Growth Inhibition By Selenium Compounds

By

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To Sou-li

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Table of Contents

Page

LIST OF FIGURES

Page

Abstract

A number of selenium compounds have been shown to have anticancer properties. The mechanism by which selenium has anticarcinogenic effects is not understood although it is likely to be related to its ability to inhibit cell growth and induce apoptosis. The mechanism by which selenium compounds induce apoptosis is also not understood although it has been hypothesised that selenite induces cell death during its reduction to hydrogen selenide by the generation of reactive oxidant species resulting in oxidative stress. Bcl-2 has been shown to inhibit the ability of a number of stimuli to cause apoptosis possibly by its ability to prevent the effects of reactive oxygen species and peroxides. The aim of this project was therefore to investigate whether Bcl-2 could inhibit cell death induced by two selenium compounds; selenodiglutathione (SDG), the primary metabolite of selenite and 1,4-phenylenebis(methylene)selenocyanate (p-XSC), a novel synthetic selenium compound.

The first part of the thesis work describes the preparation of SDG and optimisation of conditions for growth inhibition by SDG and p-XSC. The second part of the thesis describes studies to test whether over-expression of Bcl-2 could protect cells from cell death induced by selenium compounds.

MCF7 cells, a human breast cell line, were transfected with a Bcl-2 expression vector by electroporation and selected for resistance to G418, the selection marker. By western blotting of cell lysate and detection with anti-Bcl-2 antibodies, a number of clones were demonstrated to over-express Bcl-2 protein. By the use of cell proliferation and cloning assays it was demonstrated that clones over-expressing Bcl-2 protein were equally sensitive to cell death induced by selenium compounds SDG and p-XSC as cells transfected with vector control. As Bcl-2 over-expression in a haemopoetic cell line has previously been shown to protect against apoptosis induced by H_2O_2, it was also tested if the clones produced in this study to over-express Bcl-2 were able to prevent H_2O_2 induced cell death. From the results of the cell proliferation and cloning assays, cells with over-expression of bcl-2 did not appear to be more resistant to H_2O_2 induced cell death than control cells. Thus, in this study Bcl-2 over-expression was found not to provide protection against SDG, p-XSC or H_2O_2 induced cell death in MCF7 cells.

Abbreviations

AAF	2-Acetylaminofluorene
BCS	Benzyl-selenocyanate
BTC	Benzyl-thiocyanate
dCDP	Deoxy-Cytosinedinucleotidephoshate
DMBA	Dimethylbenza[a]-anthracene
DMSO	Dimethyl Sulphoxide
DNTB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
ECL	Enhanced chemi-luminescense
EDTA	Ethylenediamine-tetra-acetic acid
EF-2	Elongation Factor - 2
EIF-2	Eukaryotic Initiation Factor - 2
FCS	Foetal Calf Serum
Gpx	Glutathione Peroxidase
cGPx	Cytosolic Glutathione Peroxidase
GIGPx	Gastrointestinal Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
HO-1	Heme Oxygenase 1
HRP	Horse Radish Peroxidase
ICE	Interleukin-Beta Converting Enzyme
IDI	Iodothyronine deiodinase
IL-3	Interleukin 3
ISP	6-phenyl-7 (6H)-isoselenazolo[4,3-d] pyrimidone
JNK	Jun Kinase
LMP-1	Latent Membrane Protein 1
MEL	Mouse Erythroleukaemic
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine
MNU	N-Methyl-N-Nitrosourea
MTT	3-[4,5]-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide-adenosine-phosphate (reduced)
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NOHAAF	N-Hydroxyamino-fluorene
PBS	Phoshate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phosphate buffered ethylenediaminetetra-acetic acid
PGPx	Plasma Glutathione Peroxidase
PHGPx	Phospholipid Glutathione Peroxidase
PLC	Primary Liver Cancer
PMSF	Phenylmethylsulphonyl Fluoride
p-XSC	1,4-phenylenebis(methylene)selenocyanate
ROS	Reactive Oxygen Species
SDG	Selenodiglutathione
SDS	Sodium Doecyl Sulphate
TBS	Tris Buffered Saline
TE	Tris buffered ethylenediaminetetra-acetic acid
TEMED	Tetramethylenediamine
TLC	Thin Layer Chromatography
TNF	Tumour Necrosis Factor

1. Introduction

1.1 Selenium - an essential trace element

Selenium is an essential trace element which occurs naturally in the diet. It is best absorbed by plants as selenates and to a lesser degree as selenites (Spallholz 1994). It is particularly abundant in foods such as fish, butter, Brazil nuts and cereal crops although variation of selenium in food content occurs depending on the selenium status of the region in which it originated (Trimmer *et al.* 1988). The selenium component in plants is predominantly selenomethionine (Olson *et al.*, 1970) although selenium in the form of selenocysteine can also be obtained from animal food sources and bacterial processed foods.

A number of proteins have been identified in which selenium is incorporated as a selenocysteine molecule, including four forms of glutathione peroxidases, types-I, II and III-iodothyronine deiodinase, selenoprotein P, thioredoxin reductase and selenoprotein W. The selenocysteine in such selenoproteins differs from selenium randomly incorporated into proteins as a replacement for sulphur in cysteine in that it is encoded from a specific selenocysteine codon, UGA. The mechanism by which selenium becomes incorporated in to proteins is described in section 1.2. The selenocysteine has been shown to be essential for the activity of these proteins thus establishing a biological role for selenium (Berry *et al.* 1991; Hill *et al.* 1997).

Selenium deficiency has been associated with heart muscle damage as low selenium blood levels have been implicated in Keshan disease in rural China causing death of children aged 1-10 years old (Jackson 1987). High levels of selenium intake by cattle and sheep following consumption of seleniferous plants can cause toxic conditions known as 'alkali disease' and 'blind staggers' characterised by hair loss, loss of hoofs and deformation (Spallholz *et al.* 1994).

The US National Research Council has analysed data produced by Chinese Scientists in order to issue a Recommended Daily Allowance (RDA) of 70 μg and 55 μg of selenium per day for men and women, respectively (Levander. 1991). This dietary allowance was calculated on the basis of selenium levels required to saturate glutathione peroxidase activity and did not take in to account the levels of any other

selenoproteins or levels of selenium required to have chemopreventive effects (see section 1.4)

1.2 Selenoprotein synthesis

Glutathione peroxidase (GPX) was the first mammalian selenoprotein to be discoverved containing selenium in the form of selenocysteine (Rotruck *et al.* 1973). By sequencing the GPX gene and mRNA it was shown that the selenocysteine at the active site was encoded a by TGA codon (Chambers *et al.* 1986). What distinguishes a UGA stop codon from a UGA which codes for selenocysteine cotranslational insertion has yet to be fully determined. *E.coli* dehydrogenase is a selenoprotein encoded by the gene *fdh*F (Zinoni *et al.* 1987). A stem loop on the fdhF mRNA immediately 3' of the UGA codon has been shown to be necessary for selenocysteine incorporation (Zinoni *et al.* 1990), with changes of a single base substantially decreasing selenocysteine incorporation (Heider *et al.* 1992).

The mechanism by which selenium is incorporated in to selenoproteins is thought to be similar in eukaryotic and prokaryotic cells although this has yet to be fully determined. Inorganic forms of selenium are more easily incorporated as selenocysteine in glutathione peroxidases than selenium administered as selenocysteine (Sunde *et al.*1987). Labelling of amino acids showed that the selenocysteine in the carbon skeleton of glutathione peroxidase was derived from serine and not from selenocysteine or cysteine. This puzzle has now been explained by recent characterisation of mutant *E.coli* unable to synthesise the selenoprotein formate dehydrogenase and enabled the identification of four genes involved in selenocysteine cotranslation (Burk *et al.* 1991). SelC encodes a tRNA $^{ser\ sec}$ that associates with the UGA codon and binds L-serine catalysed by Seryl-tRNA ligase. The side chain oxygen of serine is activated before replacement with selenium. SelA mutants were unable to convert seryl-tRNA $^{ser\ sec}$ to selenocysteyl tRNA $^{ser\ sec}$. Addition of Sel A, now shown to code for selenocysteine synthase, resulted in the consumption of activated serine and the appearance of an unidentified selenium compound. Further addition of Sel D and selenite resulted in the disappearance of the unidentified selenium compound and the appearance of selenocysteine. The Sel D

product in mammals has now been identified as selenophosphate synthetase which generates selenophosphate, the active donor to the tRNA (Low *et al.* 1995). Bacterial and human selenophophate synthetases were shown to be functional in higher eukaryotes and bacteria.

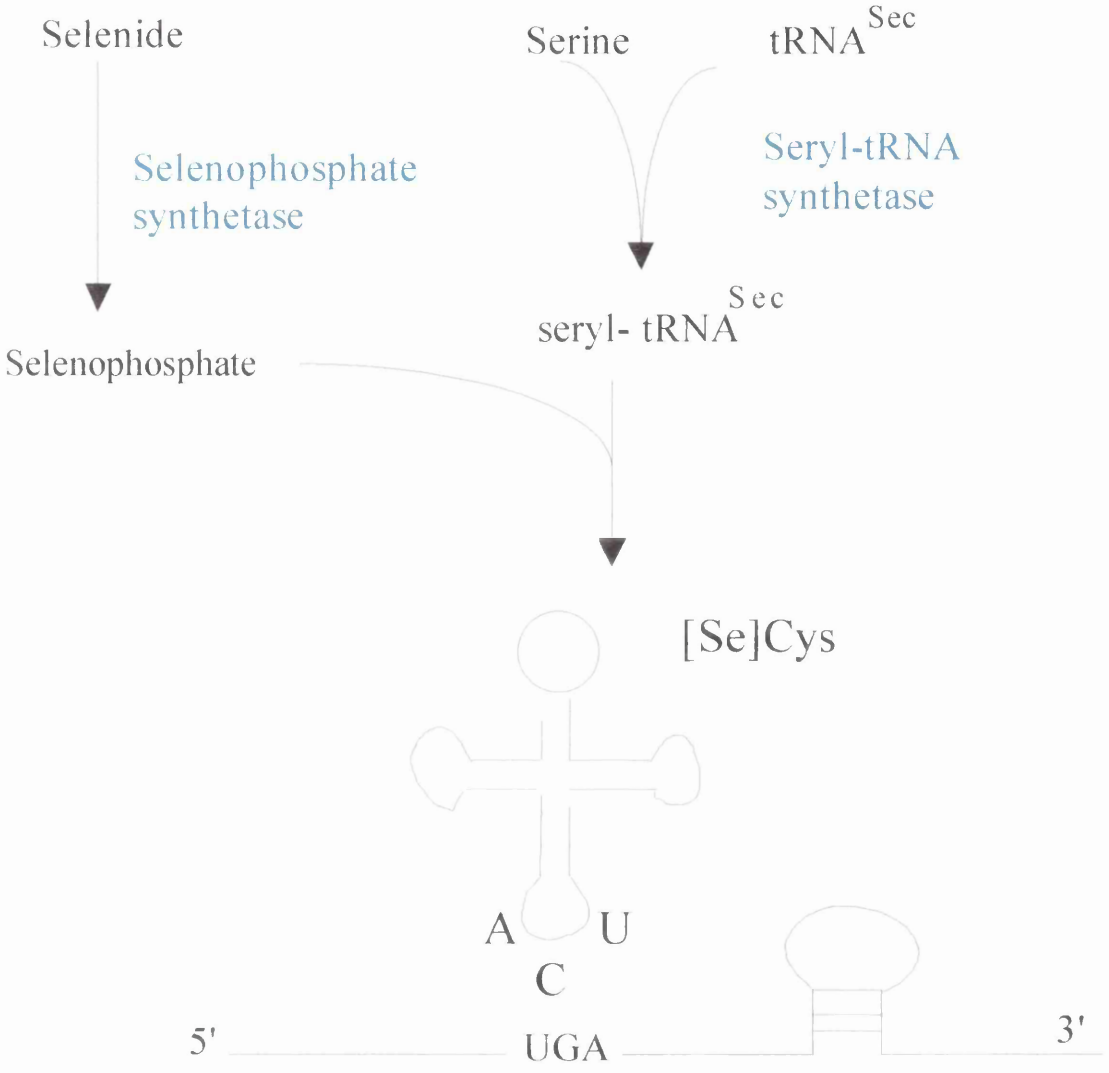


Figure 1.1

Proposed mechanism of selenocysteine incorporation into prokaryotic selenoproteins. A specific tRNA recognises the UGA sequence as a selenocysteine codon (due to the 3'loop structure) and binds L-serine. The side chain of serine is activated and replaced with selenium to form selenocysteine (Adapted from Low and Berry. 1996).

However, the human selenophosphate synthetase was only partially able to complement the bacterial system, suggesting that additional factors found in mammalian cells but not bacterial cells are required for the function of human selenophosphate synthetase activity. Sel B is a selenocysteine-specific elongation factor necessary for the incorporation of selenocysteine in to bacterial proteins.

1.3 Selenoproteins

Formation of peroxides and free radicals in the cell results in lipid peroxidation and hence loss of membrane integrity. Glutathione peroxidases utilise reduced glutathione to reduce hydrogen peroxide and organic hydroperoxides and are thus able to protect cells from oxidative damage.

There are currently four glutathione peroxidases known to exist. The first to be discovered is referred to as classical glutathione peroxidase (cGPx) and was shown in the presence of glucose to protect erythrocytes from hydroperoxide induced damage (Cohen and Hochenstein. 1963). Glucose is required to generate NADPH and thus enable the reduction of oxidised glutathione by glutathione redutase which can then be utilised by the glutathione peroxidase enzyme.

The second member of the glutathione peroxidase family to be discovered was phospholipid hydroperoxide glutathione peroxidase (PHGPx). Maiorino and coworkers were the first to identify PHGPx in human cells (Maiorino *et al.* 1991). By investgating GPx activities in 7 tumor derived cell lines they showed differential activities of cGPx and PHGPx indicating that expression of these two proteins is independent of each other. Both cGPx and PHGPx are present in the cytosol of cells.

However, the third member of the glutathione peroxidase (GPx3) is found in the plasma and is appropriately named plasma glutathione peroxidase (pGPx) (Maddiparti *et al.* 1987; Takayashi *et al.* 1986). As the concentration of glutathione is 100-fold less in the plasma than it is in the tissues the mechanism of action of pGPx is not understood and therefore raises the possibility that another thiol other than glutathione, such as thioredoxin, is utilised.

These enzymes differ in primary structure, localisation and substrates specificity. cGPx and pGPX are both tetramers consisting of 4 identical subunits each containing a single selenocysteine whereas PHGPx exists as a monomer containing

one selenocysteine molecule (Maiorino *et al.* 1991; Forstorm *et al.* 1978). Investigations of the reactions of these enzymes with various substrates revealed that they have different substrate specificity's (Yamamoto and Takahashi. 1993). Plasma GPx is able to reduce organic and hydroperoxides but has 10% of the activity that classical GPx was shown to have. Both plasma GPx and phospholipid GPx are able to cause immediate reduction of phosphatidylcholine hydroperoxide, whereas cellular GPx can not. Cytosolic GPx was shown to have little activity in reducing peroxides in erythrocyte phospholipid ghosts. Plasma GPx caused almost complete reduction of two major membrane phosholipids, phosphatidyl choline and phophatidylethanolamine but was unable to reduce cholesterol hydroperoxides. Phospholipid GPx was capable of reducing almost all of the peroxides in the erythrocyte phospholipid ghosts. The most recently identified member of the glutathione peroxidase family, gastrointestinal glutathione peroxidase (GPx-GI) was isolated from human hepatocyte cells and is thought to be a tetrameric protein localised in the cytosol (Chu *et al.* 1993). It has similar substrate specificity to cGPx as it is able to catalyze the reduction of H_2O_2, tert-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide but cannot reduce phosphatidyl hydroperoxide. Chu and coworkers (1993), demonstrated that GPx-GI is readily detected in human liver, colon, small intestine and stomach, but is not found in other tissues such as kidney, lung, heart, placenta or uterus. A possible role of GPx-GI is to protect against ingested toxic lipid peroxides.

Like glutathione peroxidase, the selenoenzyme Type 1 iodothyronine deiodinase (5'IDI) is an oxidoreductase and requires thiols for its activity. It is found mainly in the liver and kidney and is required for the conversion of thyroxine (3,5,3',5'-tetraiodothyronine, T4) to 3,5,3'-triiodothronine (T3) (Larsen *et al.* 1981). Sequencing of this enzyme determined that like the glutathione peroxidases the selenocysteine present in type 1 iodothyronine deiodinase is encoded for by a UGA codon present in the mRNA and that the selenocysteine is required for maximum activity of the enzyme (Berry *et al.* 1991).

Selenoprotein P is the first selenoprotein identified to contain more than one selenocysteine molecule. Analysis of the amino acid content suggests that each selenoprotein P molecule contains 7.5 selenocysteine molecules (Read *et al.* 1990).

However the sequence of the cDNA predicts 10 selenocysteines per selenoprotein P molecule, as the cDNA contains 10 TGA codons in its open reading frame (Hill *et al.* 1991). It is possible that some of the selenocysteines are lost during purification procedures or alternatively one of the TGA codons near the end of the sequence could behave as a stop codon giving a shorter protein than first expected. The function of selenoprotein P is not known although an important role can be inferred by the fact that it contains greater than 60% of the plasma selenium (Read *et al.* 1990). It has also been reported to incorporate selenium and be synthesized preferentially over glutathione peroxidase when selenium levels in selenium-deficient rats are repleted (Burk *et al.* 1991, Yang *et al* 1987). Selenoprotein P has been suggested to act as a transporter of selenium from the liver to the peripheral tissues based on the observation that radiolabelled selenium initially taken up by the liver is incorporated in to selenoprotein P before it accumulates in tissues (Motsenbocker and Tappel. 1982). However, since selenoprotein P contains covalently bound selenium molecules and since the selenium status of animals has no effect on the disappearance of radiolabelled selenium from the plasma, it seems unlikely that dominant role of selenoprotein P is to act as selenium transporter (Burk *et al.* 1991). Although selenoprotein P was first discovered in the plasma it has since proven to be more widespread throughout the body and has been identified in the liver, spleen, testis, kidney and heart (Motsenbocker and Tappel. 1982). Selenium-deficient rats were shown to increase selenium uptake by the brain 12-fold when injected with radiolabelled selenoprotein P, whereas no increased uptake of selenium was observed in the heart or skeletal muscle or when radiolabelled selenium was administered in the form of glutathione peroxidase, suggesting a specific mechanism of uptake of selenoprotein P by the brain (Burk *et al.*1991). A role as an antioxidant has also been suggested for selenoprotein P since administration of selenium to selenium-deficient rats protects against lipid peroxidation and liver necrosis induced by treatment with diquat (Burk *et al.* 1980). As selenoprotein P has been demonstrated to be restored more rapidly than glutathione peroxidases, it is hypothesised that it is selenoprotein P that is responsible for the observed protection against diquat following administration of selenium (Burk *et al.* 1991). The presence of many histidines and cysteines, which are known to be capable of metal binding in other proteins, and thiol groups in

selenoprotein P indicates the possibilty of redox-active sites, thus further suggesting a role for selenoprotein P as an antioxidant (Read *et al.* 1990).

Selenoprotein W was purified from rat muscle (Vendeland *et al.* 1993) and mRNA levels have been shown to alter in response to selenium status (Vendeland *et al.* 1995). The exact function of selenoprotein W is not known although its function could possibly be linked to skeletal and heart muscle myopathies observed in cases of selenium deficiency.

Although thioredoxin reductase is not a recently discovered protein it has only recently been identified as a selenoprotein (Gladyshiev *et al.* 1996). On the initial sequencing of this protein the UGA codon found close to the end of the mRNA sequence was interpreted to be a termination codon; however it is now known to encode a selenocysteine as the penultimate amino acid. This selenocysteine has recently been shown to be necessary for thioredoxin reductase activity (Hill *et al.* 1997) The thioredoxin system has an essential role in the production of DNA precursors and regulation of the activity of various proteins by reduction of protein disulphide groups.

Investigation of the activity and mRNA levels of cytosolic GPX (cGPX), PHGSH-Px and 5'IDI in rat liver following selenium depletion revealed that the abundance of the mRNA for cGPx and its activity was greatly reduced whereas the activity of PHGPx and 5'IDI and their mRNA levels were maintained (Bermano *et al.* 1995). These selenium deficiency experiments in the rat have also shown that selenoprotein activity is differentially maintained in different tissues. The activities of PHGSH-Px and 5' IDI were found to be substantially reduced in the liver whereas in the thyroid gland the activity of PHGPx was found to be maintained and the activity of 5'IDI was increased. These results demonstrate that in conditions of selenium deficiency, not only are certain selenoproteins preferentially maintained at the expense of others, but there is also differential regulation of mRNA abundance and activity of each selenoprotein between tissues. Bermano *et al.* (1995) demonstrated that the transcription of the genes for cGPx, PHGPx and 5'IDI was not effected by selenium deficiency. It was therefore suggested that differential regulation of different selenoproteins was a result of differential stability of their mRNA. Further work by Bermano *et al.* (1996) studied the effect of the untranslated region on the mRNA of

cGPx, PHGPx and 5'IDI on the efficiency and translation of 5'IDI in rat hepatoma cells. Under conditions of selenium depletion, the untranslated region of cGPx was found to cause a greater reduction in the levels of 5'IDI than the untranslated region of PHGPx or 5'IDI. On repletion of selenium levels, the translation efficiency of the cGPx and PHGPx untranslated regions were found to be similar but the efficiency of the 5"IDI untranslated region was found to be much more efficient. Thus it was demonstrated that the insertion of selenocysteine is regulated by the efficiency of the untranslated region of the mRNA.

1.4 Chemopreventive effects of selenium

1.41 Animal Work

Several studies in which selenium has been administered to the diet of animals have shown selenium to inhibit carcinogenesis in various tissues including the stomach (Kobayashi 1986), pancreas (Kise *et al.*1990), colon (Jacobs 1983; Banner *et al.* 1984), mammary gland (Ip 1981, Thompson and Becci 1980) and skin (Perchellet *et al.* 1987). Banner and co-workers (1984) showed that inhibition of tumour formation was greater in the proximal colon than in the distal colon and that this was correlated to selenium uptake and accumulation. Selenium uptake was also found to be higher in the mucosa than in muscle which is considered to be more beneficial since tumors induced by carcinogens are far more common in the mucosal tissues. In most studies, selenium is administered in the form of sodium selenite and this has been demonstrated to inhibit both chemically and virally induced carcinogenesis as well as growth of transplanted tumours (Milner 1985; Jacobs *et al.* 1983; Kobayashi 1986). Sodium selenite has been shown to inhibit carcinogenesis induced by chemical agents which require metabolic activation such as 2-acetylaminofluorene and dimethylhydrazine (Jacobs 1981; Jacobs *et al.* 1983) as well as direct acting carcinogens such as N-methyl-N-nitrosourea (MNU) (Thompson and Becci 1980) and N-methyl-N'nitro-N-nitrosoguanidine (MNNG), (Kobayashi 1986). Therefore, selenite must act to reduce tumour formation by a mechanism other than just effecting metabolic activation of carcinogens.

In a rat mammary tumour model in which carcinogenesis was initiated by DMBA and promoted by feeding the rats a high fat diet, sodium selenite was shown to inhibit both the initiation and promotional stages of carcinogenesis (Ip. 1981). Sodium selenite was able to inhibit the promotional phase of carcinogenesis but more effectively inhibited the initiation phase. Maximal inhibition of tumorigenesis was achieved by administration of sodium selenite two weeks prior to DMBA treatment and continuously during the course of the experiment. Studies investigating the stage specificity of selenium inhibition in the mouse mammary tumour model demonstrated that the initiation phase of carcinogenesis is more sensitive than the post-inititation phase (Medina and Lane 1983). The progression of established tumours was relatively insensitive (Medina and Shepherd 1981).

1.4.2 Epidemiological Studies

A number of studies comparing plasma selenium levels of cancer patients with controls matched for age, sex, and smoking. have found a lower selenium plasma concentration in cancer patients compared with controls (Salonen *et al.* 1984; Jaskievich *et al.* 1988). However other studies have found no significant difference in plasma concentrations of selenium between cases and controls (Menkes *et al.* 1986; Overad 1991). Plasma selenium concentration is subject to intra-individual variation and represents the individual's selenium consumption over a period of days to weeks. Measurement of selenium levels in toenails is a much more accurate measure of long-term selenium intake by an individual as it represents selenium intake over a period of up to 18 months prior to clipping. A case control study in patients diagnosed to have oral cancer which measured selenium levels in toenails found a negative correlation with selenium concentration and cancer risk (Rogers *et al.* 1991). A disadvantage of case control studies is that it is not possible to ascertain if the low selenium levels observed in the cancer patients is a result of the cancer rather than the cause of it. Tumours have been shown to sequester vitamins so it is possible that a large tumour could cause a decrease in plasma levels. Alternatively it may be that dietary habits of a cancer patient alter following onset of the disease.

A more reliable method to investigate the association of selenium levels and cancer risk is to set up prospective cohort studies since this type of study excludes the possibility that low selenium levels are caused by cancer. Sera collected in 1973 as part of the Hypertension Detection and Follow up Program in the USA provided a means of analysing selenium concentrations in sera, 5 years following collection from 4480 individuals. (Willett and Stampfer 1983). A small but significant increase in cancer risk was associated with low selenium levels and this effect was strongest when levels of other nutrients such as vitamin E and carotenoids were low. The strongest associations were observed for cancers of the lung, gastrointestinal tract and haematological cancers. Other prospective cohort studies have found inverse relationships with selenium levels and cancer risk in haematological, (Salonen *et al.* 1984, Ringstad *et al.* 1988), gastrointestinal (Salonen *et al.* 1984) and lung cancers (van de Brandt *et al.* 1993). An inverse relationship between serum selenium concentrations and subsequent cancer risk was observed in the stomach and oesophagus but not in the colon and rectum and this association was stronger for men than in women (Knekt *et al.*1988).

Epidemiological studies generally show only weak associations between selenium intake and cancer risk. A number of prospective studies have found no significant association between cancer risk and selenium levels (Virtamo *et al.* 1987, Nomura *et al.* 1987). This type of study has many limitations: although it is possible to select age and sex matched controls and adjust figures according to factors such as smoking and weight, there are still many variables that are unaccounted for, such as the vast variation in consumption of other foodstuffs, genetic predisposition, exercise and environmental factors. It is also argued that persons with high levels of selenium intake tend to have a healthy diet in general such as one high in vegetables and fish, and therefore have a higher intake of other vitamins and minerals which may also contribute to the observed reduction in the risk of cancer. More convincing evidence for the anticarcinogenic ability of selenium has been provided by *in vivo* studies in which variation between individual animals is more controlled.

1.4.3 Clinical Intervention Trials

A nutrition intervention trial in Linxian County, China, which has one of the world's highest rates of oesophageal and gastric cardia cancer, investigated the chemopreventive effects of four combinations of nutrients: 1) retinol and zinc, 2) riboflavin and niacin, 3) vitamin C and molybdenum and 4) beta carotene, vitamin E and selenium (Blot *et al.* 1993, Taylor *et al.* 1994). A significant decrease in mortality rate was observed in the participants receiving supplements of beta carotene, vitamin E and selenium and this reduction could be attributed mainly to a lower incidence of cancer rates, particularly cancer of the stomach. No significant effects on mortality rate were observed for any of the other treatment groups. However, as selenium was administered with vitamin E and beta carotene in this trial it is not possible to a ascertain whether selenium has chemopreventive effects *per se*.

Three intervention trials were set up in areas of China which have a high incidence of primary liver cancer (PLC) (Yu *et al.* 1991). The first trial in which table salt fortified with 15 ppm sodium selenite showed a decrease in PLC incidence of 34% after 5 years of supplementation, whereas no decrease in PLC incidence was seen in control populations which were without selenium supplementation. In the second study, 200 μg of selenium per day was given to hepatitis B virus surface antigen carriers in the form of a selenized yeast tablet. After 4 years, 5 of the 113 subjects in the control group receiving placebo had developed primary liver cancer, whereas there was no incidence of PLC in the 113 subjects taking the selenium tablets. The third intervention trial in China involved the supplementation, to persons from families with a high incidence of PLC, with 200 μg of selenium as selenium yeast tablets. At the end of a two year study 1.26% of the control group had developed PLC compared to only 0.69% in the selenium supplemented group.

An intervention trial in which 200 μg per day of selenium as yeast tablets was administered for an average of 4.5 years to approximately 1300 patients with a history of basal cell or squamous cell carcinoma found no significant effect of selenium on the further occurrence of basal cell or squamous cell cancer (Clark *et al.* 1996). However, a significant reduction in total cancer mortality and also cancers of the lung, colorectal and prostate were found to be significantly reduced on administration

of selenium. These studies provide more encouraging evidence for the potential of selenium to act as a chemopreventive agent.

1.5 Metabolism of selenite

Selenium in soils is more easily absorbed by plants as selenate than selenite (Spallholz *et al.* 1994). In plants selenate which is more toxic than selenite, is reduced to selenite and then metabolised in a pathway analogous to sulphur metabolism. In bacteria and animals a highly specific pathway for the metabolism of selenium exists. (Burk 1991).

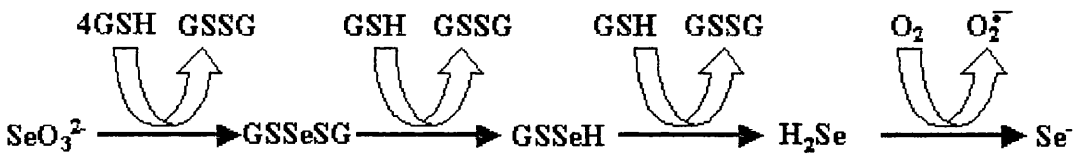


Figure 1.2

Mechanism proposed by Seko *et al.* 1989 for the generation of superoxide anion from the mixture of selenite ($Se0_3^{2-}$) and reduced glutathione GSH.

Inorganic selenite is reduced to form a selenopersulphide derivative via selenodiglutathione (SDG or GSSeSG) by the reaction proposed by Seko *et al.* (1989) (Figure 1.2). The selenopersulphide is formed by either direct reduction of selenite by glutathione reductase or spontaneously in the presence of excess glutathione (Ganther 1971). The selenopersulphide derivative undergoes a further reduction to hydrogen selenide which can be channelled in to the synthesis of selenoproteins. Alternatively it can undergo a series of methylation reactions by thiol-S-methyltransferase to form dimethylselenide and the trimethylselenonium ion in order to be excreted (Mozier *et al.* 1988; Ganther 1971) (Figure 1.3). Dimethylselenide is exhaled in the breath

whereas the trimethlyselenonium ion as well as inorganic and monomethylated forms of selenium are excreted in the urine (Vadhanavikit *et al.* 1993).

Evidence has been reported for metabolism in the back direction since animals administered trimethylselenonium have been shown to exhale dimethylselenide (Foster *et al.*1986a). Dimethylated forms of selenium such as selenobetaine and selenobetainemethylester administered to the rat has been shown to result in substantial excretion of monomethylated and inorganic forms of selenium thus providing further evidence that demethylation is an important step in the metabolism of selenium compounds (Foster *et al.* 1986b).

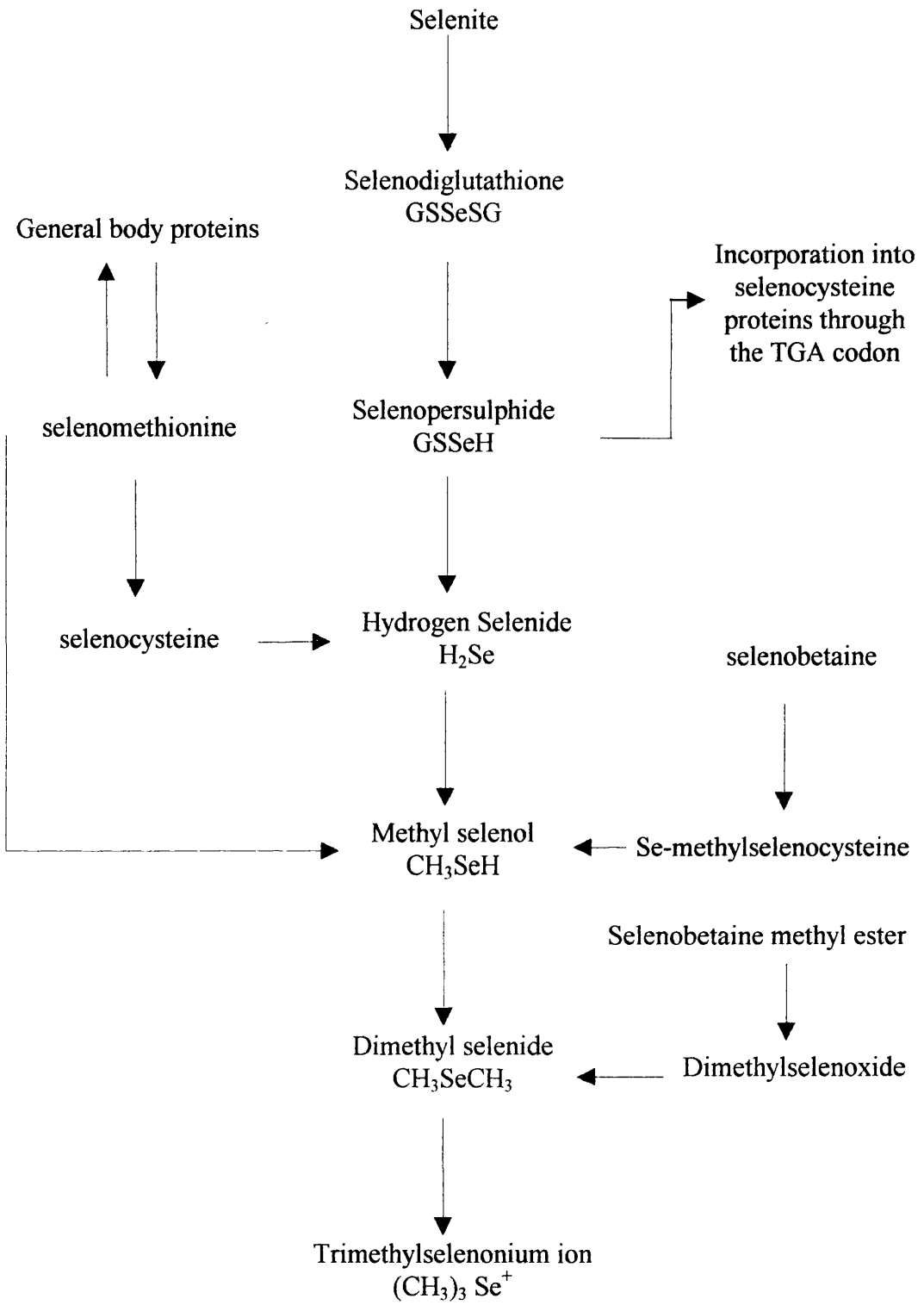


Figure 1.3

The metabolic pathway of selenite showing the entry of various seleno-compounds in to the pathway. Diagram adapted from (Ip. 1988) and (Lanfear *et al.* 1994).

1.6 Anticarcinogenic properties of selenium are related to its metabolic form

The metabolic form of selenium is critical for its ability to prevent carcinogenesis. A study of several selenium compounds in a dimethylbenz(a)anthracene (DMBA)-induced mammary tumour model in the rat found the order of potency with regard to the ability of the compound to reduce tumour incidence as follows: Se-methylselenocysteine> selenite> selenocysteine> dimethylselenoxide (Ip *et al.* 1994). This study also investigated the ability of various selenium compounds to restore glutathione peroxidase activity following selenium deprivation. Selenite and Se-methylselenocysteine were found to be the most effective in restoring glutathione peroxidase activity, whereas dimethylselenoxide was moderately effective. Trimethylselenonium was considerably less effective in the restoration of glutathione peroxidase activity, however at very high concentrations complete recovery could be achieved. As glutathione peroxidase is synthesised via hydrogen selenide this provides further evidence for demethylation as trimethylselenonium would need to be demethylated to form hydrogen selenide.

Certain mono and dimethylated selenocompounds, such as Se-methylselenocysteine and selenobetaine, have greater anticarcinogenic activity compared to selenite, yet the trimethylated selenocompound, trimethylselenonium ion, is relatively inactive. This suggests that the critical active selenium metabolite involved in the prevention of carcinogenesis is produced after selenite has been introduced in to the pathway, but before it is metabolised to the relatively inactive excretory products. This is supported by experiments in which arsenite was coadministered with selenium compounds. Arsenite inhibits the microsomal enzyme thiol-S-methyltransferase, which utilises S-adenosylmethionine to methylate hydrogen selenide or methylselenol to form dimethylselenide (Mozier *et al.* 1988) Arsenic has been shown to have no effect on mammary carcinoma formation *per se*, yet low levels of arsenite decrease the anticarcinogenic effects of inorganic selenite, whereas it potentiates the effects of methylated selenium compounds (Ip and Ganther 1990). This supports the conclusion that partially methylated selenium compounds have the greatest anti-cancer effect since coadministration of arsenic with selenium

compounds such as selenite and selenobetaine should result in the accumulation of partially methylated selenium metabolites, such as methylselenol.

1.7 Apoptosis

In living organisms there is a continuous turnover of cells in which old cells are replaced with new. Over-production of cells, or insufficient cell death, could have catastrophic effects on the organism, such as hyperplasia or tumour formation: it is therefore important that both these processes are closely regulated. There are two possible ways by which cells are known to die: necrosis and apoptosis (reviewed by Wyllie 1993). Necrosis occurs in response to a severe toxic cellular trauma, such as hypothermia or hypoxia. Swelling of the mitochondria and endoplasmic reticulum occurs followed by lysis of the cell as a result of the failure of plasma membrane ion transport mechanisms. The cytoplasmic contents of the cell are released into the extracellular fluid, including lysosmal enzymes which have the potential to damage the surrounding tissues and cause an inflammatory reaction.

In contrast, apoptosis is characterised by certain morphological cellular changes such as shrinkage in volume, rounding up caused by a loss of contact with neighbouring cells, membrane blebbing which is possibly a result of vesicles from the endoplasmic reticulum or golgi apparatus fusing with the plasma membrane and releasing their contents (Kerr *et al.* 1972). This results in a reduction in cellular volume and condensation of chromatin beneath the nuclear membrane. Apotosis is an active process, since inhibitors of RNA and protein synthesis, such as actinomycin D and cycloheximide, have been shown to inhibit apoptosis (MacDonald and Lees 1990; Martin *et al.* 1988). Activation of an endonuclease results in the cleavage of chromatin between nucleosomes which give the characteristic 'DNA ladder' when DNA from apoptosing cells is loaded on to an agarose gel and separated by electrophoresis (Duke *et al.* 1983). The endonuclease is activated by Ca^{2+} and inhibited by Mg^{2+}. Most of the organelles remain intact and the cell itself fragments into membrane bound apoptotic bodies which are phagocytosed by adjacent cells or nearby macrophages without inducing an inflammatory response. Thus, apoptosis does not result in damage to surrounding tissues.

Apoptosis is responsible for the destruction of thymocyte clones which recognise self-antigens (Macdonald *et al.* 1990) and occurs to remove excess neurons in the brain during development: those neurons unable to obtain sufficient nerve growth factor (NGF) of brain-derived neurotrophic factor undergo apoptosis in the developing brain (Martin *et al.* 1988).

A number of genes associated with the regulation of apoptosis have been shown to be involved in the formation of tumours. The ability of wild type p53 to act as a tumour supressor gene by inducing apoptosis is lost by mutation, in greater than 50% of cancers (Hollstein *et al.* 1991). Mutations of p53 are common in a wide range of cancers including cancers of the lung, colon, oesophagus, breast, liver and haemopoetic tissues (Hollstein *et al.* 1991). Overexpression of the apoptosis-inhibitory protein Bcl-2 and its homologue mcl-2 induced by the Epstein Barr protein latent membrane protein-1 (LMP-1) has been implicated in Burkitt's Lymphoma (Wang *et al.* 1996). Fas, a member of the Tumour Necrosis Factor (TNF) receptor super-family has been shown to induce apoptosis (Itoh *et al.* 1991; Oehm *et al.* 1992). A family of proteases named Interleukin-1B-converting enzyme (ICE)-like proteases, has been identified and shown to be involved in Fas and TNF mediated apoptosis since inhibitors of this proteases are able to inhibit apoptosis induced by Fas or TNF (Enari *et al.* 1995; Los *et al.* 1995).

1.8 The involvement of the Bcl-2 family of proteins in regulating apoptosis

Bcl-2 was first discovered in human follicular lymphomas containing a t(14;18) chromosomal translocation (Tsujmoto and Croce 1985). More recently Bcl-2 has been shown to prevent apoptosis in a number of cell lines including haemopoetic, human breast, rat fibroblast and Chinese Hamster ovary cell lines when induced by a variety of different stimuli, yet its mechanism of action is not yet understood (Hockenbery *et al.* 1993; Lee *et al.* 1997; Pourzand *et al.* 1997; Bissonnette *et al.* 1992). A number of apoptosis-inducing compounds have been reported to inactivate or down regulate Bcl-2, such as the differentiation- and apopotosis- inducing agent sodium phenylacetate in a human malignant breast cell line (Adam *et al.* 1995). Apoptosis in MCF7 cells induced by treatment with taxol results in phosphorylation and inactivation of Bcl-2

(Blagosklonny *et al.* 1996). It was also shown that taxol reduced the coprecipitation of Bcl-2 with Raf-1, thus activating Raf-1; moreover, Raf-1 was necessary for the taxol induced phosphorylation and inactivation of Bcl-2, since this was prevented when Raf-1 was depleted by administration of benzoquinone.

Transgenic mice producing the human bcl-2 gene in hepatocytes were shown to be greatly protected from hepatic failure due to massive hepatocyte apoptosis following treatment with the agonistic anti-Fas antibody (Lacronique *et al.* 1996).

Bcl-2 is a 25 kDa protein and it has been shown to be localised on mitochondria, nuclear membranes and endoplasmic reticulum. Since these are all sites of free radical generation it is possible that Bcl-2 may interfere with the production or effects of free radicals (Hockenberry *et al.* 1990; Krajewski *et al.* 1993). Immunolocalisation studies in subcellular fractions of cells revealed that the majority of Bcl-2 was located on the mitochondrial membrane rather than in fractions containing the plasma membrane or golgi apparatus (Hockenbery *et al.* 1990). As the major functions of the inner mitochondrial membrane include oxidative phosphorylation, as well as electron and metabolite transport mechanisms, it is not unreasonable to hypothesise that Bcl-2 could regulate one of these mechanisms. Bcl-2 contains a hydrophobic sequence in its C-terminus which has been shown to act as an anchor segment required to locate the protein to its outer mitochondrial membrane position (Nguyen *et al.* 1993). Studies with a vector expressing Bcl-2 protein lacking the C-terminal anchor sequence showed that this truncated protein was mis-localised throughout the entire cytosol in contrast to full length protein which is found to be bound to mitochondria (Hockenbery *et al.* 1993). However, although the truncated form of Bcl-2 is not localised to the mitochondrial membrane it was still able to protect the IL-3 dependant haemopoetic cell line FL5.12 from apoptosis on IL-3 withdrawal although it was not as effective as full length Bcl-2. Thus, although Bcl-2 functions more efficiently when intergrated into the mitochondrial membrane, this is not essential for its activity (Hockenberry *et al.* 1993).

Over expression of Bcl-2 has been shown to prevent c-myc induced cell death in a multi-drug resistant human breast cell line and Chinese Hamster Ovary cells (Bissonnette *et al.* 1992; Lee *et al.* 1997). Investigation of the effects of Bcl-2 on the apoptotic and mitogenic functions of c-myc was investigated in Rat-1 fibroblast cells

(Fanidi *et al.* 1992). c-myc was activated by serum depletion and addition of β-oestradiol in fibroblast cells resulting in apoptosis. However, c-myc induced apoptosis was completely inhibited in Rat-1 fibroblasts in which Bcl-2 was overexpressed, whereas the proliferation rate in low serum, under the control of c-myc activated by β-oestradiol, was not effected. These experiments suggest that Bcl-2 can selectively inhibit the apoptosis-inducing abilities of c-myc without affecting its ability to promote proliferation.

Bcl-2 is thought to inhibit apoptosis induced by UVA radiation in a rat fibroblast cell line (R6) by acting as an antioxidant. Treatment of R6 cells with UVA radiation results in the upregulation of the heme oygenase 1 (HO-1) gene by inducing oxidative stress (Keyes *et al.* 1989). Over expression of Bcl-2 has been shown to protect against UVA induced radiation and inhibit the upregulation of the HO-1 gene (Pourzand *et al.* 1997). Thus it was hypothesised that Bcl-2 can prevent the generation of reactive oxygen species or is protective against their effects.

Bcl-2 has been shown to protect a human lymphoid cell line and an IL-3 dependant murine pro-B cell line (BAF3) against apoptosis induced by a monoclonal anti-APO antibody and a neutralising monoclonal anti IL-3 antibody respectively, under hypoxic conditions (Jacobson *et al.* 1995). As the generation of reactive oxygen species is small under hypoxic conditions it was hypothesised that the protective effect of Bcl-2 may be attributed to interference with apoptotic induction pathways rather than the production of reactive oxygen species.

Bcl-2 protects against dexamethasone induced lipid peroxidation (Hockenbery *et al.* 1993). Incubation of T cell hybridoma, 2B4 cells, with the polyunsaturated fluorescent fatty acid, cis-parinoic acid, leads to its incorporation in to the lipid membrane and there it provides a measure of oxidative stress as fluorescence is lost on lipid peroxidation. Treatment of these cells with dexamethasone resulted in loss of fluorescence and therefore presumably generation of reactive oxygen species. No loss of fluorescence was observed in 2B4 cells in which Bcl-2 was overexpressed after treatment with dexamethasone thus demonstrating that Bcl-2 protects against dexamethasone induced lipid peroxidation. Using the oxidation-sensitive fluorescent compound, 5,6-carboxy-2,7- dichlorofluorescin, Hockenbery and coworkers were able to assess the production of peroxides in 2B4 cells with and without Bcl-2

overexpression following dexamethasone treatment. However, Bcl-2 did not affect peroxide production following dexamethasone treatment in cells constitutively expressing Bcl-2, compared to those which did not; this demonstrates that, although Bcl-2 can protect against the damaging effects of reactive oxygen species, it does not prevent their generation.

Jun kinase (JNK) and p38 kinase are members of the mitogen-activated protein kinase super-family and are activated in response to stimuli such as UV radiation, removal of growth factors, oxidants, osmotic shock and inflammatory cytokines (Minden *et al.*1994; Xia *et al.* 1995; Raingeaud *et al.* 1995). The stress-regulated signalling pathways that activate JNK or p38 are integrated by another MAP kinase protein, ATF2. cJun can function as a heterodimer with ATF2 which is regulated by JNK and the p38 (MAP) kinase (Livingstone *et al.* 1995; Raingeaud *et al.* 1995). Induction of JNK has been shown to activate c-Jun prior to induction of apoptosis in the PC12 pheochromocytoma cell line by withdrawal of Nerve Growth Factor (NGF) (Xia *et al.* 1995). PC12 cells transfected to constitutively overexpress Bcl-2 protein did not die on removal of serum and did not show an increase in JNK level thus suggesting that Bcl-2 works upstream from JNK, preventing its activation in order to protect cells from apoptosis (Park *et al.* 1996).

A number of other proteins have also been shown to have a role in the regulation of apoptosis such as Bax, Bad, Bcl-xS, Bcl-xL and Bag-1. Bcl-x mRNA is alternatively spliced, encoding a larger protein, Bcl-xL and a smaller protein Bcl-xS. Bcl-xL is consists of 233 amino acids and has a high degree of homology to Bcl-2, however the region with the highest homology is deleted in Bcl-xS giving a protein consisting of 170 amino acids (Boise *et al.*1993). Bcl-xL was shown to protect against growth factor withdrawal in an IL-3 dependant murine cell line FL5.12 whereas Bcl-xS was shown to inhibit the ability of Bcl-2 to protect against cell death. Bcl-xS did not have any effect on the viabilty of cells or the rate of cell death when expressed alone. Bcl-xS mRNA is found at high levels in cells which undergo a rapid turnover such as developing lymphocytes, whereas Bcl-xL is found in tissues containing long-lived post-mitotic cells such as the adult brain. Thus, regulation of the expression and splicing of Bcl-x mRNA plays an important role in the regulation of apoptosis. Bax, another member of the Bcl-2 family, has extensive homology to

Bcl-2 and forms homodimers or heterodimers with Bcl-2 (Oltavi *et al.* 1993). Overexpression of Bax resulted in acceleration of apoptosis in FL5.12 cells induced by cytokine deprivation although it did not induce cell death itself. The ratio of Bcl-2 to Bax appears to be important in determining the sensitivity of a cell to a death stimulus (Oltavi *et al.* 1993). When Bcl-2 predominates, the cells are protected from apoptosis, however when Bax is in excess, the cells are sensitive to the death stimulus. Bad has some homology to Bcl-2 but unlike the other Bcl-2 members it does not appear to form homodimers and lacks the C-terminal signal-anchor sequence responsible for positioning the other Bcl-2 family members to intergral membranes (Yang *et al.* 1995). Bad forms dimers with Bcl-xL and Bcl-2 but does not form dimers with Bax, Bcl-xS or Mcl-1. Bad competes with Bax for binding to Bcl-xL and overexpression of Bad was shown to inhibit the protective effect of Bcl-xL against programmed cell death. Bad also competes with Bax for binding to Bcl-xL but had no effect on the ability of Bcl-2 to protect against apoptosis. Overexpression of both Bcl-2 and Bag-1 gave a greater protection against a variety of death inducing stimuli than when either protein was expressed alone (Takayama *et al.* 1995). However, Bag-1 is not a member of the Bcl-2 family and does not contain any homology to Bcl-2 or any of the other Bcl-2 family members.

1.9 Selenium compounds induce apoptosis *in vitro*

In vitro studies have demonstrated the ability of various selenium compounds to induce apoptosis in a number of cell lines. Administration of selenite and the novel synthetic selenium compound p-XSC resulted in the cells displaying characteristics typical of apoptosis such as condensation of the cytoplasm and nuclei and formation of apoptotic bodies (Thompson *et al.* 1994). Electrophoresis of extracted DNA resulted in a DNA ladder which is a typical feature observed in apoptotic cells. Administration of SDG to mouse erythroleukaemic (MEL) cells resulted in membrane blebbing and many of the cells became bi- or multi-nucleate with many of the nuclei appearing damaged (Lanfear *et al.* 1994). Cells were further examined for apoptosis using two dye flow cytometry. This method involves the staining of cellular DNA with the fluorescent dyes propidium iodide and Hoechst 33242. Viable cells are able

to exclude both dyes whereas necrotic cells and cells in the late stages of apoptosis are stained with both dyes. Changes in the membrane permeability of apoptotic cells are such that Hoechst stain is able to penetrate the membrane but the propidium iodide is excluded from the nucleus. Further analysis by agarose gel electrophoresis showed that cells treated with SDG produced a DNA ladder, thus providing more evidence that SDG induces apoptosis in MEL cells. Lanfear and coworkers (1994) also demonstrated that SDG treatment caused an induction of p53 protein in the ovarian cell line A2780, which expresses wild type p53. However, it seems unlikely that the p53 pathway is essential for the induction of apoptosis since SDG induces apoptosis in MEL cells which possess mutant p53 and thus do not have the normal p53 response mechanism.

Thus, the mechanism by which selenium compounds induce apoptosis is not understood. The reduction of selenite by its reaction with glutathione is proposed to result in the generation of superoxide anion which is then converted to H_2O_2 by superoxide dimutase (Seko *et al.* 1989). Thus it has been hypothesised that selenite could induce apoptosis by the production of H_2O_2 and thereby activate oxidative stress pathways. However, comparison of apoptosis induced by SDG and H_2O_2 in the mouse mammary cell line C57 suggested that these compounds induce apoptosis by different mechanisms (Wu *et al.* 1995). The stochiometry of the reaction proposed by Seko implies that one molecule of H_2O_2 would be produced for each molecule of SDG with undergoes reduction, yet the concentration of H_2O_2 required to give an equivalent kill to that of SDG is 200 fold higher. Moreover, treatment of cells with SDG resulted in the formation of 50 and 150kb DNA fragments, whereas treatment with H_2O_2 resulted in the formation of 560kb fragments only. Wu and coworkers (1995) also demonstrated that a mutant C57 clone, B19, was resistant to SDG but was not cross resistant to cell death induced by H_2O_2 thus providing further evidence that the two compounds act by separate mechanisms.

1.10 Possible Mechanisms of chemoprevention by selenium compounds

Epidemiological studies and animal studies have shown selenium to have anticarcinogenic properties but the mechanism involved is not understood. Selenium is essential for the synthesis of glutathione peroxidases which act as antioxidants, preventing damage by peroxides and free radicals to cellular macromolecules. It has been hypothesised that increasing selenium intake could increase glutathione activity and thus inhibit carcinogenesis. The activity of cellular GPx in the mammary gland in rodents is generally saturated at approximately 0.1 ppm Se to 0.2 ppm Se for rats (Lane and Medina 1993; Ip and Daniel 1985) whereas the concentration required for inhibition of carcinogenesis is usually approximately 2 ppm. The activity of plasma glutathione peroxidase in rats was also found to be saturated at selenium adequate levels; 0.104 mg Se/kg (Bermano 1996). Therefore, it seems unlikely that the activity of these enzymes is responsible for the anti-carcinogenic properties of selenium.

Selenium was found to strongly depress the mutagenic effects of acridine orange and DMBA in the Ames Salmonella/microsome mutagenicity test (Martin *et al.* 1981). A maximum reduction of histidine revertants following acridine orange treatment was observed with 5 ppm sodium selenite which is a comparable level to that administered *in vivo.* However, a much higher concentration of 44 ppm was required to achieve maximum inhibition of the mutagenic effects of DMBA. Selenium has been shown to reduce DNA adduct formation by N-hydroxyamino-fluorene (NOHAAF) and acetylamino-fluorene (AAF) when administered to the diet of rats as 4 ppm sodium selenite 3 days before carcinogen treatment (Daoud and Griffin 1978). This study also found that selenite caused a 50% decrease in p-nitrophenol-sulphotransferase activity which is responsible for NOHAAF and AAF activation and an increase of 100% in glucoronyltransferase activity, an enzyme involved in detoxification mechanisms. The decrease in carcinogen binding to DNA on selenium administration can therefore possibly be attributed to a decrease in activation, and an increase in detoxification processes. Selenite was also shown to reduce DNA adduct formation following DMBA treatment *in vivo;* however, 3 weeks of pre-treatment with selenium was required before any inhibition was observed (Ejadi *et al.* 1989). This study also showed that selenium affected the metabolism of

the carcinogen since a decrease in the levels of anti-dihydrodiol epoxide adducts was observed.

Although the initiation phase of tumorigenesis is most sensitive to selenium administration, the post-initiation phase of tumorigenesis is also inhibited by selenium. Selenite can also inhibit tumorigenesis induced by direct acting carcinogens which do not require metabolic activation such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Kobayashi *et al.* 1986) and N-methyl-N-nitrosourea (MNU) (Thompson and Becci. 1980) as well as virally induced tumorigenesis, (Milner 1985) providing further evidence that alteration in metabolism is not solely responsible for the anticarcinogenic properties of selenium molecules.

Although at low concentrations selenium is able to stimulate cell growth, at higher concentrations it has been shown to be cytotoxic (Medina and Oborn 1984; Webber *et al.* 1985). The ability of selenium to inhibit cell growth *in vitro* may be related to its antitumorigenic properties *in vivo*. At concentrations which are growth inhibitory, selenium has been shown to inhibit the synthesis of DNA, RNA and to a lesser extent protein synthesis. (Gruenedel and Cruikshank. 1979). Selenodiglutathione (SDG), the primary metabolite of selenite has been shown to inactivate elongation factor 2 (EF2) in an *in vitro* cell free system (Vernie *et al.* 1974). It was hypothesised that SDG was able to react with the sulphydryl groups present in EF2 resulting in its deactivation. Incubation with glutathione reductase was shown to reverse the inhibition of EF2 activity (Vernie *et al.* 1975). As it can be expected that glutathione reductase is present in most cells this raises some doubt as to whether SDG would inhibit EF2 *in vivo*. Examination of protein synthesis using rabbit reticulocyte lysate found no inhibitory effect of selenite on EF2 activity, although selenite was observed to inhibit the activity of the Met-tRNA binding factor, eukaryotic initiation factor 2 (eIF-2) (Safer *et al.* 1980).

Thioredoxin is reduced by a reaction with NADPH catalysed by thioredoxin reductase and is then able to act as a hydrogen donor and reduce protein disulphides, thus regulating the activity of other protein and enzyme systems. Selenite and SDG are able to oxidise mammalian thioredoxin and thioredoxin reductase and act as competitive inhibitors of thioredoxin reductase (Bjornstedt *et al.* 1992). When selenite is added to the reaction mixture, a lag phase is observed before NADPH

oxidation occurs. However, no lag phase occurs when SDG is added to the reaction mixture, suggesting that SDG or one of its metabolites is the substrate for the enzyme, rather than selenite. Ribonucleotide reductase is required for the production of precursors for DNA replication and its activity is regulated via thiol redox control by the thioredoxin system. Thus, inhibition of the thioredoxin system could explain how selenium is able to inhibit DNA synthesis. SDG was shown to inhibit reduction of dCDP by ribonucleotide reductase, possibly by inhibition of thioredoxin reductase; however the possibility of SDG directly acting on the active site of ribonucleotide reductase can not be excluded.

1.11 Novel Selenium Compounds

Although selenium compounds have been shown to have anti-carcinogenic properties, a major problem is that toxicity occurs at levels not much higher than the therapeutic dose. Studies have been performed in which selenium is administered as naturally occurring forms such as selenium enriched garlic, with the hope of delivering selenium more safely. The garlic is grown in soil enriched with sodium selenite and results in the accumulation of high levels of an organic form of selenium in the garlic. By using a DMBA and MNU mammary carcinogenesis model in rats, selenium enriched garlic extract was found to provide significant protection against tumour formation, in contrast to natural garlic extract, which was found to have no effect on mammary carcinogenesis (Lu *et al.* 1996). It can therefore be assumed that the anti-cancer activities of the selenium-enriched garlic are provided by an active selenium compound. Selenomethyl selenocysteine has been identified as the major amino acid in selenium enriched garlic (Cai *et al.* 1995) and it has also been shown to have similar biological activities to selenium-enriched garlic extracts such as cell cycle blockade, cell morphology and DNA fragmentation patterns when examined *in vitro* (Lu *et al.* 1996). Both selenite and selenomethyl selenocysteine have been demonstrated to inhibit cell growth and induce cell death in a mouse mammary carcinoma cell line (Lu *et al.* 1995). Selenite was shown to cause single strand DNA breaks in a dose-dependant manner, which is considered a measure of genotoxicity. Selenite undergoes reductive metabolism resulting in the formation of superoxide and

other reactive oxygen species which are thought to be responsible for the DNA single strand breaks and thus cell death. Double strand breaks are also observed shortly after the appearance of single strand breaks following selenite treatment (Lu *et al.* 1995). In contrast, selenomethyl selenocysteine appears to exert its anti-cancer effects in the absence of genotoxicity as it was able to induce double strand breaks and cell death in the absence of single strand breaks. Further evidence that selenite and Se-methylselenocysteine inhibit cell growth and induce cell death by different mechanisms is provided by their ability to cause cell cycle arrest at different stages of the cell cycle. Selenite has been shown to cause cell cycle blockade in the S/G2-M phase of the cell cycle, whereas selenomethyl selenocysteine has been demonstrated to cause a G1 cell cycle arrest (Lu *et al.* 1995). Selenomethyl selenocysteine has also been shown to cause a 74% decrease in cyclin E-cdk2 content and a 57% decrease in cdk2 kinase activity whereas treatment with selenite resulted in no change in the binding of cyclins D1, E or A to cdk2 and a 30% increase in cdk2 kinase activity (Sinha *et al.* 1996).

A number of groups have tried to develop novel selenium compounds with chemopreventive activity but without toxic side effects. Replacement of the oxygen and/or sulphur atom in certain naturally occurring compounds has been shown to enhance the anti-cancer properties of that compound. A study which compared the synthetic organoselenium compound benzyl-selenocyanate (BSC) with its sulphur analogue benzyl-thiocyanate (BTC) in their ability to inhibit DMBA induced mammary carcinogenesis in rats found that the organoselenium compound BSC significantly inhibited tumour incidence and multiplicity whereas its sulphur analogue BTC had no effect on mammary carcinogenesis (Nayini *et al.* 1989). This demonstrates that the selenium molecule is essential for the anticarcinogenic properties of the compound. However, the body weights of rats fed diets containing BSC were lower than control rat thus suggesting systemic toxicity.

An analogue of BSC, 1,4-phenylenebis(methylene)selenocyanate (p-XSC), has been developed which has been shown to have greater chemopreventive properties with a lower incidence of toxicity. In a DMBA-induced mammary carcinogenesis study in the rat, p-XSC was shown to have a higher chemotherapeutic index (calculated by the ratio of the maximum tolerated dose, to the dose required to give

50% inhibition of tumour yield) than BSC, methyl selenocyanate (KSeCN) and selenite (Ip *et al.* 1994). Lung tumour multiplicity in mice in which tumours were induced by 4-(methylnitrosamino)-1-(-3-pyridyl)-1-butanone (NNK), a component of tobacco, was significantly inhibited by 5, 10 and 15 ppm p-XSC administered in the diet (El-Bayoumy *et al.* 1993). Sodium selenite was not found to have any effect on lung tumour induction by NNK. No apparent toxicty was observed at 15 ppm p-XSC which is three times the level of selenium given as selenite which can be tolerated in mice. An *in-vitro* study which compared the accumulation of p-XSC and selenite in cells demonstrated that mouse mammary carcinoma cell lines were able to tolerate higher levels of selenium when it was administered as p-XSC as opposed to selenite (Thompson *et al.* 1994).

It is not understood how p-XSC provides protection from carcinogenesis but its ability to induce apoptosis (Thompson *et al.* 1994), inhibit the activity of prostaglandin E_2 (Reddy *et al.* 1992) and prevent formation of DNA adducts (El-bayoumy *et al.* 1995), may be related to its anti-cancer properties.

A number of aliphatic selenocyantes were developed which varied only in the length of the aliphatic chain, CH_3-(CH_2)n-SeCN in which n = 0, 2, 4, or 6 and were tested for their ability to inhibit DMBA induce mammary cancer in the rat (Ip *et al.* 1995). A reduction in tumour yield and tumour incidence was observed without any effect on the growth rate of the animals. The chemopreventive potency of the compound increased with chain length, as did the ability to inhibit the formation of DNA binding adducts. The optimum aliphatic carbon side was found to be a pentyl group as there was no further increase in the chemopreventive effects by the addition of an extra carbon group.

1.12 Aims of project

The mechanism by which selenium has its anticarcinogenic effects is not understood although it is likely to be related to its ability to inhibit cell growth and induce apoptosis. The mechanism by which selenium induces apoptosis is also not understood although it has been hypothesised that selenite induces cell death during its reduction to hydrogen selenide by the generation of reactive oxidant species resulting in oxidative stress. Evidence from other studies suggests that certain organic

molecules such as selenomethyl selenocysteine may induce cell death by a different mechanism to selenite.

Bcl-2 has been shown to inhibit the ability of a number of stimuli to cause apoptosis possibly by its ability to prevent the effects of reactive oxygen species and peroxides, therefore determination of whether Bcl-2 could inhibit selenium induced apoptosis may provide a further insight in to the mechanism involved in selenium induced apoptosis.

Most studies investigating the effects of selenium on carcinogenesis have used the inorganic selenium compound selenite which is taken up from the soil by plants and converted to inorganic selenium compounds. The primary metabolite of selenite formed by its reaction with glutathione, selenodiglutathione (SDG) has been shown to be more potent than selenite and can induce apoptosis rapidly unlike selenite which induces apoptosis after a lag phase. Co-administration of glutathione with selenite results in the abolition of the lag phase thus suggesting that to induce apoptosis selenite must first be metabolised to SDG. Thus SDG was chosen to be the selenium compound used to test if Bcl-2 can protect against selenium induced apoptosis.

A number of groups have attempted to synthesize novel selenium compounds which have the anticarcinogenic properties of selenite but without the toxicty observed with selenite administration. One such compound, p-XSC has been shown to be more potent at inducing apoptosis than selenite and have greater chemopreventive properties yet appears to be less toxic than selenite. It was also therefore interesting to deduce if p-XSC induced cell death could be inhibited by overexpression of Bcl-2.

Materials and Methods

2.0 Materials

Tissue culture

Supplier: Beatson Institute Central Services

-PBS, PE

Supplier: Life Technologies Ltd, Paisley,U.K.

-Glutamine, G418, Special Liquid Medium, Trypsin

Supplier: Sigma, Poole, UK.

-RPMI Medium, Bufferall, Sodium Bicarbonate, Sodium Pyruvate

Supplier: Flowgen, Kent, U.K.

-electroporation cuvettes

Chemicals

1,4-phenylenebis(methylene)selenocyanate (p-XSC) was kindly donated by Dr. El-Bayoumy, American Health Foundation, Valhalla, New York

Supplier: Boehringer, Lewes, U.K.

-Cesium Chloride

Supplier: Dupont, Stevenage, U.K.

- ^{35}S-GSH

Supplier: Fisher Scientific, Loughborough, U.K.

-Ammonium acetate, Ammonium persulphate, Butanol, DMSO, EDTA Glacial Acetic Acid, Glucose, Glycerol, Glycine, Hydrochloric acid, Isopropanol, Methanol, Sodium Acetate, Sodium Dodecyl Sulphate, Sodium Chloride, Sodium hydroxide

Supplier: Life Technologies Ltd., Paisley, U.K.

-Tris.HCl, Protein molecular weight markers

Supplier: James Burrough (F.A.D.) Ltd.

-ethanol

Supplier: Severn Biotech Ltd.,

-30% Acrylamide

Supplier: Sigma, Poole, U.K.

-Reduced glutathione (GSH), Sodium selenite, Nickel Chloride, Ninhydrin stain TEMED, Tween 20, Ethidium Bromide, Aprotinin, Leupeptin, PhenylmethylsulphonylFluoride (PMSF)

Chromatography Materials

Supplier: Bio-rad Laboratories Ltd., Hemel Hempstead, U.K.

-AG50W H^+ resin

Supplier: Sigma, Poole, U.K.

-G10 Sephadex beads

Supplier: Camlab, Cambridge, U.K.

-TLC plates

Antibodies

Supplier: Amersham International plc, Little Chalfont, U.K.

-anti rabbit Horse Radish Peroxidase conjugated

Supplier: PharMigen Deutschland GmbH, Hamburg, Germany

-Rabbit anti-mouse Bcl-2 antiserum

Kits

Supplier: Promega, Southampton,U.K.

-MTT assay kit

ECL reagents, dyes,

Supplier: Ammersham International plc, Little Chalfont, U.K.

-ECL membranes, ECL detection reagents

Supplier: Sigma, Poole, U.K.

-bromophenol blue, ficol, Hoechst 332258 stain

Supplier: BDH Ltd.,Poole,UK.

-Giemsa Stain

Supplier: Life Technolgies Ltd., Paisley, U.K.

- 1Kb DNA ladder

Methods

2.1. Tissue culture techniques

2.1.1 Maintenance and Growth of Cell Lines

MCF7 cells were grown in a humidified incubator at 37°C in an atmosphere of 5 % (v/v) CO_2/O_2. Cells were grown in RPMI medium with 10% foetal calf serum and buffered to a constant pH using 1% Bufferal.

500 ml RPMI medium:
-400 ml distilled H_2O, 45 ml 10x RPMI, 50 ml Foetal Calf Serum,
5 ml glutamine, 5 ml sodium pyruvate, 13.3 ml sodium bicarbonate,
5 ml bufferal

Confluent cells were trypsinised every 3-4 days. For a T25 tissue culture flask growth medium was removed by aspiration and cells washed with 5 ml PBS. 0.5 ml PE containing 0.25% trypsin was added and left for 1-2 min at room temp and then gently agitated to detach cells from the flask surface. Trypsin was inactivated by the addition of RPMI/10% FCS and the cells seeded at an appropriate concentration into new flasks. Cells were grown in this manner for a maximum of 3 months after which time they were replaced from frozen stocks.

2.1.2 Storage of cells

Cells were trypsinized, resuspended in growth medium and pelleted by centrifugation for 5 min at 1000 rpm in a Centaur 2 MSE centrifuge. The cells were resuspended in RPMI/20%FCS/10%DMSO at a concentration of $5x10^6$ - $1x10^7$ per ml and alloquoted into 1 ml Nunc cryotubes. The cryotubes were wrapped in cotton wool and allowed to cool slowly overnight (about 1 °C/min) to -70°C after which they were transferred to liquid nitrogen. Ampoules of cells were thawed by placing into water at 37°C, the cells transferred to a universal container, fresh RPMI added and the cells pelleted as described above to remove DMSO. The cells were then resuspended in growth medium and seeded into dishes.

2.1.3 Testing for mycoplasma

NRK 49 fibroblast cells were seeded into 60mm dishes at 10^4 cells per dish in 4 ml SLM/10%FCS. 2 ml of media which had been in contact with MCF7 cells for a minimum of 2 days was added to the dishes. One dish of NRK cells was set up in parallel without conditioned medium as a control. The NRK cells were then incubated for 3-4 d in a humidified incubator at 37°C/5%(v/v) CO_2 .The medium was removed and the cells washed twice with PBS. 2.5 mls PBS and 2.5 mls of fixative (3 volumes methanol:1 volume glacial acetic acid) were slowly added to each dish and then poured off. Cells were then washed twice more with 5 ml fixative for 10 min and the dishes left to drain. To each dish 5mls of PBS containing 0.05μg/ml Hoechst 33258 stain was added and then left for 10 min at room temperature. The stain was removed and the dishes washed twice with distilled water. The cells were then examined for mycoplasma using a Leitz Wetzlar fluorescent microscope, using a water immersion lens.

2.2. Kill curve assays

2.2.1 MTT Assay

Cells were seeded in to 96 well microtitre plates at 500 cells per well and incubated overnight at 37°C in a humid atmosphere with 5%(v/v) CO_2. The medium was aspirated and fresh medium containing SDG or pXSC at the appropriate concentration was added to each well. Samples were prepared in quadruplicate. SDG or pXSC was added to medium immediately before addition to wells to ensure minimum degradation and plates were then incubated at 37 °C overnight. 15 μl of MTT dye was added to each well and then the plates were incubated at 37 °C for 4 h. 100 μl solubilisation stop solution was then added to each well and the plates left overnight at room temp. The optical density of each well against wells containing medium only was measured using a Dynatech MR7000 microtitre plate reader.

2.2.2 Clonogenic growth assays

250 cells were plated into 60 mm dishes and incubated overnight at 37°C to allow cells to adhere. Increasing concentrations of SDG or pXSC was then added to each dish and the cells incubated at 37°C for 14 d. The medium was then removed, the cells washed with PBS and fixed by the addition of 3 ml methanol for 20 min. The methanol was discarded, the plates gently rinsed with distilled water and 3 ml of Giemsa stain was added to the dishes and left for 15 min. The stain was then poured off and the dishes rinsed with H_20 to remove excess stain. The number of giemsa stained colonies on each dish was then counted.

2.3. Preparation of Bcl-2 expressing clones

2.3.1 Transfection of cells with Bcl-2 expressing vector

$1x10^7$ MCF7 cells were pelleted by centrifugation and resuspended in 1ml RPMI/10%FCS. 10 μg of pSSFV bcl2-2n human expression vector DNA (kindly donated by Dr Korsmeyer, Howard Hughes Medical Institute, St Louis, USA) was added and 0.8 mls was then transferred to an electroporation cuvette. Electroporation was then carried out using a Biorad gene pulser at 640 $V.cm^{-1}$, 960 μF. The contents of the electroporation tube were then transferred to a T175 flask containing RPMI/10%FCS. After 48 h, the transfected cells were selected for by the addition of the selection marker, G418, at 0.15 $mg.ml^{-1}$.

2.3.2 Ring cloning

Following electroporation, cells were plated on 90 mm petri dishes at about 50 cells per dish and incubated for 7-14 days in RPMI containing 0.15 $mg.ml^{-1}$ of the selection marker, G418, so that individual colonies, containing the pSSFV bcl2-2n human expression vector, could be selected. Medium was removed by aspiration and cells were washed with PBS. Colonies were selected using a ring to surround the colony attached to the petri dish with silica gel. Colonies were detached by the addition to the ring of 100 μl of 0.25% trypsin in PE and transferred using a pasteur pipette to a 6 mm petri dish containing RPMI/0.15$mg.ml^{-1}$ G418.

2.4. Large scale preparation of plasmid DNA

2.4.1 Transfection of *E.coli*

Competent *E.Coli* cells were obtained from Dr. C. Bartholomew (Beatson Institute For Cancer Research, Glasgow, UK). 200 µl of *E.coli* cells and 1 µl pSSFV bcl2-2n plasmid DNA were added to falcon tubes and then left on ice for 60 min. The tube was transferred to a waterbath at 42°C for 2 min to heat-shock the cells. 1ml L-broth was added and the tube left for a minimum of 5 min on ice and then transferred to a shaking 37°C incubator for 60 min. 5 or 10 µl of *E.coli* suspension was mixed with 100 µl of L-Broth and then spread across the surface of an agar plate containing 50 µg/ml ampicillin. The agar plates were then incubated upside down at 37°C. A colony was selected using a sterile toothpick and added to a universal tube containing 5 ml L-Broth with ampicillin at 50 µg/ml. The *E.coli* were grown overnight in a shaking incubator at 37°C. The *E.coli* were then added to a conical flask containing 250 ml of 'Terrific Broth' and ampicillin at 50 µg/ml and incubated overnight in a shaking incubator at 37°C.

2.4.2 Harvesting of E.coli

Solutions:

Solution 1	50 mM glucose, 25 mM Tris.Cl (pH 8), 10 mM EDTA
Solution 2	0.2 M NaOH, 1% SDS
Solution 3	60 ml potassium acetate, 11.5 ml acetic acid, 28.5 ml H_20

The *E.coli* were transferred to centrifugation tubs and spun at 5,000 rpm for 15 min at 4°C in a Sorvall RC-SB superspeed centrifuge (Dupont). The pellet was resuspended in 10 ml solution 1 and left on ice for a minimum of 5 min. 20 ml of solution 2 was added and the mixture incubated on ice for 5 min. 15 ml of solution 3 was added and the mixture incubated on ice for a further 10 min. The mixture was then centrifuged at 5,000 rpm for 15 min at 4°C. 0.6 volumes of isopropanol was added to the tub, the contents mixed well and incubated for 15 min at room temp. The DNA was recovered

by centrifugation in a Sorvall rotor at 4000 rpm for 15 min at room temp. The supernatant was discarded and the pellet resuspended in 8 mls TE.

2.4.3 Purification of plasmid DNA.

To 8 mls of DNA in TE exactly 8.8g of CsCl and 0.8 ml of 10mg/ml ethidium bromide were added. The solution was mixed well before it was transferred to an ultracentrifugation tube and centifuged at 45,000 rpm in a Sorvall Ultracentrifuge OTD combi for 36 h at 20°C. Two bands were then visible of which the lower band comprising closed circular DNA was collected. A second CsCl gradient-ethidium bromide was performed with centrifugation overnight at room temp at 55,000 rpm and again the lower band of DNA collected and transferred to fresh tube.

2.4.4 Removal of Ethidium Bromide

An equal volume of water-saturated butanol was added to the plasmid DNA and the solution mixed thoroughly. The upper red phase, containing ethidium bromide was removed and discarded. This was repeated 4-6 times until the pink colour disappeared. 4 volumes of distilled water and 2.5 volumes of ethanol was added to the extract and left for a minimum of 2 h at -20°C. The extract was then spun at 10,000 rpm in a Beckman centrifuge for 20 min at 4°C, the supernatant discarded and the DNA resuspended in 100 µl TE. The extract was transferred to dialysis tubing which had been presoaked for a minimum of 1 h in distilled water. The dialysis tubes were suspended in 0.1X TE and incubated overnight at 4°C.

2.4.5 Quantitation of DNA by spectrophotometric analysis

A 1:100 dilution of pSSFV bcl2-2n DNA in TE was transferred to a Quartz cuvette with a 1 cm pathlength and the absorbance at 260 nm and 280 nm measured against a blank of TE using a spectrophotometer(Beckman Du650). A reading of 1.0 at 260 nm was assumed to be equivalent to 50 µg/ml for plasmid DNA. DNA with a ratio of 260/280 between 1.8 and 2.0 was considered to be pure.

2.4.6 Confirmation of identity of plasmid by digestion with restriction enzymes

1-2 μg plasmid pSFFV-bcl2 2n DNA was incubated with appropriate restriction enzymes and buffer for 3 h at 37°C. 1% (w/v) agarose was dissolved in TAE (0.04M Tris-acetate, 0.001M EDTA, pH 8.0), boiled and then poured in to a gel caster and allowed to set at room temp. 4 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll) was added to each sample and loaded in to the wells of the agarose gel. A 1Kb DNA ladder was run in parallel as a size marker. Electrophoresis was carried out at 150V for 1-2 h using TAE as running buffer. After electrophoresis DNA was stained by soaking in dH_2O with 0.5 μg/ml ethidium bromide for 15 min and then soaking in dH_2O for 15 min to remove excess ethidium bromide. DNA fragments were then viewed by a UV transilluminator and recorded as a photoimage .

2.5. Western Blotting

2.5.1 Preparation of protein to test for Bcl-2 expression

MCF7 cell clones transfected with the pSSFV bcl2-2n expression vector and selected with G418 at 0.15mg.ml^{-1}, were grown to confluence in a T175 flask. The cells were trypsinised, pelleted by centrifugation at 1,000 rpm in a Centaur 2MSE centrifuge and resuspended in ice cold PBS. Cells were pelleted and reuspended in 100 μl of ice cold suspension buffer, containing protease inhibitors (Suspension buffer: 100 mM NaCl, 10 mM Tris-HCL 10 mM, pH 7.6, 1 mM EDTA, pH 8.0). and then an equal volume of 2x SDS gel loading buffer (2x SDS gel loading buffer: 100 mM Tris, 4% SDS, 4% β mercaptoethanol, 20% glycerol, 0.2% bromophenol blue) was added. The suspension buffer was previously autoclaved and protease inhibitors were added immediately before use (100 μg/μl PMSF, 1 μg/μl aprotinin, 1 μg/μl leupeptin). The sample was boiled for 5 min and then sonicated using a Soniprep 150 MSE to shear chromosomal DNA. The samples were then spun at 13,000 rpm in a microcentrifuge and the supernatant transfered to a fresh tube. Samples were frozen at -70°C until required.

2.5.2 SDS-polyacrylamide gel electrophoresis

Proteins were separated by SDS polyacrylamide gel electrophoresis according to Laemmli *et al.* (1970). For separation of Bcl-2 protein a 15% polyacylamide gel was prepared as follows.

15 % polyacrylamide gel (50ml)

	ml
H_20	11.5
30% acrylamide mixture	25
1.5 M Tris (pH 8.8)	12.5
10% SDS	0.5
10% ammonium persulphate	0.5
TEMED	0.02

The 15% acrylamide gel mixture was pippetted between two glass plates using gel casting apparatus, a layer of water saturated butanol added to the surface and the gel allowed to set at room temp. The butanol was removed and the gel surface rinsed with distilled water . A stacking gel was prepared, pippetted on top, a comb was inserted and it was allowed to set.

5% Stacking gel

	ml
H_2O	6.8
30% acrylamide mix	1.7
1.0 M Tris (pH 6.8)	1.25
10% SDS	0.1
10% ammominum persulphate	0.1
TEMED	0.01

The gel was transferred to a gel tank which was subsequently filled with running buffer (Tris glycine).

Tris Glycine - 25 mM Tris

250 mM glycine

0.1% SDS

The comb was removed and the wells flushed with Tris glycine to remove any non-polymerised acrylamide and any bubbles were removed from beneath the glass plate. Protein samples and molecular weight markers in 1x SDS loading buffer were boiled for 5 min. 20 µl lysate (from a confluent T175) or 5 µl molecular weight markers and were loaded into each well. Electrophoresis was carried out at 150-200V for 2-3h.

2.5.3 Transfer of protein to nitrocellulose membrane

12 pieces of 3 mm Whatman paper were cut to the size of the gel and 6 pieces soaked in anode 1 solution (0.3 M Tris; 20% methanol, pH 10.4), 3 pieces soaked in anode 2 solution (25 mM Tris; 20% methanol, pH 10.4) and 3 pieces soaked in anode solution 3 (40mM 6-amino-n-hexanoic acid; 20% methanol, pH 7.2) for 10 min. Hybond ECL nitrocellulose membrane was soaked for 5 min in distilled H_2O. The proteins were transferred from the acrylamide gel to the nitrocellulose membrane using a semi-dry blotter as described by Figure 2.1. Transfer was carried out at 12V for 90 min. The nitrocellulose was then blocked in TBS-0.1% Tween20 containing 10% Marvel overnight at 4°C.

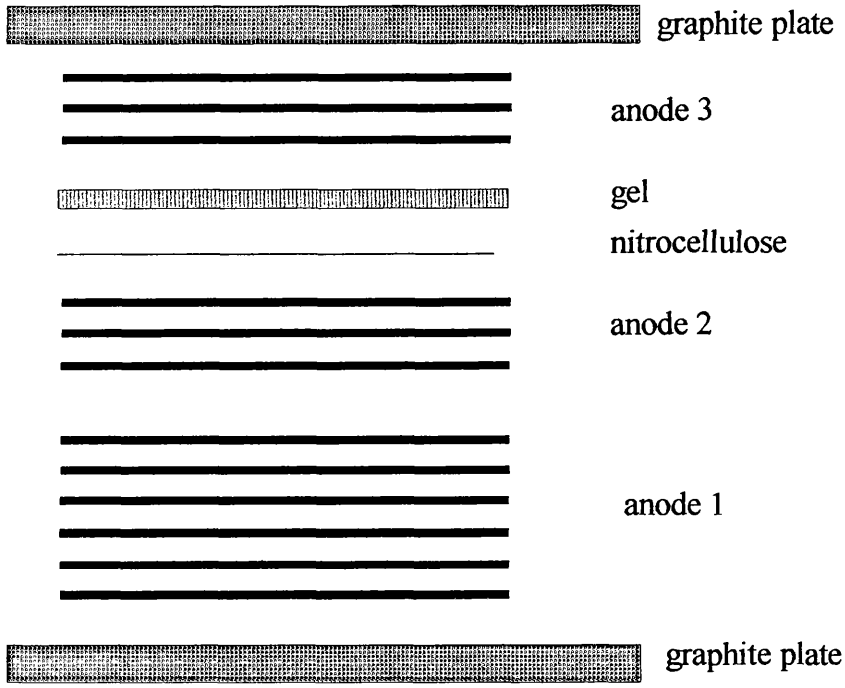
TBS- 1000 ml pH 7.4

8g NaCL

0.2g KCl

3g Tris base

Figure 2.1



2.5.4 Detection of Bcl-2 protein by Enhanced Chemi-luminescence

The blocking buffer was discarded and the nitrocellulose membrane incubated in rabbit anti-mouse Bcl-2 diluted 1:2000 with TBS-Tween 20 in 10% Marvel for 3 h at room temp. The antibody was removed and the membrane washed 3 times with TBS-Tween 20 in 10% Marvel for 20 min. The nitrocellulose membrane was incubated with anti-rabbit Horse Radish Peroxidase-conjugated antibody for 1 h at room temp and then washed 3 times with TBS-Tween in 10% marvel for 20 min and twice for 5 min in TBS-Tween 20 at room temp 5 ml of enhanced chemi-luminescence (ECL) kit solution 1 and solution 2 were mixed and added to the surface of the membrane. After 1 min the ECL mixture was removed, the membrane wrapped in Saran wrap and autoradiograhic film placed over the membrane in the dark room for 30 min. To show equal loading of protein, the nitrocellulose membrane was stained for 2 min with amido black which was then rinsed with tap H_2O.

2.6 Measurement of GSH concentration in FCS

GSH concentration was measured by the method of Tietze (1969). A calibration curve was prepared by analysis of calibration samples at 0, 2, 10, 50, 100 and 250 μM GSH dissolved in 125 mM phosphate EDTA buffer (125 mM $NaH_2PO_4.2H_2O$, 125 mM $Na_2HPO_4.2H_2O$, 6.3 mM EDTA, pH 7.5) in order to calculate the concentration of GSH in FCS. 200 μl of calibration sample or FCS (1:10 dilution) was added to 700 μl of 0.3 mM NADPH dissolved in 125 mM phosphate EDTA buffer and 100 μl of 6m M 5,5'- dithio-bis-(2-nitrobenzoic acid) (DTNB) dissolved in 125 mM phosphate EDTA buffer. Samples were equilibrated to 30°C and transferred to plastic cuvettes. The reduction of GSH was initiated by the addition of 10 μl of glutathione reductase (8.5 U/ml) to each cuvette and the reaction monitored by measuring the change in A_{412} for 1 min.

2.7. Synthesis and purification of SDG

2.7.1 Synthesis of selenodiglutathione (SDG)

SDG was synthesised and purified according to the method of Ganther (1971). A trace amount of ^{35}S-GSH (10 μl of a 3:10 dilution of ^{35}S-GSH with a specific activity of 310 Ci/mmol [3.6 μCi total]) was added to 4 ml 0.1M GSH. 0.1 M Na_2SeO_3 dissolved in 1 ml ice-cold 0.1 M HCl was then added to the GSH, the mixture was left on ice for 15 min and titrated to pH 4.5 by the addition of about 100 μl 2 M sodium acetate. This reaction results in the formation of SDG and oxidised glutathione (GSSG). Trace amount of ^{35}S-GSH was required in order to monitor SDG in subsequent purification procedures.

2.7.2 Separation of SDG and GSSG

500 g of AG50W H^+ resin was gently washed in distilled H_2O until the washes ran clear. The resin was then washed with 1 M HCl and then thoroughly washed with distilled H_2O until the pH of the washes returned to 7. The resin was then washed

with 0.1 M NaOH and again rinsed with distilled H_20 until the pH of the washes returned to 7. Approximately 150 g of the resin was set aside and later used for the concentration of SDG. The remaining AG50W resin was washed in 0.1 M sodium acetate, pH 4.7, and then soaked in 2x volumes of 0.1 M sodium acetate, 0.3 M $NiCl_2$, pH 4.7, in order to saturate the resin with nickel. The resin was poured in to a 2.6 cm diameter glass column and allowed to pack under gravity to a height of approximately 60 cm. Running buffer (0.1 M sodium acetate, 0.01 M $NiCl_2$, pH 4.7) was pulled through the column by a peristaltic pump at a rate of 2.5 ml/min for a minimum of 1 h in order to equilibrate the column. Running buffer was run into the column and the sample was loaded to the top of the column carefully using a Pasteur pipette without disturbing the resin. After the sample had run in to the column, running buffer was added to the top of the column which was then connected to the running buffer reservoir ensuring that no air was introduced in to the system. The A_{240} of the column eluate was measured by a Biorad UV monitor connected to Biorad Ecno chart recorder (model 1325) and 4 ml fractions were collected following the onset of the first peak using an Ultrurac 2070 fraction collector (LKB). 200 µL aliquots from these fractions were then measured for ^{35}S-radioactivity in order to determine which fractions contained SDG. The fractions containing SDG were then pooled and were titrated to a pH of <3 using 5 M HCL and stored overnight at 4°C .

2.7.3 Concentration of SDG and removal of nickel

150 g previously washed AG50W resin (see section 2.7.2) was poured in to a 4.5 cm glass column and allowed to pack under gravity. 0.1 M formic acid was pulled through the column at a rate of 2.5 ml/min by a peristaltic pump until the A_{240} became constant. The buffer was allowed to run from the very top of the column and the SDG was carefully loaded on the column without disturbing the resin. The SDG was then run in to the column and excess 0.1 M formic acid was run through the column until the A_{240} of the column eluate became constant. Under acidic conditions SDG becomes adsorbed to the resin; the SDG was then eluted with 0.1 M ammonium acetate pH 5.6. The A_{240} of the column eluate was measured and fractions collected and monitored as described in section 2.7.2. Fractions containing SDG were pooled and stored overnight at 4°C.

2.7.4 Buffer Change

100 g G10-sephadex beads were allowed to swell in water overnight. The G10 beads were then poured in to a 4.5 cm diameter glass column and allowed to pack under gravity. Distilled H_20 was pulled through the column by a peristaltic pump at a rate of 0.7 ml/min until the A_{240} became constant and a maximum of a third of the volume, of the column, of SDG was loaded on to the column carefully using a glass pipette. SDG was run in to the column and distilled H_20 was then run through the column at 0.7 ml/min. The A_{240} of the column eluate was again measured and fractions were collected and monitored for ^{35}S radioactivity as described above. 50 µl aliquots from fractions were used to measure the conductivity using a Osmomat 030 (Gonotec) in order to ensure that all salt had been removed from the SDG sample. The fractions containing SDG were pooled and 2 M acetic acid was added to give a final concentration of 10 mM. The SDG was then stored in aliquots at -20°C.

2.8. Analysis of SDG purity

The purity of SDG was analysed using thin layer chromatography (TLC) and by measuring the absorbance spectrum.

2.8.1 Thin Layer Chromatography

An air tight glass chamber was pre-equilibrated with a 2:1:1 mixture of butanol : acetic acid : water. 6 µl of the SDG sample was spotted on to a SIL G TLC plate and run in parallel to 3 µl of 820 µM SDG (previously synthesised in the laboratory by L.Wu), 2 µl 2 mM GSSG, 3 µl 1 mM GSH and 1µl of a 1:10 dilution of the original mixture of SDG and GSSG. Chromatography was carried out in the glass chamber until the solvent front nearly reached the top of the TLC plate. The TLC plate was then allowed to dry and the chromatography repeated. Once the solvent front had again nearly reached the top of the plate it was allowed to dry and was stained with 0.2% ninhydrin in ethanol and was then heated in the oven (Hybaid) for 15 min at 80°C to enable the pink/purple colour to develop.

2.8.2 Absorbance spectum of SDG

The spectrophotometer Du650 (Beckman) was calibrated with distilled H_20 in a quartz cuvette with a 1cm pathlength over a range of 220-420nm. The absorbance spectrum of SDG was measured over the above range and compared to that of the previously synthesised SDG.

3. Results

3.1 Aims of project

The mechanism by which selenium has its anticarcinogenic effects is not understood although it is likely to be related to its ability to inhibit cell growth and induce apoptosis. The mechanism by which selenium induces apoptosis is also not understood although it has been hypothesised that selenite induces cell death during its reduction to hydrogen selenide by the generation of reactive oxidant species resulting in oxidative stress. Evidence from other studies suggests that certain organic molecules such as selenomethyl selenocysteine may induce cell death by a different mechanism to selenite.

Bcl-2 has been shown to inhibit the ability of a number of stimuli to cause apoptosis possibly by its ability to prevent the effects of reactive oxygen species and peroxides, therefore determination of whether Bcl-2 could inhibit selenium induced apoptosis may provide a further insight in to the mechanism involved in selenium induced apoptosis.

Most studies investigating the effects of selenium on carcinogenesis have used the inorganic selenium compound selenite which is taken up from the soil by plants and converted to inorganic selenium compounds. The primary metabolite of selenite formed by its reaction with glutathione, selenodiglutathione (SDG) has been shown to be more potent than selenite and can induce apoptosis rapidly unlike selenite which induces apoptosis after a lag phase. Co-administration of glutathione with selenite results in the abolition of the lag phase thus suggesting that to induce apoptosis selenite must first be metabolised to SDG. Thus SDG was chosen to be the selenium compound used to test if Bcl-2 can protect against selenium induced apoptosis.

A number of groups have attempted to synthesize novel selenium compounds which have the anticarcinogenic properties of selenite but without the toxicty observed with selenite administration. One such compound, p-XSC has been shown to be more potent at inducing apoptosis than selenite and have greater chemopreventive properties yet appears to be less toxic than selenite. It was also therefore interesting to deduce if p-XSC induced cell death could be inhibited by overexpression of Bcl-2.

3.2 Synthesis of SDG

SDG was produced by the reaction described by Ganther (1971). Briefly, sodium selenite was reacted with glutathione at a ratio of 1:4 under acidic conditions for 15 min at 4°C. A trace amount of ^{35}S radiolabelled glutathione was included in the reaction to enable detection of SDG in subsequent purification procedures. The reaction results in the formation of SDG and oxidized glutathione (GSSG) which were separated by loading on to a chromatography column containing AG50W H^+ resin saturated with nickel chloride ($NiCl_2$). SDG has a greater affinity for nickel than GSSG and so passed through the column at a slower rate thus enabling the separation of the two compounds by collection and analysis of fractions. The A_{420} of the eluate was measured and two peaks were observed. Fractions were collected and further analysed for radioactivity (figure 3.1). The fractions of the second peak containing SDG were pooled. The SDG was concentrated and the $NiCl_2$ removed by passing through a second column containing AG50W H^+ resin. SDG binds to the resin under strong acidic conditions and can then be eluted by the addition of ammonium acetate, which is a weak acid. Measurement of the A_{420} and radioactivity enabled detection of eluate fractions containing SDG. SDG was then run through a third column consisting of G10 Sephadex beads equilibrilated with water to remove salt. Again the SDG was monitored by UV absorbance and radioactivity and the conductivity measured to ensure that salts were adequately removed.

The purity of SDG was analysed by thin layer chromatography by running in parallel with GSH, GSSG, SDG which had previously been synthesised in the lab in 1993 and the original mixture of reaction products (figure 3.2). SDG purity was further analysed by recording the UV absorbance spectrum from 220 - 420nm (figure 3.3). The UV absorbance spectrum was comparable to that published by Ganther (1971) indicating that purification was successful and the TLC analysis performed indicated that synthesis and purification of SDG had also been a success.

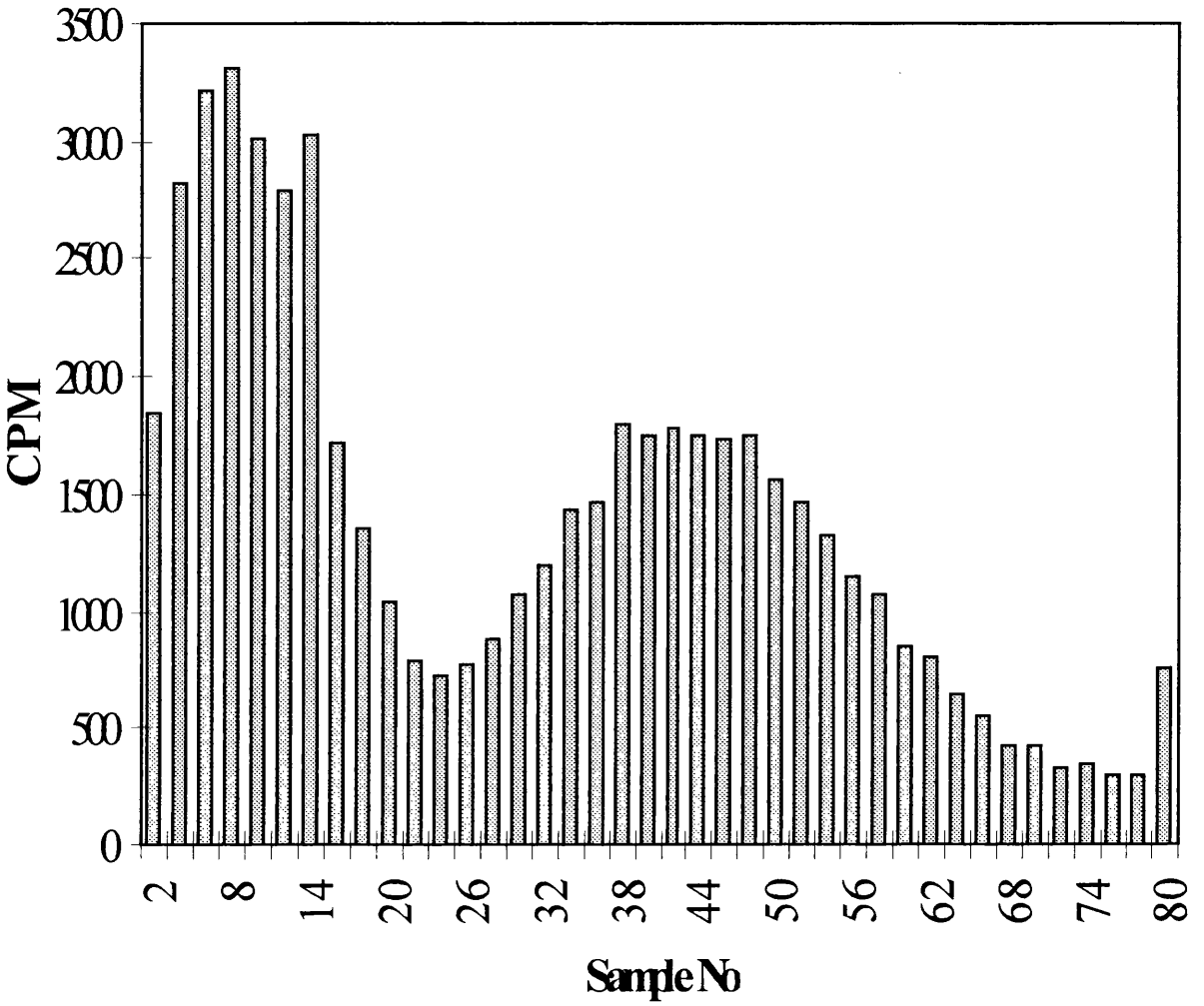


Figure 3.1

Separation of SDG and GSSG by column chromatography

Na_2SeO_3 was reacted with GSH at a ratio of 1:4 under acidic conditions on ice. The products of the reaction, SDG and GSSG were separated by column chromatography using an AG50W H+ resin saturated with nickel and 0.1M sodium acetate/0.01M $NiCl_2$, pH 4.7 as elution buffer. The A_{420} of the eluate was monitored and 4ml fractions collected of which 200µl aliquots were subsequently analysed for ^{35}S radioactivity. GSSG and SDG eluted as the 1st and 2nd peaks respectively.

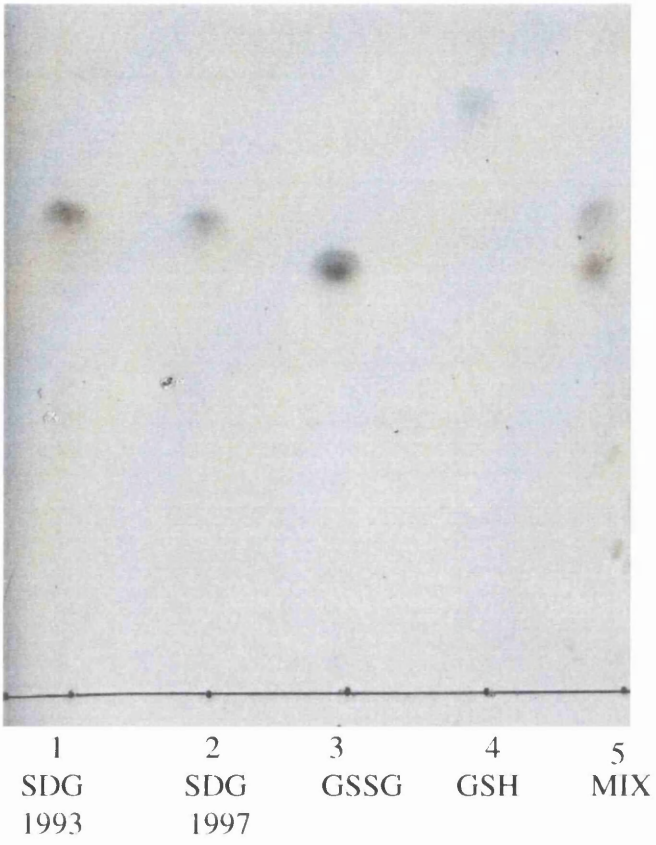


Figure 3.2 Thin layer chromatography to determine purity of SDG .

The TLC plate was stained with ninhydrin.Samples were loaded as follows:

Lane (1) 3µl 820µM SDG previously synthesised and purified by L.Wu in 1993

Lane (2) 6µl purified SDG

Lane (3) 2µl 2mM GSSG

Lane (4) 3µl 1mM GSH

Lane (5) 1µl of a 1:10 dilution of original reaction mixturecontaining GSH and sodium selenite mixed at 4:1

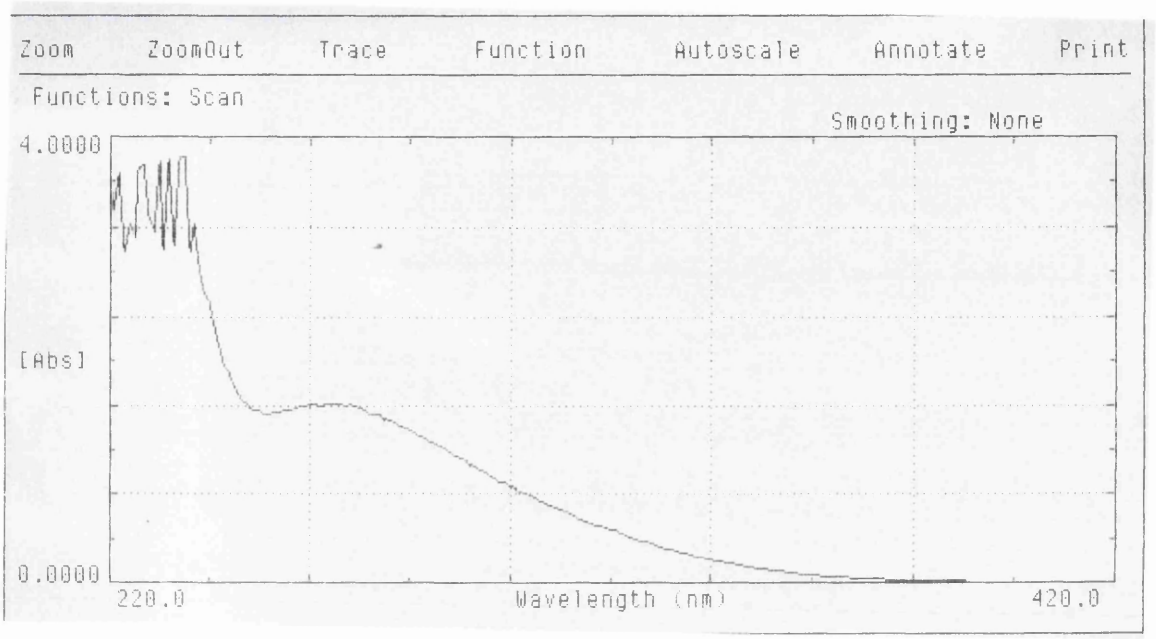


Figure 3.3
Absorbance spectrum of SDG over a range of 220 - 420 nm

3.3 Standardisation of conditions for SDG induced cell death.

Preliminary experiments showed variation in the concentration of SDG required to kill cells between experiments, despite consistent results with replicates within an experiment. It was important to determine what factors were involved in causing this inter-experimental variation in order to achieve a more standardised assay.

3.3.1 Effect of pH on SDG induced cell death

Since SDG is known to be unstable at alkaline conditions. Unavoidable changes in pH during culturing cells in bicarbonate buffers could be the cause of the variation in the apparent potency of SDG between experiments. To test this, an MTT assay was performed on MCF7 cells growing in tissue culture medium to which small quantities of HCL or NaOH were added to give pH ranging from pH 5.9 to 7.4, to determine the concentration of SDG required to kill 50% of cells at different pH (figure 3.4). An MTT assay involves the metabolism of 3-[4,5]-dimethylthiazol-[2-yl]-2,5-diphenyltetrazolium bromide, resulting in a purple colour change which is a function of the metabolic activity of cells and thus is an indirect measure of cell viability. The MTT assay performed showed that at pH 5.9 the cells were dead or had very little metabolic activity (Fig. 3.4). At pH 6.4, the cells did not appear to be growing or attach to the dish as well as they did at the higher pHs. In this experiment the highest growth rate, as determined by the MTT assay, was in medium at pH 7.4. There was little difference in the concentration of SDG (3.25-3.5μM) required to kill 50% of cells over pH range 6.4 - 7.4 thus SDG can be considered to be stable over this pH range. Future experiments were buffered at pH 7 using 1% bufferal to ensure that the pH did not rise above pH 7.4 due to fluctuating CO_2 levels and thus limiting the risk of SDG degradation which may occur at a higher pH.

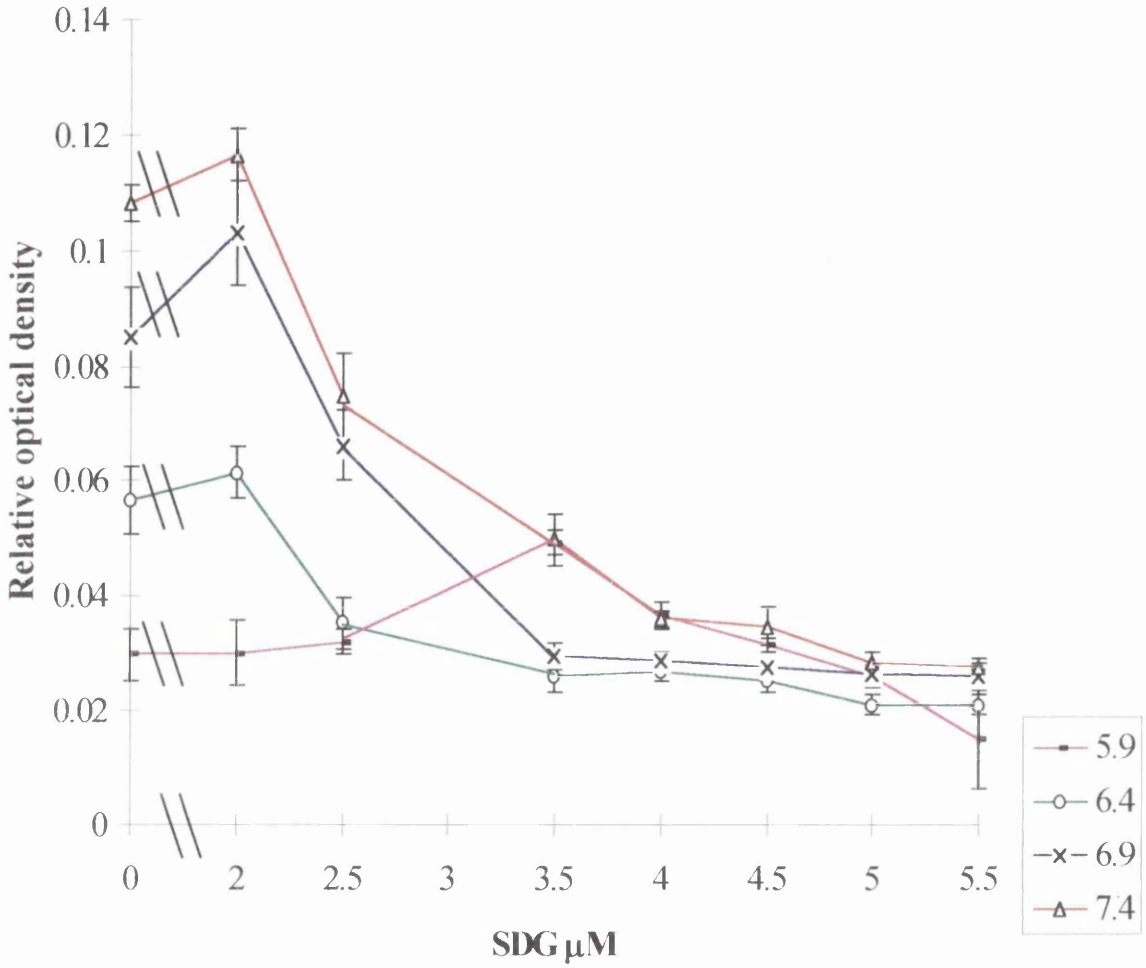


Figure 3.4

MTT assay to show the effect of pH on SDG induced cell death

500 cells were plated on to each well of a 96 well plate and incubated over night at 37°C to adhere. Tissue culture medium was prepared at pH 5.9, 6.4, 6.9 and 7.4 and the appropriate amount of SDG was added to give concentrations ranging 0-5.5µM immediately before use. The medium from each well was removed and replaced with 100µl of medium containing SDG at a specific pH and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate. 15µl of MTT dye was added to each well, the plates incubated for 4 hours at 37 °C and then 100µl solubilisation stop solution added. The optical density was measured against wells containing medium only. Error bars represent standard deviation from the mean.

3.3.2. Effect of Foetal Calf Serum concentration on SDG induced cell death

The concentration of FCS in tissue culture medium has previously been shown to effect the ability of the p-XSC to induce cell death (Ronai *et al.* 1995). To determine if FCS concentration effects SDG potency, SDG was dissolved in medium containing different concentrations of FCS and was added to cells which were incubated overnight after which time an MTT assay was performed (figure 3.5). SDG was most potent when administered to medium containing 5% FCS. A greater concentration of SDG was required to induce cell death when added to medium containing 10% FCS. With medium containing 15% FCS, SDG was unable to induce cell death over the range of concentrations studied. This experiment demonstrates that the level of FCS in the medium greatly influences the concentration of SDG required to give a 50% kill.

At low concentrations of FCS, normal cell growth can be impaired. The optimum concentration of FCS for growing MCF7 cells is considered to be approx. 10% FCS and as SDG was adequately potent at this concentration of FCS, subsequent experiments were carried out with medium containing 10% FCS in order that the same medium could be used for growing cells in mass culture and for cloning experiments.

3.3.3. Effect of Glutathione on SDG and pXSC induced cell death.

As GSH has been shown to be required for the metabolism of SDG to its persulphide derivative, it is possible that variation in GSH concentrations between batches of FCS could result in the variations in the apparent SDG potency observed between experiments (Seko 1989). To determine if co-administration of GSH could enhance SDG induced cell death an MTT assay was performed using medium containing 5 and 10μM GSH (figure 3.6). The potency of SDG was greatly increased by the addition of GSH. Addition of 5 and 10μM GSH to 3.5μM SDG reduced cell viability to approximately 35 and 15% respectively, compared to approximately 100% viability without glutathione.

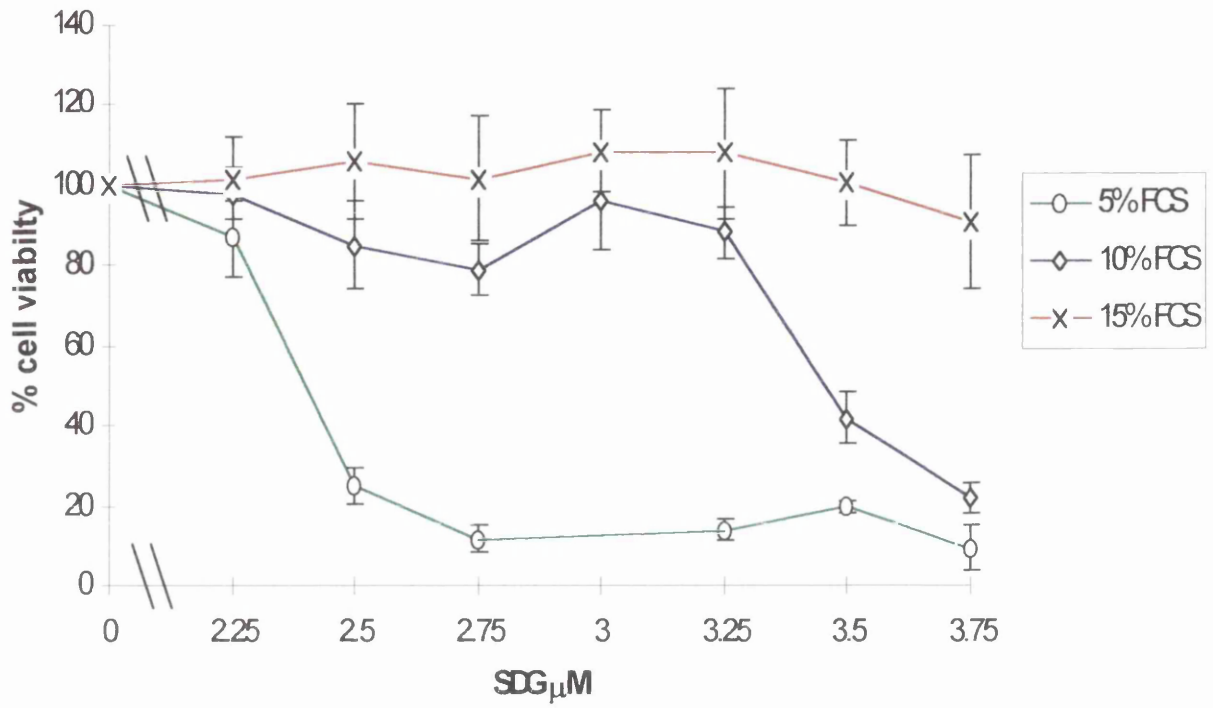


Figure 3.5
MTT assay to show the effect of foetal calf serum on SDG induced cell death
500 cells were plated into each well of a 96 well plate and left over night at 37°C to adhere. The medium from each well was removed and replaced with 100μl of medium containing SDG and 5, 10 or 15% FCS and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate and an MTT assay performed. The data are expressed relative to the optical density of the wells incubated with medium only. Error bars represent standard deviation from the mean.

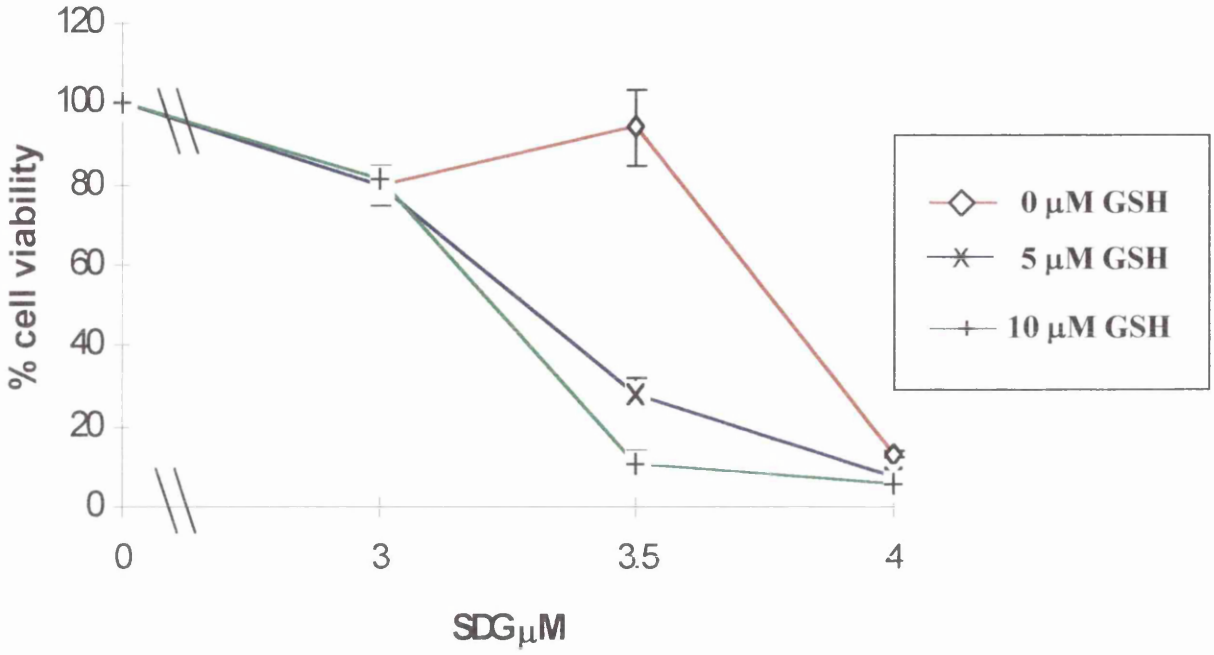


Figure 3.6
MTT assay to show the effect of GSH on SDG induced cell death
500 cells were plated on to each well of a 96 well plate and left over night at 37°C to adhere. The medium from each well was removed and replaced with 100μl of medium with GSH and SDG at the stated concentrations and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate and an MTT assay performed. The data is expressed as a percentage of the optical density of the cells incubated 0μM SDG. Error bars represent standard deviation from the mean.

As the GSH concentration has been shown to enhance the potency of SDG it was investigated if it could equally enhance the potency of the novel synthetic selenium compound pXSC by performing an MTT assay (figure 3.7). No difference in the concentration of pXSC required to kill MCF7 cells was observed when 5μM of GSH was added to the tissue culture medium. A higher concentration of pXSC was required to kill cells on addition of 20μM GSH to tissue culture medium. 50% of MCF7 cells were killed at 6μM pXSC whereas on co-administration of 20μM GSH the concentration of pXSC required to kill 50% of MCF7 cells was increased to approximately 10μM pXSC. Thus 20μM GSH was found to be protective against cell death induced by pXSC.

3.3.4. Determination of the concentration of GSH in foetal calf serum.

As alteration of GSH concentration effects the potency of SDG, it was hypothesised that fluctuations of GSH in foetal calf serum could be responsible for experimental variations in SDG potency. Calibration samples containing 0-100μM GSH were prepared and the absorbance at 412nm was measured during a reaction with glutathione reductase (Fig. 3.8). By measuring the absorbance of FCS following a reaction with glutathione reductase, FCS was found to contain negligible quantities of GSH. However, in order to demonstrate for certain that fluctuations in GSH concentration between serum bottles could not be responsible for causing the variation in SDG potency observed between previous experiments, it would be necessary to determine the concentration of GSH in the batches of FCS previously used.

3.4 Effect of Bcl-2 on cell death induced by selenium compounds

The aim of the following experiments was to determine if Bcl-2 could inhibit cell death induced by SDG and p-XSC. The ability of SDG and p-XSC to induce cell death was compared in MCF7 cells which over-expressed Bcl-2, with MCF7 cells which expressed normal levels of Bcl-2.

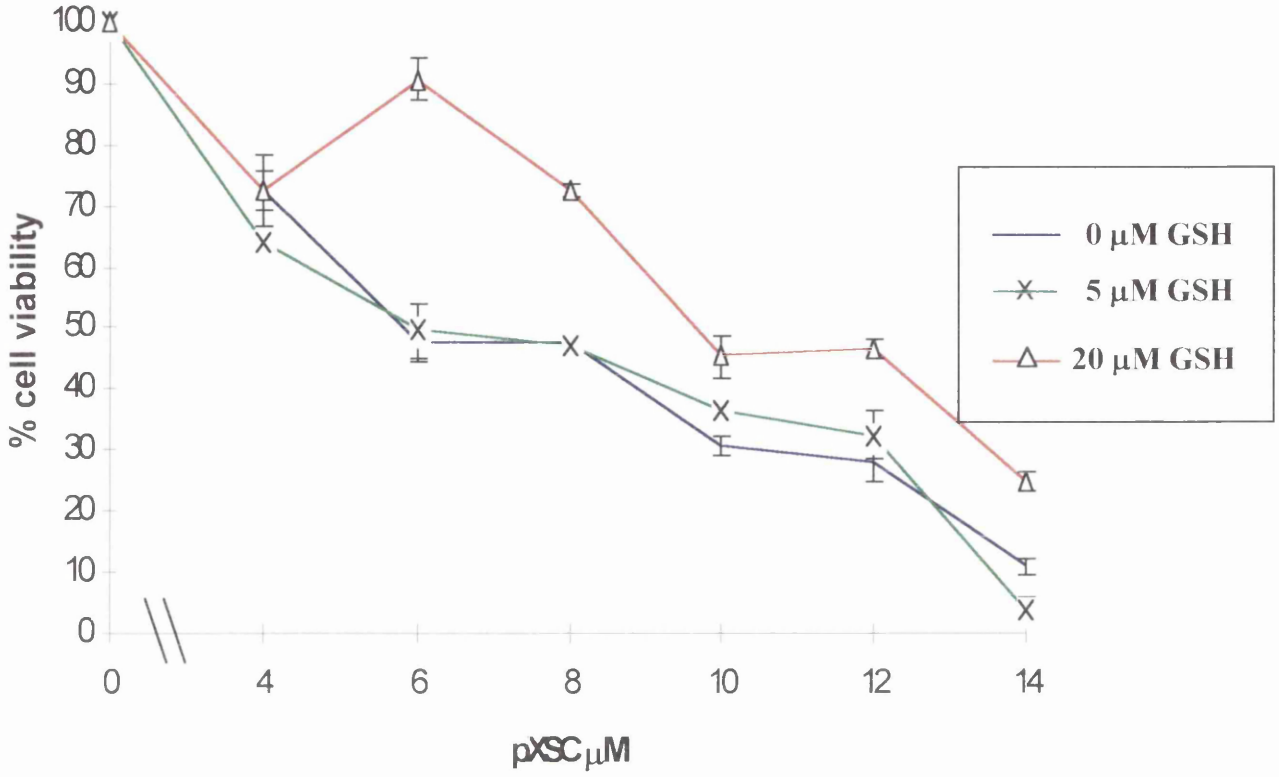


Figure 3.7
MTT assay to show the effect of GSH on pXSC induced cell death
500 cells were plated per well of a 96 well plate and left over night at 37°C to adhere. The medium from each well was removed and replaced with 100μl of medium with GSH and pXSC at the stated concentration and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate and an MTT assay performed. The data is expressed as a percentage of the optical density of the cells incubated with 0μM p-XSC. Error bars represent standard deviation from the mean.

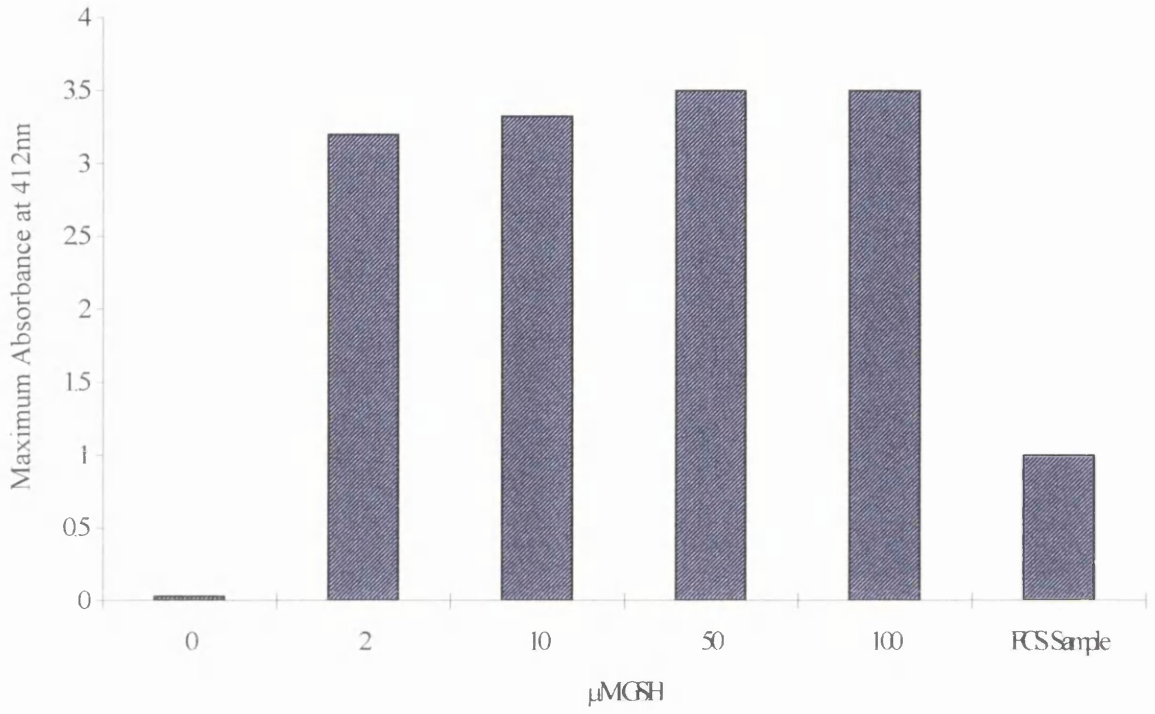


Figure 3.8

Measurement of GSH Concentration in FCS

200μl GSH (0-100μM) or 200μl of a 1:10 dilution of FCS was added to 700μl 0.3mM NADPH buffer and 100μl 6mM DTNB dissolved in 125 mM phosphate EDTA buffer. The reduction of GSH was initiated by the addition of 10μl of glutathione reductase (8.5U/ml) to each cuvette and the reaction monitored by measuring the change in A412 for 1 min. The peak absorbance for each sample over the 1 min period is shown above. The estimated absorbance of undiluted FCS is plotted.

To attain over-expression of Bcl-2, MCF7 cells were transfected with a *bcl-2* cDNA expression vector, clones containing the expression vector were selected and those demonstrated to over-express Bcl-2 protein were used in subsequent experiments. MCF7 cells were also transfected with an empty vector to act as a control.

3.4.1 Production of Bcl-2 expressing clones

The pSFFV human bcl2-2n plasmid expression vector (Hockenbery 1990) and a control pSFFV vector which did not contain the *bcl-2* cDNA, were obtained from Dr Korsmeyer (Howard Hughes Medical Institute, St Louis, USA). As only a small amount of plasmid DNA was available, it was first necessary to amplify the plasmid DNA by growing it up in *E.coli.*

Competent *E.coli* were transformed with the *bcl-2* cDNA plasmid, harvested and the plasmid DNA recovered. Plasmid DNA was purified by double cesium gradient-ethidium bromide ultra-centrifugation. A restriction digest of both plasmids with ECoRI, gave DNA fragments of the size expected from examination of the restriction enzyme map and thus verified that the correct plasmid had been isolated.

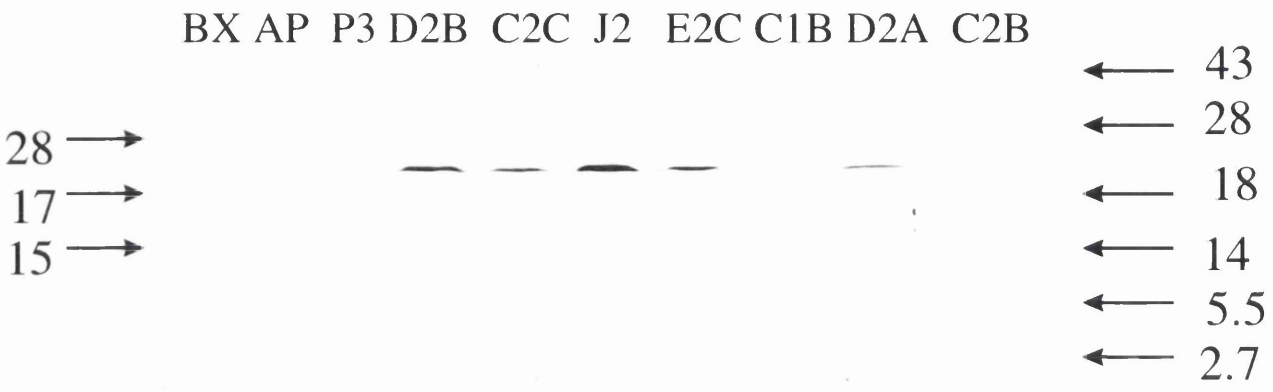
MCF7 cells were transfected with pSFFV bcl2-2n vector and vector control by electroporation and stable transfectants selected for by the addition of G418 48 h after electroporation. Since the vector contains the neomycin resistance gene, G418 resistant colonies were allowed to grow up for 3 to 4 weeks before being isolated by ring cloning. Selected colonies were trypsinised and seeded in to petri dishes and grown up in order that protein lysate could be prepared.

In order to determine which clones were expressing Bcl-2, protein lysates were prepared from 8 clones transfected with pSFFV bcl2-2n and 2 clones transfected with pSFFV vector control. The proteins in the lysate were separated on an SDS-polyacrylamide gel by electrophoresis and transferred on to a nitrocellulose membrane using a semi-dry blotter. Bcl-2 protein was detected by incubation of the nitrocellulose membrane with rabbit anti-mouse Bcl-2 antiserum, which also has affinity for human Bcl-2 protein. An incubation with anti-rabbit horse raddish peroxidase-conjugated antibody was performed enabling detection of Bcl-2 protein by

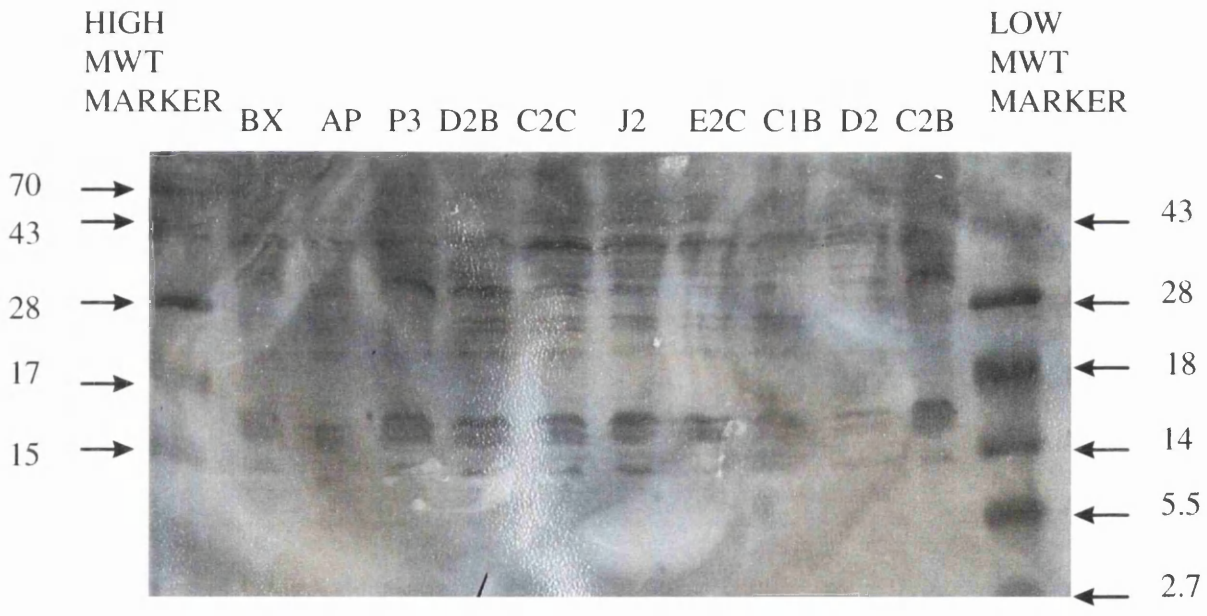
Figure 3.9
Western Blot to show Bcl-2 expression in MCF7 cell transfectants

Protein lysate from MCF7 cells transfected with pSFFV human bcl2-2n plasmid expression vector by electroporation and grown up in 0.15 $mg.ml^{-1}$ G418, was loaded on to an SDS-polyacrylamide gel and proteins separated by electroporesis. Protein was transferred to nitrocellulose membrane and incubated overnight at 4°C in blocking buffer. The nitrocellulose membrane was incubated with a 1:2000 dilution of rabbit anti-mouse Bcl-2 antibody for 3 h and then for 1 h with anti-rabbit HRP-conjugated antibody at room temp. Bcl-2 was detected by enhanced chemi-luminesence (Figure A). The nitrocellulose membrane was stained with Amido Black to show equal loading of protein (Figure B). BX and AP were transfected with empty vector controls; P3, D2B, C2C, J2, E2C, C1B, D2A and C2B were transfected with the bcl2 expression vector .

A


BX AP P3 D2B C2C J2 E2C C1B D2A C2B
28
17
15
43
28
18
14
5.5
2.7

B


HIGH MWT MARKER
BX AP P3 D2B C2C J2 E2C C1B D2 C2B
LOW MWT MARKER
70
43
28
17
15
43
28
18
14
5.5
2.7

electrochemical luminescence (ECL) (figure 3.9A). A number of clones were shown to be positive for Bcl-2 expression including D2B, C2C, J2, E2C and D2A. An amido black stain of the nitrocellulose membrane was prepared to show equal loading of protein on the gel (Fig. 3.9B).

3.4.2 Effect of Bcl-2 expression on SDG induced cell death

To determine whether Bcl-2 can prevent cell death induced by SDG, the ability of SDG to induce cell death was compared in MCF7 cells expressing normal levels of Bcl-2 and in MCF7 cells over-expressing Bcl-2. The ability of SDG to induce cell death was assessed by performing an MTT assay and a cloning assay. A cloning assay was also performed as this is a more direct method of measuring cell viability as it a measure of the cloning efficiency of cells.

MTT assay

MCF7 cell transfectants expressing Bcl-2 and MCF7 cells transfected with vector control were plated on to 96 well plates and allowed to adhere overnight. Two experiments were performed; the first compared the effects of the Bcl-2 expressing MCF7 clones, C2C and J2, with the BX MCF7 clone which was transfected with control vector only. The second experiment compared the effects of D2B, a bcl-2 positive clone, with AP, a clone containing vector control only. Tissue culture medium was removed from each well and replaced with medium containing SDG at concentrations ranging from 0 to 3.5μM SDG and the cells incubated overnight at 37°C. An MTT assay was performed and the results are shown in Figure 3.10. In both experiments there was no significant difference in the SDG concentration required to kill 50% of positive or negative Bcl-2 expressing MCF-7 clones.

Cloning Assay

Cloning assays were performed in which approximately 250 cells were seeded on to petri dishes and allowed to adhere before treatment with SDG. Three cloning assays were performed to determine if Bcl-2 can protect against SDG induced reductions in the cloning efficiency of the cells.

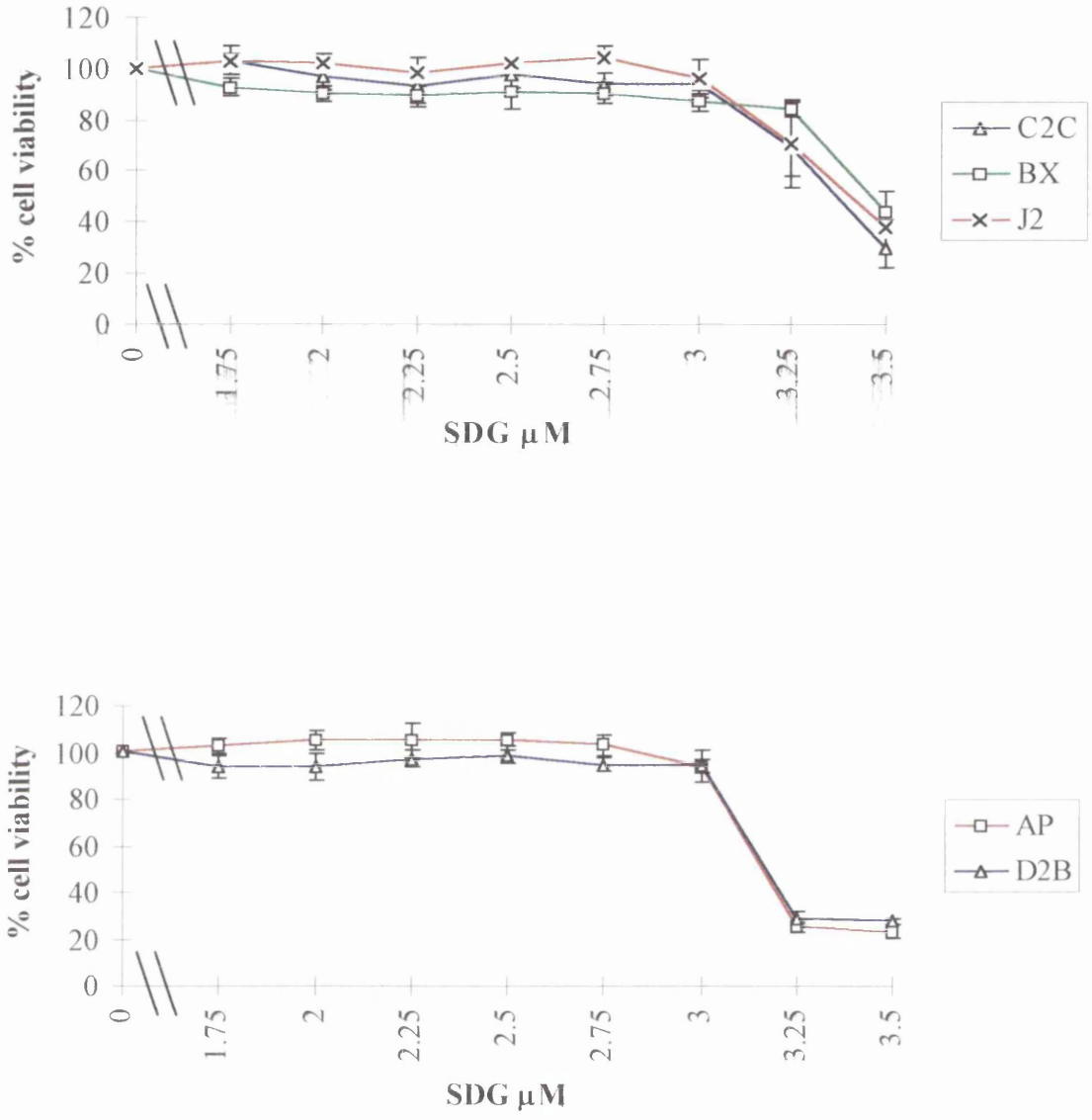


Figure 3.10
MTT assay to show the effect of Bcl-2 expression on SDG induced cell death
500 MCF7 cells expressing Bcl-2 or vector only controls were plated per well of a 96 well plate and left over night at 37°C to adhere. Bcl-2 expressing clones include J2, C2C and D2B. AP and BX are vector only controls. The medium from each well was removed and replaced with 100μl of medium containing SDG at the appropriate concentration and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate and an MTT assay performed. The data is expressed as a percentage of the optical density of the cells incubated with 0μM SDG. Error bars represent standard deviation from the mean.

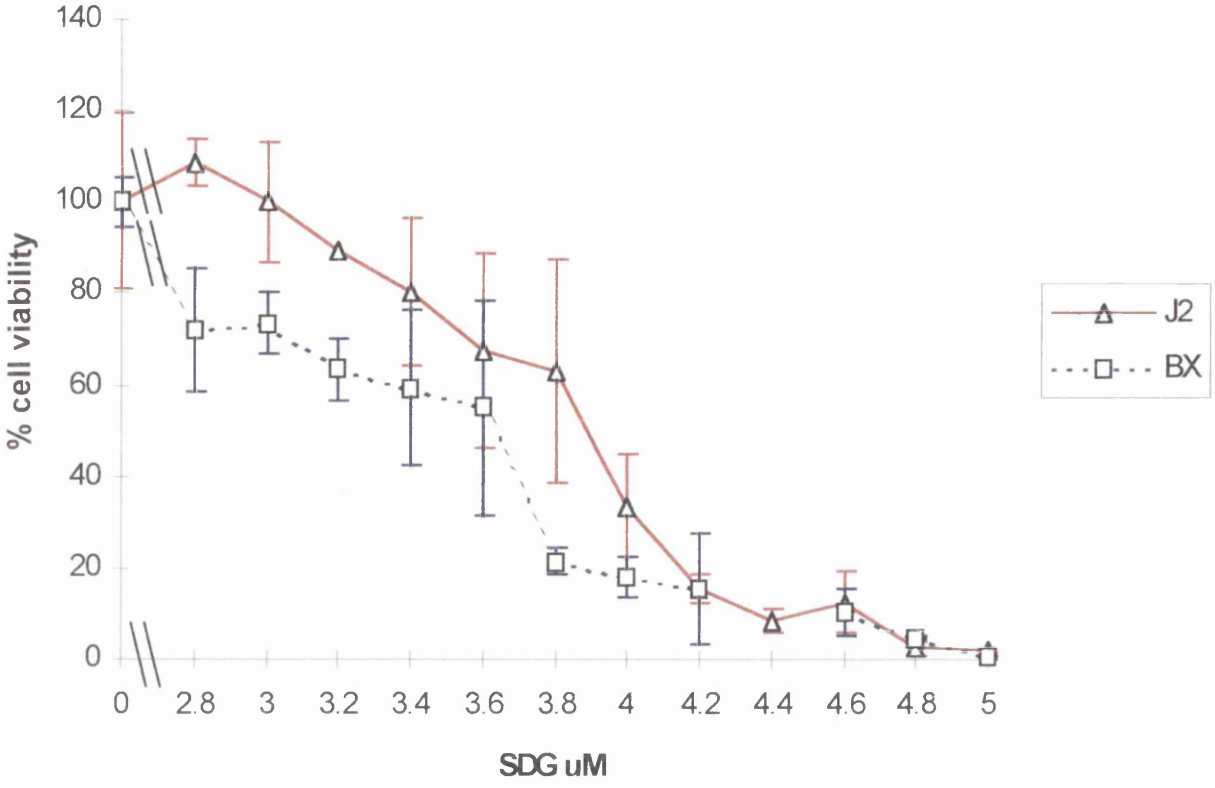


Figure 3.11 (a)
Cloning assay to show the effect of Bcl-2 expression on SDG induced cell death
Figures (a) and (b) illustrate two separate experiments comparing clones J2 and BX, and Figure (c) illustrates a comparison of clones D2b and AP. 250 MCF7 cells expressing Bcl-2 (D2B or J2) or vector only controls (AP or BX) were plated on petri dishes and left overnight at 37°C to adhere. SDG was added to each dish to give the required concentration and the cells incubated at 37°C for 14 days. Dishes at each concentration were prepared in quadruplicate. The colonies were stained with Giemsa stain and the number of colonies counted. Error bars represent standard deviation from the mean.

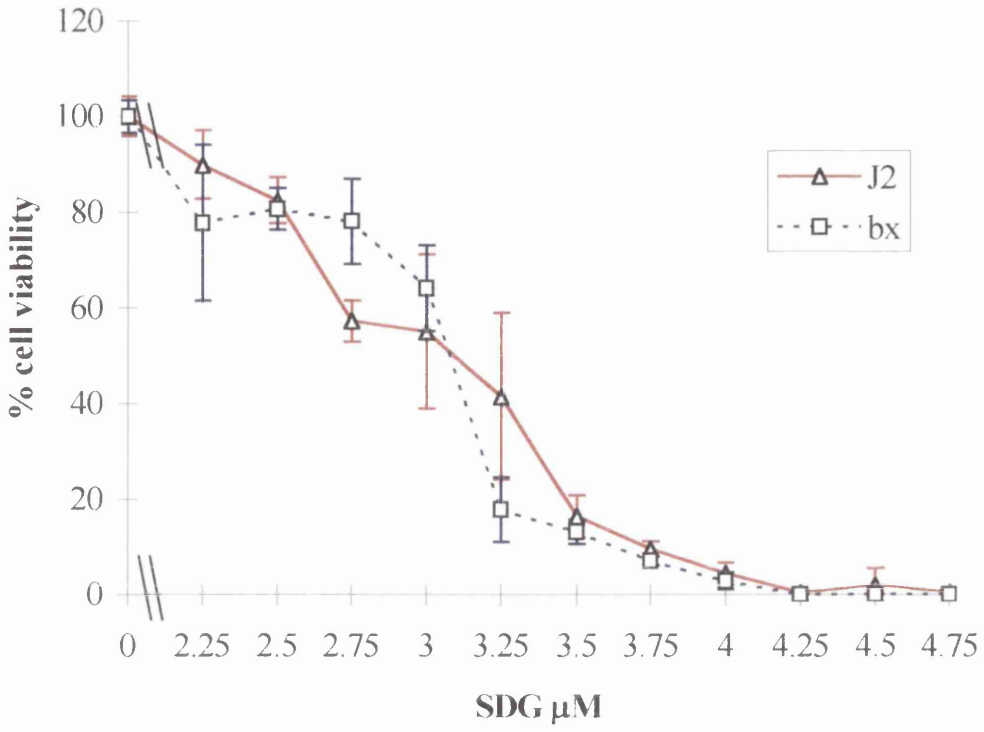

120
100
80
60
40
20
0
% cell viability
J2
bx
0
2.25
2.5
2.75
3
3.25
3.5
3.75
4
4.25
4.5
4.75
SDG µM

Figure 3.11 (b)

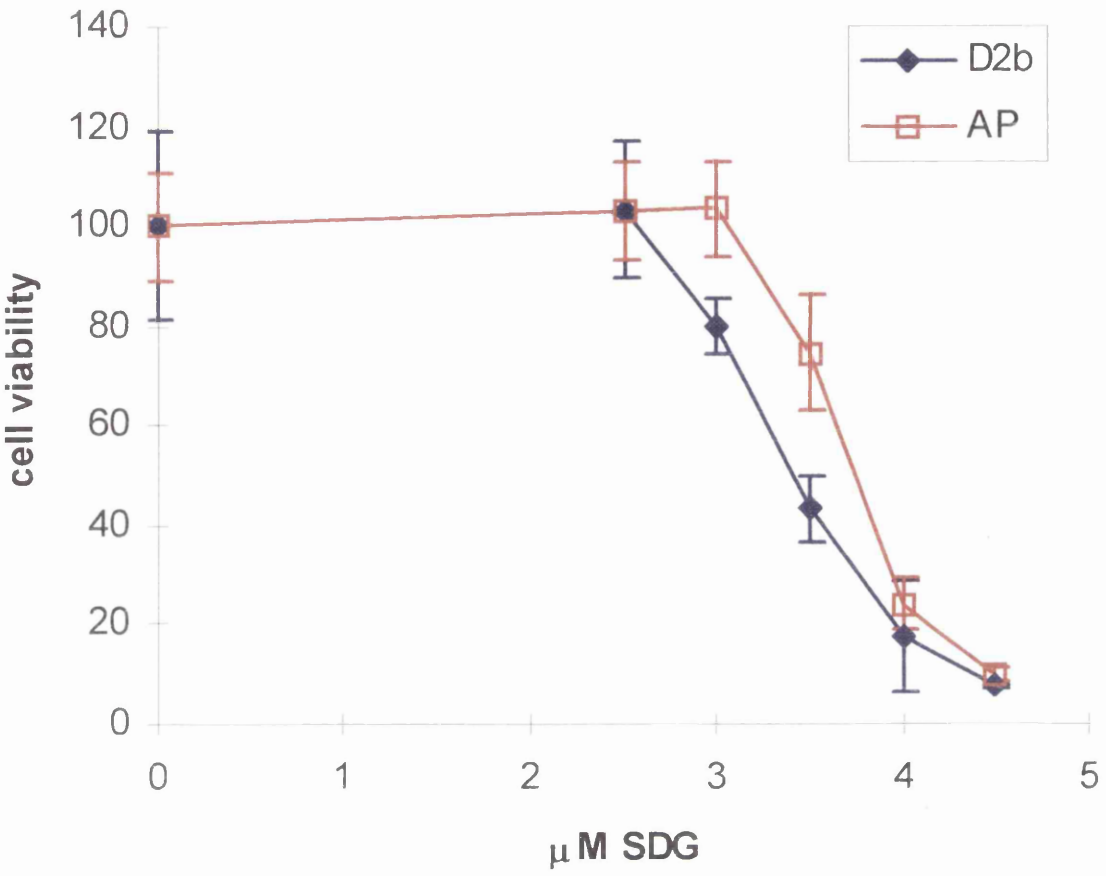


Figure 3.11 (c)

In the first cloning assay using (Figure 3.11a), the concentration of SDG required to kill 50% of the Bcl-2 positive clone, J2, was approximately 3.9μM, compared to cells containing vector control (clone BX), for which 3.65μM SDG was required to kill 50% of cells. However, on repetition of the experiment (figure 3.11b) there appeared to be no difference in the sensitivity of Bcl-2 expressing clones compared to Bcl-2 negative clones. The concentration of SDG required to kill 50% of cells was approximately 3.1μM SDG for both J2 and BX clones. A third experiment was performed in which the Bcl-2 positive clone D2B and the Bcl-2 negative clone AP were compared (Figure 3.11c). This experiment also showed no increased resistance of Bcl-2 positive clones to SDG compared to Bcl-2 negative clones.

Overall, the results of the MTT assays and the cloning assays suggest that the Bcl-2 expressing clones were equally sensitive to SDG as the Bcl-2 negative clones.

3.4.3 The Effect of Bcl-2 expression on p-XSC induced cell death

To determine whether Bcl-2 can prevent cell death induced by p-XSC, the ability of p-XSC to induce cell death was compared in MCF7 cell expressing normal levels of Bcl-2 and in MCF7 cells over-expressing Bcl-2. The ability of p-XSC to induce cell death was assessed by performing an MTT assay and a cloning assay.

MTT assay

Two MTT assays were performed in an attempt to determine if Bcl-2 expression in MCF-7 cells can protect against p-XSC induced cell death. Figure 3.12 depicts the results of an MTT assay in which the effects of p-XSC Bcl-2 positive clones J2 and C2C were compared to that on the Bcl-2 negative clone BX. Although the C2C clones appear to be slightly more resistant to p-XSC than the BX clone, the J2 clone appears to be slightly more sensitive than BX clones. A second MTT assay set up to examine the effects of p-XSC on the Bcl-2 positive and negative clones D2B and AP1 respectively, did not show any difference in the sensitivity of the two clones to pXSC.

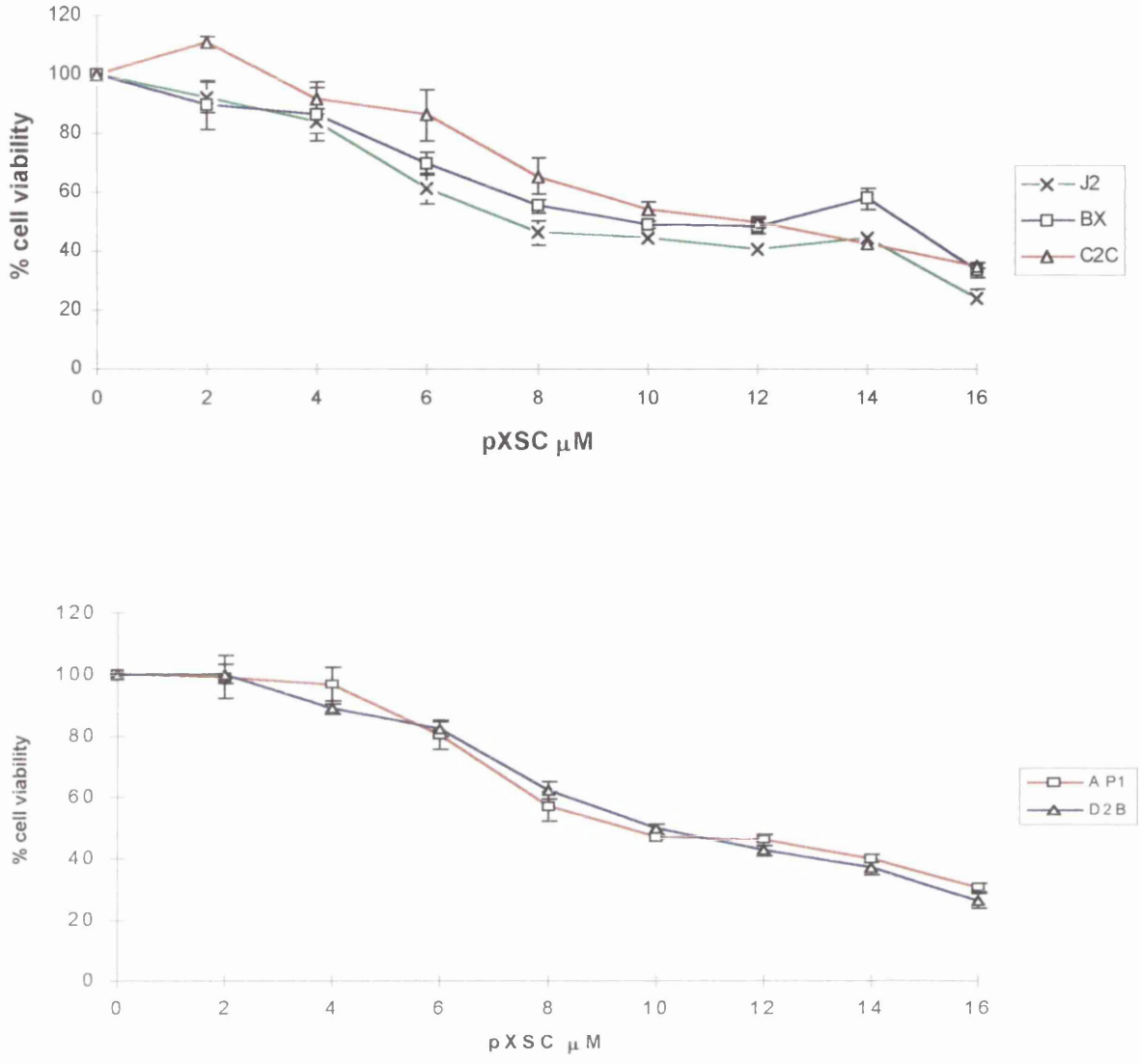


Figure 3.12
MTT assay to show the effect of Bcl-2 expression on pXSC induced cell death
500 MCF7 cells expressing Bcl-2 or vector controls were plated on to each well of a 96 well plate and left over night at 37 C to adhere. Bcl-2 expressing clones include J2, C2C and D2B. AP and BX clones are vector only controls. The medium from each well was removed and replaced with 100 μl of medium containing pXSC at the appropriate concentration and the plates incubated overnight at 37^{0}C. Samples were prepared in quadruplicate and an MTT assay performed. The data is expressed as a percentage of the optical density of the cells incubated with 0μM p-XSC. Error bars represent standard deviation from the mean.

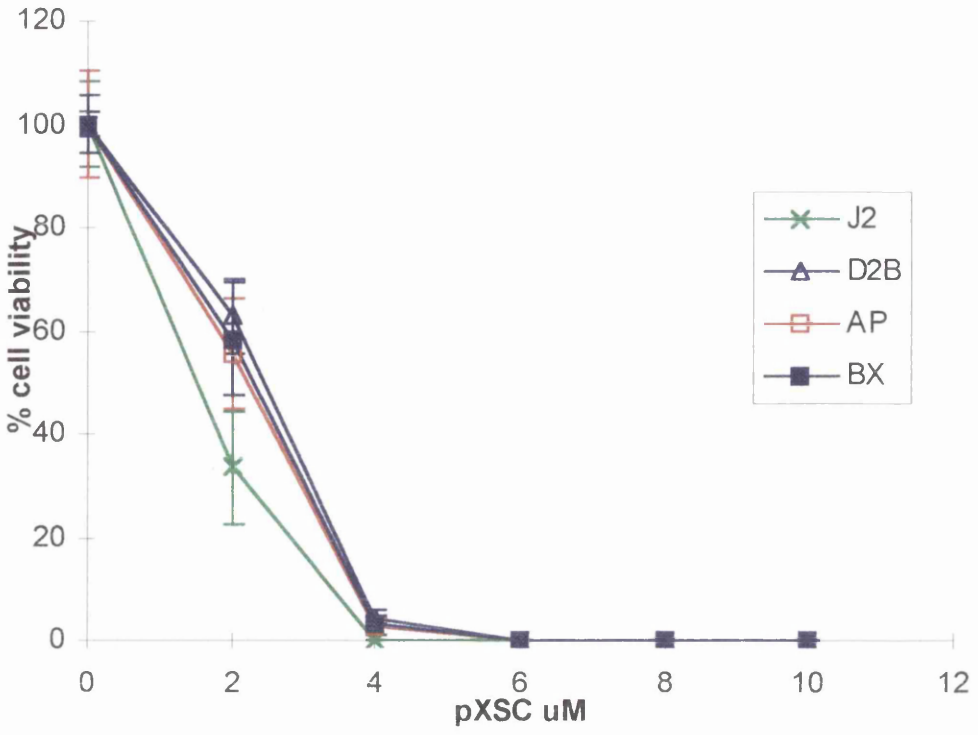


Figure 3.13
Cloning assay to show the effect of Bcl-2 expression on pXSC induced cell death
250 MCF7 cells expressing Bcl-2 (D2B and J2) or vector only controls (AP and BX) were plated on petri dishes and left overnight at 37°C to adhere. pXSC was added to each dish to give the required concentration and the cells incubated at 37°C for 14 days. Dishes at each concentration were prepared in quadruplicate. The colonies were stained with Giemsa stain and the number of colonies counted. Error bars represent standard deviation from the mean.

Cloning Assay

In order to detect any differences in the sensitivity of Bcl-2 expressing clones and Bcl-2 negative clones to pXSC, a cloning assay was performed using the Bcl-2 positive clones J2 and D2B and the Bcl-2 negative clones AP and BX. The results are shown in Figure 3.13. D2B, AP and BX were all approximately equally sensitive to pXSC with 50% of cells killed at approximately 2.5μM. The Bcl-2 positive clone J2 appeared slightly more sensitive to pXSC with 50% of cells killed at approximately 1.75μM pXSC.

3.4.4. The Effect of Bcl-2 expression on H_2O_2 induced cell death

As Bcl-2 possibly prevents cell death by it's ability to prevent the effects of reactive oxygen species and Bcl-2 has previously been shown to prevent cell death induced by H_2O_2 in a haemopoetic cell line (Hockenbery *et al.* 1990), it was investigated whether H_2O_2 induced cell death could be prevented in the MCF7 cell transfectants expressing Bcl-2. The ability of H_2O_2 to induce cell death in MCF7 cells in Bcl-2 positive and negative clones was assessed by performing an MTT assay and a cloning assay.

MTT Assay

An MTT assay was performed using BX, a Bcl-2 negative clone and J2, a Bcl-2 positive clone to help elucidate if Bcl-2 expression in MCF7 cells could protect against H_2O_2 induced cell death (Fig.3.14). The Bcl-2 positive clone J2 appeared to be slightly more resistant to H_2O_2 induced cell death than the Bcl-2 negative clone, BX. By performing a Two-Tailed Student's t-Test, the difference in sensitivity of the Bcl-2 positive and negative clones to H_2O_2 was not found to be significant at most concentrations, although the difference was found to be significant at 0.6 and 1.4mM H_2O_2.

Cloning Assay

An cloning assay was performed in which 250 D2B cells (Bcl-2 positive) and AP cells (Bcl-2 negative) were seeded on to petri dishes and left overnight to adhere. The cells were then treated with 0-1.0 mM H_2O_2 and 14 days later the percentage survival

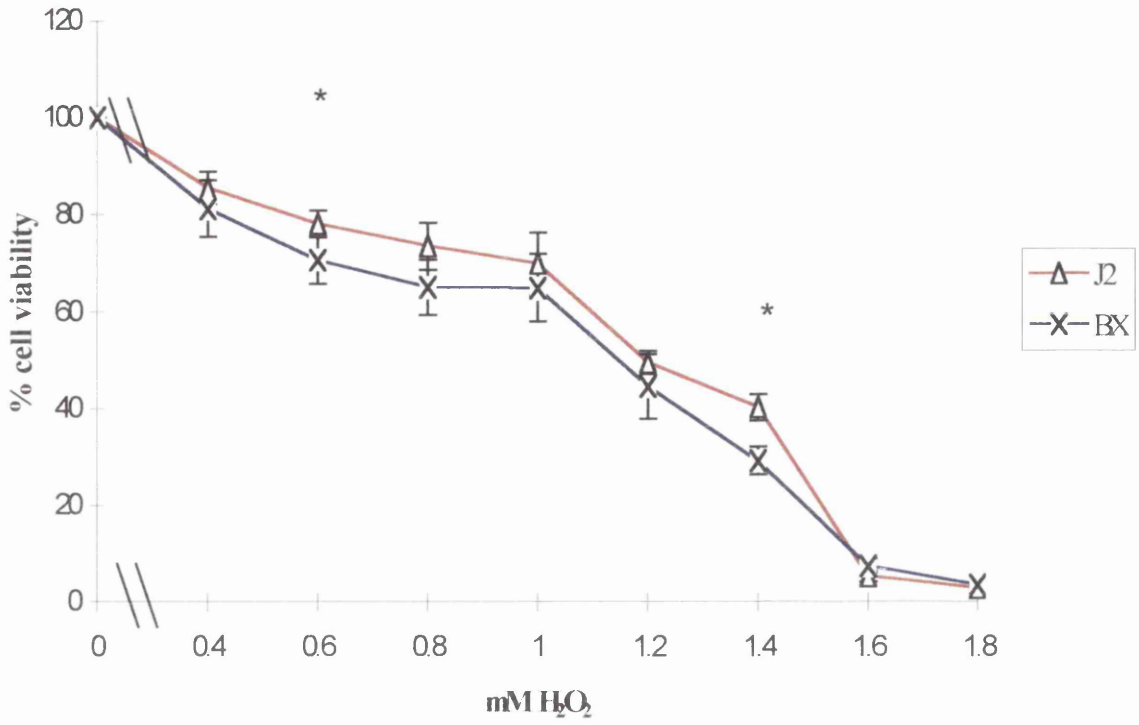


Figure 3.14
MTT assay to show the effect of Bcl-2 expression on H_2O_2 induced cell death
500 MCF7 cells expressing Bcl-2 or vector only controls were plated on to each well of a 96 well plate and left over night at 37°C to adhere. J2 and BX are a Bcl-2 expressing clone and vector only control respectively. The medium from each well was removed and replaced with 100µl of medium containing H_2O_2 at the appropriate concentration and the plates incubated overnight at 37°C. Samples were prepared in quadruplicate and an MTT assay performed. The data is expressed relative to the optical density of the cells incubated with medium only. Error bars represent standard deviation from the mean. * = significantly different i.e $p < 0.05$; Students T-test.

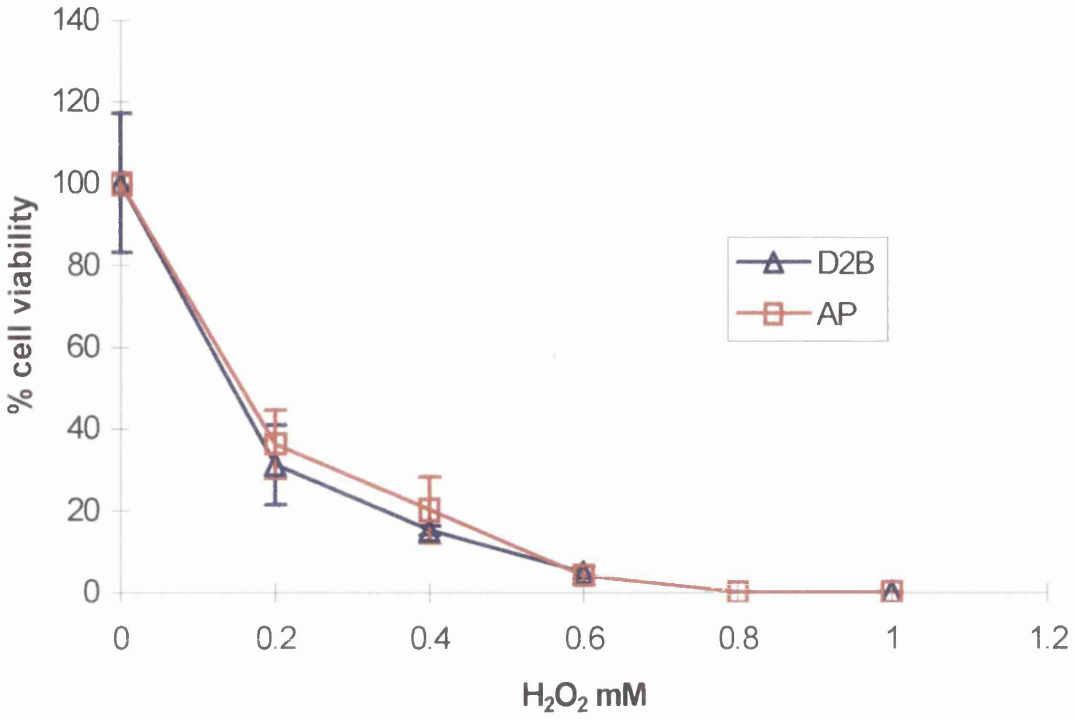


Figure 3.15

Cloning assay to show the effect of Bcl-2 expression on H_2O_2 induced cell death

250 MCF7 cells expressing Bcl-2 (D2B) or vector only controls (AP) were plated on petri dishes and left overnight at 37°C to adhere H_2O_2 was added to each dish to give the required concentration and the cells incubated at 37°C for 14 days. Dishes at each concentration were prepared in quadruplicate. The colonies were stained with Giemsa stain and the number of colonies counted. Error bars represent standard deviation from the mean.

was recorded (Fig. 3.15). The Bcl-2 positive clone does not appears to be more resistant to H_2O_2 than the Bcl-2 negative clone and no significant difference was found between the two clones using the Students t-Test.

Discussion

Standardisation of experimental conditions for SDG and p-XSC induced cell death

Sodium selenite is a natural inorganic form of selenium which is metabolised *in vivo* to organic forms which can then be incorporated in amino acids selenocysteine and selenomethionine or can undergo a series of reduction and methylation reactions in order to be excreted (see Fig. 1.3). Sodium selenite kills cells *in vitro* after a lag phase which can be eliminated by GSH coadministration. Selenodiglutathione (SDG) is the primary product of selenite, formed by the reaction of selenite and glutathione (GSH) with glutathione reductase or by non-enzymatic reduction when glutathione is in excess (Ganther *et al.* 1971). Addition of SDG to cells *in vitro* results in rapid cell death without the lag phase observed with selenite and SDG is also more potent than selenite *in vitro*. Thus it was decided that SDG would be used in order to investigate selenium induced cell death and as it was not commercially available it was necessary to synthesize and purify it in the laboratory. A novel selenium compound, p-XSC has been synthesized which has been shown to be more potent at inducing apoptosis than selenite and have greater chemopreventive activity than selenite yet appears to be less toxic than selenite (Ip *et al.* 1994; Thompson *et al.* 1994). It was also therefore interesting to investigate the mechanisms of cell death by induced by p-XSC in this study. MCF7 cells were chosen for these experiments since they are a human mammary cell line and a large number of carcinogenesis studies testing the chemopreventive effects of selenium compounds have utilised a mammary tumour models in rodents (Lane and Medina. 1985; Ip. 1981; Horvath and Ip. 1983; Thompson *et al.* 1984).

Previously, the potency of SDG was found to fluctuate substantially between experiments. Cells were sometimes killed at concentrations as low as 1.75μM SDG yet on other occasions there was complete cell survival at 10μM. This was problematic when designing experiments. It was hypothesised that SDG was subject to decomposition but the reason for the inconsistency of this decomposition remained unclear. It is unlikely to be a temperature dependant effect as this is strictly controlled: SDG is stored on ice immediately before its addition to tissue culture medium which is then incubated in a 37°C incubator.

At alkaline pH selenotrisulphides such as SDG are relatively unstable, degrading to the disulphide and elemental selenium (Ganther 1968).

RSSeSR ——————→ RSSR + Se

SDG is considered to be relatively stable at acidic pH and so is stored in 10mM acetic acid, thus minimum degradation would be expected. The pH of regular bicarbonate buffered tissue culture medium can sometimes become quite alkaline, especially when transferring micro-titre plates or open dishes from incubators with a 5% (v/v) $C0_2$ atmosphere to laminar flow hoods during manipulations and could possibly result in degradation of SDG and thus cause the variation observed between experiments. In this study, MTT assays were performed to investigate the effects of pH on SDG potency. The results of the experiment showed that the potency of SDG was stable over the pH range studied (pH 6.4-7.4). Therefore further experiments were all carried out with medium buffered at pH 7.0 using Bufferal. It is likely that without extra buffering, the pH of the bicarbonate buffered medium could rise much higher than 7.4. It may be of interest to perform further experiments at which the pH of tissue culture medium is above pH 7.4 to prove the hypothesis that SDG degrades when the pH of tissue culture medium becomes too alkaline and to also determine the threshold pH at which degradation occurs.

The addition of glutathione (GSH) to selenite has been shown to enhance cell death and abolish the lag phase observed with selenite alone. In the reaction proposed by Seko (1989) (see figure 1.1), GSH is also shown to be required for the enzymatic reduction of SDG to the persulphide derivative by glutathione reductase or by nonenzymatic reduction of SDG when GSH is in excess. If a metabolite of SDG is responsible for the toxic effect rather than SDG itself, fluctuations in GSH concentration in the tissue culture medium could be another source of variation of SDG potency. In this study an MTT assay performed to investigate the effect of GSH on SDG potency. For the first time it was shown that addition of 5 and 10μM GSH substantially increased the potency of SDG. However, addition of up to 20μM GSH did not increase the potency of the novel synthetic selenium compound pXSC. As

glutathione does not enhance the activity of pXSC and is not required for its metabolism it is likely that the ability of GSH to enhance the activity of SDG is related to its ability to reduce SDG to an active metabolite. If metabolism of SDG to an active metabolite is required to induce cell death then the metabolic capacity and hence growth status of the cells may be an important factor affecting the potency of SDG. It may be that cells in an exponential phase of growth can metabolise SDG more rapidly than quiescent cells. It is possible that the density of cells could effect their sensitivity to SDG as confluent cells may have a low metabolic rate and therefore activate SDG at only a very slow rate. However, preliminary work by J Fleming (unpublished) in C57 cells did not show any effect of density of cells on SDG potency.

In this study, co-administration of 20 μM GSH appeared to provide some protection against pXSC toxicity. Recent work by J Fleming (unpublished) has shown high concentrations of GSH to be protective against cell death induced by both SDG and pXSC; 50 μM GSH was shown to reduce C57 cell death induced by pXSC by approximately 50%, and 100 μM GSH was found to reduce SDG induced cell death by approximately 80%. SDG and pXSC are thought to induce apoptosis by oxidative stress so the presence of GSH at high concentrations may protect against cell death induced by these compounds by it's ability to act as an antioxidant (Deneke *et al.* 1985; Hagen *et al.* 1988). N-acetylcysteine (NAC) which is the biological precursor to GSH and is itself an antioxidant, has also been shown to be protective against cell death induced by SDG and p-XSC (J Fleming; unpublished).

In order to determine if variation in the amount of glutathione present in FCS was responsible for the fluctuation in potency of SDG observed between experiments, an assay was performed to determine the concentration of GSH in FCS. However, it was demonstrated here that FCS did not contain detectable levels of GSH and so this was not the cause of fluctuation in SDG potency. Glutathione peroxidases require GSH for their activity thus raising the question as to how plasma glutathione peroxidase can function if there is no GSH in serum. It may be that glutathione peroxidase utilises another thiol present in plasma other than glutathione, one possible candidate being thioredoxin.

The amount of foetal calf serum (FCS) added to the medium was previously found to be critical in altering the ability of pXSC, a novel synthetic selenium compound to induce cell death (Ronai *et al.* 1995). An MTT assay was performed here to determine if the potency of SDG was also effected by FCS concentration. The results show that, like pXSC, the potency of SDG is affected by the amount of FCS in the medium. This result is consistent with the finding of Vernie *et al.* (1979) who demonstrated that the ability of SDG to inhibit protein synthesis was reduced when the concentration of serum was increased from 5 to 20% with complete loss of inhibitory activity at 20% serum. It is possible that selenium is able to bind to selenium-binding proteins present in FCS: thus the greater the concentration of FCS, the lower the concentration of active free SDG available to cause the growth inhibitory effects. Alternatively, increasing the concentration of FCS could increase the levels of thiols which are responsible for the detoxification of SDG. In this study, subsequent experiments were carried out with medium containing 10% FCS and in experiments comparing the sensitivity of clones care was taken to use medium from the same bottle to minimise any minor fluctuations between batches of medium. However, any differences in FCS between experiments as a result of using different bottles of medium are negligible and are unlikely to account for the large fluctuations in SDG potency observed previously. The same batch of FCS was used in all experiments because variation in the content of various components such as thiols or selenium-binding proteins could occur.

Effect of Bcl-2 expression on SDG and pXSC induced cell death

Overexpression of Bcl-2 has been shown to protect against apoptosis induced by a variety of stimuli including UVA radiation, glucose deprivation, Fas, H_2O_2 and c-myc (Pourzand *et al.* 1997; Lacronique *et al.* 1996; Hockenbery *et al.* 1993; Bisonnette *et al.* 1993). Apoptosis in leukaemic cells induced by daunorubicin was delayed by overexpression of Bcl-2 but was not prevented (Allouche *et al.* 1992). In this study, to investigate whether Bcl-2 expression could protect against SDG induced cell death, MCF7 cells were transfected with the Bcl-2 expression vector pSSFV-Bcl-2n.

Overexpression of Bcl-2 protein was previously shown to protect against

apoptosis induced by H_2O_2 in a haemopoetic cell line (Hockenbery *et al.* 1993). However, in the present study, only a slight protective effect from apoptosis induced by H_2O_2 was observed in the MCF7 cells transfected with pSSFV Bcl-2nl. By performing an MTT assay, a mild protective effect against H_2O_2 induced cell death was observed in MCF7 cells overexpressing Bcl-2, although this effect was very weak and was only found to be significant at two concentrations of H_2O_2. The results of the cloning assay showed no significant difference between Bcl-2 expressing clones and vector alone controls in their sensitivity to H_2O_2 induced cell death. The weak protective effect observed suggests that either Bcl-2 expression was either not high enough in the transfected clones or that Bcl-2 is unable to protect against H_2O_2 induced cell death in MCF7 cells.

The MTT assay measures the change in metabolic activity of the whole population of cells following treatment of selenium compounds or H_2O_2, whereas the cloning assay measures the percentage of cells which are capable of proliferation. Following treatment with pXSC and H_2O_2 the metabolic activity of the population of cells appears to be less affected than the cloning efficiency. The cloning efficiency and the metabolic rate of the population of MCF7 cells, appear to be equally affected following treatment with SDG.

Although Bcl-2 overexpression was detected in this study by using anti-Bcl-2 antibodies in a Western Blot, it is conceivable that this Bcl-2 protein was not functional. For example, it is possible that a mutation could occur during the genetic manipulations carried out to produce the expression vector. To determine if a mutation in the Bcl-2 gene had occurred it would be necessary to sequence the Bcl-2 cDNA, in the vector used. However, it seems unlikely that a mutation is present in the expression vector as this vector was used sucessfully by Bissonnette *et al.* 1992 to protect against cell death induced c-Myc, therefore demonstrating that the Bcl-2 protein produced by this expression vector is functional since the vector was not manipulated in any way during the growth of the plasmid stocks.

It is therefore more likely that the levels of Bcl-2 were not sufficiently high enough to protect against H_2O_2 induced apoptosis. The western blot performed in this study to determine which clones were overexpressing Bcl-2 showed clear bands in several of the clones transfected with pSSFVbcl-2n whereas Bcl-2 protein was

undetectable in cells transfected with empty vector. This suggests that Bcl-2 expression in the Bcl-2 positive clones was several fold higher compared with the controls. However, as the levels of Bcl-2 were not sufficiently high enough to protect against H_2O_2 induced apoptosis, clones expressing higher levels of Bcl-2 would need to be produced in order to determine if over expression of Bcl-2 can prevent apoptosis induced by selenium compounds.

The mechanisms controlling cell survival and cell death are complex. The activity of Bcl-2 is inhibited by other Bcl-2 family members such as Bax and Bcl-x_S (Oltavi *et al.* 1993; Boise *et al.* 1993). It is possible that on transfection with an expression vector and an increase in the levels of Bcl-2 protein, the cell adapts by an upregulation of negative feed back pathways resulting in an increased production of inhibitory molecules, such as other bcl-2 family members, bax and bcl-xS (Oltavi *et al.* 1993; Boise *et al.* 1993. This would mean that even though levels of Bcl-2 protein were increased, its activity is kept in check. If this is the case, very high concentrations of Bcl-2 protein may be required to overcome the inhibitory action of such molecules. It is possible that the levels of Bcl-2 achieved in this study was insufficient to overcome the inhibitory action of other Bcl-2 family members. Alternatively, other proteins with which bcl-2 interacts with may not be at high enough concentrations for bcl-2 to have it's maximal effect. Coexpression of Bcl-2 and Bag-1 proteins has been shown to provide a greater protection against cell death induced by a variety of stimuli than when either protein was expressed alone (Takayama *et al.* 1995). Another explanation for the lack of protective effect by overexpression of Bcl-2 against SDG induced cell death could be that there was insufficient quantities of partner proteins such as Bag-1 which are required to enhance the activity of Bcl-2.

Future Experiments

It is possible that overpression of Bcl-2 is not able to inhibit cell death induced by H_2O_2 and selenium compounds in MCF7 cells. By using MTT assays, Bcl-2 overexpression has been shown to protect against apoptosis induced by Tumour Necrosis Factor (TNF) in MCF7 cells (Jaatella. *et al.* 1995). A simple experiment to

determine if levels of Bcl-2 are sufficient to prevent apoptosis would be to add TNF to the Bcl-2 expressing clones and see if the cells survived at concentrations which caused apoptosis in control cells. This would help determine if Bcl-2 is mechanistically unable to prevent cell death induced by H_2O_2 and selenium compounds or if the levels of Bcl-2 were simply inadequate.

The MTT assays and cloning assays used in this study were unable to demonstrate that overexpression of Bcl-2 can confer resistance to SDG and pXSC mediated cytotoxicty. However, Jaatella and coworkers (1995) used an MTT assay to demonstrate that over expression of Bcl-2 or Bcl-x conferred almost complete resistance to cytotoxicty mediated by high doses of anti-fas and TNF in MCF7 cells. MTT assays and cloning assays are able to monitor cell death yet are unable to distinguish between cells dying by necrosis and those dying by apoptosis. This study could be improved by using an assay which more directly measures cell death by apoptosis such as dye exclusion flow cytometry.

Earlier work has demonstrated the ability of SDG to induce apoptosis in mouse erythroleukaemic (MEL) cells as judged by flow cytometry and the formation of nucleosomal ladders following electrophoresis of DNA on an agarose gel. Selenite and pXSC have previously been shown to induce typical features of apoptosis in mouse mammary epithelial (MOD) cells such as condensation of the cytoplasm, formation of nucleosomal ladders and apoptotic bodies. However, it has not been proven that these compounds induce apoptosis in MCF7 cells. On treatment with SDG and pXSC condensation of the cytoplasm in MCF7 cells was observed in this study. However, further experiments such as detection DNA ladder formation, following electrophoresis on an agarose gel or two dye flow cytometry are required to demonstrate that SDG and pXSC induce apoptosis in MCF7 cells rather than causing death by necrosis.

To further investigate the mechanism of growth inhibition by selenium compounds, I would be interested to investigate whether other proteins involved in cell death pathways effect the sensitivity of cells to cell death induced by selenium compounds.

ICE proteases have been demonstrated to be required for cell death induced by a number of compounds including TNF and Fas (Itoh *et al.* 1991; Oehm *et al.* 1992).

Unlike bcl-2, ICE-family proteases inhibitors do not effect JNK activation; JNK is a member of the MAP kinase family and has been shown to be nessessary for induction of apoptosis by a number of stimuli (Park *et al.* 1996). These findings suggest that either JNK and ICE proteins are involved in separate cell death cascades or that they are both involved in same signalling pathway, with ICE proteases lying down-stream of JNK. I would like to investigate whether inhibitors of ICE proteases, could prevent cell death induced by selenium compounds.

Previous work, in our laboratory has suggested that SDG treatment of an ovarian cell line (A2780) results in accummulation of p53 protein, a known supressor of cell growth (Lanfear *et al.* 1994). However, as SDG and p-XSC have been shown to induce apoptosis in cell lines which contain mutated p53, the effect of p53 accummulation in SDG induced apoptosis remains unclear (Lanfear *et al.* 1994; Thompson *et al.* 1994). To investigate whether p53 mutations are able to effect the sensitivity of cells, to cell death induced by selenium compounds, MTT and cloning assays could be performed on A2780 cells (which have wild-type p53) and A2780 cells expressing dominant negative-mutant p53 protein (generated by A. McIlwrath *et al.* 1994; CRC Beatson Laboratories, Glasgow).

If I was to continue working on this project I would use a cell line other than MCF7 cells. MCF7 cells are highly adhesive to each other and clump together even following trypsinisation thus making it difficult to count them accurately and also separate in to single cells for cloning assays. Also, MCF7 cells grow very slowly in comparison with other cell lines which means that it can take months to grow up a tissue culture flask of cells from a single selected colony.

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