involves the excision of the damaged DNA and its replacement by newly synthesised DNA using the undamaged strand as a template. Nucleotide excision repair is thus an indirect measurement of DNA damage and can be used as a genotoxic endpoint (Waters, 1984). The most widely used assay is the measurement of unscheduled DNA synthesis (UDS) (non-semiconservative synthesis) by monitoring the uptake of [$^3$H]thymidine incorporated into DNA (Williams *et al*, 1982).

Aston University

**Content has been removed for copyright reasons**

Taken from Maron and Ames (1983) and Venitt *et al*, (1984).

The amount of repair detected in the UDS assay depends on the type of DNA damage induced, the number of lesions repaired during the period of time studied and the amount of DNA synthesis associated with the repair of each lesion. Measurement of UDS has the disadvantage that it is time consuming and relatively expensive, however it has the advantage of specificity for detecting chemicals that covalently bind DNA (Waters *et al*, 1984). For example the non-carcinogen 9-amino-acridine is a bacterial mutagen but does not induce UDS since it merely intercalates into DNA (Martin *et al*, 1978). Quantification of nuclear incorporation of [$^3$H]thymidine can be done by autoradiography or liquid scintillation counting. The former is time consuming and laborious, however allows to distinguish between repair synthesis and semi-conservative DNA synthesis, and also allows the identification of artefacts and cell death, whereas the latter is simpler and faster but does not let distinguish the above mentioned distinctions. The UDS assay can be performed in different types of cells (Martin *et al*, 1978, Waters, 1984) , but rat hepatocytes are the most popular (Butterworth *et al*, 1987). The advantages of using hepatocytes instead of other cell types are that they are essentially non-dividing (lack of semiconservative replication) and they have a high content of phase I and phase II enzymes, so that metabolism and induction of repair occurs within the same cell (Butterworth *et al*, 1987, Waters, 1984). Both *in vitro* and *in vivo* UDS are highly sensitive indicators of genotoxicity and is widely accepted and recommended to be used as a complementary test to the *S. typhimurium* assay (Carere *et al*, 1995).

### 4.1.2. Genotoxicity tests as predictors of anticarcinogenesis

Chemopreventors can inhibit carcinogenesis through various mechanisms (see section 1.4.2.) including the inhibition of mutation and cancer initiation. The genotoxicity endpoints and methods used for detecting carcinogens (see above) can also be valuable for the detection of anticarcinogens and antimutagens. For example naturally occurring flavourings (e.g. cinnamaldehyde, vanillin, coumarin) have been found to be antimutagens against 4-nitroquinoline 1-oxide in *S. typhimurium* and in *E. Coli* (Ohta, 1993) or cyclohexanol was also antimutagenic against NNK and NDEA in *S. typhimurium* strain TA100 (Espinosa-Aguirre *et al*, 1993). The UDS assay has also been used for detection of chemopreventor agents, such as the case of benzylisothiocyanate (Sugie *et al*, 1993).

This does not however imply that all anticarcinogens will be also antimutagens and therefore be detected in genotoxicity tests, since other mechanisms of chemoprevention do not involve the inhibition of mutations and genotoxicity (e.g. inhibition of cell proliferation, induction of cell differentiation, etc., see table 1.g. in general introduction). Also anti-genotoxicity might not necessarily imply inhibition of carcinogenicity, because of different conditions and endpoints, but nevertheless shows a potential for anticarcinogenicity.

Sulforaphane has been shown to inhibit CYP2E1 in rat microsomes (see 3.3.2 and 3.4.), thus suggesting that this could be a possible mechanism of anticarcinogenesis (see 1.4.2. and 1.4.3). Related isothiocyanates have also been found to inhibit the tumorigenicity of nitrosamines (metabolically activated at least in part by CYP2E1, see table 1.d.) in rodents. For example, phenethylisothiocyanate (PEITC) inhibited the tumorigenesis of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in rat lung (Morse *et al*, 1989) and of *N*-nitrosomethylbenzylamine in rat esophagus (Morse *et al*, 1993). Benzylisothiocyanate inhibited *N*-nitrosodiethylamine-induced neoplasms in the forestomach of A/J mice (Wattenberg *et al*, 1987). The ability of sulforaphane to modify the genotoxicity *in vitro* of the CYP2E1 substrate *N*-nitrosodimethylamine (NDMA) was therefore assessed in the *Salmonella typhimurium* preincubation test and in the UDS assay. NDMA is a potent hepatocarcinogen in rodents and possibly in humans (Bartsch, 1991) found in foods, industrial products and tobacco smoke (Walker, 1981; Lakritz *et al*, 1982; Biaudet *et al*, 1994), and is metabolically activated by α-hydroxylation mainly by CYP2E1 (Thomas *et al*, 1987, Yoo *et al*, 1990) to yield reactive electrophilic alkylating agents such as methyl carbonium (see figure 1.b.). NDMA is also known to induce mutations in bacteria (Bartsch, 1981) and UDS in HeLa cells (Martin *et al*, 1978).

## *4.2. Methods*

### 4.2.1. Salmonella typhimurium preincubation mutagenicity assay

The *Salmonella typhimurium* preincubation mutagenicity assay was based on Maron and Ames (1983).

#### 4.2.1.1. Plates and solutions

All solutions and preparations were sterilised by either autoclaving for 20 min (1 bar / 121 °C) or by filtration through a 0.45 μm cellulose acetate filter (Sartorius, Gottingen, Germany). All procedures were performed under sterile conditions in a Class II laminar flow cytotox safety cabinet.

4.2.1.1.1. Preparation of minimal glucose plates

Bacteriological agar (15 g) was added to distilled water (930 ml) and sterilised by autoclaving. Vogel Bonner salts (20 ml) and 40 % glucose (50 ml) were added to the hot agar solution. The solution was gently stirred thoroughly and then stood for 3 min to allow bubbles to disappear. Approximately 30 ml of agar solution was poured into plastic Petri dishes (9 cm diameter) and left to set for about 30 min. Plates were dried for about 15 min in an oven set at 40 °C. Plates were stored at 0-4 °C for no longer than 2 months. Vogel Bonner salts were prepared by dissolving magnesium sulfate ($MgSO_4 \cdot 7H_2O$, 10 g), citric acid monohydrate (100 g), potassium phosphate dibasic anhydrous ($K_2HPO_4$, 500 g), and sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$, 175g) to a final volume of one litre of distilled water and then autoclaved.

4.2.1.1.2. Top agar

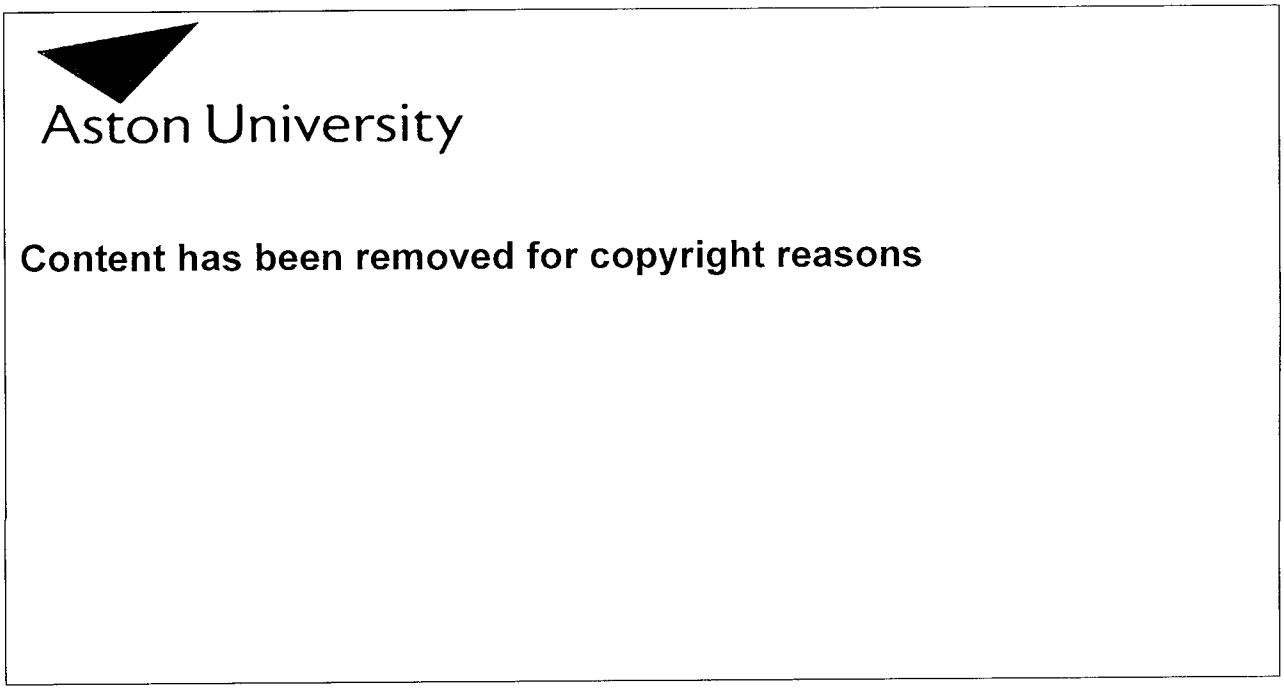
Top agar was prepared by autoclaving agar (6 g) and sodium chloride (NaCl, 5 g) in one litre of distilled water. L-Histidine / D-biotin (0.5 mM) solution ( 10 % v/v) was added after cooling and kept at 50 °C until use. Before use, 2 ml aliquots of top agar were placed in polypropylene vial inserts and kept at 45 °C in a metal heating block (Grant Instruments Ltd., Cambridge, U.K.)

### 4.2.1.2. Liver homogenate supernatant (S9 mix)

Control S9 was prepared according to Maron and Ames (1983) with livers from untreated male Balb/c mice. Animals were maintained as described in 4.2.2.1.2.. Animals were killed by dislocation of the neck and livers removed, weighted and washed with chilled KCl (0.15 M). Livers were minced and homogenised using a teflon-coated pestle in KCl (0.15 M) (3 ml/g fresh liver). The homogenate was centrifuged for 10 min at 9000 g and the supernatant (S9 fraction) was decanted and kept on ice. S9 was frozen at -196 °C, but was not refrozen. Control S9 mix was prepared on ice and contained distilled water, 0.1 mM sodium phosphate buffer (pH 7.4), 4 mM $NADP^+$, 5 mM glucose 6-phosphate, 33 mM KCl, 8 mM $MgCl_2$ and 10 % S9 (see table 4.b.).

Acetone-induced S9 mix was also prepared in a similar way as the control S9 mix, but from Balb/c mice treated with acetone (1 %) in drinking water for 7 days.

Table 4.b. Composition of control S9 mix (10 %) employed in *S. typhimurium* mutagenicity assay.




Taken from Maron and Ames (1983).

### 4.2.1.3. Procedure for growing bacteria

*Salmonella typhimurium* strain TA100 and TA1535 were obtained from B.N.Ames (Biochemistry Department, University of California, Berkeley, U.S.A.) Cultures were prepared from frozen permanents. Using a 1 µl sterile disposable culture loop, bacteria were taken from the thawed surface and inoculated in 2.5 % nutrient broth (Oxoid No. 2). Ampicillin (8 mg/ml; 15.6 µl per 5 ml) was added to the strain TA100, but not to TA1535. Cultures were incubated in a shaking water bath at 37 °C for 10 h to a density of 1-3 $\times$ $10^9$ viable cells per ml. The amount of culture prepared was based on 0.1 ml culture per plate.

### 4.2.1.4. The mutagenicity preincubation test

Mutagenicity was assessed in the *Salmonella typhimurium* pre-incubation reverse gene mutation test according to the method of Maron and Ames (1983). Triplicate plates were used for each experiment and concentration of test agent.

S9 reaction mixture prepared fresh (0.5 ml) (or phosphate buffer, pH 7.4) was delivered to sterile capped bijou tubes (5 ml, Appleton Woods Ltd.) and placed on ice. The test compound (NDMA (0.44 - 4400 µg/plate), sodium azide (5 µg/plate), or sulforaphane (0 - 2.5 mM) (in 10-20 µl), inhibitor (sulforaphane) (0 - 200 mM) when required and bacterial culture (0.1 ml) were added to the solution. The negative controls contained bacteria and S9 mixture (or phosphate buffer). The positive controls used were sodium azide (1 or 5 µg / plate) as a direct mutagen and 2-aminofluorene (10 µg / plate) as an indirect mutagen, requiring presence of S9. The contents of the tubes were mixed gently and incubated at 37 °C for 20 min (unless otherwise stated) in a moderate shaking water bath. The reaction was stopped by placing the tubes on ice. Top agar held at 45 °C in a heating block in 2ml tubes was added to the tubes. The test components were mixed by vortexing the agar for 3 sec and then poured onto minimal glucose agar plates. Plates were left to set for 30 min and then inverted and placed in a 37 °C incubator. After 48 h of incubation, the revertant colonies were counted using an automatic colony counter (Gallenkamp Colonies Counter).

The presence of an auxotrophic bacteria background lawn was confirmed on all the plates by light microscopy.

#### 4.2.1.5. Interpretation and evaluation of data

Results were expressed as number of histidine dependent ($his^+$) revertants induced per plate. Bacterial background lawn (resulting from the trace of histidine in the top agar) was routinely examined in all plates as an aid in determining the toxicity of the test chemical. A sparse background lawn on the test plates compared to the control plates indicated toxicity. Samples which showed at least twice the background number of revertants were considered mutagenic and dose-response relationships were assessed and analyzed by the statistical method of Dunnett (1955), except the inhibition of NDMA-mediated mutagenicity by sulforaphane in *Salmonella typhimurium* strain TA100, which was assessed by an analysis of variance (ANOVA).

### 4.2.2. In vitro Unscheduled DNA synthesis

#### 4.2.2.1. Preparation of isolated mouse hepatocytes

Hepatocytes were isolated by the collagenase perfusion method based on Berry and Friend (1969). All procedures, when possible, were performed under sterile conditions in a Class II laminar flow cytotox safety cabinet.

4.2.2.1.1. Solutions

1) $Ca^{2+}$-free Hanks' balanced salt solution (HBSS) (water, 10% HBSS, 5% HEPES, $NaHCO_3$ 4 mM, pH = 7.4).

2) Collagenase solution (60 ml $Ca^{2+}$-free HBSS, 45 mg collagenase A, 40 mg $CaCl_2$). Collagenase A (*Clostridium histolyticum*) from Boehringer Mannheim GmbH, (Germany).

4.2.2.1.2. Animals maintenance and anaesthesia

Male Balb/c mice (approx. 20 g, Harlan Ltd. U.K.) were used for the preparation of hepatocytes. Animals received a "Rat and Mouse maintenance diet" (Special Diet Service, U.K.). Mice were anaesthetized with a mixture of Hypnorm: Hypnovel: water (1:1:2) (1 ml / 100 g body weight) (0.2 ml) *via i.p.* injection. After a few minutes, when drowsiness was observed, sodium pentobarbitone (Sagatal, 60 mg in 1 ml, 1 ml/kg body weight, 0.02 ml) was delivered *i.p.* into the animal. Full anaesthesia was achieved after a few minutes checked by pinching tightly the tail and no response was observed. If after 5 -10 minutes the animal was not fully anaesthetised, sodium pentobarbitone (0.005-0.01 ml) was injected again.

4.2.2.1.3. Liver perfusion

A water bath was set approximately at 39 °C and a peristaltic pump fitted with 1.5 mm internal diameter flow tubing exchanger (containing a bubble trap to prevent obstruction of the portal vein during perfusion) was switched on and flush through with distilled water until the flow rate achieved 6-7 ml/min. The apparatus was then flushed through with calcium free HBSS avoiding air in tubes.

The animal was secured to the dissection board after being fully anaesthetised. A lamp was placed at few cm from the mouse in order to maintain the liver warmth throughout all the procedure. The abdomen was opened and intestines were displaced to the left of the animal. The thorax was then opened *via* diaphragm and the rib-cage was cut vertically along the left and right sides and the hepatic portal vein, heart and vena cava were exposed. A loop of thread (non-capillary braided silk suture) was tied loosely around the vena cava and the pump was switched on and a heparinized cannula (0.2 mm polypropylene tubing) was placed in the same line than the vena cava. Holding the atrium with tweezers, the hepatic portal vein was cut with fine scissors and a lateral incision was made immediately in the atrium. A cannula was then inserted into the inferior vena cava *via* the atrium and was secured in place by tightening the thread. The liver became pale and the perfusate flowed out through the portal vein. While calcium-free HBSS perfused through the liver (10 ml), collagenase and calcium chloride were dissolved in 60 ml of calcium-free HBSS and then perfused through the liver. The pump was then switched off, the cannula removed from the

vena cava and the liver was removed carefully from the carcass into a small sterile Petri dish. The gall bladder was dissected away and a few ml of incubation medium Dulbecco's Modified Eagle's Medium (DMEM) added. Cells were released by teasing the liver gently with a sterile pipette tip and filter through a nylon mesh (approx. 150 μm) with several washes of cold incubation medium (DMEM). Cells were allowed to settle on ice for 10-15 min, supernatant was removed and cells were resuspended in cold incubation medium. The washing procedure was repeated twice and finally cells were resuspended in 5 ml of DMEM.

4.2.2.1.4. Cell count and viability

Cell viability and number was determined by the trypan blue exclusion method (Moldeus et al, 1978). This method is based on the principle that viable cells do not take up the dye trypan blue, whereas dead (non-viable) cells do.

Cell suspension (0.1 ml), incubation medium DMEM (0.8 ml) and trypan blue (0.4 % w/v) were mixed thoroughly. A small volume of this mixture was transferred to the chamber of a Neubauer haemocytometer by capillarity. Non-viable cells (blue) and viable cells (non stained) were counted in the 1 mm centre square and three 1 mm corner squares. Cell count was calculated as the average count per square × dilution factor (10) × $10^4$ and viability was the % of viable cells with respect to the total number of cells.

The Unscheduled DNA synthesis assay was based on that of Williams *et al.* (1982). All the steps involving handling of live cells were performed under sterile conditions.

#### 4.2.2.2. Solutions

a) Medium I: Sterilised Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% fetal calf serum, glutamine 2.0 mM, penicillin 50 U/ml and streptomycin 50 μg/ml.

b) Medium II (serum-free modified DMEM): Sterilised Dulbecco's modified Eagle's medium (DMEM) was supplemented with glutamine 2.0 mM, penicillin 50 U/ml and streptomycin 50 μg/ml.

c) Aceto-orcein stain: 1 % (w/v) aceto-orcein in glacial acetic (180 ml) acid and distilled water (220 ml).

#### 4.2.2.3. Estimation of toxicity of the test compound

Lactate dehydrogenase leakage was used as an estimate of sulforaphane toxicity on mouse hepatocytes. The assay was based on that of Moldeus *et al.* (1978). Hepatocytes ($2\times10^6$ cells/incubation) and sulforaphane (100 μl, 0.04, 0.2, 1.0 mM) were incubated in 2 ml of DMEM for 2 h at 37 °C. Distilled water was used as the negative control and 100 μl of Triton X100 (10, 30 min) as the positive control. Incubations were then centrifuged and supernatant was kept. Phosphate buffer (100 mM, pH = 7.0) pyruvate (23 mM, 50 μl), NADH (12 mM, 10 μl) and incubations supernatants ( 20 μl) were mixed in a quartz cuvette. LDH release was measured by measuring the change of absorbance at 340 nm at 37 °C.

#### 4.2.2.4. Hepatocyte primary cell culture

Following liver perfusion and cell counting, $2\times10^5$ viable cells were plated in 2.5 ml of medium I into 35 mm 6 multiwell plates (Cel-Cult, Sterilin Ltd., Feltham, U. K.) containing

25 mm round plastic Thermanox coverslips (Lux Scientific, Flow Laboratories, U. K.). Cells were allowed to attached for 2 h at 37 °C in a humidified incubator gassed with 95 % air-5 % $CO_2$. Cultures were then examined under a microscope to ensure attachment of cells to coverslips. Non-viable cells (non-attached) were removed and the cell layer was washed once with 2 ml of medium II (pre-warmed at 37 °C).

#### 4.2.2.5. Exposure of hepatocyte cultures to the test compounds

Cultures were incubated for 20 h with test compound dissolved in distilled water (10 µl) with 5.0 µCi of [$^3$H]-thymidine (85 Ci/mmol, 2% ethanol in water, Amersham Life Science, U. K), in 2 ml of medium I (serum-free DMEM) in a humidified incubator gassed with 95 % air-5 % $CO_2$ at 37 °C. The tests compounds were sulforaphane (0.032, 0.16, 0.8, 4.0, 20.0 µM final concentration), *N*-nitrosodimethylamine (NDMA) (2.5 µg/ml final concentration) as the positive control and distilled water as the negative control.

For the inhibition studies, 10 µl of distilled water containing sulforaphane (0.032, 0.16, 0.8, 4.0, 20.0 µM final concentration), 5 µl of NDMA (1.0 mg/ml) and 5.0 µCi of [$^3$H]-thymidine were incubated for 20 h in 2 ml of medium I. Positive and negative control were the same as above.

#### 4.2.2.6. Fixation of cultures for autoradiography

After incubation, medium was removed and cultures were washed with phosphate buffer saline (PBS, pH = 7.4) to remove free radiolabel. Cells were then fixed on the coverslip by three changes of ethanol:acetic acid (3.0 ml, 3:1) for 30 min each and then they were air-dried on paper overnight and mounted onto glass microscope slides with DPX mountant (BDH Chemicals Ltd., Poole, Dorset, U. K.).

#### 4.2.2.7. Coating, development and staining of slides

All manipulations were undertaken in a dark room under safe-light conditions. Emulsion was prepared by melting K2 Nuclear Research Emulsion : distilled water (1:1) and 1.0 %

glycerol in a small plastic vessel in a water bath at 40 °C. Slides were coated individually by dipping into the emulsion, drained onto paper and subsequently dried for 3 h at 30 °C. Slides were exposed for a period of 3 days in light-proof boxes in a dessicator at + 4 °C.

Slides were developed in the dark by immersion in diluted D19 developer (Kodak, Hemel Hempstead, U. K.) (18 % v/v) for 3 min. Development was stopped by rinsing the slides in 2 % (v/v) acetic acid for 30 sec followed by fixing in 20 % (w/v) sodium thiosulfate for 8 min and finally washing under slowly running tap water for 10 min. The slides were stained in 1 % aceto-orcein (w/v) for 6 min, rinsed in tap water (twice) and dipped in absolute alcohol (dehydration). The slides were allowed to dry and then were covered with a glass coverslip (24 × 24 mm, Appleton Woods) using DPX.

#### 4.2.2.8. Quantification of UDS

Slides were examined under a light microscope (100x objective under oil immersion), the silver grains were visualised on a television monitor. Quantification of [$^3$H]-thymidine incorporation was performed by manual counting of the number of silver grains over an area of nucleus and of the maximum number over an adjacent equivalent area of the cytoplasm. Cells counted (50 per slide) were morphologically unaltered and were not in S-phase (semiconservative DNA synthesis). The cytoplasmic count was subtracted from the nuclear count to yield a net nuclear grain count (NNG) for each slide.

#### 4.2.2.9. Interpretation and evaluation of data

A positive response is usually accepted as either an increase in UDS observed with an increase of the dose of the test chemical or a statistically significant increase in NNG for at least one concentration above the negative control (Waters, 1984). There is some disagreement about what constitutes a positive result i.e. NNG>5 for Williams *et al,* (1982), NNG>3 for Casciano and Gaylor (1983). However, more recently international guidelines have defined a cell in repair as exhibiting NNG> 0 and results were evaluated analysing statistically significant dose-response effects, as recommended by international guidelines

(Kennely *et al,* 1993, Madle, *et al,* 1994). The percentage of cells in repair was calculated as the % of cells undergoing appreciative repair (NNG>5).

## *4.3. Results*

### 4.3.1. Lack of genotoxicity of sulforaphane

The genotoxicity of sulforaphane was assessed in the *Salmonella typhimurim* preincubation mutagenicity test and in the unscheduled DNA synthesis assay.

#### 4.3.1.1. Mutagenicity preincubation test

The mutagenic potency of sulforaphane was assessed using *Salmonella typhimurium* strain TA 100 preincubation mutagenicity assay with and without metabolic activation. All the incubations were in triplicate and results were expressed as the number of histidine dependent ($his^+$) revertants induced per plate (mean ± SD., n=3).

##### 4.3.1.1.1. Without metabolic activation

Sulforaphane at eight different concentrations (0.032-2500 μM) and bacteria were preincubated for 20 min prior to pouring onto agar plates.

The spontaneous reversion rate for two different analyses were 168.6 ± 17.9 and 126.3 ± 17.0 respectively. These values are within acceptable ranges of spontaneous reversion, 120-200 revertants/plate, according to Maron and Ames (1983). The direct agent sodium azide ($NaN_3$, 1 μg/plate) was used as a positive control and gave 1856±119 and 517± 4 revertants /plate for the two different analyses. Although the number of $his^+$ revertants induced by $NaN_3$ per plate differed considerably between the two experiments, a positive response was seen in both cases, as indicated by 11- and 4-fold above the spontaneous rate. This variation could be due to differences in the total number of bacteria in the overnight incubates.

Sulforaphane at 0.5 and 2.5 mM was cytotoxic as evidenced by a decrease in the background lawn of auxotrophs. Sulforaphane treatment (0.032-100 μM) failed to increase the number of $his^+$ revertants per plate compared to the spontaneous rate (Table 4.c.). All values were not statistically different form controls (analysed by Dunnett's method;

Dunnett, 1955). This indicates lack of mutagenicity of sulforaphane in *Salmonella* TA100 without metabolic acitvation.

4.3.1.1.2. With metabolic activation (control S9 and acetone-induced S9)

The mutagenic potency of sulforaphane was also assessed in the presence of metabolic activation. Uninduced mouse liver S9 and acetone-induced mouse liver S9 were used as the metabolic activation system.

Preincubations (20 min) contained sulforaphane (0.032-2500 μM), mouse liver S9 and bacteria. Control cultures gave a spontaneous rate of 133.0 ± 24.0 and 111.0 ± 13.5 revertants/plate for two different experiments. Although the value of 111.0 was slightly lower than the recomended range by Maron and Ames (1983) (120-200 spontaneous revertants/plate), it is within the normal range reported more recently (45-179 spontaneous revertants/plate without S9 and 46-184 spontaneous revertants/plate with S9) (Venitt *et al*, 1984). 2-Aminofluorene (2AF) (10 μg/plate) was the positive control and gave 1923 ± 89 and 1815 ± 97 revertants/plate in each of two separate experiments respectively. Sulforaphane at 0.5 and 2.5 mM decreased the background lawn of auxotrophs, indicating toxicity. Sulforaphane (0.032-100 μM) in the presence of uninduced mouse liver S9 was found to be not mutagenic, as indicated by the inability to increase $his^+$ revertants/plate as compared to the controls (analysed by Dunnett's method (1955)) (Table 4.d.).

The mutagenicity of sulforaphane was also assessed using acetone-induced mouse liver S9 and 45 min preincubation (this condition was also used for the inhibition studies, see section 4.3.3.). Sulforaphane (0-200 μM) was found to lack mutagenic potential. Values for $his^+$ revertants/plate were 67.34 ± 10.37, 73.50 ± 10.14 and 84.17 ± 1.18 (mean ±SD, n=2 experiments in triplicates) for sulforaphane 20, 100 and 200 μM respectively, compared to 91.5 ± 4.5 for the spontaneous rate (all values being within the recommended range 45-179).

Table 4.c. Effect of sulforaphane on *S. typhimurium strain* TA100 without metabolic activation.

| Sulforaphane (μM) | Number of his$^+$ revertants/plate[a] | |
|---|---|---|
| 0 | 168.6 ± 17.9 | 126.3 ± 17.0 |
| 0.032 | 196.0 ± 26.9 | 140.0 ± 4.4 |
| 0.16 | 206.6 ± 20.4 | 148.3 ± 13.2 |
| 0.8 | 185.0 ± 19.5 | 144.0 ± 12.7 |
| 4 | 170.0 ± 14.1 | 133.0 ± 5.2 |
| 20 | 140.3 ± 19.5 | 134.0 ± 20.0 |
| 100 | 163.3 ± 44.7 | 115.3 ± 52.0 |

[a] All values are expressed as the mean of the number of his$^+$ revertants per plate ± SD (n=3) including spontaneous revertants. All values were not statistically different from controls (analysed by Dunnett, 1955).

Table 4.d. Effect of sulforaphane on *S. typhimurium* strain TA100 with metabolic activation

| Sulforaphane (μM) | Number of $his^+$ revertants/plate[a] | |
|---|---|---|
| 0 | 133.0 ± 24.0 | 111.0 ± 13.5 |
| 0.032 | 148.3 ± 8.0 | 130 ± 6.6 |
| 0.16 | 149.0 ± 8.7 | 116.7 ± 6.7 |
| 0.8 | 158.3 ± 12.4 | 115.0 ± 14.1 |
| 4 | 161.3 ± 15.0 | 113.6 ± 7.8 |
| 20 | 126.0 ± 18.2 | 101.0 ± 10.2 |
| 100 | 123.0 ± 24.6 | 117.6 ± 13.0 |

Test compounds, mouse liver S9 and bacteria were preincubated for 20 min prior to pouring onto agar plates. [a] All values are expressed as the mean of the number of $His^+$ revertants per plate ± SD (n=3) including spontaneous revertants. All values were not statistically different from controls (analysed by Dunnett, 1955).

### 4.3.1.2. Unscheduled DNA synthesis

Cytotoxicity of sulforaphane was measured by LDH release in mouse hepatocytes. Sulforaphane was found cytotoxic at 40 μM and above (42. 86 % compared to 28.57 % for control incubations and 100 % for the positive control TritonX100). Thus concentrations of 20 μM and below of sulforaphane were used for all the studies with mouse hepatocytes.

Sulforaphane was tested at 5 different doses (0.032-20 μM to determine its ability to induce unscheduled DNA synthesis (UDS) in mouse hepatocytes. A repeat of triplicate analyses were performed as described in 4.2.2.5. section.

The cytoplasmic grain count was 14.9 ± 2.8 and 21.9 ± 6.5 (mean ±SD, n=21 slides, 50 cells/slide) for experiment 1 and 2 respectively. The percentage of hepatocytes in S phase (0.07 - 0.1 %) was similar throughout all experiments. The positive control, *N*-nitrosodimethylamine (NDMA) (33.5 μM) gave a net nuclear grain count (NNG) of 9.5 ±2.9 and 32.3 ± 13.7 for experiments 1 and 2 respectively. The percentage of cells in repair for the positive control was 84.5 % (mean of two separate hepatocytes preparations).

Sulforaphane failed to induce UDS in mouse hepatocytes. All concentrations of sulforaphane gave NNG < 0 as shown in Table 4.e. and the percentatge of cells in repair was minimal (<1.66 %) at any of the concentrations of sulforaphane tested (0, 0.032, 0.16, 0.8, 4, and 20 μM).

Table 4.e. Effect of sulforaphane in the UDS assay with mouse hepatocytes.

| Sulforaphane (μM) | Net Nuclear Grain Count (NNG)[a] | |
|---|---|---|
| 0 | -7.4 ± 1.6 | -11.9 ± 1.5 |
| 0.032 | -4.7 ± 0.5 | -5.2 ± 1.7 |
| 0.16 | -4.7 ± 1.3 | -4.9 ± 0.7 |
| 0.8 | -5.2 ± 1.1 | -5.8 ± 1.5 |
| 4 | -4.8 ± 1.5 | -6.1± 0.8 |
| 20 | -3.6 ± 0.5 | -7.1 ± 2.0 |

Sulforaphane and [$^3$H]-thymidine were incubated with mouse hepatocytes for 20 h. The positive control, NDMA (33.5 μM) gave a NNG of 9.5 ± 2.9 and 32.3 ± 13.7 for every experiment respectively. [a] For each experiment values given are the mean ± SD, n=3 slides. Nuclear and cytoplasmic grains were counted in 50 cells/slide.

## 4.3.2. Inhibition by sulforaphane of NDMA-induced mutagenicity

### 4.3.2.1. NDMA mutagenicity in the standard test (control S9 + 20 min preincubation)

The mutagenicity of the carcinogen *N*-nitrosodimethylamine (NDMA) was assessed using *Salmonella typhimurium* strain TA100 and TA1535 in a preincubation test with metabolic activation.

NDMA at 6 concentrations (44 ng/plate - 4.4 mg/plate), mouse liver S9 and *Salmonella typhimurium* strain TA100 were preincubated for 20 min (Table 4.f.). NDMA was not cytotoxic at any of the concentrations tested. The spontaneous reversion rate was 159.3 ± 22.4 and the spontaneous reversion rate with control S9 was 168.3 ± 9.3, both within the recomended ranges (see 4.3.1.1). The highest concentration of NMDA, 4.4 mg/plate, gave 250.0 ± 16.8 revertants per plate. Although this value is significantly different from the control, it is not considered positive, since it is less than double than the control and no concentration-dependent effects were observed.

NDMA at 0.044 and 4.4 mg/plate was also preincubated for 20 min with control mouse liver S9 and *Salmonella typhimurium* strain TA 1535 prior to pouring onto plates. The spontaneous reversion rate was 16.0 ± 1.9 and 12.0 ± 1.0 with and without S9 respectively, which are in agreement with values reported before (3 - 18 and 2 - 21 revertants/plate without and with S9 respectively). Although NDMA at 4.4 mg/plate gave a positive response (34.7 ± 7.1 revertants/plate) (2.2-fold), this was considered to be not high enough to be able to appreciate potential inhibitory effects by sulforaphane.

Table 4.f. Mutagenicity of NDMA in the *Salmonella typhimurium* TA100 preincubation test with control mouse liver S9.

| NDMA (µg/plate) | $his^+$ revertants/plate |
|---|---|
| 0 | 168.3 ± 9.3 |
| 0.044 | 221.6 ± 27.5 |
| 0.44 | 180.3 ±28.5 |
| 4.4 | 208.6 ± 7.5 |
| 44 | 229.0 ±25.2 |
| 440 | 185.6 ± 12.7 |
| 4400 | 250.0 ± 16.8 |

NDMA, bacteria and control mouse liver S9 were preincubated for 20 min prior to pouring onto agar plates. Values are the mean ± SD, n = triplicates. Spontaneous revertans without S9 were 159.3 ± 22.5 and the positive control 2AF (10 µg/plate) gave 2083.3 ± 308.0 revertants per plate.

### 4.3.2.2. NDMA mutagenicity using acetone-induced S9 as the metabolic system.

In order to obtain a positive response for NDMA in the *Salmonella* preincubation test, acetone-induced mouse liver S9 was prepared as described in section 4.2.2.2. The use of acetone induced mouse liver S9, relatively rich in CYP2E1 (acetone treatment induces levels of CYP2E1 (see chapter 3)), instead of untreated mouse liver S9 was expected to increase the mutagenic response to NDMA.

NDMA (0.44 µg/plate - 4.4 mg/plate), acetone-induced mouse liver S9 and bacteria (*S. typhimurium* TA 100) were preincubated for 20 min. The results are showed in table 4.g.. An increase in the number of $his^+$ revertants per plate was observed with increasing dose of NDMA. The number of $his^+$ revertants/plate of NDMA at 4.4 mg/plate was nearly triple the spontaneous rate (ratio 2.7). These results indicate that NDMA was mutagenic. No cytotoxicity was observed in any of the concentrations tested.

Table 4.g. Mutagenicity of NDMA in *S. typhimurium* TA100 with acetone-induced S9

| NDMA (μg/plate) | $his^+$ revertants/plate |
|---|---|
| 0 | 175.0 ± 11.3 |
| 0.44 | 139.0 ± 10.8 |
| 44 | 351.3 ± 8.5 |
| 4400 | 475.5 ± 6.3 |

NDMA, acetone-induced mouse liver S9 and bacteria were preincubated for 20 min prior to pouring into plates. Results represent the mean ± SD, n =3. The spontaneous rate without metabolic activation was 173.0 ± 3.5.

### 4.3.2.3. Inhibition by sulforaphane of NDMA-induced mutagenicity

The ability of sulforaphane to inhibit mutagenicity of NDMA was assessed in *S. typhimurium* strain TA100 using acetone-induced mouse liver S9. Also, in order to maximise its response in these bacteria, the preincubation time was prolonged from 20 to 45 min.

Four separate experiments were performed, with preincubations containing NDMA (4.4 mg/plate), sulforaphane (0 - 200 μM), acetone -induced S9 and bacteria. Spontaneous revertants (without S9) were 174.7 ± 27.1, 176.3 ± 13.2, 136.7 ± 13.4, and 88.3 ± 21.2 (mean ± SD, n=3) respectively for experiments 1 to 4. Spontaneous revertants in presence of acetone-induced S9 were slightly higher for all the experiments (186.7 ± 4.2, 191.0 ± 2.7, 187.0 ± 18.5 and 94.7 ± 1.5 respectively for experiments 1-4) but within the normal range reported by Maron and Ames (1983).

The mutagenic potential of NDMA differed considerably between experiments, but was a clear positive in all cases (table 4.h.). The number of $his^+$ revertants were 833.3 ± 33.3, 518.7 ± 44.9, 497.0 ± 43.5, and 235.3 ± 13.6 respectively for experiments 1-4. This variation was likely to be due to differences in the culture numbers grown overnight on different days, and are not unexpected.

Sulforaphane (0.032 -200 μM) on co-incubation with NDMA reduced the frequency of revertants compared to the positive control, NDMA alone. Table 4.h. shows the effect of sulforaphane for each of the four separate experiments with the calculated mean percentage inhibition (n = 4 experiments). There was a clear concentration-dependent effect and a significant linear trend with dose (analysed by ANOVA, $p<0.001$). Percentage of inhibition was calculated following subtraction of the spontaneous revertants. Values were significantly different from controls at 0.8 μM sulforaphane ( $p<0.05$) and above ($p<0.01$) (Students *t*-test). At the highest concentration tested, 200 μM, sulforaphane inhibited NDMA mutagenicity by 73.4%.

The ability of sulforaphane to inhibit mutagenicity of NDMA was also assessed using a lower concentration of NDMA (44 μg/plate) and under the same conditions, i.e. 45 min preincubation and acetone-induced mouse liver S9.

Spontaneous revertants with and without S9 were 186.7 ± 4.2 and 174.7 ± 27.0 respectively. NDMA at 44 μg/plate was mutagenic and gave 777.0 ± 17.1 $his^+$ revertants. The presence of sulforaphane in the incubations also reduced the frequency of revertants compared to NDMA alone (Table 4.i.).

Table 4.h. Inhibition of NDMA mutagenicity in *S. typhimurium* strain TA100 by sulforaphane

| Sulforaphane (μM) | Number $His^+$ revertants/plate [a] induced by NDMA | | | | Mean % inhibition [c] |
|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 | |
| 0 | 833.3±33.3 | 518.7±44.9 | 497.0±43.5 | 235.3±13.6 | 0 |
| 0.032 | 675.5±44.6 | 595.0±32.5 | 426.3±57.7 | 185.5±0.7[b] | 14.8 |
| 0.16 | 655.0±206.6 | 529.3±16.9 | 428.3±17.8 | 162.7±11.4 | 24.7 |
| 0.8 | 581.7±28.9 | 507.0±11.1 | 418.3±22.9 | 175.5±6.4[b] | 27.2 |
| 4 | 757.0±33.7 | 458.3±42.8 | 398.3±57.5 | 174.3±15.5 | 26.3 |
| 20 | 638.5±91.2[b] | 435.7±40.4 | 442.3±26.1 | 182.0±1.7 | 27.8 |
| 100 | 341.0±37.4 | 379.7±36.5 | 317.3±2.9 | 166.7±8.4 | 56.5 |
| 200 | 271.7±28.7 | 300.3±20.5 | 242.7±23.9 | 154.7±15.9 | 73.4 |

Sulforaphane, dimethylnitrosamine (4.4 mg/plate), acetone-induced S9 and bacteria were preincubated for 45 min before the mixture was poured onto the agar plates. Colonies were counted after 48 h.

[a] Number of $His^+$ revertants per plate including background spontaneous rate, mean±SD, (n=3; except [b] n=2). Statistics analyzed by ANOVA, significant linear trend with dose for every experiment, $p<0.01$.

[c] % Inhibition was calculated following subtraction of the spontaneous revertants. Values are significantly different from controls at 0.8 μM sulforaphane ( $p<0.05$) and above ($p<0.01$), Students *t*-test.

Table 4.i. Inhibition of NDMA (44 μg/plate) mutagenicity in *S. typhimurium* strain TA100 by sulforaphane

| Sulforaphane (μM) | Number $His^+$ revertants/plate [a] induced by NDMA | Mean % inhibition [c] |
|---|---|---|
| 0 | 777.0 ± 17.1 | 0 |
| 0.16 | 769.0 ± 28.3 [b] | 1.4 |
| 0.8 | 751.7 ± 34.8 | 4.3 |
| 4 | 662.0 ± 79.1 | 19.5 |
| 20 | 637.5 ± 113.8 [b] | 23.6 |
| 100 | 418.7 ± 73.4 | 60.7 |
| 200 | 244.0 ± 6.0 | 90.3 |

Sulforaphane, dimethylnitrosamine (4.4 mg/plate), acetone-induced S9 and bacteria were preincubated for 45 min before the mixture was poured onto the agar plates. Colonies were counted after 48 h.

[a] Number of $His^+$ revertants per plate including background spontaneous rate, mean ± SD, (n=3; except [b] n=2). A significant linear trend with dose was observed (analyzed by ANOVA, $p<0.01$).

[c] % Inhibition was calculated following subtraction of the spontaneous revertants. Values are significantly different from controls at 4 μM sulforaphane ( $p<0.05$) and above ($p<0.01$), Students *t*-test.

### 4.3.3. Inhibition by sulforaphane of NDMA-induced unscheduled DNA synthesis

The ability of sulforaphane to inhibit NDMA-induced DNA repair was assessed in the UDS assay in mouse hepatocytes. Mouse hepatocytes were incubated with sulforaphane (0.0064-20 μM), NDMA (33.5 μM) and $^{3}$H-thymidine for 20 h.

The cytoplasmic grain count (13.7 ± 2.5, mean ± SD, n=68) did not differ signicantly throughout all the experiments. Also, no difference was observed in the number of cells in S phase (approximately 0.1%) between control and treatment groups. The negative controls gave a NNG of -5.0 ± 0.9 (mean ± SD, n = 3) and the percentage of cells in repair was 0.4 ± 0.6 (mean ± SD, n = 3).

The presence of sulforaphane during treatment with NDMA reduced the NNG markedly compared to the positive control which contained NDMA alone (Figure 4.a.). The inhibition of NDMA-induced UDS by sulforaphane was clearly concentration-dependent for all concentrations tested ($p < 0.001$). NDMA (33.5 μM) induced DNA repair and gave a NNG of 17.6 ± 0.5 (mean ± SD, n = 3). NNG values varied from 10.3 ± 2.2 for sulforaphane at 6.4 nM to 1.4 ± 0.5 for sulforaphane at 20 μM.

The percentage of cells in repair was also reduced by the presence of sulforaphane as compared with NMDA alone in a concentration-dependent manner ($p < 0.01$) (Table 4.j.). The positive control NDMA (33.5 μM) gave 89.6 ± 4.2 (mean ±SD, n=3) % of cells in repair, sulforaphane at the lowest concentration tested (0.0064 μM) reduced this value to 71.1 ± 6.3 and for the highest concentration (20 μM) only 28.5 ±0.6 % of cells were in repair.

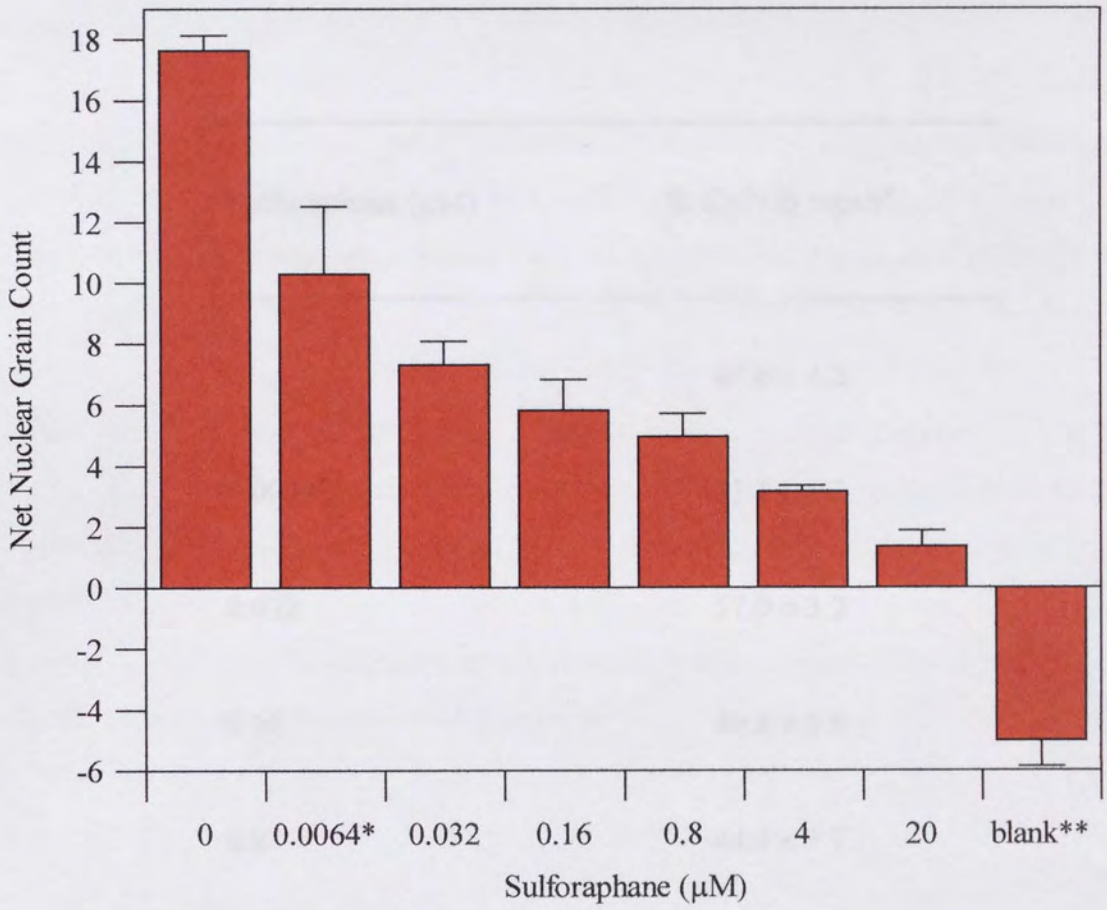



Figure 4.a. Inhibitory effect of sulforaphane on *N*-nitrosodimethylamine (NDMA)-induced UDS in mouse hepatocytes.

Mouse hepatocytes were incubated with sulforaphane, NDMA 33.5 μM and [$^3$H]-thymidine for 20 h. Values are the mean ± SD, n=3 experiments, all doses were analysed in triplicate and 50 cells were counted manually for every slide. All doses were significantly lower than the positive control NDMA alone, ($p<0.01$ by Dunnetts' method), *n=2, **distilled water was used for the blank.

Table 4.j. Effect of sulforaphane on NDMA-induced DNA repair in mouse hepatocytes.

| Sulforaphane (μM) | % Cells in repair[a] |
|---|---|
| 0 | 89.6 ± 4.2 |
| 0.0064[b] | 71.1 ± 6.3 |
| 0.032 | 57.0 ± 3.2 |
| 0.16 | 49.8 ± 9.8 |
| 0.8 | 44.4 ± 7.7 |
| 4 | 35.2 ± 0.6 |
| 20 | 28.5 ± 0.6 |

Mouse hepatocytes were incubated with sulforaphane, NDMA 33.5 μM and [$^3$H]-thymidine for 20 h. Values are the mean ± SD, n=3 experiments, all doses were analysed in triplicate and 50 cells were counted manually for every slide.
[a] % Cells in repair were calculated as the percentatge of cells undergoing appreciative repair (NNG > 5). [b] n =2.

### 4.3.4. Effect of sulforaphane on sodium azide-induced mutagenicity

The effect of sulforaphane on the mutagenicity of the direct agent sodium azide ($NaN_3$) was assessed in *S. typhimurium* strain TA100 without metabolic activation.

Sulforaphane (0 - 100 μM) was preincubated with $NaN_3$ (5 μg/plate) and bacteria for 20 min. Spontaneous revertants were 147 ± 33 (mean ± SD, n = 2 experiments, each in triplicates) and incubations containing only $NaN_3$ were clearly positive and gave 1367 ± 15 $his^+$ revertants per plate.

Co-incubation of sulforaphane (0.032 - 100 μM) with the direct mutagen did not decrease the number of $his^+$ revertants compared with the positive control, sodium azide alone (Table 4.k.). Although sulforaphane at the highest concentration (100 μM) decreased slightly the number of $his^+$ revertants per plate induced by $NaN_3$ (1164 and 1367 respectively) ($P < 0.05$, Students *t*-test), this effect was minimal and not seen at any other concentrations tested (0.5 and 0.05 μg/plate).

Table 4.k. Effect of sulforaphane on $NaN_3$ mutagenicity in *S. typhimurium* strain TA100

| Sulforaphane (μM) | $His^+$ revertants/plate induced by $NaN_3$ | |
|---|---|---|
| 0 | 1356 ± 53 | 1378 ± 65 |
| 0.032 | 1356 ± 58 | 1353 ± 71 |
| 0.16 | 1315 ± 30 | 1363 ± 125 |
| 0.8 | 1319 ± 22 | 1259 ± 73 |
| 4 | 1418 ± 151 | 1361 ± 54 |
| 20 | 1115 ± 114 | 1536 ± 211 |
| 100 | 1069 ± 60[a] | 1258 ± 34 |

Sulforaphane, $NaN_3$ (5 μg/plate) and bacteria were preincubated for 20 min prior to pouring onto agar plates. Data shown are the number of $His^+$ revertants per plate induced by $NaN_3$ and are a repeat of triplicate analyses (mean ±SD, n=3). Spontaneous revertants per plate were 171 ± 22 and 123 ± 7 respectively.

[a] Statistically different from the control group (absence of sulforaphane) ($P<0.05$).

## *4.4. Discussion*

Several isothiocyanates have been shown to inhibit the carcinogenic effects of nitrosamines in various tissues of rats and mice (see introduction section 4.1.2.) possibly by inhibition of their metabolic activation and have been classified as chemopreventors (Dragsted *et al,* 1993; Wattenberg, 1992). Following the recognition that sulforaphane can inhibit CYP2E1 in rat microsomes (chapter 3), the effect of sulforaphane on NDMA genotoxicity (CYP2E1 mediated) was assessed in two different short-term tests.

A number of isothiocyanates have also been shown to posses toxicological effects such as cytotoxicity and genotoxicity (Stoewsand, 1995; Musk and Johnson, 1993; Musk *et al,* 1995) which could override their chemopreventive properties. For example PEITC is a potential chemopreventor (see introduction) whereas allylisothiocyanate and phenylhexylisothiocyanate are mutagenic and carcinogenic (see general introduction, section 1.5.3). It thus appears that the chemoprotective activity of isothiocyanates will be the result of the balance between the potential beneficial anticarcinogenic effects and the possible genotoxic activity. The cytotoxicity and genotoxicity of sulforaphane was therefore studied in *Salmonella typhimurium* (using mouse liver homogenates as the activation system) and mouse hepatocytes prior to the study of its antigenotoxic potential. The reason for employing mouse liver preparations was that for any subsequent comparative *in vivo* studies, the amount of sulforaphane required would be relatively small.

### 4.4.1. Lack of genotoxicity of sulforaphane

Sulforaphane was found cytotoxic in *Salmonella typhimurium* strain TA100 at high concentrations (0.5 and 2.5 mM) with and without metabolic activation (mouse liver S9) as evidenced by a decrease in the background lawn of auxotrophs. This suggests that cytotoxicity could be due to sulforaphane itself (without S9). Cytotoxicity was also observed in mouse hepatocytes measured by release of lactate dehydrogenase (40 μM and above). Cytotoxicity by sulforaphane was also observed in previous studies, where sulforaphane at 18 μM reduced Hepa 1c1c7 murine hepatoma cell growth by half (Zhang *et al*, 1992). Other isothiocyanates have also been shown to be cytotoxic, such as BITC and

PEITC in Balb/c mouse 3T3 fibroblasts with the neutral red assay (50 % toxicity at 19 and 20 μM respectively) (Babich *et al*, 1993). BITC and allylisothiocyanate were both cytotoxic at 6.7 μM and 100 μM respectively in a rat liver epithelial cell line (RL-4) (Bruggeman *et al*., 1986). This toxicity may be related to the reactivity of the isothiocyanate moiety (Drobnica and Gemeiner, 1977). Furthermore, glutathione and cysteine conjugates of isothiocyanates appear to reduce the cytotoxicity of the parent compound, although allylisothiocyanate conjugates gave effects similar to the parent compound (Baillie and Slatter, 1991; Bruggeman *et al*, 1986). Sulforaphane conjugates with GSH *in vitro* both spontaneously and enzymatically by human GST isoenzymes (mainly P1-1, Zhang *et al*, 1995), and it is likely that *in vivo* will be metabolised to glutathione derivatives (in comparison with the metabolism of other isothiocyanates) (Mennicke *et al*, 1988). Indeed, sulforaphane is excreted in rats as its glutathione conjugates and as the sulfide and sulfone conjugates derivatives (Baillie, personal communication). It is therefore quite likely that glutathione conjugates of sulforaphane may be less cytotoxic than sulforaphane. In a recent study, the dithiocarbamate analog of sulforaphane, sulforamate (4-methylsulfinyl-1-(S-methyldithiocarbamyl)-butane was 3-fold less cytotoxic than sulforaphane (Gerhauser *et al*, 1997), thus further supporting the role of the isothiocyanate moiety in the toxicity of sulforaphane.

Sulforaphane at subcytotoxic concentrations was found not mutagenic in *Salmonella typhimurium* strain TA100 with or without metabolic activation (mouse liver S9 and acetone induced mouse liver S9) as evidenced by a reversion rate similar to the spontaneous reversion rate. A small decrease in the reversion rate was observed for sulforaphane 20 and 100 μM when using acetone-induced S9, but this was not significantly different from the spontaneous rate either concentration dependent and no reduction of background lawn was observed for any of the concentrations. These results contrast with the mutagenic potential (albeit weak) found for allylisothiocyanate in *S.typhimurium* TA100 (Neudecker and Henschler, 1985). *Salmonella typhimurium* strain TA100 is sensitive only to GC base pair substitutions and therefore sulforaphane did not induce this type of mutations. However the ability of sulforaphane to induce other type of mutations should be assessed.

The genotoxic potential of sulforaphane was also studied in mouse liver hepatocytes using the UDS assay. Sulforaphane at subcytotoxic concentrations failed to induce nucleotide excision repair, semiconservative DNA synthesis (S-phase) and the percentage of cells in repair, indicating lack of genotoxicity of sulforaphane in this assay. Some variation in the response of negative and positive controls (vehicle and NDMA respectively) was observed in separate hepatocyte incubations, however this variability appears to be a common factor when using rodent hepatocytes, probably due to variation in the functional state of the cells (Waters *et al,* 1984). There was a small increase in the NNG of hepatocytes exposed to sulforaphane compared to control hepatocytes, but this was not statistically significant and was not concentration-dependent. A high background of cytoplasmic labelling was also observed, which is in agreement with the findings of other authors and can be accounted for the incorporation of radiolabelled thymidine into mitochondrial DNA (Waters *et al,* 1984). The related BITC and benzylthiocyanate reduced replicative DNA synthesis (reduction of the incidence of S-phase cells) in hepatocytes after *in vivo* administration to rats compared to the control animals, which is indicative of suppression of cell proliferation (Sugie *et al.*, 1993). This reduction in S-phase cells incidence was not observed for mouse hepatocytes treated *in vitro* with sulforaphane, although the possibility that *in vivo* treatments may affect semiconservative synthesis can not be rejected. The lack of genotoxicity of sulforaphane in mouse hepatocytes *in vitro* contrasts with the clastogenic activity of related isothiocyanates. PEITC, PITC and BITC (aryl-containing isothiocyanate) induced chromosome aberrations after 24 h exposure to a SV40-transformed Indian muntjac cell line (Musk and Johnson, 1993), however allylisothiocyanate (aliphatic isothiocyanate) failed to. It is thus possible that sulforaphane, also an aliphatic isothiocyanate may not be clastogenic in this cell line. Species differences exist in the toxicological and genotoxic effects of some chemicals (Nedelcheva and Gut, 1994), so the genotoxicity potential of sulforaphane should also be examined in other species.

### 4.4.2. Genotoxic potential of NDMA

NDMA is a potent hepatocarcinogen in a wide range of animal species (Schmahl, 1981) and is also mutagenic in various systems including *S.typhimurium* (Bartsch *et al*, 1975). NDMA was not found mutagenic in the *Salmonella typhimurium* TA100 preincubation test using

mouse liver S9 (20 min preincubation time) and showed only weak mutagenicity in *S.typhimurium* TA 1535. This result contrasts with the known ability of NDMA to induce GC → AT (Guttenplan, 1987; Jiao *et al*, 1993). Nevertheless, some reports have shown that NDMA was mutagenic only when using a high concentration of rat liver S9 (28% compared with 10% used here) (Bartsch, 1981). NDMA mutagenicity appears to vary depending on the species used for the preparation of metabolic activation and rabbit liver homogenates were found to be more efficient in activating NDMA and induce mutagenicity than rat or mouse tissues (Bartsch *et al*, 1975). NDMA is an indirect carcinogen that requires metabolic activation to exert mutagenic and genotoxic effects (see figure 1.b. in general introduction) and the main enzyme involved in its activation appears to be CYP2E1 (Yang *et al*, 1990). It thus appears that Balb/c mouse liver S9 might not be very efficient at activating NDMA, although CYP2E1-dependent *p*-nitrophenol hydroxylation appears to be similar for rabbit and CR-1 mouse liver microsomes (Koop *et al*, 1989). CYP2E1 activity can be increased by pretreatment with acetone and other agents (Koop *et al*, 1989) (see 1.3.3.1.). The ability of NDMA to induce mutations was then assessed with acetone-induced mouse liver S9 and a positive response was seen in *S.typhimurium* TA100. Similar results were obtained with acetone-induced rat liver S9 (Ebata *et al*, 1993). Acetone also induces CYP2B as well as CYP2E1 in rat liver (Johansson et al, 1987). Evidence suggests that CYP2E1 is the major enzyme responsible for the low $K_M$ NDMA demethylase activity (α-activation leading to the formation of reactive methylcarbanion) in acetone-induced rat liver microsomes (Yoo *et al*, 1990, Yang *et al*, 1991), although CYP2B1 might also contribute but with less efficacy (high $K_M$) (Amelizad *et al*, 1988; Yang *et al*, 1990).

In the UDS assay in mouse hepatocytes NDMA was clearly genotoxic as evidenced by an increase in nucleotide excision repair and an increase in the percentage of cells in repair. This correlates with numerous studies using different type of cells such as HeLa cells (Martin *et al*, 1978) or rat hepatocytes (Williams *et al*, 1982; Shu and Hollenberg, 1996) where similar NNG values were reported. NDMA induced UDS in rat hepatocytes of untreated and phenobarbital-pretreated animals (induced CYP2B1) in a similar potency, but in rat hepatocytes of pyridine-pretreated animals (induced CYP2B1 and CYP2E1) NDMA was 3-4-fold more potent (Shu and Hollenberg, 1996), thus indicating that CYP2E1 was the major enzyme responsible for NDMA activation and only minor role (if any) for

CYP2B1. In mouse liver, CYP2E1 has also been shown to be the major enzyme involved in NDMA activation, although CYP2A5 may also contribute, but with less affinity (Camus *et al*, 1993). The levels of CYP2B1 in liver of rats and mice are minimal whereas constitutive levels of CYP2E1 are present (Nakajima *et al*, 1993); it is therefore clear that the activation of NDMA in mouse hepatocytes used here was mainly due to CYP2E1 with a possible contribution from other forms.

The induction of UDS by NDMA appears, therefore, not to be species-specific, which contrasts with the higher sensitive of rabbit liver homogenates in the *S.typhimurium* assay (Barsch *et al*, 1975), although correlates with the ability of NDMA to induce tumours in numerous animal species. NDMA is a methylating agent which has shown to yield $N^7$- and $O^6$-methylguanine. These are repaired by glycosylates leaving apurinic sites which in turn are repaired by endonucleases and polymerases, thus inducing the unscheduled synthesis of DNA (Guttenplan, 1987; Fan *et al*, 1991).

### 4.4.3. Inhibition by sulforaphane of NDMA genotoxicity

The effect of sulforaphane on NDMA genotoxicity was studied in both *S.typhimurium* and mouse hepatocytes. The results indicate that sulforaphane inhibited both reverse gene mutation of NDMA in *S.typhimurium* TA100 and UDS produced by NDMA in mouse hepatocytes in a concentration-dependent manner. Although sulforaphane was cytotoxic at relatively high concentrations (500 μM in bacteria and 40 μM in mouse hepatocytes), the concentrations at which sulforaphane gave a significant inhibition of NDMA genotoxicity were much lower (≥ 0.8 μM in bacteria and ≥ 0.064 μM in hepatocytes). The inhibition by sulforaphane of CYP2E1 activity in rat microsomes may be a basis for the inhibition of NDMA genotoxicity (which is mainly activated by CYP2E1, see above 4.4.2). The ability of sulforaphane to inhibit other CYPs than CYP2E1 and CYP1A has not been reported, although there is evidence indicating that sulforaphane is an effective inhibitor of nifedine oxidation in human hepatocytes (an indication of CYP3A4 activity) but not of CYP1B in rat hepatocytes (Ketterer, oral presentation at the ISSX-European Spring Workshop, 1996). It is thus likely that the modulation by sulforaphane of NDMA activation is only due to the CYP2E1 isoform.

Some isothiocyanates have been shown to be antimutagenic against UV and 4-nitroquinoline-1-oxide (4-NQO) (Kawazoe and Kato, 1982), both direct acting mutagens. These effects were observed in an excision repair proficient *E.coli* strain but not in the *uvrA*- mutant strain, thus suggesting the enhancement of excision repair as a possible mechanism. Furthermore, when *S.typhimurium* TA100 was used as the tester strain, isothiocyanates (BITC, PEITC, and PITC), dithiocarbamates and allylsulfide were devoid of antimutagenicity towards 4-NQO, which was associated only with the sulfidryl functional group (De Flora *et al*, 1994). The involvement of repair mechanisms on the antimutagenic effect of sulforaphane is thus unlikely since the bacteria strain used here, *S.typhimurium* TA100, is excision repair-deficient (Maron and Ames, 1983) and sulforaphane lacks sulfidryl groups. Furthermore, the inhibitory effects of sulforaphane in *Salmonella* were not seen with the direct acting agent sodium azide, thus supporting a critical involvement of modulation of metabolism for the inhibition of NDMA-mutagenicity. The antimutagenicity of cyclohexanol towards NNK and NDEA in *S.typhimurium* TA100 has also been attributed to the inhibition of their metabolism, since no effect was seen against direct acting agents (e.g. ethyl methanesulfonate) (Espinosa-Aguirre *et al*, 1993). The related isothiocyanate PEITC has also shown recently to inhibit NDMA mutagenicity in *S.typhimurium* TA100 *ex vivo*, i.e. using rat liver S9 from PEITC pretreated animals, by inhibiting NDMA metabolic activation (Knasmuller *et al*, 1996). In mouse hepatocytes, sulforaphane had no effect either on the incidence of S-phase cells and did not induce UDS, thus indicating the lack of effect of sulforaphane in repair systems and suggesting the modulation of metabolism as the major mechanism of inhibition of NDMA-induced UDS. The inhibition of diethylnitrosamine-induced UDS *ex vivo* by BITC was also associated with modulation of carcinogen metabolism, although suppression of cell proliferation also appeared to be involved, due to the ability of this isothiocyanate to reduce replicative DNA synthesis (S-phase) (Sugie *et al*, 1993). The latter effect was not observed with sulforaphane, thus further supporting the involvement of metabolism modulation.

It has been suggested that activation of nitrosamines might also be *via* *N*-oxidation catalysed by monoamine oxidases, however evidence indicates that this pathway is not involved in the initial step of the activation of nitrosamines to mutagenic species (Edmonson

and Bruice, 1985). Another potential mechanism of inhibition of NDMA mutagenicity is by scavenging reactive metabolites. One example of this is provided by disulfiram (Gichner and Veleminsky, 1988), however this only represents a second pathway for its inhibitory effect, modulation of metabolic activation was the major mechanism. Sulforaphane had no effect on the mutagenicity of the direct acting sodium azide, thus suggesting lack of scavenging properties. Nevertheless, the ability of sulforaphane to scavenge other reactive metabolites is unknown.

The concentrations at which sulforaphane gave statistically significant inhibition of NDMA genotoxicity were 0.064 μM in mouse hepatocytes and 0.8 μM in *S.typhimurium* and the $K_i$ for PNP hydroxylase activity in acetone-induced rat liver microsomes was about 37 μM. Several explanations exist for these differences in effective concentrations. Firstly, the inhibitory potential of sulforaphane in rat liver and mouse liver may differ. Another possible explanation could be related to incubation times; the exposure time in the UDS assay was of 20 h compared to 45 min preincubation time in bacteria (it is known that CYP450 activity in liver homogenates declines after 30 min incubation). Thus, the longer exposure time in mouse hepatocytes that in bacteria may result in an increased inhibition, which would support the hypothesis of irreversible inhibition suggested in section 3.4.. Sulforaphane was a potent inducer of GST and QR in mouse and a murine cell line (Zhang *et al*, 1992). The ability of some chemicals, such as oltipraz, to induce GSTs in cultured hepatocytes has been reported after only 4 h of incubation (highest induction after 24 h incubation, Langouët *et al*, 1996), so it is possible that during the 20 h of exposure of mouse hepatocytes to sulforaphane, induction of these enzymes might have occurred. Evidence has suggested that alkylating species generated from NDMA may be conjugated by GSH via GSTs (Frei *et al*, 1985), so it could be that the higher inhibition observed in mouse hepatocytes may partially be due to induction of detoxifying enzymes. The forms of GSTs induced by sulforaphane, however require characterization, since for example, the inducible murine GST Ya Ya has low activity towards several model substrates (McLellan and Hayes, 1989; Ketterer, 1988).

The results given here suggest that sulforaphane is unlikely to be mutagenic and that it may afford protection against carcinogens that are activated by CYP2E1 in rodent systems. However whether this will be relevant to humans is not known, since there are considerable

differences between human and rodents metabolic activation enzymes such as CYP450s (see section 1.3.6.) (Nedelvecha and Gut, 1994). The study of the ability of sulforaphane to modulate genotoxicity mediated by human CYP450s was thus necessary.

# *CHAPTER 5*

## *Inhibition by sulforaphane of NDMA- and IQ-induced DNA strand breaks in human cells expressing human CYP2E1 and CYP1A2 respectively*

## *5.1. Introduction*

The use of homogenates and subcellular fractions from rodent organs, as well as *in vivo* studies in several rodent species has provided valuable information about the metabolism and reactive intermediates produced by pro-carcinogens. However, the metabolic fate and reactivity of such chemicals in humans may be different, due to inter-species differences (section 1.3.6. in introduction), thus necessitating the assessment of these chemicals in human models. Moreover, public concern and legislation have encouraged the reduction of animal studies and the use, where possible, of cell culture systems.

The use of primary cell culture from several human tissues has provided a valuable tool for the extrapolation of laboratory animal studies to humans, together with epidemiological data (Harris,1987; Abbot, 1992). Valuable *in vitro* data have been obtained by using microsomes and homogenates, primary cell cultures or tissue slices. Primary human hepatocytes are of particular interest as a model for *in vitro* toxicological studies and have provided a closer insight into the metabolism of xenobiotics in the human liver (Butterworth *et al*, 1989; Smith and Chipman, 1988). However, the use of human hepatocytes presents several disadvantages. Firstly, they have a short life span, and secondly, cytochrome P450 activity decreases considerably after 24 h (Guillouzo *et al*, 1985). The development of specific culture media and the use of matrix or co-culture with several cell lines have improved and extended the lifetime and cytochrome P450 function of cultured human hepatocytes (Gibson-D'Ambrosio *et al*, 1993). However, the limited access to human tissue and interindividual variability makes it difficult to use isolated cell culture as a model to evaluate xenobiotics and carcinogens in humans.

### 5.1.1. Potential use of cell lines expressing human P450 cDNAs

Established human cell lines with an indefinite life span have developed over the last few years and could be used as an alternative to human tissue (Harris, 1987). However, most of these cell lines have limited or no phase I enzyme expression, therefore may not be able to metabolically activate certain carcinogens. Such is the case of HepG2 cells, which have CYP1A activity, and thus activate a range of carcinogens such benzo[a]pyrene or 2-

acetylaminofluorene, however other carcinogens such as nitrosamines are not activated due to lack of CYP2E1 activity (Rueff *et al*,1996). This defect could be overcome by addition of exogenous activation systems containing human P450s, nevertheless this also presents further additional problems. Firstly, the use of large amounts of human tissue (containing human P450s) and variability of P450 content in different samples (Smith and Chipman, 1988). Secondly, the use of "exogenous" activation systems does not reflect the *in vivo* situation accurately, since reactive intermediate metabolites are generated outside the cell, needing to be transferred across the plasma membrane to reach intracellular targets (Crespi *et al*, 1991). In an attempt to overcome these limitations, cDNAs encoding human P450 isoenzymes have been introduced into the target cell DNA. Heterologous expression of human P450s has successfully been accomplished in several expression systems, including *Saccharomyces cerevisiae*, *E. coli* and in various mammalian systems (Gonzalez and Korzekwa, 1995). Systems such as *Saccharomyces cerevisiae* also require the transfection of NADPH:cytochrome P450 reductase which is needed for the activity of CYP450.

The expression of human cDNAs into mammalian cells can be accomplished via several systems. The SV40 promoter-vector has been used to express human CYP1A1, 1A2, 3A4 and 3A5 in the COS cells (Robets-Thomson *et al,* 1993), or rat CYP1A1, CYP1A2 and CYP2B1 and human 2E1 in the V79 Chinese hamster cells (Ellard *et al*, 1991, Schmalix *et al,* 1995). The EBV-HSVTK vector has been used to express human CYP1A1, 1A2, 2A6, 2E1, and 3A4 in a human B lymphoblastoid system (AHH-1 cells) (Crespi *et al* 1993). Also, HepG2 cells have successfully been stably transfected with human CYP1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 2F1, 3A4, 3A5, and 4B1 using vaccine virus vectors (Waxman *et al*, 1991). All these systems contain endogenous NADPH:cytochrome P450 oxidoreductase and cytochrome $b_5$, ensuring cytochrome P450 activity and also express some of the phase II enzymes.

The potential use of these types of cells in drug metabolism and toxicological studies is promising. The specific forms of cytochrome P450 responsible for the activation of a drug or carcinogen can be easily demonstrated using a battery of cell lines expressing different cytochrome P450 isoenzymes. In contrast, the use of human liver microsomes or hepatocytes, which contain complex mixture forms of P450s, is a laborious study requiring

the use of inhibitors which are often not specific. Another advantage of using cell lines expressing individual P450 isoforms could be the identification of isoforms that may play a role in extrahepatic tissues, but have a minor role in the liver (Crespi *et al*, 1993). However, the information obtained from single CYP450-expressing cells should be used cautiously since they present some limitations. Looking at only one P450 isoform may be potentially misleading, since the metabolic pathway of carcinogens might be dependent on several routes (i.e. more than one P450 form might be involved). The use of human liver microsomes or hepatocytes combined with the information obtained from the cell lines would evaluate the overall metabolism. This limitation would partially be overcome by the establishment of cell lines expressing multiple forms of human CYP450s. For example Crespi *et al.* have successfully introduced four different forms of P450 and epoxide hydrolase into a human lymphoblastoid cell line (Crespi *et al*, 1991). However a limitation of this approach is that the level of relative expression of CYPs will not resemble the level in primary cells. Hence, the two methods combined are currently needed to assess the relevance of drug metabolism and carcinogen activation by human CYP450s.

The determination of the specific forms of P450 involved in metabolism can also contribute to the explanation of high interindividual variation response to drugs. Genetic polymorphisms have been identified for several CYPs (see introduction 1.3.5), so the identification of the isoforms involved in carcinogen metabolism may be helpful in predicting populations at risk of developing cancer.

Cell lines expressing individual human cytochrome P450 isoenzymes can also be used to measure a variety of toxicological endpoints (Rueff *et al*, 1996). Cytotoxicity can be assessed using the neutral red assay (Schmalix *et al*, 1993), leakage of cytoplasmic lactate dehydrogenase (Mapoles *et al*, 1995) or cell survival. Various genotoxic endpoints have also been measured in some of these cell lines. For example, mutagenicity was assessed by measurement of 6-thioguanine resistance in the V79 Chinese Hamster cells expressing CYP2E1 (Schmalix *et al*, 1995). Gene locus mutations at the hypoxanthine phosphoribosyltransferase (*hprt*) and thymidine kinase (*tk*) and induction of chromosome aberrations and micronuclei assay have been measured in the AHH-1 lymphoblastoid cell line (Langenbach *et al*, 1992). Metabolic activation of carcinogens can be studied by

analyses of DNA adducts (Crespi *et al*, 1993) or measurement of DNA strand breaks (Davies *et al*, 1995).

### 5.1.2. Human epithelial cell line expressing human P450 cDNA isoenzymes.

Human epithelial liver cells (THLE) have been established in serum-free medium (Macé *et al*, 1996a; Pfeifer *et al*, 1993 and 1995). Immortalization of primary liver cells was achieved using a recombinant Simian virus 40 large T antigen virus. The human epithelial liver cells (THLE) have an indefinite life span and are non-tumourigenic. The THLE cells have typical epithelial morphology and are hypodiploid, with most karyotypes being near-diploid (Macé *et al*, 1996). They divide rapidly in culture with an average population doubling time of 24 hours and a colony-forming efficiency of about 15 %. THLE cells express various hepatocyte-specific features, such as albumin, transferrin and fibrinogen. Cytokeratin 18, present in human hepatocytes, was uniformly expressed in early-passage cells, whereas at later passages all cells also expressed cytokeratin 19. Although cytokeratin 19 is not normally associated with hepatocytes, it has been observed in primary cultures of adult liver cells and has been linked with undifferentiated cells. These suggest that the THLE cells have a cell phenotype in between liver stem cells and hepatocytes (Pfeifer *et al*,1993 and 1995).

The THLE cells express mRNA of phase II enzymes such as epoxide hydrolase, glutathione *S*-transferase $\pi$, glutathione peroxidase, NADPH reductase, superoxide dismutase and catalase (Pfeifer *et al*, 1993). CYP1A1 expression can be induced by exposure to benzo[*a*]pyrene to a similar level to that seen in HepG2 cells (Pfeifer *et al*, 1993; Macé *et al*, 1996) but is not detectetable without induction. The cells also express CYP1A2, however levels are at least 10 times lower than hepatocytes or genetically engineered CYP1A2-expressing cells. CYP3A4 and CYP2C expression was also detected at low levels by RT-PCR, but other CYPs were not detected (Macé *et al*; 1996).

In order to increase the metabolic activity of these cells, a panel of human P450-expressing human epithelial liver cell lines have been established (Macé *et al*, 1996). Human cDNAs corresponding to CYP1A2, 2A6, 2B6, 2E1 and 3A4 have been introduced into the THLE cells (Macé *et al*, 1997). The procedure for establishment of THLE cells stably expressing

human P450 isoenzymes was similar to that used for the establishment of BEAS-2B (human bronchial epithelial cell line) cells expressing P450s (Macé *et a,l* 1994). Briefly, CYP450 cDNAs were cloned into the plasmid pCMVneo which contains the cytomegalovirus (CMV) promoter, the neomycin (*neo*) gene conferring G418 resistance and the polyadenylation site from the rabbit β-globin gene. The pCMV-CYP450 vectors were introduced into the cells by liposome-mediated transfection. After 48 hours the cells were selected for G418 resistance and subsequently cloned (Macé *et al*, 1997).

The CYP450-expressing THLE cells, like the BEAS-2B (Macé *et al*, 1997), are potential candidates for pharmaco-toxicological studies, since they have retained the characteristics of the parental cells and express various phase I (determined by the genes inserted) and phase II enzymes. CYP450 activities of the transfected enzymes are comparable to human liver CYP450 activities. The activity of various CYPs is shown in Table 5.a. Several genotoxic studies have shown promising results. For example, the activation of aflatoxin $B_1$ has been studied in the CYP1A2, 2A6 and 3A4 cell lines by measurement of formation of DNA adducts and induction of p53 mutations (Macé *et al*, 1997).

Table 5.a. Activity of several human CYPs transfected into THLE cells

| CYP | Activity measured | pmol/min/mg protein |
|---|---|---|
| CYP1A2 | Methoxyresorufin-O-deethylase | 41 ± 5 |
| CYP2A6 | Coumarin hydroxylase | 869.4 ± 100 |
| CYP2E1 | Chloroxazone-6-hydroxylase | 55.0 ± 0.8 |
| CYP3A4 | Testosterone hydroxylase | 260.4 ± 90 |

From Macé *et al*, (1996b) and personal communication.

CYP1A2 activity in the parental THLE cells was 0.17 ± 0.10 pmol/min/mg protein (Macé *et al*, 1996a).

### 5.1.3. DNA strand breaks as a sensitive, non-specific marker of DNA damage

Genotoxic carcinogens have been shown to induce mutations, which can result from several types of DNA modifications (Venitt and Parry, 1984) (see section 1.1.4.). The utility of short term tests such as mutagenicity tests (e.g. *Salmonella typhymurium* mutagenicity) or DNA repair tests (e.g. UDS) for the detection of genotoxic carcinogens has already been discussed (see section 4.1.). Another valuable endpoint in genotoxicity testing is the measurement of DNA strand breaks, which has been used as an indicator of genetic damage or mutagen exposure in several assays. Some of the most commonly used assays are based on the alkaline elution, alkaline sucrose sedimentation, nucleoid sedimentation, single cell electrophoresis assay or pulsed field gel assays (Hoffmann, 1996; Fairbairn *et al*, 1995).

DNA strand breaks can be formed as a result of the direct interaction of a carcinogen with DNA or may appear during the excision repair of DNA adducts. They may also arise from alkali labile sites introduced into the DNA and manifested at high pH. Many genotoxic and mutagenic chemicals have also been found to induce DNA strand breaks and a good correlation has been found between induction of DNA strand breaks and the carcinogenic and mutagenic potential of certain chemicals (Sina *et al*, 1983). Some examples of model agents that induce DNA strand breaks are $H_2O_2$, bleomycin, and radiation. Other chemicals that may induce DNA strand breakage include reactive metabolites of NDMA (Ashby *et al*, 1995) and the heterocyclic amine 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Davies *et al*, 1995). IQ has been found to be highly mutagenic and genotoxic as assessed in various systems (Overvik and Gustafsson, 1990) and is formed as a pyrolysis product during the cooking of meats and fish at high temperatures (Layton, 1995).

### 5.1.4. The single cell gel electrophoresis assay

The single cell gel electrophoresis assay (SCGE) is a rapid and sensitive method for measuring and analysing DNA strand breaks in mammalian cells. The (SCGE) assay was first developed by Singh *et al* (1988) as a modification of the Ostling and Johanson (1984) microgel electrophoresis. This latter technique used neutral conditions during electrophoresis to detect double-stranded DNA breaks in cells embedded in agarose gel.

One disadvantage of this technique was that single-stranded DNA breaks could not be detected. Singh's modification consisted mainly in the use of alkaline conditions, thus permitting the evaluation of single-stranded DNA breaks and alkali-labile sites. Later modifications have been introduced by several authors and numerous protocols are currently used (see Fairbairn *et al*, 1995 and McKelvey-Martin *et al*, 1993 reviews). Briefly, cells are embedded in an agarose gel on microscope slides and lysed in a mildly alkaline solution, containing salts and detergents, to allow denaturing of proteins and DNA unfolding. Electrophoresis is then performed in a highly basic solution, allowing damaged DNA to migrate towards the anode (for more details see the methods section). Cells with increased DNA damage display increased migration of DNA from the nucleus towards the anode. The SCGE assay is also named the "Comet assay" because the DNA that migrates from the nuclei resembles a comet. Several parameters can be used to quantify the DNA damage when data are analysed in a computerised system: percentage of DNA in the "tail of the comet", tail length (distance from the edge of the cell nuclei (head) to the end of migrated DNA) and tail moment (product of % tail DNA and tail length). The tail length is perhaps the most commonly used parameter, probably because it can be estimated without the computerised system. However, tail moment and percentage of tail DNA are regarded as the most appropriate parameters to quantify the extent of DNA damage (Fairbairn *et al*, 1995).

The principle of the assay seems to be related with the organisation of DNA within the nucleus. DNA in the nucleus occurs as supercoiled loops organised in nucleosomes, which are further organised to form chromatin (Simpson, 1978; Macleod, 1995). After lysing with detergents and extraction of proteins with high salt, the DNA remains in the nucleoid. The presence of DNA strand breaks relaxes the supercoiled structure and the loops are then free to extent outside the nucleoid when an electric field is applied (electrophoresis) (Ross *et al*, 1995; McKelvey-Martin *et al*, 1993). Alkaline conditions propitiate the migration of DNA fragments from the nuclei by disrupting the DNA base-pairing, resulting in denatured and flexible DNA in the open loops (McKelvey-Martin *et al*, 1993).

The Comet assay presents several advantages compared to the other techniques that detect DNA strand breakage (see above). First of all, the Comet assay is a simple, inexpensive and

rapid technique and results can be obtained in one day when data analyses are computerised. Secondly and most important, DNA strand breakage is measured in individual cells, thus allowing the detection of intercellular differences in DNA damage and repair in cell populations. Furthermore, it can be used in virtually any cell population that can be obtained as a single cell suspension and only a small number of cells is required (1000 - 10000). Another major advantage is its sensitivity. Comets have been detected at one break per $10^{10}$ daltons of DNA (Collins *et al*, 1992). However, the Comet assay also presents some limitations, such as possible induction of DNA damage due to cytotoxic effects (McKelvey-Martin *et al*, 1993) or the activation of endonucleases by $Ca^{2+}$ release (McConkey *et al*, 1989).

Despite the recent development of the Comet assay, numerous applications in different fields have already emerged, such as radiation biology (Olive *et al.*, 1994), DNA damage and repair studies (Collins *et al*, 1995, Speit and Hartmann, 1995) or biomonitoring of human populations for susceptibility to oxidative damage (Anderson *et al*, 1994, Green *et al*, 1994, Holz *et al*, 1995) or other agents, such as tobacco smoking (Sardas *et al*, 1995.). Of particular interest to this project is its potential in genotoxicity testing. The Comet assay has already proved useful in the detection of several genotoxic agents in different cell types and rodents, and issues such as metabolic activation, cell-specificity, or dose-response relationships can be studied using this technique (Fairbairn *et al*, 1995; Mckelvey-Martin *et al*, 1993; Monteith and Vanstone, 1995; Vaghef and Hellman, 1995).

In order to assess the effect of sulforaphane on human CYP1A2 and CYP2E1, the different cell lines T5-1A2 cl5.3 and T5-2E1 cl2 expressing human CYP1A2 and CYP2E1 respectively were used. Also a control cell line lacking transfected CYP450-cDNA, T5-neo cl5.16 was used. The latter cell line was established by transfection with a control plasmid containing the neomycin resistance gene but no cytochrome P450. Because of the advantages of the Comet assay mentioned above, this was chosen for the analyses of genotoxicity of NDMA (CYP2E1 activated) and IQ (CYP1A2 activated) and the ability of sulforaphane to modify it in the THLE cell lines expressing human CYP cDNAs. Due to the length of this chapter, the results sections were subdivided into Part I (cell culture) and Part II (genotoxicity studies with the Comet assay).

## *5..2. Methods*

The chemicals used in these experiments were purchased from Sigma-Aldrich or BDH unless otherwise stated. IQ was from Toronto Research Chemicals Inc., Ontario, Canada. Fully frosted microscope slides were purchased form Appleton Woods, Birmingham, UK.

### 5.2.1. Cell culture

#### 5.2.1.1. Solutions

All solutions were prepared under sterile conditions in a Class II laminar flow Cytotox safety cabinet.

Coating medium

Coating medium used for precoating cell culture flasks (see 5.2.1.2.) was kindly provided by A.M.A.Pfeifer, Nestlé Research Centre, Lausanne, Switzerland. LHC basal medium (Biofluids, Rockville, USA) contained human fibronectin (0.01 mg/ml), Vitrogen 100 (purified bovine collagen for cell culture) (Collagen Corp. Palo Alto) (0.01ml/ml) and bovine serum albumin (BSA) (Biofluids, Inc., Rockville) (0.1 mg/ml). The solution was filtered through a 0.22 μm filter.

Culture medium

The culture medium was PMFR-4 low calcium (Biofluids) and contained several additives, as described in Table 5.b.. The solution was incubated at 37 °C for 10 minutes in order to dissolve all the different ingredients and then filtered a 0.2 μm disposable sterile bottle top filter (Corning). The complete culture media was kept below 4 °C for up to two weeks.

Freezing medium

The cell culture freezing media was prepared by mixing equal volumes of solution A and B. Solution A was L-15 medium containing Hepes (20 mM), FCS (20 %), 200 units penicillin (200 units/ml) and streptomycin (200 μg/ml). Solution B was L-15 medium containing Hepes (20 mM), DMSO (15 %) and polyvinylpyrrolidone (2 %).

Phosphate Buffer Saline (PBS)

Sterile PBS was prepared by dissolving one tablet of phosphate buffer saline (Oxoid) in 100 ml of distilled water and then autoclaving for 20 min.

Trypsin-EDTA solution

EDTA-disodium salt (0.025 %) was dissolved overnight at 37 °C in a sterile PBS solution. Then trypsin (0.025 %) (from Porcine pancreas, Sigma) was added to the solution and left at 37 °C until it was dissolved. The final solution was filter-sterilised through a filter membrane (0.2 μm)

Table 5.b. Additives to PMFR-4 Culture medium

| Compound | final conc. | volume | stock conc. |
|---|---|---|---|
| L-glutamine (Gibco) | 2 mM | 5 ml | 200 mM |
| Gentamycin (Gibco) | 50 μg/ml | 2.5 ml | 10 mg/ml |
| Insulin (Biofluids) | 1.75 μM | 2.5 ml | 0.35 mM |
| Hydrocortisone (Biofluids.) | 0.2 μM | 10 μl | 10 mM |
| EGF[1] (Sigma) | 5 ng/ml | 0.5 ml | 5 μg/ml |
| Transferrin (Sigma) | 10 μg/ml | 1.0 ml | 5 mg/ml |
| P/E[2] (Biofluids) | 50 nM | 2.5 ml | 0.1 mM |
| $T_3$[3] (Biofluids) | 50 nM | 25 μl | 1 mM |
| BPE[4] (Sigma) | 7.5 μg/ml | 1.25 ml | 6 mg/ml |
| RA[5] (Sigma) | 0.33 nM | 50 μl | 3.3 μM |
| FFS[6] (UBI) | 3 % | 15 ml | 100 % |

1: Epidermal growth factors
2: Phosphoethanolamine
3: Triiodothyronine, thyroid hormone, promotes intracellular metabolism.
4: Bovine pituitary extract
5: Retinoic acid
6: Factor Free Serum (chemically denatured fetal bovine serum)

### 5.2.1.2. Cell Culture

All procedures were performed under sterile conditions in a Class II laminar flow cytotox safety cabinet.

Cell culture flasks (Falcon, 25 $cm^2$, 50 ml) containing coating media (1 ml) were incubated at 37 °C for 30 min. The exceeding coating medium was kept in a new container for later use (coating media was reused up to 3 times).

The frozen cells contained in a cryovial were left for 1 min at room temperature and then at 37 °C for 2-3 min until completely thawed. The cells were gently mixed using a 1 ml sterile pipette and then plated into three precoated flasks, containing 3-5ml of culture media at 37 °C. The cells were grown at 37 °C and 3.5 % $CO_2$ in a $CO_2$ incubator (Sanyo, model MCO-17A). When the cells were attached to the bottom surface of the flasks (examined under a microscope), approximately after two to three hours of seeding them, the culture medium was removed and fresh medium (7 ml) prewarmed at 37 °C was added into the flasks. The culture medium was removed when required and fresh medium was added. All flasks containing cells were examined daily under the microscope.

The cells were subcultured when they had reached a monolayer (3 - 5 days). The culture medium was removed and cells were washed once with prewarmed PBS (1-2 ml). One ml of prewarmed trypsin-EDTA solution was added into the flask and was left for 5 minutes at room temperature until most of the cells detached from the flask surface. After addition of HBBS containing 2.5 % FCS (to stop trypsination), cells were centrifuged at low speed in a bench centrifuge for 3 minutes. The medium was removed and the cells were either resuspended in freezing media (1 ml) or resuspended in culture medium (1 ml) for subculturing. The cells were split at 1:3 to 1:5. The cells were gently mixed with a 1 ml plastic pipette and plated into precoated flasks as described above.

## 5.2.2. Cell count

Cells were trypsinised as described in the previous section and resuspended in 1 ml of culture medium or PBS. A small volume of cell suspension was transferred to the chamber of a Neubauer haemocytometer by capillarity. For samples containing > $10^6$ cells/flask, cell suspension was diluted 1:10. The cells were counted in five 1mm centre squares. Cell count was calculated as the average count per square × $10^4$ (× dilution factor when needed).

## 5.2.3. EROD activity and protein content in 96-well plate

EROD activity and protein content were determined in T5-1A2 cells cultured in 96-well plates (Packard, UK) for 24 hours. The method was based in that of Lorenzen and Kennedy, (1993) and Kennedy and Jones, (1994) with some modifications.

Sodium phosphate buffer 50 mM, pH 7.4 was used for the determination of EROD activity. Sodium phosphate buffer 50mM, pH 8.0 was used for the determination of protein content. Reagents ethoxyresorufin and dicumarol were dissolved in DMSO to a final concentration of 1 mM and then diluted to the desired concentration (50 µM and 100 µM respectively) with sodium phosphate buffer 50 mM, pH 7.4. Fluorescamine was dissolved in acetonitrile (300 µg/ml). Resorufin standard was dissolved in MeOH (25 µM) and BSA was dissolved in phosphate buffer.

Cells were rinsed twice with PBS in order to eliminate traces of phenol red from the culture medium. Cells were incubated at 37 °C with phosphate buffer 50 mM, pH 7.4, ethoxyresorufin (0 - 20 µM), and dicumarol (10 µM) for 15, 30, 45 and 60 min. When the reaction was concluded, media was transferred into clean 96-well plates and fluorescence was measured at excitation and emission wavelengths of 530 and 590 nm respectively in a plate reader (Perkin Elmer Luminiscence Spectrometer LS50B). A resorufin standard curve was prepared (0 - 1 µM) in phosphate buffer 50 mM, pH 7.4.

Cells were washed with PBS and phosphate buffer 50mM, pH 8.0 and fluorescamine (100 µg/ml) were added and incubated at 37 °c for 15 min. A protein standard curve was

prepared with BSA (0 - 75 μg/ml) to which fluorescamine was also added. Fluorescence was measured at excitation and emission wavelengths of 400 and 460 nm respectively (slit widths of 15 and 20 nm respectively).

In attempt to measure total resorufin produced (i.e. free-resorufin and conjugation products of resorufin) β-glucoronidase (type H-1, Sigma) (1mg/ml) dissolved in acetate buffer, 0.1 M, pH 4.5 was added to the supernatant and incubated for 2 h (Donato *et al*, 1993). This was not useful since turbidity due to the enzymes interfered with fluorescence measurements.

### 5.2.4. Protein Determination

Protein content of the cells was determined by the method of Bradford (1976). Biorad solution was prepared from Bio-rad Protein assay solution and was diluted 1:5 in distilled water and filtered. The solution was stored in the fridge for up to two weeks. Protein standard was prepared with bovine serum albumin (BSA) (Sigma). A solution of 1mg/ml was prepared and then diluted 1:10 to obtain a stock solution of 0.1 mg/ml

Cells were washed with PBS twice and processed for protein determination (or frozen until needed). One ml of PBS was added to 6-well plates containing cells and the cells were scraped with a rubber policeman. Cells were sonicated (10 s), 50 μl of cell sample was diluted into 100 μl of PBS and 1 ml of diluted Biorad solution was added. A standard curve was prepared from BSA 0.1 mg/ml solution (0-50 μg/ml) and 1 ml of biorad solution was also added. Samples were mixed and left standing for 15 min. Absorbance at 595nm was recorded between 15 and 30 min of addition of the Biorad solution.

### 5.2.5. EROD activity in 6-well plates.

T5-neo and T5-1A2 cells were cultured in 35-mm (6-well) plates as described in section 5.2.1. Ethoxyresorufin deethylase activity was measured after 24 hours when cells were nearly confluent. All solutions and reagents were warmed to 37°C prior to addition to the cells. Phosphate buffer (50 mM, pH 7.5) was prepared by addition of a solution of

$NaH_2PO_4$ (50 mM) to a solution of $Na_2HPO_4$ (50 mM) until required pH was reached. Ethoxyresorufin and resorufin were dissolved in MeOH (1 mM) and then diluted to the desired concentration in distilled water. Dicumarol was dissolved in DMSO (10 mM) and then diluted in distilled water. Sulforaphane was dissolved in distilled water.

Plates were preincubated for 30 min in fresh PMFR-4 with or without 10 μM sulforaphane (10 μl of 1 mM solution). The plates were washed twice with PBS at 37 °C to remove phenol red (present in culture medium) and sulforaphane. The substrate, ethoxyresorufin (10 μM) (100 μl of 100 μM solution), dicumarol (10 μM) (10 μl of 1 mM solution) and when required 10 μM sulforaphane (10 μl of 1 mM solution) were added to the cells in 1 ml of phosphate buffer. The reaction proceeded for 30 min at 37 °C. The reaction mixture was removed from the plates and the cell monolayers were rinsed twice with PBS. The cells were frozen and saved for protein determination. The supernatant was centrifuged for 2 min at low speed in order to remove cells. The fluorescence of the resorufin product was measured in the supernatant with an excitation wavelength of 530 and an emission wavelength of 590 nm. A standard curve was prepared using resorufin (0 - 0.15 μM, stock solution 5 μM).

### 5.2.6. PNP hydroxylase activity

This assay was based on that one of Koop *et al* (1985) previously described (see section 3.2.4), with some modifications (Gomez-Lechón, personal communication). Activity was measured after incubation of cells for 30 min with HEPES-saline solution (pH 7.5) containing $CaCl_2$ (1 mM), sodium pyruvate (20 mM) ascorbic acid (1 mM), p-nitrophenol (0.5 mM).

## 5.2.7. Standard Comet assay

This was based on the method of Singh *et al*, (1988) with some modifications.

### 5.2.7.1. Solutions

Agarose: "Normal Melting Point" (NMP) agarose was used at the concentration of 0.5 % in calcium and magnesium free phosphate buffer saline (PBS) (see section 5.2.1). "Low Melting Point" (LMP) agarose was prepared at 0.5% and 0.75% in PBS. Agarose was dissolved by heating to boiling in a microwave and stored at 4 ° C until needed.

Lysing solution: A solution of distilled $H_2O$ containing NaCl (146.1 g, 2.5 M), EDTA disodium salt (37.2 g, 100 mM), Tris base (1.2 g, 10 mM) was prepared (concentrations are final concentration for a volume of 1 l). The solution pH was set to 10.0 by addition of solid NaOH (approximately 12 g). *N*-Lauroylsarcosinate (10 g, 1%) was added to the solution, which was heated until the *N*-lauroylsarcosinate was completely dissolved. The volume was adjusted to 1 l and then the solution was filter-sterilised (0.45 μm disposable sterile bottle top filter, Corning) and stored at 0-4 ° C. The complete lysing solution was prepared fresh before use by adding 10 % DMSO and 1 % Triton X-100 to the lysing solution and then refrigerated for 60min prior to slide addition.

Electrophoresis buffer: The electrophoresis buffer freshly prepared contained NaOH (75 mM) (60 ml NaOH 2.5 M) and EDTA (1 mM) (10 ml EDTA 200 mM) in UHQ water (2 l) and was stored at less than 15 ° C until use.

Neutralising solution: The neutralising solution was 0.4 M Tris HCl, pH 7.5.

Staining solution: Ethidium bromide (20 μg/ml) was freshly prepared by diluting ethidium bromide 200 μg/ml in UHQ water (1:10) and then filter-sterilised through a filter membrane (0.2μm).

### 5.2.7.2. Preparation of slides

Single cell suspensions (T5-neo, T5-2E1 and T5-1A2 cells) were obtained from the monolayers either by trypsination (see section 5.2.1.) or by scraping with a rubber policeman. Cells were centrifuged at low speed in a bench centrifuge and resuspended by adding 10 µl of PBS.

LMP and NMP agarose were melted and kept at 37 °C and 50 °C respectively. NMP agarose (100 µl) was added to the fully frosted microscope slide and a glass coverslip was added carefully to minimise bubbles. The slide was placed on a metallic tray on ice and the agarose was left to solidify for 10 min. The coverslips were gently removed and 75 µl of LMP agarose were added to the cells, mixed gently and the mixture (~85 µl) was added to the slides on top of the LMP agarose. The coverslips were replaced and the slides placed on the frosted tray for 10 min. The coverslips were gently slid off and a third layer of LMP agarose (75 µl) was added, coverslips were replaced and the slides returned to the frosted tray for another 10 min. The coverslips were again removed and the slides were placed into cold complete lysing solution for 1 h at 4 °C in the dark.

### 5.2.7.3. Electrophoresis

Slides were removed from the lysing solution and placed on the horizontal tray of the electrophoresis tank (Pharmacia, GNA200). The tank was then filled up with cold electrophoresis buffer (2 l). The slides were maintained in the alkaline buffer for 20 minutes to allow unwinding of DNA at room temperature.

Electrophoresis was then run at room temperature for 20 min at 25 V and approximately 300 mA (Power Pack from Pharmacia, LKB, GPS200/400). The slides were removed gently and washed three times with "Neutralisation solution" (5 min each wash). All these steps were performed under yellow light.

### 5.2.7.4. Staining

Slides were stained with 50 µl of freshly diluted (×10), filtered ethidium bromide (excess was dried off) and covered with a glass coverslip. Slides were kept in a moist environment and scored within 24-48 hours of electrophoresis.

### 5.2.7.5. Evaluation of DNA damage

The slides were analysed under a fluorescent microscope Axiovert 10 (Zeiss, Germany) at 250× magnification (20/0.45) with an excitation filter of 515-560 nm, from a mercury lamp and a barrier filter of 590 nm.

Parameters of comets (% tail DNA, tail moment and tail length (µm)) were automatically analysed on a computer using a "Komet" (Kinetic Imaging Ltd.) image analysis system. The system setup for these experiments was as follows: head threshold 5, tail threshold 10, smoothing value 1, background height 20 and tail break length 10.

## 5.2.8. Modified Comet assay

### 5.2.8.1. Cell culture

Fully frosted microscope slides were cut to fit in a 35mm 6-well cell culture flasks and were placed in 6-well cell culture flasks and precoated with coating media (see section 5.2.1.) for 30 min at 37 °C. Meanwhile confluent cells from T25 flasks were trypsinised (see section 5.2.1.), counted and resuspended in fresh media. Coating media was removed and slides were washed 2-3 times with PBS in order to eliminate phenol red. Cells ($1\text{-}5\times10^4$ cells/slide) were plated on to the slides in 2ml of complete PMFR-4 media and cultured for 2-4 h. Cells were treated with $H_2O_2$ (25 µM) for the positive controls, NDMA (0.01 -1 µg/ml), IQ (0.1 -10 µg/ml), or aflatoxin (0.1- 10ng/ml) as required and sulforaphane (0.1 - 10 µM) when required.

#### 5.2.8.2. Agarose slides preparation

Slides were washed 2-3 times with PBS in order to eliminate residues of phenol red from the incubation media. LMP agarose (0.75%, 100 µl) was added to the slides and covered with a coverslip. After 10 min on a frosted metallic tray the coverslip was removed and the slides were placed into cold complete lysing solution for 30 min at 4 °C in the dark.

#### 5.2.8.3. Electrophoresis, staining and evaluation of DNA damage

Electrophoresis and evaluation of DNA damage was as described previously in section 5.2.7. Slides were stained with 25 µl of diluted ethidium bromide (20µg/ml).

### 5.2.9. Statistical analyses

Data were analysed using parametric tests (ANOVA, two sample *t*-test, linear regression) and non-parametric tests (Kruskal-Wallis and Mann-Whitney tests) using Minitab software. Analyses were performed using n=3 and n= total cell population. In the results section, when there is only one *P* value, this means that both tests gave the same results. If there were any differences, the test used was indicated.

## *5.3.I. Result Part I. The culturing of THLE cells expressing human CYPs s*

### 5.3.I.1. Cell culture growth

T5-neo/1A2/2E1 cells were grown in monolayers as described in section 5.2.1. Cells were plated ($10^5$ cells/flask) and attached to the flasks surface within 2 - 3 hours after seeding. The cell growth curve for the T5-neo cells is shown in Figure 5.I.a. Cell growth was characterised by an initial lag phase (days 0 - 1) followed by an exponential growth phase (days 2 - 5) where the cell doubling time was approximately 24 hours, and finally reaching a stationary phase to reach a terminal cell density of approximately $10^6$ cells/flask. Cells in stationary phase lasted up to one day. After this time, cells started to detach form the surface and consequently die. Cells were then trypsinised and subcultured.

T5-2E1 cell growth curve was similar to that of T5-neo cells (Figure 5.I.b). An initial lag phase (days 0 - 2) was followed by an exponential growth phase (days 3 - 5) reaching the stationary phase by day 6 ($1.2 \times 10^6$ cells/flask). T5-1A2 growth was also similar to the above described, with cells reaching the stationary phase by day 4 - 5. During the course of the following experiments (also chapter 6), T5-neo, 1A2 and 2E1 cells were seeded at a higher density ($2 \times 10^5$ cells/flask), so the stationary phase was reached in a shorter period (3 -5 days).

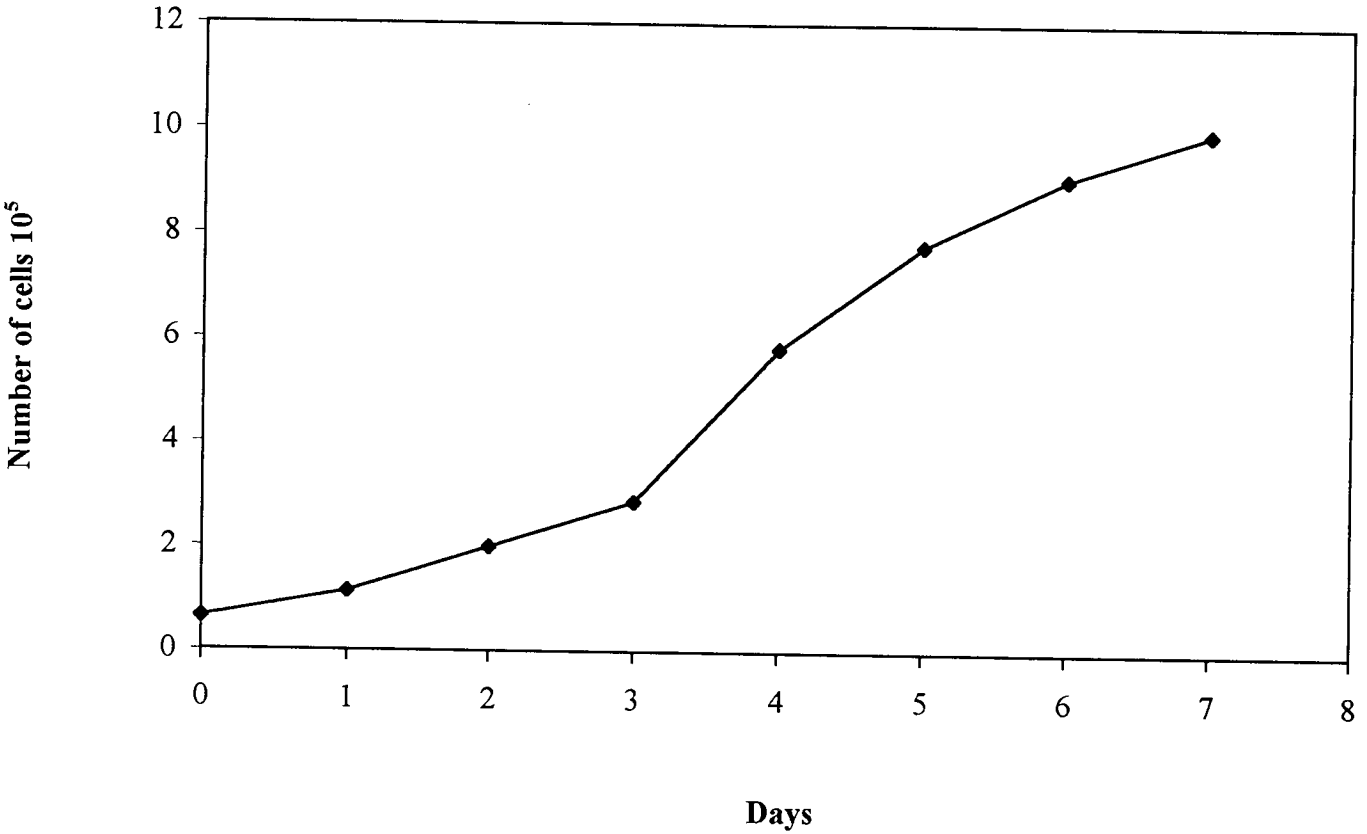



Figure 5.I.a. Cell growth curve of T5-neo cells

Cells were grown in monolayers as described in section 5.2.1., trypsinised and counted. Values are the mean of n = 2 flasks.

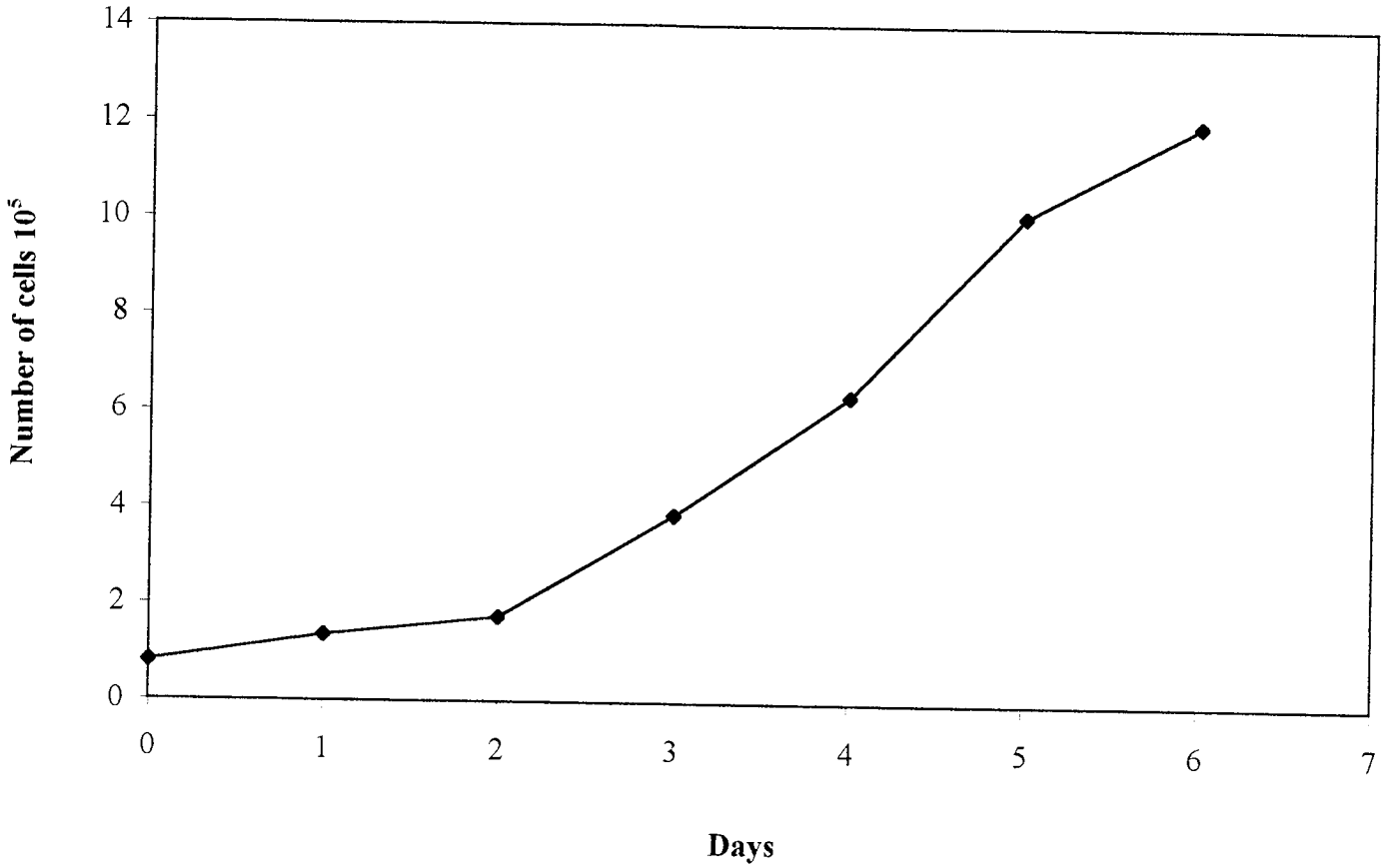



Figure 5.I.b. Cell growth curve of T5-2E1 cells

T5-2E1 cells were grown in monolayers as described in section 5.2.1., trypsinised and counted. Values are the mean of n = 2.

### 5.3.I.2. EROD activity of T5-1A2 cells in 96-well plates

EROD activity was measured in T5-1A2 cell cultures for 24 h in 96-well plates as described in methods 5.2.3. A typical resorufin standard curve is shown in Figure 5.I.c.. Data correlated well with a linear regression (r = 0.998). Protein calibration curve using BSA is shown in Figure 5.I.d. and a good correlation between data and a linear fit was also observed (r = 0.998).

Ethoxyresorufin was originally used at the concentration range of 0 - 20 μM, however ethoxyresorufin 20 μM gave fluorescence readings much lower than the other concentrations, and a decrease on protein content was also observed, suggesting that it was cytotoxic. Therefore, the maximum concentration used of ethoxyresorufin was 15 μM. EROD activity was initially studied at different incubation times (15, 30, 45 and 60 min) (Figure 5.I.e.). Incubations at 15 and 30 min gave higher fluorescence readings (see Figure 5.I.e..) than 45 and 60 min, however 15 min did not give a saturation curve as seen with the 30 min incubation and therefore determination of EROD activity was carried out with 30 min incubations.

EROD activity in T5-1A2 cells (Figure 5.I.f..) was characterised with a Vm of 28.48 ± 1.36 pmol/min/mg and a Km of 4.3 ± 0.72 μM (mean ± SD, n = 2 separate experiments). These values are consistent with those reported by Macé *et al*, (1996b) (methoxyresorufin O-deethylase activity was 41 ± 5 pmol/min/mg protein).

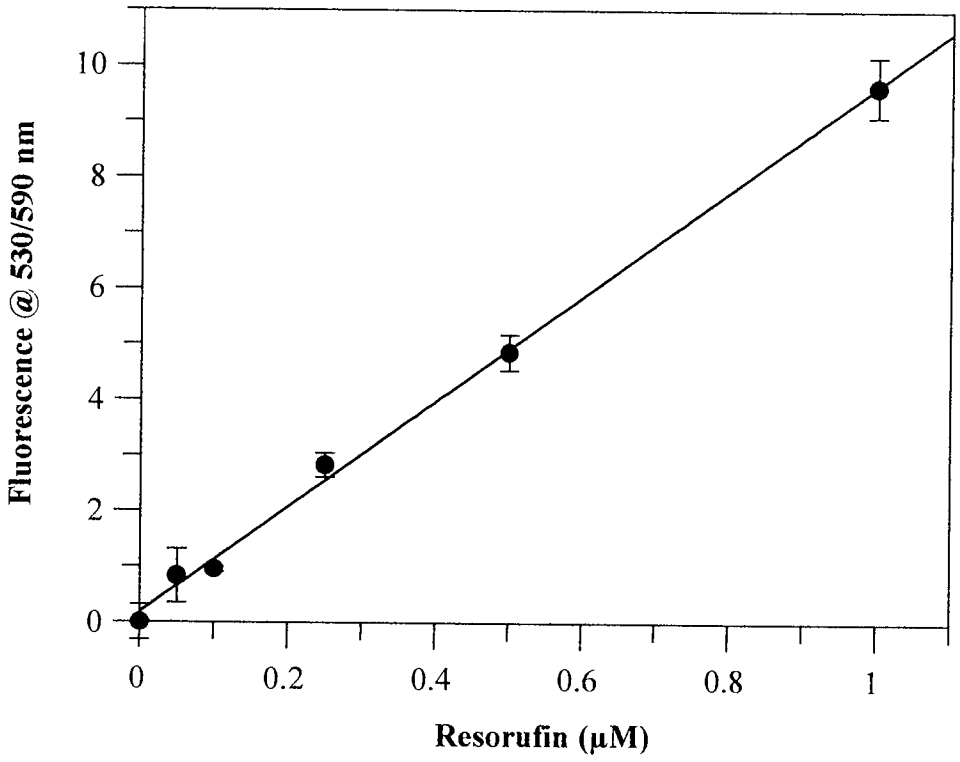



Figure 5.I.c. Resorufin standard curve in 96-well plates

Resorufin standards were prepared in sodium phosphate buffer (50 mM, pH 7.4) in 96-well plates and fluorescence was measured using excitation and emission wavelength 530 and 590 nm respectively. Values are the mean ± SD, n=3.

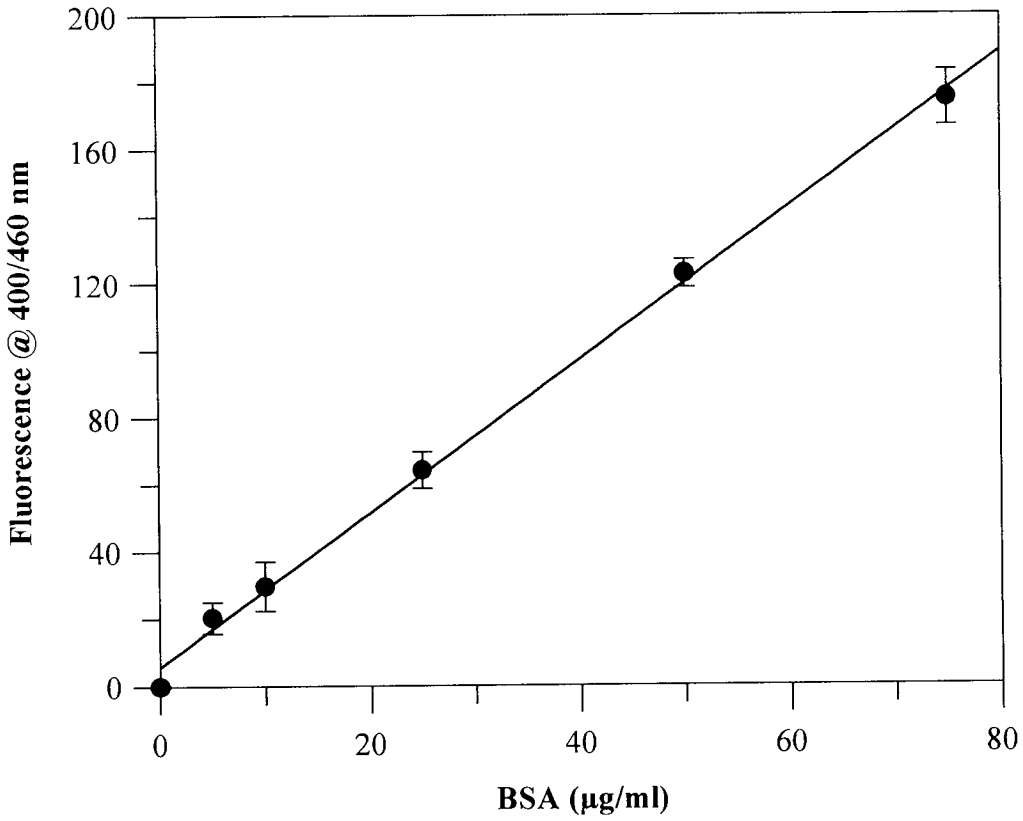



Figure 5.I.d. Protein standard curve in 96-well plates

Bovine serum albumin (BSA) standards were prepared in phosphate buffer pH 8.0 in 96-well plates and fluorescamine was added to this. Values are the mean ± SD, n=3.

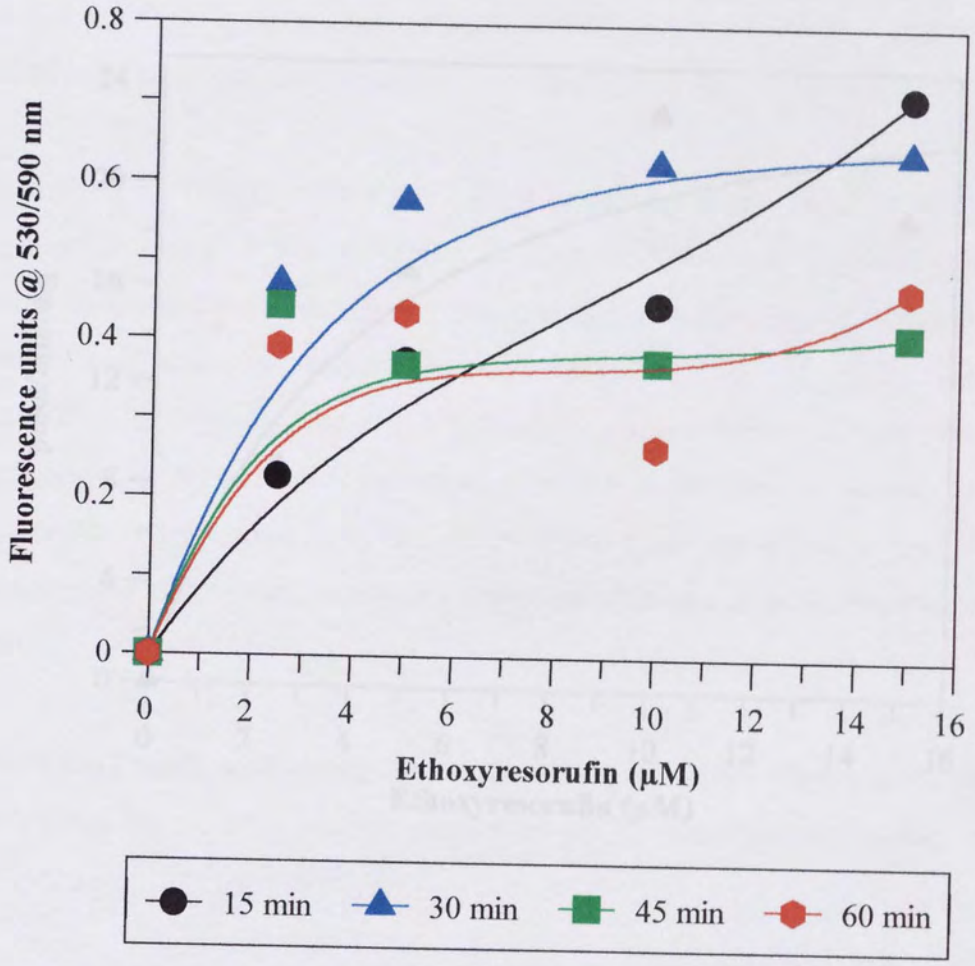



Figure 5.I.e. Effect of incubation time on resorufin production in T5-1A2 cells.

T5-1A2 cells cultured in 96-well plates for 24 h, were incubated with ethoxyresorufin (0 -15 μM) and dicumarol (10 μM) at 37 °C for 15, 30, 45 and 60 min. Values are the mean of triplicate measurements.

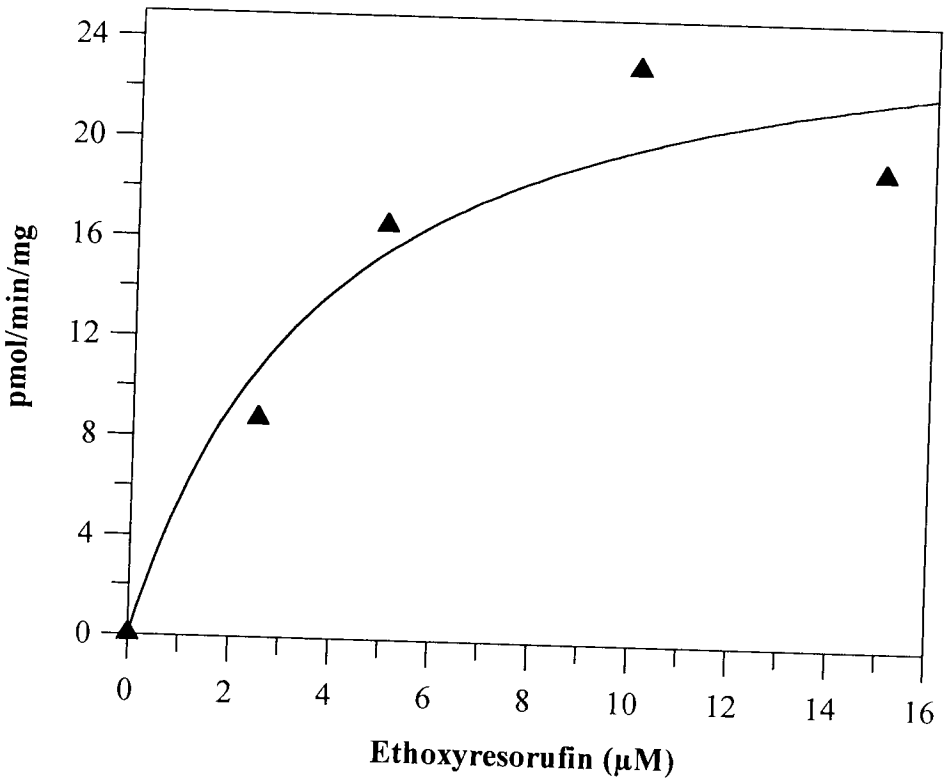



Figure 5.I.f. EROD activity in T5-1A2 cells in 96-well plates

Cells were cultured for 24 h in 96-well plates, and then assessed for EROD activity. Ethoxyresorufin (0 -15 μM) and dicumarol (10 μM) were incubated at 37 °C for 30 min. Activity and protein content were measured simultaneously at 530/593 and 400/460 nm respectively (methods 5.2.3.). Values are the mean of triplicate analyses.

### 5.3.I.3. EROD activity in T5-1A2 cells plated in 6-well plates

T5-neo and 1A2 cells were cultured in 6-well culture plates for 24 h were analysed for EROD activity with or without sulforaphane (10 μM). EROD activity was quantified fluorimetrically by comparison to a calibration curve from resorufin (0 - 0.15 μM) (see section 3.3.4.). Protein content was assessed according to the method of Bradford and was quantified by comparison to a calibration curve from BSA (0 - 30 μg/ml) (Figure 5.I.g.).

EROD activity in T5-1A2 cells was 17.63 ± 3.8 pmol/mg/min (mean ± SD, n=3) when ethoxyresorufin (10 μM) was incubated for 30 min. EROD activity in T5-neo cells was below the detection limit. This values were similar to those previously reported (see above and table 5.a.) although slightly lower, possibly due to the higher specificity of methoxyresorufin towards human CYP1A2 than ethoxyresorufin (Burke *et al*, 1995). Furthermore, it is also likely that the actual activity is higher than the values reported here, since resorufin is conjugated by several phase II enzymes, and only free-resorufin product can be measured, unless supernatants are incubated with deconjugating enzymes (Donato *et al*, 1993).

In a preliminary study, incubations containing sulforaphane (10 μM) decreased significantly EROD activity compared to control incubations (17.63 pmol/min/mg), giving 15.04 ± 2.6 pmol/mg/min ($P < 0.05$, paired-*t*-test).

### 5.3.I.4. CYP2E1 activity in T5-2E1 cells

The measurement of PNP hydroxylase activity in T5-2E1 cells was attempted but was unsuccessful, possibly due to an incubation time not long enough (30 min) (other authors have measured this activity in cell lines over a period of 24 h; Dai *et al*, 1993), or due to the sensitivity of the technique. CYP2E1 activity in T5-2E1 cells was nevertheless measured elsewhere by measuring chlorzoxazone 6-hydroxylase activity using HPLC (55 pmol/min/mg protein) (Berthou, F., and Macé, K., personal communication).

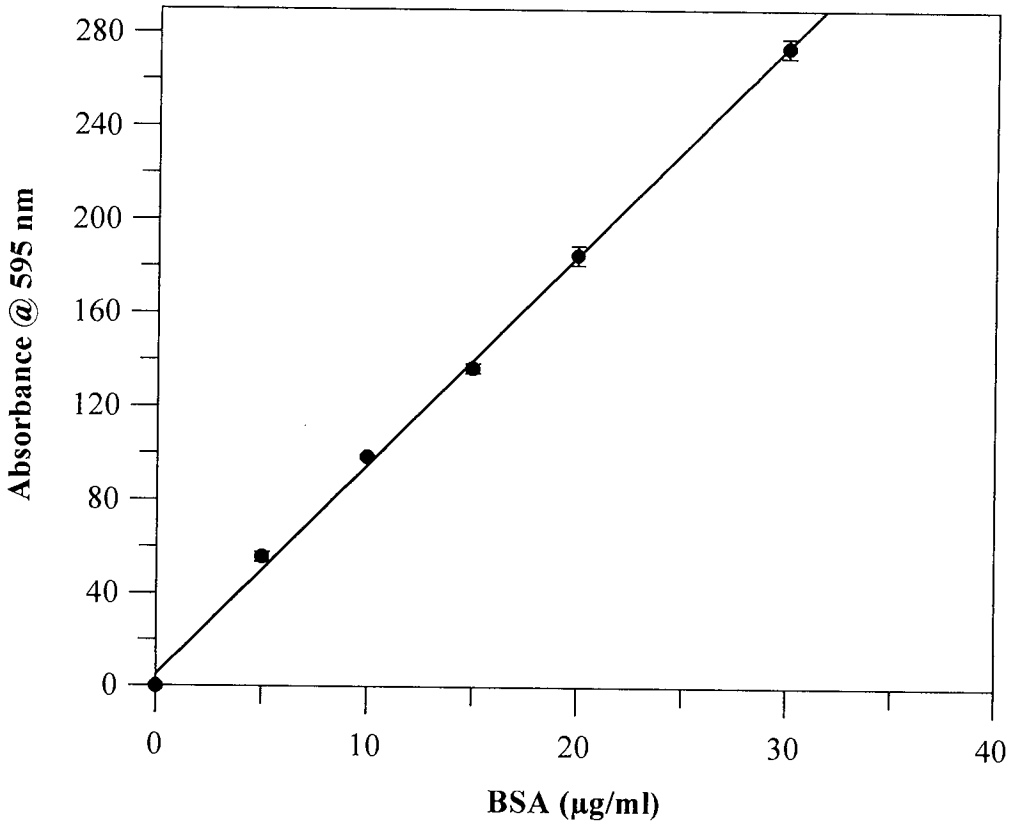



Figure 5.I.g. Bovine serun albumin (BSA) standard curve

Biorad solution (1 ml) was added to BSA standards, mixed and left standing for 15 min at room temperature. Absorbance was measrued at 595 nm. Values are the mean ± SD, n=2.

## *5.3.II. Results Part II. Production of DNA strand breaks by NDMA and IQ and its inhibition by sulforaphane*

### 5.3.II.1. Refinement and modification of the standard Comet assay.

#### 5.3.II.1.1. Effect of trypsination and scraping on DNA damage

The Comet assay requires a single cell suspension. Human epithelial liver cells expressing human CYPs grow as a monolayer. The preparation of a single cell suspension from a monolayer is usually achieved by trypsination. Trypsination however is not desirable as it requires incubation at 37 °C and could alter the effect of previous incubations with test compound such as allowing for repair of DNA damage. Moreover, trypsination has been reported to induce DNA damage in the Comet assay (Singh *et al*, 1991) by an unknown mechanism. An alternative technique is scraping, which consists of physically detaching the cells from the surface using a rubber policeman. To determine which technique was more appropriate, the effect of trypsination and scraping on DNA damage in the Comet assay was studied using the human epithelial liver cell line expressing human CYP1A2 (T5-1A2) and the cell line expressing human CYP2E1 (T5-2E1).

T5-1A2 and T5-2E1 were grown as monolayers as described in section 5.2.1. Cells were incubated in PFMR-4 medium alone for 1 h at 37 °C in 3.5 % $CO_2$. To obtain a single cell suspension, the cells were either scraped or trypsinised. The viability of the cells obtained by the two methods was determined by the Trypan blue assay (see methods 4.2.2.1.4.). Cells were then centrifuged, resuspended in LMP agarose at 37 °C (100 µl) and processed for the Comet assay (see section 5.2.7.).

The results are shown in Table 5.II.a. The viability of the cells (based on membrane integrity) obtained by trypsination was much higher than those obtained by scraping in both lines. For the T5-2E1 cell line viability after trypsination was 99 % whereas after scraping was 64 %. The same trend was observed for the T5-1A2 cell line (97.5 % for trypsination compared to 54 % for scraping). On the other hand, cells obtained by trypsination exhibited much higher DNA damage than cells obtained by scraping. The three parameters most

commonly used in the comet assay are shown in Table 5.II.a. In both cell lines, the % tail DNA, tail moment and tail length was significantly higher for the cells obtained by trypsination compared to scraping ($P<0.05$ for T5-2E1 and $P<0.01$ for T5-1A2 cells). For T5-2E1 cells, % tail DNA was 26.5 ± 6.3 % for trypsination compared with 20.4 ± 2.1 % for scraping and tail moment was 40.2 ± 15.4 and 26.4 ± 2.3 respectively for trypsination and scraping. These differences were even more accentuated in T5-1A2 cells, in which a % tail DNA of 38.2 ± 1.7 was obtained for trypsinised cells and only 18.3 ± 5.1 for scraped cells. Tail moment and tail length followed the same trend ( 63.9 ± 6.0 and 22.2 ± 6.1 for trypsination and scraping respectively).

The effect of trypsination and scraping on DNA damage in the T5-2E1 cell line is shown in Figure 5.II.a. Cells were classified into five different populations according to their degree of damage: minimum: 0-10 % tail DNA, low: 10-25 % tail DNA, medium: 25-40 % tail DNA, high: 40-75 % tail DNA and maximum: >75 % tail DNA. For the cells obtained by trypsination, there were more cells that fell in the categories of high and maximum damage compared to the cells obtained by scraping. For T5-1A2 cells obtained by trypsination (Figure 5.II.b.), there was a relatively large number of cells in the high and maximum damage categories with less cells in the categories with minimum and low damage, whereas the cells obtained by scraping were mainly in the categories of minimum and low damage.

This indicates that both trypsination and scraping gave a high background of percentage of tail DNA and tail moment in the comet assay for the cell lines used. Induction of DNA migration in the comet assay by trypsination and scraping in a fibroblast cell line has also been reported elsewhere (Singh *et al*, 1991). The technique of scraping gave a much lower background readings for the three parameters indicative of strand breakage than did trypsination, but on the other hand cell viability was much lower for scraping than trypsination and only few single cells were obtained when scraping. The establishment of adequate sensitivity whilst maintaining a limited background value in the controls was therefore necessary.

Table 5.II.a. Effect of trypsination and scraping on viability (Trypan blue) and parameters in the Comet assay in the T5-2E1 and 1A2 cell lines.

| Cell line | Treatment | Viability | % Tail DNA | Tail moment | Tail length |
|---|---|---|---|---|---|
| T5-2E1 | trypsin | 99.0 % | 26.5 ± 6.3* | 40.2 ± 15.4 | 99.2 ± 20.1 |
| | scraping | 64.0 % | 20.4 ± 2.1 | 26.4 ± 2.3 | 84.8 ± 2.1 |
| T5-1A2 | trypsin | 97.5 % | 38.2 ± 1.7** | 63.9 ± 6.0 | 139.4 ± 13.2 |
| | scraping | 54.0 % | 18.3 ± 5.1 | 22.2 ± 6.1 | 89.2 ± 13.3 |

T5-2E1 and T5-1A2 cells were cultured as described in section 5.2.1., trypsinised or scraped and processed for the comet assay (section 5.2.7.) Viability was determined by the Trypan blue assay (section 4.2.2.1.4.). Values are the mean ± S.D. (n=3, 100 cells analysed in total). * Significantly different from T5-2E1 cells obtained by scraping, $P<0.05$ analysed by two sample *t*-test. ** Significantly different from T5-1A2 cells obtained by scraping, $P<0.01$ analysed by two sample *t*-test and Mann-Whitney test.

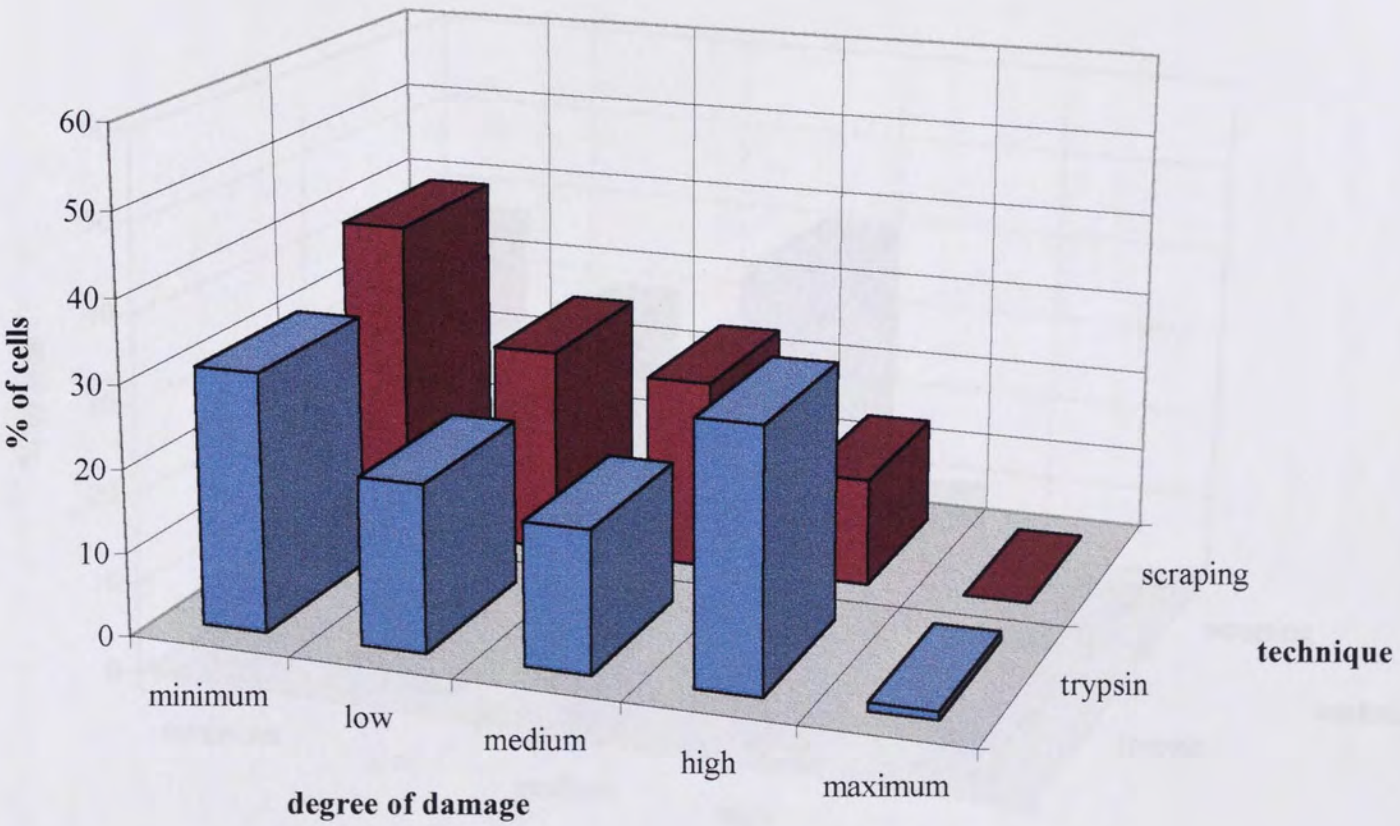



Figure 5.II.a. Effect of trypsination and scraping on DNA damage in T5-2E1 individual cells

Cultured cells were either trypsinised or scraped to give a suspension of single cells and then embedded in agarose and processed for the "standard" Comet assay. One hundred cells were analysed.

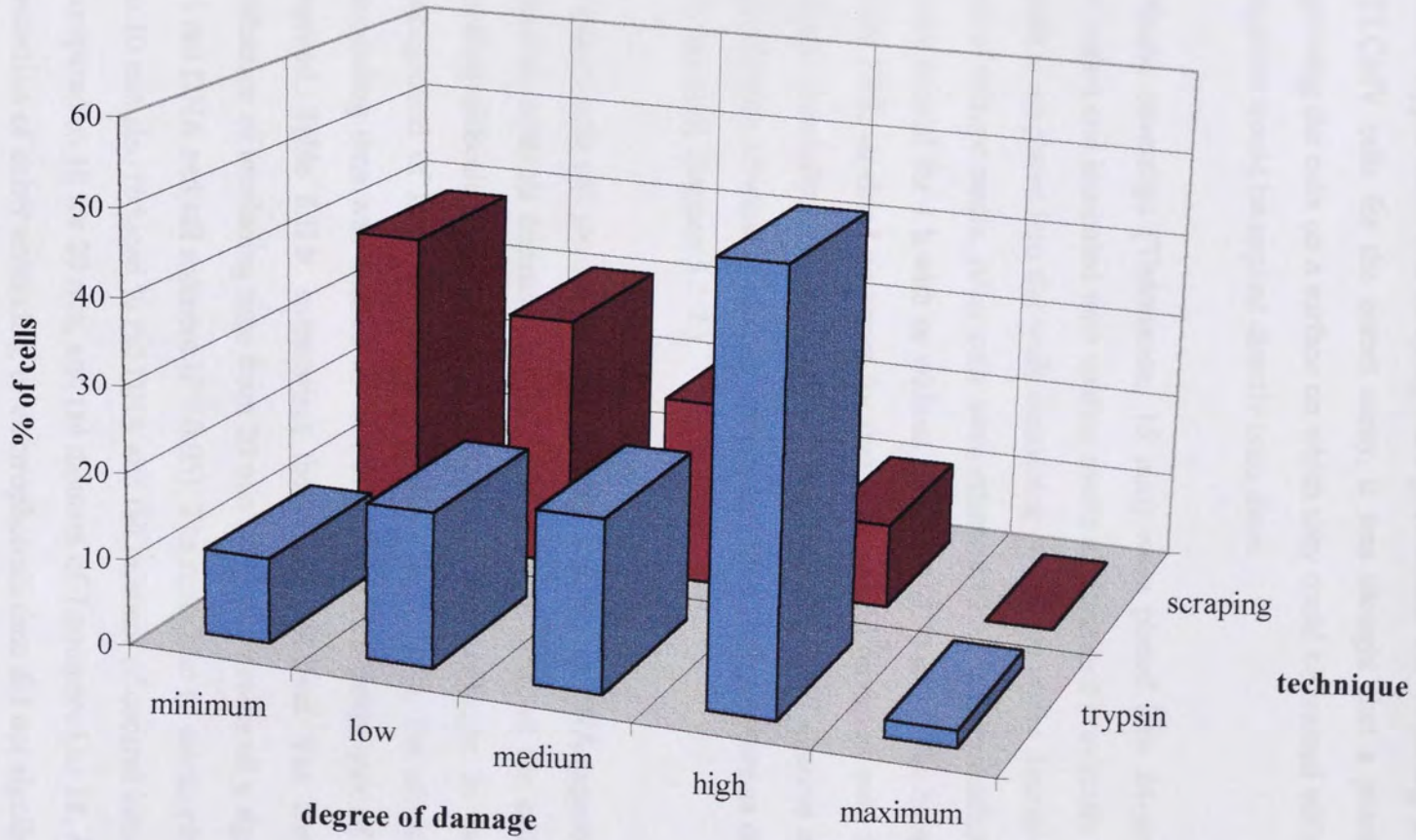



Figure 5.II.b. Effect of trypsination and scraping on DNA damage in T5-1A2 individual cells

Cells were either trypsinised or scraped and processed for the standard comet assay (section 5.2.7.) One hundred cells were analysed.

### 5.3.II.1.2. Adaptation of the comet assay method for the human liver epithelial cell lines.

Since trypsination and scraping were problematic for obtaining a single cell suspension of T5.CMV cells for the comet assay, it was thought that a possible alternative could be growing the cells on a surface on which they could be treated with the test compounds and agarose would be applied directly onto them.

Plastic coverslips (Thermanox, 15 mm) were placed into 24-well tissue culture cluster (Costar) and incubated with coating media as described previously (section 5.2.1.). T5-1A2 cells were plated into the wells containing coverslips at the density of 5000 cells/well in 0.5 ml of culture media. After cells were attached (2 h), culture medium was changed and cells were treated for 1 h with or without $H_2O_2$ (50 -100 μM). The coverslips were then washed with PBS, air-dried on paper for two minutes and mounted onto frosted glass microscope slides. Coverslips were covered with two layers of LMP agarose at 37 °C (200 μl and 100 μl). Lysing, unwinding electrophoresis and slide analyses were as described for the standard comet assay (section 5.2.7.).

Control cells still gave relatively high values of % tail DNA (approximately 20 %). For the positive controls treated with $H_2O_2$, 50 and 100 μM, all the cells were highly cometed, making difficult an accurate quantification of the damage. In an attempt to reduce the background of DNA strand breakage in control cells, the effect of electrophoresis and unwinding time was studied in control and positive treatments of T5-1A2 cells using this method. Table 5.II.b. summarises the results obtained. For the negative controls, the reduction of unwinding time from 20 min to 10 min produced a significant decreased in the % tail DNA and tail moment ($P< 0.05$). The reduction of electrophoresis time from 20 min to 10 min also reduced % tail DNA and tail moment of control incubations (tail DNA of 13 compared to 18 for 20 min, and tail moment of 7 compared to 18, $P < 0.05$). However, the reduction of either unwinding or electrophoresis time did not significantly change the % tail DNA and tail moment of the incubations with $H_2O_2$ (50 μM). The tail length was reduced for both control and positive treatments when reducing unwinding and electrophoresis time ($P < 0.01$), indicating a dependence of the tail length on the assay conditions.

Table 5.II.b. Effect of variation of unwinding and electrophoresis times on the comet assay parameters

| Treatment | Unwinding time (min) | Electrophoresis time (min) | %Tail DNA | Tail moment | Tail length (μm) |
|---|---|---|---|---|---|
| Control | 20 | 20 [a] | 18.14 | 18.32 | 77.20 |
| | 10 | 20 [b] | 14.37 * | 9.87 ** | 56.82 ** |
| | 20 | 10 [c] | 12.72 * | 6.79 ** | 40.04 ** |
| $H_2O_2$ | 20 | 20 [b] | 39.76 | 46.95 | 108.05 |
| | 10 | 20 [b] | 41.56 | 43.39 | 92.77 ** |
| | 20 | 10 [d] | 44.35 | 41.6 | 87.22 ** |

T5-1A2 cells were grown on plastic coverslips, treated with or without $H_2O_2$ (50 μM), and then covered with L.M.P. agarose. Comet assay was performed as in section 6.2.1. Values were the mean of : [a] one slide, n=50 cells; [b] two slides, n=100 cells; [c] one slide, n=55 cells and [d] one slide, n=20 cells. * Significant different from experiment performed at 20 min unwinding time and 20 min electrophoresis time ($P < 0.05$, Students *t*-test); ** $P < 0.01$.

Although this method seemed useful originally, the agarose layers on the coverslips were lost very often before the end of the electrophoresis, and often only one (or zero) slides were left instead of triplicates (see table 5.II.b. footnote). This modified method was therefore not reproducible and had to be abandoned.

### 5.3.II.1.3. Definitive adaptation of the comet assay

A modification of the method of Singh *et al,* (1991) for culturing human fibroblasts onto frosted microscope slides was attempted.

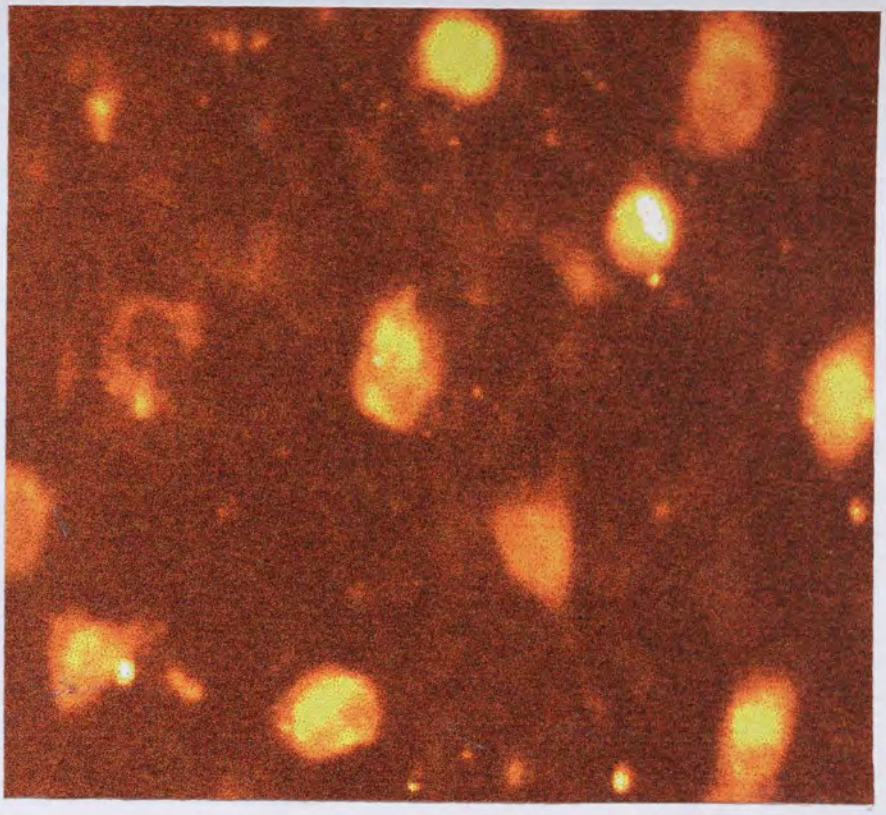
Fully frosted slides were cut to fit 35 mm 6-well cell culture plates and were incubated for 30 min with coating media at 37 °C. T5-1A2 cells were plated into the 6-well plates (~$10^4$ cells/well). After 4 h the slides were washed with PBS, ethidium bromide was added ( 5 µl, 20 µg/ml) and were analysed under the fluorescent microscope (details in section 5.2.8.). Cells were present and attached onto the microscope slides, as evidenced by the characteristic shape of epithelial cells. This is shown in Figure 5.II.c.i. To make sure that the cells on frosted microscope slides were fully functional, they were left to grow overnight onto the microscope slides in the culture conditions described in section 5.2.1. and analysed the following day as described above. Figure 5.II.c.ii. shows a higher density of cells, thus indicating that the cells were able to replicate and function as if they were grown on a plastic surface.

For the comet assay, the cells plated on precoated fully frosted microscope slides were treated after they were attached and acquired their characteristic shape of epithelial cells (2 - 4 h). The culture medium was removed and fresh medium was added containing $H_2O_2$ (50 µM) for the positive control or culture media alone for the negative control. Two layers of LMP agarose (0.5 %, 50 µl) were originally added to the microscope slides, but agarose layers were often lost throughout the experiment, and also a high fluorescence background was seen, probably due to traces of phenol red from the incubation media. Therefore, slides were washed three times with PBS in order to avoid this problem, and one single layer of LMP agarose (0.75 %, 100 µl) was added to the slides, which proved to be attached well on the slide and survived the different steps of the assay.

To determine whether the lysing time had any effect on the % tail DNA of control slides, different slides were set and processed for the comet assay as described in section 5.2.8. with a lysing time of 15, 30 and 60 min. For 60 min lysing time, control slides gave a % tail DNA of 19.7 and for the treatments with hydrogen peroxide 68 % tail DNA. These values did not differ significantly from the assays where lysing time was 15 min (control 18.2 and positive 62.3) or 30 min (control 17.6 and positive 64). Since the lysing time did not have an effect on the results, it was reduced to 30 min instead of 1 h.

Figure 5.II.d. shows the main differences between the standard comet assay and the modified version used here. Summarising, cells were plated onto precoated fully frosted microscope slides and left to attach for 2-4 h. An extra plate without microscope slide was set in order to follow up attachment of cells. After treatment, slides were rinsed three times with PBS and one layer of LMP agarose (0.75 %, 100 µl) was added and cells were lysed for 30 min. Unwinding and electrophoresis were performed as described for the standard comet assay, 20 min and 20 min. Since positive controls, $H_2O_2$ (50 µM), gave a very high degree of damage (i.e. many cells were highly cometed and the nuclei was no longer visible), which was difficult to analyse, and therefore not accurate, treatments were reduced to the concentration of 25 µM $H_2O_2$.

i)



ii)

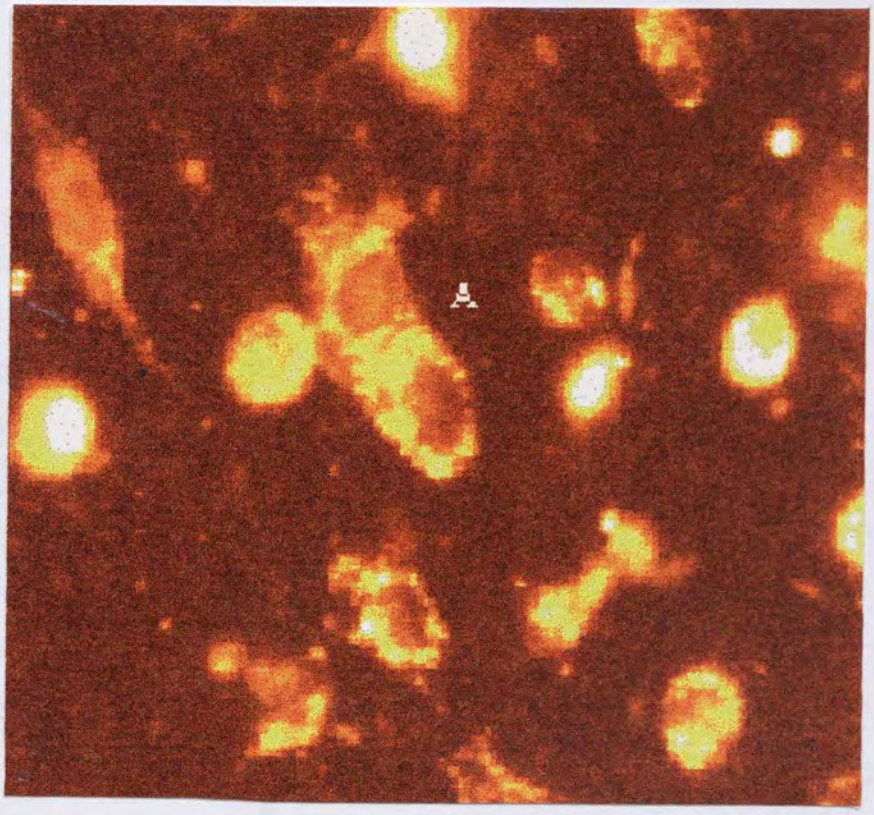


Figure 5.II.c. T5-1A2 cells attached onto fully frosted microscope slides

Cells were grown as described in section 5.2., trypsinised and plated onto precoated fully frosted microscope slides. Cells were stained with ethidium bromide and analysed under a fluorescent microscope i) after 4 hours and ii) after 12 hours, showing some cells dividing (A).

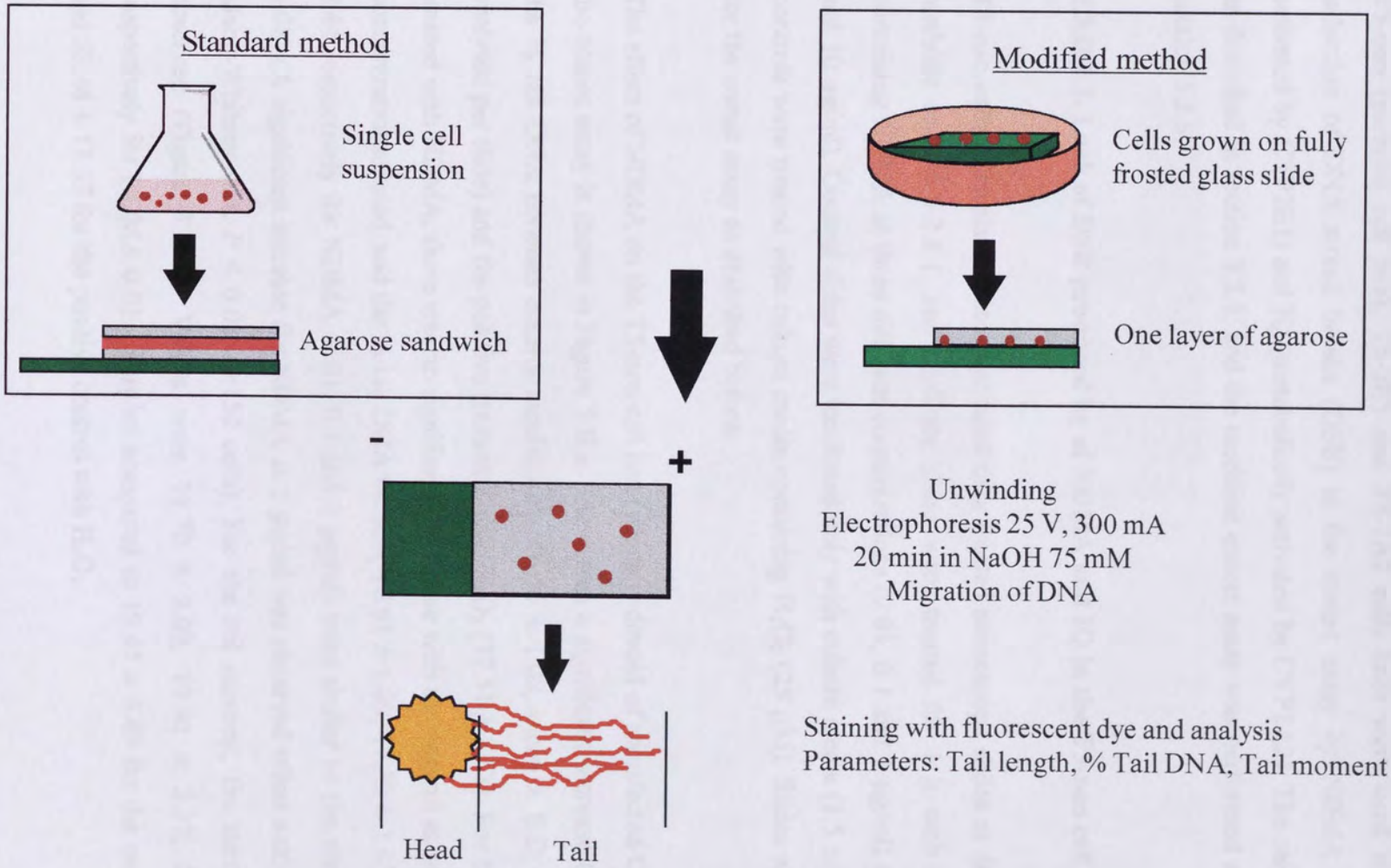



Figure 5.II.d. "Standard" and modified Comet assay

## 5.3.II.2. DNA strand breaks produced by NDMA and IQ in the human liver epithelial cell lines expressing human CYPs.

T5-neo (parental cell line), T5-2E1 and T5-1A2 cells lines were used to evaluate the induction of DNA strand breaks (DSB) in the comet assay by NDMA (metabolically activated by CYP2E1) and IQ (metabolically activated by CYP1A2). The cells were grown as described in section 5.2.1. and the modified comet assay was performed as described in section 5.2.8.

### 5.3.II.2.1. Lack of DSB produced by of NDMA and IQ in the T5-neo cell line

T5-neo cells were plated onto precoated fully frosted microscope slides as described in the methods section 5.2.8.1. and triplicate slides were treated for 1 h with culture media containing NDMA at three different concentrations (0.01, 0.1 and 1 μg/ml) and IQ (0.1, 1 and 10 μg/ml). Control slides were incubated only with culture media (1.5 ml) and positive controls were treated with culture media containing $H_2O_2$ (25 μM). Slides were processed for the comet assay as described before.

The effect of NDMA on the T5-neo cell line (which is devoid of transfected CYP cDNA) in the comet assay is shown in Figure 5.II.e. There was a significant increase ($P < 0.001$) in the % tail DNA between control incubations (16.56 ± 1.08, mean ± S.D, n=3, 50 cells analysed per slide) and the positive treatment with $H_2O_2$ (37.51 ± 3.53). For the incubations treated with NDMA, there was no significant difference with the control at any of the three concentrations tested, and the % tail DNA values ( 16.61 ± 1.89, 17.52 ± 1.03, and 19.40 ± 2.44 respectively for NDMA 0.01, 0.1 and 1 μg/ml) were similar to the control (16.56 ± 1.08) (A significant increase for NDMA at 1 μg/ml was observed when analysed using the Mann-Whitney test, $P < 0.05$, n=150 cells). For the tail moment, the same trends were observed (Figure 5.II.e.). Values were 18.70 ± 3.03, 19.92 ± 2.37, 21.31 ± 4.01 respectively for NDMA 0.01 - 1 μg/ml compared to 19.45 ± 3.49 for the negative control and 53.46 ± 11.37 for the positive control with $H_2O_2$.

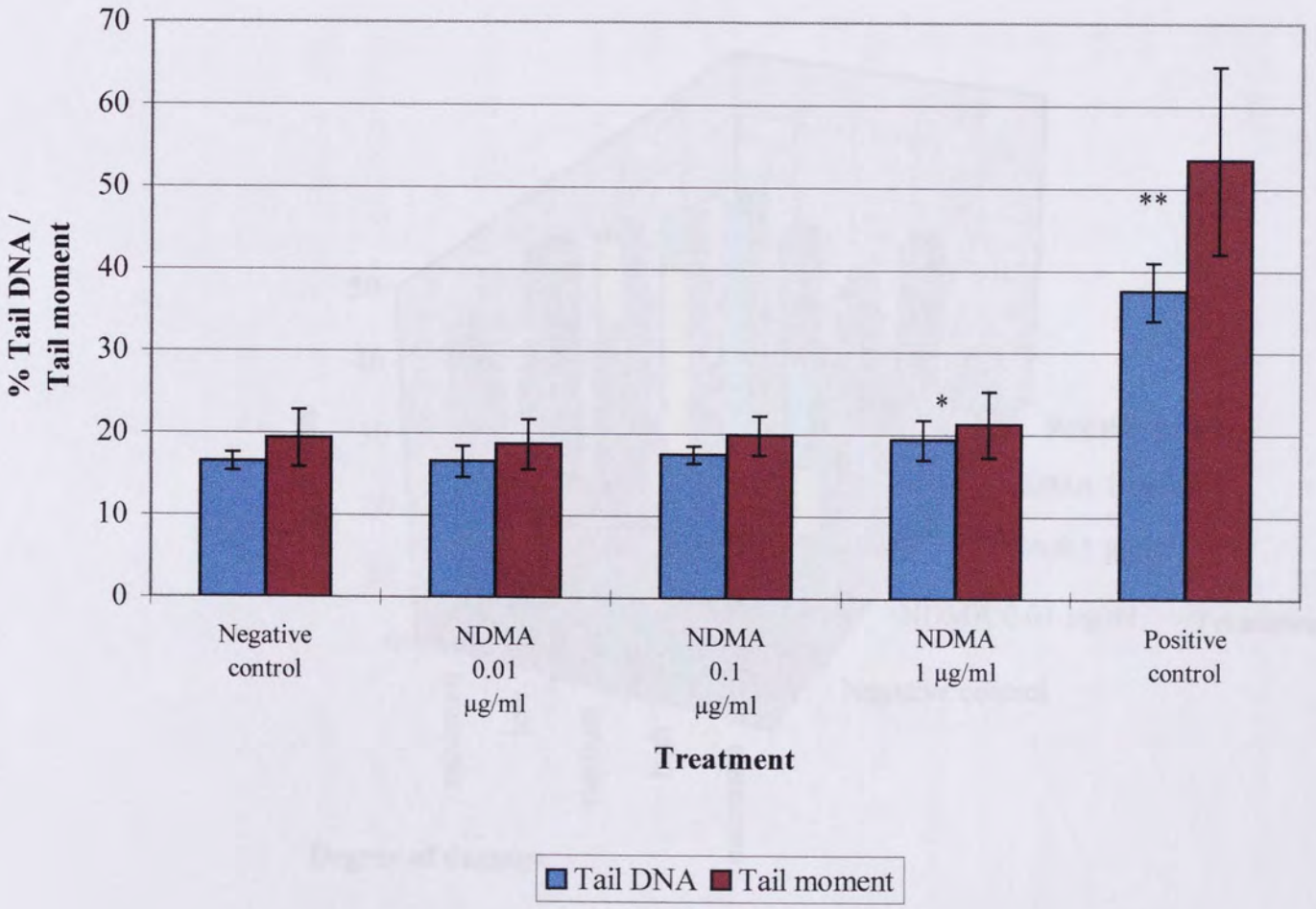



Figure 5.II.e. Lack of NDMA-induced DNA strand breaks in the T5-neo cell line

T5-neo cells were treated with NDMA (0-1 µg/ml) and hydrogen peroxide (25 µM) as the positive control for 1 h. Values are the mean ± S.D., n=3, 50 cells analysed per slide. * $P < 0.05$ for non-parametric tests using all the cells for analyses, ** $P < 0.01$.

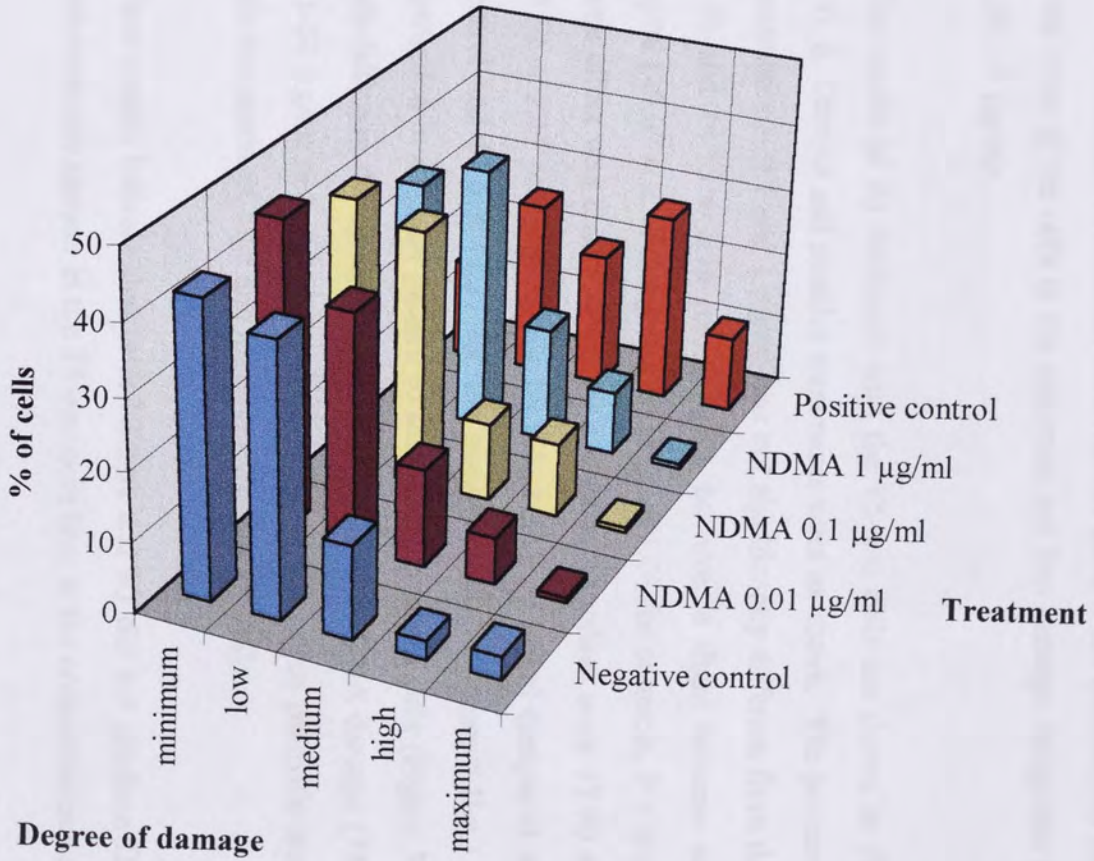



Figure 5.II.f. Lack of NDMA-induced DNA damage in T5-neo individual cells

Cells were incubated for 1h with NDMA (0.01-1 µg/ml), hydrogen peroxide (25 µM) as the positive control or medium (vehicle) alone for the negative control, and processed for the comet assay (section 5.2.8.). One hundred and fifty cells were analysed.

The response of individual cells to the different treatments is shown in Figure 5.II.f. Cells were classified into different populations according to their degree of DNA damage (see section 5.3.II.1.1.). For untreated cells (control) the majority of cells fell in the categories of minimum and low damage (81 % of cells), whereas for the positive control the population of cells with minimum and low damage decreased (41 % of cells) and populations with medium, high and maximum damage increased considerably (59 % of cells compared to 19 % for controls). Treatments with NDMA show a cell distribution very similar to the control, with most of the cells in the minimum and low damage categories (73 - 79 %, for NDMA 0.01 - 1 μg/ml).

The results of IQ treatment with the T5-neo cells are shown in Figure 5.II.g. and Figure 5.II..h. Control and positive treatments were as above. The percentage of tail DNA for IQ treatments at 0.1 and 1 μg/ml was not significantly different from that of controls (16.01 ± 2.30 and 14.73 ± 2.74 respectively), however a slight increase was observed for IQ 10 μg/ml (20.07 ± 2.42 compared to 16.56 ± 1.08 for controls, $P < 0.05$) (Figure 5.II.g.). The same effect was observed for the tail moment; values were 17.90 ± 4.29, 14.93 ± 5.4 and 23.01 ± 5.37 respectively for IQ 0.1, 1 and 10 μg/ml compared to 19.45 ± 3.49 for the control and 53.6 ± 11.37 for the positive control (Figure 5.II.g.). Cell populations were distributed in a similar pattern to that seen in control cells (Figure 5.II.h.) with most of the cells falling in the categories of minimum and low DNA damage (75 - 80 % of cells for IQ 0.1-10 μg/ml respectively), whereas for the hydrogen peroxide treatment the majority of cells had medium, high and maximum damage (59 %).

These results indicate that both NDMA and IQ did not produce DNA strand breaks in a dose-response manner in the T5-neo cells line, at the concentrations tested.

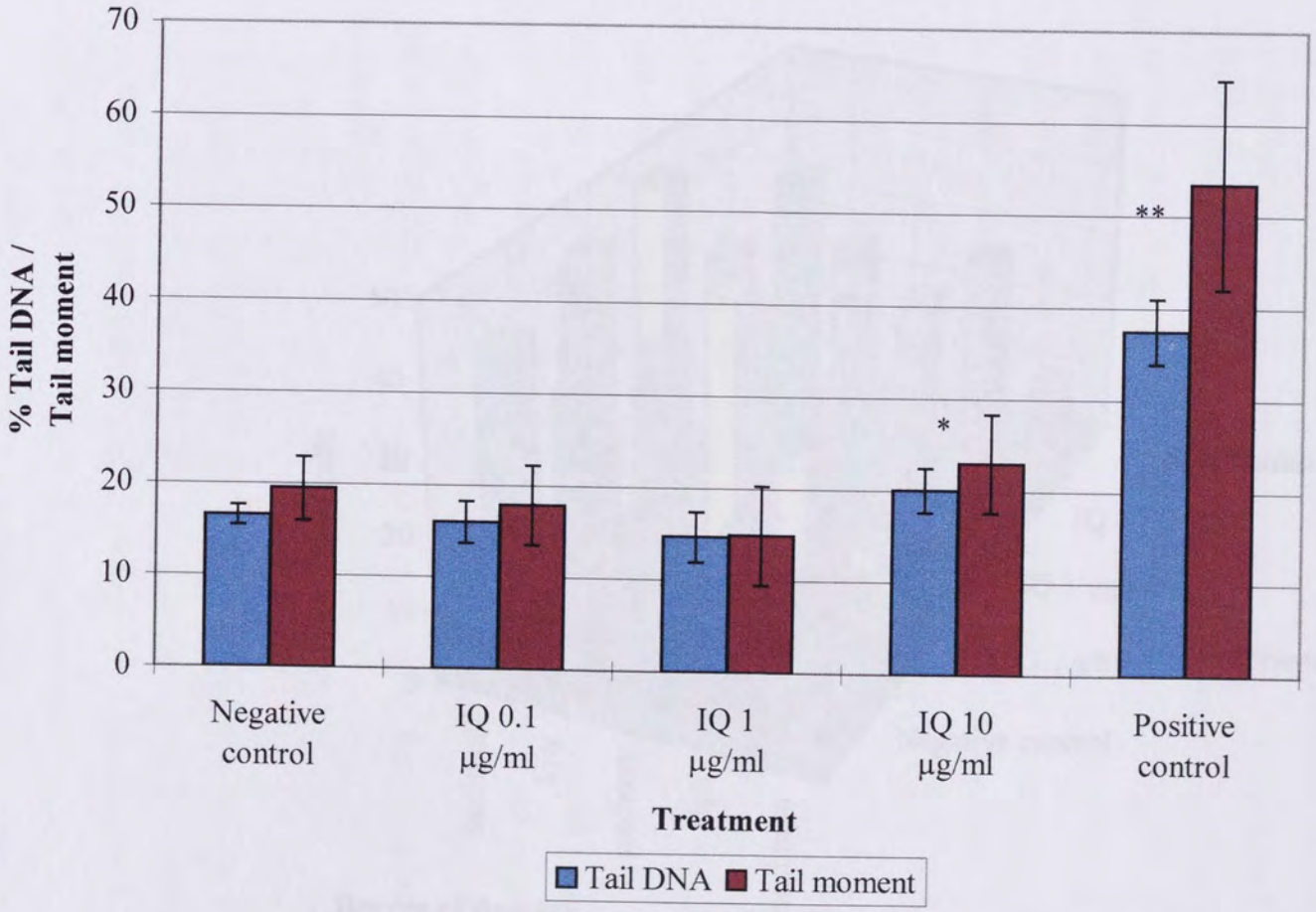



Figure 5.II.g. Lack of IQ-induced DNA strand breaks in the T5-neo cell line

T5-neo cells were incubated with IQ (0-10 µg/ml) for 1h and processed for the Comet assay (section 5.2.8.). The positive control was hydrogen peroxide (25 µM). Values are the mean ± S.D., n=3, 50 cells analysed per slide. * $P < 0.05$ for non-parametric tests using all the cells for analyses; ** $P < 0.01$.

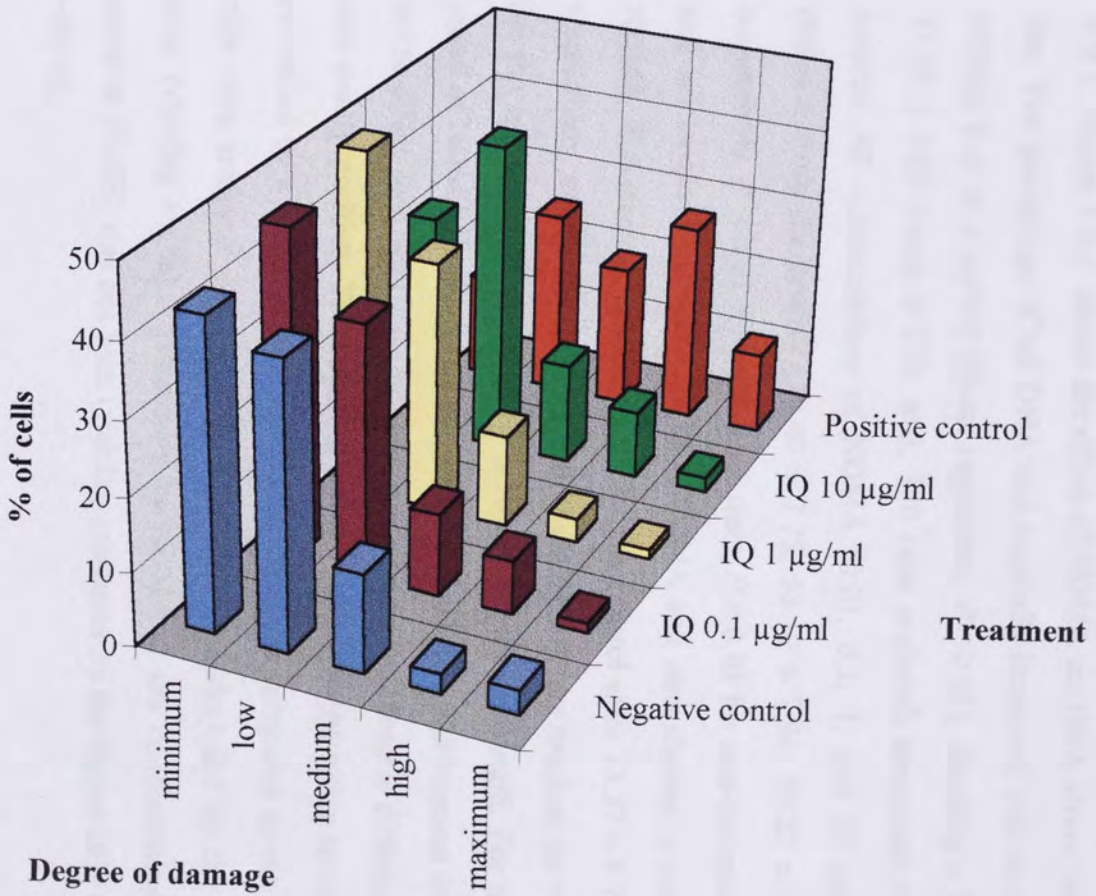



Figure 5.II.h.. Lack of IQ-induced DNA damage in T5-neo individual cells

T5-neo cells were treated for 1 h with IQ (0.1-10 µg/ml), hydrogen peroxide (25 µM) as the positive control or culture medium (vehicle) alone for the control and processed for the comet assay (5.2.8.). One hundred and fifty cells were analysed.

### 5.3.II.2.2. Production of DNA stand breaks by NDMA in the T5-2E1 cell line

T5-2E1 cells were treated with fresh media containing NDMA at 0, 0.01, 0.1, 1 and 10 μg/ml for 1 h at 37 °C. All treatments were triplicates and 50 cells were analysed per slide.

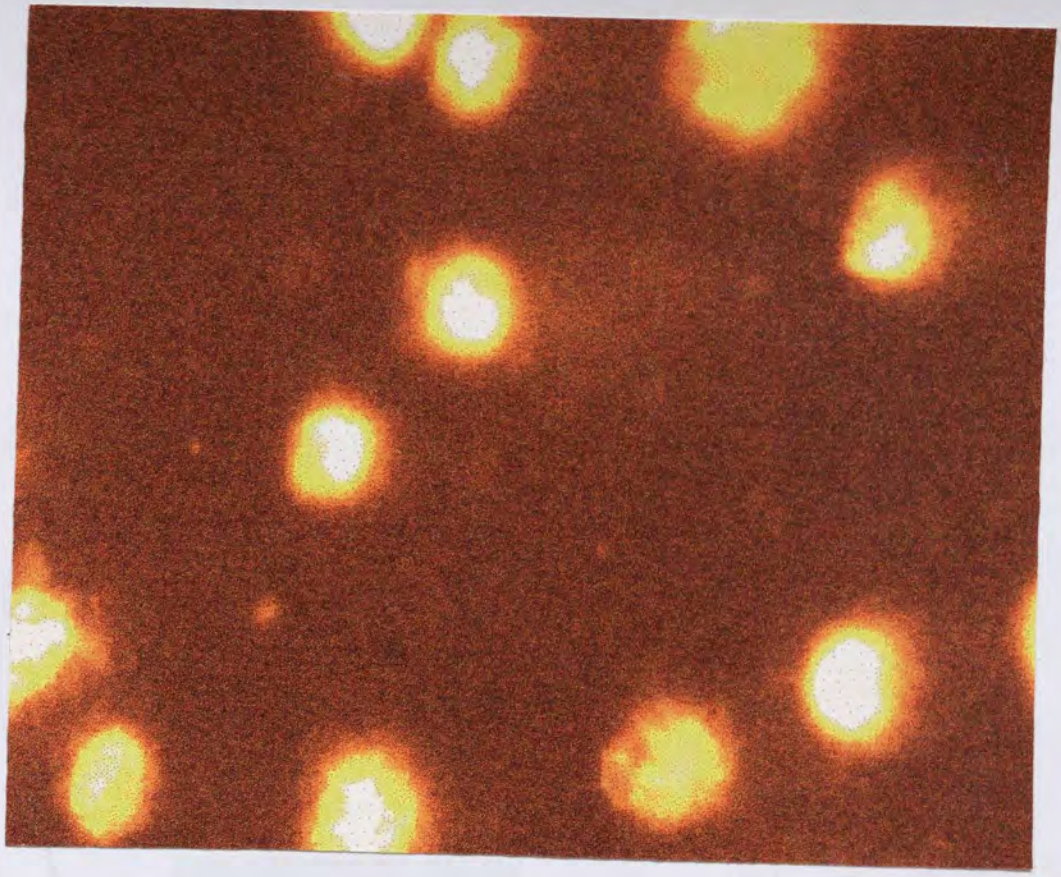
Typical comet images from control and NDMA (1 μg/ml) treatments are shown in Figure 5.II.i.. Figure 5.II.j. shows the effect of NDMA on DNA strand breaks in the T5-2E1 cell line. The percentage of tail DNA was markedly increased with increasing concentration of NDMA (up to 1 μg/ml) (linear regression, $P < 0.01$), reaching a % tail DNA as high as 73.27 ± 3.03 (mean ± S.D, n=3, 150 cells analysed) compared to 13.62 ± 1.77 for the control. All concentrations of NDMA (0.01, 0.1, 1, and 10 μg/ml) were significantly different from the control (39.40 ± 7.75, 58.19 ± 7.94, 73.27 ± 3.03 and 58.07 ± 3.11 respectively, $P < 0.01$ for parametric tests, $P < 0.05$ for non-parametric tests). Tail moment and tail length correlated with % tail DNA and also showed a marked dose-response of NDMA. Tail moment and tail length for the control were 13.37 ± 1.77 and 51.95 ± 2.72 μm respectively, and for NDMA 1 μg/ml these parameters reached the values of 117.96 ± 2.86 for the tail moment and 154.12 ± 3.58 μm for the tail length. For the concentration of 10 μg/ml of NDMA there was a decrease of % tail DNA, tail moment and tail length compared to the effect seen with 1 μg/ml, however, it was significantly different from the control and was clearly positive. The apparent reduced effect might relate to cytotoxicity, which was evidenced by a lower density of cells on the slides compared to the other slides (only 100 cells were analysed, compared to 150 for other slides) and by the trypan blue exclusion assay (viability 89 %). Cytotoxicity by NDMA at the concentrations of 0.01 to 1 μg/ml tested in T5-2E1 was not seen (viability measured by the trypan blue exclusion method was > 98 %).

Figure 5.II.k. shows the DNA damage produced by NDMA in individual cells. For the control treatment, most of the cells fell in the categories of minimum and low damage (85 %). With increasing concentration of NDMA, the cell populations with minimum and low damage decreased as whereas a % of populations with medium, high and maximum degree of damage increased. For the concentration of 0.01 there was a reduction from 85 % (control) to 46 % of cells with minimum and low damage. This difference in cell populations

was even more accentuated for the concentration of 1 μg/ml, where only 2 % of cells presented minimum or low damage and 98 % of cells where highly cometed, of which 8 % had medium damage, 32 % high and 58 % had total damage (% tail DNA >75).

These results clearly indicate that NDMA produced DNA strand breaks in the T5-2E1 cell line. Figure 5.II.1. compares the results obtained for the T5-2E1 and T5-neo cell lines. Production of DNA strand breaks (measured as % tail DNA) by NDMA was very potent when using T5-2E1 cells (transfected with human CYP2E1), whereas when using T5-neo cells, DNA strand breaks were not produced.

i)



ii)

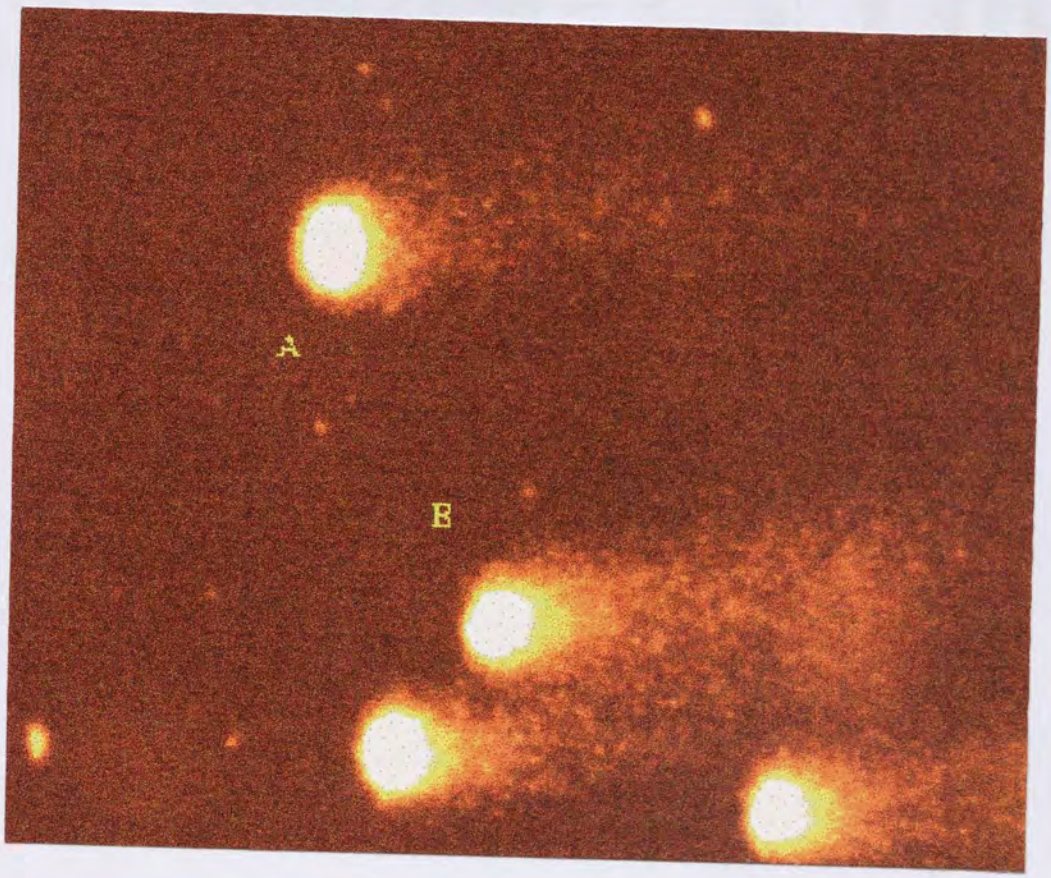



Figure 5.II.i. Typical images of cells processed for the Comet assay
i) T5-2E1 control cells and ii) T5-2E1 cells treated with NDMA (1 μg/ml) for 1 h at 37 °C, showing different degrees of damage; A: medium damage and B high damage.

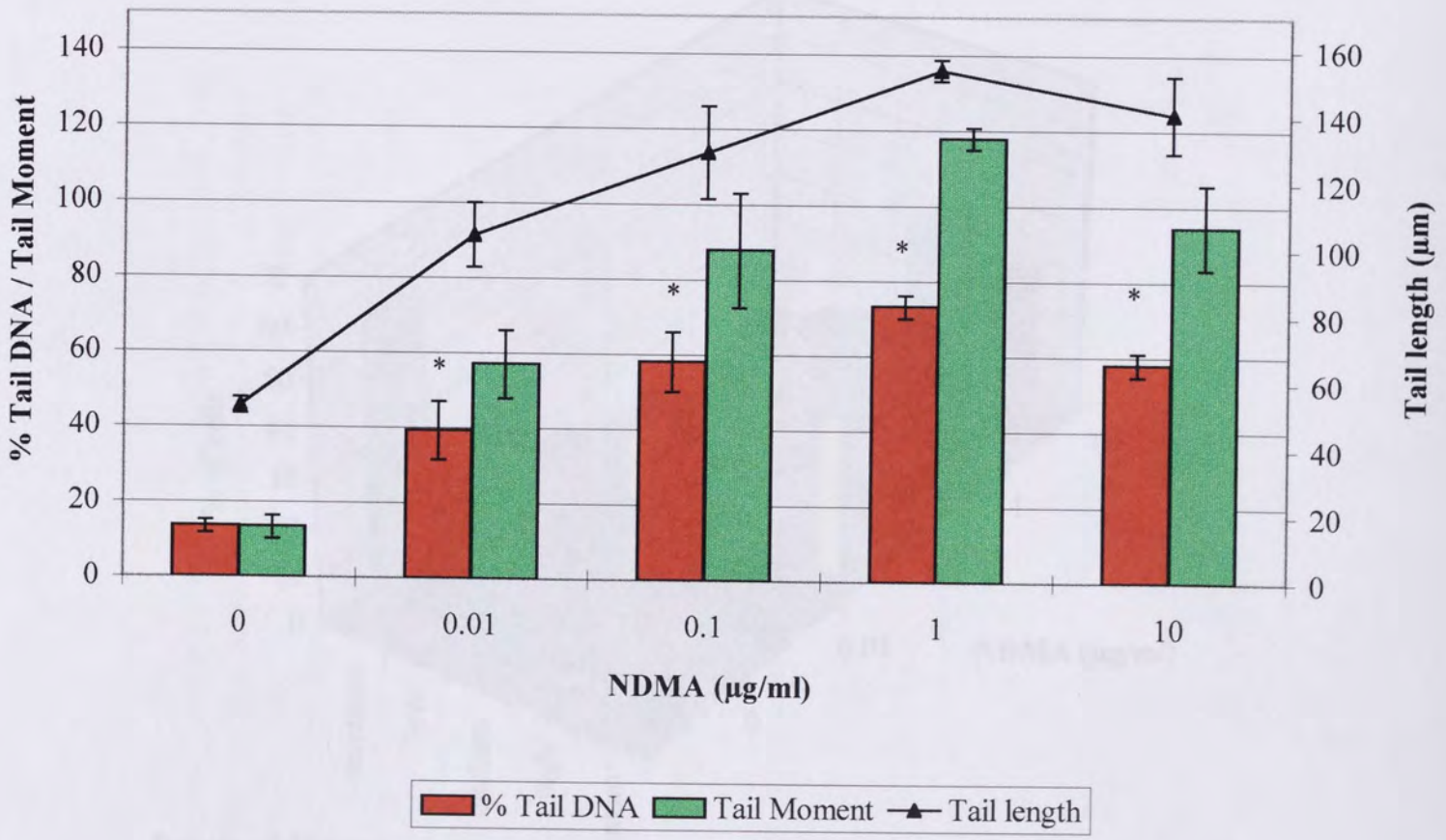



Figure 5.II.j. Induction of % tail DNA, tail moment and length by NDMA in T5-2E1 cells

T5-2E1 cells were incubated for 1 h with NDMA (0-10 µm/ml) and processed for the comet assay (section 5.2.8.). Values are the mean ± S.D., n=3, 50 cells analysed per slide. *Statistically different from control ($P < 0.01$ for parametric tests and $P < 0.05$ for non-parametric).

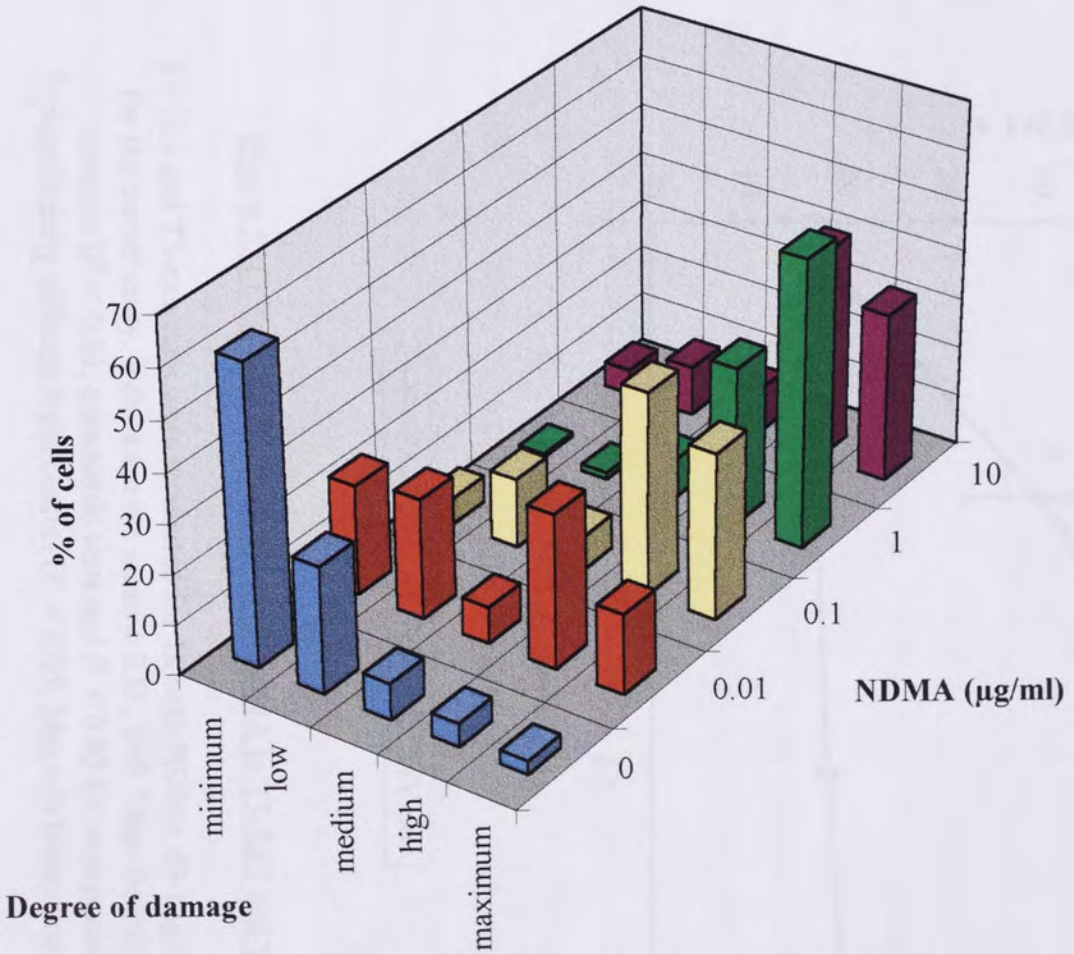



Figure 5.II.k. Comet response of T-2E1 individual cells to NDMA

T5-2E1 cells were incubated for 1h with NDMA (0 - 10 µg/ml) and processed for the comet assay (section 5.2.8.). One hundred and fifty cells were analysed, except for 10 µg/ml, only 100 cells were analysed.

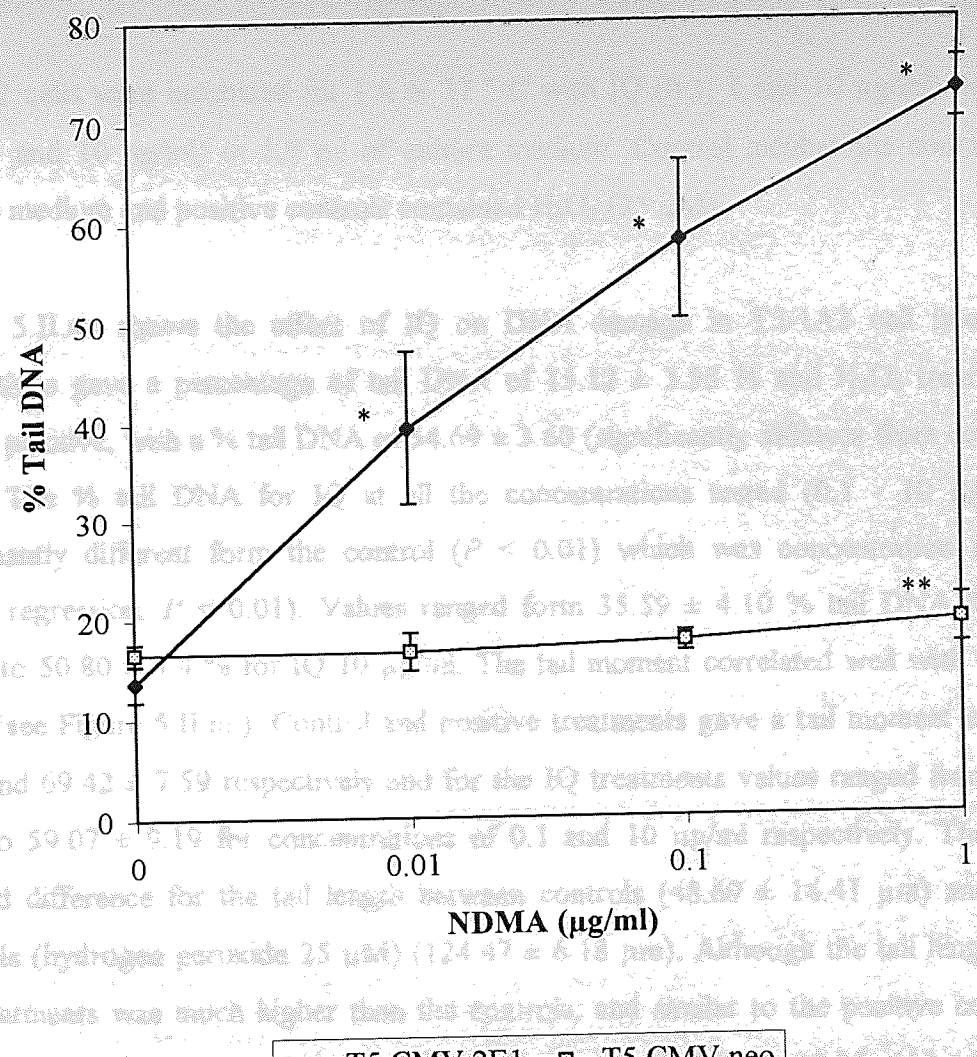



Figure 5.II.1. Effect of NDMA on % Tail DNA in T5-2E1 and T5-neo cells

T5-2E1 and T5-neo cells were incubated for 1h with NDMA (0-1µg/ml) and processed for the comet assay. Values are the mean ± S.D., n=3.*Significantly different from controls ($P < 0.01$, parametric tests and $P < 0.05$ for non-parametric tests); **Significantly different from control ($P < 0.05$, Mann-Whitney test for n=150 cells)

### 5.3.II.2.3. Production of DNA stand breaks by IQ and aflatoxin in T5-1A2 cells.

T5-1A2 cells were incubated for 1 h at 37 ° C with IQ (0.1, 1 and 10 μg/ml) or aflatoxin (0.1, 1 and 10 ng/ml) in 1.5 ml of culture medium. Control incubations contained only culture medium and positive controls contained $H_2O_2$ (25 μM).

Figure 5.II.m. shows the effect of IQ on DNA damage in T5-1A2 cell line. Control incubations gave a percentage of tail DNA of 15.12 ± 3.55 % and $H_2O_2$ treatment was clearly positive, with a % tail DNA of 54.69 ± 3.60 (significantly different from control, $P < 0.01$). The % tail DNA for IQ at all the concentrations tested (0.1 - 10 μg/ml) was significantly different form the control ($P < 0.01$) which was concentration dependent (linear regression, $P < 0.01$). Values ranged form 35.59 ± 4.10 % tail DNA for IQ 0.1 μg/ml to 50.80 ± 3.4 % for IQ 10 μg/ml. The tail moment correlated well with the % tail DNA (see Figure 5.II.m.). Control and positive treatments gave a tail moment of 11.24 ± 5.60 and 69.42 ± 7.59 respectively and for the IQ treatments values ranged from 39.48 ± 6.68 to 59.07 ± 9.19 for concentrations of 0.1 and 10 μg/ml respectively. There was a marked difference for the tail length between controls (48.69 ± 14.41 μm) and positive controls (hydrogen peroxide 25 μM) (124.47 ± 6.18 μm). Although the tail length for the IQ treatments was much higher than the controls, and similar to the positive control, the values did not increase with increasing concentration (117.25 ± 20.05, 113.44 ± 32.92, 116.96 ± 11.54 for IQ 0.1, 1, and 10 μg/ml respectively). Nevertheless, the % tail DNA and tail moment did increase with increasing concentration of IQ, suggesting that the tail length reaches a maximum value which depends on the electrophoresis conditions rather the DNA damage. The dependence between tail length and electrophoresis conditions was also seen in section 5.3.II.1.2. Consequently, for the following experiments only the % tail DNA and tail moment will be used as measurements of DNA strand breaks in the comet assay. No cytotoxic effects were observed (viability for IQ 0.1 - 10 μg/ml > 96 %)

The distribution of cells in different populations according to their degree of damage is shown in Figure 5.II.n. For the control treatments, most of the cells did not have comets (82 % of cells in minimum and low categories) whereas for $H_2O_2$ treatments most of the cells were highly cometed (82 % of cells in medium, high and maximum degree of damage

categories). The distribution of cells treated with IQ was closer to the $H_2O_2$ treatment than to the controls. With increasing concentration of IQ there were less cells in the categories of minimum and low damage (38 - 18 % cells for IQ 0.1 - 10 µg/ml, compared to 82 % for controls) and an increasing number of cells in the categories of medium, high and maximum damage ( 62 - 82 % cells, similar to 82 % for the positive control).

The results of IQ treatment on DNA strand breaks in the T5-1A2 and T5-neo cell lines are compared in Figure 5.II.o.. It is clear that IQ produced DNA strand breaks (measured as % tail DNA) in the T5-1A2 cell line, whereas there was hardly any production in the T5-neo cell line. Although there was a slight increase in the % tail DNA in the T5-neo cells with IQ 10 µg/ml (also see section 5.3.II.2.1.), this is not biologically significant, if it is compared with the effect seen at the same concentration for T5-1A2 cells. The results indicate that IQ induced DNA strand breaks in the T5-1A2 cell line but not in the T5-neo cell line.

The treatments with aflatoxin (0.1 - 10 ng/ml) also resulted in the induction of DNA strand breaks in the T5-1A2 cell line. However, aflatoxin at 1 and 10 ng/ml was cytotoxic, as evidenced by a decrease of cells left onto the slides. The percentage of tail DNA, tail moment and tail length for aflatoxin 0.1 ng/ml were 33.49 ± 7.21 %, 39.78 ± 11.76 and 86.02 ± 17.16 respectively, which were significantly different from the control ($P < 0.01$) (15.12 ± 3.55 %, 11.24 ± 5.60 and 48.69 ± 14.41 respectively). These results indicate that aflatoxin was a very potent inducer of DNA strand breaks in the T5-1A2.

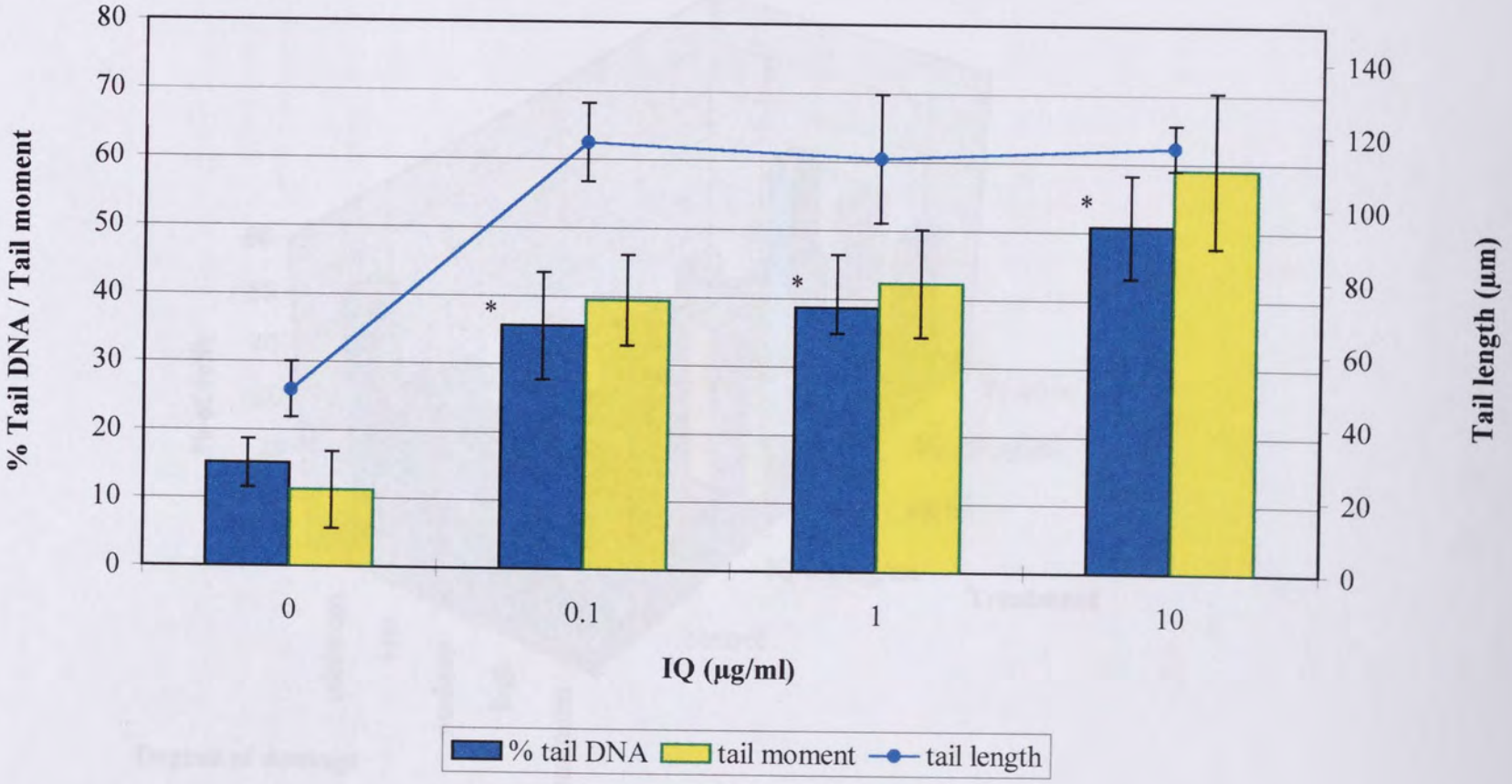



Figure 5.II.m. Induction of % tail DNA, tail moment and tail length by IQ in T5-1A2 cells

T5-1A2 cells were incubated for 1 h with IQ (0-10 µg/ml) and processed for the comet assay (section 5.2.8.) Values are the mean ± S.D., n=3, 50 cells analysed per slide. * $P < 0.01$ for parametric tests and $P < 0.05$ for non-parametric.

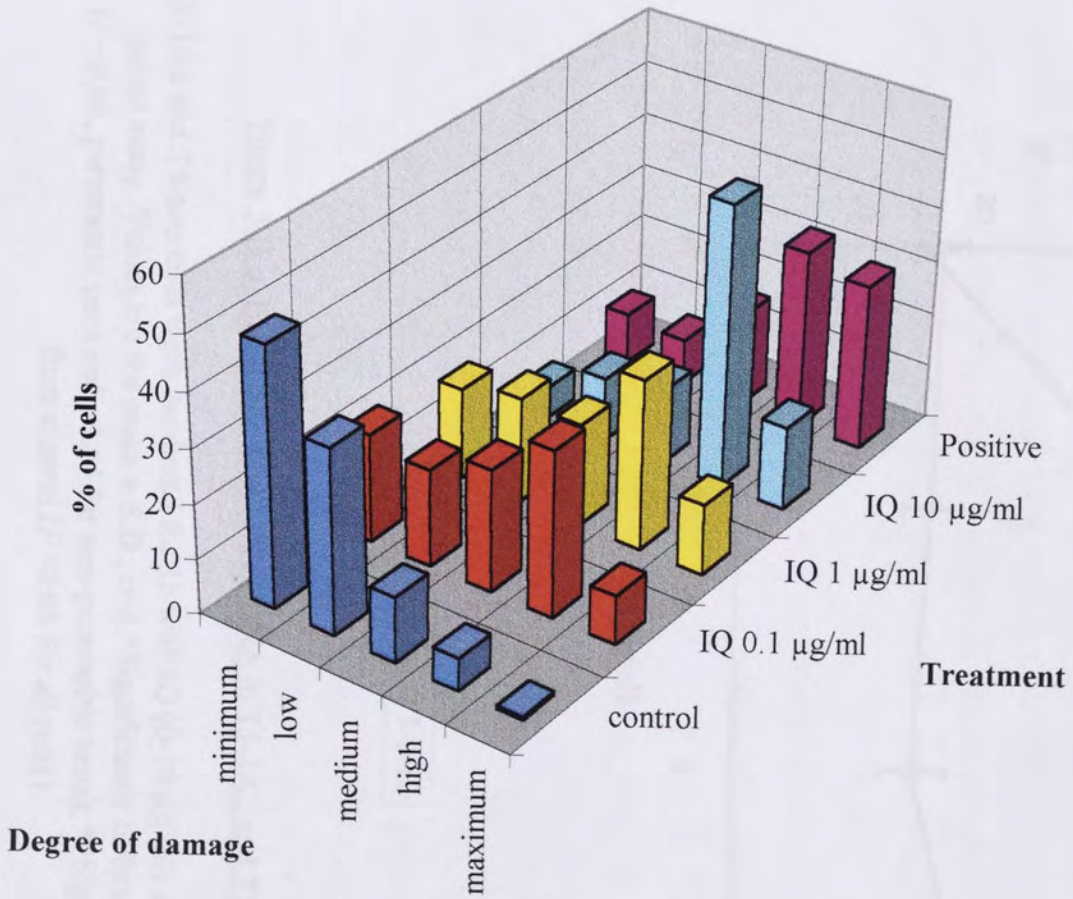



Figure 5.II.n. Comet response of T5-1A2 individual cells to IQ

T5-1A2 cells were incubated for 1 h with IQ (0-10 µg/ml) or hydrogen peroxide (25 µM) as the positive control and processed for the comet assay. One hundred and fifty cells were analysed.

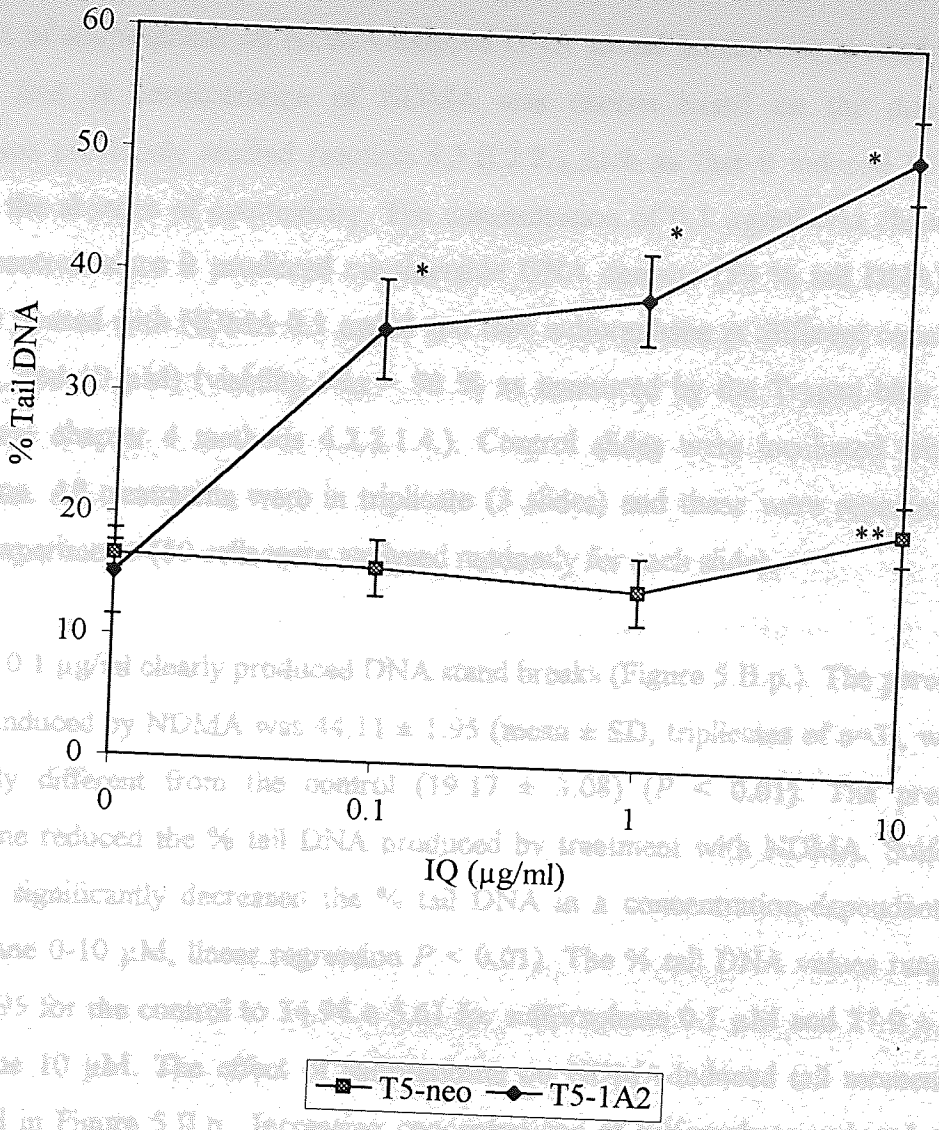



Figure 5.II.o. Effect of IQ on % Tail DNA in T5-1A2 and T5-neo cells

T5-1A2 and T5-neo cells were incubated for 1h with IQ (0-10 µg/ml) and processed for the comet assay. Values are the mean ± S.D., n=3.*Significantly different from controls ($P$ <0.01, parametric tests and <0.05 for non-parametric tests); **Significantly different from control ($P$ <0.05 for all tests).

## 5.3.II.3. Inhibition by sulforaphane of NDMA-induced DNA strand breaks

The effect of sulforaphane on NDMA-induced DNA strand breaks was studied in the T5-2E1 cell line. A concentration of NDMA was chosen based on the dose-response relationships previously studied (section 5.3.II.2.2.) such as that it induced DNA strand breaks in the absence of cytotoxicity. The concentration of 0.1 μg/ml was chosen for the positive control, since it produced considerable DNA damage (59 % tail DNA). T5-2E1 cells were treated with NDMA 0.1 μg/ml and then sulforaphane at different concentrations (0, 0,1, 1, and 10 μM) (viability was > 98 % as measured by the Trypan blue exclusion method (see chapter 4 methods 4.2.2.1.4.). Control slides were incubated with culture media alone. All treatments were in triplicate (3 slides) and these were repeated in three different experiments (50 cells were analysed randomly for each slide).

NDMA at 0.1 μg/ml clearly produced DNA stand breaks (Figure 5.II.p.). The percentage of tail DNA induced by NDMA was 44.11 ± 1.95 (mean ± SD, triplicates of n=3), which was significantly different from the control (19.17 ± 3.08) ($P < 0.01$). The presence of sulforaphane reduced the % tail DNA produced by treatment with NDMA. Sulforaphane treatments significantly decreased the % tail DNA in a concentration-dependent manner (sulforaphane 0-10 μM, linear regression $P < 0.01$). The % tail DNA values ranged from 44.11 ± 1.95 for the control to 34.94 ± 5.61 for sulforaphane 0.1 μM and 27.9 ± 1.38 for sulforaphane 10 μM. The effect of sulforaphane on NDMA-induced tail moment is also represented in Figure 5.II.p.. Increasing concentrations of sulforaphane reduced markedly the tail moment of the incubations containing NDMA alone. Tail moment values for sulforaphane ranged from 44.76 ± 9.08 to 30.47 ± 4.71 for sulforaphane 0.1 to 10 μM respectively, compared to 63.84 ± 4.18 for NDMA alone (0.1 μg/ml). For the control treatments, tail moment was 17.77 ± 7.19.

These results can also be expressed as percentage of inhibition of NDMA-induced DNA strand breaks for the % of tail DNA (see Table 5.II.c.). The percentage of inhibition was calculated after subtraction of the control values (treatment with culture media alone). Sulforaphane inhibited the % of tail DNA induced by NDMA from a 36.77 % inhibition for sulforaphane 0.1 μM up to 65.0 % inhibition for sulforaphane 10 μM ($P < 0.01$).

Figure 5.II.q. shows the distribution of cells according to DNA damage (% tail DNA). Cells without treatment (control) were mainly distributed in the categories of minimum and low damage (72 % of cells), whereas for the treatment with NDMA alone, only 35 % of cells were in the minimum and low damage categories and a high population of cells had high and maximum damage (49 %). Increasing concentration of sulforaphane increased the number of cells in the minimum and low damage categories (44 %, 55 % and 58 % of cells for sulforaphane 0.1, 1, and 10 μM respectively compared to 35 % for NDMA treatment). At the same time, increasing concentrations of sulforaphane reduced the number of cells highly cometed (high and maximum damage) giving 36 %, 27 % and 23 % of cells for sulforaphane 0.1, 1, and 10 μM respectively compared to a 49 % of cells with high and maximum damage for the NDMA treatments. It also can be seen in figure 5.II.q. that with increasing concentration of sulforaphane, the pattern of the distribution of cells becomes more similar to the distribution of the control treatment.

These results indicate that sulforaphane was able to reduce the percentage of tail DNA and tail moment (measurements of DNA strand breaks) of cells treated with NDMA and also increased the populations of cells with low DNA damage and decreased populations of cells highly cometed. This indicates that sulforaphane inhibited NDMA-induced DNA strand breaks in the T5-2E1 cell line.

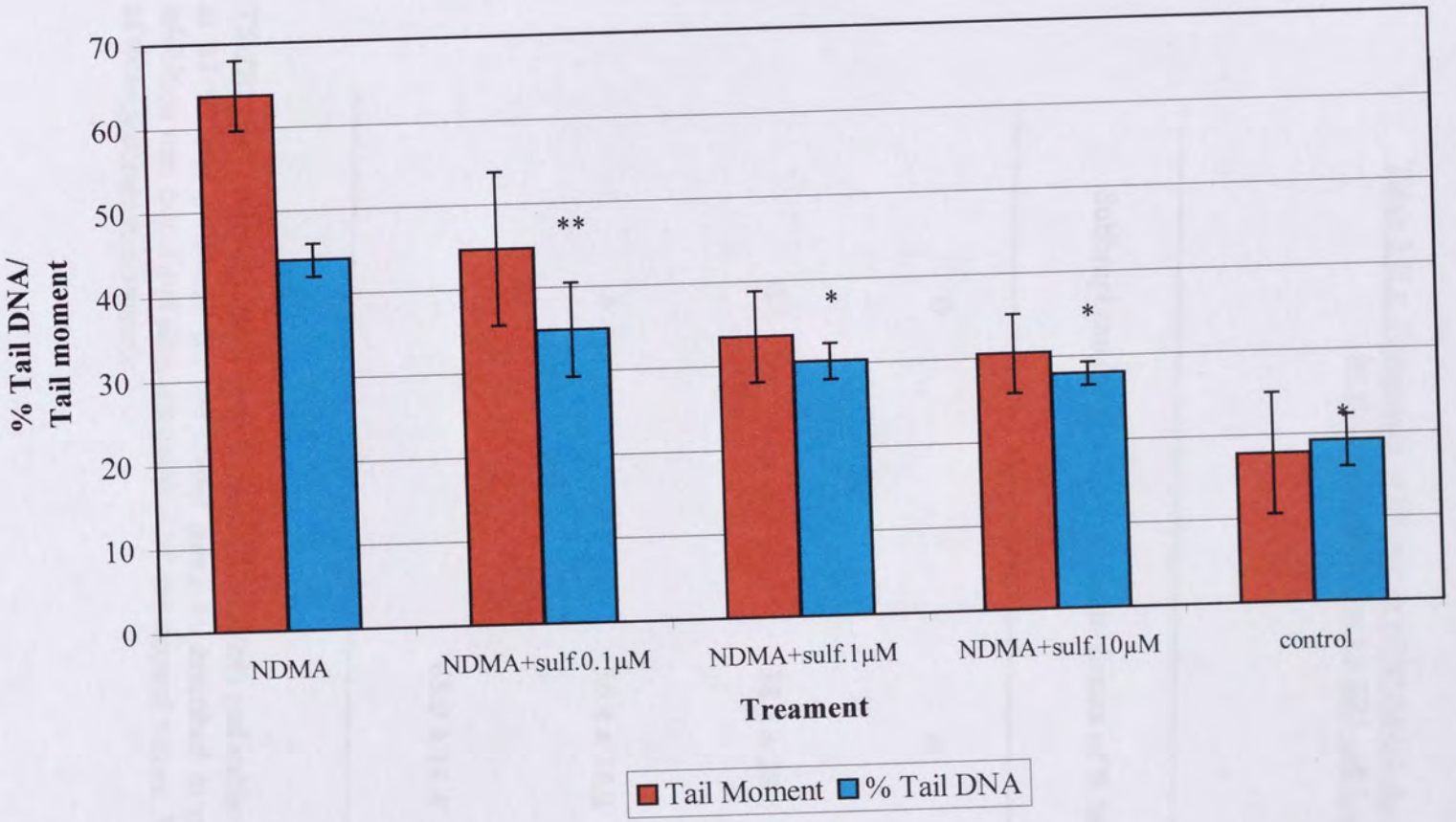



Figure 5.II.p. Inhibition by sulforaphane of NDMA-induced % tail DNA and tail moment in the T5-2E1 cell line

Cells were incubated for 1 h with NDMA (0.1 μg/ml) and sulforaphane (sulf.) (0-10 μM) or culture media alone for the controls. Values are the mean ± S.D. of three separate experiments, each in triplicate. * $P < 0.01$ for parametric tests and $P < 0.05$ for non-parametric; ** $P < 0.05$ for all tests

Table 5.II.c. Percentage of inhibition of NDMA-induced strand breaks by sulforaphane in the T5-2E1 cell line

| Sulforaphane (μM) | % inhibition of % tail DNA |
|---|---|
| 0 | 0 |
| 0.1 | 38.3 ± 28.5 |
| 1 | 56.4 ± 16.2 |
| 10 | 65.9 ± 14.4 |

T5-2E1 cells were incubated with NDMA (0.1 μg/ml) and sulforaphane (0-10 μM) for 1 h at 37 °C and processed for the comet assay as described in methods 5.2.8. The % of inhibition was calculated after subtraction of the control values. Values are the mean ± SD of three separate experiments.

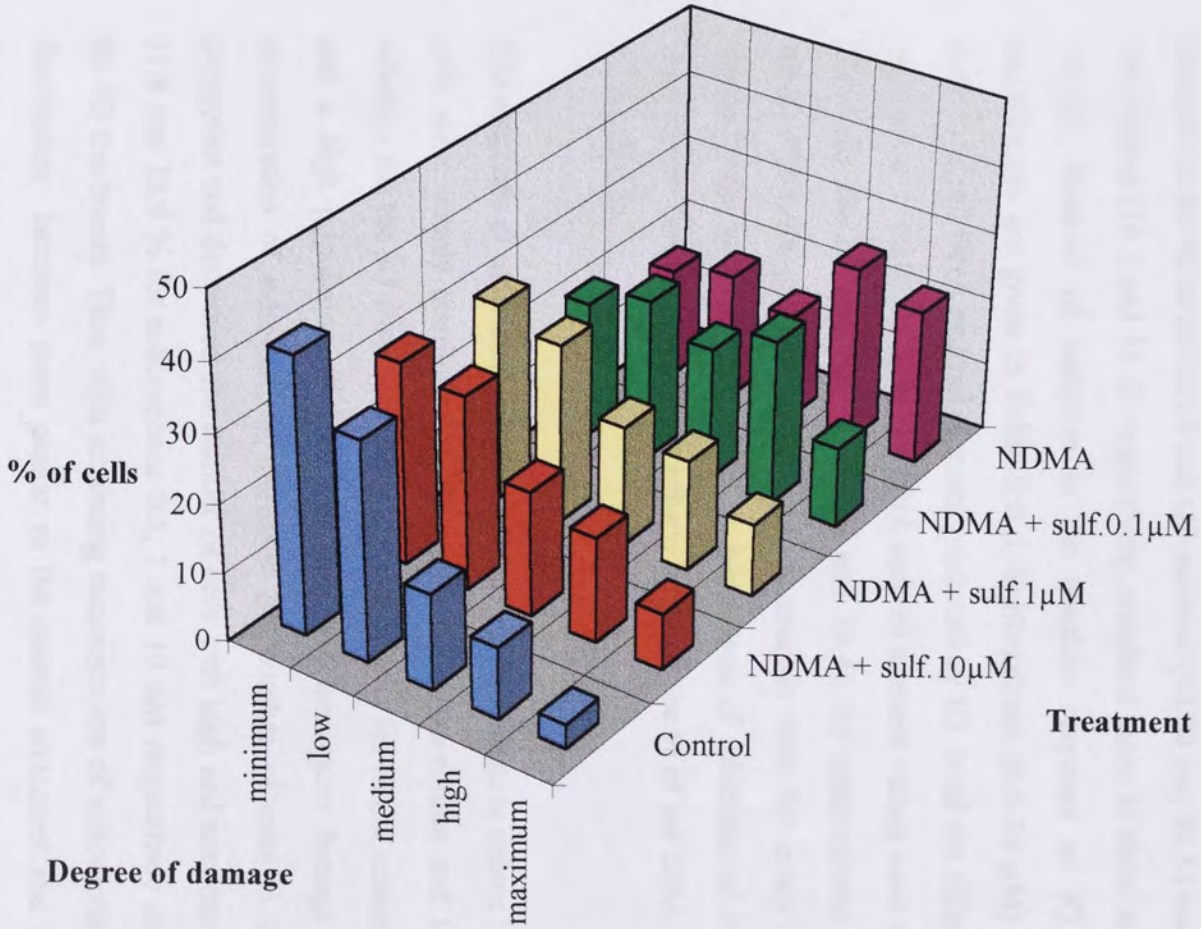



Figure 5.II.q. Effect of sulforaphane on NDMA-induced DNA damage in T5-2E1 individual cells

Cells were treated with NDMA (0.1 μg/ml) and sulforaphane (sulf.) (0-10 μM) or medium alone for the controls. Four hundred and fifty cells cells were analysed in total (three separate cell preparations).

## 5.3.II.4. Effect of sulforaphane on IQ-induced DNA strand breaks in the T5-1A2 cell line.

The effect of sulforaphane on IQ-induced DNA strand breaks was studied in the T5-1A2. T5-1A2 cells were incubated with IQ (1 μg/ml) and with sulforaphane (0, 0.1, 1, and 10 μM) for 1 h at 37 °C.

IQ at 1 μg/ml produced DNA strand breaks in the T5-1A2 cell line as evidenced by an increase in the % of tail DNA and tail moment (40.90 and 39.54 respectively) compared to the control (16.5 and 11.55 respectively, weighted mean of three separate experiments) ($P$ <0.01). Because of variation in the absolute response to IQ, results of individual experiments are given in Table 5.II.d. Sulforaphane (0.1-10 μM) decreased significantly both % of tail DNA and tail moment produced by IQ in all the different experiments (linear regression, $P$ <0.05). The % tail DNA and tail moment values were reduced from 40.90 and 39.54 for the IQ treatment to 24.48 and 19.46 for sulforaphane 10 μM ($P$ < 0.01 for parametric tests or $P$ < 0.05 for non-parametric tests for every individual experiment). These results were also expressed as percentage of inhibition of IQ-induced DNA strand breaks (see Table 5.II.e.). Sulforaphane inhibited the % of tail DNA induced by IQ by up to 71.7 ±19.0 %.

The response of individual cells to the different treatments is shown in Figure 5.II.r. Control cells were mainly distributed in the categories of minimum and low damage (63.0 %), whereas for the IQ treatments only 37.3 % of cells fell in the minimum and low categories and a high population of cells had high and maximum damage (48.3 %). Increasing concentration of sulforaphane increased the number of cells in the minimum and low categories and decreased the number of cells with high and maximum damage giving 37.0, 31.8 and 19.4 % for sulforaphane 0.1, 1 and 10 μM respectively compared to 48.3 % for the IQ treatments. Thus, with increasing concentration of sulforaphane the cell populations distribution becomes more similar to the control treatment and less similar to the IQ distribution (Figure 5.II.r.). The results thus indicate that sulforaphane reduced the % of tail DNA and tail moment of T5-1A2 cells treated with IQ, indicating that sulforaphane inhibited IQ-induced DNA strand breaks in this cell line.

Table 5.II.d. Inhibition by sulforaphane of IQ-induced DNA strand breaks

| Sulforaphane (μM) | % Tail DNA | | | Tail moment | | |
|---|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 1 | Experiment 2 | Experiment 3 |
| 0 | 28.97 ± 3.19 | 38.70 ± 3.38 | 50.98 ± 3.38 | 20.37 ± 5.39 | 32.91 ± 3.66 | 57.36 ± 16.47 |
| 0.1 | 22.29 ± 0.63 | 28.63 ± 7.98 | 43.80 ± 5.86 | 16.37 ± 3.59 | 24.02 ± 9.63 | 40.01 ± 3.55 |
| 1 | 20.97 ± 3.26 | 31.45 ± 2.52 | 41.77 ± 0.22 | 13.64 ± 6.81 | 25.56 ± 4.25 | 44.17 ± 4.90 |
| 10 | 14.00 ± 0.96 | 23.34 ± 3.19 | 32.90 ± 3.5 | 6.70 ± 0.39 | 16.50 ± 1.55 | 30.51 ± 4.41 |

IQ (1 μg/ml) and sulforaphane were incubated with T5-1A2 cells for 1 h at 37 °C and processed for the Comet assay. Because of variation in the absolute response to IQ results for individual experiments are shown. Values are the mean ± SD, n=3, except for experiment 3, n=2. Control incubations gave % tail DNA of 12.98 ± 2.37, 13.41 ± 5.80 and 21.22 ± 0.55 respectively for experiments 1-3 and tail DNA of 5.20 ± 1.82, 6.31 ± 3.93, and 18.92 ± 2.26. Statistical difference for sulforaphane at 0.1 and 1 μM varied depending on the test, but at 10 μM all tests for all experiments were significantly different, $P < 0.05$. Linear trend was observed in all experiments ($P < 0.05$).

Table 5.II.e. Percentage of inhibition by sulforaphane of IQ-induced DNA strand breaks in T5-1A2 cells

| Sulforaphane (μM) | % inhibition of tail DNA |
|---|---|
| 0 | 0 |
| 0.1 | 35.3 ± 9.8 |
| 1 | 44.1 ± 24.6 |
| 10 | 71.7 ± 19.0 |

T5-1A2 cells were incubated with IQ (1 μg/ml) and sulforaphane (0 -10 μM) for 1 h at 37 °C and processed for the Comet assay as described in methods 5.2.8. The % of inhibition was calculated after subtraction of the control values. Values are the mean ± SD of three separate experiments.

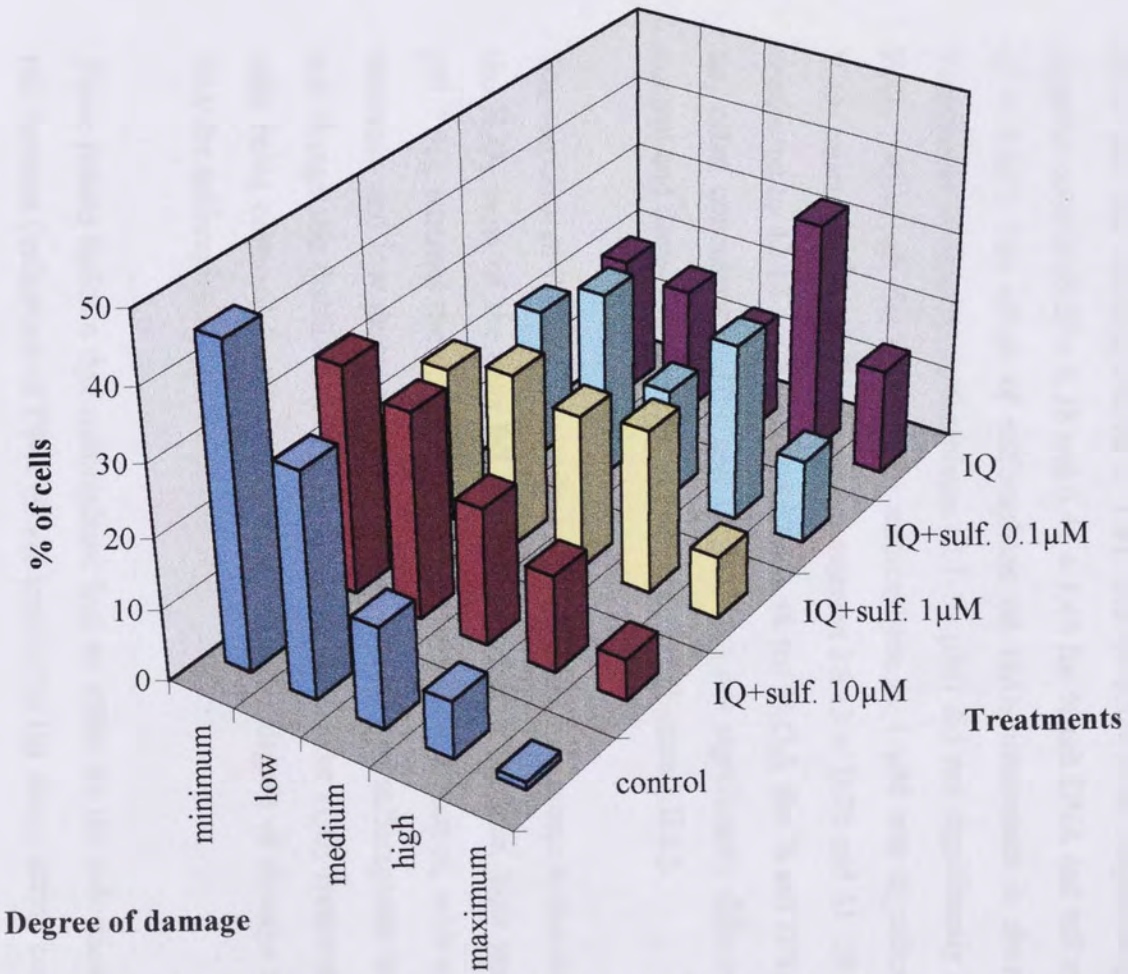



Figure 5.II.r. Inhibition by sulforaphane of IQ-induced DNA damage in T5-1A2 indiviudal cells

Cells were treated for 1 h with IQ (1 μg/ml) and sulforaphane (sulf.) (0-10 μM) or culture medium alone for the controls. One hundred and fifty cells were analysed in three separate cell preparations.

## 5.3.II.5. Effect of sulforaphane on $H_2O_2$-induced DNA strand breaks in the T5-neo cell line.

The effect of sulforaphane on $H_2O_2$-induced DNA strand breaks was studied in the T5-neo cell line. Cells were incubated with $H_2O_2$ (25 μM) and sulforaphane (0-10 μM) for 1h at 37 °C in two separate experiments.

$H_2O_2$ (25 μM) induced DNA damage in T5-neo cells as seen by an increase in % of tail DNA and tail moment (42.18 ± 1.51 and 64.57 ± 4.48 respectively) compared to the negative control (8.23 ± 0.38 and 6.41 ± 1.48 for % tail DNA and tail moment respectively) ($P < 0.01$). The effect of sulforaphane on $H_2O_2$ treatments is shown in Figure 5.II.s. Treatments containing sulforaphane (0.1-10 μM) did not significantly reduce the effect of $H_2O_2$ . Although the response to sulforaphane at 1 μM was significantly lower than the $H_2O_2$ treatment (% tail DNA and tail moment 36.13 ± 0.70 and 51. 24 ± 1.91 respectively, compared to 42.18 ± 1.51 and 64.57 ± 4.48 for $H_2O_2$), the % tail DNA of sulforaphane at any other concentration (0.1 and 10 μM) was not significantly different from the positive control and dose-response effects were not seen (Figure 5.II.s.).

The response of the individual cells to the different treatments is shown in Figure 5.II.t. For the $H_2O_2$ most of the cells fell in the categories of medium, high and maximum damage (71.7 %), whereas the opposite effect was seen for the control, with most of the cells with minimum and low damage (92.7 %). The presence of sulforaphane in the incubations did not change the distribution of cells with respect to the $H_2O_2$ treatment, with most of the cells being cometed (medium, high and maximum degree of damage ranged from 65.8 to 70.9 for sulforaphane 0.1-10 respectively).

These results indicate that sulforaphane had no effect on the induction of % tail DNA and tail moment (indicators of DNA strand breaks) by the direct acting compound $H_2O_2$ in the T5-neo cell line.

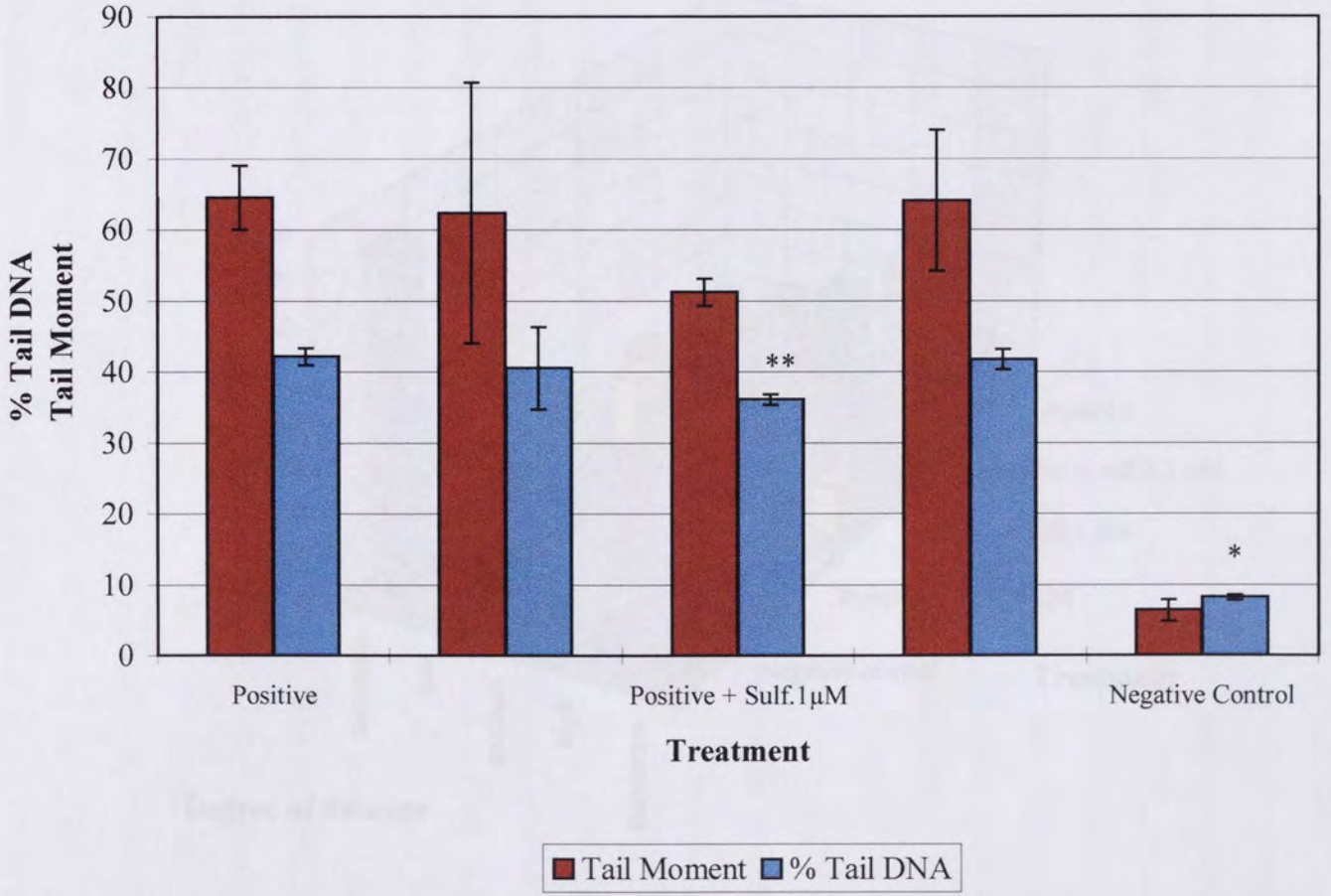



Figure 5.II.s. Effect of sulforaphane on $H_2O_2$-induced DNA strand breaks in the T5-neo cell line

T5-neo cells were incubated for 1h with $H_2O_2$ (25 μM) (positive) and sulforaphane (0-10 μM) or culture media alone for the negative control. Values are the weighted mean ± SD of two separate experiments, each in triplicate. * $P < 0.01$. ** $P < 0.05$.

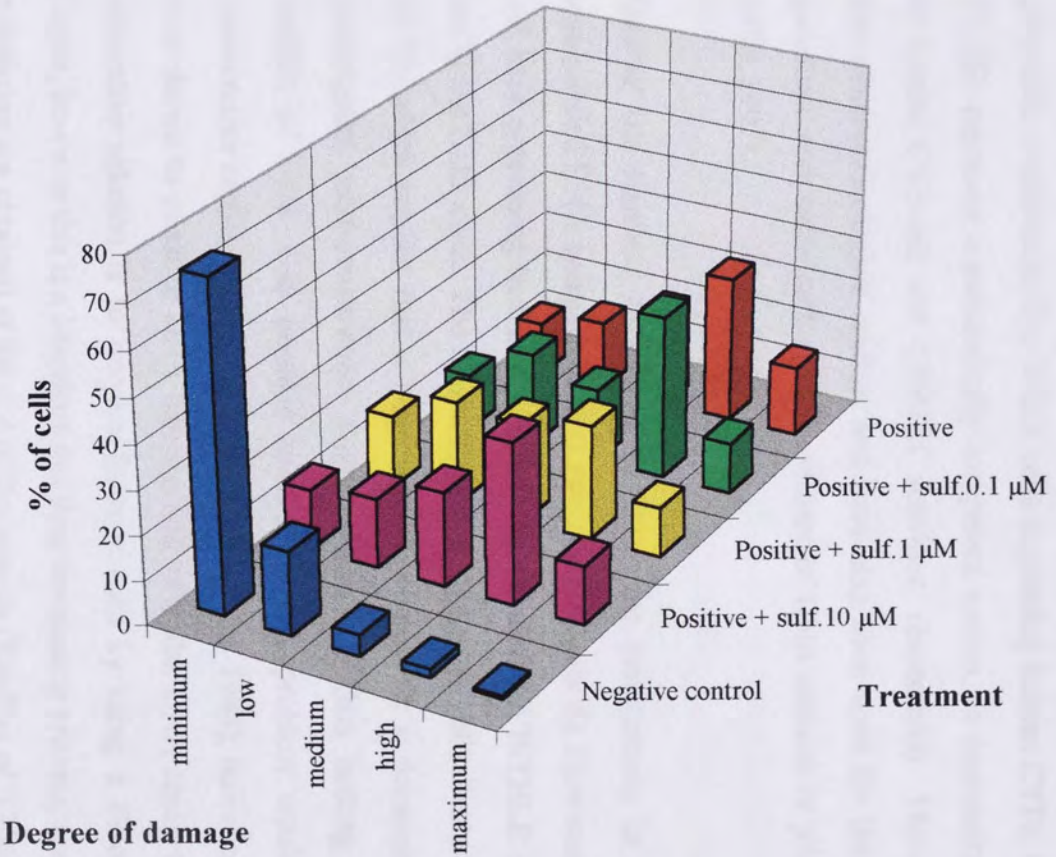



Figure 5.II.t. Effect of sulforaphane on hydrogen peroxide-induced DNA damage in T5-neo individual cells

T5-neo cells were incubated for 1 h with hydrogen peroxide (Positive) (25 μM) and sulforaphane (sulf.) (0-10 μM) or culture media alone for the negative control. Three hundred and fifty cells were analysed.

## 5.4. Discussion

Heterologous expression systems for human cytochrome P450 isoenzymes are valuable tools to aid in extrapolation of data from laboratory animals to humans (Langenbach *et al*, 1992; Ellard *et al*, 1991; Gonzalez and Korzekwa, 1995) and have been used to measure a variety of genotoxic endpoints (see section 5.1.). In this study we used a human liver epithelial cell line (THLE) expressing human CYPs to detect the activation of procarcinogens and to assess the ability of sulforaphane to modify the production of genotoxic metabolites. The THLE cells expressing human CYPs, in particular T5-1A2 and T5-2E1 represent a metabolically competent system, as demonstrated by the measurement of human CYP1A2 and CYP2E1 activities, respectively. These cells can be used for pharmaco-toxicological studies, and have also been used for the measurement of several genotoxic endpoints such as measurement of DNA adducts or p53 mutations (Macé *et al*, 1996, 1997).

One of the standard techniques to determine genotoxicity in mammalian cells is the unscheduled DNA synthesis (UDS) assay (see chapter 4). However, most of the established cell lines expressing human CYPs, including human CYP-THLE cells, are not suitable for use in the UDS assay. The reason for this is that these cells divide rapidly (doubling time < 24 h), which implies that a large number of cells in a determined time are in S-phase (undergoing semi-conservative DNA replication), thus making impossible an accurate analysis of UDS. One possible solution to this problem would be to suppress semi-conservative replication with hydroxyurea (Waters, 1984), however this compound has also been shown to produce DNA damage and to inhibit DNA repair (Collins *et al*, 1977). An alternative solution is to remove S-phase cells by using a Fluorescence Activated Cell Sorter, however this is a laborious and time consuming process, and only few cells of a sub-population are obtained at the end of the process (Rueff *et al*, 1996).

An alternative genotoxicity test could be the SCGE or Comet assay, which measures DNA strand breaks as the end point (see section 5.1.4.). Strand breakage is non-specific and can be produced directly (e.g. uv light) or indirectly (through alkali-labile sites or excission repair) by a wide range of genotoxic agents such as chlorobenzene (Vaghef and Hellman,

1995), benzo[*a*]pyrene (Monteith and Vanstone, 1995), 4-nitroquinoline-1-oxide or DMBA (Speit and Hartmann, 1995). One of the several advantages of the Comet assay is that it can be used in any nucleated cell population, independently of their position in the cell cycle (McKelvey-Martin *et al*, 1993). The Comet assay has been used in several cell lines such as murine lymphoma subline LY-R (Kruszewski *et al*, 1994), HeLa cells (Collins *et al*, 1995) or human fibroblasts (Singh *et al*, 1991), suggesting that it may be a useful technique for measurement of genotoxicity in the THLE cells expressing human CYPs.

### 5.4.1. Adaptation of the Comet assay

The Comet assay requires isolated cells. Since T5-neo, 2E1 and 1A2 cells grow in monolayers (see section 5.2.1.), a single cell suspension was generated either by trypsination or by scraping. This study indicates that these techniques were not suitable for these cells, since both cell suspensions obtained by trypsination and scraping in two different cell types (T5-2E1 and T5-1A2) gave a high background of percentage of tail DNA, tail moment and tail length, parameters indicative of DNA strand breakage. Furthermore, a high percentage of cells exhibited medium and high damage (Figures 5.II.a. and b.), particularly those cells obtained by trypsination. These findings were in agreement with those of Singh *et al*, (1991), who reported induction of DNA damage by trypsination and scraping in human-foreskin derived fibroblasts using the Comet assay. They also suggested that these effects could be the result of cell membrane damage and the subsequent elevation of intracellular free calcium which has the potential to activate endodeoxyribonucleases (Singh *et al*, 1991). However, the induction of DNA damage was not reported in other cell types, such as HeLa cells using a mixture of trypsin-versene (Collins *et al*, 1995) or in human cell lines MRC5CV1 and xeroderma pigmentosum cell line XP12ROSV using trypsin (Speit and Hartmann, 1995). This suggests that the enhanced migration of DNA in the Comet assay following trypsination might depend on the type of cells used (see below). The establishment of a method with adequate sensitivity whilst maintaining a low background for control cells was thus required, and was achieved by using a modification of the method by Singh *et al*, (1991), in which cells were grown on microscope slides.

A relatively high background was still present in control treatments of T5-1A2, 2E1 and neo cells, however the sensitivity was adequate for detecting positive responses (see following sections). Several reports have indicated that certain cell types such as human and mouse sperm (Singh *et al,* 1989) or mouse kidney cells (Fairbairn *et al,* 1994) showed DNA damage in the Comet assay due to the presence of alkali-labile sites (DNA damage was not observed under neutral conditions) rather than preexisting DNA strand breaks, thus suggesting that this background represents a functional characteristic of the chromatin in these cell types rather than DNA damage. As part of the technique modification described above (section 5.3.II.1.2. and table 5.II.b), the effect of unwinding and electrophoresis time on DNA damage in control cells was studied. The reduction of unwinding and electrophoresis time (both under alkaline conditions) decreased significantly the percentage of tail DNA, tail moment and tail length of control T5-1A2 cells. This suggests that the background observed in these cells (and probably in T5-2E1 and neo cells) may (as with the sperm and kidney cells; see above), also be a consequence of the presence of alkali-labile sites rather than DNA damage.

### 5.4.2. Induction of DNA strand breaks by carcinogens

NDMA is a potent genotoxicant in rodents and possibly in humans (Bartsch, 1991) and has been shown to be metabolically activated by human CYP2E1, although other enzymes such as CYP2A6 may also contribute to the activation with less affinity in the AHH-1 cDNA expression system (Crespi *et al,* 1990; Bellec *et al,* 1996). NDMA (0.01 - 1 μg/ml) produced DNA strand breaks in the T5-2E1 cell line (expressing human CYP2E1) in a concentration-dependent manner but had a minimal effect on the T5-neo cells (lacking transfected human CYP isoenzymes). The small increase in percentage of tail DNA for NDMA at 1 μg/ml in T5-neo cells (only significantly different from the control when the whole cell population was used for the statistical analyses) might be explained by a low level of residual CYP2E1 (or CYP2A6, although levels of both enzymes were not detected by Western blotting, there is evidence of production of low levels of $N^7$-methyldeoxyguanosine adducts; Pfeifer *et al,* 1993). The production of DNA strand breaks by NDMA in T5-2E1 was also clearly indicated by an increase in the percentage of cells cometed (i.e. with % tail DNA > 40, corresponding to high and maximum categories, Figure 5.II.k.). On the other

hand, the majority of T5-neo cells treated with NDMA remained within the categories of minimum and low damage. These results are in accord with the fact that T5-neo cells are relatively inefficient in the metabolic activation of NDMA, whereas T5-2E1 cells activate NDMA via CYP2E1 to a reactive metabolite, and thus demonstrating the applicability of these cells.

It has been suggested that cytotoxicity under some circumstances may increase the parameters measured in the Comet assay (McKelvey-Martin *et al*, 1993). However, in this study NDMA was not found to be cytotoxic at the concentrations tested (0.01 - 1 μg/ml) (measured by trypan blue exclusion) in T5-2E1 cells. NDMA at 10 μg/ml was, however, found to be cytotoxic, which resulted in a decrease of DNA damage with respect to the previous concentration. These results indicate that the DNA strand breakage observed after treatment of T5-2E1 cells with NDMA (0.01- 1 μg/ml) is not secondary to cytotoxicity. The production of DNA strand breaks by NDMA has also been observed in rat hepatocytes using the Comet assay, although at a higher concentration than the one used here (1.85 mg/ml) (Ashby *et al*, 1995) and *in vivo* in hamster and rat hepatocytes using the alkaline elution assay (Jorquera *et al*, 1994). NDMA is a methylating agent which has shown to yield $N^7$-, $O^6$-methylguanine, and may yield apurinic sites during DNA repair (Guttenplan, 1987). The repair of alkylated bases involves the sequential action of an endonuclease, DNA polymerase and ligase (see section 1.1.3.1.), during which process DNA strand breaks may be formed (Jorquera *et al*, 1994). The positive response of NDMA in the Comet assay may therefore be a result of DNA strand breaks induced during repair and/or production of alkali labile sites (apurinic sites). NDMA in mouse hepatocytes induced UDS at the concentration of 2.5 μg/ml (see chapter 4), thus the sensitivity of the Comet assay was comparable to that of other standard genotoxicity tests.

IQ is a potent mutagen in various systems (bacteria, laboratory animals) and a carcinogen in rodents and primates (Overvik and Gustafsson, 1990), however its risk to humans is still unclear, although it is thought that it may play a role in the aetiology of human cancers (Overvik and Gustafsson, 1990). IQ has been found to be activated by the human CYP1A2 (Thompson *et al*, 1991; Aoyama *et al*, 1990). IQ (0.1- 10 μg/ml) produced DNA strand breaks in a concentration-dependent manner in T5-1A2 (expressing CYP1A2) whilst no

concentration-response was seen in T5-neo cells, thus suggesting that T5-1A2 cells were capable of metabolic activation of IQ via CYP1A2. These results were further supported by the fact that the percentage of T5-1A2 cells cometed increased with increasing concentration of IQ (Figure 5.II.n.), whereas T5-neo cells remained with minimum and low damage for both control and IQ treatments. A small increase in DNA damage parameters was observed in T5-neo cells with IQ 10 μg/ml, however that was not biologically significant if compared with the response at the same concentration in T5-1A2 cells. IQ was not cytotoxic in T5-1A2 cells, thus indicating that production of DNA strand breaks by IQ was the result of genotoxicity. These results are comparable to those of Davies *et al,* (1995), where IQ produced DNA strand breaks at 2.5 μM and above in V79 cells genetically engineered to express rat CYP1A2. The mechanisms of formation of these breaks are not fully understood, but may reflect alkali labile sites or excision repair of DNA adducts derived from IQ. Aflatoxin $B_1$, which has recently been shown to be metabolically activated by human CYP1A2, CYP3A4 and CYP2A6 (CYP1A2 was the isoenzyme with the greatest affinity) in AHH-1TK cells expressing human enzymes (Crespi *et al,* 1990, 1991) and in T5 cells (Mace *et al,* 1996b, c), also produced DNA strand breaks in T5-1A2 cells. Aflatoxin $B_1$ produced DNA strand breaks at the concentration of 0.1 ng/ml, which is 1000-fold lower than the concentration used for IQ (0.1 μg/ml). Thus, T5-1A2 cells appeared to be much more sensitive to aflatoxin $B_1$ than to IQ. This contrasts with the fact that IQ is a more potent mutagen than aflatoxin $B_1$ in bacterial mutagenicity assays. IQ has shown to induce up to 400 revertants/ng in *Salmonella typhymurium* TA98 (Rumney *et al,* 1993; Overvik and Gustafson, 1990) whereas the mutagenic potential of aflatoxin $B_1$, was lower (18 revertants/ng in *S.typhymurium* TA100, Ames *et al,* 1975). The different DNA damaging potencies of aflatoxin $B_1$ and IQ in T5-1A2 cells might be explained in terms of metabolic activation of these compounds. Aflatoxin $B_1$ is activated to the highly reactive 8,9-epoxide by several human CYPs, including CYP1A2 (see above) which can then covalently bind to DNA to form DNA adducts (Guengerich *et al,* 1992) and DNA strand breaks, presumably produced as a consequence of excision repair or alkali labile sites. IQ is metabolised by CYP1A2 to *N*-hydroxy-IQ which is further metabolised to the ultimate reactive form IQ-$NH^+$ (see figure 1.b. for structure) via *N-O*-sulphatation or *N-O*-acetylation (mainly by NAT2, see general introduction section 1.2.3; Overvik and Gustafsson, 1990) which then can bind to DNA to produce *N*-(deoxyguanosin-8-yl)-IQ

adducts (Zu and Schut, 1991). Thus, the activation of aflatoxin $B_1$ involves one step whereas IQ requires two steps (two enzymes) which could explain the relatively low response of this compound in T5-1A2 cells. No information is available on the content of NAT2 in T5-1A2 cells; nevertheless, IQ was effective in producing DNA damage in T5-1A2 cells in the Comet assay.

Some controversy exists on the issue of which parameters should be used in the Comet assay to reflect DNA strand breakage. Whereas the majority of authors have used the tail length (or tail migration), which is easily measured, other authors have suggested that the percentage of tail DNA (Anderson *et al*, 1994; Collins *et al*, 1995; Ashby *et al* 1995) or tail moment (Vaghef and Hellman, 1995; Speit and Hartmann, 1995; Kruszewski *et al*, 1994; Hellman *et al*, 1995) are more accurate and correlate better with the amount of DNA damage produced (these two parameters require the use of a computerised system in order to be quantified). In this study, a correlation of tail length with percentage of tail DNA and tail moment was seen for NDMA in T5-2E1 cells, however for IQ treatments in T5-1A2 cells the tail length did not correlate with the other parameters. In fact, the tail length increased for the lowest concentration of IQ compared to the control treatment, but did not increase with increasing dose of IQ reaching a plateau. This agrees with the observations of other authors (Fairbairn *et al*, 1995; McKelvey-Martin *et al*, 1993), who suggested that the tail length is largely defined by the electrophoresis conditions rather than the DNA damage. This was further supported by the results obtained in an attempt to improve the "Standard" Comet assay for the cells used here (section 5.3.II.1.2. and table 5.II.b.). The reduction of unwinding and electrophoresis time did not change significantly the percentage of tail DNA and tail moment of T5-1A2 cells treated with hydrogen peroxide, however the tail length decreased, thus clearly indicating that this parameter depends on the electrophoresis conditions and for certain compounds may not reflect DNA damage.

Since the Comet assay measures DNA damage in individual cells, one of its advantages is that it allows the detection of cell sub-populations with different responses to the test agent. T5-neo, T5-2E1 and T5-1A2 are genetically "identical" and therefore should respond to genotoxins in an equal manner. However, cells showed heterogeneity of response to all the different agents tested (NDMA, IQ, aflatoxin $B_1$ and hydrogen peroxide). Nevertheless, a

gradual shift of cells from low damage to high damage was observed with increasing concentrations of test compound, as indicated in T5-2E1 treated with NDMA 1 μg/ml, where most of the cells had medium, high and maximum damage (Figure 5.II.k.). One possible explanation for this heterogeneous response is that the cells are asynchronous, i.e. they will be at different stages of the cell cycle (some cells may be in S-phase, $G_1$ or $G_2$-phase), and they might respond in a different manner to the genotoxic agent. This has been suggested by some authors (Fairbairn *et al*, 1995), however others have indicated that under alkaline conditions (as used in this work) no differences were seen, although when the assay was performed under neutral conditions (this allows detection of double strand breaks only) cell-cycle differences were observed (McKelvey-Martin *et al*, 1993; Olive *et al*, 1991). The theoretical basis behind these cell cycle differences might be that chromatin structure varies throughout the cycle and thus may affect the formation of comets. Furthermore, DNA repair systems may behave differently depending on the cell cycle position, which may result in a different production of DNA strand breakage, especially for the agents used here where excision repair may contribute to the induction of DNA strand breaks. Another possible explanation to the observation of cell heterogeneity is that enzyme activity (CYP1A2 and CYP2E1 for T5-1A2 and T5-2E1 cells respectively) may be affected by the stage of cell division, although no evidence for this exists. The variable responses to hydrogen peroxide, a direct acting genotoxicant not requiring metabolic activation, with all the different cell types suggests that heterogeneity is not simply a feature of variation in metabolic activation.

The Comet assay has been proposed as a screen for genotoxicity testing due to its sensitivity and non-specificity (measurement of DNA strand breaks is non-specific), although it also presents some minor limitations (i.e. false positives due to cytotoxicity or $Ca^{2+}$ release) (see introduction 5.1.4) (Fairbairn *et al*, 1995, McKelvey-Martin *et al*, 1993). The results shown here indicate that NDMA and IQ-production of DNA strand breaks was mediated by the human CYP2E1 and CYP1A2 isoenzymes respectively. These findings also suggest that the Comet assay may also be a valuable tool to aid mechanistic work on the role of CYP450 in the activation of chemical carcinogens.

### 5.4.3. Inhibition by sulforaphane of NDMA- and IQ-induced DNA strand breaks

The main purpose of this chapter was to determine whether sulforaphane was able to modulate genotoxicity of NDMA and IQ in human cells expressing human CYP2E1 and CYP1A2 and to aid the extrapolation of data from rodent-derived metabolic activation systems from previous chapters (chapter 4) to humans. Sulforaphane inhibited both NDMA and IQ-induced DNA strand breakage in T5-2E1 and T5-1A2 cells respectively. This was evidenced by a decrease on the percentage of tail DNA and tail moment with respect to the positive controls (NDMA and IQ) and also by a decrease of cell populations highly cometed and increasing populations with low damage. This inhibition by sulforaphane was not related to cytotoxicity effects, which were not evident in any of the cell lines. Sulforaphane had previously been shown to inhibit NDMA genotoxicity using rodent enzymes for activation (see chapter 4). The inhibition of IQ mutagenicity by several cruciferous vegetable juices has been reported (Edenharder *et al,* 1994) and sulforaphane has been found in some of these vegetables (Zhang *et al,* 1992), however it might be ambitious to link these two events, since vegetable juices contain a large number of known and probably unknown components. Recently, Knasmuller *et al,* 1996 reported the inhibition of the related amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (also activated by CYP1A2) genotoxicity by PEITC in various systems.

The inhibition of genotoxicity of NDMA and IQ in T5-2E1 cells and T5-1A2 cells respectively by sulforaphane is considered to reflect the ability of sulforaphane to inhibit both human CYP2E1 and human CYP1A2. Nevertheless it could be argued that sulforaphane may inhibit genotoxicity via modulation of DNA repair systems or other mechanisms or may interfere with the Comet assay itself. In order to clarify the mechanism of inhibition of DNA strand breakage by sulforaphane, the ability of sulforaphane to modulate genotoxicity by an indirect acting agent was assessed in T5-neo cells in the Comet assay. Hydrogen peroxide has been shown to produce DNA strand breaks *per se* in numerous cell types (Anderson *et al*, 1994) and was also effective in the T5-neo cells. Although a small decrease in DNA damage by sulforaphane was observed at the concentration of 1 μg/ml, no effect was seen at the other concentrations and concentration-dependent effects were not seen. Moreover, the distribution of cell populations did not

change with respect to the hydrogen peroxide treatment. This indicates that sulforaphane did not interfere with the Comet assay itself and did not protect against damage produced by $H_2O_2$ suggesting that sulforaphane may be devoid of antioxidant properties, although this should be confirmed in other systems and further experiments. The lack of sulforaphane to influence hydrogen peroxide-induced DNA strand breakage thus supports the inhibition of metabolic activation as a basis for the ability of sulforaphane to inhibit NDMA- and IQ-induced DNA strand breaks in T5-2E1 and T5-1A2 cells and suggests that such inhibition is relevant to the human forms of CYP2E1 and CYP1A2.

Interspecies variability regarding CYP450 expression, substrate specificity or inhibition are common (see section 1.3. 6). For example, furafylline is a potent inhibitor of human CYP1A2 but is far less potent in rat. Sulforaphane inhibited rat CYP2E1 and CYP1A2 (chapter 3), and also, genotoxcity of NDMA mediated by mouse enzymes (mainly CYP2E1) (chapter 4). The results showed here indicate that sulforaphane is also an effective inhibitor of the human orthologous CYP2E1 and CYP1A2. It has been suggested that inhibition of the metabolic activation (generally CYP450-mediated) of carcinogens may be a mechanism of chemoprevention (see 3.4. and general introduction 1.4.3). Furthermore, some chemopreventor agents that are specific inhibitors of some human CYP450 isoenzymes such as oltipraz (inhibits human CYP1A2 and CYP3A4; Langouët *et al*, 1995), are currently undergoing clinical trials (see general discussion, chapter 6). The ability of sulforaphane to inhibit genotoxicity of NDMA and IQ mediated by human enzymes suggests that this agent might be chemoprotective in humans.

# CHAPTER 6

# General discussion

Cruciferous vegetables are one of the major groups of vegetables associated with a decreased risk of certain cancers (Steinmetz *et al*, 1993) (see section 1.4.1). The epidemiological data are supported with experimental data from rodents (see 1.5.) and it has been suggested that this protective effect may be attributed, at least in part, to certain micronutrients (chemopreventors) (Wattenberg, 1983, 1992). Although the mechanisms of such protection are unknown and probably multifactorial, it has been suggested that the modulation of carcinogen metabolism may contribute (see section 1.4.3.). Sulforaphane, a degradation product of the glucosinolate gluconaphanin present in various cruciferous vegetables, was recently proposed as a potential chemopreventor agent, due to its ability to suppress DMBA tumorigenicity in rats, which was attributed to the ability of sulforaphane to induce enzymes involved in the detoxification of this carcinogen (Zhang *et al*, 1992, 1994). We wished to test the hypothesis that sulforaphane protection may also involve the modulation of metabolism via inhibition of activation pathways, i.e. CYP450. Sulforaphane inhibited rat CYP1A and CYP2E1 (chapter 3) and also inhibited the genotoxic effects of the potent carcinogen NDMA (mainly activated by CYP2E1) mediated by mouse metabolic activation systems (chapter 4). Furthermore, genotoxicity of NDMA and IQ mediated by human CYP2E1 and CYP1A2 was also reduced by sulforaphane. The inhibitory effects of sulforaphane were measurable at concentrations much lower than those found cytotoxic.

Human and rodent cytochrome P450 enzymes play a major role in the activation of carcinogens (see section 1.3.3.). Human CYP1A2 is involved in the activation of several carcinogenic arylamines (including IQ) and aflatoxin $B_1$ (Guengerich and Shimada, 1991, Crespi *et al*, 1991), and CYP2E1 activates NDMA, halogenated hydrocarbons and many low molecular weight environmental pollutants (Yang *et al*, 1990). The inhibition of CYP2E1 and 1A2 is thus expected to inhibit metabolic activation of these carcinogens and their fatal effects. Such is the case of allium organosulfides (diallylsulfide, sulfoxide and sulfone), which inhibited DMH-induced hepatotoxicity and colon carcinogenesis in rats (Yang *et al*, 1994). The inhibition of CYP2E1-dependent activation of DMH has been proposed as the most likely mechanism behind this effect (Brady *et al*, 1991, Reicks and Crankshaw, 1996). The inhibition of NDMA-induced hepatocarcinogenesis by the drug disulfiram, as well as the inhibition of tumorigenicity of various nitrosamines (CYP2E1 activated, at least in part) by various isothiocyanates (see section 4.1.2.) has also been

attributed to their ability to inhibit CYP2E1 (Yang *et al*, 1994, Ishizaki *et al*, 1990, Guo *et al*, 1992, Morse *et al*, 1993). In addition, the inhibition of NNK lung tumorigenicity in mice and rats by PEITC and other isothiocyanates (Morse *et al*, 1989, Jiao *et al*, 1994) was also associated with the inhibition of CYP1A2, responsible, at least in part for its activation (Smith *et al*, 1996, Guo *et al*, 1991, 1993). The inhibition of other forms of CYP450, such as CYP3A4 by oltipraz, has been associated with the inhibition of carcinogen activation by this enzyme (i.e. aflatoxin $B_1$; Langouët *et al*, 1995). The ability of sulforaphane to modulate human CYP2E1 and CYP1A2 mediated genotoxicity, thus suggests that it may afford chemoprotection against carcinogenic substrates of these two enzymes. Sulforaphane was also a potent inducer of GST in a murine cell line and in various organs of mice (15 μmol/mouse/day increased levels of GST and QR 2-3-fold; Zhang *et al*, 1992), enzymes involved mainly (see below) in the detoxification of carcinogens (see section 1.2.4. and 1.2.5.). In a recent study, sulforaphane has shown to regulate transcription of the Ya subunit of rat GST (Fei *et al*, 1996) however, studies concerning other subunits have not been reported yet. The induction of phase II enzymes and inhibition of CYP450s are therefore complementary mechanisms, since both will contribute to blocking certain carcinogens from reaching or reacting with cellular targets. Tumorigenicity of DMBA was reduced by sulforaphane in rats *in vivo* (Zhang *et al*, 1994). However, it can not be concluded whether this effect was due to the induction of phase II enzymes (GSTs and QRs) alone (Zhang *et al*, 1992) or whether inhibition of rat CYP1A (chapter 3) also contributed. The study of the inhibitory effects of sulforaphane *in vivo* thus requires attention.

CYP450 enzymes are also known to metabolically detoxify certain carcinogens (see table 1.d. in general introduction). To cite some examples, 1,3-dinitropyrene and trichloroethylene can be detoxified by CYP1A2 and CYP2E1 respectively (Guengerich, 1994). On the other hand, phase II GSTs (see section 1.2.4), whose major role is to detoxify reactive intermediates, can result in increased reactivity in some instances (e.g. ethylene dibromide) (Guengerich, 1994). As a result of this, the inhibition of CYP450 isoenzymes, and induction of GST isoforms, may not always result in a protective effect, in fact it could increase carcinogenicity for compounds such as the above mentioned. The protective effects of any chemopreventor agent, including sulforaphane, towards one or

various carcinogens should therefore not be extrapolated to other carcinogens, unless there is sufficient experimental evidence.

The chemoprotective action of a chemical can be viewed as the result of the balance between beneficial and adverse effects. Isothiocyanates are an interesting class of compounds that illustrate this point, since they appear to have both anticarcinogenic and mutagenic/carcinogenic properties. The overall balance of these effects dictates their final action; for instance, allylisothiocyanate is under certain circumstances mutagenic and carcinogenic (Neudecker and Hernschler, 1985, Dunnick *et al,* 1982), whereas PEITC and BITC are regarded as chemopreventive agents, although they have also been found genotoxic (Babich *et al,* 1993). Sulforaphane was not genotoxic itself in two different systems (see section 4.4.1.), thus suggesting an overall beneficial balance. It is also encouraging that no adverse effects were seen when administered to rats (50-150 μmol/rat/day by gavage) previous to DMBA administration (Zhang *et al,* 1994).

Another potential adverse effect to chemoprevention might be the induction of CYP450 forms following *in vivo* exposure. Since CYP450s play key roles in the activation of carcinogens (see above) (Guengerich and Shimada, 1991, Gonzalez and Gelboin, 1994), it has been suggested that their induction may result in enhanced carcinogen activation and carcinogenicity (Ioannides and Parke, 1993). This may be the case for indole-3-carbinol, a glucosinolate degradation product also found in brassica vegetables which has been shown to induce AHH activity (Loub *et al,* 1975). In contrast to this, isothiocyanates derived from glucosinolates, such as PEITC did not induce CYP1A, CYP2E1 or CYP3A in rats, although increased levels of CYP2B were observed (Ishizaki *et al,* 1991, Guo *et al,* 1992). Nevertheless, the role of the human orthologous CYP2B6 in carcinogen activation is minimal (Guengerich, 1995). The ability of sulforaphane to induce CYP450 is not known. However, sulforaphane appears to be a monofunctional inducer, i.e. induces phase II enzymes without interaction with the Ah receptor, which is responsible for the induction of CYP1A1 and CYP1A2 (see sections 1.3.4.1.1. and 1.4.4. of general introduction). Evidence for this is provided by the induction of QR in Hepa 1c1c7 cells defective in the Ah receptor (Zhang *et al,* 1992). The induction of GST and QR by sulforaphane appears to be mediated by the ARE responsive element (Prestera and Talalay, 1995). This was confirmed

in a recent study, where sulforaphane induced CAT-expression in HepG2 human hepatoma cells transfected with the 5' regulatory region of rat QR linked to the reporter gene CAT, by interacting with ARE, but no induction was observed with XRE (Gerhauser *et al*, 1997). XRE sequences are present in the enhancer region of various phase II enzymes and also in those of CYP1A1 and CYP1A2, whereas ARE sequences are present only in the phase II detoxification enzymes but not in CYP1A (see 1.3.4.1.1. and 1.4.4.). The findings of Gerhauser *et al*, (1997) thus suggest further that sulforaphane does not induce CYP1A. A more complete assessment of P450 induction has not been reported for sulforaphane but it is of interest that the related glucosinolate hydrolysis product, 1-isothiocyanate-3-methylsulfinylpropane gave no significant induction of phase I enzymes at up to 100 μmol/kg (gavage for 7 days) in rats, while it induced GST and QR in the intestine (Kore *et al*, 1993).

The potential ability of sulforaphane to modulate carcinogenesis by other mechanisms is not known, but should not be ignored. Administration of dried broccoli to rats resulted in the induction of antioxidant enzymes in several organs (Vang *et al*, 1995). The induction of glutathione reductase in liver and glutathione peroxidase in kidney was associated with indole glucosinolates and glucoerucin, and the induction of SOD in liver correlated with the glucosinolate glucoerysolin (Vang *et al*, 1995). Glucoerucin and glucoerysolin are the glucosinolate precursors of 1-isothiocyanate-4-methyl-thiono-butane ($CH_3$-S-$(CH_2)_4$-NCS) and 1-isothiocyanate-4-methylsulfonylbutane ($CH_3$-$SO_2$-$(CH_2)_4$-NCS) respectively, whose structure highly resembles that of sulforaphane ($CH_3$-SO-$(CH_2)_4$-NCS). The induction observed was probably due to the isothiocyanate (at least in part), since the broccoli powder samples were incubated at pH 7.5 for 2 h, conditions known to degradate the glucosinolates by endogenous myrosinase into isothiocyanates (see introduction 1.5.2. and figure 1.e.) (Vang *et al*, 1995). It is thus conceivable that sulforaphane might exert similar effects, due to their related structures. Furthermore, sulforaphane has been shown to be transformed to its sulfide and sulfone derivatives *in vivo* (see chapter 3 discussion 3.4.), which are in fact the compounds mentioned above. The potential of sulforaphane as an inducer of antioxidant enzymes is thus promising, and should therefore be investigated.

The protective effect of chemopreventors against carcinogenesis should be analysed with caution, since adverse effects may occur under certain conditions. One of the major forethoughts concerns tissue specificity. The ability of a certain agent to inhibit carcinogen activation in a particular tissue, e.g. liver, may result in greater delivery of the carcinogen to a different organ. One example of this is the inhibition by ethanol or disulfiram of NDMA hepatocarcinogenesis yet enhanced tumorigenicity in other tissues (Yang *et al,* 1994, De Flora and Ramel, 1988). The extrapolation of a certain protective effect should therefore be avoided, especially when different carcinogenic agents and different tissues are involved. The case of 6-phenylhexylisothiocyanate (PHITC) illustrates this well. This compound is a potent chemopreventive agent of NNK tumourigenesis in the lung of mice (Jiao *et al,* 1994), but it enhanced azoxymethane-induced colon tumourigenesis (Rao *et al,* 1995). In addition to the carcinogen and tissue differences within the previous studies, the sequence of administration of the isothiocyanate may also account for the increase in tumours, since rats were fed PHITC during the initiation and post-initiation phases of carcinogenesis (Rao *et al,* 1995). Similar effects were observed with chlorophyllin, which inhibited IQ carcinogenesis in three different organs in rats when co-administered with IQ, but increased the number of aberrant crypts during post-initiation exposure (Bertram, 1995). Carcinogenesis is a complex multistage process (see general introduction 1.1.2.) and different events are involved in the initiation and promotion stage. Depending on the stage at which chemopreventors exert their action (see section 1.4.2), the sequence of their administration may affect the outcome of their inhibitory potential.

The ability of sulforaphane to modulate the promotion stage of carcinogenesis has so far not been investigated. In a recent study, sulforaphane induced c-Jun transcription factor mRNA (Fei *et al,* 1996). This could contribute to the induction of expression of phase II enzymes, since AP-1 like sites are present in ARE (Fei *et al,* 1996), although this is controversial (see general introduction 1.4.4.). The implications of this induction (*c-Jun*) on the possible influence on cell proliferation have not been addressed. Nevertheless, related chemicals to sulforaphane had an antiproliferative effect. Cysteine derivates of PEITC, BITC, and phenypropyl-ITC (glutathione conjugate products) had *in vitro* selective antiproliferative activities against human leukemia 60 (HL60) cells, but were not effective against differentiated cells (Adesida *et al,* 1996). The related glucosinolate degradation products of

glucoerucin (see above), glucocheirolin ($CH_3$-$SO_2$-$(CH_2)_3$-NCS), and glucoraphenin ($CH_3$-SO-CH=$CHCH_2CH_2$-NCS) also inhibited human erythroleukemic K562 cell proliferative growth (Nastruzzi *et al,* 1996). The latter was also active towards other tumour cells, such as human T-lymphoid cells or human cervix carcinoma cells (Nastruzzi *et al,* 1996). Bearing in mind structure activity relationships (the above chemicals have a similar structure to sulforaphane with only one difference (or two) in their structures, i.e. sulfide group, sulfone + three carbon chain, and double bond, respectively instead of a sulfoxide group and four carbon chain), one could envisage a potential antiproliferative activity for sulforaphane, although it does require study.

Figure 6 summarises the effects of sulforaphane known to date. The potential but unknown mechanisms are marked with a question mark. It thus appears that the global effect of sulforaphane might be that of chemoprotection, since to date, major adverse effects have not been found.

The question that arises is whether the effects of sulforaphane seen here will be relevant to humans. Sulforaphane is present as the glucosinolate precursor glucoraphanin in a variety of cruciferous vegetables (see general introduction 1.5.2), which are regularly consumed in a well balanced diet. The impact of sulforaphane in the human diet has not been assessed, although it has been estimated that the mean content of glucoiberin in an U.S.A diet is 1 μmol/kg/day (Kore *et al,* 1993). In the UK, it has been estimated that up to 46 mg of glucosinolate might be consumed daily (Sones et al, 1984). Glucoiberin and glucoraphanin represent approximately a 10% of the total glucosinolate content in broccoli (Loft et al, 1992), and approximately 14 μg of sulforaphane was isolated from fresh broccoli (calculated from Zhang et al, 1992), it is thus conceivable that a few mg of glucoraphanin might be consumed daily. Both inhibition of CYP450s and induction of phase II enzymes *in vitro* by sulforaphane were observed at μM concentrations, which are potentially achieved from the human diet. Little is known regarding the stability of glucosinolates and isothiocyanates, and its absorption in the gut, however there is evidence indicating that glucosinolates are degraded to isothiocyanates by the microflora (see 1.5.2.) and also during the process of cooking and vegetable processing (Johnson, 1997).

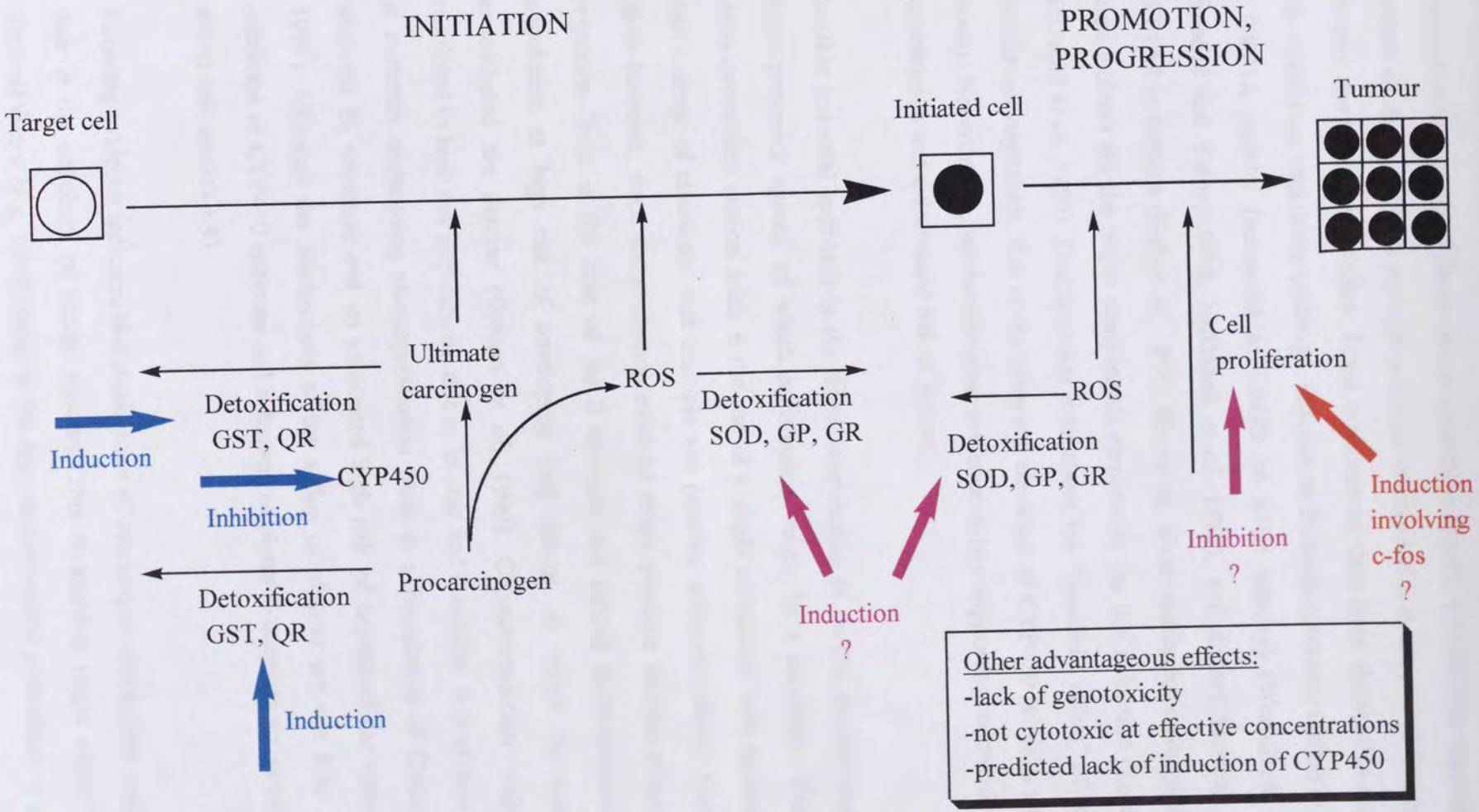



Figure 6. Summary of known and potential effects of sulforaphane in the carcinogenesis process

Blue indicates a known beneficial mechanism, purple possible beneficial action (limited evidence), and red potential unknown adverse effect. GST: glutathione-*S*-transferase; QR: quinone reductase; CYP450: cytochrome P450; ROS: reactive oxygen species; SOD: superoxide dismutase; GP: glutathione peroxidase; GR: glutathione reductase

One potential approach to chemoprevention is to promote dietary changes leading to an increased consumption of foods rich in protective factors. Cruciferous vegetables contain a plethora of chemicals with multiple potential effects and it is the overall balance which will influence the human population. Some experimental data have shown that feeding animals with cruciferous vegetables (cabbage, broccoli or Brussels sprouts) resulted in the induction of CYP1A activity (measured as EROD or AHH activity) (Wortelboer *et al*, 1992, Ramsdell and Eaton, 1988, McDanell *et al*, 1989), and dietary broccoli also induced CYP1A2 in humans (Kall *et al*, 1996). However, some studies have suggested that indole glucosinolates are the major components responsible for this induction (Loub *et al*, 1975, McDanell *et al*, 1989). Discrepancies exist about the "beneficial effect" of consumption of cruciferous vegetables, due to the observed elevation of CYP450 levels in some cases (see above). Nevertheless, epidemiological evidence exists supporting correlation between its consumption and a decreased risk of cancer.

Another potential approach is the direct intervention of human populations with putative chemopreventor agents, of which sulforaphane might be a candidate. The advantage of chemoprevention clinical trials is the use of a single compound with known effects rather than a range of chemicals with multiple and possibly unknown effects. Caution should be taken however, since some chemopreventors might produce adverse effects in the human organism. Such is the case of the β-carotene and retinol chemoprevention study in a population at high risk of developing lung cancer, in which the treatment actually exacerbated the disease (Omenn *et al*, 1996). Chemoprevention trials are currently restricted to high risk populations, due to its cost and feasibility. It is of interest that oltipraz is currently undergoing chemoprevention trials in a population of China (Qidong) with aflatoxin $B_1$ exposure and an associated high risk of hepatocellular carcinoma (Kensler, 1996). Although the mechanisms of the action of oltipraz are not fully understood, the inhibition of CYP450 enzymes and induction of phase II enzymes are good candidates (see above and section 3.4).

Growing evidence indicates that modulation of carcinogen metabolism enzymes may play a role in the aetiology of human cancers. This modulation might occur by means of an external factor (e.g. components in the diet, environmental pollutants) or as a consequence

of genetic factors. In fact, it is now recognised that genetic polymorphisms in carcinogen metabolising enzymes (e.g. CYP450s, GSTs, NAT2, see 1.2 and 1.3.5.) influence susceptibility to cancer. Of particular interest are the combined polymorphisms, such as CYP1A1 and GSTM1 related to smoking and lung cancer risk (Hayasi *et al*, 1992, Nakachi *et al*, 1993). Also the recent findings of Lear *et al*, suggest that CYP2D6 EM (or CYP1A1 m1m1) and GSTM1 null genotypes for individuals with skin type 1 were correlated with a relatively high risk of developing cutaneous basal cell carcinomas (Yengi *et al*, 1996; Lear *et al*, 1996). The modulation of CYP450 enzymes and GST by sulforaphane (and other agents) could therefore be viewed in parallel to the influence of polymorphisms on carcinogen metabolising enzymes.

# ***References***

Abbot, P.J. (1992) Carcinogen chemicals in food : evaluating the health risk. *Fd. Chem. Toxic.*, **30**, 4, 327-332.

Adam-Rodwell,G., Morse M.A. and Stoner,G.D. (1993) The effects of phenethylisothiocyanate on benzo[a]pyrene-induced tumors and DNA adducts in A/J mouse lung. *Cancer Letters* , **71**, 35-42.

Adesida,A., Edwards,L.G. and Thornalley,P.J. (1996) Inhibition of human leukaemia Go cell growth by mercapturic acid metabolites of phenethyl isothiocyanate. *Food Ceh. Tox.*, **34**, 385-392.

Amelizad,Z., Appee,K.E., Oesch,F. and Hildebrand,A.G. (1988) Effect of antibodies against cytochrome P450 on demethylation and denitrosation of *N*-nitrosodimethylamine and *N*-nitrosomethylaniline. *J.Cancer Res.Clin. Oncol.*, **114**, 380-384.

Ames.B.N. and Gold, L.S. (1991) Endogenous mutagens and the causes of aging and cancer. *Mutation Research*, **250**, 3-16.

Ames,B.N., McCann,J. and Yamasaki,E. (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research,* **31**, 347-364.

Anderson,D., Yu,T-W., Phillips,B.J. and Schemezer,P. (1994) The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. *Mut. Res.*, **307**, 261-271.

Ashby,J., Tinwell,H., Lefevre,P.A. and Browe,M.A. (1995) The single cell gel electrophoresis assay for induced DNA damage (Comet assay): measurement of tail length and moment. *Mutagenesis*, **10**, 2, 85-90.

Aoyama,T., Gelboin,H.V. and Gonzalez,F.J. (1990) Mutagenic activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline by complementary DNA-expressed human liver P-450. *Cancer Research,* **50**, 2060-2963.

Atallah,A., Landolph,J.R. and Hochstein,P. (1987) DT-Diaphorase and quinone detoxification. *Chemica Scripta*, **27**A, 141-144.

Babich,H., Borenfreund,E. and Stern,A. (1993) Comparative cytotoxicities of selected minor dietary non-nutrients with chemopreventive properties. *Cancer Letters*, **73**, 127-133.

Baillie,T.A. and Slatter,J.G. (1991) Glutathione: A vehicle for the transport of chemically reactive metabolites *in vivo*. *Acc. Chem. Res.*, **24**, 264-270.

Balenovic,K., Deljac,A., Monkovic,I. and Stefanac, Z. (1966) Synthesis of sulforaphene. *Tetrahedron*, **22**, 2139-2143.

Bartsch,H. (1981) Detection of N-nitroso compounds as bacterial mutagens - past experience. Form Safety evaluation of nitrosatable drugs and chemicals. Edited by Gibson,G.G. and Ioannides,C. Taylor & Francis Ltd, 185-205.

Bartsch,H. (1991) *N*-Nitroso compounds and human cancer: Where do we stand?. From: Relevance to Human cancer of *N*-nitrosocompounds, tobacco smoke and mycotoxins. IARC Monographs, Edited by O'Neill, Chen and Bartsch, 1-9.

Bartsch,H., Malaveille,C. and Montesano,R. (1975) *In vitro* metabolism and microsome-mediated mutagenicity of dialkylnitrosamines in rat, hamster and mouse tissues. *Cancer Research*, **35**, 644-651.

Baxter,J.N., Cymerman-Craig,J., Moyle,M. and White,R.A. (1956). *J.Chem.Soc.*, 659.

Bellec,G., Dreano,Y., Lozach,P., Menez,J.F. and Berthou,F. (1996) cytochrome P450 metabolic dealkylation of nine *N*-nitrosodialkylamines by human liver microsomes. *Carcinogenesis*, **17**, 9, 2029-2034.

Berry, C.L. (1993) Epigenic carcinogenesis. From: General and Applied Toxicology, volume 2. By Brian Ballantyne, Timothy Marrs and Paul Turner. Stochton Press. Macmillan Publishers, 979-987.

Berry,M.N. and Friend,D.S. (1969) High yield preparation of isolated rat liver parenchymal cells. *The Journal of Cell Biol.*, **43**, 506-520.

Bertram,J.S. (1995) Fifth Heidelberger Conference on Targets for Cancer Research Prevention, Differentiation and Selective Therapy. *Cancer Research*, **55**, 705-709.

Biaudet, H., Mavelle,T. and Derby,G. (1994) Mean daily intake of *N*-nitrosodimethylamine from foods and beverages in France in 1987-1992. *Fd.Chem.Toxic.*, **32**, 5, 417-421.

Bierley,C.H. and Burchell,B. (1993) Human UDP-glucuronosyl transferases-chemical defense, jaundice and gene therapy. *Bioessays*, **11**, 749-754.

Bolt,H.M. (1988) Roles of etheno-DNA adducts in tumorigenicity of olefins. *CRC Crit. Rev.Toxicol.*, **18**, 299-309.

Bose,A.K., Greer,F. and Price,C.C. (1958) A procedure for phthaloylation under mild conditions. *J. Org. Chem.*, **23**, 1335.

Boyd,J.N., Babish,J.G. and Stoewsand,G.S. (1982) Modification by beet and cabbage diets of aflatoxin $B_1$ induced rat plasma α-foetoprotein elevation, hepatic tumorigenesis and mutagenicity of urine. *Food Chem. Tox.*, **20**, 47-52.

Bradfield,C.A., Chang,Y. and Bjeldanes,L.F. (1985) Effects of commonly consumed vegetables on hepatic xenobiotic-metabolizing enzymes in the mouse. *Food and Chemical Toxicology*, **23**, 899-904.

Bradford,M.M. (1976) A rapid method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

Brady,J.F., Ishizaki,H., Fukuto,J.M., Lin,M.C., Fadel,A., Gapac,J.M. and Yang,C.S. (1991) Inhibition of cytochrome P450IIE1 by diallylsulfide and its metabolites. *Chem. Res. Toxicol.*, **4**, 642-647.

Bridges,B. (1988) Genetic toxicology at the crossroads-personal view on the development of short-term tests for predicting carcinogenicity. *Mutation Research*, **205,** 25-31.

Brockman, H.E. and De Marini, D.M. (1988) Utility of short term tests for genetic toxicity in the aftermaths of the NTP's analysis of 73 chemicals. *Envir. Mol. Mutagenesis*, **11**, 421-435.

Bruggeman,I.M., Temmink,J.H.M. and Bladeren,P.J. (1986). Glutathione and cysteine-mediated cytotoxicity of allyl and benzyl isothiocyanate. *Toxicology and Applied Pharmacology*, **83**, 349-359.

Burbach, K.M., Poland,A. and Bradfield,C.A. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *PNAS.*, **89**, 8185-8189

Burke,M.D., Thompson,S., Elcombe,C.R., Halpert,J., Haaparante,T. and Mayer,R.T.(1985) Ethoxy-, pentoxy- and benzoxy phenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.*, **34**, 18, 3337-3345.

Burke,M.D., Thompson,S., Weaver,R.J., Wolf,C.R. and Mayer,R.T.(1994) Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochemical Pharmacology*, **48**, 5, 923-936.

Butterworth,B.E., Ashby,J., Bermudez,E., Casciano,D., Mirsalis,J., Probst,G. and Williams,G. (1987) A protocol and guide for the in vitro rat hepatocyte DNA-repair assay. *Mutation Research*, **189**, 113-121.

Butterworth, B.E., Smith-Oliver, T., Earle, L., Loury, D.J., White, R.D., Doolittle, D.J., Working, P.K., Cattley, R.C., Jirtle, R., Michaloupos, and Strom, S. (1989) Use of primary cultures of human hepatocytes in toxicology studies. *Cancer Research*, **49**, 1075-1084.

Byers,T. (1993) Dietary trends in the U.S. Relevance to cancer prevention. *Cancer -suppl.*, **72**, 3, 1015-1018.

Caldwell,J. (1996) Importance of stereospecific bioanalytical monitoring in drug development. *Journal of chromatography*, **719**, 1, 3 13.

Camoirano,A., Balansky,R.M., Bennicelli,c., Izzotti,A., D'Agostini,F. and De Flora,S. (1994) Experimental databases on inhibition of the bacterial mutagenicity of 4-nitroquinoline-1-oxide and cigarette-smoke. *Mutation Research*, **317**, 2, 89-109.

Camus,A.M., Geneste,O., Honkakoski,P., Bereziat,J.C., Henderson,C.J., Wolf,C.R., Barsch,H. and Lang,M.A. (1993) High variability of nitrosamine metabolism among individuals- role of cytochromes 2A6 and 2E1 in the dealkylation of *N*-

nitrosodimethylamine and *N*-nitrosodiethylamine in mice and humans. *Molecular Carcinogenesis*, 7,4, 268-275.

Carere,A., Mohn,G.R., Parry,J.M., Sors,A.I. and Nolan,C.V. (1995) Methods and testing strategies for evaluating the genotoxic properties of chemicals. European Commission Report. Published by European Commisssion, Luxembourg.

Casciano,D.A. and Gaylor,D>W. (1983) Statistical criteria for evaluating chemicals as positive or negative in the hepatocyte / DNA repair assay. *Mutation Research*, **122**, 81-86.

Catteau,A., Bechtel,Y.C., Poisson,N., Bechtel and Bonatïti-Pellie,C. (1995) A population and family study of CYP1A2 using caffeine urinary metabolites. *Eur. J. Clin. Pharmacology*, **47**, 423-430.

Cheng,K.C. and Loeb,L.A. (1997) Genomic stability and instability: A working paradigm. In: Genetic stability and tumorigenesis. *Current Topics in Microbiology and Immunology*, **221**, 5-18.

Chipman,J.K. (1995) Connexin genes as tumour suppressor genes. *Human and Experimental Toxicology*, **14**, 539-540.

Clayson,D.B., Mehta,R. and Iverson,F. (1994) Oxidative DNA damage. The effects of certain genotoxic and operational non-genotoxic carcinogens. *Mutation Research*, **317**, 25-42.

Coles,B. and Ketterer,B. (1990) the role of glutathione and glutathione transferases in chemical carcinogenesis. *Critical Reviews in Biochemistry and Molecular Biology*, **25**, 1, 47-69.

Collins,A.R., Ai-Guo,Ma. and Duthie,S.J. (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. *Mutation Research*, **336**, 69-77.

Collins,A.R.S., Schor,S.L. and Johnson,R.T. (1977) The inhibition or repair in UV irradiated human cells. *Mut. Res.*, **42**, 413

Correia,M.A. (1995) Rat and human liver cytochromes P450. In: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortiz de Montellano, Plenum Press, 1995, 607-630.

Cosma,G., Crofts,F., Taioli,E., Toniolo,P. and Garte,S. (1993) Relationships between genotype and function of the human CYP1A1 gene. *Jouranl of Toxicology and Enviromental Health*, **40**, 309-316.

Corey,E.J. and Chaykovsky,M. (1965) Methylsulfinylcarbanion formation. *J. Am.Chem. Soc.*, **87**, 1345-1350.

Cortés,F., Piñero,J. and Ortiz,T. (1993) Importance of replication fork progression for hte induction of chromosome damage and SCE by inhibitors of DNA topoisomerases. *Mut. Res.*, **303**, 71-76.

Crathon,A.R. and Roberts,J.J. (1966) Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature*, **21**, 150-153.

Crespi,C.l, Gonzalez,F.J., Steimel,D.T. et al (1991) A metabolically competent human cell line expressing five cDNAs encoding procarcinogen-activating enzymes. *Chem.Res.Tox.*, **4**, 566-572.

Crespi, C.C., Penman, B.W., Gonzalez, F.J., Gelboin, H.V., Galvin, M. and Langenbach, R. (1993) Genetic toxicology using human cell lines expressing human P-450. *Biochemical Society Transactions,* **21**, 1023-1028.

Crespi,C.L., Steimel,D.T., Aoyama,T., Gelboin,H.V. and Gonzalez,F.J. (1990) Stable expression of human cytochrome P4501A2 cDNA in a human lymphoblastoid cell line: role of the enzyme in metabolic activation of aflatoxin $B_1$. *Mol. Carcino.*, **11**, 65-73.

Dai,Y., Rashba-Step,J. and Cederbaun,A.I. (1993) Stable expression of human cytochrome P4502E1 in HepG2 cells: characterization of catalytic activities and production of reactive oxygen intermediates. *Biochemistry*, **32**, 6928-6937.

Daly,A.K., Cholerton,S., Armstrong,M. and Idle,J.R. (1994) Genotyping for polymorphisms in xenobiotic metabolism as a predictor of disease susceptibility. *Environmental Health Perspectives*, **102** suppl., 9, 55-61.

Damai,L.A. (1987) Metabolism of sulfur containing drugs. 581-603

Davies,J.E., Dashn,P., Doehmer,J. and Chipman,J.K. Metabolic activation of 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) and DNA strand breakage (Comet assay) in V79NHr1A2 cells. (Abstract) *Proceedings of the UKEMS Meeting*, July 1995, Leicester.

Day,G.L., Shore,R.E., Blot,W.J., Mclaughlin,J.K., Austin,D.F., Greenberg,R.S., Liff,J.M., Preston-Martin,S., Sarkar,S., Schoenberg,J.B. and Fraumeni,J.F. (1994) Dietary factors and second primary cancers: a follow-up of oral and pharyngeal cancer patients. *Nutrition Cancer*, **21**, 3, 223-232.

De Flora,S. and Ramel,C. (1988) Mechanisms of inhibitors of mutagenesis and carcinogenesis. Classification and overview. *Mutation Research*, **202**, 285-306.

De Flora,S., Rosenkranz,H.S. and Klopman,G. (1994) Structural basis of antimutagenicity of chemicals towards 4-nitroquinoline 1-oxide in *Salmonella typhimurium. Mutagenesis*, **9**, 1, 39-45.

De Flora,S., Balansky,R., Bennicelli,C., Camoirano,A., D'Agostini,F., Izzotti,A. and Cesarone,C.F. (1995) Mechanisms of anticarcinogenesis: the example of *N*-acetylcysteine.

From: *Drugs, Diet and Disease*. Volume 1. Mechanistic approaches to cancer. Edited by C.Ioannides and D.F.V.Lewis. Ellis Horwood Ltd., 151-202.

DeMatteis,F. (1978) Loss of liver cytochrome P450 caused by chemicals. In: Heme and Hemoproteins, Handbook of experimental Pharmacology (F.De Matteis and W.N. Aldrige, eds), Springer-Verlag, Berlin, 44, 95-127.

Doll,R. (1992) The lessons of life: Keynote Adress to the Nutrition and cancer conference. *Cancer Research* suppl. **52**, 2024s-2029s.

Doll,R. and Peto,R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, **66**, 1191-1309.

Donato,M.T., Gómez-Lechón,M.J. and Castell,J.V. (1993) Amicroassay for measuring cytochrome P4501A1 and P4502B1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Analytical Biochemistry*, **213**, 29-33.

Dragsted,L.O., Strube.M. and Larsen,J.C. (1993) Cancer-Protective factors in fruits and vegetables: Biochemical and Biological background. *Pharmacology and Toxicology*, **72**, s1, 116s-135s.

Drobnica,L. and Gemeiner,P. (1977) The chemistry of the NCS-group. In: The chemistry of cyanates and their thio-derivatives. Edited by Patai, Wiley and Sons, Chistester, 1003-1221.

Dunnett,C.W. (1955) A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.*, **50**, 1096-1121.

Dunnick,J.K., Prejean,J.D., Haseman,J., Thompson,R.B., Giles,H.D. and McDonnell,E.E. (1982). Carcinogenesis bioassy of allyl isothiocyanate. *Fundam. Appl. Toxicol.*, **2**, 114-120.

Eaton,D.L. and Gallagher,E.P. (1994) Mechanisms of aflatoxin carcinogenesis. *Ann. Rev. Pharmacol.Toxicol.*, **34**, 135-172.

Ebata,J., Toyohara,M., Kitahara,S., Kawai,K. and Furukawa,H. (1993) Enhancement of mutagenicity of N-nitrosodimethylamine (NDMA) by a modified microsomal / *Salmonella* pre-incubation assay using acetone and fasting-inducible S9 form rat liver. (Abstract). *Mutation Research*, **292**, 262-263.

Edenharder, R., Kurz, P., John,K., Burgard, S. and Seeger, K. (1994) In vitro effect of vegetable and fruit juices on the mutagenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline. *Food and Chemical Toxicology*, **32**, 5, 443-459.

Edmonson,D.E. and Bruice,T.C. (1985) N-Nitrosodialkylamines do not function as substrates for liver monoamine oxidase. *Proc.Natl.Acad.Sci.*, **82**, 682-683.

Ellard,S., Mohammed.Y., Dogna,S., Wölfel,C., Doehmer,J. and Parry,J.M. (1991) the use of genetically engineered V79 chinese hamster cultures expressing rat liver CYP1A1, 1A2and 2B1 cDNAs in micronucleus assays. *Mutagenesis*, **6**, 6, 461-470.

Espinosa-Aguirre,J.J., Vilchis,C., Ostrosky-Wegman,P., Benitez,L., Lores,I. and Rubio, J. (1993) Antimutagenicity of cyclohexanol towards *NNK* in *S.typhimurium* TA100. *Mutation Research*, **300**, 151-154.

Essigmann,J.M. and Wood,M.L. (1993) The relationship between the chemical structures and mutagenic specificities of the DNA lesions formed by chemical and physical mutagens. *Toxicology Letters*, **67**, 29-39.

Evans,D.A.P. (1992) N-Acetyltransferase In Kalow W Pharmacogenetics of Drug Metabolism. New York, Pergamon, 95-178.

Fairbairn,D.W., Olive,P.C. and O'Neill,K.L. (1995) The comet assay: a comprehensive review. *Mutation research*, **339**, 37-59.

Fairbairn,D.W., Reyes,W.A. and O'Neill,K.L. (1994) Alkali-labile sites are prevalent in kidney tissue DNA. *Cancer Letters*, **81**,67-76.

Fan,C.Y., Butler,W.H. and O'Connor,P.J. (1991) Promutagenic lesions persist in the DNA of target cells for nitrosamine-induced carcinogenesis. From: Relevance to Human cancer of *N*-nitrosocompounds, tobacco smoke and mycotoxins. IARC, 119-122.

Favreau,L.V. and Pickett,C.B. (1995) The rat quinone reductase antioxidant response element. *Journal of Biological Chemistry*, **270**, 4, 24468-24474.

Fearon,E.R. and Vogelstein,B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.

Fei,P., Matwushyn,G.A., Rushmore,T.H. and Kong,A.-H.T. (1996) Transcription regulation of rat glutathione S-transferase Ya subunit gene expression by chemopreventive agents. Pharmaceutical Research, 13, 7, 1043-1048.

Fenwick,G.R., Heaney,R.K. and Mullin,W.J. (1982) Glucosinolates and their breakdown products in food and food plans. *CRC Crit. Rev.Food Sci. Nutr.*, **18**, 123-201.

Ferguson,L.R. (1994) Antimutagens as cancer chemoprotective agents in the diet. *Mutation Research*, **307**, 395-410.

Floyd,R.A. (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB Journal*, **4**, 2587-2597.

Frei,E., Bertram,B. and Wiessler,M (1985) Reduced glutathione inhibits the alkylation by N-nitrosodimethylamine of liver DNA in vivo and microsomal fraction in vitro. *Chem.-Biol.Inter.*, **55**, 123-137.

Friedberg,E.C. (1992) Xeroderma Pigmentosum, Cockayne's Syndrome, Helicases and DNA repair: What's the relationship?. *Cell*, **71**, 887-889.

Gerhäuser, C., You, M., Liu, J., Moriarty, Hawthorne, M., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. (1997) Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug metabolising enzymes. *Cancer Research*, **57**, 272-278.

Gibson-D'Ambrosio,R.E., Crowne,D.L., Shuler,C.E. D'Ambrosio,S.M. (1993) The establishment and continous subculturing of normal human adult hepatocytes: expression of differentiated liver functions. *Cell Biol.Toxicol.*, **9**, 385-403.

Gichner,T. and Veleminsky,J. (1988) Mechanisms of inhibition of *N*-nitroso compounds-induced mutagenicity. *Mutation Research*, **202**, 325-334.

Gonzalez, F.J., Crespi, C.L. and Gelboin, H.V. (1991) cDNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. *Mutation Research*, **247**, 113-127.

Gonzalez,F.J. and Gelboin,H.V. (1992) Human cytochromes P450: evolution and cDNA-directed expression. *Environmental Health Perspectives*, **98**, 81-85.

Gonzalez,F.J. and Gelboin,H.V. (1994) Role of human cytochrome P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metabolism Reviews*, **26** (1&2), 165-183.

Gonzalez,F.J. and Korzekwa,K.R. (1995) Cytochromes P450 expression systems. *Annual Review Pharmacology Toxicology*, **35**, 369-390.

Graham,S. (1983) Results of case-control studies of diet and cancer in Buffalo, New York. *Cancer Research* (suppl.), **43**, 2409s-2413s.

Grant,D.M., Blum,M. and Meyers,U.A.(1992) Polymorphisms of N-acetyltransferase genes. *Xenobiotica*, **22**, 1073-1081.

Green,M.H.C., Lowe,J.E., Wangh,A.P.W., Aldridge,K.E., Cole,J. and Arlett,C.F. (1994) Effect of diet and vitamin C on DNA strand breakage in freshly-isolated human white blood cells. *Mutation Research*, **316**, 91-102.

Green, S. (1991) The search for molecular mechanisms of non-genotoxic carcinogens. *Mutation Research*, **248**, 371-374.

Groves, J.T. and Han, Y.-Z. (1995) Models and mechanisms of cytochrome P450 action. In: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortisz de Montellano, Plenum Press, 1995, 3-48.

Guengerich,F.P. (1984) Factors involved in the regulation of the levels and activities of rat liver cytochromes *P*-450. Biochemical Society Transactions, 12, 68-70.

Guengerich,F.P. and Shimada, T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chemical Research in Toxicology*, **4**, 4, 391-407.

Guengerich,F.P. (1992) Characterization of human cytochrome P450 enzymes. *FASEB Journal*, **6**, 745-748.

Guengerich,F.P. (1994) Mechanisms of formation of DNA adducts from ethylene dihalides, vinyl halides and arylamines. *Drug Metabolism Reviews*, **26** (1&2), 47-66.

Guengerich, F.P. (1995) Human cytochrome P450 enzymes. In: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortiz de Montellano, Plenum Press, 1995, 473-535.

Guenguerich, F.P., Bell, L.C. and Okazaki O. (1995) Interpretations of cytochrome P450 mechanisms from kinetics studies. *Biochimie*, 77, 573-580.

Guengerich,F.P., Shimada,T., Ravey,K.D., Yun,C.-H., Meyer,D.J., Ketterer,B., Harris,T.H., Groopman,J.D. and Kadlubar,F.F. (1992) Elucidation of catalytic specificities of human cytochrome P450 and Glutathione S-transferase enzymes and relevance to molecular epidemiology. *Environmental Health Perspectives*, **98**, 75-80.

Guillouzo,A., Beaune,P., Gascoin, M.-N., Begue,J.-M., Campion, J.-P., Guengerich, F.P. and Guguen-Guillouzo, C. (1985) Maintenance of cytochrome P450 in cultured adult human hepatocytes. *Biochemical Pharmacology*, **34**, 16, 2991-2995.

Guo,Z., Smith,T.J., Wang,E., Sadrieh,Ma., Thomas,P.E. and Yang,C.S. (1992) Effects of phenethyl isothiocyanate, a carcinogenesis inhibitor, on xenobiotic metabolising enzymes and nitrosamine metabolism in rats. *Carcinogenesis*, **13**, 12, 2205-2210.

Guo,Z., Smith,T.J., Wang,E., Eklind,K.I., Chung,F-L. and Yang,C.S. (1993) Structure-activity relationships of arylalkyl isothiocyanates for the inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (*NNK*) metabolism and the modulation of xenobiotic-metabolizing enzymes in rats and mice. *Carcinogenesis*, **14**,6, 1167-1173.

Guttenplan,J.B. (1987) *N*-Nitrosamines: bacterial mutagenesis and in vitro metabolism. *Mutation Research*, **186**, 81-134.

Harper,B.L., Rinkus,S.J., Scott,M., Ammenhauser,M. et al. (1983). In Castellani Ed. The use of human cells for the evaluation of risk from physical and chemical agents, Nato ASI Series, vol 60,Plenum Press, N.Y., pp 353-424.

Harris, C.C. (1987) Human tissues and cells in carcinogenesis research. *Cancer Research*, **47**, 1-10.

Hayashi,S., Watanabe,J. and Kawajiri,K. (1992) High susceptibility to lung cancer analyzed in terms of combined genotypes of P4501A1 and Mu-class glutathione S-transferase genes. *Japanese Journal Cancer Research*, **83**, 866-870.

Hellman,B., Vaghef,H., and Boström,B. (1995) The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. *Mutation Research*, **336**, 123-131.

Hoeijmakers,J.H.J. and Boostma, D. (1994) Incision for excision. Nature, 371, 654-655.

Hoffmann,G.R. (1996) Genetic Toxicology. In Casarett and Doull's Toxicology. The basic science of poisons. Edited by C.C.Klaassen. McGrawhill, 9, 269-300.

Holz,O., Jörres,R., Kästner,A. and Magnussen,H. (1995) Differences in basal and induced DNA single-strand breaks between human peripheral monocytes and lymphocytes. *Mutation Research*, **332**, 55-62.

Ioannides,C., Lewis,D.F.V. and Parke.D.V. (1995) Mechanisms of chemical carcinogenesis and molecular parametric analysis in the safety evaluation of chemicals. From "*Drugs, diet and disease*" Vol. 1, Mechanistic Approaches to cancer. Edited by C.Ioannides and D.F.V.Lewis. Ed. Ellis Horwood, 3-46.

Ioannides,C. and Parke.D.V. (1993) Induction of cytochrome P4501 as an indicator of potential chemical carcinogenesis. *Drug Metabolism Reviews*, **25** (4), 485-501.

Ishizaki,H., Brady,J.F., Ning,S.M. and Yang,C.S. (1990) Effect of phenethyl isothiocyanate on microsomal *N*-nitrosodimethylamine metabolism and other monooxygenase activities. *Xenobiotica*, **20**, 255-264.

Izzotti,A., D'Agostini,F., Bagnasco,M., Scatolini,L., Rovida,A., Balansky,R.M., Cesarone,C.F. and De Flora,S. (1994) Chemoprevention of carcinogen-DNA adducts and chronic degenarative diseases. *Cancer Research* suppl, **54**, 1994s-1998s.

Jaiswal,B.K., Burmett,P., Adeshi,K.M. and McBride,O.W. (1990) Nucleotide and deduced aminoacid sequence of a humam cDNA ($NQO_2$) corrresponding to a second member of the NADPH:Quinone oxidoreductase gene family. Extensive polymorphisms at the $NQO_2$ gene locus on chromosome 6. *Biochemistry*, **26,** 1899-1906.

Jiao, D., Conaway, C.C., Wang, M.-H., Yang, C.S., Koehl, W. and Chung, F.-L. (1996) Inhibition of *N*-nitrosodimethylamine demethylase in rat and human liver microsomes by isothiocyanates and their glutathione, L-cysteine and *N*-acetyl-L-cysteine conjugates. *Chemical Research Toxicology*, **9**, 932-938.

Jiao,D., Eklind,K.I., Choi,C-I., Desai,D.H., Amin,S.G. and Chung,F-L. (1994) Structure-activity relationships of isothiocyanates as mechanism-based inhibitors of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice. *Cancer Res.*, **54**, 4327-4333.

Jiao,J., Glickman,B.W., Anderson,M.W. and Zielinska,M. (1993) Mutational specificity of *N*-nitrosodimethylamine: comparison between in vivo and in vitro assays. *Mutation Research*, **301**, 27-31.

Johansson,I., Ekstrom,G., Scholte,B., Puzycki,D., Jornvall,H. and Ingelman-Sundberg,M. (1987) Ethanol-, fasting-, and acetone-inducible cytochromes P450 in rat liver: Regulation and characteristics of enzyme belonging to the IIB and IIE gene subfamilies. *Biochemistry*, **27**, 1925-1934.

Johnson,I. (1997) Secondary plant metabolites in the human diet: mechanisms of influence on cancer. (Abstract). *Mutagenesis*, **12**, 2, 100.

Jorquera,R., Castonguay,A. and Schuller,H.M. (1994) DNA single-strand breaks and toxicity induced by 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone or N-nitroso-dimethylamine in hamsters and rat liver. *Carcinogenesis*, **15**, 2, 389-394.

Kadlubar, F.F. (1994) Biochemical individuality and its implications for drug and carcinogen metabolism: Recent insights from acethyltransferase and cytochrome P450 1A2 phenotyping and genotyping in humans. *Drug Metabolism Reviews*, **26** (1&2) 37-46.

Kadlubar, F.F., Butter,M.A., Kaderlik,K.R., Chou,H.-C. and Lang,N.P. (1992) Polymorphisms for aromatic amine metabolism in humans: Relevance for human carcinogenesis. *Enviromental Health Perspectives*, **98**, 69-74.

Kall,M.A., Vang,O. and Clausen.J. (1996) Effect of dietary broccoli on human *in vivo* drug metaboliszing enzymes: evaluation of caffeine, oestrone and chlorzoxazone metabolism. *Carcinogenesis*, **17**, 793-799.

Kao,T.C., Meyer,W.J. and Post,J.F.M. (1993) Inhibitory effects of ascorbic acid on growth of leukemic and lymphoma cell lines. *Cancer Letters*, **70**, 101-106.

Katoh,T., Iratomi,H, Nagaoka,A. and Sugita,A. (1995) Cytochrome P4501A1 gene polymorphism and homozygous deletion of the glutathione-S-transferase M1 gene in urothelial cancer patients. *Carcinogenesis*, **16**, 3, 655-657.

Kawajiri,K., Nakachi,K., Imai,K., Yoshii,A., Shinoda,N. and Watanabe,J. (1990) Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the human cytochrome P4501A1 gene. *FEBS Lett.*, **263**, 131-133.

Kawazoe,Y. and Kato,M (1982) Antimutagenic effect of isothiocyanates and related compounds in *Escherichia Coli*. *GANN*, **73**, 255-263.

Kennedy,S.W. and Jones,S.P. (1994) Simultaneous measurement of cytochrome P4501A1 catalytic activity and total protein concentration with a fluorescence plate reader. *Analytical Biochemistry*, **222**, 217-223.

Kennelly,J.C., Waters,R., Ashby,J., Lefevre,P.A., Burlinson,B., Benford,D.J., Dean,S.W. and Mitchell,I. de G. (1993) *In vivo* rat liver UDS assay. *In*: D.J. Kirkland and M. Fox (eds.), *Supplementary mutagenicity tests, UKEMS recommended procedures*, pp. 52-73. Cambridge; University Press.

Kensler,T.W. (1996) Oltipraz: Clinical opportunities for cancer chemoprevention. (Abstract) *Proceedings of the 5th International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis*, Japan, December 1996, 51.

Ketterer,B. (1988) Protective role of glutathione and glutathione transferases in mutagenesis and carcinogenesis. *Mutation Research*, **202**, 343-361.

Kim and Yi (1985) Preparation and reactivity of di-2-pyridylthionocarbonate. *Tetraedron Letters*, **26**, 1661-1664.

Kjaer,A. and Christensen,B. (1958) *Acta Chem., Scand*, **12**, 833-838.

Knasmuller, S., Friesen,M.D., Holme,J.A., Alexander,J., Sanyal,R., Kassie,F. and Bartsch,H. (1996) Effects of phenethyl isothiocyanate on metabolism and on genotoxicity of dimethylnitrosamine and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Mutation Research*, **350**, 93-102.

Koop,D.R., Laethem,C.L. and Tierney,D.J. (1989) The utility of *p*-nitrophenol hydroxylation in P4502E1 analysis. *Drug Metabolism Reviews*, **20** (2-4), 541-551.

Koop,D.R. and Tierney,D.J. (1990) Multiple mechanisms in the regulation of ethanol-inducible cytochrome P4502E1. *Bioessays*, **12**, 429-435.

Kore,A.M., Jeffery,E.H. and Walling, M.A. (1993) Effects of 1-isothiocyanato-3-methylsulfinyl-propane on xenobiotic metabolising enzymes in rats. *Food and Chemical Toxicology*, **31**, 10, 723-729.

Kritchersky,D. (1993) Dietary Guidelines. *Cancer* , **72**, 3, 1011-1014.

Kruszewski,M., Green,M.H.L., Lowe,J.E. and Szumiel,I. (1994) DNA strand breakage, cytotoxicity and mutagenicity of hydrogen peroxide treatment at 4°C and 37°C in L5178 Y sublines. *Mutation Research*, **308**, 233-241.

Kune,S., Kune,G.A. and Watson,L.F. (1987) Case-control study of dietary etiological factors: the Melbourne colorectal cancer study. *Nutr. Cancer*, **9**, 21-42.

Kune G.A., Bannerman,S., Field,B., Watson,L.F., Cleland,H., Merenstein,D. and Vitetta,L. (1992) Diet, alcohol, smoking, serum, β-carotene and vitamin A in male nonmelanocytic skin cancer patients and controls. *Nutrition and Cancer*, **18**, 3, 237-244.

Kwak, M.K., Kim, S.G., Kwak, J.Y., Novak, R.F. and Kim, N.D. (1994) Inhibition of cytochrome P4502E1 expression by organosulfur compounds allylsulfide, allylmercaptan and allylmethylsulfide in rats. *Biochemical Pharmacology*, **47**, 3, 531-539.

Lakritz,L., Gates,A., Gugger,A.M. and Wasserman,A.E. (1982) Nitrosamine levels in blood, urine and gastric aspirate following ingestion of foods containing potential nitrosamine precursors or preformed nitrosamines. *Food and chemical Toxicology*, **20**, 455-459.

Lambard,S.E., Burnett,A.K., Wolf, C.R. and Craft,J.A. (1991) The role of specific cytochromes P450 in the formation of 7,12-dimethylbenz[a]anthracene-protein adducts in rat liver microsomes in vitro. Biochemical Pharmacology,42, 8, 1529-1535.

Langenbach, R., Blaise Smith, P. and Crespi, C. (1992) Recombinant DNA approaches for the development of metabolic systems used in *in vitro* toxicology. *Mutation Research*, **277**, 251-275.

Langouët,S., Coles,B., Morel,F., Becquemont.L., Beaune,P., Guengerich,F.P., Ketterer,B. and Guillouzo,A. (1995) Inhibition of CYP1A2 and CYP3A4 by Oltipraz results in reduction of aflatoxin $B_1$ metabolism in human hepatocytes in primary culture. *Cancer Research*, **55**, 5574-5579.

Langouët,S., Morel,F. Meyer,D.J., Fardel,O., Corcos,L., Ketterer,B. and Guillouzo,A. (1996) A comparison of the effect of inducers on the expression of glutathione-S-transferases in the liver of the intact rat and in hepatocytes in primary culture. *Hepatology*, **23**, 881-887.

Layton,O.W., Bogen,K.T., Knize,M.G., Hatch,F.T., Johnson,V.M. and Felton,J.S. (1995) Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis*, **16**, 1, 39-52.

Lear,J.T., Heagerty,A.H.M., Smith,A., Bowers,B., Payne,C.R., Smith,C.A.D., Jones,P.W., Gilford,J., Yengi,L., Allderssea,J., Fryer,A.A. and Strange,R.C. (1996) Multiple cutaneous basal-cell carcinomas- Glutathione-S-transferase (GSTM1, GSTT1) and cytochrome-P450 (CYP2D6, CYP1A1) polymorphisms influence tumour numbers and accrual. *Carcinogenesis*, **17**, 9, 1891-1896.

Lee,M.-S. (1994) Oxidative conversion of isothiocyanates to isocyanates by rat liver. *Environ. Health Perspect.*, **102** (suppl 6), 115-118.

Levine,A.J., Momand,J. and Finlay,C.A. (1991) The p53 tumor suppressor gene. *Nature*, **351**, 6326, 453-456.

Lewis,D.F.V., Ioannides,C. and Parke,D.V. (1993) Validation of a novel molecular orbital approach (COMPACT) for the prospective safety evaluation of chemicals, by comparison with rodent carcinogenicity and *Salmonella* mutagenicity data evaluated by the USNCI/NTP. *Mutation Research*, 291, 61-77.

Lewis,D.F.V., Moereels,H., Lake,B.G., Ioannides,C. and Parke,D.V. (1994) Molecular modeling of enzymes and receptors involved in carcinogenesis: QSARs and compact 3-D. *Drug Metabolism Reviews*, **26** (1&2), 261-285.

Li,Y. and Jaiswal,A.K. (1992) Regulation of human NADPH:Quinone Oxidoreductase gene. *The Journal of Biological Chemistry*, **26**, 21, 15097-15104.

Lindsay,D.G. (1996) Dietary Contribution to Genotoxic risk and its control. *Food and Chemical Toxicology*, **34**, 423-431.

Loft,S., Otte,J., Poulsen, H.E. and Sørensen,H. (1992) Influence of intact and myrosinase-treated indoyl glucosinates on the metabolism in vivo of metronidazole and antipyrine in the rat. *Food and Chemical Toxicology*, **30**, 11, 927-935.

Lorenzen, A. and Kennedy,S.W. (1993) A fluorescence-based protein assay for use with a microplate reader. *Anal. Biochem.*, **214**, 346-348.

Loub,W.D., Wattenberg,L.W. and Davis,D.W. (1975). Aryl hydrocarbon hydroxylase induction in rat tissues, by naturally occurring indoles of cruciferous plants. *Journal of the National Cancer Institute*, **54**, 4, 985-988.

Lowry,O.M., Rosenbourgh,N.L., Farr,L. and Randall,R.J. (1951) Protein estimation with the folin phenol reagent. *J.Biol.Chem*, **193**, 265-275.

Macé,K., Gonzalez,F.J., McConnell,I.R., Garner,R.C., Avanti,O., Harris,C.C. and Pfeifer,A.M.A. (1994) Activation of promutagens in a human bronchial epithelial cell line stably expressing human P450 1A2. *Mol.Carcinog.*, **11**, 65-73.

Macé, K., Harris, C.C., Lipsky, M.M. and Pfeifer, A.M.A. (1996a) Human hepatocytes. From: Culture of Immortalized cells. Edited by R. Ian Freshney and Mary G. Freshner. Wiley-Liss, Inc., 161-181.

Macé, K., Vautravers, P., Gonzalez, F., Harris, C.C. and Pfeifer, A.M.A. (1996b) Metabolism-competent human liver epithelial cell lines for in vitro toxicological applications. (Abstract). STCS, Lausanne, Switzerland, March 1996.

Macé, K., Aguilar, F., Vautravers, P., Gonzalez, F., Wang, J.-S., Groopman, J.D., Harris, C.C. and Pfeifer, A.M.A. (1996c) AFB1 metabolism and p53 mutation in metabolically competent human liver epithelial cell lines. (Abstract). *Procceedings of the American Association for Cancer Research*, Washington D.C. 1996.

Macé, K., Aguilar, F., Wang, J-S., Vautravers, P., Gomez-Lechón, M., Gonzalez, F.J., Groopman, J., Harris, C.C. and Pfeifer, A.M.A. (1997) Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis*, in press.

MacLeod,M.C. (1995) Interactions of bulky chemicals carcinogens with DNA in chromatin. *Carcinogenesis*, **16**, 9, 2009-2014.

Madle,S., Dean,S.W., Andrae,U., Brambilla,G., Burlinson,B., Doolittle,D.J., Furihata,C., Hertner,T., McQueen,C.A. and Mori,H. (1994) Recommendations for the performance of UDS tests *in vitro* and *in vivo*. *Mut. Res.*, **312**, 263-285.

Mannervik,B., Awasthi,Y.C., Board,P.G., Hayes,J.D., Di Ilio,C., Ketterer,B., Listowsky,I., Morgenstern,R., Muramatsu,M., Pearson,W.R., Pickett,C.B., Sato,K., Widersten,M. and Wolf,C.R. (1992) Nomenclature for human glutathione transferases. *Biochemical Journal*, **282**, 305-306.

Mapoles,J., Berthon,F., Alexander,A., Simon,F. and Menez,J.F. (1993) Mammalian PC-12 cell genetically engineered for human cytochrome P4502E1 expression. *European Journal of Biochemistry*, **214**, 735-745.

Maron,D.M. and Ames,B.N. (1983) Revised methods for the Salmonella mutagenicity test. *Mut. Res.*, **113**, 173-215.

Martin,C.N., McDermid,A.C.and Garner,R.C. (1978) Testing of known carcinogens and noncarcinogens for their ability to induce unscheduled DNA synthesis in HeLa cells. *Cancer Research*, **38**, 2621-2627.

McConkey,D.J., Hartzell,P., Nicotera,P, Wyllie,A.H. and Orrenius,S. (1988) Stimulation of endogenous endonuclease activity in hepatocytes exposed to oxidative stress. *Toxicology Letters*, **42**, 2, 123-130.

McDanell,R., McLean,A.E.M., Hanley,A.B.,Heaney,R.K. and Fenwick,G.R. (1988) Chemical and biological properties of indole glucosinolates (Glucobrassicins): A review. *Food and Chemical Toxicology*, **26**, 1, 59-70.

McDanell, R., McLean, A.E.M., Hanley, A.B., Heaney, R.K. and Fenwick, G.R. (1989) The effetc of feeding brassica vegetables and intact glucosinolates on mixed-function-oxidase activity in the livers and intestines of rats. *Food and Chemical Toxicology*, **27**, 5, 289-293.

McKelvey-Martin,V.J., Green,M.H.L., Schmezer,P., Pool-Zobel,B., De Meo,M.P. and Collins,A. (1993) The single cell gel electrophoresis assay (comet assay): An European review. *Mutation Research*, **288**, 47-63.

McLellan,L.I. and Hayes,J.D. (1989) Differential induction of class-alpha glutathione S-transferases in mouse-liver by the anticarcinogenic antioxidant butylated hydroxyanisole - purification and characterization of glutathione S-transferase YA1 YA1. *Biochemical Journal*, **263**, 393-402.

Meister,A. (1994) Glutathione, Ascorbate and cellular protection. *Cancer Research*, **54**, 1969s-1975s.

Melnick,R.L. (1992) Does chemically-induced hepatocyte proliferation predict liver carcinogenesis. *FASEB Journal*, **6**, 9, 2698-2706.

Mennicke,W.H., Goeler,K., Krumbiegel,G., Lorenz,D. and Rittmann,N. (1988) Studies on the metabolism and excretion of benzyl isothiocyanate in man. *Xenobiotica*, **18**, 4, 441-447.

Meyer,D.J., Crease,D.J. and Ketterer,B. (1995) Forward and reverse catalysis and product sequestration by human glutathione-S-transferases in the reaction of GSH witn dietary aralkyl isothiocyanates. *Biochemical Journal*, **306**, 565-569.

Moldeus,P., Hodberg,J. and Orrenius,S. (1978) Isolation and use of liver cells. *Methods Enzymol.*, **52**, 60-71.

Monteith,D.K. and Vanstone,J. (1995) Comparison of Dna damage from genotoxicants using the microgel elctrophoresis assay with primary cultures of rat and human hepatocytes. *Mutation Research*, **345**, 79-86.

Morse,M.A., Wang,C-G., Stoner,G.D., Mandal,S., Conran,P.B., Admin,S.G., Hetch,S.S. and Chung,F-L. (1989) Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-l-butanone-

induced DNA adduct formation and tumorigenicity in the lung of F344 rat by dietary phenethyl isothiocyanate. *Cancer Res.*, **49**, 549-553.

Morse,M.A., Zu,H., Galati,A.J., Schmidt,C.J. and Stoner,G.D. (1993) Dose-related inhibition by dietary phenethyl isothiocyanate of esophageal tumorigenesis and DNA methylation induced by *N*-nitrosomethylbenzylamine in rats. *Cancer Letters*, **72**, 103-110.

Moslen,M.T. (1996) Toxic responses to the liver. In Casarett and Doull's Toxicology. The basic science of poisons. Edited by C.C.Klaassen. McGrawhill, **3,** 403-416.

Murray,M. and Reidy,G.F. (1990) Selectivity in the inhibition of mammalian cytochromes P450 by chemical agents. *Pharmacological Reviews*, **42**, 2, 85-101.

Musk,S.R.R., Astley,S.B., Edwards,S.M., Stephenson,P., Hubert,R.B. and Johnson,I.T. (1995) Cytotoxic and clastogenic effects of benzylisothiocyanate towards cultured mammalian cells. *Food and Chemical Toxicology*, **33**, 1, 31-37.

Musk,S.R.R. and Johnson,I.T. (1993) The clastogenic effects of isothiocyanates. *Mut. Res.*, **300**, 111-117.

Nakachi,K., Imai,K., Mayashi,S.-I. and Kawajiri,K. (1993) Polymorphisms of the CYP1A1 and glutathione-S-transferase genes associated with susceptibility to lung cancer in relation to cigarette dose in a Japanese population. *Cancer Research*, **53**, 2994-2999.

Nakajima,T., Wang,R.-S., Elovaara,E., Park,S.S., Gelboin,H.V. and Vainio,H. (1993) Cytochrome P450-related differences between rats and mice in the metabolism of benzene, toluene and trichloroethylene in liver microsomes. *Biochemical Pharmacology*, **45**, 5, 1079-1085.

Nastruzzi,C., Cortesi,R., Esposito,E., Menegatti,E., Leoni,O., Iori,R. and Palmieri,S. (1996) *In vitro* cytotoxic activity of some glucosinolate-derived products generated by myrosinase hydrolysis. *J. Agric. Food Chem.*, **44**, 1014-1021.

Nedelcheva,V. and Gut,I. (1994). P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer. *Xenobiotica*, **24**, 12, 1151-1175.

Nelson,D.R. (1995) Cytochorme P450 nomenclature and alignment of selected sequences. From: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortiz de Montellano, Plenum Press, 1995, 575-606.

Nelson, D.R., Kamataki, T., Waxman,D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA and Cell Biology*, **12**, 1, 1-51.

Neudecker,T. and Henschler,D. (1985) Allyl isothiocyanate is mutagenic in *Salmonella typhimurium*. *Mutat. Res.*, **156**, 33-37.

Nicolau,K.C. (1992) Deprotection of amines. *J.Am.Chem.Soc.*, **114**, 3134.

Nguyen,Y., Rushmore,T.H. and Pickett,C.B. (1994) Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. *The Journal of Biological Chemistry*, **269,** 18, 13656-13662.

Oberley,L.W. and Oberley,T.D. (1995) Reactive oxygen species in the aetiology of cancer. From: *Drugs, diet and disease*. Volume 1. Mechanistic approaches to cancer. Edited by C.Ioannides and D.F.V.Lewis. Ellis Horwood Ltd.
*Carcinogenesis*, **6**, 9, 2057-2062.

Ohta,T. (1993) Modification of genotoxicity by naturally occurring flavonoids and their derivatites. *Critical Reviews in Toxicology*, **23**,2, 127-146.

Oinonen,T., Saarikoski,S., Husgafvelpursiainene,K., Hirvonen,A., Lindros,K.O. (1994) Pretranslational induction of cytochrome P4501A enzymes by beta-naphthoflavone and 3-methylcholanthrene occurs in different liver zones. *Biochemical Pharmacology*, **48**, 12, 2189-2197.

Okey,A.B., Riddick,D.S. and Harper,P.A. (1994) The Ah receptor: mediator of the toxicity of 2, 3, 7, 8-tetrechlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicology letters*, **70**, 1-22.

Olive,P.L. and Banath,J.P. (1993) Induction of and rejoining of radiation-induced DNA single-strand breaks: tail moment aa a function of possition in the cell cycle. *Mut. Res.*, **294,** 275-283.

Olive,P.L., Charlene,M., Vikse,A.A. and Durand,R.E. (1994) Hypoxic fractions measured in murine tumors and normal tissues using the comet assay. *International Journal Radiation Oncology Biology Physics*, **29**, 3, 487-491.

Omenn,G.S., Goodman,G.E., Thornquist,M.D., Balmes,J., Cullen,M.R., Glass,A., Keogh,J.P., Meyskens,F.L., Valanis,B., Williams,J.H., Barnhart,S., Cherniack,M.G., Brodkin,C.A. and Hammar,S. (1996) Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J.Natl.Cancer Inst.*, **88**, 1550-1559.

Omura,T. and Sato,R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., 239, 2370-2378.

Ortiz de Montellano, P.R. (1995) Arylhydrazines as probes of hemoprotein structure and function. *Biochimie*, **77**, 581-593.

Ortiz de Montellano,P.R. (1995) Oxygen actication and reactivity. From: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortiz de Montellano, Plenum Press, 1995, 245-303.

Ortiz de Montellano,P.R.and Correia,M.A. (1995) Inhibition of cytochrome P450 enzymes. From: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortiz de Montellano, Plenum Press, 1995, 305-364.

Overvik,E. and Gustafsson,J.-A. (1990) Cooked-food mutagens: current knowledge of formation and biological significance. *Mutagenesis*, **5**, 437-446.

Paine,A.J. (1995) Heterogeneity of cytochrome P450 and its toxicological significance. *Human and Experimental Toxicology*, **14**, 1-7.

Pascale,R.M., Smile,M.M. and Feo,F. (1993) Genomic abnoramlities in hepatocarcinogenesis. Implications for a chemopreventive strategy. *Anticancer Research*, **13**, 1341-1356.

Parkinson,A. (1996) Biotransformation of xenobiotics. In In Casarett and Doull's Toxicology. The basic science of poisons. Edited by C.C.Klaassen. McGrawhill, **6,** 113-186.

Pegg,A.E. and Byers,T.L. (1992) Repair of DNA containing $O^6$-alkylguanine. *FASEB Journal*, **6**, 2302-2310.

Pfeifer,A.M.A., Cole,K.E., Smoot,D.T., Weston,A., Groopman,J.D. Shields,P.G., Vignaud,J.-M., Juillerat,M., Lipsky,M.M., Trumps,B.F., Lechner,J.F. and Harris,C.C. (1993) Simian virus 40 large tumor antigen-immortalized normal human liver epothelial cells express hepatocyte characteristics and metabolize chemical carcinogens. *PNAS*, **90**, 5123-5127.

Pfeifer, A.M.A., Mace, K., Tromvoukis, Y. and Lipsky, M.M. (1995) Highly efficient establishment of immortalized cells from adult human liver. *Methods in Cell Science*, **17**, 83-89.

Pickett,C.B. and Lu,A.Y.N. (1989) Glutathione-S-transferases: gene structure, regulation and biological function. *Annual Reviews Biochemistry*, **58**, 743-764.

Pitot,H.C. and Dragan,Y.P. (1994) The multistage nature of chemically induced hepatocarcinogenesis in the rat. *Drug Metabolism Reviews*, **26** (1&2), 209-220.

Pitot,H.C. and Dragan,Y.P.(1996) Chemical carcinogenesis. In Casarett and Doull's Toxicology. The basic science of poisons. Edited by C.C.Klaassen. McGrawhill, **8,** 201-267.

Pohl,R.J. and Fouts,J.R. (1980) A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Analytical Biochemistry*, **107**, 150-155.

Poland,A., Palen,D., and Glover,E.D. (1994) Analysis of the four alleles of the murine arylhydrocarbon receptor. *Molecular Pharmacology*, **46**, 915-921.

Porter,T.D. and Coon,M.J. (1991) Cytochrome P4540. Multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. *J.Biol.Chem.*, **266,** 13469-72.

Poulsen,H.E., Loft,S. and Wassermann,K. (1993) Cancer risk related to genetic polymorphisms in carcinogen metabolism and DNA repair. *Pharmacology and Toxicology*, **72**, s1, s93-s103.

Prestera,T. and Talalay,P. (1995) Electrophile and antioxidant regulation of enzymes that detoxify carcinogens. *Proccedings of the National Academy of Sciences*, **92**, 8965-8969.

Primiano,T., Egner,P.A., Sutter,T.R., Kelloff, G.J., Roebuck,B.D. and Kensler,T.W. (1995) Intermittent dosing with oltipraz: Relationship between chemoprevention of aflatoxin-induced tumorigenesis and induction of glutathione S-transferases. *Cancer Research*, **55**, 4319-4324.

Prochazka, Z. (1959) *Collect.Czech.Chem.Commun.*, **24**, 2429-2430

Prochaska,H.J., Santamaria,A.B. and Talalay,P. (1992) Rapid detection of inducers of enzymes that protect againswt carcinogenesis. *PNAS*, **89**, 2394-2398.

Prochaska,H.J. and Talalay,P. (1988) Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.*, **48**, 4776-4782.

Purchase,I.F.H. (1994) Current knowledge of mechanisms of carcinogenecity: genotoxins versus non-genotoxins. *Human and Experimental Toxicology*, **13**, 17-28.

Quintanilla,M., Brown,K., Ramsden,M. and Balmain,A. (1986) Carcinogen-specific mutations and amplifications of Ha-*ras* during mouse skin carcinogenesis. *Nature*, 322, 78-80.

Ramsdell, H.S. and Eaton, D.L. (1988) Modification of aflatoxin $B_1$ biotransformation *in vitro* and DNA binding *in vivo* by dietary broccoli in rats. *Journal of Toxicology and Environmental Health*, **25**, 269-284.

Rannug,A., Alexandrie,A.-K., Persson,I and IngelmanpSundberg,M.I. (1995) Genetic polymorphism of cytochromes P450 1A1, 2D6 and 2E1: Regulation and toxicological significance. *Journal of Occupational and Environmental Medicine*, **37**, 1, 25-36.

Rao,C.V., Riverson,A., Simi,B., Zang,E., Hamid,R., Kelloff,G.J., Steele,V., and Reddy,B.S. (1995) Enhancement of experimental colon caricnogenesis by dietary 6-phenylhexylisothiocyanate. *Cancer Research*, **55**, 4311-4318.

Raunio,H. and Pelkonen,O. (1995) Cancer genetics. Genetics factors in the activation and inactivation of chemical carcinogens. From: *Drugs ,diet and disease*. Volume 1. Mechanistic approaches to cancer. Edited by C.Ioannides and D.F.V.Lewis. Ellis Horwood Ltd.

Raunio,H., Husgafnel-Pursiainen,K., Anttila,S., Hietanen,E., Hirronen,A. and Pelkoren,O. (1995) Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility- a review. *Gene*, **159**, 113-121.

Regan,J.d. and Setlow,R.B. (1974) Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. Cancer Research, 34, 3318-3325.

Reicks,M.M. and Crankshaw,D.L. (1996) Modulation of rat hepatic cytochrome P-450 activity by garlic organosulful compounds. Nutr. Cancer, 25, 241-248.

Reinke,L.A. and Moyer,M.J. (1985) *p*-Nitrophenol hydroxylation. A microsomal oxidation which is highly inducible by ethanol. *Drug Metab. Dispos.*, **13**, 548-552.

Rice-Evans,C.A. and Miller,N.J. (1996) Antioxidant activities of flavonoids as bioactive componenets of food. *Biochemical Society Transactions,* 24, 790-795.

Roberts-Thomson, S.J., McManus, M.E., Tukey, R.H., gonzalez, F.F. and Holder, G.M. (1993) The catalytic activity of four expressed human cytochrome P450s towards benzo[a]pyrene and the isomers of its proximate carcinogens. *Biochemical and Biophysical Research Communications*, **192**, 3, 1373-1379.

Ross, G.M., McMillan,T.J., Wilcox,P. and Collins,A.R. (1995) the single cell microgel electrophoresis assay (comet assay): technical aspects and applications. Report on the 5th LH Gray Trust Workshop, Institute of Cancer Research, 1994. *Mutation Research*, **337**, 57-60.

Rueff, J., Chiapella, C., Chipman, J.K., Darroudi, F., Duarte Silvia, I., Duverger-van Bogaert, M., Fonti, E., Glatt, H.R., Isern, P., Laires, A., Leonard, A., Llagostera, M., Mossesso, P., Natarajan, A.T., Palitti, F., Rodrigues, A.S., Schinoppi, A., Turchi, G. and Werle-Schneider. (1996) development and validation of alternative metabolic systems for mutagenicity testing of short-term assays. *Mutation Research*, **353**, 151-176.

Ruggeri,B., Caamano,J., Goodrow,T., Dirado,M., Bianchi,A, Trono,D., Conti,C.J. and Klein-Szanto,A.J.P. (1991) Alterations of the *p53* tumor suppressor gene during mouse skin tumor progression. *Cancer Research,* **51**, 6615-6621.

Rumney,C.J., Rowland,I.R. and O'Neill,I.K. (1993) Conversion of IQ to 7-OHIQ by gut flora. *Nutrition and Cancer*, **19**, 67-76.

Sardas,S., Walker,D., Akyol,D. and Karakaya,A.E. (1995) Assessment of smoking-induced DNA damage in lymphocytes of smoking mothers of newborn infants using the alkaline single-cell gel elctrophoresis technique. *Mutation Research*, **335**, 213-217.

Schmahl,D. (1981) The role of nitrosamines in carcinogenesis - an overview. Form Safety evaluation of nitrosatable drugs and chemicals. Edited by Gibson,G.G. and Ioannides,C. Taylor & Francis Ltd, 1-15.

Schmalix,W.A., Barrenscheen,M., Landsiedel,R., Janzowskic,C., Eisenbrand,G., Gonzalez,F., Eliasson,E., Ingelman-Sundberg et al. (1995) Stable expression of human cytochrome P4502E1 in V79 Chinese hamster cells. *European Journal of Pharmacology*, **293**, 123-131.

Schmid,H. and Karrer,P. (1948) Synthesis of sulforaphane. *Helv. Chim. Acta*, **31**, 1495-1505.

Schulte-Hermann, Timmermann-Trosiener,I., Barthel,G. and Bursch,W. (1990). DNA synthesis, apoptosis and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. *Cancer Research*, **50**, 5127-5135.

Schwartz,J.L. (1996) The dual roles of nutrients as antioxidants and prooxidants: Their effects on tumor cell growth. *Journal of Nutrition*, **126**, 1221S-1227S.

Seree, E.M., Botto, F., Pisano, P., Lechevalier, E., Desobry, A. and Barra, Y. (1995) Evidence for the existence of two CYP2E1 cDNAs using different polyadenylation signals. *Biochemical and Biophysical Research Communications*, **209**, 2, 717-722.

de Serres,F.S. and Shelby,M.D. (1979) Recomendations on data production and analysis using the *Salmonella* / microsome mutagenicity assay. *Mutation Research*, **64**, 159-165.

Setlow,R.B. (1978) Repair deficient human disorders and cancer. *Nature*, **271**, 713-717.

Sevrioukova, I.F. and Peterson, J.A. (1995) NADPH-P450 reductase: Structural and functional comparisons of the eukaryotic and prokaryotic isoforms. *Biochimie*, **77**, 562-572.

Shimada,T. and Nakamura,S.-I. (1987) Cytochrome P450-mediated activation of procarcinogens and promutagens to DNA-damaging products by measuring expression ot *umu* gene in Salmonella typhimurium TA1535/pSK1002. *Biochemical Pharmacology*, **36**, 7, 1979-1987.

Shimada,T., Yamazaki,H., Mimura,M. and Guenguerich,F.P. (1992a) Rat pulmonary microsomal cyrochrome P450 enzymes involved in the activation of procarcinogens. *Mutation Research*, **284**, 233-241.

Shimada,T., Yun,C.-H., Yamazi,H., Gautier,J.-C., Beaune,P.H. and Guengerich,F.P. (1992b) Characterization of the human lung microsomal cytochrome P-450 1A1 and its role in the oxidation of chemical carcinogens. *Molecular Pharmacology*, **41**, 856-864.

Shu,L. and Hollenberg, P. (1996) Role of cytochrome P450 in DNA damage induced by N-nitrosodialkylamines in cultured rat hepatocytes. *Carcinogenesis*, **17**, 3, 569-576.

Simpson,R.T. (1978) Structure of the chromatosome, a chromatin particle containing 160 base pairs of DNA and all the histones. *Biochemistry*, **17**, 25, 5524-5531.

Sina,J.F., Bean,C.L.., Dysart,G.R., Taylor,V.I. and Bradley,M.O. (1983) Evaluation of the alkaline elution / rat hepatocyte assay as a predictor of carcinogenic / mutagenic potential. *Mutation Res.*, **113**, 357-391.

Singer,B. (1986) O-Alkylpyrimidines in mutagenesis and carcinogenesis: Ocurrence and significance. *Cancer Research*, **46**, 4879-4885.

Singh,N.P., Danner,D.B., Tice,R.R., McCoy,M.T., Collins,G.D. and Schreider,E.L. (1989) Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Experimental Cell Research*, **184**, 461-470.

Singh,N.P., McCoy,M.T., Tice,R.R. and Scheneider,E.L. (1988) A simple thecnique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, **175**, 184-191.

Singh,N.P., Tice,R.R., Stephens,R.E. and Scheneider,E.L. (1991) A microgel elctrophoresis technique for the direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides. *Mutation Research*, **252**, 289-296.

Smith,A.J. and Chipman,J.K. (1988) Inter-individual variation in the mutagenic activation of 2-acetylaminofluorene by human liver in relation to animal metabolic models. *Mutagenesis*, **3**,4, 323-328.

Smith,G., Harrison,D.J., East,N. Rae,F., Wolf,H. and Wolf,C.R. (1993) Regulation of cytochrome P450 expression in human colon and breast tumour xenografts. *British Journal of Cancer*, **68**, 57-63.

Smith, T.J., Guo, Z., Guengerich, F.P. and Yang, C.S. (1996) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-1butanone (NNK) by human cytochrome P450 1A2 and its inhibition by phenethylisothiocyanate. *Carcinogenesis*, **17**, 4, 809-813.

Sones,K., Heaney,R.K. and Fenwick,G.R. (1984) An estimate of the mean daily intake of glucosinolates from cruciferous vegetables in the UK. *J.Science Food and Agriculture*, **35,** 712-720.

Speit,G. and Hartmann,A. (1995) the contribution of excision repair to the DNA effects seen in the alkali single cell gel test (comet assay). *Mutagenesis*, **10**, 6, 555-559.

Sporn,M.B., Dunlop,N.M., Newton,D.L. and Smith,J.M. (1976) Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids) *Fed. Proc.*, 35, 1332-1338.

Song, B.-J., Veech,R.L., Park,S.S., Gelboin,H.V. and Gonzalez,F.J. (1989) Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *The Journal of Biological Chemistry*, **246**, 6, 3568-3572.

Staab,H.A. and Walker,G. (1962). Reaction of *N,N'*-thionocarbonyldiimidazole with amines. *Justus Liebigs Ann. Chem.*, **657**, 104.

Stanley,L.A. (1995) Review article. Molecular aspects of chemical carcinogenesis: The role of oncogenes and tumour suppressor genes. *Toxicology*, **96**, 173-194.

Stavric,B. (1994) Antimutagens and anticarcinogens in foods. *Food and Chemical Toxicology*, **32**, 1, 79-90.

Steinmetz,K.A. and Potter,J.D. (1991) Vegetables, fruit and cancer. *Epidemiology, Cancer Causes and Control*, **2**, 325-57.

Steinmetz, K.A., Potter, J.D. and Folsom, A.R. (1993) Vegetables, fruit and lung cancer in the Iowa Women's Health Study. *Cancer Research*, **53**, 536-543.

Still,W.C., Khan,M., and Mitra,A. (1978) A rapid chromatographic technique for preparative separations with moderate resolution. *Journal Organic Chemistry*, **43**, 2923.

Stoewsand,G.S. (1995) Bioactive organosulfur phytochemicals in *Brassica oleracea* vegetables - A reveiw. *Food and Chemical Toxicology*, **33**, 6, 537-543.

Strauss,D.S. (1981) Somatic mutation, cellular differentiation, and cancer causation. *Journal National Cancer Institute*, **67**, 233-239.

Sugie,S., Yoshimi,N., Okumana,A., Tauka,T. and Morie,H. (1993) Modifying effects of benzyl isothiocyanate and benzyl thiocyanate on DNA synthesis in primary cultures of rat hepatocytes. *Carcinogenesis*, **14**, 2, 281-283.

Talalay,P. (1989) Mechanisms of induction of enzymes that protect against chemical carcinogensis. *Advanced Enzyme Regulation*, **28**, 237-250.

Tan,Y., White,S.P., Paranawithana,S.R. and Yang,C.S. (1997) A hypothetical model for the active site of human cytochrome P4502E1. *Xenobiotica*, **27**, 3, 287-299.

Tawfiq,N., Heaney,R.K., Plumb,J.A., Fenwich,G.R., Musk,R.R., and Williamson,G. (1995) Dietary glucosinates as blocking agents against carcinogenesis glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hepa 1c1c7 cells. *Carcinogenesis*, **16**, 5, 1191-1194.

Tennant,R.W. (1993) Perspective on nonmutagenic mechanisms in carcinogeneis. *Environm. Heath. Perspectives*, **101** suppl., 231-236.

Tew,K.D. (1994) Glutathione-associated enzymes in anticancer drug resistance. *Cancer Research*, 54, 4313-4320.

Thomas,P.E., Bandiera,S., Maines,S.L., Ryan,D.E. and Levin,W. (1987) Regulation of cytochrome P450 j, a high-affinity *N*-nitrosodimethylamine demethylase, in rat hepatic micrososmes. *Biochemistry*, 26, 2280-2289.

Thompson,L.H., Wu,R.W. and Felton,J.S. (1991) Introduction of cytochrome P4501A2 metabolic capacity into cell lines genetically matched for DNA repair proficiency/deficinecy. *Proceedings of the National Academy of Science*, **88**, 3827-3831.

Tlsty,T.D.(1997) Genomic instability and its role in neoplasia. In: Genetic stability and tumorigenesis. *Current Topics in Microbiology and Immunology*, **221**, 37-46.

Trosko, J.E. (1989) Towards understanding carcinogen hazards: A crisis in paradigms. *Journal of the American College of Toxicology*, **8**, 6, 1121-1131.

Vaghef,H. and Hellman,B. (1995) Demonstration of chlorobenzene-induced DNA damage in mouse lymphocytes using the single cell gel electrophoresis assay. *Toxicology*, **96**, 19-28.

Vang,O., Rasmussen,B.F., Sorensen,H., Clausen,J., Andersen,O. (1995) Effects of dietary broccoli on antioxidant enzymes. *Clinical Chemistry*, **41**, 12, 1910-1911.

Vatsis,K.P., Weber,W.W., Bell,D.A. *et al.* (1995) Nomenclature for *N*-acetyltransferases. *Pharmacogenetics*, **5**, 1-17.

Venitt, S., Crofton-Sleigh, C. and Forster, R. (1984). Bacterial mutation assays using reverse mutation. From: Mutagenicity testing: a practical approach. Edited by S.Venitt and J.M. Parry. Irl Press, Oxford, 1985. **3**, 45-98.

Venitt,S. and Parry,J.M. (1984). Background to mutagenicity testing. From: Mutagenicity testing: a practical approach. Edited by S.Venitt and J.M. Parry. Irl Press, Oxford, 1985. **3**, 1-24.

Venitt,S., Forster,R. and Longstaff,E. (1983) Bacterial mutagencity assays. From: Report of the UKEMS Sub-Committee on guidelines for mutagenicity testing, Part I. Edited by Dean,B.J., 1, 5-40.

Vile,G.R. and Morris,G.A. (1992) The multiple molecular mechanisms of cancer. In search of unification. In Introduction to the molecular genetics of cancer. Edited by R.G.Vile. Published by J.Wiley and sons Ltd., 1-31.

Walker,R. (1981) Nitrosamines and nitrosatable drugs in food. Form Safety evaluation of nitrosatable drugs and chemicals. Edited by Gibson,G.G. and Ioannides,C. Taylor & Francis Ltd, 220-233.

Warwick,A., Sarhanis,P., Redman,C., Pemble,S., Taylor,J.B., Ketterer,B., Jones,P., Alldersea,J., Gilford,J., Yengi,L., Fryer,A. and Strange,R.C. (1994) Theta class glutathione-s-tranferase GSTT1 genotypes and susceptibility to cervical neoplasia- Interactions with GSTM1, CYP2D6 and smoking. *Carcinogenesis*, **15**, 12, 2841-2845.

Waters, R. (1984) DNA repair tests in cultured mammalian cells. From: Mutagenicity testing: a practical approach. Edited by S.Venitt and J.M. Parry. Irl Press, Oxford, 1985. **3**, 99-117.

Waters,R., Ashby,J., Burlinson,B., Lefevre,P., Barret,R. and Martin,C. (1984) Unscheduled DNA synthesis. From: UKEMS sub-committee on guidelines for mutagenicity testing (PartII A), Edited by Dean,B.J., 4, 63-83.

Wattenberg,L.W. (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res. Suppl.*, **52**, 2085s-2091s.

Wattenberg,L.W. (1983) Inhibition of neoplasia by minor dietary constituents. *Cancer Res. Suppl.*, **43**, 2448s-2453s.

Wattenberg,L.W. (1996) Chemoprevention of cancer. *Preventive medicine*, **25**, 44-45.

Wattenberg,L.W. (1987) Inhibitory effects of benzyl isothiocyanate adminstered shortly before diethylnitrosamines or benzo(a)pyrene on pulmonary and forestomach neoplasia in A/J mice. *Carcinogenesis*, **8**, 1971-1073.

Waxman,D.J., Lapenson,D.P. Aoyama,T., Gelboin,H.V., Gonzalez., F.J. and Korzekwa,K. (1991) Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochromes P450s.*Arch.Biochem.Biophys*, **209**, 160-166.

Weaver,R.J., Dunbar,B., Dickins,M., Melvin,W.T., Fothergill,J. and Burke,M.D. (1994) Evidence for a new cytochrome P450 form induced by 3-methylcholanthrene in rats. *Biochemical Pharmacology*, **47**, 8, 1457-1460.

Weinstein,B. (1991) Cancer Prevention: Recent progress and future opportunities. *Cancer Research*, suppl. **51**, 5080s-5085s.

Whitlock,J.P. and Denison,M.S. (1995) Induction of cytochrome P450 enzymes that metabolize xenobiotics. From: Cytochrome P450: Structure, mechanisms and biochemistry. 2nd edition. Edited by Ortisz de Montellano, Plenum Press, 1995, 367-389.

Wilbourn,J., Haroun,L., Heseltine,E., Kaldor,J., Partensky,C. and Vainio,H. (1986) Response of experimental animals to human carcinogens. An analysis based upon thge IARC monographs program. *Carcinogenesis*, 7, 11, 1853-1863.

Williams,G.M., Laspia,M.F. and Durkel,V.C. (1982) Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and non-carcinogens. *Mutation Research*, **97**,

Wortelboer,H.M., de Kruif,C.A., van Iersel,A.A.J., Noordhoek,J., Blaauboer,B.J., van Bladerens,P.J. and Falke,H.E. (1992) Effects of cooked brussels sprouts on cytochrome P450 profile and phase II enzymes in liver and small intestinal mucosa of the rat. *Food and Chemical Toxicology*, **30**, 1, 17-27.

Wrighton,S.A. and Stevens, J.C. (1992) The human hepatic cytochromes p450 involved in drug metabolism. *Critical Reviews in Toxicology*, **22**, 1, 1-21.

Xiao,G.-H. and Russell,A.P. (1995) Induction of xenobiotic metabolizing enzymes regulated by arylhydrocarbon (*Ah*) receptor dependent and independent mechanisms. *Proceedings of the Fourth International ISSX Meeting, Seattle, Washington, USA*, **8**, 394.

Yamasaki,H., Meshil, M., Omori, Y., Mironov, N. and Krutovskikh, V. (1995) Intercellular communication and carcinogenesis. *Mutation Research*, **333**, 181-188.

Yang,C.S., Smith,T.J. and Hong,J.Y. (1994) Cytochrome P450 enzymes as targets for chemoprevention against chemical carcinogenesis and toxicity:opportunities and limitations. *Cancer Research*, supl. **54**, 1982s-1986s.

Yang,C.S., Yoo,J-S.H., Ishazaki,H. and Hong,J. (1990) Cytochrome P450 2E1. Roles of nitrosamine metabolism and mechanisms of regulation. *Drug Metab. Rev.*, **22**, 147-159.

Yengi,L., Inskip,A., Gilford,J., Alldersea,J., Bailey,L., Hand,P., Hayes,J.D., Jones,P.W., Strange,R.C. and Fryer,A.A. (1996) Polymorphisms at the glutathione-S-transferases locus GSTM3: interactions with cytochrome P450 and glutathione-S-transferase genotypes as risk factors for multiple cutaneous basal cell carcinoma. *Cancer Research*, **56**, 9, 1974-1977.

Yoo,J-S.H., Ishizaki,H. and Yang,C.S. (1990) Roles of cytochrome P450IIE1 in the dealkylation and denitrosation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in rat liver microsomes. *Carcinogenesis*, **11**, 2239-2243.

Yun, C.-H., Jeong,H.G., Jhon,J.W. and Guengerich,F.P. (1995) Non-specific inhibition of cytochrome P450 activities by chlorophyllin in human and rat liver microsomes. *Carcinogenesis*, **16**, 6, 1437-1440.

Zhang,Y. and Talalay,P. (1994) Anticarcinogenic activities of organic isothiocyanates: Chemistry and mechansims. *Cancer Research*, **54**, 1976s-1981s.

Zhang,Y., Kolm,R.H., Mannervik,B. and Talalay,P. (1995) Reversible conjugation of iosthiocyanates with GSH catalyzed by human GSTs. *Biochemical and biophysical Research communications*, **206**, 2, 748-755.

Zhang,Y., Talalay,P., Cho,C-G. and Posner,G.H. (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci.*, **89**, 2399-2403.

Zu,H.-X. and Schut,A.J. (1991) Formation and persistence of DNA adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline in male Fischer-344 rats. *Cancer Research*, **51**, 5636-5641.

## *LIST OF PUBLICATIONS*

S. Barcelo, J.M. Gardiner, A. Gescher and J.K. Chipman. (1996) CYP2E1-mediated mechanism of anti-genotoxicity of the broccoli constituent sulforaphane. *Carcinogenesis*, **17**, 277-282.

S. Barceló, K. Macé, A.M.A. Pfeifer and J.K. Chipman (1997) Production of DNA strand breaks by *N*-nitrosodimethylamine (NDMA) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) in THLE cells expressing human CYP isoenzymes and inhibition by sulforaphane. *Mutation Research*, in press.