MOLECULAR MECHANISMS OF SELENIUM-INDUCED GROWTH INHIBITION

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To Mum and Dad

Abstract

Selenium is a nutritional essential trace element. It is required for the activity of a number of selenoproteins which contain one or more selenocysteine residues that are encoded by in-frame UGA codons. Epidemiological studies also show that there is an inverse correlation between the dietary intake of selenium and the incidence of cancer. In animals, supra-nutritional but sub-toxic dietary levels of selenium have also been shown to have anti-carcinogenic activity in many chemical- and viral-induced tumour models and is effective at inhibiting both initiation and post-initiation (promotion) stages of tumour progression suggesting that selenium may act by inhibiting mutagenic events and also the growth of initiated cells. The anti-carcinogenic efficacy of selenium is also highly dependent on chemical form, suggesting metabolism is important for its chemoprotective effect.

Selenium at nanomolar concentrations is also essential for the optimum growth of cells in culture. However, at higher concentrations, selenium is a potent inhibitor of cell growth and it is thought that the growth inhibitory effects of selenium *in vitro*, may be mechanistically relevant to the post-initiation chemopreventive effects of selenium.

The mechanism of selenium-induced growth inhibition has been studied in a mouse mammary cell line, C57. Of a number of seleno-compounds that have varying degrees of chemopreventive activity, only sodium selenite had a significant effect on cell growth and caused cell death after a lag period of 48h. This lag period could be abolished if glutathione (GSH) was added simultaneously with selenite, suggesting that selenite needed to be reduced by GSH to exert its effect. Consistent with this, selenodiglutathione (SDG), the primary metabolite from the reaction of selenite and GSH, was found to be far more cytotoxic than selenite with micromolar concentrations of SDG causing a significant decrease in cloning efficiency within 1h.

SDG- and H_2O_2-induced cytotoxicity were compared since the reaction between selenite and GSH has been shown to generate reactive oxygen species. H_2O_2 also had a rapid effect and reduced the cloning efficiency of C57 cells within 1h. However, SDG and H_2O_2 appeared to be inducing cell death by distinct mechanisms as judged by the following observations: (1) SDG was found to reduce the mRNA levels of phospholipid hydroperoxide glutathione peroxidase (PHGPX), glutathione peroxidase (GPX) and glutathione S-transferase Ya subunit (GST Ya) whereas H_2O_2 had no effect; and (2) SDG induces both 560Kb and 50Kb DNA fragments whereas H_2O_2 only induces the formation of 560Kb fragments. The cleavage of DNA into high molecular weight fragments has been suggested to be indicative of apoptosis. However, key morphological markers of apoptosis were not observed with treatment of either agent.

To further investigate the mechanism of selenium-induced growth inhibition, a genetic approach was adopted. Using a one-step selection strategy, an SDG-resistant cell line (B19) was generated from C57 cells. B19 cells were found to be cross-resistant to selenite but equally sensitive to H_2O_2 as C57 cells which is consistent with the conclusions that selenite is acting through the production of SDG and that SDG and H_2O_2 induce cell death by distinct mechanisms. C57 and B19 cells were compared to gain some insight into the possible mechanisms of selenium-resistance. Selenium-uptake and GSH concentrations were eliminated as possible modes of resistance since these two parameters were unchanged in the B19 cells. However, the levels of a number of mRNAs were found to be different between

the two cell lines. GST Ya and GST Yc were 2-fold higher in B19 cells whereas PHGPX and Bcl-x_S were 2-fold lower in B19 cells. Additionally, the selenium-labelling protein complement of the two cell lines were compared. Both increases and decreases in the labelling or levels of a number of proteins were found to be altered in the B19 cells. The most striking differences were the absence in C57 cells of two 72KDa selenium-labelling proteins.

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Declaration

The work described in this thesis was performed personally, unless otherwise acknowledged.

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Abbreviations

2D	2-dimensional
A_x	Absorbance (x=wavelength)
AAF	2-acetylaminofluorene
AOM	azoxymethane
bp	base pairs
BOP	bis(2-oxopropyl)-nitrosamine
BSA	bovine serum albumin
BSO	buthionine sulphoximine
cDNA	complementary deoxyribonucleic acid
Ci	curie
cpm	counts per minute
Da	Dalton(s)
DAB	3-methylaminoazobenzene
DAPI	4,6-diamidino-2-phenylindole (DAPI)
DH	ductal hyperplasias
dH_2O	distilled water
DMBA	7,12-dimethylbenz[*a*]anthracene
DMH	dimethylhydrazine
DMS	dimethyl selenoxide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	3'deoxyribonucleoside 5'triphosphate
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal growth factor
EMS	ethylmethanesulphonate
FCS	foetal calf serum
FDH	formate dehydrogenase
g	gram(s)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione *S*-transferase
GTP	guanosine tri-phosphate
HAN	hyperplastic alveolar nodule
HOG	hyperplastic outgrowth
ICE	interleukin Iβ converting enzyme
ID-I	type I tetraiodothyronine deiodinase
kb	kilobase(s)
kDa	kiloDaltons
L-FABP	liver fatty acid binding protein
μ	micro
M	molar
MAM	methoxylazoxymethanol acetate

MDA	malondialdehyde
min	minute(s)
MNNG	N-alkyl-N'-nitro-N-nitrosoguanidine
MNU	methylnitrsourea
MOPS	4-morpholinepropanesulphonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
n	nano
NADPH	reduced nicotinamide adenine diphosphate
NLAL	nodule-like alveolar lesion(s)
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NOHAAF	*N*-hydroxy-2-acetylaminofluorene
oligo	oligonucleotide
P	plasma
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PGPX	plasma glutathione peroxidase
PHGPX	phospholipid hydroperoxide glutathione peroxidase
pI	isoelectric point
PLC	primary liver cancer
p-XSC	1,4-phenylenebis(methylene)selenocyanate
ROS	reactive oxygen species
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
R/T	room temperature
SB	selenobetaine
SBME	selenobetaine methyl ester
SDG	selenodiglutathione
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
[Se]Cys	selenocysteine
[Se]Met	selenomethionine
SMSC	seleno-methylselenocysteine
SOD	superoxide dismutase
TEMED	tetramethylenediamine
TLC	thin layer chromatography
TMS^+	trimethyl selenonium ion
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
tRNA	transfer ribonucleic acid
TRX	thioredoxin
U	unit(s)
UV	ultraviolet
v/v	volume for volume
W	watts
w/v	weight for volume

Chapter1

Selenium: Essential trace element

1.1 Introduction

In the last 40 years it has been recognised that selenium is an essential dietary element with syndromes such as Keshan disease, a juvenile cardiomyopathy, being attributed to selenium-deficient diets (reviewed in Ge and Yang, 1993). Before a biological role had been assigned to selenium it was believed that selenium primarily functioned as an antioxidant since it could prevent liver necrosis and exudative diathesis, a condition characterised by leakage of plasma into sub-cutaneous spaces, induced by a vitamin E-deficient diet (Patterson *et al.*, 1957; Schwarz and Foltz, 1957). The first biochemical role for selenium was recognised when it was discovered that selenium forms an integral part of the antioxidant enzyme, glutathione peroxidase (GPX) (Rotruck *et al.*, 1973). The selenium moiety was subsequently found to be in the active site in the form of the amino acid residue selenocysteine ([Se]Cys) encoded by an in-frame termination UGA codon (Chambers *et al.*, 1986). A number of selenoproteins have since been identified that contain one or more UGA-encoded [Se]Cys. These include three other glutathione peroxidases, type I tetraiodothyronine deiodinase (ID-I) which is primarily expressed in the liver and deiodinates the pro-hormone thyroxine (T_4) to 3,5,3'-triodothyronine (T_3), and a plasma glycoprotein, selenoprotein P, the function of which is presently unknown (reviewed in Sunde, 1990).

In addition to being an essential trace element, a number of epidemiological studies indicate that dietary selenium can inhibit carcinogenesis (reviewed in Willett and Stamper, 1986). This is strongly supported by a wealth of animal studies which show that supra-nutritional, but sub-toxic, levels of selenium are protective in a number of different tumour model systems (reviewed in Ip, 1986).

In cell culture, selenium is also an essential trace element and is required in the nanomolar range for the optimum growth of cells (McKeehan *et al.*, 1976; Medina and Oborn, 1981). However, in the micromolar range selenium is a potent

inhibitor of cell growth (Fico *et al.*, 1986). The mechanism by which selenium inhibits cell growth is unknown but it has been suggested that the *in vivo* chemopreventive and *in vitro* anti-proliferative properties of selenium are mechanistically related. The purpose of this study has therefore been to investigate the growth inhibitory effects of selenium *in vitro* to identify mechanisms that may be potentially important for the chemopreventive properties of selenium.

1.2 Selenium metabolism

Selenium lies in the same group as sulphur in the periodic table and is thus chemically similar. In plants and bacteria, inorganic forms of selenium are readily metabolised into the seleno-amino acids, selenomethionine ([Se]Met) and [Se]Cys, which can substitute for their sulphur analogues in protein synthesis (Burnell and Whatley, 1977). However, while selenium may follow pathways of sulphur metabolism, the existence of a unique mechanism of [Se]Cys incorporation into selenoproteins indicate that selenium-specific pathways exist.

Sulphur is normally metabolised by oxidation whereas selenium generally is reduced. The pathways of selenium metabolism are illustrated in figure 1.1. The most common form of selenium used in supplementation studies is selenite. Selenite is reduced to H_2Se following the reaction pathway characterised by Hsieh and Ganther (1977). The intermediate products, selenodiglutathione (SDG) and selenopersulphide, have been synthesised and characterised and found to rapidly decompose at neutral pH (Ganther, 1968). This suggests that *in vivo* these products are relatively short lived. The reduction of selenite is catalysed by glutathione reductase and NADPH but can occur non-enzymatically in the presence of excess glutathione (GSH) (Hsieh and Ganther, 1977). H_2Se is highly toxic and is either rapidly incorporated into selenoproteins (see section 1.3 and 1.4) or detoxified by methylation and excreted as dimethyl selenide in exhaled air or in urine as monomethylated seleno-compounds and trimethylselenonium ion (figure 1.1) (Ganther, 1986; Vadhanavikit *et al.*, 1993). A Se-methyltransferase located in

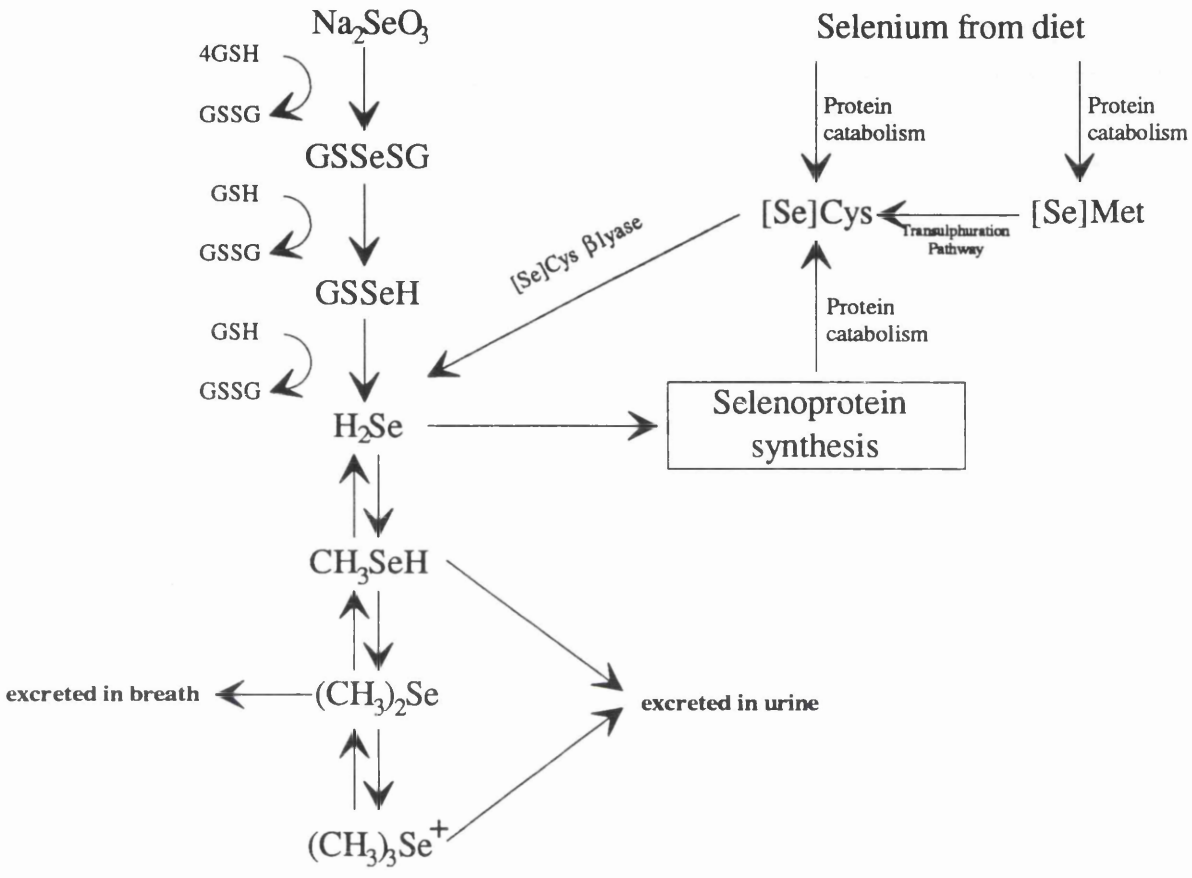


Figure 1.1
Intermediary selenium metabolism. Diagram showing essential pathways by which inorganic and organic selenium is metabolised into selenoproteins or excreted. (G represents the thiol-containing tri-peptide, glutathione). See text for details.

rat liver microsomes which is sensitive to inhibition by arsenite can form dimethylselenide from selenite in a cell-free system (Ganther, 1966, Hsieh and Ganther, 1977). There also exists a cytosolic methyl-transferase that is less sensitive to arsenite inhibition (Hsieh and Ganther, 1977). This arsenite-insensitive methyl-transferase may also be responsible for the final methylation step to form trimethyl selenonium ion (Mozier *et al.*, 1988). Although methylation is a detoxification process, this is to some extent reversible since the selenium atom in methylated Se-compounds can be incorporated into GPX indicating that demethylation can occur (figure 1.1) (Ip *et al.*, 1991).

[Se]Cys is obtained from the diet or generated either during selenoprotein synthesis or from [Se]Met. In mammals, [Se]Met cannot be synthesised from inorganic forms of selenium (Cummins and Martin, 1967) but is obtained from plants, such as wheat, in the diet (Meltzer *et al.*, 1992). [Se]Met can be converted into [Se]Cys by the transulphuration pathway using cystathione β synthase and cystathione γ lyase (Esaki *et al.*, 1981) (figure 1.1). [Se]Cys, which is toxic, is reduced by selenocysteine β lyase to H_2Se (Esaki *et al.*, 1982), thus allowing Se to be recycled into selenoproteins or excreted.

1.3 Selenoprotein synthesis

The mechanism of selenoprotein synthesis has been best characterised in bacteria. *Eschericia coli* have three selenopolypeptides that constitute the isozymes of formate dehydrogenase (FDH_H, FDH_N and FDH_O) (Cox *et al.*, 1981; Sawyers *et al.*, 1991), each containing [Se]Cys encoded by a UGA codon. The incorporation of [Se]Cys is co-translational (Zinoni *et al.*, 1987) and four genes have been identified (*sel A,B,C* and *D*) that are required for the correct translation of UGA into [Se]Cys (Leinfelder *et al.*, 1988a, 1988b) (Figure 1.2). The *sel C* gene product encodes a tRNA molecule, $tRNA^{sec}$, that is charged with L-serine by seryl-tRNA synthetase to form seryl-$tRNA^{sec}$ (Leinfelder *et al.*, 1988b). However, the amino-acylation of $tRNA^{sec}$ exhibits K_{cat} and K_M values that are

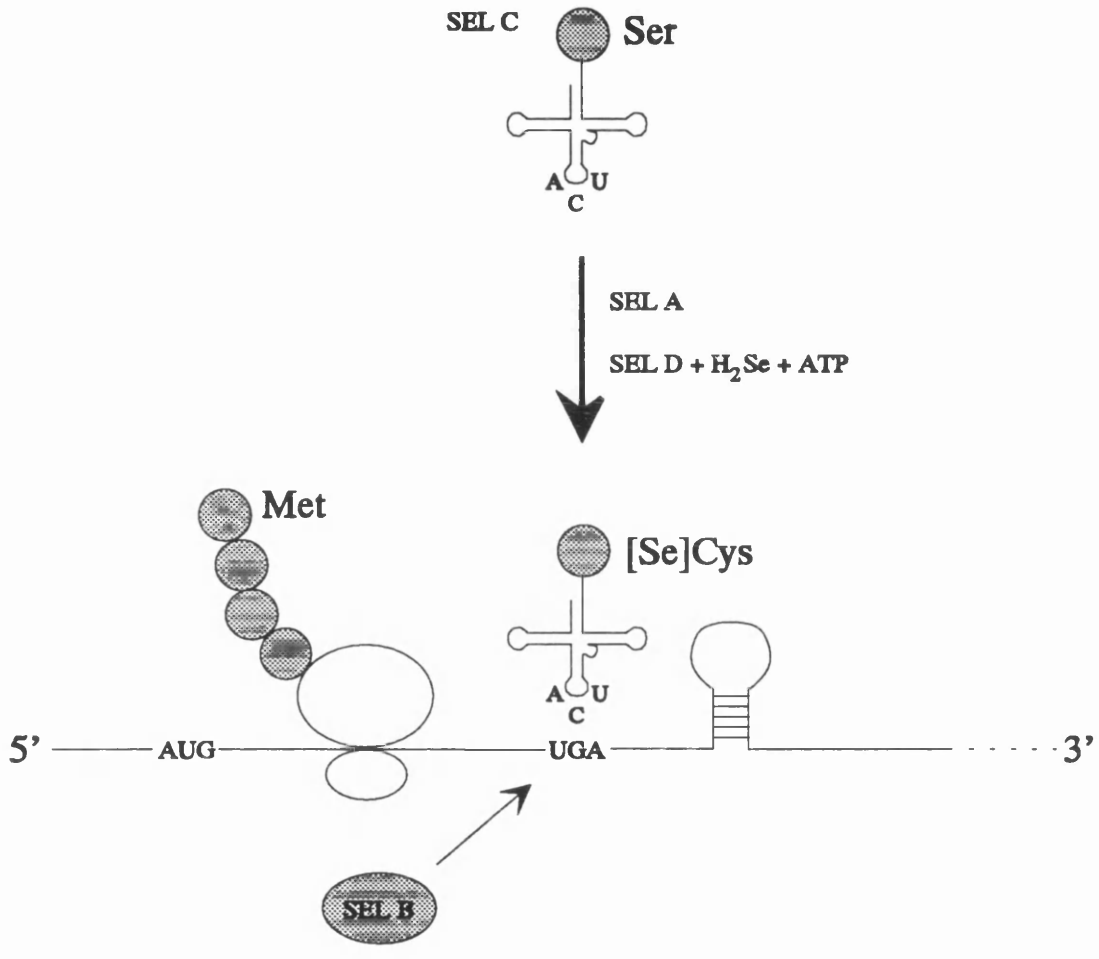


Figure 1.2
Co-translational insertion of selenocysteine during selenoprotein synthesis in prokaryotes. Schematic representation of the gene products and other factors required for selenoprotein synthesis. See text for details.

10-fold lower than those of $tRNA^{ser}$ (Baron and Böck, 1991). A major structural determinant distinguishing $tRNA^{sec}$ from $tRNA^{ser}$ is the amino acid acceptor stem which consists of 8 bp in $tRNA^{sec}$ and 7 bp in $tRNA^{ser}$ and all other tRNAs of *E.coli* (Leinfelder *et al.*, 1988b).

Seryl-$tRNA^{sec}$ is converted to selenocysteyl-$tRNA^{sec}$ by a two-step process catalysed by the *sel A* and *sel D* gene products. *sel A* encodes [Se]Cys synthase which is a 600kDa homo-oligomer composed of 50kDa subunits (Forchhammer *et al.*, 1991). [Se]Cys synthase is responsible for the 2,3-elimination of water from the seryl residue giving rise to an aminoacrylyl-$tRNA^{sec}$ intermediate. The addition of H_2Se with the concomitant hydrolysis of one molecule of ATP is catalysed by the *sel D* gene product (figure 1.2) (Forchhammer and Böck, 1991). The *sel B* gene encodes a protein that functions in the translation step and has considerable homology with the elongation factor EF-Tu (Forchhammer *et al.*, 1989, 1990). Both proteins bind GTP and probably serve analogous functions. However, while SEL B binds $tRNA^{sec}$ stoichiometrically, EF-Tu binds with low affinity (Förster *et al.*, 1990). It is therefore thought that SEL B is an elongation factor specific for [Se]Cys incorporation (figure 1.2).

Readthrough of the normally terminating UGA codon also requires structural features of the selenoprotein mRNA (figure 1.2). Deletion experiments with the *fdhF* gene from *E.coli* demonstrated that a sequence of about 40 bases directly downstream of the UGA codon, which could fold into a putative stem-loop structure, was required for efficient [Se]Cys incorporation (Zinoni *et al.*, 1990). Searches of different prokaryotic selenoprotein mRNAs also revealed similar structures in the vicinity of the UGA codon (Zinoni *et al.*, 1990). Point mutational experiments are consistent with the idea that the correct folding of this structure and sequence elements within the loop region are absolutely required and it has been hypothesised that SEL B interacts with this structure to form a quaternary complex at the mRNA together with GTP and selenocysteyl-$tRNA^{sec}$ (figure 1.2) (Heider *et al.*, 1992).

The process of selenoprotein synthesis in eukaryotes is less well understood. However, evidence suggests that the mechanism may be similar to the prokaryotic situation. Eukaryotes contain a seryl-tRNA that recognises the UGA codon and is converted to selenocysteyl-tRNA *in vivo* (Hatfield, 1985; Lee *et al.*, 1989). Additionally, the selenocysteyl $tRNA^{sec}$ is ubiquitous in the animal kingdom (Lee *et al.*, 1990) and the human homologue can complement a lesion in the $tRNA^{sec}$ gene in *E.coli*, despite there being little sequence homology between the two tRNAs, suggesting functional conservation (Baron *et al.*, 1994). Putative stem-loop structures identified in selenoprotein mRNAs are also thought to be required for eukaryotic selenoprotein synthesis (Berry *et al.*, 1991a). Deletion experiments have shown that a 200 nucleotide stretch in the 3' untranslated region of type I tetraiodothyronine deiodinase is essential for [Se]Cys incorporation and that it can be functionally substituted for by the 3' untranslated region of rat GPX mRNA. The ID-I and GPX 3' untranslated regions share little sequence homology suggesting that it is a structural, rather than a sequence determinant of the 3' untranslated region required for [Se]Cys incorporation (Berry *et al.*, 1991a). The fact that these are located in the 3' untranslated region of the mRNA rather than immediately downstream of the UGA codon, as is the case in prokaryotes, could suggest there are fundamental mechanistic differences between pro- and eukaryotic selenoprotein synthesis. However, in both cases it is believed that the stem-loop structure must fold back and interact with the translational machinery to facilitate [Se]Cys incorporation. The different spatial positioning of the stem-loop structures in relation to the UGA codon may therefore be incidental since the size of the stretch of the mRNA looped out is unlikely to be functionally significant.

1.4 Mammalian selenoproteins

In rats, 80% of the body's selenium is in the form of [Se]Cys (Hawkes *et al.*, 1985). Probably therefore, selenium primarily functions through selenoproteins. A number of mammalian selenoproteins have been identified. ID-I

is a selenium-dependent enzyme identified as the enzyme being responsible for the decreased production of T_3 in selenium-deficient animals (Arthur and Beckett, 1989; Arthur *et al.*, 1991). Classification of ID-I as a selenoprotein was confirmed by cloning and sequencing of the ID-I cDNA which was found to contain an in-frame UGA codon (Berry *et al.*, 1991b). Selenoprotein P is located in the plasma and contains a putative 10 [Se]Cys residues as judged by sequencing (Hill *et al.*, 1991). However, amino acid analysis and measurement of selenium content show an average of 7.5 [Se]Cys residues/polypeptide suggesting that some of the in-frame UGA codons may act as termination codons (Read *et al.*, 1990). The function of selenoprotein P is unknown. However, it contains 60% of plasma selenium (Read *et al.*, 1990) and it has been postulated that it may be involved in selenium transport since in selenium-deficient rats, selenoprotein P is rapidly synthesised in preference to other selenoproteins, such as GPX, has a rapid turnover and its synthesis precedes the appearance of selenium in tissues (Motsenbocker and Tappel, 1982; Burk and Gregory, 1982). However, the fact that selenium is covalently incorporated into selenoprotein P and that dietary selenium status does not affect its turnover argues against selenoprotein P being specifically involved in selenium transport (Burk *et al.*, 1991; Read *et al.*, 1990). Alternatively, it has been suggested that selenoprotein P may have an antioxidant function since the appearance of selenoprotein P, but not GPX, correlates with the protective action of selenite against diquat-induced liver necrosis and lipid peroxidation, when injected into selenium-deficient animals (Burk *et al.*, 1980; Burk *et al.*, 1991).

The most extensively characterised selenoproteins are the [Se]Cys-containing glutathione peroxidases. These function to protect against the generation and effects of reactive oxygen species and are related by the fact that they all utilise GSH to reduce hydroperoxides (Combs and Combs, 1986). The family consists of four members, each with subunits ~20KDa in size, that differ in their expression patterns, subcellular localisation and substrate specificity. Three

members, namely GPX, PGPX and GPX-GI, act as homo-tetramers (Epp *et al.*, 1983; Takahashi *et al.*, 1987; Chu *et al.*, 1993) whereas a fourth member, PHGPX, acts as a monomer (Ursini *et al.*, 1985).

GPX, which is cytosolic and ubiquitously expressed, is active against hydrogen peroxide and organic peroxides, such as fatty acid hydrperoxides. It is not, however, active against phospholipid hydroperoxides (Sevanian *et al.*, 1983; van Kuijk *et al.*, 1986).

GPX-GI has similar a substrate specificity to GPX. However, GPX-GI is only 61% homologous to GPX at the nucleotide level and is antigenically distinct from GPX. GPX-GI also has a more restricted tissue distribution than GPX, with levels highest in liver and colon in humans and the gastro-intestinal tract in rodents (Chu *et al.*, 1993).

PGPX is a plasma glycoprotein primarily synthesised in kidney in rodents and kidney, liver, heart lung and breast in humans (Chu *et al.*, 1992; Yoshimura *et al.*, 1991). It is immunologically distinct from GPX (Takahashi *et al.*, 1987) and can reduce hydrogen peroxide and both fatty acid and lipid hydroperoxides (Yamamoto and Takahashi, 1993).

PHGPX is mostly cytosolic but is also bound to some extent to cell membranes. It is ubiquitously expressed and can reduce a wide range of hydroperoxides and is active on cholesterol hydroperoxides as well as many phospholipid hydroperoxides (Maiorino *et al.*, 1990).

1.5 Selenium-binding proteins

SDS/PAGE and 2D gel analysis of proteins from cells labelled *in vivo* and *in vitro* with [75Se]selenium reveal a number of selenium labelled proteins (Danielson and Medina, 1986; Behne *et al.*, 1988). While many of these are probably, as yet, unidentified selenoproteins, some have been molecularly cloned and shown to bind selenium in an as yet unidentified form and not as UGA-encoded [Se]Cys.

A 14KDa liver selenium-associated protein was purified and identified as liver fatty acid binding protein (L-FABP) (Bansal *et al.*, 1989). L-FABP is a member of a family of fatty acid-binding proteins that interact with long chain fatty acids. Their exact function *in vivo* is unknown. However, L-FABP has been associated with hepatocyte growth since the level of immunostained L-FABP is markedly increased during mitosis of all dividing hepatocytes of rat newborn, immature, adult and regenerating livers (Custer and Sorof, 1984, 1985; Bassuk *et al.*, 1987). Additionally, linoleic acid, which is a ligand for L-FABP, requires the overexpression of L-FABP to stimulate the growth of hepatocytes (Keller et al., 1992). The functional significance of selenium-binding is unknown.

Another group of selenium-binding proteins are of molecular weight 56-58kDa. One has been shown to be protein disulphide isomerase (PDI) which has dithiol-disulphide oxidoreductase activity and is involved in assisting protein folding. Interestingly, the increased levels or labelling of PDI correlated with selenite-induced inhibition of DNA synthesis (Morrison *et al.*, 1988; Sinha *et al.*, 1993). However, it is unclear how functionally significant this association is since liver PDI activity is unaffected by selenium-deficiency (Arthur *et al.*, 1991) and in cultured mammary cells PDI activity is independent of selenium concentration in the medium (Sinha *et al.*, 1993). However, under conditions of iodine deficiency liver PDI activity can be increased by selenium supplementation (Arthur *et al.*, 1991).

Another liver selenium-associated protein of molecular weight 56kDa (SP56) has been molecularly cloned by Medina's group (Bansal *et al.*, 1990). The function of SP56 is unknown but it does share a high degree of homology (97%) with an acetominophen-binding protein (AP56) (Lanfear *et al.*, 1993). AP56 was previously identified as a protein that binds acetominphen metabolites during acetominophen-induced hepatoxicity (Pumford *et al.*, 1992; Bartolone *et al.*, 1992). However, it is not known if AP56 binds selenium and what role selenium has for either of these proteins. AP56 has been postulated to be involved in

acetominophen toxicity. However, it is unknown if AP56 is involved in acetominophen detoxification *per se*, or whether it provides some vital function that is targeted by acetominophen, resulting in its inactivation and toxicity. The high degree of homology between SP56 and AP56 suggests that SP56 probably serves a similar function. However, both proteins are differentially regulated with SP56 primarily expressed in liver, kidney and lung and AP56 expressed mainly in liver (Lanfear *et al.*, 1993).

Chapter 2

Selenium: Anti-carcinogenic agent

2.1 Anti-carcinogenic effects of selenium in humans

A number of studies have shown selenium to have anti-carcinogenic properties in humans. Ecological studies indicate an inverse correlation between geographical areas of low selenium intake with the incidence of certain cancers such as oesophageal and primary liver cancer (Jaskiewicz *et al.*, 1988; Yu *et al.*, 1988). In a more comprehensive study, Schrauzer *et al.* (1977) showed that the dietary selenium intakes in 27 countries inversely correlates with cancer mortalities from tumours of colon, rectum, prostate, breast, ovary, lung and leukaemia. Weaker correlations were also noted for pancreas, bladder and skin. One possible limitation of this study is that dietary intakes were calculated from average selenium concentrations of food sources. Since selenium content of food can vary widely depending on the soil levels in which they are grown, putative selenium intake, calculated from average selenium concentrations of food sources, may not necessarily be a reliable reflection of actual selenium intake. However, when blood selenium levels, which are a more accurate indicator of selenium intake, from normal donors from 22 different countries and 19 states were measured and compared, similar conclusions were obtained (Schrauzer *et al.*, 1977). Blood levels are therefore often used to assess selenium intake, although the exact dose-effect relationship is not determined. An alternative measurement of selenium intake is levels of selenium in toenail tissue. This may have several advantages over serum selenium measurements. Serum selenium levels are a short term measure of selenium-intake and may fluctuate over periods of days to weeks (Thompson and Robinson, 1989). In contrast, toenail selenium levels represent the selenium status over several months and so the intra-individual variation in toenail levels for individuals having the same intake may be less than those for serum (Morris *et al.*, 1983).

From the ecological studies it might be expected that selenium levels of cancer patients would be lower than those of healthy individuals. Indeed, in such case-control studies, using both serum and toenail levels as indicators of intake, inverse relationships between selenium levels and cancer has been observed (Broghamer *et al.*,1976; Rogers *et al.*, 1991). However, since serum selenium levels are measured post-diagnosis, the possibility exists that lower selenium levels are due to the disease rather than a cause. Cancer often leads to decreased intake of nutrients and dietary intake levels of certain nutrients may thus be lowered due to a consequence of the disease, Additionally, selenium has been shown to localise and become concentrated in tumours (Cavalieri *et al.*, 1966). A large tumour could therefore have a marked effect on measured selenium intake levels.

The use of prospective studies overcomes any confounding effects cancer may have on blood selenium levels. Such studies measure selenium levels before the diagnosis of cancer and then in follow-up studies during which cancer is diagnosed, any association between selenium levels and the risk of developing cancer can be assessed. Many studies have shown that individuals having low selenium levels have an increased risk of developing cancer. Where specific sites have been analysed, selenium has been shown to be protective against gastro-intestinal, prostate, lung and haematological tumours (Willett *et al.*, 1983; Ringstad *et al.*, 1988; van den Brandt *et al.*, 1993). However, this is not a completely consistent finding and some studies have found selenium levels to have no effect on cancer risk (Nomura *et al.*, 1987). One explanation for these conflicting studies is confounding factors that are difficult to control for, such as interaction of selenium with other nutrients. For example, the anti-carcinogenic effect of selenium is most significant in diets low in vitamin E, C or β carotene (Rogers *et al.*, 1991; van den Brandt *et al.*, 1993; Willett *et al.*, 1983). Therefore in populations consuming high doses of vitamins, the protective effect of selenium may be diminished. The influence of dietary factors may also explain why there does not appear to be an absolute intake of selenium required for a

chemopreventive effect. For example, using serum selenium levels as an indicator of selenium intake, both 5 and 6 year follow-up prospective studies in the U.S.A. and Finland, respectively, found low selenium intakes to be associated with an increased risk of developing gastro-intestinal cancers (Willett *et al.*, 1983; Salonen *et al.*, 1984). However, in the U.S.A. study, the lowest quintile selenium level, which had the highest risk of developing cancer, was actually 2-fold higher than the healthy control levels in the Finland study (Willett *et al.*, 1983; Salonen *et al.*, 1984). Therefore, while the protective effect of selenium may be qualitatively similar between populations, the absolute levels required to give an effect will be dependent on other factors such as diet which will vary between populations. Another confounding factor may be gender since some studies have found the chemopreventive effect of selenium to be greatest in males (Kok *et al.*, 1987). Different cancers may also have different confounding effects. Therefore, in studies where total cancer incidence has been analysed, protective effects against individual cancers may be masked by the occurrence of other cancers which are less sensitive to selenium inhibition. The form of selenium ingested is also likely to be an important confounding factor for the chemopreventive effect and it is unknown what different forms exist in different food sources. It might be argued that toenail or serum selenium levels is a measure of bioavailability. However, it does not necessarily follow that bioavailability correlates with chemopreventive activity.

Taking into consideration the limitations of epidemiological studies, the evidence for a chemopreventive function for selenium is suggestive but is by no means conclusive. A more direct approach to address this question is the use of intervention trials. Linxian County, China, has the world's highest rate of oesophageal/stomach cancer. In a randomised trial involving 29,584 adults, supplements of 1-2 times the U.S. recommended daily amount of vitamin E, β carotene and selenium for a period of 5.25 years were found to significantly decrease mortality rates and that this was mainly due to a lower incidence of

stomach cancer (Blot *et al.*, 1993; Taylor *et al.*, 1994). However, selenium alone was not used and so it is difficult to attribute any significant function to selenium. Another study conducted in Qidong County, which has the second highest rate of primary liver cancer (PLC) in China, used selenium alone in intervention trials. Either inorganic selenium in the form of anhydrous sodium selenite or selenium in the form of selenized yeast was used to supplement either (1) the general population of Qidong County, (2) individuals who were positive for the Hepatitis B virus surface antigen or (3) members of families with a history of high rates of PLC (Yu *et al.*, 1991). Supplementation periods lasted for 5,4 and 2 years respectively. The results showed that both forms of selenium had a significant inhibitory effect on PLC incidence (Yu *et al.*, 1991).

2.2 Anti-carcinogenic effects of selenium in animals

Much evidence for the chemopreventive effects of selenium has come from animal studies which show that dietary selenium at levels 10-20 fold higher than the dietary minimum can inhibit a variety of chemical- and virus-induced cancers without any adverse toxic effects (reviewed in Ip, 1986; Medina, 1986; Vernie, 1984). The carcinogens against which selenium is active include both direct-acting chemicals, such as methylnitrosourea (MNU), and those requiring metabolic activation, such as 7,12-dimethylbenz[*a*]anthracene (DMBA). Generally, selenium is found to reduce tumour incidence, latency and yield, and target organs include skin (Shamberger, 1970), colon (Birt *et al.*, 1982), liver (Griffin and Jacobs, 1977) mammary gland (Welsch *et al.*, 1981), pancreas (Kise *et al.*, 1990) and lung (El-Bayoumy *et al.*, 1993) (table 1.1).

2.3 Mammary tumorigenesis as a model for studying the anti-carcinogenic effects of selenium

Most studies measuring the chemopreventive effects of selenium have administered selenium during the whole experiment and therefore not examined the

Species	Organ	Carcinogen
Rat	Liver	DAB, AAF
Rat	Colon	DMH, MAM, BOP
Rat	Mammary gland	AAF,MNU, DMBA
Mouse	Mammary gland	MMTV, DMBA
Mouse	Skin	DMBA
Hamster	Pancreas	BOP
Mouse	Lung	NNK

Table 1.1
Models of chemical-induced carcinogenesis in which selenium supplementation has been shown to have an inhibitory effect (adapted from Medina, (1986) and El-Bayoumy *et al.* (1993)).

Abbreviations:
DAB, 3-methylaminoazobenzene; AAF, 2-acetylaminofluorine; DMH, dimethylhydrazine; MAM, methylazoxymethanol acetate; AOM, azoxymethane; BOP, bis(2-oxopropyl)-nitrosamine; MNU, methylnitrosourea; DMBA, 7,12-dimethylbenz[a]anthracene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

stages of neoplastic progression at which selenium is most active. In theory, because of the multistage nature of carcinogenesis, selenium could act at a number of stages by a variety of mechanisms. Initiation may be inhibited by selenium preventing mutations induced by carcinogens. Alternatively, selenium could prevent the outgrowth of initiated cells or may directly inhibit tumour cell growth. Due to its multi-step nature, mouse mammary tumorigenesis has been a useful system in studying the stage(s) of carcinogenesis at which selenium is most effective.

2.3.1 The biology of mouse mammary tumorigenesis

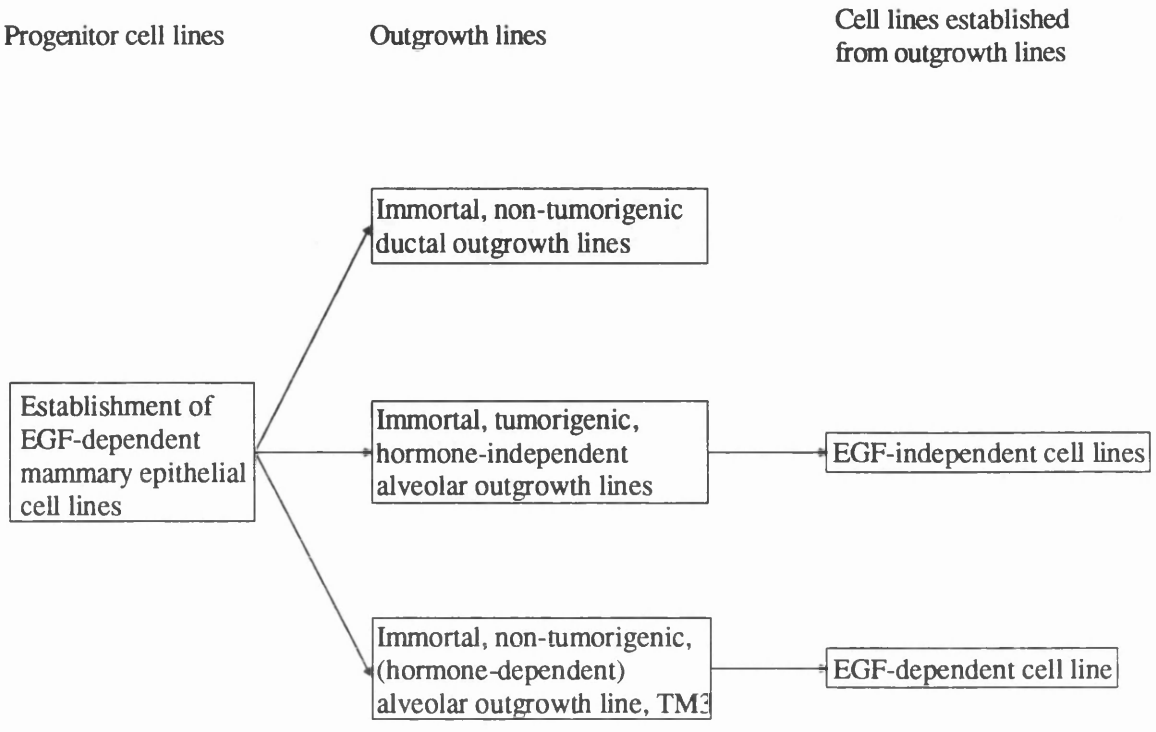
Mouse mammary tumours develop through a number of characteristic preneoplastic states (DeOme *et al.*, 1959, 1978). Preneoplastic lesions arise spontaneously at a low frequency (Medina and Kittrell, 1987) but this can be increased by chemical carcinogens (Medina and Warner, 1976; Medina, 1976), hormone treatment (Medina, 1973; Lanari *et al.*, 1986) and mouse mammary tumour virus (MMTV) (DeOme *et al.*, 1959). The study of these preneoplastic lesions has been greatly facilitated by the ability of mammary tissue to be transplanted into syngeneic mice. This involves "clearing" the mammary fat pad by surgically removing the host mammary epithelium. The cleared mammary fat pad then acts as an *in vivo* culture system for the transplantation of foreign isogenic mammary epithelial tissue. The two most commonly found preneoplastic lesions are hyperplastic alveolar nodules (HAN) and ductal hyperplasias (DH). HAN and DH are believed to be precursors to a neoplastic state since they both develop into adenocarcinomas when transplanted into the cleared mammary fat pad of syngeneic mice at a higher frequency than normal mammary tissue (DeOme *et al.*, 1959; Medina, 1973). The frequency of HAN and DH development is highly dependent on the strain of mouse and inducing agent. Of the two preneoplastic lesions, most studies have been conducted with HAN. In addition to the increased tumorigenic potential of HAN, there are a number of other features that distinguish the HAN

preneoplastic state from normal mammary tissue. HAN are immortal and can be serially transplanted indefinitely into cleared mammary fat pads of syngeneic mice. Some of these transplants, termed hyperplastic outgrowth (HOG) lines, have been serially transplanted for over 20 years (Medina, 1988). In contrast, normal mammary cells can only be transplanted for 5-7 generations after which they lose their growth potential (Daniel *et al.*, 1975). Morphologically, HOG lines derived from primary HAN resemble differentiated alveolar cells of the pregnant mammary gland. However, this differentiated state is not ovarian hormone-dependent since HOG lines retain their alveolar morphology when transplanted into virgin mice (Medina, 1988). HAN-derived HOG lines are therefore generally characterised as a population of immortal cells with a differentiated alveolar morphology with a higher than normal potential to form tumours.

HOG lines differ from neoplastic cells in that they are still responsive to growth controls that regulate normal mammary cells. For example, HOG lines are dependent on fat pad stroma and do not overgrow uncleared normal mammary pads (DeOme *et al.*, 1959). Furthermore, normal mammary cells when transplanted with preneoplastic alveolar nodule cells significantly inhibit the tumorigenic potential of the alveolar cells (Medina *et al.*, 1978). HOG lines therefore require additional alterations in order to escape this growth regulation and become neoplastic. This transition occurs spontaneously to varying degrees for different HOG lines but can be induced by treatment with various carcinogens or MMTV (Medina, 1973; Medina *et al.*, 1978).

The development of HAN and DH from normal mammary cells is unlikely to involve a single genetic event. Indeed, a number of studies have demonstrated that the development of preneoplastic lesions involves at least four dissociable events. A series of cell lines derived from normal mammary epithelial tissue gave rise to both ductal and alveolar outgrowth lines (figure 1.3A) (Kittrell *et al.*, 1992). Transplantation assays revealed that the ductal outgrowth lines and one alveolar outgrowth line (TM3) were immortal but did not go on to develop tumours

A



B

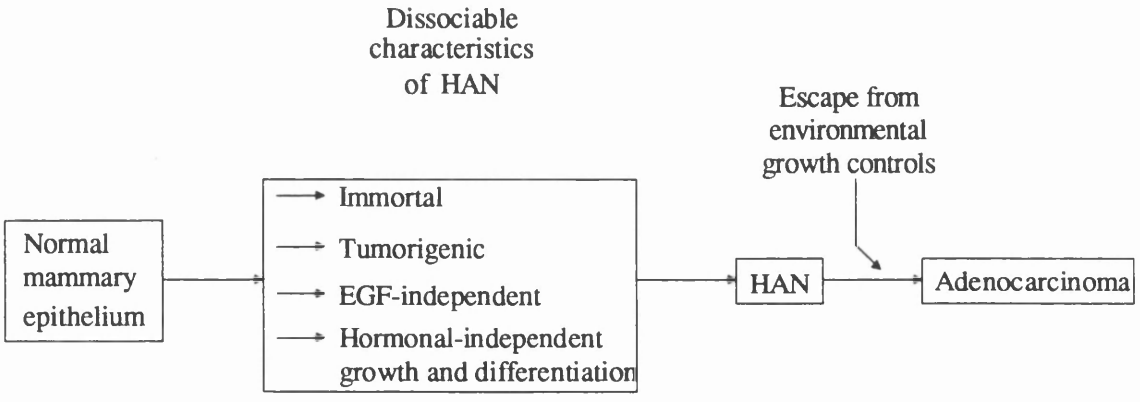


Figure 1.3
(A) Summary of charcteristics of mammary epithelial cell lines and outgrowth lines as derived by Medina and Kittrell (1993) and Medina *et al.* (1993a,b).

(B) Biological states of mouse mammary tumour progression. Based on data from Medina (1988), Medina and Kittrell (1993) and Medina *et al.* (1993a,b).

(Medina and Kittrell, 1993; Medina *et al.*, 1993a). This indicates that immortality and tumorigenicity arise from at least two separate genetic alterations. The converse situation has also been demonstrated, whereby tumorigenic alveolar hyperplasias have been generated that have finite life-spans (Miyamoto *et al.*, 1990). This suggests that the phenotypic features of preneoplasia may arise independently and do not need to occur in any particular chronological order during mammary tumour progression. The outgrowth line, TM3, also differed in other respects from the other alveolar outgrowth lines, which were typically preneoplastic (Medina *et al.*, 1993a). Although TM3 outgrowth was morphologically alveolar in non-pregnant female mice, this phenotype was hormone-dependent to a certain degree since when TM3 cells were transplanted into ovarectomized mice, both growth and alveolar differentiation were markedly diminished (Medina *et al.*, 1993a). In contrast, growth and differentiation of the other alveolar outgrowth lines was unaffected by ovarectomy (figure 1.3A) (Medina *et al.*, 1993a). Epidermal growth factor (EGF) independence also appeared to be a characteristic associated with preneoplastic development. EGF-dependence for growth was displayed by the progenitor cell lines that gave rise to the outgrowth lines (figure 1.3A) (Kittrell *et al.*, 1992). However, cell lines established from the outgrowth lines, displayed a diminished dependence for EGF in comparison to their respective progenitor cell lines (figure 1.3A) (Medina *et al.*, 1993b). In contrast, the TM3 progenitor cell line and TM3 cell line established from the TM3 outgrowth line displayed an equal dependence for EGF (figure 1.3A) (Medina *et al.*, 1993b). The characterisation of preneoplasic lines has enabled mammary tumorigenesis to be divided into discrete identifiable stages (figure 1.3B)

2.3.2 Stage-specific effects of selenium

Mouse mammary tumours that develop through characteristic preneoplastic states can be induced by the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA)

(Medina, 1978). The reproducibility of this model has been used to assess the efficacy of selenium to inhibit the three definable stages of mammary tumour development: preneoplasia development, progression of preneoplasia to a neoplasic state and growth of the neoplastic state. A number of studies indicate that selenium is most effective during the early stages of mammary tumorigenesis, that is initiation and preneoplasia development. Medina and Shepherd (1981) demonstrated that the formation of DMBA-induced preneplastic lesions is significantly inhibited by selenium supplementation. Additionally, selenium had an inhibitory effect on tumour yield if given either only during DMBA administration or after DMBA treatment. This indicates that selenium is effective at both initiation and promotion stages. Later stages of tumour progression appear less sensitve to the inhibitory effects of selenium since mammary tumour development from only 5 of 14 preneoplastic outgrowth lines was inhibited (Medina and Lane, 1983). In contrast, the growth of mammary tumour cells appears relatively refractory to growth inhibition by selenium since only 3 of 36 mammary tumours transplanted into syngeneic mice were found to be inhibited by selenium (Medina and Lane, 1983). The ability of selenium to inhibit the early stages of tumorigenesis has also been demonstrated in organ culture. Treatment with DMBA of whole mammary gland, cultured *in vitro*, results in the appearance of nodule-like alveolar lesions (NLAL) which can be identified microscopically (Kundu *et al.*, 1978). These lesions are believed to be analogous to the *in vivo* ocurring HAN since NLAL retain their alveolar morphology when serially transplanted into virgin mice and have an increased tumour-producing capability (Telang *et al.*, 1979). Selenium in the culture medium during or after DMBA administration was found to inhibit both the yield and incidence of NLAL formation suggesting that both initiation and promotional stages of mammary tumorigenesis are sensitive to selenium inhibition (Chatterjee and Banerjee, 1982).

Similar stage-specific effects of selenium have also been demonstrated in DMBA-induced mammary tumours in the rat. Rat mammary tumours are thought

to develop in a similar manner to mouse mammary tumours. Ductal and alveolar lesions that resemble those of the mouse can be induced by DMBA (Beuving *et al.*, 1967a). Additionally, both types of lesions retain their respective morphological structure when transplanted into cleared mammary fat pads and develop into tumours (Beuving *et al.*, 1967b; Beuving, 1968). Ip (1981) showed by giving rats restricted periods of selenium supplementation, that selenium was effective when administered during the 2 weeks before and after DMBA treatment, or when given continuously for 2-24 weeks after DMBA treatment. The inhibitory effect of selenium when given during tumour promotion, that is after DMBA treatment, was reversible since selenium given for a shorter period of 2-12 weeks after DMBA treatment resulted in a similar tumour yield as unsupplemented control groups. In contrast to the effect on tumour yield of selenium given during or early after DMBA treatment, selenium given late (12-24 weeks) after carcinogen treatment had little effect. The maximum protective effect of selenium was observed when selenium was given continuously for the duration of the experiment starting 2 weeks prior to the administration of DMBA (Ip, 1981). The efficacy of selenium to inhibit various stages of DMBA-induced mammary tumorigenesis can thus be summarised (figure 1.4).

While it is apparent that selenium can act in a stage-specific manner it is not clear if this applies to all tumour models. In different tumour models tumour progression occurs by a number of different pathways involving the activation and inactivation of different oncogenes and tumour suppressor genes, respectively. One of the genetic events associated with DMBA-induced mammary tumours is an A-T transversion at codon 61 of the H-*ras* gene (Mangues and Pellicer, 1992). However, if DMBA-induced HAN are serially transplanted as outgrowth lines, the resulting tumours contain the wild-type codon 61 of H-*ras* (Kumar *et al.*, 1990). This implies that DMBA-induced mammary tumours can arise from at least two different mechanisms, with only one pathway requiring activation of H-*ras*. Therefore, while selenium is most effective at inhibiting early events of

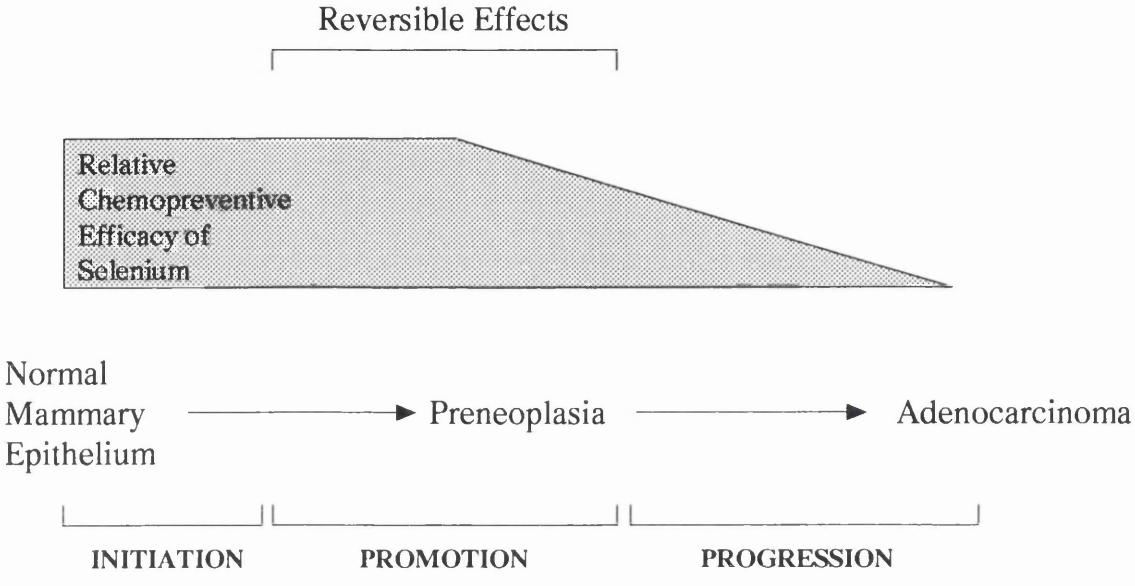


Figure 1.4
Schematic representation showing the relative chemopreventive activity of selenium when administered during different stages of DMBA-induced mammary tumour development. Adapted from Medina (1986).

DMBA-induced mammary tumours, this may not apply to all tumours that have arisen through different pathways. For example, triphenylselenonium chloride is effective at inhibiting both initiation and post-initiation events of DMBA-induced mammary tumorigenesis but is only effective during the post-initiation phase of methylnitrosourea-induced mammary tumours (Ip *et al.*, 1994a). Additionally, later stages of tumorigenesis may be sensitive to selenium in other tumour models since selenium has been shown to inhibit the *in vivo* growth of a number of fully malignant tumour cells including human mammary MCF-7 tumour cells (Watrach *et al.*, 1984), transplantable rat mammary tumour MT-W9B (Ip *et al.*, 1981), canine mammary carcinoma cells (Watrach *et al.*, 1982), Ehrlich ascites cells (Greeder and Milner, 1980) and L1210 leukaemia cells (Milner and Hsu, 1981).

2.3.3 The anti-carcinogenic effects of selenium is dependent on chemical form

The biological activity of selenium is highly dependent on chemical form. For example, while levels of four or more parts per million (ppm) of selenium in the diet in the form of selenite causes a depression in growth rate of rats, suggestive of a toxic effect, triphenylselenonium may be tolerated up to levels of 200ppm without any adverse effects (Ip *et al.*, 1994a, 1994b).

Regarding the chemopreventive effects of selenium, a number of chemical forms, such as 6-phenyl (6H)-isoselenazolo [4,3-d] pyrimidone, *p*-methoxybenzeneselenol and 1,4-Phenylenebis(methylene)selenocyanate (*p*-XSC) have been shown to be effective at inhibiting the growth of leukaemia cells and reducing the yield of chemical-induced stomach and colon tumours, respectively (Ito *et al.*, 1990; El-Bayoumy, 1985; Reddy *et al.*, 1992). *p*-XSC has also been shown to inhibit chemically-induced mouse lung tumours (El-Bayoumy *et al.*, 1993). The relative chemopreventive activities of different selenium compounds has been most extensively studied using the DMBA-induced mammary tumour model. Selenite is one of the most commonly tested forms and is generally one of the more active compounds, being more effective than [Se]Met (Thompson *et al.*,

1984; Ip and Hayes, 1989). This is probably, in part, due to the non-specific substitution of seleno-amino acids for their sulphur analogues in sulphur metabolism since under conditions of limiting dietary methionine, the chemopreventive effect of [Se]Met was diminished whereas selenite was not (Ip, 1988). This suggests that [Se]Met can be diverted into methionine metabolism and therefore reduce the quantity of selenium available for anti-carcinogenic purposes. Consistent with this, tissue selenium levels increased under methionine limiting conditions in [Se]Met-supplemented rats (Ip, 1988). The absolute tissue selenium levels are higher in [Se]Met-supplemented rats than selenite-supplemented rats at equal dietary leves of selenium (Ip and Hayes, 1989). This indicates that while there is a general dose-dependent effect between dietary and tissue selenium levels, absolute tissue selenium levels may be a poor indicator of chemopreventive efficacy. Indeed, triphenylselenonium chloride administered in the diet at 30 ppm reduced the yield of DMBA-induced mammary tumours by 69% but did not significantly affect tissue selenium levels (Ip *et al.*, 1994a). A poor correlation between tissue selenium levels and chemopreventive efficacy was also observed for selenium-enriched garlic and onion and brazil nut which is a naturally selenium-rich food (Ip and Lisk, 1994a, 1994b). It is therefore clear that the chemopreventive action of selenium is not due to the element *per se* but rather is highly dependent on its chemical form implying that selenium metabolism is important. However, it is unclear what these critical metabolic pathways and chemical forms are.

Using the DMBA-induced mammary tumour model, a series of experiments, set out to identify metabolites of selenium important for its chemopreventive effect, compared the anti-carcinogenic effects of inorganic selenite with a number of synthetic compounds that enter the organic selenium pool after selenite reduction to H_2Se. These included Se-methylselenocysteine (SMSC), selenobetaine (SB), selenobetaine methyl ester (SBME), dimethyl selenoxide (DMS) and trimethyl selenonium ion (TMS^+) which is the product from the final methylation step in selenium detoxification. Based on metabolic studies

using double-labelled ^{14}C and ^{75}Se seleno-compounds and analysis of excretory products, SMSC and SB form monomethyl selenol and SBME and DMS feed into dimethyl selenide (Foster *et al.*, 1986a, 1986b; Vadhanavikit *et al.*, 1993) (figure 1.5). The relative chemopreventive efficiencies for the various compounds are as follows (Ip and Ganther, 1988, 1990; Ip *et al.*, 1991):

$$SB \cong SBME \cong SMSC \geq Selenite > DMS > TMS^{+}$$

The chemopreventive activities of these various compounds could be markedly affected when co-administered with arsenite. Interestingly, the effect of selenite was found to be diminished by arsenite whereas the effect of SB, SBME, SMSC and TMS^{+} were enhanced (Ip and Ganther, 1988, 1990; Ip *et al.*, 1991). This observation, together with the fact that (1) selenite enters selenium metabolism prior to H_2Se formation whereas the other compounds enter selenium metabolism after H_2Se methylation and (2) in cell-free systems, H_2Se methylation is sensitive to inhibition by arsenite (Hsieh and Ganther, 1977), suggested that a methylated selenium compound may be responsible for the chemopreventive effect of selenium (figure 1.5). Analysis by high pressure liquid chromatography of selenium excretory products resulting from oral feeding of all six compounds at chemopreventive doses revealed that the mono-, di- and tri-methylated compounds resulted in higher levels of excreted methylated metabolites compared to selenite feeding and that this reflected their expected entry point into intermediary selenium metabolism (figure 1.5) (Vadhanavikit *et al.*, 1993). However, there was no clear correlation between the levels or form of excreted methylated metabolites and the chemopreventive efficacy of the various compounds added to the diet. Moreover, arsenite had no significant effect on the profile of excreted methylated metabolites for any of the seleno-compounds (Vadhanavikit *et al.*, 1993). Thus, it is presently unclear why arsenite has differential effects on the chemopreventive activity of selenite and other methylated seleno-compounds. However, the data presently

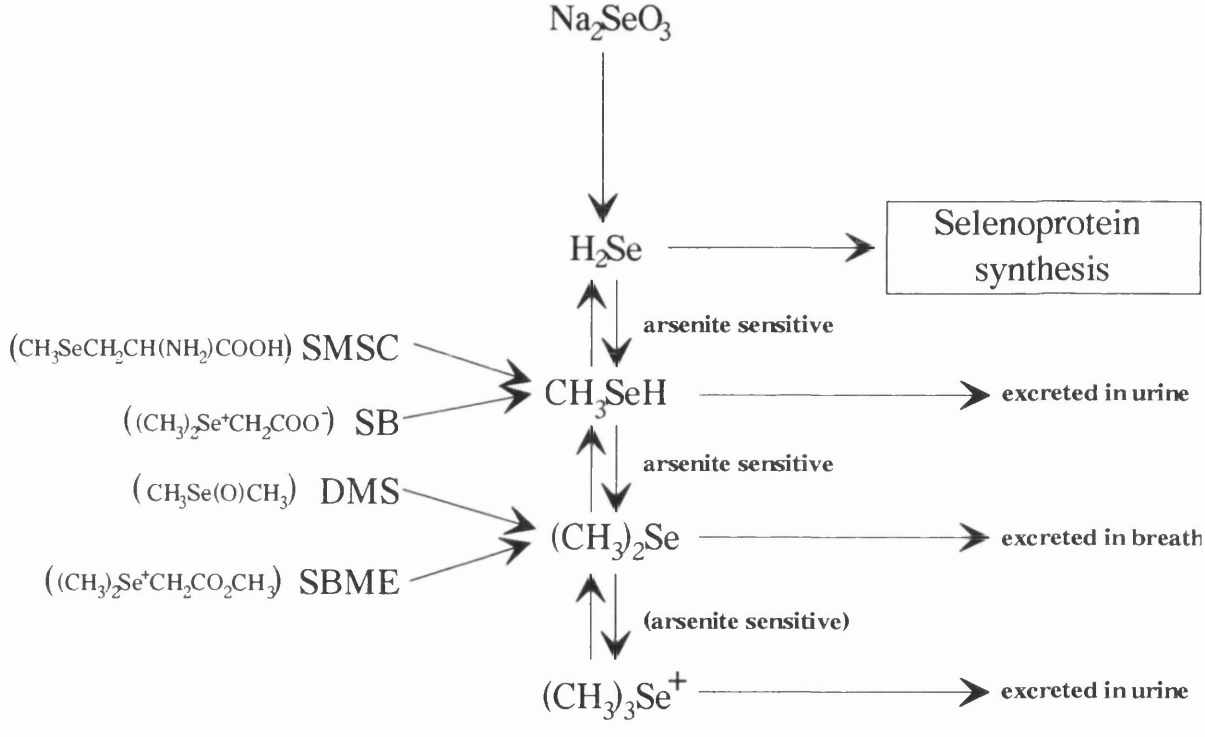


Figure 1.5
Synthetic methylated selenium compounds and their point of entry into intermediary selenium metabolism.

available suggests that arsenite does not alter selenium metabolism but could directly interact with seleno-metabolites.

Another important factor determining the efficacy of selenium to act as an anti-carcinogen in the DMBA-induced mammary model appears to be the bioavailability of the selenium atom for incorporation into selenoproteins such as GPX. In GPX activity repletion assays, in which selenium compounds are tested for their ability to restore GPX activity in selenium deficient animals, the selenium in active chemopreventive compounds such as selenite, TMS^+, SMSC, DMS, Brazil nut-derived selenium and *p*-XSC can be incorporated into GPX (Ip *et al.*, 1991; Reddy *et al.*, 1992; Ip and Lisk, 1994b; Ip and Ganther, 1992). In contrast, the selenium in ebselen (2-phenyl-1,2-benzisoselenazol-3-(2H)-one), which has no anti-carcinogenic activity, is metabolically inert and thus not available for incorporation into GPX (Ip *et al.*, 1991). This could imply that selenium is acting through a selenoprotein. However, as is the case with tissue selenium levels, the extent to which a seleno-compound can restore GPX activity is not a good indicator of chemopreventive efficacy (Ip *et al.*, 1991, see also section 2.4.2). Selenium may need to act through a seleno-compound which is generated from the metabolically available selenium pool which would imply that it is essential that selenium enter intermediary metabolic pathways to have a chemopreventive effect. Biosynthesis of selenoproteins may therefore just be an indicator of the bioavailability of selenium in a particular compound but not necessarily be of any functional significance with respect to chemopreventive activity.

The chemopreventive efficacy of selenium is clearly dependent on a number of factors but exactly what these are is still unclear. However, the point of entry of a particular selenium compound into intermediary metabolism and to some extent nutritional bioavailability are evidently important constraints on the chemopreventive effects of selenium. Furthermore, these factors may differ for different tumour models. For example, selenium administered as selenite and *p*-XSC at a dietary level of 2.5 and 5ppm, respectively, is equally effective at

inhibiting DMBA-induced mammary tumours (Ip *et al.*, 1994b). However, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced mouse lung tumours are refractory to the effect of 5ppm selenium administered as selenite but are sensitive to 5ppm selenium administered as *p*-XSC (El-Bayoumy *et al.*, 1993). This suggests that different selenium compounds may be active against different pathways of tumour progression.

2.4 Anticarcinogenic effects of selenium: Possible mechanisms

The mechanism of selenium-induced inhibition of carcinogenesis is unknown. Any hypothesis must take into account the ability of selenium to inhibit tumours at a number of different sites, that selenium can inhibit tumours induced by a wide range of chemical and viral agents that have different modes of action and that selenium is active at both initiation and promotion, two mechanistically distinct stages of carcinogenesis. The most likely explanation is that selenium inhibits tumorigenesis by a number of mechanisms. A number of postulated mechanisms as to how selenium inhibits tumorigenesis are briefly covered in the following sections.

2.4.1 Alteration of carcinogen metabolism

The ability of selenium to act during the initiation stage of a number of chemical-induced tumours suggests that selenium somehow inhibits mutagenic events. Indeed, in micro-organisms, selenium has been demonstrated to be protective against both spontaneous (Rosin, 1981) and chemical-induced mutations caused by a variety of agents such as malondialdehyde (MDA), β-propiolactone (Shamberger *et al.*, 1979), acetylamino fluorene (AAF) (Jacobs *et al.*, 1977), acridine orange, DMBA (Martin *et al.*, 1981) and N-alkyl-N'-nitro-N-nitrosoguanidine (MNNG) (Rosin and Stich, 1979).

Carcinogen metabolism, involving activation and detoxification reactions, is critical in modifying the mutagenicity of certain carcinogens. Carcinogen

metabolism involves two classes of enzymes, termed phase I and phase II enzymes. Phase I enzymes, which include the microsomal monooxygenase system and P-450 cytochrome oxidases, introduce polar groups onto xenobiotics, such as drugs, poisons and carcinogens (Guengerich, 1988). The reactions are on the whole oxidations but also include some reductions (Guengerich, 1991). While these reactions are largely protective for the cell by facilitating detoxification of the xenobiotic, many pro-carcinogens are activated by phase I enzymes (Guengerich, 1988). The ultimate carcinogenic forms are positively charged electrophilic species. The electrophilic nature of active carcinogens, either those that act directly or those activated by phase I enzymes, can induce phase II enzymes, which include glucuronyltransferase, NADP(H):quinone reductase and a family of glutathione *S*-transferases (GSTs) (Prochaska and Talalay, 1988). Phase II reactions involve conjugations resulting in the formation of glucuronides, GSH conjugates and sulphates which are excreted (Wattenberg, 1985).

Many chemopreventive compounds are thought to act by modifying levels of phase I and phase II enzymes and thereby inhibit activation or increase detoxification of carcinogens (Wattenberg, 1983, 1992; Zhang and Talalay, 1994; Yang *et al.*, 1994). Evidence suggests that the chemopreventive effects of selenium could involve similar mechanisms. For example, the activity of the phase I enzyme, aryl hydrocarbon hydroxylase, can be inhibited by selenium in cultures of human lymphocytes (Rasco *et al.*, 1977). Additionally, when rats were fed the liver carcinogen, *N*-hydroxy-2-acetylaminofluorene (NOHAAF), selenium supplementation was shown to increase the hepatic activities of the phase II enzyme, glucuronyltransferase and inhibit the activity of *p*-nitrophenyl-sulphotransferase, the enzyme responsible for producing the ultimate carcinogenic metabolite of NOHAAF (Daoud and Griffin, 1978; DeBaun *et al.*, 1970). This shift in NOHAAF metabolism favouring detoxification was reflected by a decrease in the amount of hepatic NOHAAF and reduction in the amount of liver DNA-bound carcinogen (Daoud and Griffin, 1978). The effect on

NOHAAF-DNA binding appeared quite specific since AAF-DNA adduct formation was unaffected by selenium supplementation (Daoud and Griffin, 1978).

Other indirect evidence for the effect of selenium on carcinogen metabolism has been shown for 1,2-dimethylhydrazine (DMH). Selenium supplemented rats fed [^{14}C]DMH, were found to exhale larger amounts of [^{14}C]azomethane, a pro-carcinogenic metabolite of DMH, than control rats on low selenium diets (Harbach and Swenberg, 1981). Again, this was reflected in a lower amount of liver DNA alkylation products (Harbach and Swenberg, 1981).

The formation of DMBA-DNA adducts in mammary tissue is also reduced in selenium supplemented rats (Ip *et al.*, 1995; Liu *et al.*, 1991). Additionally, DMBA-DNA adduct formation is reduced in mammary epithelial cells cultured in supplemental selenium or in mammary epithelial cells derived from rats fed high selenium diets as compared to the unsupplemented control cells and rats, respectively (Ejadi *et al.*, 1989). This suggests that increased carcinogen detoxification may occur in the target organ and not necessarily require systemic detoxification by, say, the liver.

Inhibition of mutagenic events may also be achieved by increased repair of mutagenic lesions. In rats fed AAF, hepatic AAF-DNA adduct formation was unaffected by supplemental selenium in the diet, in agreement with the findings of Daoud and Griffin (1978), suggesting that AAF metabolism was unaltered (Wortzman *et al.*, 1980). However, AAF was found to induce single strand DNA breaks. This occurred to a greater degree in rats on a low selenium diet as compared to rats on a selenium supplemented diet suggesting that although AAF metabolism was unaltered, AAF-induced single strand DNA breaks were repaired more efficiently in selenium supplemented rats (Wortzman *et al.*, 1980).

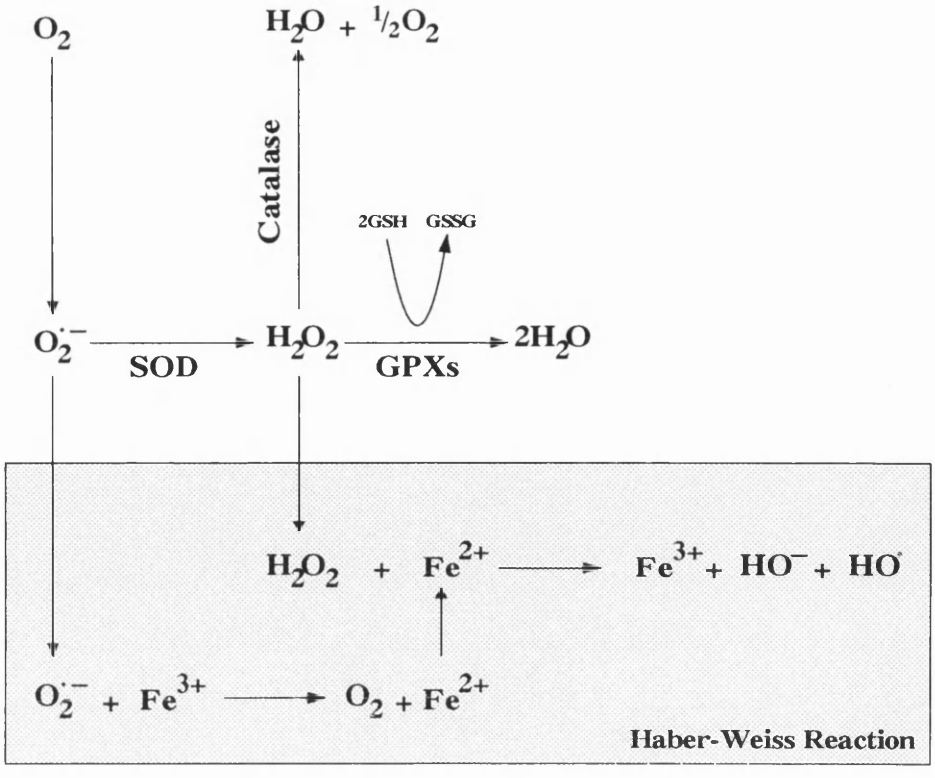
2.4.2 Inhibition of oxidative damage by production of glutathione peroxidase

Cells are constantly exposed to reactive oxygen species and their harmful effects. Activated oxygen species may arise endogenously as a result of oxidative

phosphorylation. Although this is a very efficient process, ~2% of the oxygen becomes uncoupled from the mitochondrial respiratory chain resulting in partially reduced oxygen (Chance *et al.*, 1979). Other endogenous sources include peroxisomes (Kassai *et al.*, 1989) and cytosolic oxidases (Kehrer and Smith, 1994). The most common activated oxygen species are superoxide anion and hydrogen peroxide. These can further react together with a metal catalyst, such as ferric or cupric ions, to generate the highly reactive hydroxyl radical by the Haber-Weiss reaction (figure 1.6). The consequence of reactive oxygen species generation is oxidative damage to DNA and proteins (Frei, 1994). Additionally, reactive oxygen species may cause the auto-oxidation of polyunsaturated fatty acids, which is termed lipid peroxidation (Logani and Davies, 1980). This type of oxidation is initiated by allylic hydrogen abstraction followed by oxygen attack on the carbon radical thus generated. The lipid peroxyl radical can then react with an adjacent polyusaturated fatty acid side chain to form another lipid radical (figure 1.6). The self-propagating nature of lipid peroxidation means that, uncontrolled, lipid peroxidation can be very detrimental to the cell resulting in the production of toxic by-products and loss of membrane conformation and structural integrity (Kappus and Sies, 1981).

There is strong evidence for the role of oxygen free radicals in carcinogenesis (Sun, 1990). Indeed, many carcinogens participate in redox cycling which generate reactive oxygen species (ROS) (Kappus and Sies, 1981). Cells have thus evolved a battery of antioxidant enzymes to protect against the generation and effects of ROS. Superoxide dismutases dismute superoxide to hydrogen peroxide and catalase reduces hydrogen peroxide to water (figure 1.6) (Frei, 1994). The family of GPXs also reduce hydrogen peroxide, in addition to various organic and lipid hydroperoxides (figure 1.6). The discovery that selenium formed an integral part of GPX (Rotruck *et al.*, 1973) thus provided a possible mechanism for the chemopreventive activity of selenium. According to this hypothesis, selenium could act in an antioxidant manner by increasing the levels of

A



B

$RH + O_2 \longrightarrow R^{\cdot} + HO^{\cdot}$

$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$

$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$

$R^{\cdot} + R^{\cdot} \longrightarrow RR$

$R^{\cdot} + ROO^{\cdot} \longrightarrow ROOR$

$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$

Figure 1.6
(A) Schematic representation of some of the reactions leading to the production of reactive oxygen species and their subsequent detoxification by antioxidant enzymes

(B) Lipid peroxidation: RH represents a fat molecule in which H is an allylic hydrogen

(see text for more details)

GPX and thus reduce the levels of peroxides and subsequent DNA damage and lipid peroxidation which is also believed to play a role in carcinogenesis (Cerutti, 1985). Evidence suggested that the chemopreventive activity of selenium could be mediated through an antioxidant function. The incidence of DMBA-induced mammary tumours in rats increases under conditions of selenium-deficiency (Ip and Sinha, 1981a). However, this augmentation is only apparent in animals on a high polyunsaturated diet and not in rats on a low polyunsaturated fat or high saturated fat diet suggesting that the tumour-enhancing effect of selenium-deficiency may involve increased lipid peroxidation (Ip and Sinha, 1981a). Additionally, vitamin E, with known antioxidant functions, did not have an inhibitory effect on DMBA-induced mammary tumours when administered alone but could synergize and enhance the anti-carcinogenic effect of selenite, further suggesting an antioxidant role in the chemopreventive effect of selenium (Horvath and Ip, 1983). However, the chemoprotective effect of supra-nutritional levels of selenium-supplementation in the DMBA-induced mammary tumour model is independent of the level and type of fat in the diet with selenium having a protective effect in rats fed low or high polyunsaturated fat and high saturated fat diets (Ip and Sinha, 1981b). Additionally, the levels of malondialdehyde (MDA), a marker of lipid peroxidation, was unaffected by selenium-supplementation, suggesting that selenium does not act by reducing lipid peroxidation (Ip and Sinha, 1981b). Lane and Medina (1983) also demonstrated that mammary and liver GPX was maximally active at dietary levels of selenium that are ~20-fold lower than the minimum dose required for a chemopreventive effect in DMBA-induced mammary tumours. Additionally, as mentioned above, although GPX activity is generally dependent on dietary levels of selenium, the extent to which different anti-carcinogenic selenium compounds can restore GPX activity in selenium-deficient animals is not necessarily a reliable indicator of chemopreventive efficacy in the DMBA-induced mammary tumour model (Ip *et al.*, 1991). These findings suggest that selenium does not act through the activation of GPX and

subsequent reduction in lipid peroxidation in the DMBA-induced mammary tumour model. However, the role of other GPX family members in the anti-carcinogenic effects of selenium is relatively unexplored. For example, PHGPX, which is ubiquitously expressed, has a broader substrate specificity than GPX and therefore may detoxify hydroperoxides critical in carcinogenesis that are not substrates for GPX. This would require that selenium be differentially channelled into the synthesis of these two enzymes. Indeed, in selenium-deficient animals, PHGPX and GPX activities are differentially maintained suggesting that the supply of selenium to these two enzymes is different (Weitzel *et al.*, 1990).

Nevertheless, GPX activity could play a role in different, non-mammary, tumour models. For example, while GPX activity in the liver and mammary tissue is saturated at 0.1 ppm selenite in the diet (Lane and Medina, 1983), colon GPX activity in azoxymethane-treated rats could be further increased (~3-fold) by supplementation with 20ppm *p*-XSC in the diet (Reddy *et al.*, 1992). This correlated with a 21% inhibition in the incidence of azoxymethane-induced colon tumours (Reddy *et al.*, 1992).

2.4.3 Inhibition of cell proliferation

While selenium may be able to inhibit the mutagenic events of tumour initiation, the ability of selenium to inhibit post-initiation stages of tumorigenesis suggests that selenium somehow inhibits the growth of initiated tumour cells. This could be achieved systemically by, say, stimulation of the immune system which has been reported for selenium supplementation (Kiremidjian-Schumacher and Stotzky, 1987). However, selenium can inhibit the formation of nodule-like alveolar lesions in mammary gland organ culture (Chatterjee and Banerjee, 1982) and inhibit UV-induced skin lesions and tumours when applied topically (Burke *et al.*, 1982) suggesting that selenium may act directly on cells.

In vitro, selenium is also an essential trace element and is required for the maximum growth of cells (McKeehan *et al.*, 1976; Medina and Oborn, 1981).

However, at higher concentrations, selenium is a potent inhibitor of cell growth and, similar to the *in vivo* situation, the growth suppressing effect is highly dependent on chemical form (Fico *et al.*, 1986; Yan *et al.*, 1991). *In vitro*, the most commonly used form is selenite, which is both anti-carcinogenic and anti-proliferative. Intracellularly, selenite is reduced by GSH to H_2Se (Hsieh and Ganther, 1977) (Figure 1.1). It is thought that for selenite to exert it's effect it must undergo this metabolic reduction since the simultaneous addition of GSH can enhance the growth suppressing effect of selenite (Yan et al., 1991; Kuchan and Miller, 1991). Furthermore, SDG, the primary product from the reaction between selenite and GSH (Figure 1.1) is a potent inhibitor of cell growth and is more effective than selenite (Fico *et al.*, 1986).

The mechanism by which selenite acts as an anti-proliferative agent is unclear. Selenite has been shown to have a number of biological effects such as inhibiting protein kinase C (Su *et al.*, 1986), which is believed to be an effector of various tumour promotors such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Additionally, *c-myc*, which is thought to regulate a number of genes involved in cell proliferation, including ornithine decarboxylase (Bello-Fernandez *et al.*, 1993) and α-prothymosin (Gaubatz *et al.*, 1994), is down regulated by selenite both *in vitro* and *in vivo* (Yu *et al.*, 1990).

Selenium is also an inhibitor of DNA, RNA and protein synthesis (Gruenwedel and Cruikshank, 1979) and has been shown to inhibit the activity of a number of enzymes involved in all three processes. Thymidine kinase activity can be inhibited in cultured cells and in cell-extracts selenium has been shown to inhibit the activity of DNA methylase, DNA polymerase α and RNA polymerase II (Tillotson *et al.*, 1994; Cox and Goorha, 1986; Frenkel *et al.*, 1987). Additionally, in cell-free assays, selenite and SDG are potent oxidants of the thioredoxin system (Björnstedt *et al.*, 1992; Kumar *et al.*, 1992) which acts as a hydrogen donor for ribonucleotide reductase (Holmgren 1985). SDG is also a potent inhibitor of protein synthesis in cell-free systems and in intact cells (Vernie *et al.*, 1983) and it

has been shown to inhibit mammalian elongation factor 2 (Vernie *et al.*, 1975) and initiation factor 2 (Safer *et al.*, 1980). While it is apparent that selenium can directly inhibit the synthesis of DNA, RNA and protein synthesis, it is unclear what functional significance this has for the growth inhibitory effects of selenium. For example, Fico *et al.* (1986) have demonstrated that the inhibition of RNA synthesis, but not DNA or protein synthesis, correlates with selenium-induced growth suppression.

Selenium has also been shown to affect progression through the cell cycle. In cultured hepatoma cells, flow cytometry analysis indicated that selenite increased the proportion of cells in G1 of the cell cycle and increased cell doubling time (LeBoeuf *et al.*, 1985). From the doubling time and proportion of cells in various phases of the cell cycle, the duration of each phase could be calculated and selenite was found to increase the length of G1, S and G2, but not M, of the cell cycle (LeBoeuf *et al.*, 1985). However, this may be a cell-type specific effect, since in cultured mammary cells, flow cytometry analysis showed selenite induced a specific block in S-G2 of the cell cycle (Medina *et al.*, 1983).

2.4.4 Activation of apoptosis and possible involvement of oxidative stress

Cancer occurs due to a loss in tissue homeostasis such that there is a net gain in cell number. This may be accomplished by an increase in proliferation. However, it is becoming increasingly clear that cell death plays an important role in tissue homeostasis and that cancer may arise due to an imbalance of cell division and cell death.

Apoptosis is a genetically programmed, delineated set of events that lead to cell death which can be induced by a variety of physical and chemical treatments such as irradiation, growth factor withdrawal and exposure to a number of anti-neoplastic drugs (Barry *et al.*, 1990; Williams *et al.*, 1990; Warters, 1992). Characteristic features include chromatin condensation, fragmentation of the nucleus and convolution of the cell membrane (Wyllie, 1985). Chromatin

condensation is believed to result from the cleavage of genomic DNA into 600-50Kb or smaller polynucleosomal fragments (Wyllie, 1985; Oberhammer *et al.*, 1993; Bicknell *et al.*, 1994). The final stages of apoptosis involve the "pinching" off of cell contents in membrane encapsulated apoptotic bodies (Wyllie, 1985). This is in contrast to necrosis, where the cell membrane is ruptured and the cells contents are released into the extracellular environment.

Apoptosis is an active process as exemplified by the fact that some cells require protein synthesis to initiate apoptosis (Story *et al.*, 1992). Genetic approaches designed to identify genes involved in regulating apoptosis have been most informative in the nematode worm, *Caernorhabditis elegans*. During its development, certain cells are programmed to die by apoptosis. This has been shown to require the activity of two genes, *ced3* and *ced-4* (Ellis and Horvitz, 1986), whereas a third gene product, *ced-9*, inhibits apoptosis (Hengartner et al., 1992). Mammalian homologues of *ced-3* and *ced-9* have been identified suggesting that apoptosis is evolutionary conserved. The mammalian *ced-3* homologue encodes interleukin-1β (Il-1β) converting enzyme (ICE) and is involved in processing of the growth-factor Il-1β (Yuan *et al.*, 1993). Both Ced-3 and murine ICE can induce apoptosis when transfected into rat fibroblasts demonstrating functional as well as structural conservation (Miura *et al.*, 1993). The mammalian protein, Bcl-2, the homologue of *ced-9*, can substitute functionally for Ced-9 in *C. elegans* (Hengartner and Horvitz, 1994) and it also inhibits apoptosis in mammalian cells induced by a variety of chemical treatments (Zhong *et al.*, 1993). Bcl-2, which is localised in membranes (Hockenberry *et al.*, 1990), has no defined biochemical function. However, it has been postulated to have antioxidant activity since overexpression of Bcl-2 in T-cells has been shown to inhibit dexamethasone-induced lipid peroxidation (Hockenberry *et al.*, 1993). A number of other *bcl-2*-like genes have also been identified (Boise *et al.*, 1993; Kozopas *et al.*, 1993; Lin *et al.*, 1993; Oltvai *et al.*, 1993). Of particular interest are *bax* and *bcl-x*, the functions of which have been studied in a B-cell line which

upon withdrawal of the cytokine Il-3 undergoes an apoptosis that is prevented by Bcl-2 overexpression. Bax can heterodimerise and inhibit the protective function of Bcl-2 (Oltvai *et al.*, 1993). *bcl-x* encodes a transcript that is differentially spliced to give two mRNAs, Bcl-x_L and Bcl-x_S. Bcl-x_L has similar anti-apoptotic activity to Bcl-2 whereas Bcl-x_S has no effect on apoptosis induction when overexpressed alone but can antagonise Bcl-2 function when co-expressed in a Bcl-2 expressing cell line (Boise *et al.*, 1993). The existence of a number of apoptosis-regulating genes that have been evolutionary conserved demonstrates that programmed cell death is an important process in multi-cellular organisms for the removal of unwanted cells which is subject to a complex control involving multiple pathways and gene products.

If the anti-carcinogenic effects of selenium involve the inhibition of cell proliferation, one way this could be achieved could be through the activation of apoptosis. Indeed, both organic and inorganic seleno-compounds have been shown to induce apoptosis in diverse systems such as leukaemia and mammary cultured cells as judged by morphological criteria, formation of nucleosomal ladders and flow cytometry, which utilises the principle of differential uptake of two DNA-binding fluorochromes by apoptotic but not necrotic cells (Lanfear *et al.*, 1994; Lu *et al.*, 1994; Thompson *et al.*, 1994). The mechanism of induction is unknown; however oxidative stress has been postulated to be involved since the generation of reactive oxygen species, superoxide anion and hydrogen peroxide, have been shown to be generated from the reaction of selenite and thiols using the chemiluminescent compounds lucigenin and luminol (Yan and Spallholz, 1993). This is consistent with selenite's requirement for GSH to mediate its growth-inhibitory effect. Moreover, selenite-induced DNA strand breaks have been shown to be oxygen-dependent (Garberg *et al.*, 1988). Selenite can also deplete GSH stores (Anundi, *et al.*, 1984), which may also contribute to a pro-oxidant state.

Generally, oxidative stress is believed to play a causative role in apoptosis (Buttke and Sandstrom, 1994). Neurons when cultured in a high oxygen atmosphere undergo apoptosis (Enokido and Hatanaka, 1993). Similarly, hydrogen peroxide can induce apoptosis in a variety of cell systems as judged by morphological criteria and the formation of nucleosomal DNA ladders (Pierce *et al.*, 1991; Forrest *et al.*, 1994). Consistent with this, antioxidants such as *N*-acetylcysteine and GSH can block apoptosis induced by a variety of signals such as growth factor withdrawal (Hockenberry *et al.*, 1993), the endotoxin lipopolysaccharide (Abello *et al.*, 1994) and T-cell receptor activation (Sandstrom *et al.*, 1994). Additionally, overexpression of the antioxidant enzyme GPX inhibits Il-3 deprivation-induced apoptosis (Hockenberry *et al.*, 1993).

2.5 Aims of project

The broad aims of this project are to investigate the molecular mechanisms by which selenium inhibits cell growth *in vitro* since similar mechanisms may account for some of the chemopreventive effects of selenium. A mouse mammary cell line, C57, has been used in these studies since the chemopreventive effects of selenium have been most extensively characterised in mammary tumour models. A number of approaches have been adopted. Since the *in vivo* effects of selenium are highly dependent on chemical form, the response of C57 cells to various selenium compounds with varying degrees of chemopreventive activity has been assessed. Regarding possible mechanisms of growth inhibition, the strong link between selenium metabolism, oxidative stress and the induction of apoptosis has been investigated. Also, a genetic approach has been taken using an SDG-resistant variant to identify other and possibly novel mechanisms of growth inhibition induced by selenium.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Cell lines, media and tissue culture supplies

Mouse mammary epithelial cell line, C57, had been previously derived (Vaidya *et al.*, 1978). NRK49 fibroblasts were from laboratory stocks.

Supplier: *Beatson Institute Central Services*

- sterile dH_2O
- sterile PBS
- sterile glassware and glass pipettes

Supplier: *Becton Dickinson Labware, Plymouth, U.K.*

- 60mm and 100mm diameter tissue culture dishes

Supplier: *Fisons Scientific Equipment, Loughborough, Leics., U.K.*

- dimethylsulphoxide (DMSO)

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, U.K.*

- Eagle's minimal essential medium
- Foetal calf serum (FCS)
- 200mM glutamine
- 1M HEPES

Supplier: *A/S Nunc, Roskilde, Denmark*

- Tissue culture flasks
- Nunc tubes

Supplier: *Worthington, New Jersey, USA*

- trypsin

3.1.2 Chemicals

Supplier: *Amersham International plc, Amersham, Bucks., U.K.*

-[α-^{32}P] dCTP ~ 3mCi/mmol

Supplier: *BDH Chemicals Ltd., Poole, Dorset, U.K.*

Unless otherwise stated, all chemicals were obtained from BDH and were of AnalaR grade or better.

Supplier: *Bethesda Research Laboratories, Life Technologies, Inc., USA*

- agarose, ultrapure electrophoresis grade
- LMP agarose, ultrapure electrophoresis grade

Supplier: *James Burrough Ltd., Witham, Essex, U.K.*

- ethanol

Supplier: *DuPont UK Ltd., Stevenage, Herts., U.K.*

- ^{35}S-GSH

Supplier: *Fisons Scientific Equipment, Loughborough, U.K.*

- acetic acid
- chloroform
- 38% (w/v) formaldehyde
- glycerol
-hyperchloric acid ($HClO_4$)
- methanol
- propan-2-ol

Supplier: *Fluka Chemika-Biochemika AG, Buchs, Switzerland*

- formamide

Supplier: *Merck, Darmstadt, Germany*

- ninhydrin

Supplier: *Millipore UK Ltd., Watford, Herts., U.K.*

- all reagents for 2D gel electrophoresis

Supplier: *National Diagnostics, Manville, New Jersey, USA*

- ecoscint A

Supplier: *Pharmacia Ltd., milton Keynes, Bucks., U.K.*

- 50mM dNTPs
- Sephadex G-50
- Sephadex G-100

Supplier: *Pierce and Warringer, Chester, U.K.*

- Coomassie protein assay reagent

Supplier: *Rathburn Chemicals Ltd., Wakeburn, U.K.*

- phenol

Supplier: *Sigma Chemical Co., Ltd., Poole, Dorset, U.K.*

- bromophenol blue
- bovine serum albumin (BSA) (fraction V)
- ethidium bromide
- ethylmethanesulphonate (EMS)
- 4,6-diamidino-2-phenylindole (DAPI)

- Dowex-50 Na^+ resin
- DTNB
- Giemsa
- reduced glutathione (GSH)
- oxidized glutathione (GSSG)
- Hoechst 33258
- MOPS
- nickel chloride
- polyvinylpyrolidone
- salmon sperm DNA
- selenomethionine ([Se]Met)
- sodium selenite
- TEMED
- triton X-100

Supplier: *Surgipath, St. Neots, Cambridgeshire, U.K.*

- hematoxylin
- eosin
- scots tap water

Supplier: *University of Missouri, Columbia, USA*

- [^{75}Se]-sodium selenite ~430μCi/μg Se

Supplier: *Vector Labs Inc., Burlingame, California, USA*

- Vectashield

3.1.3 Enzymes and Inhibitors

Supplier: *Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.*

- Klenow

- MoMuLV reverse transcriptase
- Proteinase K

Supplier: *Promega, Southhampton, Hampshire, U.K.*

- Taq DNA polymerase + reaction buffer
- RNASin

Supplier: *Sigma Chemical Co., Ltd., Poole, Dorset, U.K.*

- glutathione reductase

3.1.4 Kits

Supplier: *Bio 101 Inc., Stratatech Scientific, Luton, U.K.*

- Geneclean kit

Supplier: *Appied Biosystems, Warrington, U.K.*

- Dyedeoxy Terminator Cycle Sequencing Prism Kit

Supplier: *Boehringer Manheim UK, Lewes, East Sussex, U.K.*

- DNA random priming labelling kit

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, U.K.*

- Trizol

3.1.5 Markers

Supplier: *Bethesda Laboratories Research, Gibco Ltd., Paisley, U.K.*

- kb DNA ladder
- RNA ladder
- λ phage DNA

Supplier: *Bio-RAD , Hemel Hempstead, Herts., U.K.*

- yeast chromosome markers

3.1.6 Membranes, paper, TLC plates and X-ray film

Supplier: *Amersham international plc, Amersham Bucks., U.K.*

- Hybond N^+

Supplier: *Eastman Kodak Co., Rochester, New York, USA*

- X-ray film (XAR)

Supplier: *Camlab Ltd., Cambridge, U.K.*

- Polygram TLC plates

Supplier: *Whatman International Ltd., Maidstone, Kent, U.K.*

- 3MM chromatography paper

3.1.7 Water

Distilled water for buffers and all general solutions were purified through a Millipore MillRO 15 system. Water for protein of nucleic acid procedures which required further purification was obtained from a Millipore MilliQ system at 18MΩ cm.

3.2 Methods

3.2.1 Tissue culture

All cell lines were grown in Eagle's minimal essential medium supplemented with 10% foetal calf serum (FCS), 1mM glutamine and 1mM HEPES buffer. Cells were kept in a moist atmosphere containing 5% CO_2 (v/v) at 37°C. Cells were passaged at sub-confluency by rinsing with phosphate buffered saline (PBS) and removing cells from flasks with trypsin (1mg/ml PBS). Trypsin was inactivated by

10% FCS and the cell suspension was diluted as required and seeded into fresh culture medium.

To reduce chances of genetic drift, cells were replaced from frozen stocks at least every 3 months. Frozen stocks were made from logarithmically growing cells in culture medium with 10% dimethyl sulphoxide (DMSO) added. Ampoules were slowly cooled to -70°C o/n by wrapping in cotton wool. Ampoules were then transferred to liquid nitrogen for long term storage. When required, cells were thawed rapidly at 37°C. DMSO was removed by centrifugation at 1000 rpm, 5 min, and resuspension in fresh culture medium and cells were then transferred to flasks for routine culture.

3.2.2 Mycoplasma testing of cell lines

Cells were checked for mycoplasma contamination. Conditioned medium was collected from cells which had been cultured for at least 2-3d. ~2ml of the conditioned medium was co-incubated with 2 x 10^5 NRK49 fibroblasts in 2ml fresh culture medium. NRK49 fibroblasts are known to be mycoplasma-free although a negative control was always set up in parallel: NRK49 cells without conditioned medium. After incubation in a moist atmosphere containing 5% CO_2 (v/v) at 37°C for 3d, cells were fixed by the addition of fixative (1 volume glacial acetic acid: 3 volumes methanol) for 5min. This was followed with a further two incubations in fresh fixative for 5min and the cells allowed to air-dry. Hoechst 33258 is a fluorescent stain which detects DNA and was prepared from a 1mg/ml stock by diluting 1:20 000 in PBS. This solution was added to the fixed cells and incubated at R/T for 10min. After staining, excess stain was removed and cells washed twice with dH2O, then viewed by fluorescent microscopy using a water immersion lens. Mycoplasma contamination was detected as fluorescence in the cytoplasm of NRK49 cells.

3.2.3 Growth curves

~5 x 10^4 cells were seeded into 25cm^2 flasks and allowed to adhere o/n. Fresh culture medium was added containing the test agent. At the indicated times, cell numbers were determined by rinsing cells with PBS and removing adherent cells with trypsin (1mg/ml PBS) to obtain a single cell suspension. These were then counted using a Coulter counter (Coulter Electronics, Ltd.). Alternatively, after rinsing cells with PBS, cell number was determined indirectly by lysing adherent cells *in situ* with 0.5-3 ml 0.1M NaOH. Nucleic acid concentration of cell lysates was then determined spectrophotometrically by measuring absorbance of cell lysates at 260nm. The spectrophotometer was calibrated with a 0.1M NaOH blank and samples were read in a quartz cuvette with a 1cm pathlength. This figure was assumed to be linearly proportional to cell number.

3.2.4 Clonogenic assays

Logarithmically growing cells were seeded into 6cm diameter dishes at a concentration of 100 cells/dish and allowed to adhere o/n. Cells were then exposed to increasing concentrations of either SDG or H_2O_2. H_2O_2 was removed after 1h and replaced with fresh culture medium. SDG was left in the medium for the entire duration of the experiment unless otherwise stated. Plates were then incubated in a moist atmosphere containing 5% CO_2 (v/v) at 37°C for 10-14d. Colonies were rinsed with PBS and fixed and stained with 0.6% Giemsa in 50% glycerol/50% methanol (v/v). Plates were rinsed in H_2O and Giemsa-stained colonies counted. The plating efficiency of untreated cells was usually in the range of 50-80%.

3.2.5 Cell staining

5 x 10^5 cells were seeded into 25cm^2 flasks and allowed to adhere o/n. Cells were then treated with with varying concentrations of either SDG or H_2O_2 for the indicated times. Detached cells were pooled with adherent cells, that were

removed by trypsinisation, and ~10^4 cells were centrifuged onto microscope slides in a Cryo-Tek cytospin centrifuge (500 r.p.m. for 5min) (Miles Scientific). Cells were then fixed in methanol for 5min. Cells were then stained with DAPI at a concentration of 20ng/ml in Vectashield (Vector Laboratories) and viewed by fluorescent microscopy. Cells were stained with hematoxylin and eosin in the following way: Cells were re-hydrated by 30s sequential soaks in ethanol, 70% ethanol and H_2O and stained in hematoxylin for 4min. Hematoxylin (Surgipath) stain was rinsed off with H_2O before cells were immersed in scots tap water (Surgipath) for 1min, rinsed in H_2O and stained with eosin by five 0.5s dips. Eosin stain was then rinsed off with H_2O and cells were dehydrated by sequential 30s soaks in 70% ethanol, twice in ethanol and finally xylene. Cells were viewed under light microscopy.

3.2.6 Derivation of SDG-resistant cell lines

3.2.6.1 Mutagenesis

Conditions for mutagenesis were determined by constructing an ethylmethanesulphonate (EMS) dose-response kill-curve. 10^6 cells were seeded into 75cm^2 flasks and allowed to adhere o/n. Cells were then exposed to increasing concentrations of EMS in serum-free medium which was removed after 2h and replaced with fresh culture medium containing 10% FCS. Cells were left to recover for 24h before being seeded into 6cm diameter dishes at a concentration of 100 cells/dish. Colonies were rinsed with PBS and fixed and stained with 0.6% Giemsa in 50% glycerol/50% methanol (v/v) for 15min. Plates were rinsed in H_2O and Giemsa-stained colonies counted. A concentration of 1.75 mg/ml EMS was chosen since this gave a survival rate of 20%.

3.2.6.2 Selection of SDG-resistant cells

1-4 x 10^5 viable cells either after mutagenesis with 1.75 mg/ml EMS or untreated, were seeded into 10cm diameter Petri dishes. Cell viablilty was

estimated from the plating efficiency (50-80%) and an assumed EMS kill of 80%. Cells were allowed to adhere o/n after which they were exposed to varying concentrations of SDG. Plates were then incubated in a moist atmosphere containing 5% CO_2 (v/v) at 37°C for 10-14d. Colonies were located by microscopy and ring cloned into fresh culture medium without SDG and maintained in routine culture.

3.2.7 DNA methodologies

3.2.7.1 Agarose Gel Electrophoresis

DNA fragments were resolved according to apparent molecular weights on agarose gels. 1% (w/v) agarose was dissolved in 1x TAE buffer (40mM tris acetate, 20mM sodium acetate, 2mM EDTA, pH7.4) and after microwaving, the solution was allowed to cool to ~50°C before the addition of ethidium bromide at a final concentration of 0.5μg/ml. Gels were cast in the appropriate gel former and allowed to set at R/T. Samples for electrophoresis were mixed with sample loading buffer, to a final concentration of 1 x (10 x loading buffer: 50% (v/v) glycerol, 0.4% (w/v)bromophenolblue in TE buffer), loaded into sample wells and electrophoresed in 1 x TAE buffer. kb ladder molecular weight markers were run in parallel (Gibco BRL). After electrophoresis, resolved DNA was visualised by UV transillumination and a permanent record kept as either a polaroid or photoimage.

3.2.7.2 Purification of DNA fragments from agarose gels

The appropriate band was exised from the gel with the aid of UV illumination. DNA fragments were recovered from agarose by the methods of "Genecleaning" or centrfiugation. The procedure of "Genecleaning" was according to the manufacturer's instructions. For centrifugation, the gel slice was cut up into smaller pieces and placed on top of a glass wool plug contained in a 0.5ml eppendorf tube. This 0.5ml tube was previously pierced at the base using a

syringe needle. The plugged tube with the gel slice was then placed in a 1.5ml eppendorf tube and centrifuged for 5-10 min. at 5K, R/T in a benchtop microfuge. this resulted in the DNA being eluted from the agarose in the gel buffer and collected in the 1.5ml eppendorf tube. The agarose remained in the glass wool plug. the DNA was further purified by phenol/chloroform extraction and ethanol precipitation when necessary.

3.2.7.3 Spectrophotometric quantitation of DNA

Absorbance readings at 260nm and 280nm were taken of samples diluted in water in a quartz cuvette with a 1cm pathlength. The spectrophtometer was calibrated with a water blank. An absorbance of 1 at 260nm was taken to be equivalent to 50mg/ml DNA and A_{260}/A_{280} ratio of 1.8 indicative of an essentially pure preparation of DNA.

3.2.7.4 Preparation and quantitation of oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesizer. 5' trityl groups were removed on the synthesizer before the oligonucleotide was eluted from the column in concentrated (29%) ammonia solution for 1.5h at room temperature. Oligonucleotides were deprotected by incubation in ammonia in a sealed glass vial o/n at 55°C. This was followed by ethanol precipitation with extensive washing of the pellet with 70% ethanol to remove salt. Oligonucleotides were resuspended in water and quantified spectrophotometrically as in section 3.2.7.3. An A_{260} value of 1 was taken to equal 33μg/ml of oligonucleotide. A ratio of A_{260}/A_{280} was ~1.8 when sufficiently purified.

3.2.7.5 Sequencing

DNA was sequenced using cycle sequencing. DNA PCR products were sequenced on both strands using the same primers used in the initial PCR reaction.

3.2 pmoles of primer was added to approximately 1µg DNA and the total volume made up to 10.5µl using dH_2O. To this mixture was added 9.5µl of the Dyedeoxy Terminator Cycle Sequencing Prism Kit (Applied Bio-Systems). Reactions were carried out in a Perkin-Elmer 9600 thermal-cycler using 25 cycles of 96°C for 15s, 50°C for 1s and 60°C for 4min. 80µl dH_20 was added to the reaction mixture and was extracted twice with phenol/chloroform/dH_2O (68:18:14). The DNA was then precipitated by adding 15µl 2M sodium acetate, pH 4.5 and 300µl ethanol to the final aqueous phase and incubated at -70°C for 15min. The DNA was pelleted by centrifugation at 35 000 rpm for 15min in a microfuge and washed in 0.5ml 70% ethanol. After allowing the pellet to air-dry, it was then redissolved in 3-4µl of loading buffer (1:5 dilution of : 5parts deionized formamide, 1 part 50mM EDTA containing 30mg/ml blue dextran). Running and analysis of sequencing gels was expertly performed by Robert McFarlane (Beatson Institute, Glasgow, U.K.) as follows: Samples were run on a 6% polyacrylamide gel (made from a 40% stock [19:1 ratio of acrylamide:bis-acrylamide]) in 1 x TBE buffer using an ABI 373A DNA Sequencer (Applied Biosystems) at 30W for 12h. Gels were analysed using 373A software version 1.2.1.

3.2.7.6 Pulsed-field gel electrophoresis

2 x 10^6 cells were seeded into 75cm^2 flasks and allowed to adhere o/n. Cells were then exposed to either 8µM SDG or 1.3mM H_2O_2. At the indicated times, detached cells were pooled with adherent cells, which were removed by trypsinisation. Cells were washed three times in PBS by centrifugation at 1000rpm for 5min and resuspension in PBS. The cell pellet was then resuspended in 50µl ice-cold L-buffer (0.1M EDTA, [pH 8.0], 0.01M tris.HCl [pH 7.6], 0.02M NaCl) and warmed to 55°C. The cell suspension was then mixed with an equal volume of 2% (w/v) low melting-point agarose in L-buffer, which had previously been boiled by microwaving and then cooled to 55°C. The molten mixture was then pipetted into 100µl sample mould plugs (Bio-Rad) and allowed to set on ice. Agarose

blocks were then transferred to 50 volumes of L-buffer containing 1mg/ml proteinase K and 1% sarkosyl and incubated for 24h at 50°C. The original digestion mixture was then replaced with fresh digestion mixture and incubated for a further 24h at 50°C. Agarose blocks were then sliced into blocks containing approximately 10^6 cells and set in 1% (w/v) agarose in TBE buffer (90mM tris-base, 90mM boric acid, 2mM EDTA, pH 8.0), which had previously been boiled by microwaving and cooled to 65°C, or transferred to 50mM EDTA for long-term storage at 4°C. Yeast chromosome DNA size markers (Bio-Rad) and λ phage DNA (Gibco BRL) were loaded in parallel to samples. Electrophoresis was carried out in 0.5 x TBE buffer at 14°C for 16h using the CHEF-DR II pulsed-field gel elctrophoresis system (Bio-Rad).

3.2.8 RNA methodologies

3.2.8.1 RNA extraction, purification and quantitation

In preparations of RNA and handling of RNA, some precautionary steps were taken in order to avoid degradation by contaminating RNases. All manipulations were carried out wearing gloves, tubes and solutions were pre-cooled on ice, centrifugations carried out at 4°C and aerosol-resistant, DNase- and RNase-free tips were used for all manipulations.

RNA extraction and purification using Trizol (Gibco BRL) was carried out according to the manufacturer's instructions. The concentration of aqueous solutions of RNA was measured spectrophotometrically as in section 3.2.7.3. An A_{260} value of 1 was taken to equal 40μg/ml RNA.

3.2.8.2 RNA electrophoresis

RNA samples (~20-80μg) were added to 5 x sample volume of sample buffer (0.7ml formamide, 0.15ml 10 x MOPS buffer [10 x MOPS buffer: 200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH7.0], 0.24ml formaldehyde, 0.1ml H_2O, 0.1ml glycerol, o.05% (w/v) bromophenol blue). RNA samples were

denatured at 65°C for 15min., chilled on ice for 10min., followed with a brief microfuge. RNA ladder (Gibco BRL) molecular weight markers were run in parallel. Electrophoresis was performed in a 1% (w/v) agarose gel containing 2.2M formaldehyde in 1 x MOPS buffer with recirculation using a peristaltic pump.

3.2.8.3 Northern transfer and hybridisation

Following electrophoresis, northern gels were washed for 30min in 20 x SSC (3M NaCl, 0.6M sodium citrate, pH 7.0) before capillary action transfer to Hybond N^+ membrane. Transfer buffer was 20 x SSC, and transfer was allowed to proceed for a minimum of 16h. After transfer, the membrane was UV cross-linked. Lanes containing RNA markers were fixed in 0.5% acetic acid for 5min and stained with 0.04% methylene blue in 0.5M sodium acetate, pH5.2.

Hybridization buffer (5 x SSPE [20 x SSPE: 3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA, pH8.3], 5 x Denhardts [50 x Denhardts: 1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (BSA)], 50% (v/v) formamide, 0.5% (w/v) sodium dodecyl sulphate), was supplemented with denatured, sonicated salmon sperm DNA to a final concentration of 215µg/ml and incubated with the membrane at 42°C for a minimum of 4h with constant rotation in a Hybaid oven. Following pre-hybridization, radioisotopically labelled probe was boiled for 5min, chilled on ice for 5min and added to the hybridization buffer. The membrane was subsequently hybridized for a minimum of 16h at 42°C. After hybridization, the membrane was washed under increasingly stringent conditions with the following wash solutions:

15min at room temperature with 1 x SSC, 0.1% (w/v) SDS
15min at 65°C with 1 x SSC, 0.1% (w/v) SDS
15min at 65°C with 0.1 x SSC, 0.1% (w/v) SDS

The membrane was then exposed to X-ray film at -70°C for the required times.

3.2.8.4 cDNA synthesis and polymerase chain reaction (RT/PCR)

cDNA was synthesised as follows: Reverse transcription was carried out in a 20µl volume containing 1 x PCR buffer (Promega), 1mM dNTPS, 20U RNAsin (Promega), 100pmoles oligo(dT) primer, RNA (1-3µg) and 200U BRL MoMuLV reverse transcriptase. The reaction was incubated at R/T for 10min and then heated to 95°C for 5min and chilled on ice to denature the reverse transcriptase. To the 20µl reaction mixture was added 80µl 1 x PCR buffer (Promega) containing ~1µg of each primer and 1-2U Taq DNA polymerase. The reaction mixture was overlayed with 100µl paraffin oil to prevent evaporation. Reaction conditions were 40 cycles of : 94°C for 1min, annealing at 60°C for 1min and extension at 72°C for 1min. This was followed by a final extension step of 72°C for 5min. Analysis of PCR products by agarose gel electrophoresis was as previously described in section 3.2.7.1. For amplifying sequences using DNA as a starting template, similar PCR conditions were employed using ~1µg genomic DNA from human foreskin fibroblasts (kindly provided by Kevin Ryan, Beatson Institute, Glasgow, U.K.).

3.2.8.4.1 Oligonucleotide sequences for RT/PCR

Prepared as previously described in section 3.2.7.4

GST Ya:

5' ATGGCCGGGAAGCCCGTGCTT 3'

5' CTGAATCTTGAAAGCCTTCCT 3'

TRX:

5' AGTAGTCTCGAGGGCCCAAAATGGTGAAGCTGATCG 3'

5' AGTAGTTCTAGAGGTACCATGATTAGGAATATTCAG 3'

Bcl-x

5' ATGTCTCAGAGCAACCGGGAG 3'

5' AAAGCTCTGATATGCTGTCCC 3'

3.2.8.5 Radioisotopic labelling using random priming

Random priming probes for Northern analyses used the commercially available kit marketed by Boehringer Manheim. The protocol followed was according to the manufacturer's instructions, incorporating radioisotope [α-^{32}P] dCTP. Probes were seperated from unincorporated radioisotope by chromatography on G-50 sephadex columns in 1 x TE (10mM tris-HCl, 1mM EDTA, pH7.0), 0.1% SDS.

3.2.9 2D protein gels

18.5μCi [^{75}Se]-selenite was added to logarithmically growing cells and incubated in a moist atmosphere containing 5% CO_2 (v/v) at 37°C. Culture medium was removed, cells rinsed twice with PBS and lysed in 0.5ml lysis buffer (9.9M urea, 4% (v/v) NP-40, 2.2% (v/v) ampholytes pH 3-10/2D, 100mM DTT). The 2D gels were expertly performed by Lyn McGarry (Beatson Institute, Glasgow, U.K.) as follows: first dimension gels contained: 9.5M urea, 2% (v/v) NP-40, 4.1% (v/v) acrylamide (from stock acrylamide solution containing 30.8% (w/v) acrylamide, 2.6% (w/v) bis-acrylamide), 2% (v/v) ampholytes pH3-10/"D and polymerised with 0.67% (w/v) ammonium persulphate. These gels were overlayed with: 0.5M urea, 0.2% (v/v) NP-40), 0.1% (v/v) ampholytes pH 3-10/2D, 5mM DTT, 0.7M β-mercaptoethanol, then prefocused to 2000V using a constant current of 110μA per gel. Samples were then applied beneath the overlay buffer, each sample contained ~25 000cpm in 50μl sample buffer. Focussing was carried out for 18000 volt-hours (17.5h at 1000V then 30min at 2000V), after which the gels were extruded into gel equilibration buffer (0.3M tris-base, 75mM tris-HCl, 3% (w/v) SDS, 50mM DTT, 0.001% (w/v) bromophenol blue) and

allowed to equilibrate for 2min. before being placed horizontally on the upper surface of a vertical SDS polyacrylamide gel (10% (v/v) acrylamide/bis-acrylamide (stock: 30.8%/1.6%), 0.37M tris pH 8.8, 0.1% (w/v) SDS and polymerised with 0.05% (v/v) TEMED and 0.025% (w/v) ammonium persulphate). Electrophoresis was continued for ~4h at a constant power of 16W per gel. All 2D gel chemicals were supplied by Millipore (UK) Ltd. Gels were dried onto blotting paper under vacuum at 80°C for 2h and exposed to X-ray film at room temperature for ~70d.

3.2.10 Synthesis and purification of selenodiglutathione (SDG)

SDG was synthesised by mixing the following reactants: 4ml 0.1M GSH, 1ml 0.1M Na_2SeO_3 and 1ml H_2O. A trace amount of ^{35}S-GSH (9.5µl of a 1:10 dilution of ^{35}S-GSH [specific activity 501.9 Ci/mmol]) was also included in the reaction to enable the SDG to be monitored in subsequent purification steps. The reaction mixture was incubated for 30min at R/T.

3.2.10.1 Separation of SDG and GSSG

Separation of the reaction products, SDG and GSSG, was achieved by chromatography primarily according to the method of Ganther (1971). Approximately 500g Dowex-50 resin Na^+ was washed by gentle agitation in dH_2O until washes ran clear. The resin was then gently washed with 1M HCl and rinsed with dH_2O until the pH of the washes returned to 7. This was repeated using 1M NaOH. 150g of the Dowex-resin was set aside and used for the subsequent concentration of SDG (see section 3.2.10.2) and the remaining 350g Dowex-resin was finally washed in 0.1M sodium acetate, pH4.7 and was saturated with $NiCl_2$ by suspension in 2 x volumes of 0.1M sodium acetate, 0.3M $NiCl_2$, pH4.7. The suspension was poured into a 3cm diameter jacketed column and allowed to pack under gravity to a height of about 60cm and then equilibrated with running buffer (0.1M sodium acetate, 0.01M $NiCl_2$, pH4.7). Running buffer was pulled through the column under a vacuum, set up by a peristaltic pump, at a rate of 2.5ml/min.

The sample was loaded onto the column by way of a three-way valve which temporarily disconnected the buffer reservoir from the column without introducing air into the system. The A_{240} of column eluate was measured by a UVcord II, UV monitor (LKB) connected to a chart recorder before 2ml fractions were collected in an Ultrurac 2070, fraction collector (LKB). This gave an estimation of which fractions contained the SDG which eluted as the second A_{240} peak. 200µl aliquots from each of these fractions was then measured for ^{35}S-radioactivity to identify exactly which fractions contained SDG. These were then pooled and 5M HCl was added to give a final pH of ≤ 3.

3.2.10.2 Concentration of SDG

150g washed Dowex-50 resin (see section 3.2.10.1) was poured into a 8cm diameter column and allowed to pack under gravity. 0.1M formic acid was pulled through the column under vacuum, set up by a peristaltic pump, at a rate of 2.5ml/min, until the A_{240} of the column eluate became constant. The SDG was then loaded gently onto the column with a Pasteur pipette ensuring the resin was not disturbed. The SDG was then run into the column; under the acidic conditions SDG becomes adsorbed to the resin matrix. The column was then washed through with excess 0.1M formic acid until the A_{240} of the column eluate became constant again. The SDG was then eluted with 0.1M ammonium acetate, pH5.6 and the fractions containing SDG were monitored as described in section 3.2.10.1.

3.2.10.3 Buffer change

The SDG was loaded onto a G10-sephadex column using dH_2O as a running buffer. The column eluate was pulled through the column under a vacuum, set up by a peristaltic pump, at a rate of 0.7ml/min. SDG-containing fractions were monitored as described in sections 3.2.10.1 and pooled. Acetic acid was then added to give a final concentration of 10mM and 5ml aliquots were stored at -20°C. The amount of SDG purified was estimated by measuring the

^{35}S-radioactivity recovered, after purification, assuming the initial reaction had gone to completion.

The entire process of SDG synthesis and purification was performed over a period of 2-3d. Between each chromatographic step, SDG was stored at 4°C to minimise degradation of SDG.

3.2.10.4 Analysis of SDG purity

The purity of SDG was finally confirmed using absorbance spectra and thin layer chromatography (TLC). The absorbance spectrum of SDG was measured over a range of 220-420nm using a quartz cuvette with a 1cm pathlength. The spectrophotometer was calibrated with a dH_2O blank. For TLC, ~15µl SDG was spotted onto Polygram SIL G TLC plates (Camlab) and chromatographed in a 2:1:1 mixture of butanol:acetic acid:H_2O in an air-tight glass chamber that had been pre-equilibrated with the solvent buffer. Run in parallel to SDG was 1µl of 4mM GSH, 1µl of 2mM GSSG and 3µl of a mixture of 13.3mM GSH and 3.3mM Na_2SeO_3 that had been incubated for 30min at R/T. Chromatography was continued until the buffer front reached the top of the TLC plate. The TLC plate was then allowed to dry and chromatography was repeated , again until the solvent buffer reached the top of the TLC plate. After drying, the TLC plate was stained with 0.2% ninhydrin in ethanol and developed for 5min at 80°C.

3.2.11 Measurement of selenium uptake

2 x 10^5 cells were seeded into 25cm^2 flasks and allowed to adhere o/n. 13.5µCi [^{75}Se]-selenite (specific activity: 3.5µCi/µl) was added to the culture medium and cells were incubated at 37°C. At the indicated times, cells were washed three times in PBS and adherent cells were lysed *in situ* with 1ml 0.1M NaOH. 0.7ml of each cell lysate was then measured for ^{75}Se-radioactivity in a γ-radiation counter.

3.2.12Measurement of GSH concentration

GSH concentration was measured essentially using the method of Tietze (1969). Logarithmically growing cells were removed by trypsinisation and washed twice with PBS by centrifugation (1000rpm, 5min) and resuspension. The final cell pellet was resuspended in1ml PBS and was centrifuged at 6000rpm for 1min. The PBS was removed and the cells were lysed in 1ml 1M $HClO_4$, 2mM EDTA and centrifuged at 6000rpm for 1min. The cell lysate was retained and the pellet redissolved in 1ml 0.3M NaOH and assayed for protein concentration by mixing 5µl of the redissolved protein with 1ml of coomassie protein assay reagent (Pierce and Warringer) diluted 1:2 with dH_2O and measuring the A_{595}. Protein concentration was calculated from a standard curve, constructed previously, using known concentrations of bovine serum albumin. 0.5ml of the cell lysate was added to 0.36ml 2M KOH, 0.3M MOPS and centrifuged at 6000 rpm for 1min. 200µl of the supernatant was added to 700µl of 0.3mM NADPH dissolved in 125mM phosphate-EDTA buffer (125mM $NaH_2PO_4.2H2O$, 125mM $Na_2HPO_4.2H_2O$, 6.3mM EDTA, pH 7.5) and 100µl of 6mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) dissolved in phosphate-EDTA buffer. Samples were equilibrated to 30°C and transferred to a plastic cuvette. 10µl glutathione reductase (8.5U/ml) was added and the reaction monitored in a spectrophotometer by measuring the change in A_{412} over a period of 60s. GSH concentration was calculated from a standard curve, constructed previously, using known concentrations of GSH dissolved in phosphate-EDTA buffer. Intracellular GSH concentration was then calculated and expressed as mg GSH/ g protein.

3.2.13 Quantitative analysis of autoradiographs

Autoradiographs were scanned by laser densitometer (Molecular Dynamics) and image analysis carried out using the PDQuest and Quantity 1 software from Protein Databases Incorporated.

3.2.14 Statistical analysis

The students *t*-test was used to determine statistical significance of data. For northern analyses, comparing relative levels of mRNA levels between C57 and B19 cells, the null hypothesis to be tested was that the ratio of B19/C57 for a particular mRNA is 1. This was calculated using :

$$t=\frac{\bar{x}-\mu}{s/n^{1/2}}$$

where,

$\bar{x}$= the mean value of the B19/C57 ratio

μ= 1

$s^2=1/(n-1)\left\{ \Sigma x^2 - 1/n(\Sigma x)^2 \right\}$

n= sample number

For all other tests, where absolute values were being compared, the null hypothesis tested is that the means are equal. This was tested using:

$$t= \frac{\bar{x}_1 - \bar{x}_2}{s(1/n_1 + 1/n_2)^{1/2}}$$

where,

$\bar{x}_1$= the mean B19 values

$\bar{x}_2$= the mean C57 values

n_1= the number of B19 values

n_2= the number of C57 values

$$s^2= \frac{\Sigma_1(x-\bar{x}_1)^2 + \Sigma_2(x-\bar{x}_2)^2}{n_1+n_2-2}$$

Chapter 4

Mechanisms of growth inhibition by selenium

4.1 Anti-proliferative potential of selenium compounds *in vitro*

The efficiency of selenium to act as an anti-carcinogen is highly dependent on the chemical form in which it is administered suggesting that its metabolism is an important step in exerting its action (Chapter 2, section 2.3.3). Two forms of selenium commonly used in chemopreventive studies are sodium selenite and [Se]Met. Both compounds are converted by different pathways to H_2Se which is a precursor molecule for the formation of [Se]Cys (Chapter 1, section 1.2). H_2Se is also metabolised to form mono-, di- and tri-methylated selenium excretory products (figure 1.1). Compounds such as seleno-betaine (SB) and Se-methylselenocysteine (SMSC), which are converted to mono-methyl selenol, or dimethyl selenide (DMS), which is metabolised to dimethyl selenide, have also been demonstrated to have anti-carcinogenic activity (figure 1.5) (Ip and Ganther, 1990; Ip *et al.*, 1991). The relative efficiencies of these various compounds to inhibit carcinogenesis in the DMBA-induced mammary tumour system are as follows (Ip and Ganther, 1990; Ip *et al.*, 1991):

$$SB \cong SMSC > Selenite > DMS$$

$$Selenite > [Se]Met$$

It has been postulated that the anti-carcinogenic effects of selenium may be mechanistically related to its *in vitro* properties as an inhibitor of cell growth. It was therefore of interest to examine the growth inhibitory effects of these various selenium compounds *in vitro* and determine if there was any relationship between a compound's *in vivo* anti-carcinogenic and *in vitro* anti-proliferative properties. Seleno-compounds: SB, SBME, SMSC and DMS were a kind gift from Dr. Howard E. Ganther (Dept. Preventive Medicine, University of Texas, Galveston, Texas, USA). A mouse mammary cell line C57 (Vaidya *et al.*, 1978), that had

been derived from normal mammary tissue, was used for this study since the chemopreventive effect of selenium has been most extensively studied in mammary tumour models. The anti-proliferative potential was examined for sodium selenite, [Se]Met, DMS, SB and SMSC. After addition of selenium to logarithmically growing C57 cells, growth was monitored over a period of days by removing adherent cells with trypsin and measuring cell numbers using a Coulter counter. 50 μM sodium selenite had a marked inhibitory effect on cell growth after a lag phase of 48h (figure 4.1). Between 48h and 96h after selenite addition, cell number did not significantly increase whereas cells grown in the control medium continued to grow logarithmically. After 96h in selenite, cells began to die as indicated by a decrease in cell number (figure 4.1). The effect of selenite appeared quite specific, since [Se]Met, DMS, SB and SMSC at a concentration of 100μM had little effect on the growth of C57 cells with only a 10-25% depression in cell number in comparison to the controls measured after 3d (table 4.1).

The initial delay of 48h before selenite began to inhibit cell growth suggested selenite may need to be metabolised to exert its action. Intracellularly, selenite is reduced to H_2Se by GSH (Hsieh and Ganther, 1977). This is believed to be an important step in exerting its anti-proliferative effect since the addition of exogenous glutathione can potentiate the inhibitory effect of selenite on cell colony formation (Caffrey and Frenkel, 1990). The simultaneous addition of 50μM GSH with 50μM selenite was found to abolish the delay in growth inhibition observed with selenite alone, causing a decrease in cell number within 48h indicating cell death (figure 4.2). 50μM GSH alone had no effect on growth (figure 4.2).

4.2 Synthesis of SDG

The enhancing effect of GSH on the growth inhibitory effect of selenite suggested that selenite and GSH were reacting together to form a highly cytotoxic compound and that this was a rate limiting step in the effect of selenite. To test this hypothesis, the response of C57 cells to selenodiglutathione (SDG), the

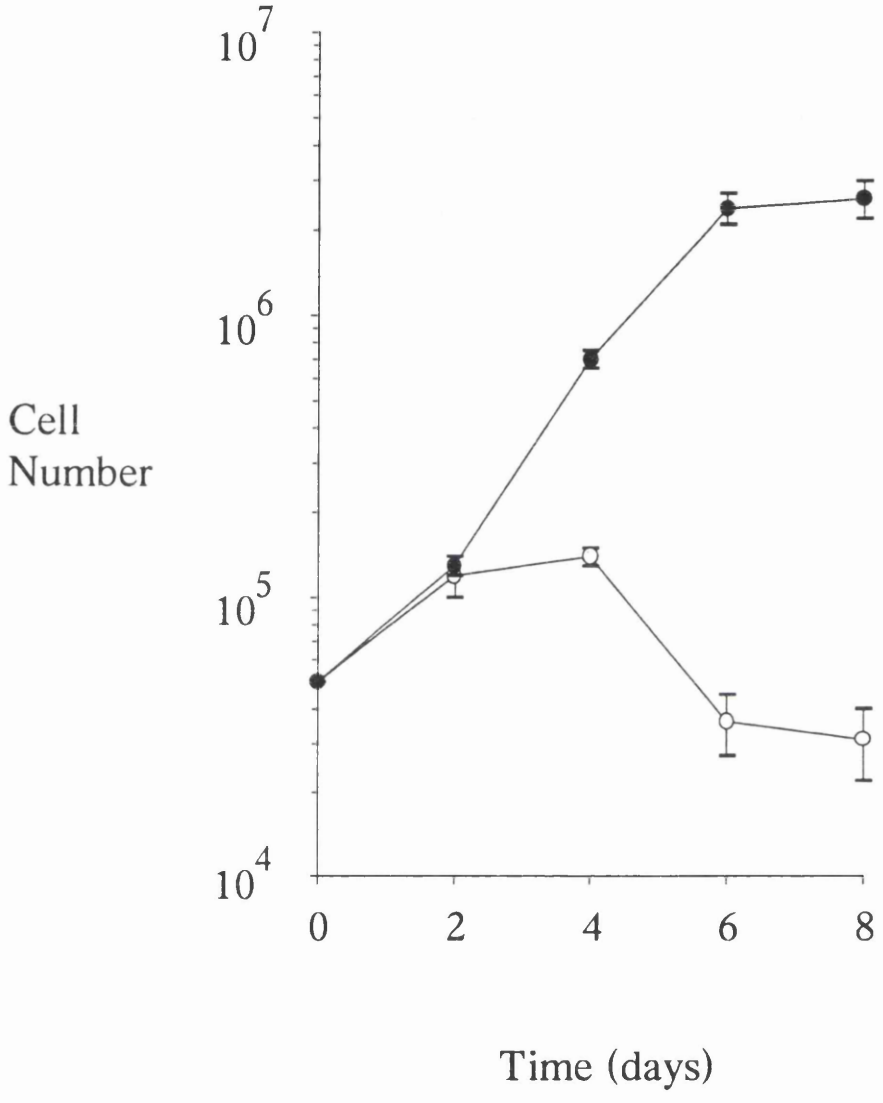


Figure 4.1
Growth of C57 cells as measured by cell number in control medium (●) or 50μM sodium selenite (○). Points are mean ± S.D. of three measurements

	Cell number ($x10^{-4}$)			
	Days in culture			
Compound	**0**	**1**	**3**	**4**
Control	8.9	24 ± 2	170 ± 30	180 ± 10
[Se]Met	8.9	23 ± 3	150 ± 10	140 ± 10
SB	8.9	23 ± 3	150 ± 10	140 ± 10
SMSC	8.9	23 ± 3	150 ± 20	130 ± 10
DMS	8.9	23 ± 1	130 ± 10	150 ± 10

Table 4.1
Cell growth of C57 cells, as measured by cell number, in control growth medium or 100μM selenium. Values are mean ± S.D. of three measurements.

Abbreviations: [Se]Met - *selenomethionine*, SB - *selenobetaine*, SMSC - *selenomethylselenocysteine* and DMS - *dimethylselenoxide*.

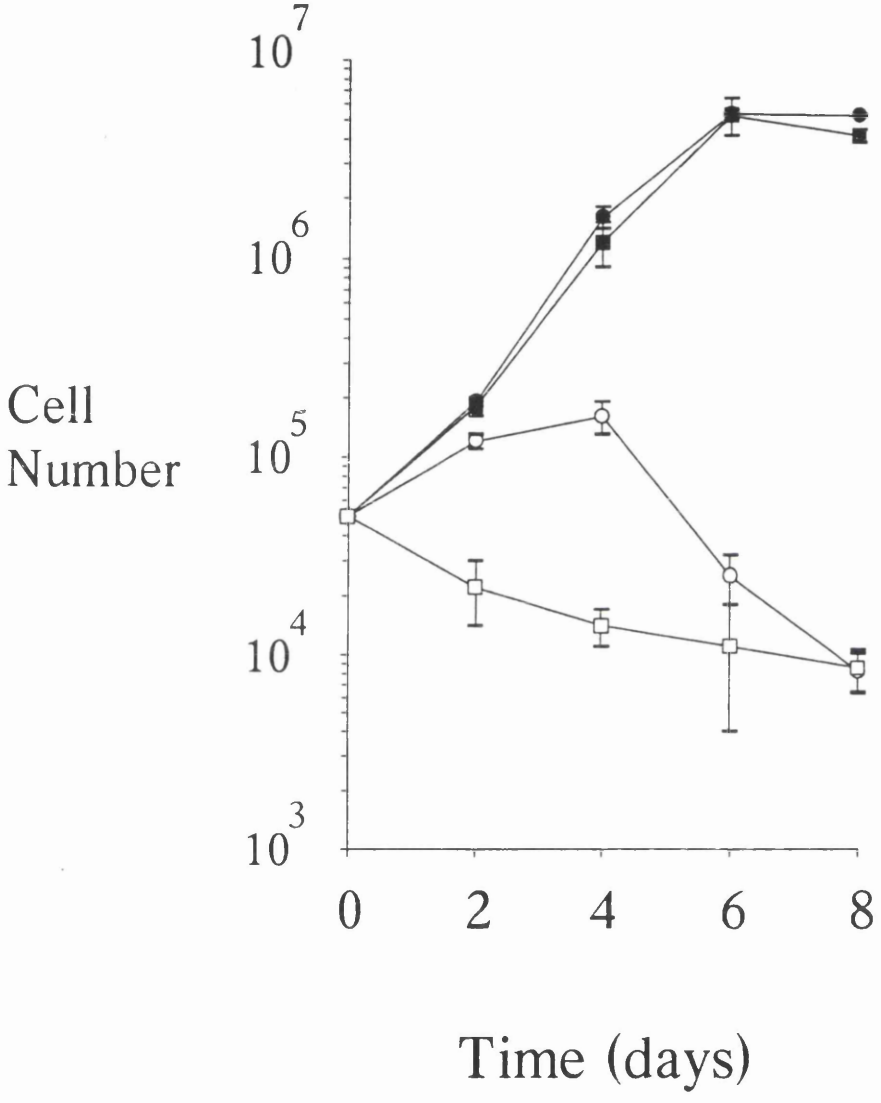


Figure 4.2
Growth of C57 cells as measured by cell number in control culture medium (●), 50μM GSH (■), 50μM sodium selenite (○) or 50μM sodium selenite and 50 μM GSH (□). Points are mean ± S.D. of three measurements.

primary product from the reaction between selenite and GSH, was investigated. SDG was synthesised essentially according to the method of Ganther (1971) (see chapter 3 section 3.2.10 for more details). In outline, SDG was synthesised *in vitro* by incubating selenite and GSH at a ratio of 1:4 at room temperature for 30 min. A trace amount of ^{35}S-GSH was also included in the reaction to enable the SDG to be monitored during subsequent purification steps. The reaction products, SDG and GSSG, were separated by ion exchange chromatography on Dowex-50 resin columns saturated with nickel chloride. Nickel is incorporated into the column since it produces a differential retardation of SDG and GSSG such that the two products can be separated from the column as two distinct peaks with SDG eluting as the second peak (figure 4.3). Fractions from the second peak were pooled and adsorbed onto a second Dowex-50 resin column and washed with formic acid. This served to concentrate the SDG and remove the nickel. SDG was then eluted with ammonium acetate buffer. The buffer was exchanged for H_2O by chromatography of SDG-containing fractions on a G100-sephadex column and acetic acid was added to give a final concentration of 10mM. The purity of the SDG was confirmed using ultraviolet spectrum absorption analysis and thin layer chromatography (TLC). The ultraviolet absorption spectrum (figure 4.4) was identical to that reported previously for SDG (Ganther, 1971) with a peak at 263nm and an absorbance extending to 400nm. TLC analysis of the SDG run in parallel against standards which contained GSSG, GSH or a mixture of selenite and GSH (figure 4.5) followed by ninhydrin staining, showed that SDG ran as a single spot and had the predicted mobility in relation to the other standards as reported by Ganther (1968).

4.3 Comparison of the effects of SDG and H_2O_2

A cloning assay was performed to determine the effect of SDG on cell viability. SDG was found to have a potent dose-dependent cytotoxic effect on C57 cells with a significant reduction in cloning efficiency after a 1h treatment in

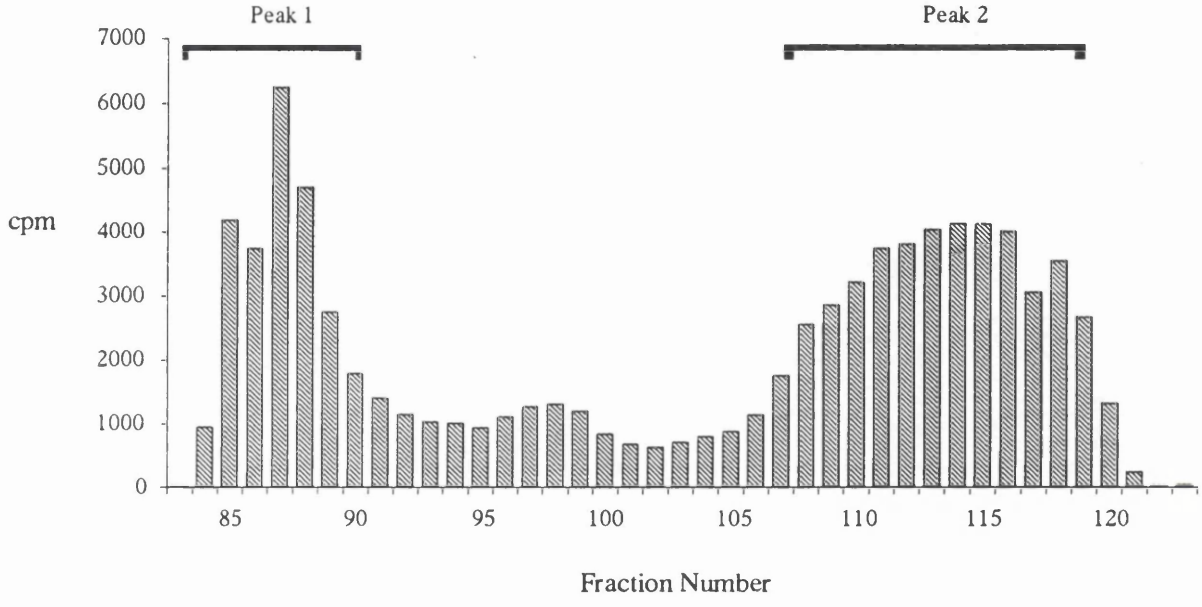


Figure 4.3
Separation of SDG and GSSG by chromatography on nickel chloride-saturated dowex-50 resin columns. 2ml fractions were collected and an aliquot measured for ^{35}S radioactivity. GSSG and SDG eluted as peaks 1and 2, respectively.

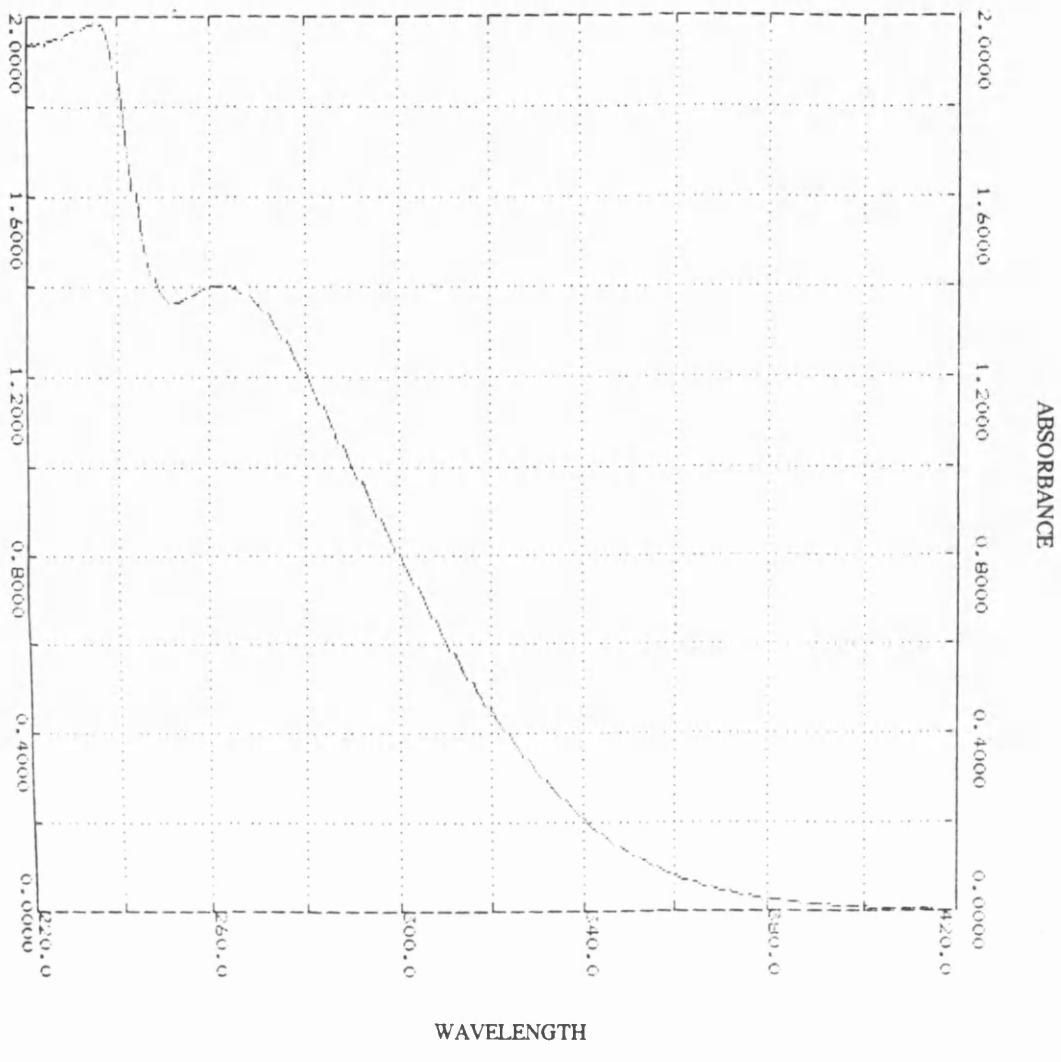
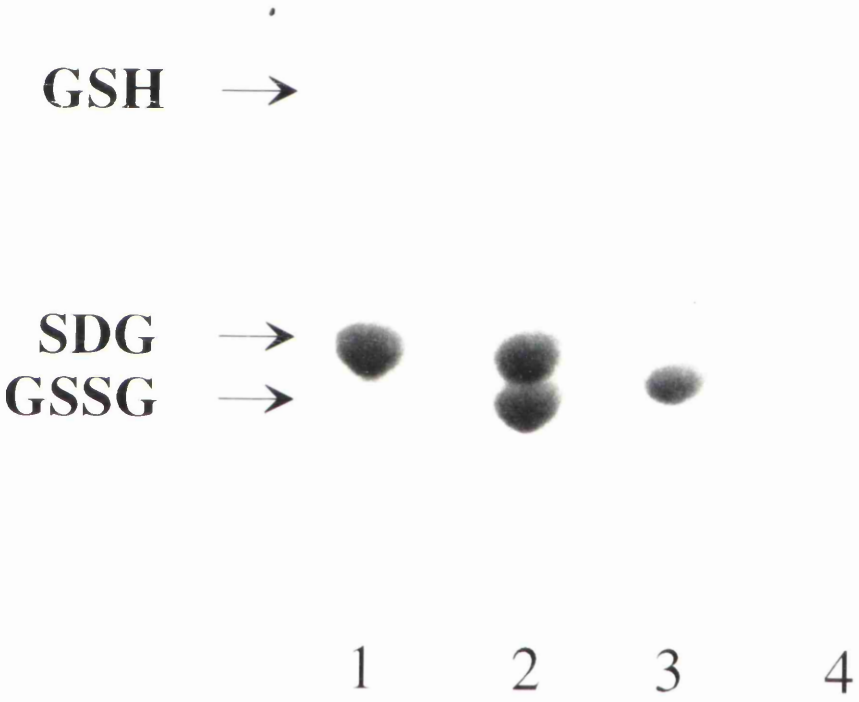


Figure 4.4
Absorbance spectrum of SDG

figure 4.5

Figure 4.5
Thin layer chromatography followed by ninhydrin staining. Positions of GSH, GSSG and SDG were deduced from known standards (lanes 2-4) and are indicated by arrows. (1) purified SDG, (2) selenite and GSH mixed at a 4:1 ratio and incubated for 0.5h at room temperature, (3) GSSG, (4) GSH


GSH
SDG
GSSG
1
2
3
4

increasing concentrations of SDG (figure 4.6). It has been proposed that selenium may act by inducing a state of oxidative stress since addition of selenite to cells has been shown to generate superoxide anion and hydrogen peroxide (Yan and Spallholz, 1993) and selenite-induced DNA strand breaks are oxygen dependent (Garberg *et al.*, 1988). It was therefore of interest to compare the cytotoxic effect of SDG and H_2O_2 on C57 cells. H_2O_2 was found to have a similar dose-dependent cytotoxic effect on C57 cells with a significant reduction in cloning efficiency after a 1h treatment (figure 4.6).

4.3.1 SDG and H_2O_2 induce similar morphological changes

Light microscopic analysis of cells treated with SDG or H_2O_2 and stained with hematoxylin and eosin, revealed similar morphological changes. Both 8μM SDG and 1.3mM H_2O_2 induced the appearance of cytoplasmic convolutions within 2h treatments (figures 4.7). However, no changes in gross nuclear and chromatin structure were evident at 2 or 4h (stained with the DNA-binding fluorochrome, DAPI) after treatment with either agent (figure 4.7). These morphological changes are suggestive of apoptosis but not in any way conclusive.

4.3.2 Induction of 50kb genomic DNA fragments is specific to SDG treatment

A common feature of apoptosis is the cleavage of genomic DNA into high molecular weight fragments (Oberhammer *et al.*, 1993; Bicknell *et al.*, 1994). Pulsed-field gel electrophoresis was used to analyse genomic DNA from C57 cells before and after SDG treatment since this technique enables separation and resolution of large DNA fragments ranging in size from 20kb to 2Mb. At time 0h, C57 genomic DNA consisted solely of high molecular weight material which did not migrate out of the well (figure 4.8A, lane 1). However, within 1h treatment with 8μM SDG there was a clear induction of cleavage of genomic DNA into 50Kb fragments (figure 4.8A, lanes 2-5) which could still be detected at 2,3 and 4h. To a much lesser extent, SDG also caused cleavage of DNA into 560Kb

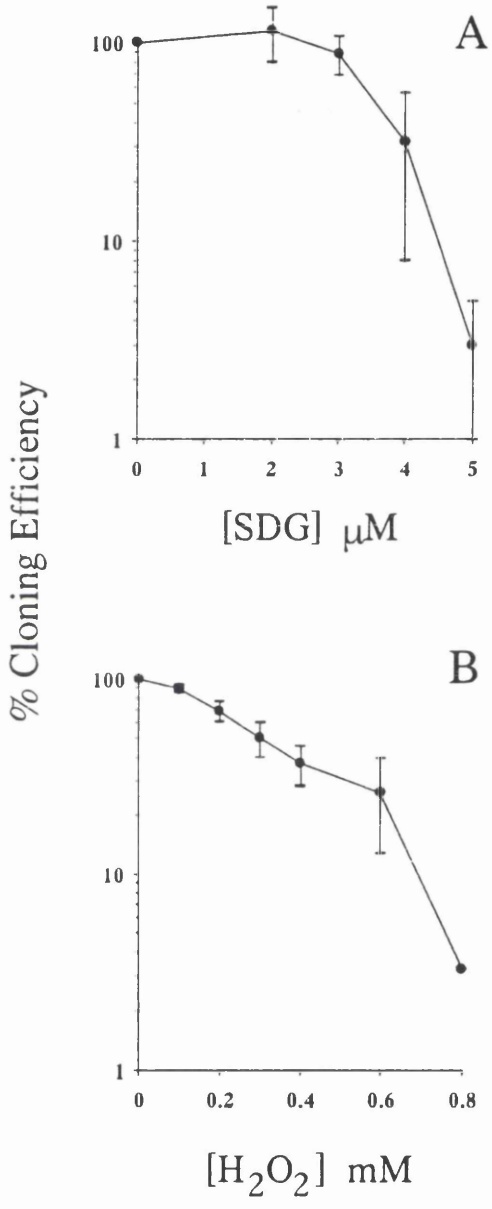


Figure 4.6
Cloning efficiency of C57 cells after 1h treatments with (A) SDG and (B) H_2O_2. Points are mean ± S.D. of three measurements

figure 4.7

Figure 4.7
Hematoxylin- and eosin-stained cytospins of C57 cells before (A) and after 2h treatments with (B) 8μM SDG and (C) 1.3mM H_2O_2. DAPI-stained cytospins of C57 cells before (D) and after 4h treatments with (E) 8μM SDG and (F) 1.3mM H_2O_2.

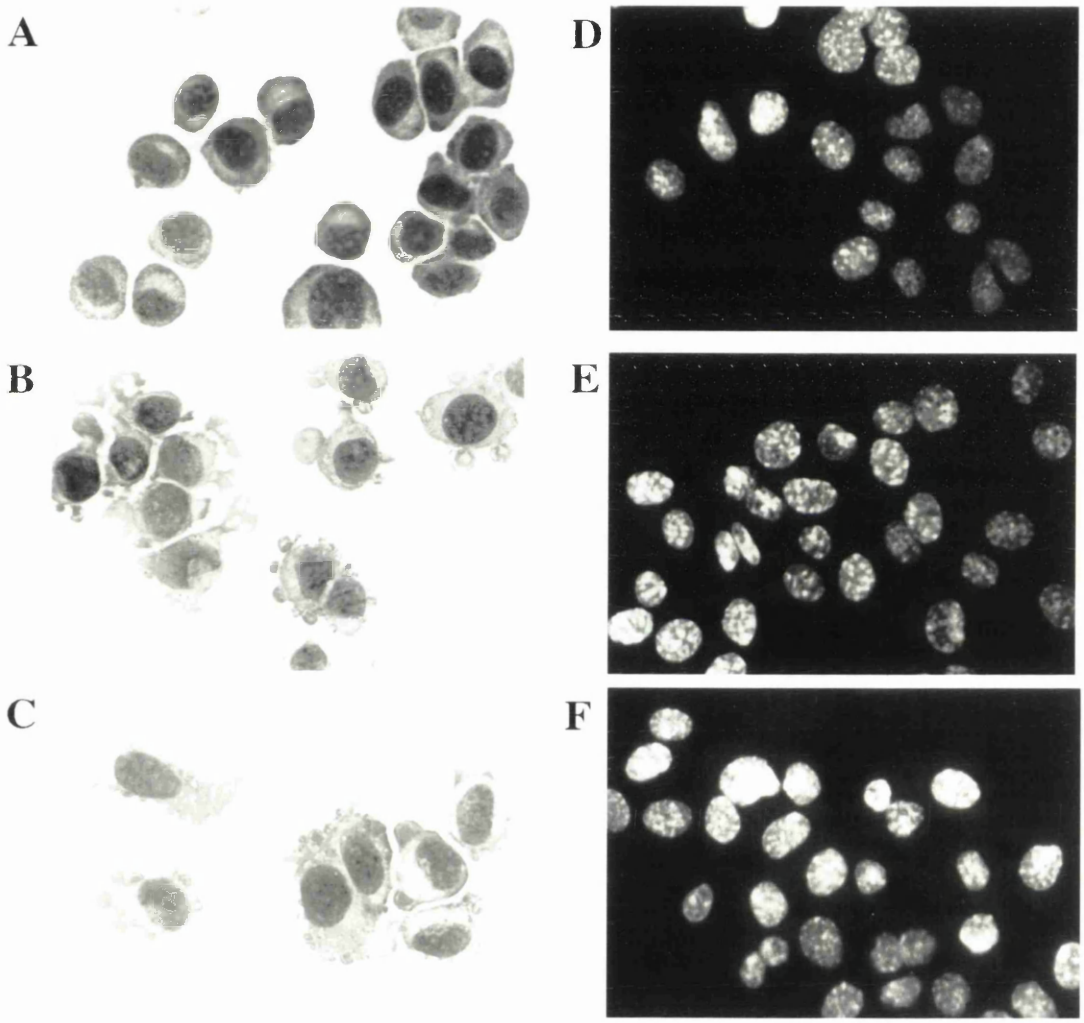
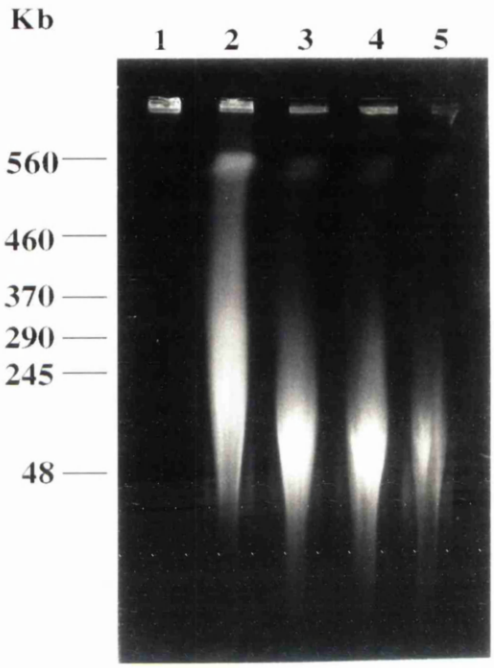

A
B
C
D
E
F
10 μm

figure 4.8

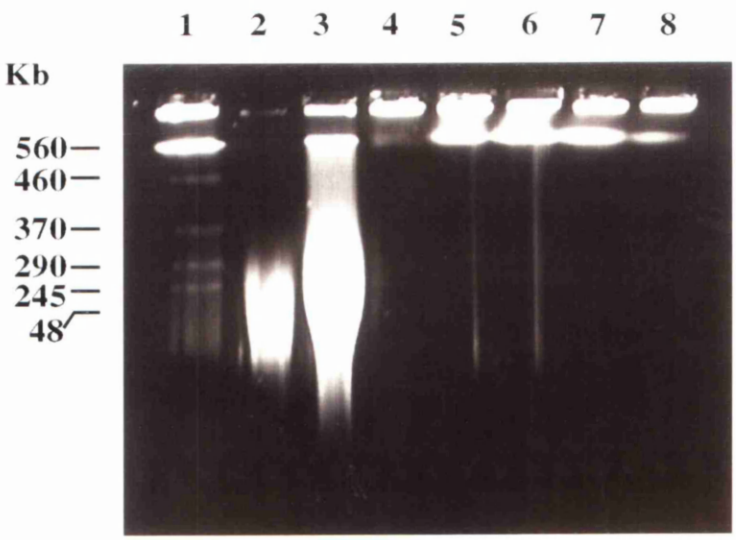
Figure 4.8

Pulsed field gel electrophoresis of DNA from C57 cells treated with (A) 8μM SDG for 0,1,2,3 and 4 h (lanes 1-5 respectively) and (B) 1.3mM H_2O_2 for 0,1,4,8 and 24h (lanes 4-8 respectively). In (B), as a control, C57 cells were treated with 8μM SDG for 2h (lane 3). Lanes 1 and 2 are molecular weight markers and λ DNA (48Kb) respectively.

A


Kb
1
2
3
4
5
560
460
370
290
245
48

B


1
2
3
4
5
6
7
8
Kb
560
460
370
290
245
48

fragments (figure 4.8A, lanes 2-5). The specific cleavage of genomic DNA into 50Kb fragments is highly characteristic of apoptosis (Oberhammer *et al.*, 1993). Cleavage of genomic DNA into 50Kb fragments, however, could not be detected in C57 cells treated with 1.3mM H_2O_2 for 1,4,8 and 24h (figure 4.8B, lanes 4-8) although this concentration of H_2O_2 is known to kill >95% of the cells (figure 4.6). There did however appear to be induction of cleavage of DNA into 560Kb fragments (figure 4.8B, lanes 4-8).

4.3.3 Differential effects of SDG and H_2O_2 on gene expression

To investigate further the cytotoxic effects of SDG and H_2O_2, the expression of a number of genes were studied in response to the two compounds. Northern analysis was used to determine the mRNA levels of PHGPX and GPX, seleno-enzymes responsible for H_2O_2 detoxification, and the GST Ya subunit of GST α which has selenium-independent glutathione peroxidase activity. A full-length cDNA probe for rat PHGPX was kindly provided by Dr. Roger Sunde (Nutrition Cluster, University of Missouri, Columbia, U.S.A.). For GPX, a cloned 700bp EcoR1 fragment from the mouse GPX gene containing exon 2 was used as a probe (Chambers *et al.*, 1986). The probe for GST Ya detection was made as described in chapter 3, section 3.2.8.4. Briefly, reverse transcription was used to synthesise first strand cDNA from C57 total RNA using an oligo(dT) oligonucleotide as a primer. GST Ya cDNA was then amplified using primers designed from sequences that contain the translation initiation and termination codons, thus amplifying the entire coding region. The resulting 650bp fragment was sequenced using an automated sequencer and was found to agree with the published sequence of GST Ya (Daniel *et al.*, 1987). Re-hybridisation of stripped filters with a cDNA probe for 7s rRNA was used as an internal loading control. 7s rRNA which is a component of the ribosome is relatively stable in comparison to mRNAs and was therefore a suitable choice as a measure of total RNA. The

figure 4.9

Figure 4.9
Northern analysis to determine the levels of GPX, PHGPX and GST Ya mRNA levels after treatment with 1.3 mM H_2O_2 for the indicated times. Figures below the autoradiographs indicate levels of mRNA relative to time zero using 7S rRNA as a loading control. Similar results have been obtained in two other experiments.

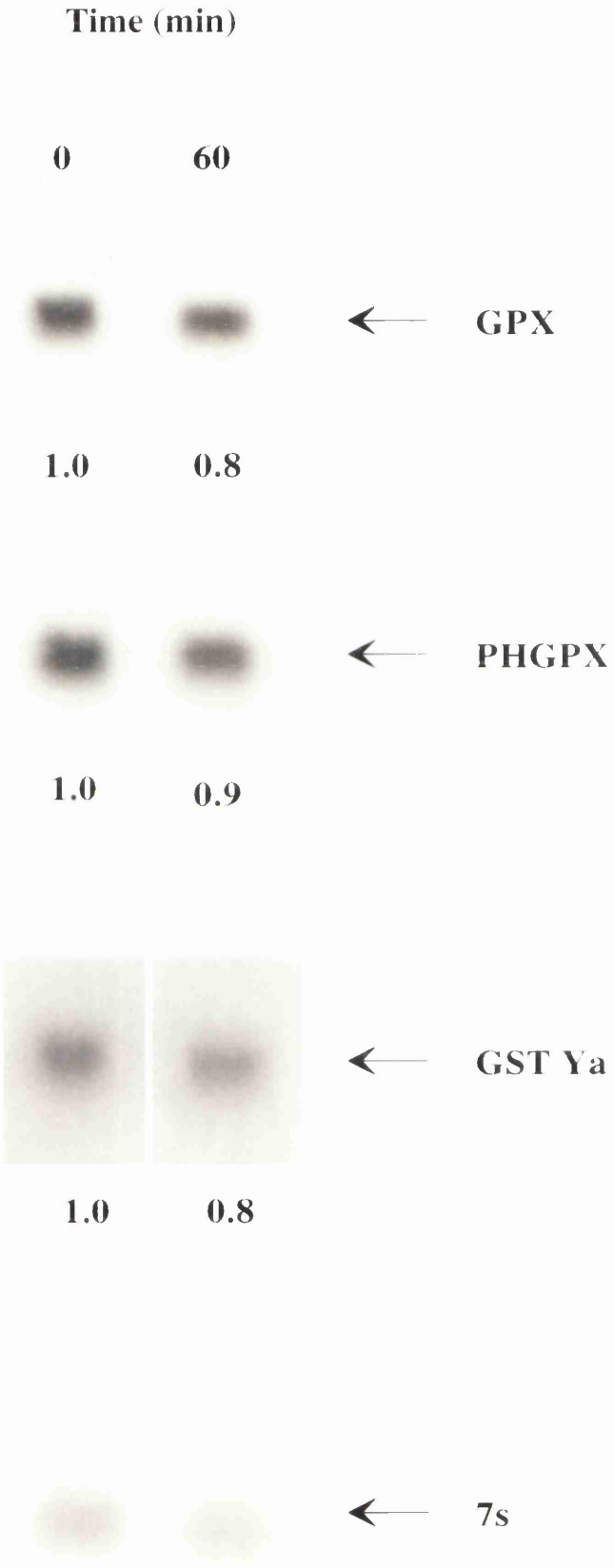
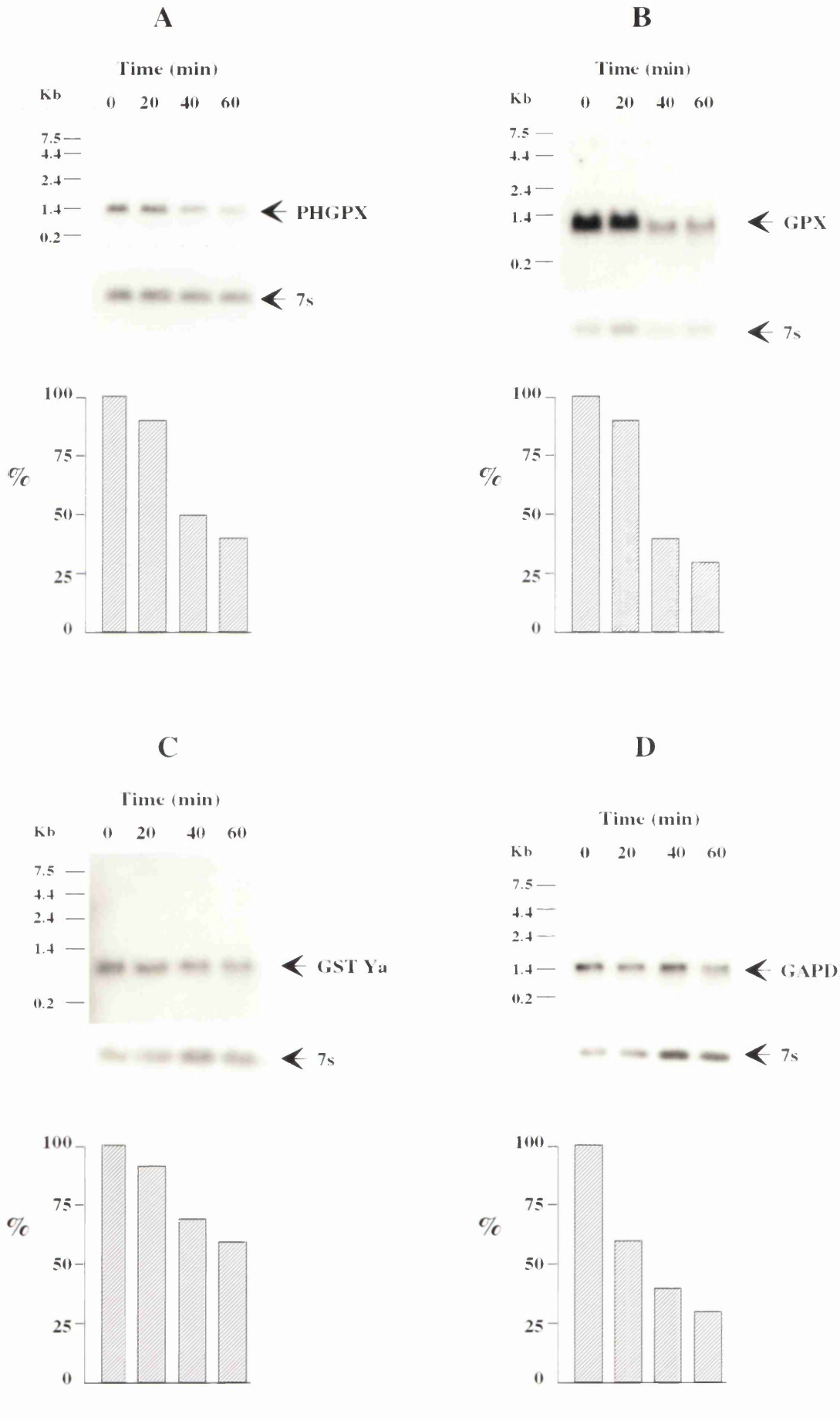

Time (min)
0
60
GPX
1.0
0.8
PHGPX
1.0
0.9
GST Ya
1.0
0.8
7s

figure 4.10

Figure 4.10
Northern analysis to determine the mRNA levels of (A) PHGPX, (B) GPX, (C) GST Ya and (D) GAPDH after treatment with 8μM SDG for the indicated times. Hatched bars indicate % levels of mRNA relative to time zero using 7S rRNA as a loading control. Similar results have been obtained in 2-5 other experiments.


A
Time (min)
Kb
0 20 40 60
7.5
4.4
2.4
1.4
0.2
PHGPX
7s
100
75
50
25
0
%
B
Time (min)
Kb
0 20 40 60
7.5
4.4
2.4
1.4
0.2
GPX
7s
100
75
50
25
0
%
C
Time (min)
Kb
0 20 40 60
7.5
4.4
2.4
1.4
0.2
GST Ya
7s
100
75
50
25
0
%
D
Time (min)
Kb
0 20 40 60
7.5
4.4
2.4
1.4
0.2
GAPDH
7s
100
75
50
25
0
%

relative amounts of test mRNA to 7s rRNA were calculated by densitometric analysis of autoradiographs.

Treatment of C57 cells with 1.3mM H_2O_2 for 1h had no significant effect on the levels of any of the tested mRNAs (figure 4.9). In contrast, treatment with 8μ M SDG caused a down regulation of all three mRNAs by ~50% within 40 min (figures 4.10). The northern blots were re-probed with a cDNA for the house-keeping gene GAPDH to determine if down-regulation of GPX, PHGPX and GST Ya mRNAs was specific or just a consequence of a general lowering of mRNAs. GAPDH mRNA levels were found to be down-regulated in a similar manner with ~50% reduction by 40 min (figure 4.10). This suggested that SDG was inducing a general down-regulation of mRNA levels. The different effects of SDG and H_2O_2 on gene expression suggests that these two compounds act by distinct mechanisms.

4.4 Summary

The growth inhibitory potential on mammary C57 cells of a number of seleno-compounds that have varying degrees of anti-carcinogenic activities in animal tumour models were studied. Sodium selenite was found to be the most potent compound. However, the fact that (1) selenite only had an effect after a lag period of 48h, (2) GSH could abolish this delay in growth inhibition, and (3) SDG had a rapid cytotoxic effect and was more effective than selenite, suggested that selenite needed to be metabolised by reduction with GSH to exert a growth inhibitory effect.

The reduction of selenite by GSH has been shown to generate reactive oxygen species (Yan and Spallholz, 1993). Additionally, we and others have previously shown seleno-compounds, including SDG, to induce apoptosis (Lanfear *et al.*, 1994; Lu *et al.*, 1994; Thompson *et al.*, 1994) and oxidative stress has been implicated in mediating apoptosis (Buttke and Sandstrom, 1994). The strong link between selenium metabolism, oxidative stress and apoptosis prompted us to test

the hypothesis that SDG induces apoptosis in C57 cells and that this involves the generation of reactive oxygen species. SDG was found to induce the formation of 560 and 50Kb DNA fragments which is suggestive of apoptosis (Oberhammer *et al.*, 1993; Bicknell *et al.*, 1994). However, other key features of an apoptotic cell, notably chromatin condensation and nuclear fragmentation, could not be observed. SDG appeared to induce a cell death distinct from H_2O_2-induced oxidative stress as judged by (1) the maintenance of PHGPX, GPX and GST Ya mRNAs and (2) the absence of 50Kb DNA fragments during H_2O_2-induced cell death.

Chapter 5

Generation of selenium-resistant mutants

5.1 Introduction

Since SDG appeared to be inducing cell death by a mechanism distinct from H_2O_2, a genetic approach was adopted to analyse the mechanism of SDG-induced cell death. A common strategy to studying mechanisms of drug action is the generation of drug-resistant cell lines derived from a drug-sensitive parental cell line. These lines can then be studied to determine biochemical differences that may be important for drug resistance with the possible identification of resistance genes. This approach minimises the problems of interpretation that occur when differences are being compared between cell lines derived from different sources, since changes are being identified against a common genetic background. Additionally, it is possible to identify genes that affect drug sensitivity that may not necessarily be affected by drug administration and so would not be detected when studying drug-induced effects.

A strategy was therefore devised to isolate selenium-resistant variants of C57 cells. Traditionally, one approach to isolating drug-resistant cell lines is by growing cells in the presence of drug and increasing the concentration by small increments over a period of time. Partially resistant variants are therefore selected for with each successive increase in drug concentration. One disadvantage of this approach is that the resulting resistant cell line is likely to be heterogeneous and therefore consist of a mixture of different resistant variants. Furthermore, because the strategy involves multiple rounds of selection the mode of resistance may involve a number of mechanisms making interpretation of any differences between sensitive and resistant cell lines complex.

An alternative approach is a one-step selection whereby drug resistant cells are selected for in a high dose of drug. This strategy overcomes the problems associated with the multi-stage selection approach since the resistant variants are

clonal and have arisen from one round of selection. A further advantage is that a number of independently arising resistant variants with different resistant mechanisms may be selected for in this manner. However, if the actions of a drug is bimodal with a high drug concentration having different effects from low drug concentrations, the resistance mechanisms that are selected for at a high drug concentration may not necessarily be the same as those that might be selected for in an incremental-dose selection strategy.

5.2 Choice of selective agent

A number of possible strategies for selecting selenium-resistant cells were evaluated. Initially, C57 cells were grown continuously in the presence of 50μM sodium selenite. However, the delay of 24-48h before selenite had an effect proved problematical with this approach since after each refeeding, there would be no selective pressure for 24-48h during which non-resistant cells could recover and proliferate, thus reducing the enrichment procedure for potentially selenium-resistant cells. Additionally, other work in the group (Dr. A. Wong, unpublished data) demonstrated that the effect of selenite was cell density-dependent. A similar cell-density dependent effect of selenite-induced growth inhibition has also been demonstrated in other cell systems (Medina *et al.*, 1985; LeBoeuf *et al.*, 1985). This explained the observed delay before selenite had an effect and suggested that selenite acted through the production of a secreted growth inhibitor that needed to reach a certain threshold concentration in the medium before having an effect. The fact that selenite toxicity was dependent on cell-density made selenite an unsuitable agent to select for selenium-resistant cells since it was impossible to ensure constant selective conditions. In view of the evidence that SDG is the active metabolite of selenite, SDG appeared to be a more suitable choice of agent for selection of selenium-resistant cells since it had a rapid cytotoxic effect and appeared to be cell density-independent. However, SDG is unstable in culture medium and loses all growth inhibitory activity within a 24h

incubation period in 10% FCS at 37°C, compared to SDG added freshly to medium (figure 5.1). This obviously presented practical difficulties in maintaining a constant SDG concentration during selection. This would be critical for an incremental dose-selection strategy, since a drop in selective pressure would allow non-resistant cells to proliferate. This would be particularly problematical during the early rounds of selection when the kill-rate would be low. A one-step selection using a high SDG dose was therefore chosen to generate SDG-resistant variants of C57 cells.

5.3 Optimisation of conditions for selection

The frequency of a SDG-resistant cell spontaneously arising within a population of cells is dependent on a number of factors such as the mutation frequency, the spectrum of mutations or number of potentially mutable genes that may give rise to resistance, and whether the mutation is dominant or recessive. It was therefore necessary to determine the dose of SDG required to select for such a rare event. Conditions were therefore chosen to isolate a variant arising at a frequency of 1 SDG-resistant variant/10^5-10^6 viable cells.

A dose-response kill curve for C57 cells with SDG treatment was determined by measuring the cloning efficiency of C57 cells in the presence of increasing doses of SDG. This produced a more sensitive and accurate measurement of cell viability than other assays such as growth curves or ^{3}H-thymidine uptake which are often used to assess cell survival. C57 cells were seeded into Petri dishes at a density of 1-4 x 10^5 cells/dish and allowed to adhere for 24h. The cells were then treated with a range of SDG concentrations and incubated for 10-14 days. In parallel, the plating efficiency of cells was determined by counting the number of colonies arising from 100 cells seeded into non-selective growth medium. The number of clonogenic cells seeded before SDG treatment could then be determined and the cloning efficiencies calculated and expressed as the number of colonies/viable cell. SDG produced a steep dose-response curve which was variable between

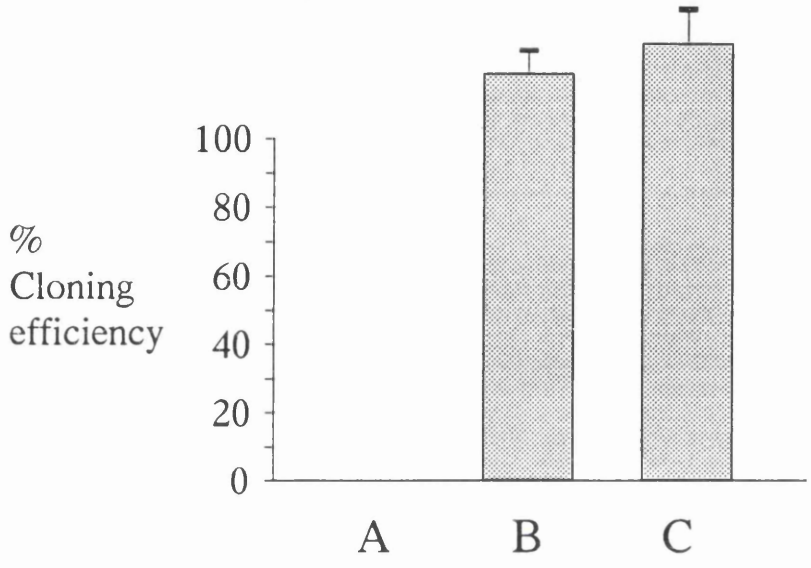


Figure 5.1
Cloning efficiency of C57 cells in (A) fresh culture medium with 4.5μM SDG, (B) culture medium with 4.5μM SDG after pre-incubation at 37°C for 24h and (C) culture medium after pre-incubation at 37°C for 24h. Values are expressed as a percentage of the cloning efficiency of C57 cells in fresh culture medium. Bars are mean ± S.D. of four measurements.

successive experiments (table 5.1). This fluctuation in kill-rate is probably due to factors in the culture medium that affect the intrinsic instability of SDG. These could include the ratio of GSH:GSSG which may fluctuate with culture medium age and therefore affect SDG degradation, or pH changes that occur when a large number of Petri dishes are being manipulated in the low CO_2 atmosphere of a tissue culture hood. A number of steps were therefore taken in order to try and standardise the SDG dose-response curve. Foetal calf serum was aliquoted, stored at -20°C and thawed only as required to minimise the time it was kept at 4°C. HEPES buffer was also added at10mM to minimise pH fluctuations in the culture medium. However, despite these precautions, consistent dose-response curves were unobtainable. In each experiment, a range of SDG concentrations was therefore chosen to select for resistant mutants. This ensured that for any given experiment a dose of SDG that gave the desired kill would be included.

5.4 Selection of SDG-resistant mutants

From experiment 1 (table 5.1) 3 colonies (C57A, B and G) that had arisen in 7 μM SDG at a frequency of $1.3/10^5$ viable cells were isolated. These were tested for an increased resistance to SDG as compared with the parental C57 cells by measuring cell growth over a period of 3 days in the presence and absence of 7μM SDG. For convenience, growth was monitored by measuring nucleic acid concentration of cell lysates, as determined by measuring absorbance at 260nm. This figure was assumed to be linearly related to cell number. In the absence of SDG all three clones grew at a similar rate to that of C57 cells. However, in the presence of 7μM SDG, one clone, C57A, had a significantly higher number of cells 1 and 3 days after SDG addition as compared with C57 cells and the other 2 clones (figure 5.2). Additionally, under cloning conditions, C57A cells were also more resistant (figure 5.3). However, this difference was marginal and was only stable for about 40 days growth in non-selective media after which C57 and C57A cells displayed similar SDG sensitivities (figure 5.3). This suggested that C57A cells

Cloning Efficiency ($x10^{-5}$) (colonies/viable cell)

	[SDG] μM					
	2	**4**	**5**	**6**	**7**	**10**
Expt.1:	Confluent		Confluent		1.3	<0.1
Expt 2:		500	46	7.8	0.25	

Table 5.1
Cloning efficiency of C57 cells in the presence of SDG. Values were calculated as a mean of 5-10 plates.

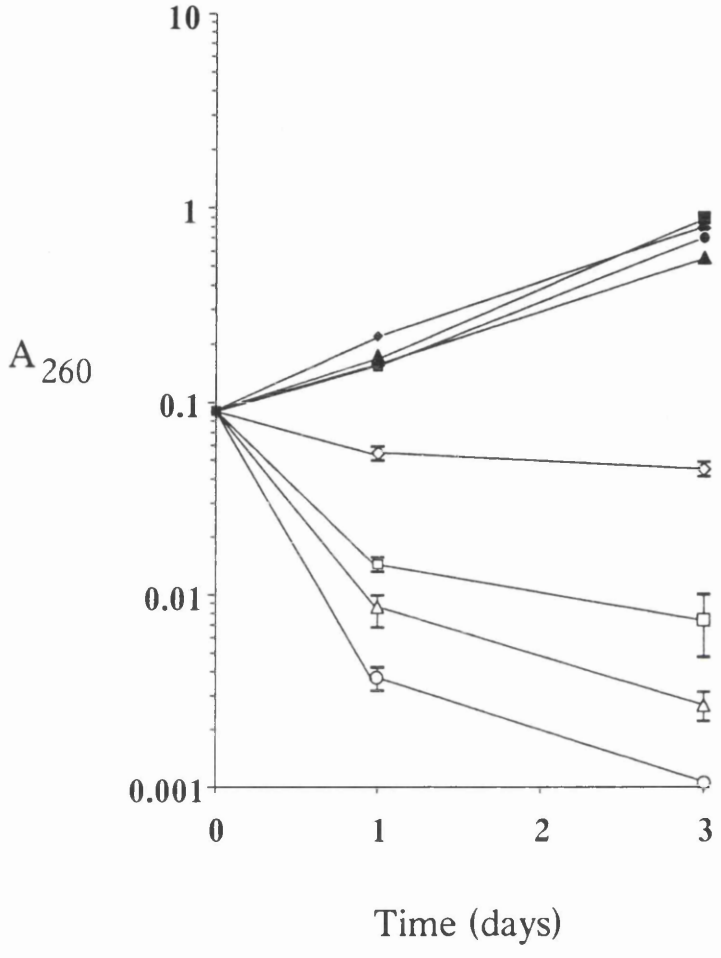


Figure 5.2
Cell growth, as measured by absorbance of cell lysates at 260nm, of C57 (circles), C57A (diamonds), C57B (triangles) and C57G (squares) cells in control culture medium (solid shapes) and 7μM SDG (open shapes). Points are mean ± S.D. of three measurements.

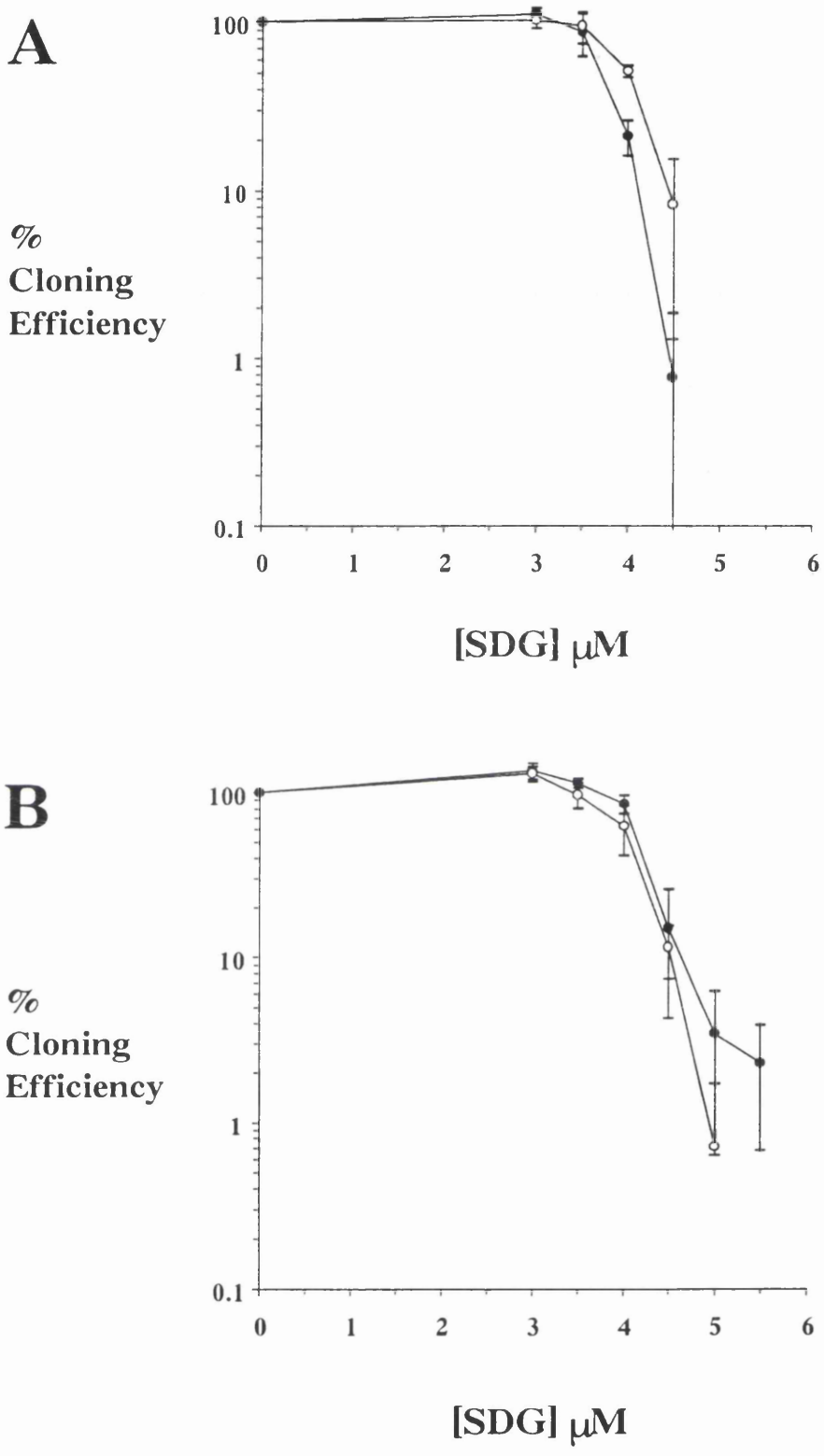


Figure 5.3
Cloning efficiency of C57 (•) and C57A (○) cells in SDG after (A) 20 and (B) 40 days culture in the absence of SDG. Points are mean ± S.D. of three measurements.

had acquired some unstable epigenetic change that conferred a SDG-resistant phenotype only transiently. C57A cells were therefore an unsuitable system in which to study mechanisms of SDG resistance.

5.5 Mutagenesis of C57 cells prior to selection in SDG

In the generation of drug resistant lines, cells are commonly treated with mutagen prior to selection to increase the mutation frequency. A commonly used mutagen is ethyl methanesulphonate (EMS) which is a monofunctional ethylating agent. EMS has been shown to cause a wide range of mutations covering base pair transitions, insertions and deletions to more extensive intragenic deletions and chromosome breaks (Sega, 1984). Since it is unknown what type of mutations are likely to give rise to SDG resistant mutants, EMS seemed a suitable choice of mutagen for use in the single step selection approach.

5.5.1 Optimisation of conditions for mutagenesis

The dose of EMS with which to mutagenize C57 cells prior to selection was decided by constructing a dose-response kill-curve. The cloning efficiency of logarithmically growing C57 cells was determined after treatment with increasing concentrations of EMS for 2h in serum-free growth medium (figure 5.4). A concentration of 1.75mg/ml EMS was chosen as this gave a survival rate of 20%, a figure which is commonly used by other workers.

5.5.2 Selection of SDG-resistant mutants after mutagenesis with EMS

After mutagenesis with EMS, C57 cells were incubated for 24h in the absence of mutagen. Cells were then seeded into Petri dishes at a density of 2 x 10^5 cells/dish and incubated for a further 24h. A total of 48h incubation in non-selective growth medium post EMS treatment allowed the cells to both recover from the mutagenic insult and express any mutations, such as transcription of genes that have been activated or degradation of mRNA from genes that have

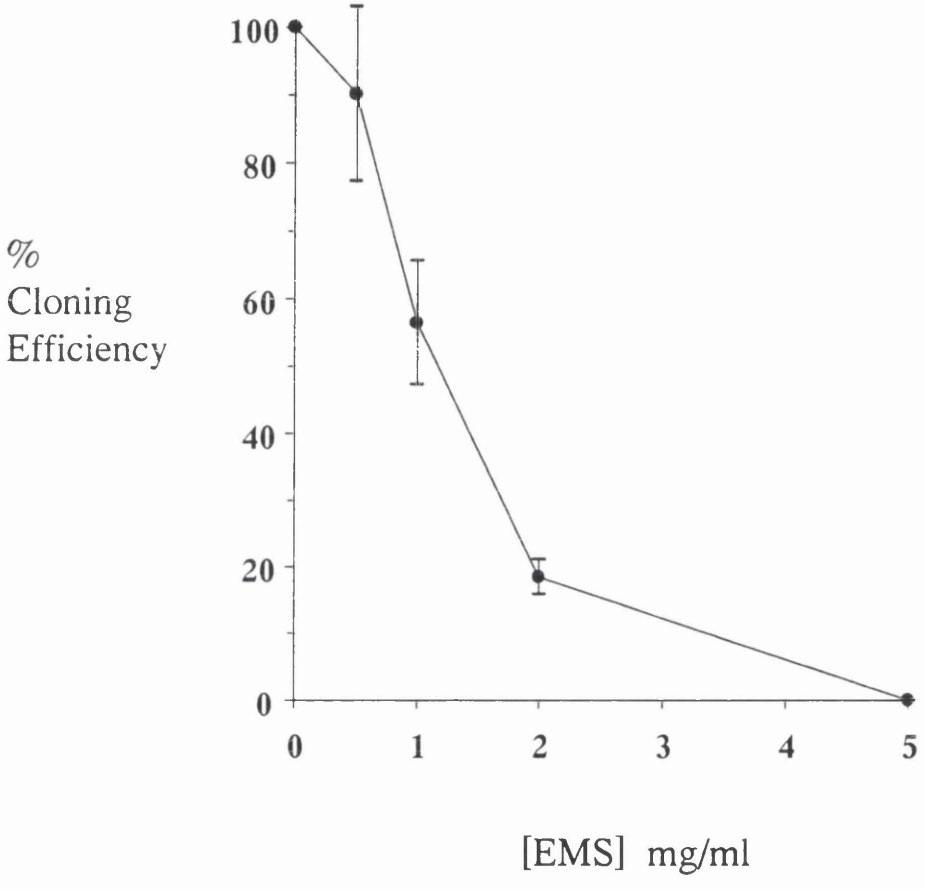


Figure 5.4
Cloning efficiency of C57 cells after a 2h exposure to EMS in serum-free culture medium. Points are mean ± S.D. of three measurements.

been silenced. Mutagenized cells were then exposed to either 5 or 6μM SDG and incubated for 10-14 days. From cells treated with 6μM SDG, five colonies were isolated from a total of 1.8 x 10^6 viable cells plated. All five clones displayed varying degrees of resistance to SDG when re-cloned in SDG (table 5.2). However, only 2 clones (B15 and B19) remained stably resistant after at least 50 days growth in non-selective growth medium suggesting that the resistant phenotype was due to some stable genetic change (figure 5.5). Differences in cloning efficiencies in SDG of C57 cells compared to B15 or B19 cells were statistically significant over a range of SDG-concentrations (figure 5.5).

5.6 Comparison of selenium-resistant and -sensitive cell lines

Drug resistance may be acquired by a variety of mechanisms such as decreased drug uptake or metabolic activation, or increased detoxification. Additionally, pathways through which the drug induces cell death may be altered and this could affect drug sensitivity indirectly. A number of approaches were therefore adopted to identify biochemical changes conferring resistance to SDG. B19 cells were chosen for this purpose as they displayed the greatest resistance to SDG.

5.6.1 B19 cells are cross-resistant to sodium selenite but not H_2O_2

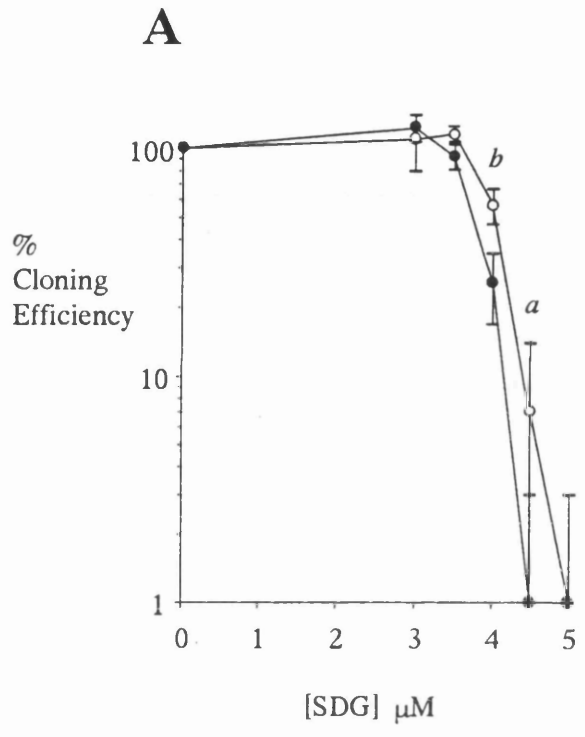
Sensitivity to growth inhibition by selenite was measured for C57 and B19 cells to determine if the mutation(s) acquired by B19 cells specifically conferred resistance to SDG or affected sensitivity to other selenium compounds. Logarithmically growing C57 and B19 cells were exposed to 50μM sodium selenite and cell growth monitored over a period of days by measuring cell number. Both cell lines displayed the same delay of 24h before selenite began to inhibit cell growth. However, B19 cells were inhibited to a significantly lesser extent thereafter (figure 5.6A). B19 cells were therefore resistant to the growth inhibitory effects of both SDG and selenite.

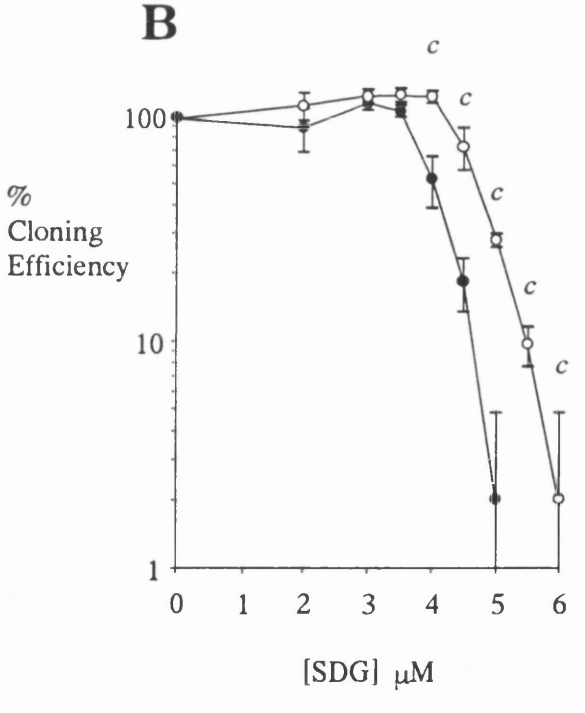
	Cloning Efficiency		
	[SDG] μM		
Cell Line	**0**	**3.5**	**4.5**
B15	100	49	1
B16	100	47	4
B17	100	105	2
B18	100	67	0
B19	100	100	20
C57	100	21	0

Table 5.2
Cloning efficiency of C57 cells and four clones selected in 6μM SDG. Values are mean ± S.D. of three measurements.

figure 5.5

Figure 5.5
Cloning efficiency of C57 cells (closed circles) in SDG compared to (A) B15 and (B) B19 cells. Poins are mean ± S.D. of three measurements. (*a*, $p<0.1$; *b*, $p<0.05$; *c*, $p<0.001$, students *t*-test.)


A
100
10
1
% Cloning Efficiency
b
a
0
1
2
3
4
5
[SDG] μM


B
100
10
1
% Cloning Efficiency
c
c
c
c
c
0
1
2
3
4
5
6
[SDG] μM

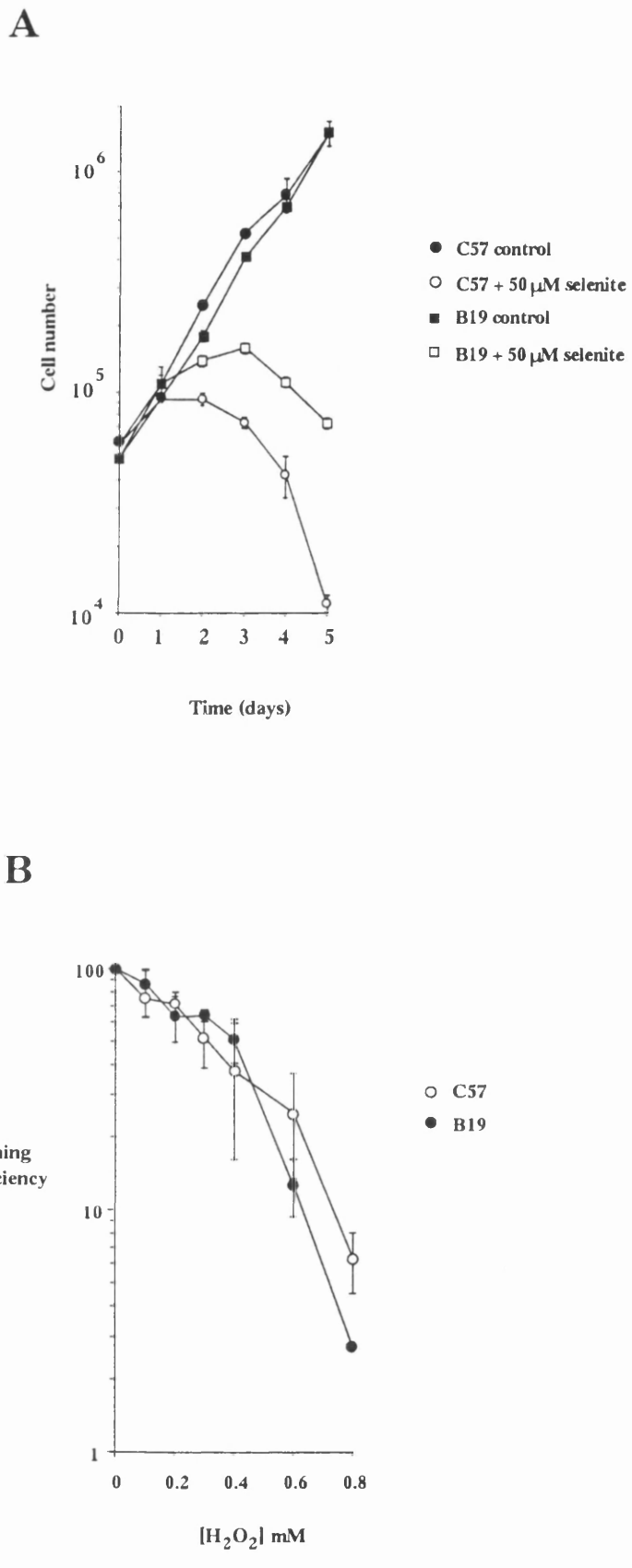


Figure 5.6
Comparison of the sensitivity of C57 and B19 cells to (A) 50μM selenite as judged by cell number and (B) H_2O_2 as judged by cloning efficiency. Points are mean ± S.D. of three measurements.

Since SDG had different biochemical effects in C57 cells from H_2O_2, such as production of 50Kb fragments and down-regulation of PHGPX, GPX and GST Ya mRNA levels, it was also of interest to determine if sensitivity to H_2O_2 was altered in B19 cells. However, cloning efficiencies after a 1h treatment with increasing concentrations of H_2O_2 were not significantly different between the two cell lines (figure 5.6B).

5.6.2 Rates of selenium-uptake are similar between B19 and C57 cells

Selenite uptake was measured between the two cell lines to determine if different rates of selenium uptake could be responsible for SDG-resistance. ~70nM [^{75}Se]-sodium selenite was added to logarithmically growing C57 and B19 cells and incubated at 37°C. At various time points, the culture medium was removed and cells were rinsed with phosphate-buffered saline. Adherent cells were then lysed *in situ* with 0.1M NaOH and the ^{75}Se radioactivity in the cell lysates was measured in a γ-radiation counter. The selenium uptake of cell lysates was measured over a period of 48h. During this period no significant difference in selenite uptake could be observed between the two cell lines (figure 5.7).

5.6.3 Intracellular GSH concentration is similar in C57 and B19 cells

The initial fate of selenite upon entry into a cell is reduction to selenide by GSH. This reduction appeared to be a rate-limiting step in the anti-proliferative effect of selenite since GSH potentiated the effect of selenite and SDG was a much more potent growth-inhibitor than selenite (compare figures 4.2 and 4.6). However, it is unknown if SDG has a direct effect or it too requires further reduction by GSH. It was therefore possible that B19 cells had a lower GSH concentration and that this affected the metabolism of selenite and SDG. The GSH levels in lysates from logarithmically growing C57 and B19 cells were therefore measured using an enzymatically recycling reaction in which glutathione is sequentially oxidized by 5,5'-dithio-bis-(2-nitrobenzoic acid) and reduced by

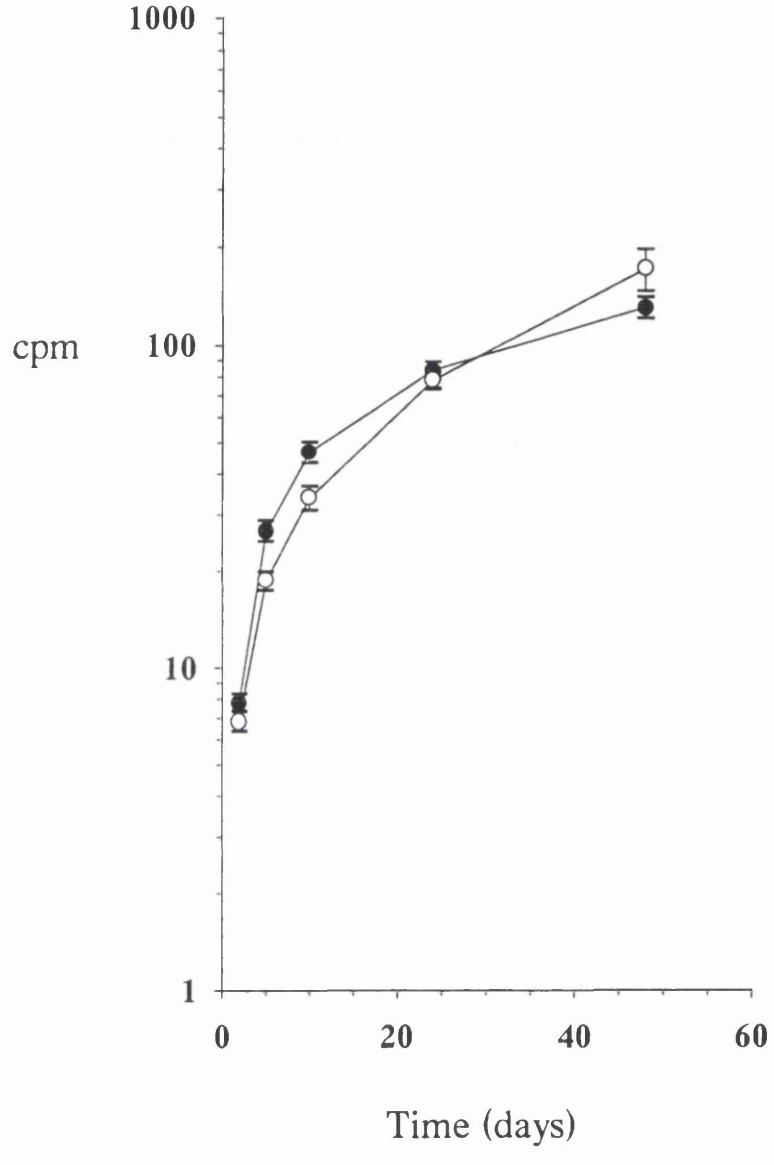


Figure 5.7
Selenite uptake in C57 (○) and B19 (●) cells as measured by cpm of lysates from cells grown in [^{75}Se]-sodium selenite. Figures are mean ± S.D. of three measurements.

NADPH and glutathione reductase. The production of the chromophoric product, 2-nitro-5-thiobenzoic acid, is thus directly proportional to the glutathione concentration and can be detected by absorbance at 412nm (see chapter 3, section 3.2.12 for details). Intracellular GSH concentrations were found to fluctuate between experiments for reasons that remain unclear. However, within experiments, where cells were exposed to identical growth conditions, no statistically significant difference (students *t*-test) in intracellular GSH concentrations between C57 and B19 cells could be detected (table 5.3).

5.6.4 Differences in gene expression between C57 and B19 cells

To further investigate the mechanism of selenium-resistance, the mRNA levels of a number of genes were compared between C57 and B19 cells. Although SDG did not appear to mediate all its effects through the generation of H_2O_2, the role of other ROS and organic peroxides, which are substrates for these enzymes, could not be eliminated. The mRNA levels of PHGPX, GPX and glutathione S-transferase α (GST α), which displays a selenium-independent glutathione peroxidase activity towards organic peroxides, were therefore investigated. Another GST family member, GST π, was also of interest since it is commonly involved in resistance against a wide spectrum of drugs. The mRNA levels of thioredoxin (TRX) was also investigated since both selenite and SDG in cell-free systems are potent inhibitors of this redox factor (Björnstedt *et al.*, 1992; Kumar *et al.*, 1992). The probes used to determine PHGPX, GPX and GST Ya mRNA levels were those previously described in chapter 4, section 4.3.3. The probe for TRX was made as described in chapter 3, section 3.2.8.4. Briefly, reverse transcription was used to synthesise first strand cDNA from C57 total RNA using an oligo(dT) oligonucleotide as a primer. TRX cDNA was then amplified using primers designed from sequences that contain the translation initiation and termination codons, thus amplifying the entire coding region. The sequence of the resulting 340bp fragment was confirmed by automated sequencing. Full length

	Cell line	[GSH] mg GSH/ g protein
Expt 1:	C57	2.2 ± 0.4
	B19	3.0 ± 0.5
Expt 2:	C57	1.0 ± 0.4
	B19	1.5 ± 0.3
Expt. 3:	C57	4.3 ± 1.0
	B19	4.6 ± 1.0

Table 5.3
Comparison of glutathione concentrations between C57 and B19 cells. Values are mean ± S.D. of four measurements for expt.1 and three measurements for expts. 2 and 3.

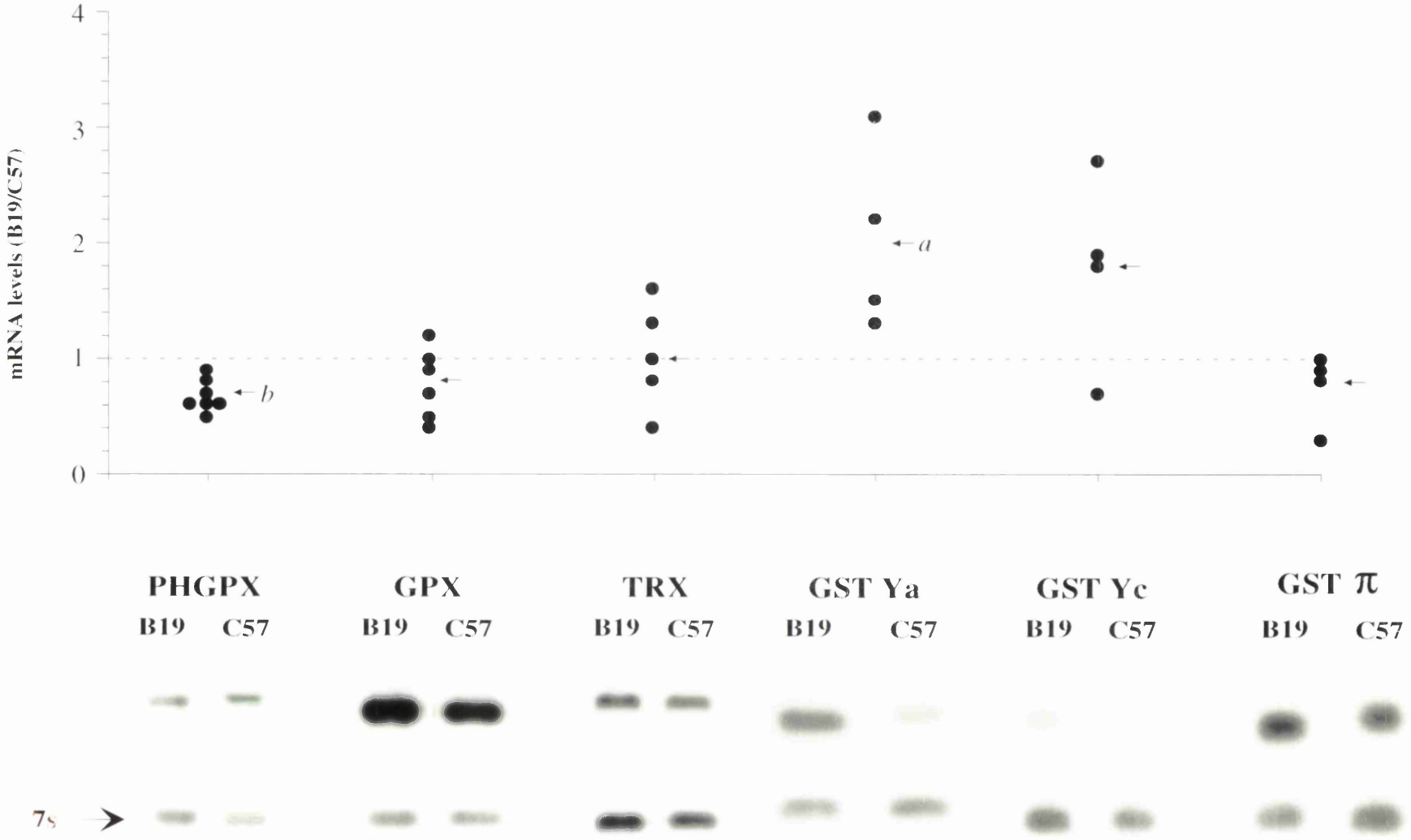
cDNA probes for GST π and GST Yc were kindly provided by Professor Roland Wolfe and Dr. John Hayes (both of ICRF, Biomedical Research Centre, Dundee, U.K.), respectively. Quantitation of mRNA levels was performed as described in chapter 4, section 4.3.3 using 7S rRNA as an internal loading control. mRNA levels were analysed in untreated C57 and B19 cells since it was possible that the mechanism of selenium resistance is constitutively active. Analyses were carried out on at least four independent RNA preparations for each cell line and the students *t*-test was used to determine any statistical significance for any differences detected between the two cell lines.

No significant differences were apparent for GPX, TRX, GST Yc and GST π mRNA levels between the two cell lines (figure 5.8). However, a significant ($p<0.01$) 2-fold down-regulation of PHGPX mRNA in B19 cells was observed. In contrast, GST Ya mRNA levels were significantly ($p<0.1$) 2-fold higher in the B19 cells. Although the GST Yc mRNA levels in the two cell lines were not significantly different at the $p<0.1$ level, the mean level (4 experiments) of GST Yc mRNA in B19 cells was 1.8-fold higher than the level observed in C57. Of the various GSTs, GST π mRNA levels appeared to be considerably higher than GST Ya and GST Yc mRNA levels as judged by the period of autoradiography required for a signal to be detected for the various mRNA species (<24h for GST π versus 3-7d for GST Ya and GST Yc).

Since SDG appeared to induce a cell death reminiscent of apoptosis, as judged by the appearance of 50Kb DNA fragments, the mRNA levels of a number of apoptosis-regulating genes were also analysed, namely *bcl-2* and *bcl-x*. BCL-2 is a membrane associated protein which can inhibit apoptosis induced under a number of conditions. The *bcl-x* gene encodes two gene products that are homologous to BCL-2 and result from differential splicing of the *bcl-x* transcript to give *bcl-x*$_S$ and *bcl-x*$_L$ mRNAs. *bcl-x*$_L$ encodes a protein that has similar apoptosis-inhibiting functions as BCL-2 whereas BCL-X$_S$ can antagonise BCL-2 function. A mouse *bcl-2* cDNA was kindly provided by Dr. Christopher Bartholomew (Beatson

figure 5.8

Figure 5.8
Northern analysis to determine the relative mRNA levels of a number of genes in C57 and B19 cells. Points are from independent experiments and indicate B19:C57 ratio of band intensities relative to 7s rRNA. Arrows indicate mean ratio for each mRNA examined (*a*, $p<0.1$; *b*, $p<0.01$ students *t*-test)


mRNA levels (B19/C57)
4
3
2
1
0
b
a
PHGPX
GPX
TRX
GST Ya
GST Yc
GST π
B19
C57
7s

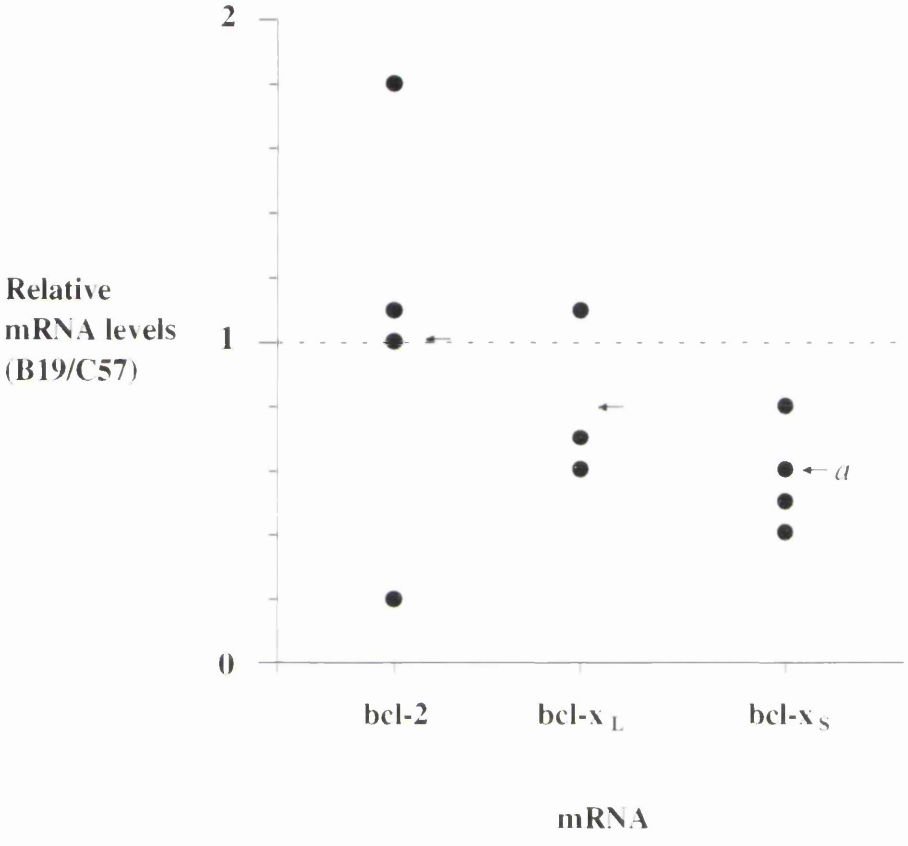
Institute for Cancer Research, Glasgow, U.K.). PCR was used to amplify the first 430bp of the *bcl-x* coding region from human genomic DNA (kindly provided by Kevin Ryan, Beatson Institute for Cancer Research, Glasgow, U.K.) since its sequence is common to both splice products. The sequence was confirmed by automated sequencing. *bcl-2* and *bcl-x*$_L$ mRNA levels were not significantly different between the two cell lines (figure 5.9). However, there was a significant ($p<0.05$) 2-fold reduction in *bcl-x*$_S$ mRNA in B19 cells (figure 5.9).

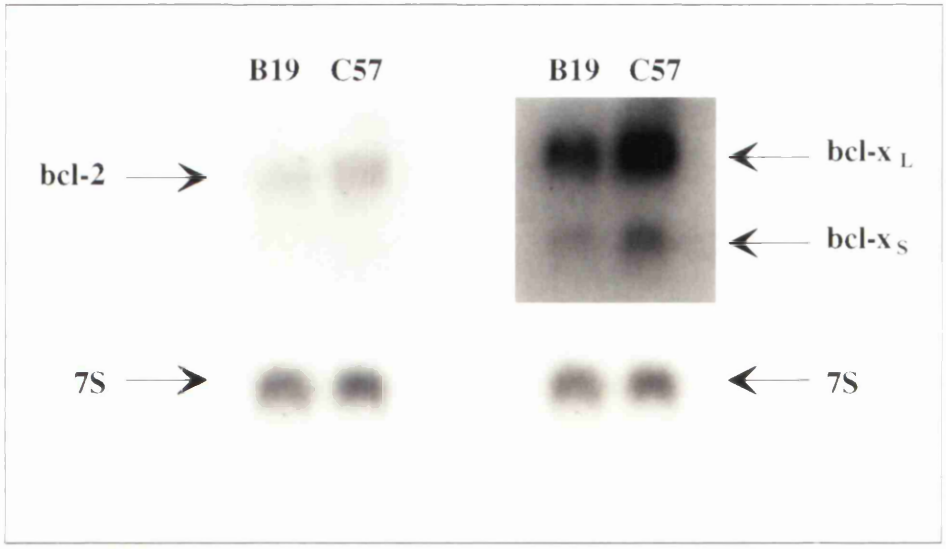
5.6.5 Differences in selenoprotein levels between C57 and B19 cells

Since selenite uptake and GSH concentration levels were similar between the two cell lines, the mechanism of selenium resistance may involve steps further downstream of selenium metabolism. Selenium functions primarily through the production of selenoproteins, such as GPXs, which contain [Se]Cys encoded by an in-frame UGA codon. Additionally, selenium-binding proteins exist that bind selenium in an unidentified manner. One hypothesis, therefore, as to how selenium acts as an anti-proliferative agent is through the production of a growth inhibitory selenoprotein(s) or selenium-binding protein(s). To investigate this hypothesis, the selenoprotein and selenium-binding protein complement of C57 and B19 cells was analysed. 18.5 μCi [75Se]-selenite was added to logarithmically growing cells and incubated for 96h. Culture medium was then removed, cells were rinsed with phosphate-buffered saline and adherent cells were lysed *in situ*. In collaboration with Lynn McGarry, two samples each from duplicate flasks of each cell line were analysed by 2D gel electrophoresis and autoradiographs of each gel were quantitated by densitometric analysis. Visual inspection of the autoradiographs (four per cell line) revealed that the complement of selenium-labelled proteins from the two cell lines are very similar with a total of 26 spots detected that ranged in size from 19 - 101KDa (table 5.4). A more detailed analysis was performed using densitometric quantitation between the two cell lines. For each cell line, gels were normalised to each other by expressing the intensity of each spot relative to that of

figure 5.9

Figure 5.9
Northern analysis to determine the relative mRNA levels of a number of genes in C57 and B19 cells. Points are from independent experiments and indicate B19:C57 ratio of band intensities relative to 7S rRNA. Arrows indicate mean ratio for each mRNA examined (*a*, $p<0.05$, students *t*-test)


2
1
0
Relative
mRNA levels
(B19/C57)
a
bcl-2
bcl-x L
bcl-x S
mRNA


B19 C57
B19 C57
bcl-2
bcl-x L
bcl-x S
7S
7S

Spot Number	Apparent MW (kDa)	Approximate pI
1	77.2	7.42
2	18.5	5.38
3 (↑)	29.0	5.10
4	49.1	5.02
5	66.2	6.02
6 (↑)	19.0	6.88
7 (↑)	35.0	5.08
8	47.0	6.85
9	54.5	5.04
10	42.3	5.03
11	21.1	5.27
12	22.5	6.13
13 (*)	72.2	6.33
14 (*)	72.5	6.45
15	82.8	6.49
16	23.4	5.27
17 (↑)	71.3	6.64
18 (↓)	101.3	5.63
19	66.0	6.38
20	25.9	5.27
21 (↓)	99.8	5.40
22	83.0	6.70
23	19.0	4.94
24	22.5	7.17
25	22.5	6.87
26	82.8	6.84

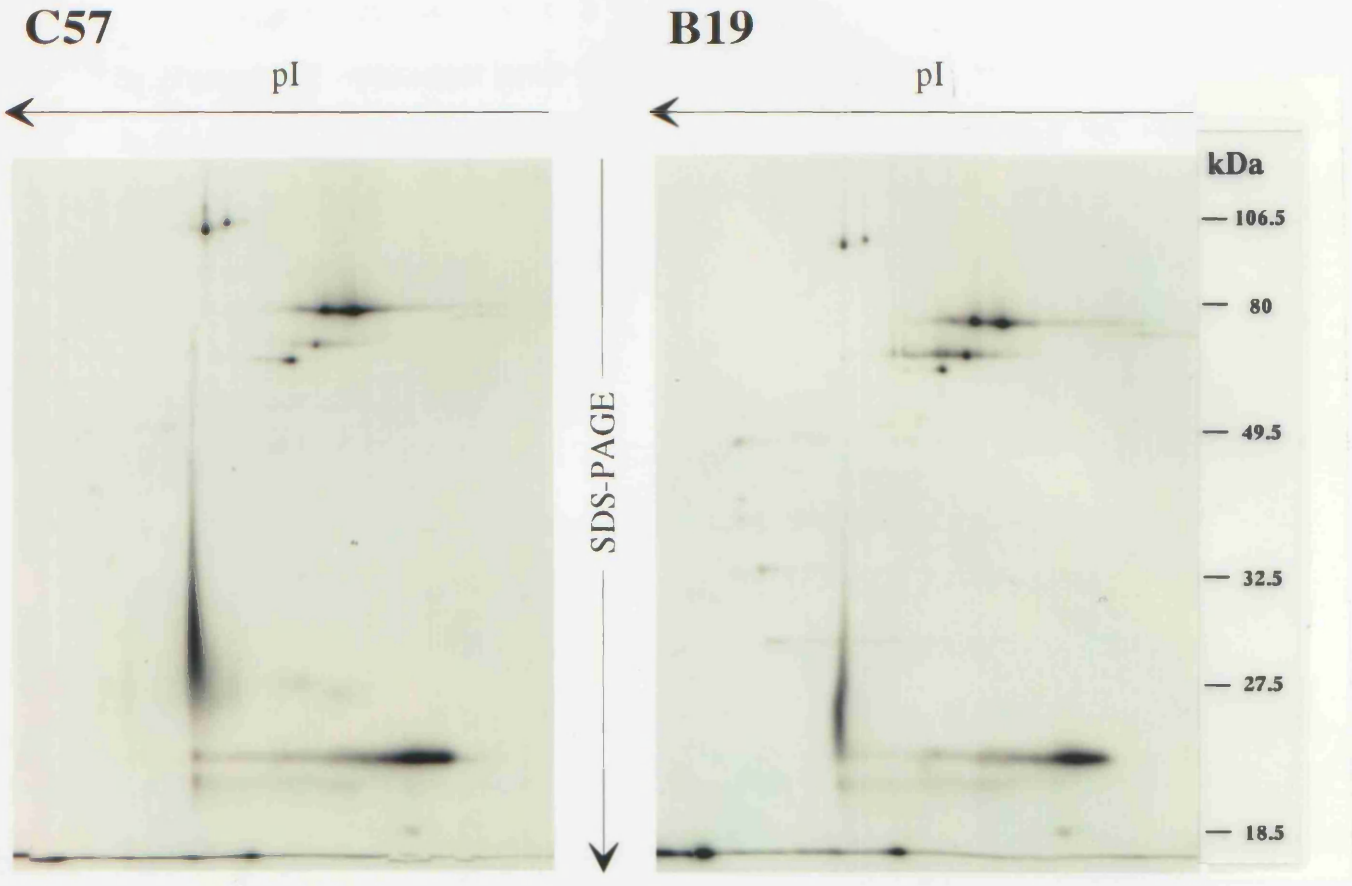
Table 5.4
Estimated molecular weights and isoelectric points of selenium-labelled proteins identified by 2D gel analysis. (symbols in parentheses: ↑, spot-intensity increased in B19 cells; ↓, spot-intensity decreased in B19 cells; *, spot absent from C57 cells; see section 5.6.5 and figure 5.10 for details)

spot 19 which was taken as 1 (figure 5.10D). Spot 19 was chosen as the reference spot since it appeared as a discrete spot in all 8 gels and did not appear to be markedly different between the two cell lines by visual inspection. The intensity of each spot in relation to spot 19 was then calculated for all four gels. The set of values obtained for each spot for each cell line was compared between the two cell lines and the students *t*-test used to determine any statistically significant differences.

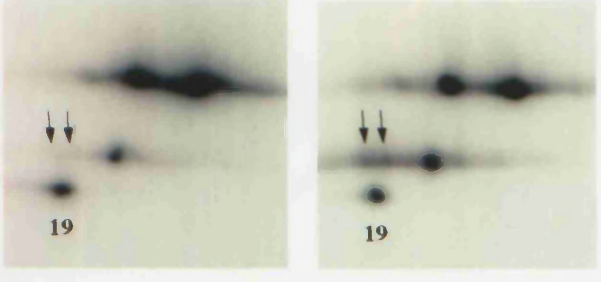
Both qualitative and quantitative differences were observed between the two cell lines. Two proteins having a molecular weight of 72Kda (spots 13 and 14) which were present in B19 cells could not be detected in C57 cells (figure 5.10B). Additionally, spots 3, 6, 7 and 17 (molecular weights 29, 19, 35 and 73KDa, respectively) were significantly more intense in B19 cells than the corresponding spots in C57 cells by 2-5 fold ($p<0.05$ for spots 3,6 and 7; $p<0.002$ for spot 17) (figure 5.10D). In contrast, spots 18 and 21 (molecular weights 101 and 100KDa, respectively) were significantly more intense in C57 cells than in B19 cells by approximately 2-fold ($p<0.01$, $p<0.02$ for spots 18 and 21, respectively) (figure 5.10D).

A number of spots, notably 11, 16, 20, 24 and 25 with molecular weights 21, 24, 27 23 and 23 KDa, ran as smears (figure 5.10A). Spots 24 and 25 most likely contained the subunits of PHGPX and GPX which could be detected at relatively high levels by northern analysis (figure 4.9). Interestingly, the intensity of these two spots were not significantly different between the two cell lines suggesting that the difference detected in the levels of PHGPX mRNA between the two cell lines may not be biologically significant. However, spots 24 and 25 did not fully resolve and ran as a smear and it is therefore debatable if a 2-fold difference could be detected for either of these spots.

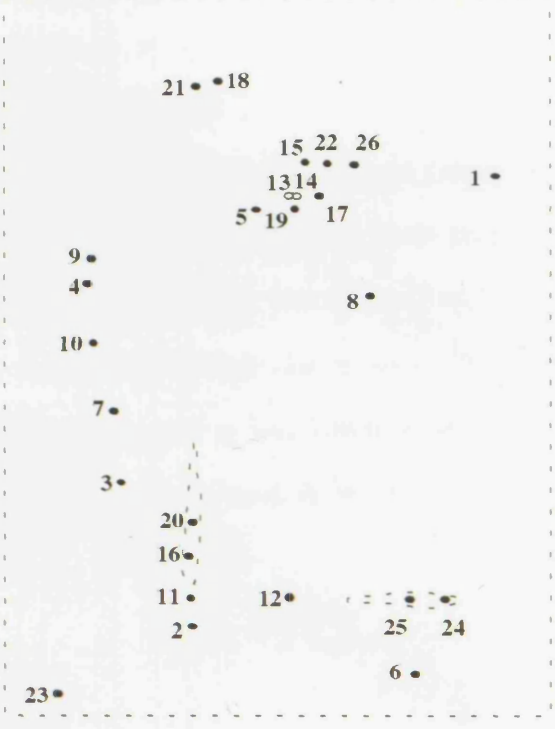
A


C57
pI
B19
pI
SDS-PAGE
kDa
106.5
80
49.5
32.5
27.5
18.5

B


C57
B19
19
19

C


21
18
15
22
26
1
13
14
5
19
17
9
4
8
10
7
3
20
16
11
12
2
25
24
6
23

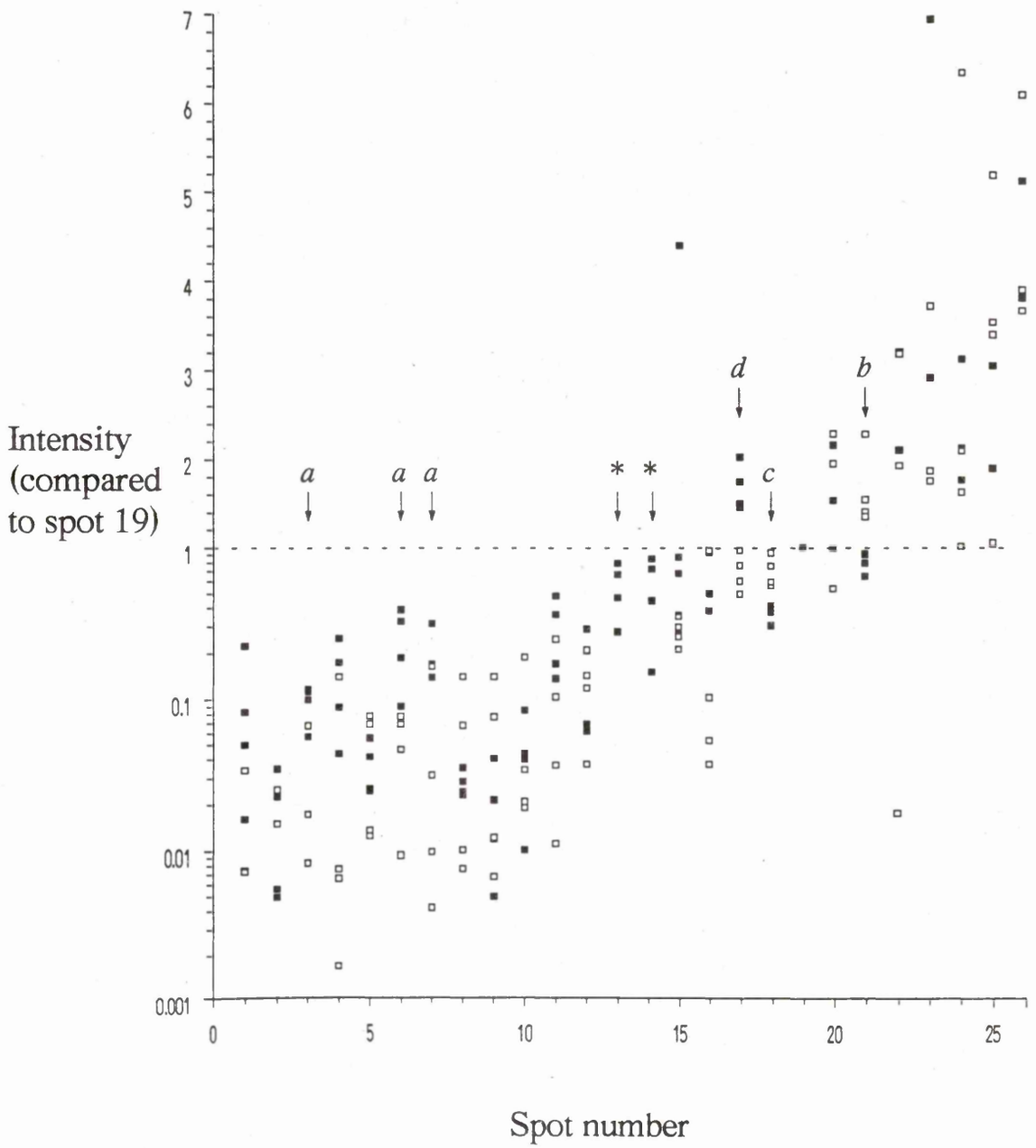


Figure 5.10
2D gel analysis of selenium-labelled proteins.

(A) Autoradiographs of 2D protein gels from C57 and B19 cells labelled with [75Se]-selenite. A representative gel, from a total of four, for each cell line is shown.

(B) Expanded area of gels from (A) showing the absence of two 72KDa spots from C57 cells and their presence in B19 cells. Spot 19 is indicated to aid orientation.

(C) Composite diagram of gel spots from both cell lines. Spots are numbered in order of increasing intensity. Open circles indicate those spots only found in B19 cells. Dashed lines encompass protein spots that ran as smears.

(D) Spot intensities relative to spot 19 for each of four gels for C57 (open squares) and B19 (filled squares) cells. Arrows indicate spots, the intensities of which are statistically significantly different between the two cell lines. (*a*, $p<0.05$; *b*, $p<0.02$; *c*, $p<0.01$; *d*, $p<0.002$, students *t*-test; *, not present in C57 cells).

5.7 Summary

A genetic approach has been taken to investigate the mechanisms of selenium-induced growth inhibition by generating selenium-resistant variants. A number of strategies were evaluated with which to select potential resistant-cells and a one-step clonal selection using a high dose of SDG seemed the most suitable. Initially, conditions were devised to isolate clones arising at a frequency of 1 clone/10^5-10^6 viable cells. However, using this kill-rate was unsuccessful and it only gave rise to one variant that was only transiently resistant. Increasing the mutation frequency by treatment with EMS prior to selection in SDG gave rise to two SDG-resistant clones that were stable for at least 50 doubling times. The resistant variant, B19, was chosen for further analysis since it displayed the greatest degree of resistance.

B19 cells were also resistant to selenite cytotoxicity. However, the lag period before selenite had an effect was the same for both cell lines. The fact that B19 cells are cross-resistant to selenite supports (1) the conclusion that B19 is selenium-resistant and (2) the hypothesis that selenite is acting through the production of SDG. Differential selenium uptake and GSH concentrations, which might affect selenite/SDG metabolism, were eliminated as possible mechanisms of resistance since these parameters were unchanged in the B19 cells. However, other comparative studies revealed a number of differences between C57 and B19 cells.

The mRNA levels of a number of candidate genes that could be involved in selenium-resistance was investigated. GST α subunits mRNA levels were approximately 2-fold higher in B19 cells. In contrast, PHGPX and Bcl-xS mRNA levels were found to be approximately 2-fold lower in B19 cells.

The selenium-labelling protein complements of C57 and B19 cells were also compared to identify selenoproteins or selenium-binding proteins that may be functionally involved in the resistant phenotype. A number of proteins were identified, the labelling of which were altered in the B19 cells. The most striking

difference was the presence of two 72KDa proteins which were not apparent in C57 cells.

Chapter 6
Discussion

6.1 Cytotoxic effects of selenite requires metabolic activation

In this study, the molecular mechanisms of selenium-induced growth inhibition have been investigated using a mouse mammary cell line, C57. Of a number of seleno-compounds, sodium selenite appeared to be the only compound that significantly affected the growth of C57 cells at the concentrations tested. Selenite had a cytotoxic effect after a lag period that could be abolished by the simultaneous addition of GSH. Other work in the group suggested that selenite toxicity was cell density-dependent (Dr. A. Wong, unpublished data), which has also been shown for other cell lines (Medina *et al.*, 1985; LeBoeuf *et al.*, 1985) and that selenite was acting through a secreted growth inhibitor (Dr. A. Wong, unpublished data). Additionally, SDG, the primary metabolite from the reaction of selenite and GSH was found to be a far more potent cytotoxic agent than selenite and had a cell density-independent effect as judged by its ability to act on C57 cells under cloning conditions. Other workers have also demonstrated SDG to be a potent cytotoxic agent (Santoro and Milner, 1991) and more effective than selenite *in vitro* (Fico *et al.*, 1986). The simplest interpretation of these results is that selenite needs to be metabolised by way of its reduction by GSH to have a toxic effect. This is in agreement with a number of studies that have also shown that the simultaneous addition of GSH can enhance the toxic effects of selenite on human and canine mammary tumour cells and HL60 cells (Yan *et al.*, 1991; Kuchan and Milner, 1991; Batist *et al.*, 1986). One question arising from the enhancing effect of exogenous GSH on selenite toxicity is why concentrations of 50-250 μM GSH (50 μM GSH in this study) increase the severity of the effect of selenite so dramatically since selenite can permeate C57 cells within 2h upon addition to the culture medium and intracellular concentrations of GSH are generally in the millimolar range (Meister, 1988). Furthermore, hepatocytes begin

to produce dimethyl selenide within 4h of selenite addition (Annundi *et al.*, 1984). Selenite is therefore rapidly taken up by cells and enters intermediary metabolism almost immediately. It is therefore puzzling as to why the high intracellular concentration of GSH does not have the same enhancing effect on selenite as relatively low extracellular concentrations of GSH.

Studies to determine the effect of intracellular GSH on selenite toxicity have been inconsistent. Buthionine sulphoximine (BSO), an agent that inhibits γ-glutamylcysteine synthetase, an enzyme involved in GSH synthesis, has been shown to enhance and inhibit selenite toxicity in human mammary cells (Yan *et al.*, 1991) and HeLa cells (Caffrey and Frenkel, 1991), respectively. Additionally, selenite and SDG treatment can affect intracellular levels of GSH; however, increases, decreases and no effect at all on GSH levels have been observed depending on cell type (Santoro and Milner, 1991; Yan *et al.*, 1991; Kuchan and Milner, 1991). These conflicting findings may be explained by the fact that while selenite reduction is required for its activation to more toxic compounds such as SDG, intracellularly, selenite reduction is also involved in cellular selenite detoxification since selenite needs to be reduced to selenide before it can be methylated and excreted (figure 1.1). The intracellular ratio of GSH:selenite could therefore critically affect selenite toxicity. Any changes in this ratio would not only affect the relative levels of potentially toxic seleno-metabolites, such as SDG, but also the rate of their detoxification. Ganther (1971) showed that under cell-free conditions, SDG is most favourably produced when the GSH:selenite ratio is 4 or less and that when the ratio exceeds 4, glutathione selenopersulphide becomes the dominant species. Additionally, Kuchan and Milner (1992) find that the molar ratio of GSH:selenite negatively correlates with the extent of growth inhibition by selenite. The GSH:selenite ratio may also be affected by cell density which has been shown to lower GSH concentration (Kuchan and Milner, 1992) and increase selenite toxicity (Medina *et al.*, 1985; Kuchan and Milner, 1992). The consequence of altering the intracellular GSH levels will not solely depend on the

GSH:selenite ratio but will also be influenced by a number of factors such as other cellular sulphydryls which may compete with GSH to react with selenite to form other selenotrisulphides. Under conditions where GSH levels have been depleted, selenotrisulphides such as selenodicystine [$(NH_2(COOH)CHCH_2S)_2Se$] or selenodimercaptoethylamine [$(NH_2CH_2CH_2S)_2Se$] are produced, which may have similar or even different toxic effects to SDG (Frenkel *et al.*, 1991). For example, Frenkel and Falvey (1988) showed that diethylmaleate, which produces a non-specific depletion of thiols, could decrease the the degree of DNA synthesis inhibition induced by selenite whereas BSO, which specifically depletes GSH, did not. It was therefore suggested that a selenotrisulphide, but not SDG, was responsible for selenite inhibition of DNA synthesis. The formation of SDG or other selenotrisulphides may thus be a mechanism by which selenite is metabolised to a more toxic agent but this may not occur in some cell systems due to intracellular physiological conditions favouring detoxification. The lag period of selenite's effect on C57 cells may thus represent a period when GSH levels are being altered either as a process of selenite metabolism or increased cell density. During this period the GSH:selenite ratio may favour selenite detoxification. However, the GSH:selenite ratio may reach a certain threshold where toxic seleno-compounds may begin to accumulate and at this point selenite begins to have its cytotoxic effect.

Whether selenite actually kills C57 cells by the accumulation of SDG or whether other selenotrisulphides are involved is unknown. Additionally, assuming SDG is the active metabolite of selenite, it is unknown if adding SDG exogenously kills cells by the same mechanism as selenite since presumably selenite generates SDG intracellularly, yet it is unknown if exogenous SDG enters cells or acts extracellularly. However, the fact that B19 cells are resistant to both SDG and selenite strongly suggests that SDG is exerting some if not all of the effects of selenite and that their site of action, that is whether they act intra- or

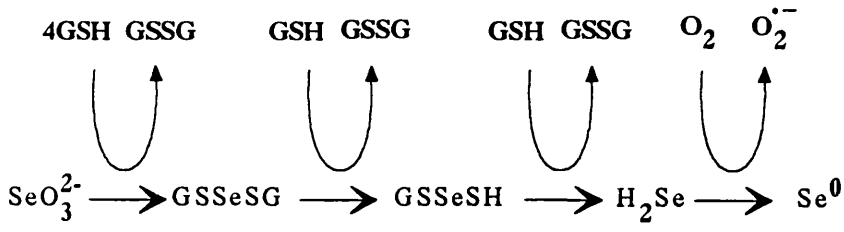
extracellularly, results in the activation of similar if not identical pathways leading to cell death.

6.2 Selenite and SDG induce cell death by a mechanism distinct from H_2O_2 toxicity

Selenite reduction by GSH has been shown to generate superoxide and H_2O_2 (Yan and Spallholz, 1993) and Seko *et al.* (1989) has proposed a reaction sequence for the reduction of selenite by GSH resulting in the production of superoxide anion, which is converted to H_2O_2 by superoxide dismutases (figure 6.1A). Additionally, selenite-induced DNA strand breaks have been shown to be oxygen-dependent (Garberg *et al.*, 1988). Oxidative stress is therefore strongly implicated in the toxic effects of selenite (Spallholz, 1994). In this study, this hypothesis has been tested by comparing the various biochemical effects of SDG and H_2O_2.

Both agents had a rapid effect and markedly reduced cloning efficiency within 1h. Interestingly, H_2O_2 was found to be required at concentrations reaching 200 fold higher than SDG to have a similar cytotoxic effect. This is contrary to what would be expected from the reaction proposed by Seko *et al.* (1989) which indicates that one molecule of SDG should give rise to one molecule of superoxide anion which would give rise to one molecule of H_2O_2 (figure 6.1A). This might suggest that SDG is acting through a very specific mechanism rather than just generating reactive oxygen species as by-products of its metabolism. This stoichiometric anomaly has been addressed in a hepatocyte system by Högberg and his colleagues: the metabolic conversion of selenite to dimethyl selenide occured after a lag period during which selenium accumulated in the cell and changes in the GSH redox system took place, as judged by oxygen consumption, oxidation of GSH and depletion of NADPH (Annundi *et al.*, 1984). Together with the suggestion that SDG and Se^{2-} may autooxidize (Tsen and Tappel, 1958; Ganther, 1979; Dilworth and Bandurski, 1977) they postulated a reaction scheme whereby

A



B

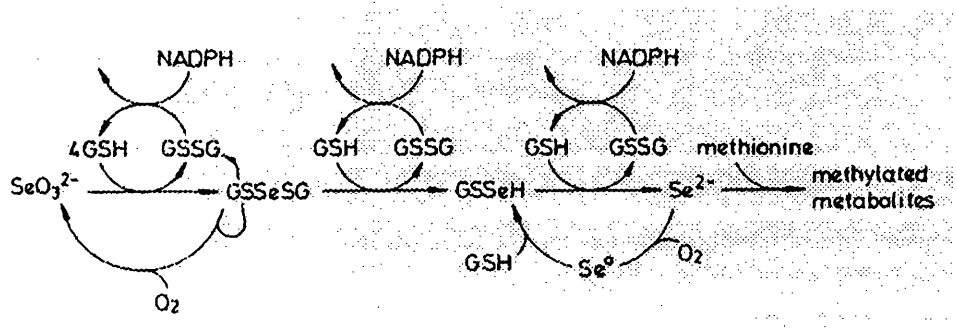


Figure 6.1
(A) Reaction scheme showing the metabolic reduction of selenite with the concomittant conversion of reduced glutathione (GSH) to oxidised glutathione (GSSG) and generation of superoxide. Adapted from Seko *et al.* (1989).

(B) Reaction scheme showing selenite and selenite-metabolites entering into redox cycles and the concommitant consumption of oxygen. Adapted from Annundi *et al.* (1984)

selenite metabolites induce a state of hypoxia (Garberg and Högberg, 1987) by entering redox cycles from which reactive oxygen species (ROS) may be generated (figure 6.1B). Therefore it is possible that the metabolism of low concentrations of selenite/SDG could result in the formation of toxic quantities of ROS, such as H_2O_2 which can mediate some of the effects of SDG in C57 cells such as formation of 560Kb DNA fragments and induction of similar morphological changes. However, other data strongly suggests that selenite/SDG cytotoxicity requires signals other than the production of H_2O_2. The observation that B19 cells are resistant to both SDG and selenite but not H_2O_2 demonstrate that these two pathways resulting in cell death can be segregated genetically and that SDG has additional effects besides generating H_2O_2. However, since H_2O_2 is thought to arise from the reductive metabolism of selenium, an alternative interpretation could be that the B19 cells have acquired some mutation that inhibits events upstream of selenite/SDG induced production of H_2O_2 such as selenium uptake or metabolism. In such a situation, H_2O_2 could mediate the effects of SDG but B19 cells would still be SDG-resistant due to the fact that less H_2O_2 were produced. However, this seems excluded since the rate of selenite uptake and the glutathione concentration of the two cell lines are not significantly different. Furthermore, although B19 cells are resistant to the eventual toxic effects of selenite, the lag period of selenite toxicity is similar between C57 and B19 cells suggesting that the reductive pathway of selenite metabolism is intact in the B19 cells and that the mutation(s) conferring selenium resistance affects steps after selenite conversion to more toxic seleno-metabolites. The absence of 50Kb DNA fragments and maintenance of PHGPX, GPX and GST Ya mRNA levels during H_2O_2-induced cell death further indicates that there are fundamental mechanistic differences between the cytotoxic effects of SDG and H_2O_2.

The fact that H_2O_2 toxicity cannot account for the effects mediated by SDG does not exclude the involvement of oxidative stress in SDG toxicity. For example, superoxide which is also generated from GSH reduction of selenite (Yan

and Spallholz, 1993) may have additional or different effects to H_2O_2. Although superoxide presumably is converted to H_2O_2 this will be dependent on the levels and activity of superoxide dismutases. Regarding the role of other ROS, the SDG-mediated down-regulation of PHGPX, GPX and GST Ya mRNAs in relation to 7s rRNA may be of functional significance. Since cells are continuously generating ROS as by-products of general oxidative metabolism, any lowering of the oxidant defences of the cell would augment the intracellular levels of not only H_2O_2 but also other ROS and organic peroxides which may elicit different or additional effects from H_2O_2 alone. Such a mechanism has been proposed for the growth inhibition of rat hepatocytes by TGF-β1, which down-regulated the mRNA levels of Mn and Cu-Zn superoxide dismutases, catalase and GST subunits 1 and 2 and caused a concomitant increase in the level of intracellular peroxides (Kayanoki *et al.*, 1994). SDG may therefore be inducing a pro-oxidant state by a similar mechanism. However, the lowering of GAPDH mRNA levels suggests that SDG-induced down-regulation of PHGPX, GPX and GST Ya is non-specific and that SDG is most likely causing a general inhibition of RNA synthesis, as has been reported for selenite. The fact that the levels of 7S rRNA are unaffected after SDG treatment probably reflects the higher stability of rRNAs over mRNAs. The functional significance of inhibition of RNA synthesis by SDG is unclear. For example, it is unknown if RNA inhibition is a direct effect of SDG or is affected indirectly as a consequence of, say, protein or DNA synthesis inhibition which has been reported for selenite in other systems (Vernie *et al.*, 1983; Frenkel and Falvey, 1988)). However, Fico *et al.* (1986) has demonstrated that the inhibition of RNA synthesis, but not DNA or protein synthesis correlates with selenium-induced growth suppression. Whatever role down-regulation of PHGPX, GPX and GST Ya plays during SDG-induced cell death, it appears to be an early event since it precedes detachment of cells from the monolayer.

Inhibition of cellular oxidant defences could alternatively be achieved post-transcriptionally by inhibiting the activity of antioxidant enzymes. Indeed,

selenite and SDG may induce a pro-oxidant state by such a mechanism as they are both powerful inhibitors in cell-free systems of the redox factor, thioredoxin (Björnstedt *et al.*, 1992; Kumar *et al.*, 1992), which is involved in free radical defence (Nonogaki *et al.*, 1991).

6.3 SDG induces an apoptotic-like cell death

In comparing further the effects of SDG and H_2O_2, the mode of cell death induced by these agents has been assessed. A cell may die by one of two pathways, necrosis or apoptosis. Necrosis generally occurs in response to cell injury and results in cell swelling, loss of membrane integrity and ultimate lysis of the cell and release of its contents into the extracellular environment which elicits an inflammatory response. In contrast, apoptosis is a genetically programmed, delineated set of events that can be activated by numerous stimuli: it generally involves cell shrinkage, maintenance of membrane integrity and does not induce an inflammatory response since cell contents bud off in membrane bound vesicles, termed apoptotic bodies, which are phagocytosed (Wyllie, 1993). The morphological and biochemical characteristics of apoptosis have been extensively studied in glucocorticoid-induced apoptosis in thymocytes (Compton and Cidlowski, 1992). When treated with dexamethasone, rat thymocytes undergo apoptosis with the most notable characteristics being condensation of chromatin forming a crescentic mass at the edge of the nuclear membrane (Wyllie *et al.*, 1980) and formation of internucleosomal 180-200bp DNA fragments (Wyllie, 1980). While these morphological and biochemical characteristics have acted as standard criteria for defining apoptosis it is debatable as to how applicable these criteria are for assessing apoptosis in all cell systems induced with a variety of different treatments. For example, hepatocytes (Oberhammer *et al.*, 1993) and oligodendrocytes (Barres *et al.*, 1992) can undergo morphological apoptotic death in the absence of internucleosomal DNA ladder formation. Therefore, in defining apoptosis many workers have adopted additional biochemical assays with which to

assess apoptosis. For example, the formation of high molecular weight DNA fragments ranging from 50-600Kb can be detected during apoptosis (Walker *et al.*, 1991; Roy *et al.*, 1992; Bicknell *et al.*, 1994) and it has been suggested that this represents an earlier and more consistent step in apoptosis (Oberhammer *et al.*, 1993). Additionally, since apoptosis is an active process, anti-metabolites such as cycloheximide and actinomycin D, which inhibit protein and RNA synthesis, respectively, can prevent apoptosis (Zaleski *et al.*, 1994). This, however, is not a consistent finding; other reports have found both agents to have either no effect (Kruman *et al.*, 1992) or even induce apoptosis (Martin, 1993). The involvement of signal transduction pathways may also play a role in apoptosis since inhibitors of tyrosine kinases can prevent apoptosis (Migita *et al.*, 1994; Yousefi *et al.*, 1994). Additionally, the endonuclease activity thought to be responsible for apoptotic DNA fragmentation is dependent on Ca^{2+} and Mg^{2+} and this can be inhibited by metal chelators such as EDTA or Zn^{2+} (Compton, 1992). While these assays implicate an active cell death rather than necrosis, there is presently no single definitive biochemical test for the occurrence of apoptosis. For this reason, many workers rely mostly on morphological criteria to assess apoptosis, notably chromatin condensation, since this is the most striking feature of an apoptotic cell that can be detected by light microscopy. However, Kung *et al.* (1991), in studying the cytotoxic effects of aphidicolin and vincristine, showed that cell death could be prevented by cycloheximide, suggestive of apoptosis, but observed that as cells died they took up a number of different morphologies, not all relating to the apoptotic model of the thymocyte. Indeed, nearly 50% of the cells had no gross nuclear abnormalities. The role of nuclear changes in apoptosis is also brought into doubt by the ability of apoptosis to occur in cells lacking a nucleus (Jacobson *et al.*, 1994). This suggests that while nuclear changes may be a diagnostic marker for apoptosis they may not be universal or functionally significant. Therefore, true apoptosis, as defined in the thymocyte model, may only occur under some conditions whereas other apoptotic-like deaths may occur elsewhere; the form and

extent to which cell death resembles classical apoptosis being dependent on the cell type and inducing agent.

We and others have shown that both organic and inorganic seleno-compounds can induce apoptosis in diverse systems such as leukaemia and mammary cultured cells as judged by morphological criteria, formation of nucleosomal ladders and flow cytometry, which utilises the principle of differential uptake of two DNA-binding fluorochromes by apoptotic but not necrotic cells (Lanfear *et al.*, 1994; Lu *et al.*, 1994; Thompson *et al.*, 1994). Similarly, hydrogen peroxide can induce apoptosis in a variety of cell systems as judged by morphological criteria and the formation of nucleosomal DNA ladders (Pierce *et al.*, 1991; Sandstrom *et al.*, 1993; Nosseri *et al.*, 1994; Forrest *et al.*, 1994). In this study, SDG and H_2O_2 were found to induce the formation of 560Kb DNA fragments and SDG caused further cleavage to 50Kb DNA fragments. It has been suggested that chromatin condensation during apoptosis reflects the cleavage of DNA into high molecular weight fragments (Oberhammer *et al.*, 1993). However, in the present study cleavage of DNA occurred in the absence of any gross nuclear structure or chromatin changes. Fragmentation in the absence of chromatin condensation has also been observed by other workers when cells are treated with exogenous proteases or nucleases (Duke *et al.*, 1994; Williams *et al.*, 1994). While no nuclear morphological changes were observed, SDG and H_2O_2 did however cause the appearance of cytoplasmic convolutions which is suggestive of apoptosis (Wyllie, 1992). These morphological and biochemical data therefore suggests that an apoptotic-like death may be induced in C57 cells by SDG and H_2O_2 but that the pathways differ with respect to DNA fragmentation. However, in the absence of classical diagnostic morphological markers of apoptosis additional biochemical tests are required to further define the mode of cell death in this cell system.

6.4 Mechanisms of selenium-resistance

A number of comparative studies of C57 and B19 cells were carried out in order to determine the possible mode of resistance displayed by the B19 cells. As mentioned earlier, the mutations affecting selenium-resistance appeared to be acting downstream of early events in selenium-cytotoxicity such as selenite entry into the cell and reduction to SDG. One possibility therefore was that selenoproteins may be involved in selenium-resistance. The fact that the selenoprotein complement of C57 and B19 cells were very similar suggests that there are no gross changes in B19 cells affecting selenoprotein or selenium-binding protein synthesis and that the machinery for selenoprotein synthesis is probably intact. There were however a number of proteins whose levels appeared to differ between the two cell lines suggesting that selenium-resistance displayed by the B19 cells may involve selenium-labelling proteins. Their respective molecular weights do not correspond to those of any known selenoproteins suggesting these are as yet uncharacterised selenium-labelling proteins. It is also unknown if these proteins are true selenoproteins that contain [Se]Cys encoded by a UGA codon or selenium-binding proteins. Regarding the latter class of proteins, the differential labelling of these spots between the two cell lines may reflect selenium-binding activity rather than actual protein levels. Additionally, proteins 13 and 14 which appear to be only present in the B19 cells could represent post-translationally modified forms of protein 17 which has the same molecular weight but a different pI value. Such modifications could include phosphorylation which would be expected to alter charge of the protein without any significant change in molecular weight. In such a situation, absence of spots 13 and 14 from C57 cells may not necessarily represent an absence of protein but rather a different phosphorylation status.

A number of differences were also observed in gene expression levels between the two cell lines. The fact that both increases and decreases in mRNA levels were detected and that the levels of some mRNAs such as GPX, TRX, GST

Yc, GSt π, bcl-2 and bcl-x_L were unaltered suggests that the differences observed are specific and not just a result of some change affecting, say, the general transcription of RNA polymerase II dependent genes.

The mRNA levels of GST Ya and GST Yc, the sub-units that constitute GSt α, were approximately 2-fold higher in B19 cells. GST α overexpression is associated with resistance against nitrogen mustards (Lewis *et al.*, 1988; Puchalski and Fahl, 1990; Giaccia *et al.*, 1991). A 2-fold overexpression of GST α could thus be functionally significant in conferring selenium-resistance. This would not necessarily require selenite, SDG or potentially other toxic seleno-metabolites to be substrates for GST α since GST α could be involved in downstream events of selenium-cytotoxicity such as detoxification of organic peroxides which might be generated. In this context, it should be noted that GST π mRNA levels were found to be considerably higher than GST α mRNA and therefore any role GST α may play in selenium-resistance might be expected to involve substrates specific to GST α rather than other GSTs, such as GST π.

Since SDG induced an apoptotic-like death it was of interest to examine the levels of various genes that are known to affect sensitivity to induction of apoptosis. bcl-2 mRNA levels were unaltered between the two cell lines. However, bcl-x_S mRNA, the product of which antagonises the protective function of BCL-2, is down-regulated in B19 cells. Thus, if BCL-2 were to be involved in determining susceptibility to SDG-induced cell death, down regulation of bcl-x_S mRNA could confer selenium-resistance. The differential maintenance of bcl-x_L and bcl-x_S mRNAs suggests that the down-regulation of bcl-x_S mRNA is due to a post-transcriptional mechanism since bcl-x_L and bcl-x_S mRNAs are splice products from the same transcript (Boise *et al.*, 1993).

Down-regulation of PHGPX mRNA in B19 cells was surprising since lowering the levels of PHGPX might be expected to sensitise a cell to both H_2O_2- and SDG-induced cytotoxicity. However, neither of these phenotypes were observed. Although PHGPX could not be definitively identified on the 2D protein

gels, the most likely candidates are spots 24 and 25 since these were the major selenoproteins running at approximately 20KDa, which corresponds to the molecular weight of PHGPX. Interestingly, the intensity of these two spots was not significantly different between the two cell lines suggesting that the differences in mRNA levels may not be biologically significant.

6.5 Physiological relevance of *in vitro* effects to *in vivo* anti-carcinogenic activity of selenium

Of a number of seleno-compounds, namely SBME, SMSC, DMS, selenite and [Se]Met, which have varying degrees of chemopreventive activity, only selenite had a significant effect on the growth of C57 cells. The relative ineffectiveness of SBME in comparison to selenite has also been shown on L1210 leukaemia cells (Wilson *et al.*, 1991). The lack of correlation between the relative efficiencies of seleno-compounds to inhibit growth *in vitro* and their relative effectiveness to inhibit tumorigenesis *in vivo* might argue against the hypothesis that the *in vitro* effects of selenium are mechanistically relevant to the anti-carcinogenic effects. However, it is becoming increasingly apparent that metabolism is an essential step in the anti-carcinogenic action of selenium. Although these critical metabolic pathways and metabolites have yet to be identified it is unlikely that all cells possess the metabolic machinery to generate active seleno-compounds. For example, the methyl-transferase thought to be involved in the final methylation step of selenium detoxification is mainly expressed in lung and liver (Mozier *et al.*, 1988). Therefore one explanation for the lack of correlation between *in vitro* and *in vivo* studies could be that C57 cells cannot process all selenium forms to toxic metabolites whereas *in vivo* such metabolites may be generated by distant organs and then secreted and delivered to the site of action. Kato *et al.* (1992) showed that both inorganic (selenite) and organic ([Se]Cys) forms of selenium after absorption are converted to a molecule designated A-Se which is highly extracted by liver but not other organs such as

lungs or kidney. Additionally, absorbed selenium was excreted to a higher rate if the liver was bypassed using a portacaval shunt. The liver therefore plays a central role in selenium metabolism and homeostasis in the organism and may be important in metabolising selenium compounds to active chemopreventive agents.

It has been suggested that the *in vivo* anti-carcinogenic and toxic effects of selenium are directly related (Spallholz, 1994). However, these two properties of selenium have been shown to be dissociable. Ip *et al.* (1994a) introduced a chemopreventive index which gives an indication of the safety margin between the anti-carcinogenic and toxic doses of selenium. The chemopreventive index of a particular selenium compound is calculated as a ratio of the maximum tolerable dose, defined as the dose which produces the first indication of a significant decrease in growth of the animal, to the dose which produces a 50% reduction in tumour yield in the DMBA-induced mammary tumour model. If the anti-carcinogenic and *in vivo* toxic effects of selenium were mechanistically related, it would be expected that the chemopreventive index of selenium compounds would be constant. However, this is clearly not the case with chemopreventive indices of 1, 1.3, 4 and >10 being reported for [Se]met, selenite, 1,4-phenylene-bis(methylene)selenocyanate (p-XSC) and triphenylselenonium chloride, respectively (Ip *et al.*, 1994a, 1994b). This raises the question of whether the *in vitro* cytotoxic effects of selenium are mechanistically relevant to the *in vivo* anti-carcinogenic action or toxic effects of selenium. While it is well established that selenite is cytotoxic *in vitro*, a number of studies have also claimed selenite to be a reversible inhibitor of growth and that this cytostatic action of selenite may be more relevant to the chemopreventive activity of selenium. Medina and Oborn (1984) showed that selenite inhibited cell growth, as measured by cell number and DNA synthesis, but that growth resumed after selenite removal. However, since the clonogenic potential of cells after selenite removal was not assessed and recovery began only after a lag period of 2-4 cell doublings, it is therefore possible that resumption of cell growth was actually a measure of a sub-population of cells

surviving selenite treatment rather than a true reversible effect. Similarly, Gruenwedel and Cruickshank (1979) demonstrated recovery of DNA, RNA and protein synthesis after selenite removal but this too only occurred after a lag period exceeding 3-4 generation times. While they also did not assess the clonogenic potential of cells after selenite removal, cell viability, as measured by trypan blue exclusion, indicated that more than 90% of the cells had intact membranes after selenite treatment. However, since selenium can induce apoptosis in some systems, membrane integrity may be an unsuitable assessment of cell viability since cell membranes of apoptotic cells remain intact until the later stages of apoptosis (Wyllie, 1985). Indeed, Lu *et al.* (1994) showed that during selenite-induced apoptosis of L1210 leukaemic cells, trypan blue exclusion was a poor indicator of long-term proliferative potential as measured by clonogenic assays.

The *in vitro* growth inhibitory effects of selenium appear to be dependent on its chemical form. While selenite cytotoxicity is associated with the induction of DNA strand breaks, SB and SBME at doses 5-fold higher than those used in the present study or 100μM triphenylselenonium chloride can inhibit growth in the absence of DNA strand breaks (Wilson *et al.*, 1992; Ip *et al.*, 1994b). All three compounds are also superior to selenite with respect to chemopreventive efficacy (Ip and Ganther, 1990; Ip *et al.*, 1994a). The ability to inhibit growth in the absence of DNA strand breaks might suggest a specific mechanism which may be relevant to the anti-carcinogenic activity of selenium. On the other hand, the effects of selenite and SDG, such as the induction of apoptosis, may reflect a general response to cellular damage, such as DNA strand breaks, and therefore may be more relevant to the *in vivo* toxic effects of selenium. However, p-XSC which has a chemopreventive index that is 4-fold higher than selenite induces apoptosis more effectively than selenite *in vitro* suggesting that for these two compounds, at least, chemopreventive efficacy correlates with their ability to induce apoptosis *in vitro* (Thompson *et al.*, 1994). Additionally, the *in vivo* toxic effects of selenium, which is usually assessed as a decrease in body weight, may be due to a systemic

mechanism rather than a direct effect on cells since growth retardation induced by feeding rats with high levels of selenite in the drinking water correlated with suppresion of growth hormone levels (Thorlacius-Ussing *et al.*, 1989). Moreover, selenium-induced inhibition of growth rates could be reversed by administration of exogenous growth hormone (Thorlacius-Ussing *et al.*, 1989). It is therefore presently unclear exactly how the *in vitro* and *in vivo* effects of selenium are mechanistically related since there is no clear correlation between the chemopreventive efficacy of a particular compound and the mechanism by which cell growth is inhibited *in vitro*. It is however possible that different selenium compounds may exert different anti-carcinogenic effects depending on their metabolic fates with some compounds inducing cell-death and others possibly having a cytostatic effect (Lu *et al.*, 1995).

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