Selenium as a Modulator of Efficacy and Toxicity of Chemotherapy and Radiation

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Richard John Lobb

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Abstract

Selenium (Se) is an essential trace mineral required in maintaining health in humans. Recent research has demonstrated that Se shows promise as an agent that can reduce the harmful side-effects of chemotherapy (CT) and radiotherapy (RT), while not compromising the effectiveness of treatment. Clinical evaluations using varying doses of Se have demonstrated a significant reduction in toxicity of CT and RT.

Se supplementation appears to mediate its biological effects through four main mechanisms: Se treatment promotes the death of malignant cells, but not normal cells, through the differential effects on the endoplasmic reticulum stress response; it inhibits hypoxia-induced angiogenesis which is required by tumours for their blood supply; it enhances DNA repair in normal but not malignant cells; and it reverses cellular resistance to some cytotoxic drugs.

The objectives of this thesis were to evaluate the biological characteristics of the differential impact of a Se compound, methylseleninic acid (MSA), on malignant and non-malignant human cells and how that modulates the effects of cisplatin chemotherapy and/or radiation. In parallel with this, similar evaluations were undertaken in patients receiving a Se compound or placebo in conjunction with cisplatin and radiation for head and neck squamous cell carcinoma (HNSCC).

MSA induced differential effects in peripheral blood mononuclear cells (PBMC) obtained from healthy individuals compared to the monocytic leukaemia THP-1 cell-line. While MSA did not change PBMC viability, it reduced that of THP-1 cells in a concentration-dependent manner. Furthermore, MSA treatment enhanced the cytotoxicity of CT and RT toward THP-1 cells without compromising the survival of PBMC. This increased sensitivity in THP-1 cells is likely to be related to significantly

reduced levels of intracellular glutathione (GSH) in response to MSA. In contrast, GSH levels in PBMC rose substantially in response to MSA.

Assessment of ER stress response proteins revealed that pro-survival proteins (GRP78, phospho-eIF2α, and spliced XBP-1) were upregulated in PBMC in response to MSA treatment. The malignant THP-1 cells had high levels of pro-survival proteins at baseline, which increased to a variable degree, especially caspase-8, in response to MSA.

Putative pharmacodynamic markers of biological activity of Se were evaluated in plasma samples and PBMC from HNSCC patients in a randomised, placebo-controlled, double-blind phase II trial. Patients were treated with a 7-week radical course of concurrent cisplatin and RT (CRT) and were randomised to receive either selenomethionine (SLM) or matching placebo for 11 weeks, starting a week prior to CRT treatment. Because the trial is not yet complete, treatment allocation remains blinded and thus the pharmacodynamic results according to treatment with SLM or placebo cannot be made at the time of writing this thesis. However, markers of angiogenesis and the selenoprotein glutathione peroxidise 3 were measured and shown to significantly change during the course of treatment in a patient-specific manner. Lastly, ER stress markers were shown to be detected in PBMC obtained from patients during treatment.

Se compounds differentially affect non-malignant and malignant cells. This results in increased therapeutic efficacy of cisplatin and RT targeting malignant cells. These effects appear to be mediated, at least in part, by modulation of intracellular GSH and ER stress response proteins.

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ASK1	apoptosis signal-regulating kinase
ATF6	activating transcription factor 6
BCA	bicinchoninic acid
CHOP	homologous protein/growth arrest DNA damage protein
CR	complete response
CT	chemotherapy
CRT	chemoradiation
DDR	DNA-damage response
eEFSEC	sec-specific elongation factor
ELISA	enzyme-linked immunosorbent assay
eIF2α	eukaryotic initiation factor 2α
ER	endoplasmic reticulum
ERSE	ER stress response element
GGR	global genomic repair
GPX	glutathione peroxidase
GSH	glutathione
Gy	Gray
HCC	hepatocellular carcinoma
HIF	hypoxia-inducible factor
HMG	high-mobility-group proteins
HNSCC	head and neck squamous cell carcinoma
IRE1	inositol-requiring enzyme 1
IRES	internal ribosomal entry site
JNK	c-Jun N-terminal kinase
MSA	methylseleninic acid
MTT	methyl-thiazol-tetrazolium cell viability assay
NER	nucleotide excision repair pathway

NPC	Nutritional Prevention of Cancer trial
PBMC	peripheral blood mononuclear cells
PERK	pancreatic ER kinase (PKR)-like ER kinase
PHD	prolyl hydroxylase
ROS	reactive oxygen species
RT	radiotherapy
S1P	Site 1 protease
S2P	Site 2 protease
Se	selenium
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SLA	soluble liver antigen
SLM	selenomethionine
TCR	transcription-coupled repair
TNF	tumour necrosis factor
TRAF2	TNF-receptor-associated factor 2
UPR	unfolded protein response
UTR	untranslated region
VEGFR-1	tyrosine-kinase receptor of VEGF-A
VEGF	vascular endothelial growth factor
XBP1	X box-binding protein 1
XPC	xeroderma pigmentosum complement group C
XPE	xeroderma pigmentosum complement group E

Chapter 1

Introduction and literature review

1.1 Introduction

Selenium (Se) is a remarkable element which has proven useful to humanity in a number of ways. Discovered in 1817 by the Swedish chemist Berzelius, Se was first considered to be solely toxic to humans, but this villainous view of Se has changed dramatically since it was recognized as an essential trace mineral required for maintaining health in humans and other species.

1.2 Biogeography of Selenium

Se is a ubiquitous element derived from the rocks and soils of our terrestrial environment. Most soils contain between 0.1 and 2.0 µg Se/kg (Swaine, 1955; Rosenfeld & Beath, 1964) however Se is unevenly distributed, which can result in Se-deficient areas or seleniferous (Se-rich) areas. Soils in some countries such as New Zealand, Denmark, Finland and in parts of China are known to be Se-deficient. In contrast, other areas such as the great plains of the USA, Canada, Columbia, Venezuela, certain regions of China and Ireland have high levels of Se in soil and are therefore considered seleniferous. Biogeochemical mapping of top soils in New Zealand has shown that most soils are Se-deficient (Wells, 1967) due to either volcanic soil or excessive leaching which occur in the North and South Islands respectively. Most of the Se in food systems ultimately resides in the soil, entering the food system through plants. The biological availability of Se to plants depends on a number of factors including the species of Se, the pH of the soil, and the presence of other elements which can inhibit the uptake of Se. These factors ultimately influence the availability of Se to animals, including humans.

1.3 Human Intake

The dietary intake of Se in human populations varies greatly throughout the world due to the uneven distribution of Se in soil and the varying nature of its bioavailability. Se was first recognized as an essential micronutrient when it was demonstrated Se could be used to replace vitamin E in rats and chicks to prevent vascular, muscular and hepatic lesions (Schwarz & Foltz, 1957; Schwarz et al. 1957). Se deficiency-linked diseases in humans were subsequently identified in Se-deficient areas of China where dietary intake of Se was extremely low (≤ 10 µg/day). Se deficiency has also been linked to the aetiology of Kashin-Beck disease, a deforming arthritis, and Keshan disease, an endemic cardiomyopathy prevalent in children (Zhou, et al., 2003). Keshan disease is believed to be triggered by coxsackie B viral infection, but Se supplementation remarkably prevents and reverses this disease. A number of other conditions have also been linked to Se deficiency, including male infertility, decreased immune and thyroid function, and increased cancer risk.

1.4 Selenoproteins

The beneficial impact of Se on human health has been attributed largely to the presence of Se in proteins termed selenoproteins. It is therefore crucial to understand the role and regulation of these proteins and their impact on human health. Selenium is unlike most other metals which interact with proteins as cofactors; instead Se is cotranslationally incorporated into a polypeptide chain in the form of selenocysteine (Sec). Sec is now well established as the 21st genetically encoded amino acid, incorporated at UGA codons, which are normally read as stop codons (Berry, et al., 1991; Kryukov, et al., 2003b). This unique situation of codon duality is controlled by the presence of evolutionary conserved *cis-* and *trans*-acting elements as well as a myriad of proteins dedicated to decoding UGA into Sec. Proteins which contain Sec as part of their polypeptide chain are defined as selenoproteins. The human selenoproteome consists of 25 identified selenoproteins, which are diversely expressed in tissues. These include glutathione peroxidases (5 genes), iodothyronine deiodinases (3 genes),

thioredoxin reductases (3 genes), selenophosphate synthetase 2, 15kDa-selenoprotein as well as a host of other selenoproteins which have been annotated in alphabetical order (SelH, SelI, SelK, SelM, SelN, SelO, SelP/SepP, SelR, SelS, SelT, SelV, and SelW) (Kryukov, et al., 2003a).

1.4.1 Selenocysteine biosynthesis

Sec is unlike other amino acids being entirely synthesized on its own tRNA, designated tRNA [Ser]Sec, using serine as an intermediate (B. J. Lee, Worland, Davis, Stadtman, & Hatfield, 1989). This unique tRNA has an anticodon that is complimentary to UGA, incorporating Sec in the presence of specialized secondary structures in the 3' untranslated region (UTR) of selenoprotein mRNAs. One of the quirks in Sec biosynthesis is that it requires SPS2, which is itself a selenoprotein, suggesting that it is involved in the autoregulation of its own biosynthesis. SPS2 forms the active Se donor monoselenophosphate, which replaces the phosphate group of phosphoseryl-tRNA [Ser]Sec thereby forming selenocysteyl-tRNA [Ser]Sec which incorporates Sec into the polypeptide chain (Papp, et al., 2007).

1.4.2 tRNA [Ser]Sec

In humans, and all other mammals examined to date there is a single tRNA [Ser]Sec gene (*trsp*) that encodes two isoforms differing by a single methyl group (D. L. Hatfield & Gladyshev, 2002), which changes the tertiary structure of the tRNA (D. Hatfield, Lee, Hampton, & Diamond, 1991). The relative distribution of the two isoforms is dependent on cell type, and can be altered in response to Se supplementation. Cell culture work has demonstrated that Se treatment induces the production of the methylated isoform (5-methylcarboxymethyluridine-2'-*O*-methylribose) over the unmethylated isoform 5-methylcarboxymethyluridine (Chittum, et al., 1997; Diamond, et al., 1993; D. Hatfield, et al., 1991).

1.4.3 Selenocysteine insertion sequence

Though deletion studies have shown the requirement for tRNA [Ser]Sec in UGA recoding (Bosl, Takaku, Oshima, Nishimura, & Taketo, 1997), this unique tRNA

however, is not sufficient as the sole cause of UGA readthrough. The presence of specialized secondary structures in the 3' UTR of selenoprotein mRNAs termed the selenocysteine insertion sequence (SECIS) element is the universal determinant of UGA recoding and can be located up to several kilobases away from the UGA codon (Berry, et al., 1991; Berry, Banu, Harney, & Larsen, 1993; Wilting, Schorling, Persson, & Bock, 1997). Studies have revealed that SECIS elements possess a conserved AAR motif with an apical loop which contains either two consecutive unpaired AA or CC (Korotkov, Novoselov, Hatfield, & Gladyshev, 2002; Kryukov, et al., 2003a) residues. Furthermore, SECIS elements contain what is termed the SECIS core, a stem structure containing non-Watson-Crick G to A base pairing with either A or G residues preceding the core (Papp, et al., 2007). There are two SECIS elements depending on their structure shown in Figure 1.
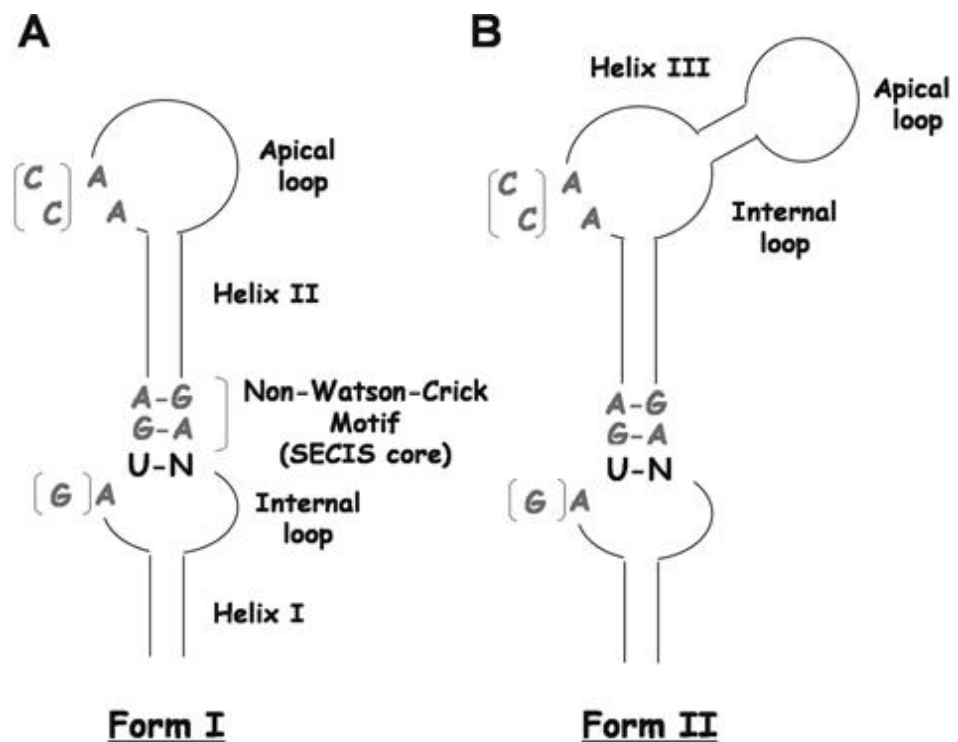
Figure 1: Two forms of eukaryotic SECIS elements (from Papp *et al.,* 2007)

All selenoproteins depend on the presence of one SECIS element for Sec incorporation, except SelP, which requires two SECIS elements due to the presence of up to 18 UGA codons in its mRNA, and thus multiple sites for potential termination. *In vitro* mutational analysis of conserved SECIS sequence has shown that this element is absolutely essential for Sec incorporation, while mutations in non-conserved areas partially reduce recoding (Krol, 2002). This may mean that mutations in selenoprotein SECIS may contribute to the aetiology of disease in humans. In the 15 kDA selenoprotein the 1125G/A SNP (dbSNP refSNP: rs5859) in the SECIS of humans is distributed at a different frequency in individuals with cancer compared to apparently healthy individuals (Hu, et al., 2001; Kumaraswamy, et al., 2000). Kumaraswamy and colleagues found that AA homozygotes were twice as frequent as GG homozygotes in cancer patients. Interestingly *in vitro* studies showed that the AA genotype was twice as efficient at promoting UGA recoding, but far less responsive to increased Se levels as well as the cells being more resistant to selenite-induced apoptosis (Apostolou, et al., 2004; Kumaraswamy, et al., 2000).

UGA readthrough additionally requires the cooperative action of several proteins. In mammals this process is rather complex involving SBP2 (Copeland & Driscoll, 1999; Copeland, Fletcher, Carlson, Hatfield, & Driscoll, 2000), Sec-specific elongation factor (eEFSEC) (Fagegaltier, et al., 2000), ribosomal protein L30 (Chavatte, Brown, & Driscoll, 2005), Secp43 (a 43 kDa RNA-binding protein), and soluble liver antigen (SLA) (Xu, et al., 2005). SBP2 is perhaps the most well-characterized Sec incorporation factor. SBP2 was initially discovered to be involved in Sec incorporation in cell lysates. Cell lysates depleted of SBP2 lacked translational activity of selenoprotein synthesis, which was restored upon SBP2 repletion (Copeland *et al.* 2000). Further studies reinforced the requirement of SBP2 for Sec incorporation by using siRNAs against SBP2 which caused global selenoprotein depletion (Papp, et al., 2007). *In vitro* studies with cell lysates have shown that SLA, Secp43 and tRNA [Ser]Sec form a complex and depletion of either protein factor decreases the binding of the other to tRNA [Ser]Sec and depletion of both causes depression of selenoprotein synthesis (Papp, et al., 2007).

1.4.4 Selenoprotein biology

To date, most selenoproteins characterized have oxidoreductase activity with Sec found at the active site, where it is essential for efficient catalytic function. Sec differs from cysteine by a single atom (Se instead of S) which confers a lower pKa (5.2 v. 8.3), thus higher reactivity (Kim & Gladyshev, 2005). Selenoprotein homologues that have cysteine in place of Sec at their active site exhibit a 100-fold decrease in their catalytic activity (Kim & Gladyshev, 2005). The bioavailability of Se affects both the expression and activity of many selenoproteins (Bermano, et al., 1995; Muller & Pallauf, 2003). Supplementation with Se results in increases in both selenoprotein expression, at mRNA and protein levels, and activity (Sunde, Raines, Barnes, & Evenson, 2009). Indeed, the current recommended daily allowance intakes of Se are based on the amount of dietary Se required to reach maximal activity of glutathione peroxidase (GPX). In human health, GPX 1 activity is lower in individuals with inadequate Se intake. Lei *et al.* (2007) showed that the activity of GPX is dependent on the Se concentration, with deficient intake reducing GPX 1 expression and activity.

1.5 Selenium Chemoprevention

Evidence for the anticancer properties of Se has come from a number of sources. Epidemiological studies have indicated inverse association of Se status and cancer risk; animal experiments have shown that supra-nutritional doses of Se reduce tumorigenesis; and clinical intervention trials have indicated Se supplementation may reduce cancer risk.

1.5.1 Epidemiology

Se deficiency was first proposed to be involved in the aetiology of cancer in the 1960's. Based on the observation that cancer mortality in the United States was inversely associated with Se content in forage crops (Kubota, Allaway, Carter, Gary, & Lazar, 1967), Shamberger and Frost (1969) postulated that Se had a role in the prevention of cancer. Other epidemiological studies have echoed these findings. Shamberger and Willis (1971) found an increased risk of cancer

mortality from lymphomas and cancers of the gastrointestinal tract, peritoneum, lung, and breast in areas of the United States that have low Se content in forage crops. Using the same forage crop data, Clark et al. (1991) showed an increase risk of colorectal mortality in areas of low forage Se. These findings are yet further supported by an analysis of apparent Se intake in 27 countries that indicated an inverse association of Se intake and total age-adjusted cancer mortality due to leukaemia and cancers of the colon, rectum, breast, ovary, and lung (Schrauzer, White, & Schneider, 1977). Moreover, a study conducted in 8 provinces of China indicated a significant inverse association of blood Se levels and total cancer mortality rates (Yu, Li, Zhu, Yu, & Hou, 1989). These data generated a great deal on interest in Se as they indicated that this trace element could be a naturally-occurring anticarcinogen.

1.5.2 Animal studies

There have been numerous animal studies which have investigated the chemoprevention hypothesis of Se supplementation. Supranutritional doses of Se when supplemented in the diet, or drinking water, have been demonstrated by various researchers to protect laboratory animals from the development of mammary carcinomas (Ip, 1981; Ip & Ip, 1981; Ip & Sinha, 1981; Medina, Lane, & Shepherd, 1981; Medina & Shepherd, 1980; Schrauzer, White, & Schneider, 1976; Tappel, 1965). Se supplementation has also been shown to prevent prostate cancer in rats (McCormick & Rao, 1999) as well as reduce the effect of DNA damage-inducing agents in canine prostate cells (Waters, et al., 2003). In a study involving male beagle dogs, which develop spontaneous prostate cancer, supplementation with SLM or selenized yeast for 7 months revealed up-regulated epithelial cell apoptosis in their prostates in Se supplemented dogs compared with controls (Waters, et al., 2003). Although several mechanisms for the chemopreventative effects of Se compounds have been proposed, Se-induced apoptosis is regarded as the most feasible mechanism (Rayman, 2005).

1.5.3 In vitro studies

Using cell culture systems, several research groups have demonstrated that Se treatment inhibits cellular growth and induces apoptosis in a dose-dependent

manner (Cho, Jung, & Chung, 1999; Jung, Zheng, Yoon, & Chung, 2001; Zeng, 2002). Se compounds have been shown to be potent inducers of apoptosis in HL-60 cells (Cho, et al., 1999; Jung, et al., 2001; Zeng, 2002), which was shown to be dependent on caspase activation. Furthermore, Se compound inhibition of cell growth and induction apoptosis appears to preferentially impact malignant cells over normal cells (Menter, Sabichi, & Lippman, 2000). Se compounds have also been shown to induce apoptosis in mammary cancer cell lines. Given these results, the manipulation of apoptosis can provide novel strategies in cancer chemoprevention, or possibly in cancer chemotherapeutics.

1.5.4 Clinical prevention trials

Intervention trials have been carried out in order to assess whether Se supplementation reduces the risk of particular cancers. Some studies have investigated if hepatocellular carcinoma (HCC) incidence was reduced in response to Se supplementation. One study in Qidong County in China involving 226 hepatitis B antigen carriers - who are approximately 200 times more likely to develop HCC than non-carriers - showed no cases of HCC in individuals supplemented with 200 µg of selenized yeast, while 7 HCC cases developed in the placebo group over a four year period (Yu, Zhu, & Li, 1997). In another study by the same group, HCC incidence fell by 35% in townships that had their salt fortified with sodium selenite (Na_2SeO_3) at 15 mg/kg while the incidence of HCC remained unchanged in control townships (Yu, et al., 1997).

The Nutritional Prevention of Cancer (NPC) trial was the first double-blind placebo-controlled Se intervention trial carried out in a western (US) population. In this trial, 1312 individuals with a history of non-melanoma skin cancer were randomized to either placebo or 200 µg of Se per day as selenized yeast. There was no effect on the primary endpoint of non-melanoma skin cancer; however, secondary endpoints indicated fewer cancers of the prostate, colon and lung (Duffield-Lillico, Dalkin, et al., 2003; Duffield-Lillico, Slate, et al., 2003). Analysis of the NPC data showed that individuals in the lowest tertile of plasma Se (<106 ng/ml) at entry to the trial achieved the greatest effect with Se supplementation ((Duffield-Lillico, Dalkin, et al., 2003; Duffield-Lillico, Slate, et al., 2003).

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was designed to specifically investigate the chemoprevention of prostate cancer. 35,533 healthy men, $\geq$ 55 years old ($\geq$ 50 years if African American), with normal rectal exams and prostate-specific antigen <4 ng/ml, were randomized to one of four arms: (i) 200 µg/day l-selenomethionine, (ii) 400 IU/day all-rac-alpha-tocopheryl acetate (vitamin E), (iii) both supplements, or (iv) placebo for 7 to 12 years (Dunn, Richmond, Minasian, Ryan, & Ford, 2010). This study was however terminated prematurely due to concerns over safety and futility when interim analysis showed that neither supplement, alone or in combination, prevented prostate cancer in this population of men. These results provided a significant blow to the chemoprevention hypothesis regarding Se. It should be noted however, the NPC trial showed no benefit of Se supplementation in individuals with a baseline Se > 123.2 ng/ml and prevention of prostate cancer was only evident after more than 5 years (Duffield-Lillico, Dalkin, et al., 2003; Duffield-Lillico, Slate, et al., 2003). In the SELECT trial the upper 3 quartiles of both the placebo and treatment groups had a Se baseline > 123.2 ng/ml (Dunn, et al., 2010) and was terminated on an interim analysis conducted when median trial participation was less than 5 years. These features indicate that this study, which was originally thought of as well-planned, had obvious faults. Furthermore, SELECT showed that one chemical form of Se was ineffective at protecting against prostate cancer. The chemical form used in this study was l-selenomethionine (SLM), which had already been shown in animal studies to be less effective in preventing prostate cancer in rats (McCormick & Rao, 1999) and DNA damage in canines (Waters, et al., 2003) compared with selenized yeast, which interestingly is the form used by the NPC trial (Duffield-Lillico, Dalkin, et al., 2003; Duffield-Lillico, Slate, et al., 2003).

1.6 Selenium and cancer treatment

Although the vast majority of investigations involving Se have focused on chemoprevention, there are a number of studies which have illuminated the potential value of Se in conjunction with chemotherapeutic agents. The dose-limiting toxicity of anticancer therapies is a major ongoing clinical issue.

Therefore establishing whether particular agents can usefully modulate the toxicity and efficacy of chemotherapy (CT) and radiotherapy (RT) is important. Preclinical work has demonstrated that Se compounds can reduce the toxicity of CT and RT while not compromising the efficacy of treatment. Se compounds have been shown to provide significant benefits in reducing the development of drug resistance (Caffrey & Frenkel, 2000a; Frenkel & Caffrey, 2001; Siddik, 2003), toxicity (S. S. Cao, A. Durrani, & Y. M. Rustum, 2004; Francescato, et al., 2001), and in improving antitumour efficacy (S. S. Cao, et al., 2004; M. G. Fakih, et al., 2008; Fischer, Mihelc, Pollok, & Smith, 2007). The doses of Se which improve the narrow therapeutic index of anticancer treatments are far higher than those required to achieve homeostatic activity of selenoproteins, suggesting that different (non-selenoprotein-mediated) mechanisms come into play, such as disruption of protein folding.

1.6.1 Animal studies

In preclinical trials, supra-nutritional doses of Se compounds were found to both reduce irinotecan-induced toxicity and increase irinotecan's antitumour efficacy in nude mice bearing human tumour xenografts (S. S. Cao, et al., 2004). This effect was not specific to a particular chemotherapeutic drug or tumour. Indeed, the effect of Se supplementation appears to be broad, enhancing the antitumour efficacy of cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin. Moreover, Se supplementation has been successfully used to treat a variety of human tumour xenografts, including small cell and non-small cell lung carcinoma, colorectal carcinoma and head and neck squamous cell carcinoma (HNSCC) (Caffrey & Frenkel, 2000a; S. S. Cao, et al., 2004; M. G. Fakih, et al., 2008; Fischer, et al., 2007; Frenkel & Caffrey, 2001). Pretreating tumour-bearing rats with methylselenocysteine (MSC) for 14 days before treatment with cisplatin 6mg/kg increased the tumour complete response (CR) rate from 25% to 75% while preventing stomatitis and diarrhoea. Furthermore, 9mg/kg of cisplatin was lethal in non-treated rats but tolerated in Se-treated rats, with a CR rate of 88% (S. S. Cao, et al., 2004).

1.6.2 Human studies

Only a few small clinical trials have evaluated the effect of Se supplementation in combination with anticancer therapies. In one study, 62 women receiving chemotherapy for ovarian cancer (cisplatin 100 mg/m^2 and cyclophosphamide 600 mg/m^2 every three weeks) were recruited into a double-blind trial. Patients were randomized to capsules with or without selenized yeast (200 µg per day) for three months, starting concurrently with chemotherapy. At three months patients supplemented with Se exhibited a significant reduction in toxicities compared to the control group (Sieja & Talerczyk, 2004). Furthermore, neutrophil counts at three months in the Se group were better maintained than in the untreated group (3.39 vs. 2.52 x 10^9/l, p = 0.048) (Sieja & Talerczyk, 2004). In another randomized trial, women receiving adjuvant RT for gynaecological malignancies as well as oral selenite (500µg daily with RT and 300µg on non-RT days) were shown to have a significant increase in blood Se from 62.8 to 86.9 µg/l and reduced the incidence of ≥ grade 2 diarrhoea from 46.6% to 21.0%. Other clinical randomized studies have reported similar findings regarding the reduction of measured toxicities in patients (Asfour, et al., 2009; Asfour, et al., 2006; Asfour, et al., 2007; Hu, et al., 1997).

Fakih et al. (2008) conducted a phase I clinical trial to determine an appropriate oral dose of SLM required to achieve a plasma Se concentration of 15 µM or greater after one week. The authors demonstrated that doses of 4800 µg twice daily resulted in plasma Se greater than 15 µM at day eight, and that doses of up to 7200 µg twice daily for a week then once daily thereafter were generally well tolerated and resulted in even higher plasma Se concentrations. A plasma Se concentration of 15 µM or more is thought to provide therapeutic benefit, based on plasma Se concentrations in mice at which therapeutic effects were observed (S. S. Cao, et al., 2004).

1.7 Mechanisms

Se supplementation appears to mediate its biological effects through four main mechanisms. Se treatment: 1) enhances DNA repair in normal but not malignant

cells (Fischer, et al., 2007; Seo, Kelley, & Smith, 2002; Seo, Sweeney, & Smith, 2002; Smith, Lancia, Mercer, & Ip, 2004); 2) promotes the death of malignant cells, but not normal cells, through the differential effects on the endoplasmic reticulum stress response (Wu, Zhang, Dong, Park, & Ip, 2005); 3) inhibits hypoxia-induced angiogenesis (C. Jiang, Ganther, & Lu, 2000; C. Jiang, Jiang, Ip, Ganther, & Lu, 1999); and 4) reverses cellular resistance to cytotoxic drugs (Caffrey & Frenkel, 1998, 2000a, 2000b; Frenkel & Caffrey, 2001).

1.7.1 DNA repair

The human body contains approximately 10^{13} cells that are exposed to an inordinate number of DNA lesions per day (Jackson & Bartek, 2009). This represents a dynamic equilibrium where cells have to maintain their nucleotide sequence in the face of processes which generate such lesions. There are three main influences on the magnitude and diversity of lesions. First, the physicochemical properties of DNA do not guarantee permanent stability, as some bonds can spontaneously break under physiological conditions. Spontaneous hydrolytic or deamination reactions can occur leaving non-instructive abasic sites or miscoding bases respectively (Hoeijmakers, 2001). Second, the genesis of lesions can be due to normal physiological processes such as DNA mismatches introduced through DNA replication, or via the action of reactive oxygen species (ROS) generated through aerobic metabolism. Finally, DNA lesions can also be introduced through external environmental factors. The most pervasive external source of DNA damage is ultraviolet light, in which residual rays of ultraviolet A and B can induce 100,000 lesions per cell per hour (Jackson & Bartek, 2009). Other prevalent environmental DNA damaging agents are ionizing radiation and environmental genotoxins. The most prevalent environmental cancer-causing agent is, of course, tobacco smoke, which is known to cause various cancers, in particular those of the lung, oral cavity and adjacent tissues.

Many DNA lesions can block genome replication and transcription that in turn can lead to mutations or genome-wide DNA aberrations if they are not repaired correctly. Due to there being a multitude of different DNA lesions induced by various factors, cells have evolved multiple DNA-repair mechanisms – known collectively as the DNA-damage response (DDR) – in order to detect and repair

DNA lesions. Deficiency in the DDR pathway is a known causative factor in a number of human diseases, including some cancers.

There are a number of DDR pathways operating in mammals that respond to different lesions but generally follow a common program of response as illustrated in Figure 2. While there are some lesions that can corrected through direct protein-mediated reversal, most DNA lesions require the synchrony of multiple and sophisticated protein interactions and catalytic events.

It is evident that the wide diversity of DNA lesions had lead to the evolution of a diverse repertoire of DDR pathways (Table 1), however only the nucleotide excision repair (NER) pathway will be discussed in further detail.
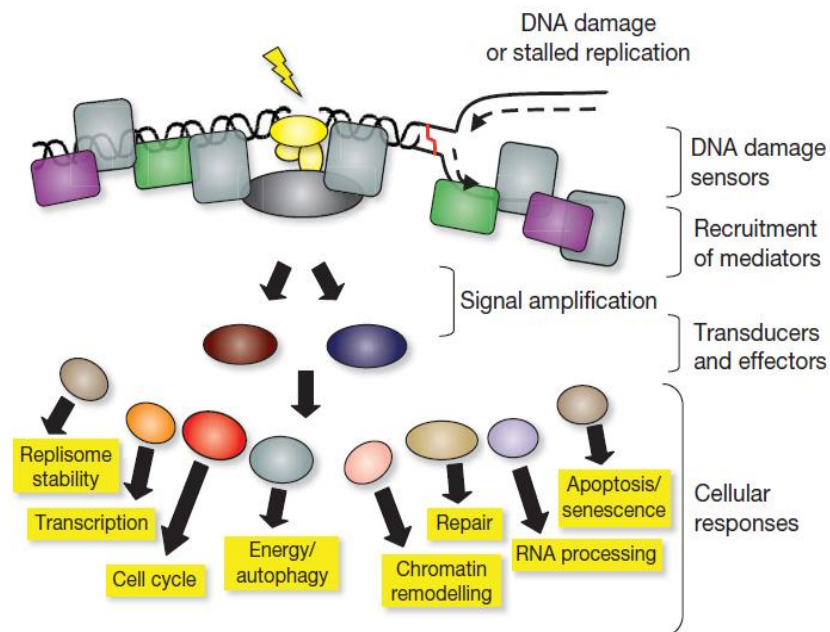
Figure 2: Model for the DNA-damage response. The presence of DNA lesions are recognised by various sensors that initiate signalling pathways (adapted from Jackson & Bartek 2009)

Table 1: DNA-damage response pathways (adapted from Jackson & Bartek 2009)

Pathway	Type of damage	Damaging agents/causes
Nucleotide-excision repair (NER)	CPD; (6-4) photoproducts	UV; cisplatin; 4-nitroquinoline oxide; and other oxidative damages
Base-excision repair (BER)	Single-base DNA damage (short-patch BER); single-strand break (long-patch BER)	Oxidizing, methylating, alkylating agents; ionizing radiation
Mismatch repair (MMR)	Mispaired nucleotides; insertion/deletion loops	Slippage of polymerase during replication
Non-homologous end-joining (NHEJ)	Double-strand break	Ionizing radiation; chemical agents such as neocarsinostatin
Homologous recombination (HR)	Double-strand break	Ionizing radiation; chemical agents such as neocarsinostatin

NER is perhaps the most versatile pathway of the DDR, recognizing a wide class of helix-distorting lesions that interfere with DNA replication and transcription. There are three main syndromes that are known to be caused by inborn mutations of NER factors: xeroderma pigmentosum, Cockayne syndrome, and trichothriodystrophy which are all characterized by sun sensitivity. NER is responsible for removing bulky lesions such as the 6-4 photoproducts caused by UV radiation which results in NER-defective xeroderma pigmentosum individuals exhibiting greater than 1000-fold increase in sun-induced cancers (Jackson & Bartek, 2009)

NER consists of two sub-pathways which differ only in their mechanism of lesion recognition: global genomic repair (GGR) which surveys the entire genome, and transcription-coupled repair (TCR) which targets transcription-blocking DNA lesions. The initial recognition step of damaged DNA in these pathways is different, but subsequent steps involve the same proteins. The tumour suppressor p53 is a tetrameric transcription factor which mediates the induction of numerous genes involved in DDR pathways. In particular p53 directly modulates

GGR by recognizing NER-type DNA lesions thereby initiating their repair. p53 modulates GGR through the expression of p48 and GADD45 which have roles in DNA lesion recognition, and chromatin assembly and repair respectively. Defects or overexpression of these factors can contribute to the sensitivity of normal and malignant cells to current therapies.

1.7.2 Cancer and DNA repair

The most prevalent non-surgical interventions for cancer are radiotherapy and chemotherapy. Both of these therapies function by generating DNA damage, thus it is essential to understand DDR pathways in order to increase efficacy of anticancer treatment.

1.7.3 Cisplatin and DNA repair

Presently, cisplatin (*cis*-diamminedichloroplatinum(II)) is one of the most widely used anticancer drugs, and displays clinical activity towards a number of tumours, including head and neck, testicular, small cell lung, and a number of other solid tumours (Jamieson & Lippard, 1999). The use of cisplatin as an anticancer drug has its origins in a serendipitous discovery by Rosenberg and colleagues in 1965 studying *Escherichia coli* cell division in response to an electrical current applied to the growth medium. It was quickly realized that the inhibition of cell division was not due to the electrical current, but rather due to the formation of tetravalent complexes from the reaction of platinum derived from the electrodes with ammonium chloride from the growth medium (Rosenberg, Vancamp, & Krigas, 1965). The cytotoxicity of cisplatin is ascribed to its interaction with nucleophilic N7 sites of purine bases in DNA. Cisplatin has the potential to form DNA-protein and DNA-DNA intrastrand and interstrand crosslinks. The current consensus view is that the cytotoxic effect of cisplatin can be largely attributed to DNA-DNA intrastrand crosslinks. This is consistent with the observation of Kelland (1993) that 1,2-intrastrand ApG and CpG crosslinks account for approximately 90% of total lesions.

Platinum-DNA adducts formed in response to platinum-containing chemotherapeutics are repaired by the NER pathway. However, this repair is very

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inefficient *in vivo* and can be attributed to the binding of high-mobility-group (HMG) proteins to cisplatin adducts, which inhibits their repair (Moggs, Szymkowski, Yamada, Karran, & Wood, 1997; Zamble, Mu, Reardon, Sancar, & Lippard, 1996) and the low level of DNA-repair proteins XPA and ERCC1-XPF in particular tissues (D. Wang & Lippard, 2005). Inhibition of repair by HMG proteins is probably due to shielding of adducts from NER damage-recognition factors. However, this shielding is incomplete, as cells with endogenous levels of HMG1 can repair G^G adducts, whereas addition of excess HMG1 completely abrogates repair (Evans, Moggs, Hwang, Egly, & Wood, 1997; Huang, Zamble, Reardon, Lippard, & Sancar, 1994). The DNA lesion recognition protein p48 is upregulated by BRAC1 in a p53-dependent manner following cisplatin treatment (D. Wang & Lippard, 2005) which may in part serve to explain why cells deficient in GGR display heightened sensitivity towards cisplatin treatment. Conversely, cells which express enhanced levels of NER factors have been shown to be resistant to cisplatin treatment (Dabholkar, Vionnet, Bostickbruton, Yu, & Reed, 1994; Q. D. Li, et al., 1998; States & Reed, 1996)

1.7.4 Se and DNA repair

The initial recognition step for GGR is controlled by p53 through its transcriptional control of the DNA damage-recognition proteins xeroderma pigmentosum complement groups C (XPC) and E (XPE). Interestingly, Se administration induces the expression of NER damage-recognition factors in a p53-dependent manner (Fischer, et al., 2007). Using two cell lines, one which contained active p53, the other containing an inactive mutant, Fischer and colleagues showed that treating cells with Se induced the expression of repair factors XPC and p48XPE only in $p53^{+/+}$ cells. Furthermore, this selective induction of NER repair factors in p53 cells provided enhanced repair rates and protection against UV radiation and cisplatin.

Redox regulation is critical for numerous proteins, including the activity of p53. SLM has been shown to modulate p53 activity independent of DNA damage through redox mechanisms (Seo, Kelley, et al., 2002). By using an assay based on reacting free sulfhydryl groups with *N*-ethylmaleimide, SLM was shown to promote the reduction of p53 cysteine residues. The authors then demonstrated

that this redox mechanism requires Ref1. Cells depleted of Ref1 with either RNAi or a dominant-negative Ref1 abrogated p53 reduction. p53 activity can be modulated by an antioxidant mechanism involving Se compounds, thereby protecting cells from DNA damage. Indeed, Seo and colleagues showed using UV radiation that SLM treatment protects from DNA damage only in cells which contain a functional copy of p53.

Smith and colleagues (2004) using a p53 reporter gene showed that SLM, methylseleninic acid (MSA), and sodium selenite all affected p53 activity through transactivation of a reporter construct. However, it would appear that different chemical forms of Se appear to have differential impacts on p53 post-translational modification. As shown previously (Seo, Kelley, et al., 2002), SLM modifies key p53 cysteine residues. MSA was shown to induce phosphorylation of one or more p53 threonine residues while having no impact on p53 serine phosphorylation. Selenite however, induced phosphorylation of serine 20, 37, and 46, residues known to modulate apoptosis (Smith, et al., 2004). These results illustrate that protection from DNA damage provided by Se treatment is dependent on the species of Se used and the p53 status of the cell. Since by definition cells without functional p53 are malignant, Se treatment may provide a unique way of differentiating normal from malignant cells, through promoting the death of malignant cells while enhancing the DNA repair capacity of non-malignant cells.

1.8 Apoptosis

Multicellular organisms require a dedicated program that removes excess or potentially dangerous cells. Programmed cell death, or apoptosis, is an essential cellular process required to maintain homeostasis. Apoptosis is characterized by many distinct morphological changes that distinguish this process from necrosis. Given that induction of apoptosis is considered an essential process in the therapeutic efficacy of anticancer therapies, it is essential to understand the molecular mechanisms that govern this pathway. A family of cysteine proteases are responsible for most of the morphological changes observed in cells undergoing apoptosis (Hengartner, 2000). These death proteases are part of a large protein family know as caspases(Hengartner, 2000). Caspases are the central

executioners of apoptosis and inhibition of effecter caspase using small pharmacological agents has been shown to inhibit apoptotic cell death (Chau, Cheng, Kerr, & Hardwick, 2000). There are two main apoptotic pathways; the extrinsic death-receptor pathway, and the intrinsic mitochondrial pathway. There is however, a third pathway, involving the endoplasmic reticulum (ER) that will be discussed later. Caspase-8 is the key initiator of the extrinsic death-receptor pathway, and caspase-9 initiates the intrinsic mitochondrial pathway. Interestingly, Se treatment has been shown to activate apoptosis through caspase-8 activation.

1.8.1 Se and caspase-8

Previous studies have shown Se treatment induces apoptosis in prostate cancer cells through capase-8 activation (Jiang *et al*. 2001). Li et al. (2007) showed enhanced apoptosis in MCF-7 breast cancer cells in response to Se and doxorubicin treatment. The authors were able to show that this synergistic effect was caused by indirect activation of both intrinsic and extrinsic pathways. In another study Maiko et al. (2010), using four cell lines and various Se compounds, demonstrated the presence of high levels of caspase-8 during apoptosis. Finally, Li et al. (2008) showed that Se-enhanced apoptosis of breast cancer cells involved caspase-8 activation. It should be noted however, that the authors demonstrated that capsase-8 activation followed caspase-9 activation, indicating Se-induced activation of caspase-8 may require cross-talk between intrinsic and extrinsic apoptotic pathways. These results indicate that the Se-induced apoptosis is, in part, reliant on the activation of caspase-8.

1.8.2 Cell cycle and apoptosis

Advancements in our basic understanding of cell cycle physiology and apoptosis have lead to an increased understanding of the effects of chemotherapy and radiation on normal and malignant cells. The mechanism by which cells divide is known as the cell cycle and is characterized by four distinct phases (G_1, S, G_2, M). It is apparent that sensitivity towards various chemotherapy drugs depends on the cell cycle. Apoptosis (discussed in section 1.8) and the cell cycle are intimately

related as cells that undergo growth arrest may be resistant to cytotoxic agents (Shah & Schwartz, 2001).

Se compounds are well known to inhibit cell cycle progression, and induce apoptosis in malignant cell lines (Jung, et al., 2001; Z. S. Wang, Jiang, & Lu, 2002; Zeng, 2002). The active moiety of the observed effects of Se compounds is considered to be methylselenol (Evans, et al., 1997; Ip, Dong, & Ganther, 2002), and precursors that directly generate methylselenol have been shown to be very effective in tumour inhibition (Ip, Thompson, Zhu, & Ganther, 2000). MSA is useful in cell culture studies since it does not require a beta-lyase reaction to release the active methylselenol moiety, instead, MSA directly provides methylselenol through non-enzymatic reduction (Ip, et al., 2000).

1.9 ER stress

Among the myriad of cellular processes required to maintain cellular homeostasis it is essential that all newly-synthesized proteins fold correctly and localize to the correct cellular compartments. In eukaryotic cells the ER is the organelle responsible for folding, export, secretion, and initial post-translational modifications of newly-synthesized proteins. Nascent proteins enter into the ER lumen through the translocon as unfolded polypeptide chains where they are directed down productive folding pathways by ER-resident chaperones. There are several factors involved in the efficient folding of proteins in the ER, and the ER is very sensitive to any factors which perturb normal protein folding, which may result in the accumulation of naive or aberrantly-folded proteins.

The demand on the folding capacity of the ER will vary amongst different cell types and through the cellular life cycle in response to extracellular stimuli or cell cycle progression. The folding capacity of the ER can be overwhelmed transiently as extracellular stimuli alter a cell's expression patterns, or more permanently due to genetic mutations preventing the correct folding of their respective proteins (Bernales, Papa, & Walter, 2006). Perturbations in protein folding can also result from alterations in ER homeostasis in response to redox reactions, nutrient deprivation, hypoxia, alterations in calcium balance, viral infection, and heat-

induced protein denaturation. This is known as ER stress, a state which is toxic to cells, thus cells have evolved various protective strategies in order to cope with and counteract the deleterious effects of ER stress. These various protective strategies are collectively amalgamated as an intracellular ER-to-nucleus signal transduction pathway termed the unfolded protein response (UPR).

This complex signalling pathway is induced in order to help cells cope with ER stress, though if large accumulations of aberrantly-folded proteins persist degradative pathways will be induced, causing cells to undergo apoptosis. The pro-survival effects of the UPR are mediated through three main mechanisms (i) inhibition of eukaryotic initiation factor 2α (eIF2α) or cap-dependent protein translation, (ii) enhanced translation of ER chaperones to enhance ER folding capacity, and (iii) ER-associated degradation which degrades misfolded proteins within the ER.

The UPR is governed through three transmembrane receptors: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). All three transmembrane receptors are maintained in an inactivated state through the association of their N-terminal domains with the ER chaperone GRP78, which is considered the gatekeeper to activation of all three branches of the UPR. Free GRP78 is present at low levels however, upon accumulation of unfolded proteins in the ER, GRP78 dissociates from the three receptors in order to bind aberrantly-folded proteins, thus leading to their activation (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000). The UPR pathway is illustrated in Figure 3.
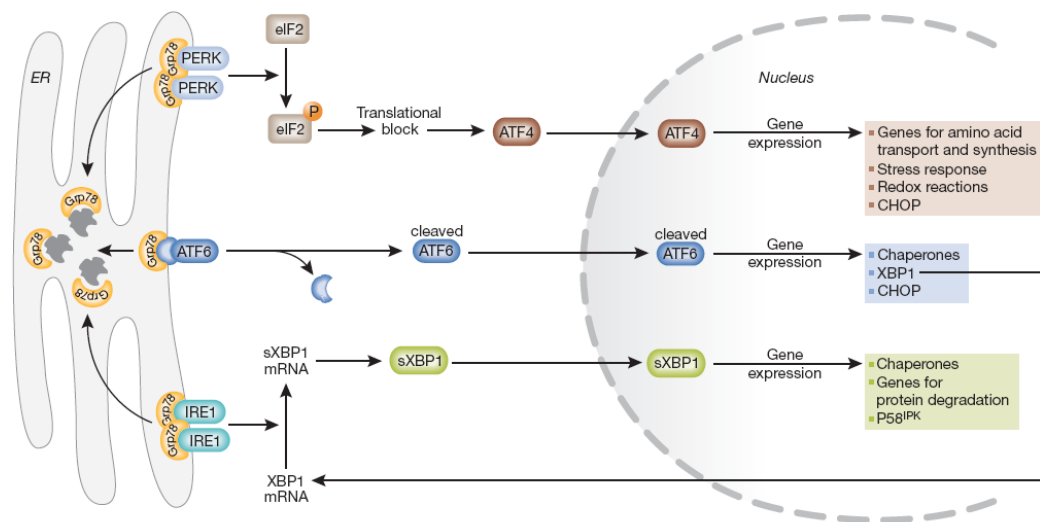
Figure 3: Unfolded protein response. On aggregation of unfolded proteins in the ER lumen GRP78 dissociates from the three transmembrane receptors (IRE1, ATF6 and PERK), resulting in their activation (from Szegezdi *et al.*, 2006).

1.9.1 Survival signalling pathways

1.9.1.1 PERK

Dissociation of GRP78 allows the ER-localized type 1 membrane protein PERK to oligomerize, resulting in its activation through autophosphorylation of the serine/threonine kinase domains (Bernales, et al., 2006). Active PERK phosphorylates Ser 51 of eIF2α causing global inhibition of eIF2α and cap-dependent translation by inhibiting the guanine nucleotide exchange factor eIF2β which recycles eIF2 to its active GTP-bound form (Krishnamoorthy, Pavitt, Zhang, Dever, & Hinnebusch, 2001). Inactivation of eIF2α lessens the burden of naïve and aberrantly-folded proteins in the ER and causes a rapid decrease in the concentration of cyclin D1 resulting in G1 cell cycle arrest (Brewer & Diehl, 2000; Brewer, Hendershot, Sherr, & Diehl, 1999; Niwa & Walter, 2000). The importance of temporal translation inhibition for cell survival has been demonstrated in PERK^{-/-} cells. Phosphorylation of eIF2α and reduced protein synthesis in response to ER stress is lost in Perk^{-/-} cells (Harding, Zhang, Bertolotti, Zeng, & Ron, 2000; Koumenis, et al., 2002) and mutant cells are more susceptible to cell death in response to ER stress-inducing agents. Furthermore,

cell death due to ER stress in PERK-deficient cells was reduced when protein synthesis was inhibited by cyclohexamide (Harding, et al., 2000).

Transcription of UPR target genes is also activated in response to the PERK-mediated survival pathway. Translational inhibition induced by phosphorylation of eIF2α is not universal to all transcripts, and can be bypassed if mRNA transcripts contain certain regulatory sequences in their 5' untranslated region (UTR), such as an internal ribosomal entry site (IRES). In this way expression of the UPR transcription factor ATF4 is induced by PERK activation (Harding, et al., 2000; Scheuner, et al., 2001). ATF4 is a cAMP response element-binding transcription factor (C/EBP) that promotes cell survival by inducing genes involved in redox reactions, amino-acid metabolism, stress response, and protein secretion (Harding, et al., 2003). However not all proteins induced by ATF4 are pro-survival; the expression of the transcription factor C/EBP induces homologous protein/growth arrest DNA damage (CHOP/GADD153), which promotes apoptosis, is highly dependent on ATF4 and will be detailed later.

1.9.1.2 **ATF6**

Transcriptional induction of pro-survival genes is mediated by the *cis*-acting ER stress response element (ERSE) in their promoter, with the consensus sequence CCAAT-N9-CCACG9 (Yoshida, Haze, Yanagi, Yura, & Mori, 1998). Two mammalian proteins, XBP1 and ATF6, have been identified as ERSE-binding proteins. ATF6 is a basic leucine zipper protein constitutively expressed as a type II transmembrane protein in the ER. In response to ER stress, ATF6 translocates from the ER membrane to the Golgi apparatus upon GRP78 dissociation. Upon arrival in the Golgi apparatus ATF6 is processed into its active form by site 1 (S1P) and site 2 (S2P) proteases. S1P and S2P cleavage of ATF6 liberates the N-terminal cytoplasmic domain - which contains a bZIP domain - after which the activated transcription factor translocates to the nucleus and, in conjunction with the nuclear factor Y transcription factors, induces genes with ERSE containing promoters. ATF6 has been shown to induce ER-resident chaperones such as GRP78 (Hong, Li, Mao, & Lee, 2004), GRP94 (Yoshida, et al., 1998), and protein disulfide isomerases (Szegezdi, Logue, Gorman, & Samali, 2006) thereby preventing the aggregation of aberrantly-folded proteins and enhancing the folding capacity of the ER. Other identified targets to date include the

transcription factors CHOP and X box-binding protein 1 (XBP1). Although CHOP expression is induced there are no reports of ATF6-induced apoptosis (Szegezdi, et al., 2006)

1.9.1.3 **IRE1**

IRE1 is a type 1 transmembrane protein which possesses a serine–threonine kinase domain and an endoribonuclease domain (Sidrauski & Walter, 1997). On activation, IRE1 oligomerizes in the ER membrane allowing the juxtaposed kinase domains to autophosphorylate. Activation of IRE1 may be caused by either binding of naïve or aberrantly-folded proteins to the IRE1 lumenal domain, or the release of repressing chaperones, or both (Ron & Hubbard, 2008). Subsequently, the *trans*-autophosphorylation of the IRE1 kinase domain activates the endonuclease activity of IRE1. IRE1 is then free to participate in an unconventional cytosolic mRNA-splicing reaction, removing a 26-nucleotide intron from the XBP1 mRNA induced previously by ATF6. The XBP1 mRNA encodes a nonfunctional protein which is activated by this unconventional splicing event, resulting in a frameshift, thus generating a mature XBP1 protein with a potent transactivation domain (Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). This spliced variant is a potent activator of ER chaperones and HSP40 family member P58^{IPK} (A. H. Lee, Iwakoshi, & Glimcher, 2003), which also has the ERSE.

The activation of P58^{IPK} is not an immediate event but occurs somewhat after the initial ER stress. P58^{IPK} expression represents a negative feedback loop in the UPR as P58^{IPK} binds and inhibits PERK, thus relieving the translational block. P58^{IPK} induction therefore represents the end of the UPR where, if the UPR has been successful, cells will return to normal function or, if unsuccessful, allow the synthesis of proteins involved in apoptotic pathways. However, the pro-apoptotic nature of P58^{IPK} has not been confirmed as P58$^{IPK\ -/-}$ mice exhibited increased pancreatic apoptosis leading to diabetes (Ladiges, et al., 2005). This is most likely due to prolonged PERK inactivation leading to the depletion of essential proteins that are eIF2α- and cap-dependent.

1.9.2 Apoptosis Signalling Pathways

Prolonged ER stress can trigger pro-apoptotic signalling pathways mediated by all three UPR transmembrane receptors. Cell death is not initiated directly by these receptors, but rather executed by downstream effectors induced by the UPR signalling cascades. Induction of CHOP was initially identified in response to DNA damage, but subsequently shown to also mediate ER stress-induced apoptosis, the latter probably being a more potent activator of CHOP induction (Oyadomari & Mori, 2004; Zinszner, et al., 1998). The role of CHOP in ER stress-induced apoptosis has been demonstrated in CHOP [-/-] mouse embryonic fibroblasts which show an increased resistance to ER stress-induced apoptosis (Zinszner, et al., 1998). CHOP expression may be induced by all three branches of the UPR, but PERK mediated induction of ATF4 is essential. Heterodimerization of ATF4 and CCAAT/enhancer binding protein β (C/EBP-β) activates the expression of CHOP (Fawcett, Martindale, Guyton, Hai, & Holbrook, 1999) resulting in the regulation of various genes involved in cell death.

Activated IRE1 is also involved in ER stress-induced apoptosis. Overexpression of IRE1 in HEK293T cells was demonstrated to induce apoptosis (X. Z. Wang, et al., 1998). IRE1 is thought to promote apoptosis through the activation of the c-Jun N-terminal kinase (JNK) pathway (Szegezdi, et al., 2006). ER stress-induced JNK activation, which regulates apoptotic machinery through BCL2 proteins (Davis, 2000), has been shown to be IRE1- and TNF-receptor-associated factor 2 (TRAF2)-dependent. IRE1 further potentiates apoptosis through association with the adaptor TRAF2. This complex recruits apoptosis signal-regulating kinase (ASK1). Neurons from *Ask1*^{-/-} mice have been shown to be resistant to ER stress-induced apoptosis, while overexpression of ASK1 was shown to enhance apoptosis in various cell culture models (Hatai, et al., 2000; Nishitoh, et al., 2002).

1.9.3 Selenium and ER stress

Se compounds have been shown to induce ER stress through the induction of UPR proteins. *In vitro* investigations in PC-3 human prostate cancer showed that low doses of MSA preferentially activated the pro-survival branch of the UPR (Wu, et al., 2005). Using three signature ER stress markers (phospho-PERK,

phospho-eIF2α, and GRP78) Wu and colleagues showed that MSA induced these targets in a time- and dose-dependent manner. Prolonged exposure, as well as high concentrations of MSA induced an apoptotic response through the induction of CHOP and cleaved caspase 12 (Wu, et al., 2005).

1.10   Angiogenesis

Angiogenesis is defined as the formation of new blood vessels from existing vasculature. Although angiogenesis is fundamental in homeostasis through tissue repair, embryonic development and fertility (Carmeliet, 2005), it is also well known to be involved in promoting tumour growth and metastasis (Avraamides, Garmy-Susini, & Varner, 2008; Carmeliet, 2005). The family of transcription factors known as hypoxia-inducible factors (HIF) are essential for regulating cellular pathways involved in angiogenesis (Giaccia, Siim, & Johnson, 2003), promoting the transcription of genes such as vascular endothelial growth factor (VEGF) under low oxygen conditions.

At the onset of tumour growth expanding lesions are able to uptake nutrients and expel waste through diffusion. However, if these micro-tumours are to grow beyond approximately $3mm^3$, diffusion alone is not sufficient to support growth. For lesions to grow beyond the size limit imposed by diffusion, tumour growth is fully reliant on the ability to promote new blood vessel formation. Therefore, there have been intensive efforts to develop strategies to inhibit angiogenesis in cancer. Agents which inhibit angiogenesis are of clinical importance and interest and have been used successfully against particular types of cancers in various clinical trials (Carmeliet, 2005).

1.10.1 HIF-1 complex

The regulatory subunits of HIF which are involved in the hypoxia response pathway are HIF-1α and HIF-2α. Under normoxic conditions the HIF-1α and HIF-2α subunits are short-lived due to prolyl hydroxylase (PHD) proteins mediating their degradation. HIF-1α and HIF-2α subunits interact with the von Hippel-Lindau tumour suppressor protein (VHL) under normoxic conditions, in which

VHL targets HIF-1α and HIF-2α subunits for proteosomal degradation. This interaction though relies on the hydroxylation of critical HIF-1 proline residues by PHD enzymes (J. W. Lee, Bae, Jeong, Kim, & Kim, 2004). HIF-1α and HIF-2α can form heterodimers with HIF-1β (G. L. Wang, Jiang, Rue, & Semenza, 1995), inducing the transcription of genes which contain hypoxia-response elements in their promoters (Benest & Augustin, 2009).

To date, VEGF-A and its receptors are the most well characterized signalling pathway in angiogenesis (Carmeliet, 2005). VEGF-A implements its biological effects by binding one of two receptor tyrosine kinases, VEGFR-1, and VEGFR-2 (which is known to directly mediate the angiogenic signals of VEGF-A). The role of VEGFR-1 in mediating VEGF-A signals is more complex. VEGFR-1 may function as an anti-angiogenic receptor through sequestering VEGF-A, and thus preventing VEGF-A binding to VEGFR-2 (Fong, Rossant, Gertsenstein, & Breitman, 1995). However, other studies have demonstrated the importance of VEGFR-1 in haematopoiesis and recruitment of bone marrow-derived cells such as monocytes (Barleon, et al., 1996; Huusko, et al., 2010; Kearney, Kappas, Ellerstrom, DiPaola, & Bautch, 2004; Kerber, et al., 2008)

1.10.2 Selenium and angiogenesis

Importantly, Se compounds have been shown by various researchers to inhibit angiogenesis. *In vitro* experimentation using human DU 145 prostate cancer cells revealed that cellular and secreted VEGF levels were decreased in response to MSA in a dose-dependent manner (C. Jiang, et al., 2000).

In vivo research has shown that Se compounds were able to reduce microvessel density in mammary carcinomas in rats injected with methylnitrosourea to initiate mammary carcinogenesis (C. Jiang, et al., 1999) indicating that Se may inhibit neovascularisation. Using Western blot analysis the same workers demonstrated that VEGF expression in some, but not all, mammary carcinomas exhibited a marked decrease in (C. Jiang, et al., 2000). Reduction in microvessel density in response to Se supplementation has also been observed in nude mice bearing MCF-7 breast cancer xenografts (Z. Li, et al., 2009).

Vasculature in solid tumours is characterized by its disordered arrangement as a result of excessive expression of angiogenic factors (Folkman, 1995). Interestingly Se supplementation has been shown to increase vessel maturation in (Bhattacharya, et al., 2008) resulting in improved tumour perfusion and thus improved tumour concentrations of irinotecan, and its active metabolite SN-38, (Yin, et al., 2006) as well as doxorubicin and capecitabine (Bhattacharya, et al., 2009). The observed antiangiogenic effects of Se were demonstrated to be a result of VEGF depression mediated through inhibition of COX-2, HIF-1α and i-NOS due to stabilization of PHD enzymes (Y Rustum, unpublished results), which are typically inhibited under hypoxic conditions. These observations in part serve to explain the improved tumour response rates observed in these studies.

1.11   Drug resistance

Resistance to anticancer therapies can arise from a number of factors, either intrinsic, or acquired by malignant cells during treatment. The major mechanism responsible for acquired drug resistance to a broad variety of CT agents is the expression of multi-drug transporters (Gottesman, 2002). This is an energy-dependent mechanism that detects the presence of, and enhances the ejection of, drugs out of the cell (Gottesman, 2002). But other mechanisms that involve insensitivity towards drug-induced apoptosis, and cell-detoxifying mechanisms, also play a pivotal role in acquired drug resistance.

Endogenous nucleophiles in the cytoplasm such as glutathione (GSH) have been extensively investigated for their role in the development of resistance to anticancer treatments (Biaglow, et al., 1989; Bump & Brown, 1990; Coleman, Bump, & Kramer, 1988; Mitchell & Russo, 1987; Tew, 1994). In ovarian cancer xenograft-bearing mice, Caffrey & Frenkel (2000a) demonstrated that pretreatment with SLM or selenite prevented an increase in intracellular glutathione levels in response to cisplatin or melphalan. Using the same model the authors demonstrated that this strategy could prevent resistance developing to cisplatin, carboplatin or melphalan (Caffrey & Frenkel, 1998, 2000a, 2000b; Frenkel & Caffrey, 2001). UPR stress modulators have also been linked to CT resistance. Enhanced levels of GRP78 have been demonstrated to be involved

with resistance to various types of anticancer therapies (J. Li & Lee, 2006), whereas, reduction in GRP78 levels has been shown to increase the sensitivity of malignant cells to treatment (Rahmani, et al., ; Y. Wang, et al., 2008).

1.12   Objectives of this thesis

The overall objective of this thesis was to conduct an analysis of the biological characteristics of the differential impact of Se on malignant (THP-1 cells) and non-malignant cells (PBMC) in humans, with and without CT and RT. This investigation aimed to provide insights into the biological markers that may be informative in clinical trials using Se compounds to modulate the efficacy and toxicity of CT and/or RT.

In order to investigate this, the biological effects of Se, cisplatin and RT were evaluated *in vitro* in cell culture models, to provide a framework of which markers to investigate clinically. The information gathered from the *in vitro* studies was used in part to evaluate the pharmacodynamic studies in humans undergoing anticancer therapy with or without Se.

Specific objectives are listed in each chapter.

Chapter 2

General methods

2.1 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from buffy coats obtained from blood donations given by healthy individuals, and supplied by the NZ Blood branch at Waikato Hospital. In order to isolate the mononuclear cell fraction via density gradient centrifugation, buffy coats were diluted 1:10 with sterile phosphate buffer saline (PBS) pH 7.4. Subsequently, 3ml of the diluted buffy coat suspension was layered over an equal volume of Histopaque $^®$ (Sigma-Aldrich) in a 15 ml falcon tube (CELLSTAR$^®$). The tube(s) were placed in a Heraeus multifuge 1 S-R centrifuge and spun at 450 x g for 30 min to separate out the component of the buffy coat. Using a sterile transfer pipette the top layer was removed to just above the interphase which contained the PBMC. Using a new sterile pipette the interphase was removed and placed in a new 15 ml falcon tube. 10 ml of sterile PBS pH 7.4 was then gently added and gently mixed with the PBMC. The tube(s) were then placed back in the Heraeus multifuge and centrifuged for 10 minutes at 250 x g in order to wash the cells of contaminating platelets. The solution was carefully tipped off taking care not to disturb the pellet. The pellet was then resuspended in 5ml of sterile PBS pH 7.4 and centrifuged as before. After a total of 3 washes the pellet was resuspended in 2 ml of RPMI-1640 culture media supplemented with 10% foetal calf serum, 1% penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml).

2.2 Measurement of cell concentration

Cell concentration was measured using an Axiostar plus microscope and a haemocytometer. The haemocytometer was cleaned with 70% ethanol and allowed to dry. The shoulders of the haemocytometer were moistened and the cover slip affixed gently using small circular motions. The cover slip was considered properly affixed if Newton's rings could be observed; this ensured the correct depths of the chamber. A 100 µl aliquot of the cell suspension in the culture media was transferred to a 1.7 ml Eppendorf tube and diluted 1:10 with sterile PBS pH 7.4. A 100 µl aliquot of this diluted suspension was then transferred to a new 1.7 ml tube and mixed gently with 100 µl of 0.4% (w/v) trypan blue solution and allowed to sit for 2 minutes. The cell suspension was gently mixed again, then using a pipette an aliquot of the cell suspension containing the trypan blue was drawn up and used to fill up the haemocytometer by resting the tip at the edge of the chambers and allowing capillary action to draw in the sample. The pipette was re-filled and the procedure performed again with the second chamber.

The dimensions of the haemocytometer are as follows:

Dimensions	Area	Volume at 0.1 mm depth
1 x 1 mm	1 mm^2	100 nl
0.25 x 0.25 mm	0.0625 mm^2	6.25 nl
0.25 x 0.20 mm	0.05 mm^2	5 nl
0.20 x 0.20 mm	0.04 mm^2	4 nl
0.05 x 0.05 mm	0.0025 mm^2	0.25 nl

Using an Axiostar Plus light microscope the gridlines of the set of squares with a volume of 6.25 nl were focused upon using a 10X objective lens. These squares correspond to a set of 16 corner squares which measure a total volume of 100 nl when all are counted. In both chambers the 4 corners were used giving a total of 128 6.25 nl squares or a volume of 800 nl. Using a tally counter, cells which were positioned within squares or on the bottom and right hand boundaries, and were

not stained blue by trypan blue, were counted. Cells which were stained were counted separately for viability. The number of cells in the 16 squares is equivalent to the number of cells x 10^4/ml. Therefore, to obtain a count of cells, the total number of cells in 8 corner squares was calculated according to the following equation:

$$\left(\frac{\text{Total cells counted}}{4} \right) \quad X\ 10 \quad = \text{cells/ml x } 10^4$$

This accounts for both dilution steps employed when preparing the sample to be counted.

2.3 Trypan blue cell viability

Trypan blue is used as a measure of cell viability. If cells have lost their membrane integrity trypan blue can permeate the cell causing the cytoplasm to be stained dark blue. These cells are assumed to be unviable and considered dead. Cells that were faintly or strongly stained blue were counted as dead cells and were counted in the same method as live cells. To calculate the viability of cells the following equation was used:

$$\left(\frac{\text{Unstained cells}}{\text{Total cells}} \right) \quad X\ 100 \quad = \text{percentage viability}$$

2.4 Media preparation and PBMC culture conditions

RPMI-1640 Medium was prepared as described by the manufacturers. All media was filter-sterilized with 0.22 μm filters. Foetal bovine serum was heat-inactivated by incubation at 56 °C for 30 minutes. After isolation, PBMC were diluted to an appropriate density for particular experiments and cultured at 37 °C in 5% CO_2 in sterile greiner culture plates.

2.5 THP-1 culture

Cells were maintained in the aforementioned RPMI-1640 culture media supplemented with 10% foetal calf serum, 1% penicillin (10,000 units/ml) and streptomycin (10,000 µg/ml). The passage number of the cells since their original isolation was unknown, however, for experiments carried out, only cells passaged no more than 10 times were used in experiments. Cells were maintained at 37°C in a greiner 40 ml airtight culture flask. As recommended by the website, cells were maintained at a density below the 9×10^6 cells/ml, which equated to sub-culturing every two or three days. Cells were passaged by adding approximately six ml of fresh media (at 37°C) to approximately one ml of cell suspension. For experimentation, cells were aliquoted at appropriate densities into sterile greiner culture plates and cultured at 37 °C in 5% CO_2.

2.6 Comet assay

The single cell gel electrophoresis assay, otherwise known as the comet assay because of the appearance of a "head" with a "tail" of DNA fragments, was first described by Ostling and Johanson in 1984 and later adapted by Singh *et al* (1988) to include an alkaline step, which is the method used here. Preparation of fresh blood slides was carried out by topping a pre-agarose-coated slide (1% normal melting point in MQ water) prepared the day before, with a suspension of 5 µl of blood in 75 µl of 0.5% low melting point agarose in PBS pH 7.4. A 20 mm x 40 mm coverslip was placed gently on top, and slides are kept at 4°C for 10 minutes to allow the gel to solidify. After gels were set, the coverslips were gently removed.

The gels were then lysed at 4°C in a solution containing 1% Triton X-100, 10% DMSO, 2.5 M NaCl, 100 mM EDTA, and 10 mM Tris (pH 10.0) for four hours. The resulting "nucleoid" was subsequently denatured for 20 min in an alkaline buffer [300mM NaOH and 1mM EDTA (pH > 13)] and electrophoresed using an Owl separation systems gel rig powered by Lightning Volt™ OSP-250L power supply for 20 min at 25 V and 300 mA in the same buffer.

After electrophoresis, slides were rinsed with distilled water before staining with SYBR Gold. 300 µl of SYBR Gold (1/10,000) was pipetted directly onto the slides and incubated for 10 minutes. The slides were then rinsed with distilled water to remove excess stain. Comets were examined by using Axiostar plus microscope, scoring 50 comet images from each slide and assessing DNA damage via the five classes of comets (0-4) described by Collins (2004). Slides were prepared in duplicate giving a total of 100 comets per sample (total score range of 0-400).

2.7 ER stress response markers

Quantification of intracellular proteins that are markers of the ER stress response pathway (GRP78, phosphorylated elF2α, CHOP, spliced XBP1 and caspase-8) required a number of steps: firstly, protein isolation from PBMC derived from buffy coats or trial patient blood samples, or from cultured THP-1 cells; secondly, protein quantification from the cell lysates; thirdly, separation of protein lysates by gel electrophoresis; and fourthly, western blotting for the proteins of interest. Each of these steps is described below.

2.7.1 Protein isolation from cell culture

Total cell protein was isolated by lysing cells using RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, phosphatase inhibitors (PhosSTOP purchased from Roche, catalogue number: 04 906 845 001), protease cocktail inhibitors (Purchased from Roche, catalogue number: 11 836 170 001), and 1 mM phenylmethanesulfonylfluoride (added just prior to lysis)), followed by centrifugation to remove cellular debris. Cells from each experiment were transferred from culture plates into 1.7ml tubes and sedimented with an Eppendorf centrifuge (5415 R) at 4 ° C for 10 minutes at 1600rpm. The supernatant was pipetted off using a transfer pipette and cells were washed by resuspended the cell pellet in sterile PBS pH 7.4. This procedure was repeated to ensure cells were properly washed. The cells were pelleted once more and resuspended in 150ul of RIPA buffer and vortexed for approximately 5 seconds before being put on ice. Samples were allowed to lyse in ice for 30

minutes. Following lysis, lysates were centrifuged at 13,200 rpm for 10 minutes at 4˚C. Finally, the supernatants were transferred to a new 1.7ml tube and stored at – 80˚C until further analysis.

2.7.2 Protein quantification from cell lysates

Protein concentration was estimated using a modification of the Biuret assay. The bicinchoninic acid (BCA) assay was purchased from Thermo Scientific Pierce (catalogue number 23227) and stored at room temperature. This method utilizes the reduction of Cu^{2+} ions to Cu^{1+} by protein in an alkaline medium. Cu^{1+} ions are detected by the BCA reagent, forming a purple-coloured reaction that is spectrophotometrically detected at 562 nm. Standards containing bovine serum albumin were prepared at various concentrations in RIPA buffer and stored at -20 ℃ until required. The BCA working reagent was prepared by combining 5 ml of BCA reagent A (Product number 23227) with 100 µl of BCA reagent B (4% cupric sulfate). 25 µl of standards and unknown samples were aliquoted into 0.6 ml tubes and 200 µl of the working reagent added to each tube. Tubes were then incubated at 37 ˚C for 20 minutes before being placed on ice. The OD_{562} was measured by aliquoting 2 µl of standard, or sample, onto the pedestal of a NanoDrop ND-1000 Spectrophotometer. Standards were measured first in triplicate to prepare a standard curve. Sample values were then measured in triplicate, and protein concentrations calculated using the prepared standard curve.

2.7.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing polyacrylamide gel electrophoresis was performed for the separation of whole cell proteins lysates using a Hoefer mighty small SE250/SE260 gel rig. The procedure consists of three main stages: gel preparation, sample preparation and electrophoresis. The glass plates and spacers were cleaned with tap water, wiped dry with paper towels, and then cleaned again with 70% ethanol. Prepared plates were placed in a Hoefer five gel caster. Gels were prepared according to the appropriate composition shown in Table 2. Ammonium persulfate and TEMED (N, N,N,N',N'-tetramethyl-ethylenediamine) were not added until immediately

before casting the gel. The separating gel was prepared in a 50 ml falcon tube (CELLSTAR®) then poured immediately into the five gel caster and overlaid with a thin layer of isopropanol. The gels were allowed to polymerise before pouring off the isopropanol and carefully drying off any remaining liquid on top of the gels with filter paper (Whatman No. 1). The stacking gel was then poured and combs (10 tooth) were put in place. The stacking gels were left to polymerise with a wet paper towel covering the top in order to prevent the gels from drying out. The apparatus was then dismantled and gels were wrapped with a wet paper towel and stored in zip lock plastic bag at 4 ˚C for no longer than 4 weeks.

Table 2: Composition of stacking and separating gels

Reagent	Concentration		Volume*	
	Stacking	Separating	Stacking	Separating
37:1 Acrylamide:bis (22%)	4%	10%	1.98 ml	20.4 ml
0.5M Tris HCl pH 6.8			3.75 ml	n/a
3M Tris HCl pH 8.8			n/a	6 ml
10 % SDS (w/v)			150 μl	450 μl
TEMED			15 μl	45 μl
10 % APS (w/v)			150 μl	450 μl
H2O			9 ml	17.7 ml

*Sufficient to prepare 5 gels

When required, gels were unwrapped and clamped to a Hoefer mighty small II (SE250/SE260) gel electrophoresis unit. The comb was removed and wells washed to remove any unpolymerised acrylamide out of the wells with MQ water using a syringe with an 18 gauge hypodermic needle. The buffer chambers were filled to the appropriate levels with SDS running buffer (25 mM Tris HCl, 192 mM glycine, 0.1% SDS). The prepared samples were loaded with a 20ul pipette.

2.7.4 Western blotting

To detect the presence and expression patterns of proteins involved in ER stress responses, gels were western blotted. Western blotting consists of three main procedures: protein transfer, antibody binding, and immunodetection.

2.7.4.1 Transfer

Proteins were electrophorectically transferred with either a nitrocellulose, or polyvinylidene fluoride membrane, using a fully submerged Biorad Mini-Trans-Blot® blotting apparatus. The arrangement of the blot is illustrated in Figure 4. The membrane, and 4 pieces of 3MM filter paper were cut slightly bigger that the gel to ensure effective protein transfer. The sandwich was assembled as in Figure 4 and transferred to the Biorad Mini-Trans-Blot® tank, along with an ice container and stirrer magnet. Transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS) was added so the membrane was fully submerged and run for 1 hour at 100 V with constant stirring.

Black Perspex	
Sponge	
Filter paper x2	
Gel	
Membrane	
Filter paper x2	
Sponge	
Clear Perspex	
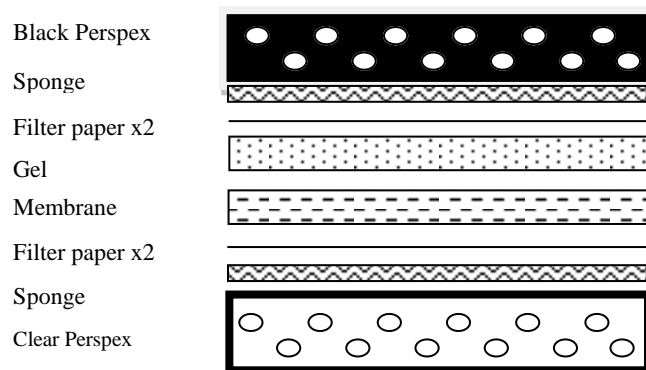
Figure 4: Arrangements of components used in western blotting

2.7.4.2 Antibody binding

After the proteins were transferred to the membrane the blotting apparatus was carefully disassembled and transfer efficiency checked by ponceau (0.2% ponceau, 1% acetic acid) staining for 5 minutes. The following procedure is summarized in Table 3. The membrane was rinsed with distilled water and transferred to a new container with approximately 20ml of BLOTTO (10% skim milk in TBS-T) to be blocked for one hour at room temperature. Once the

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membrane was blocked the membrane was transferred onto a 2 ml drop of primary antibody (appropriate dilution in BLOTTO) and incubated for one hour as illustrated in Figure 5.

Table 3: Procedure for immunoblotting

Step	Solution	Volume (ml)	Duration
Block	10% (w/v) skim milk powder in TBS-T[1] (BLOTTO)	30 ml	60 minutes
1°Ab Binding	Diluted antibody (rabbit anti-human) in BLOTTO	2 ml	60 minutes
Wash	Three changes of TBS-T	20 ml	10 minutes each
2°Ab Binding	Diluted antibody (goat anti-rabbit) in BLOTTO	2 ml	60 minutes
Wash	Three changes of TBS-T	20 ml	10 minutes each

[1] TBS-T (Tris buffered saline-Tween 20) contains 20 mM Tris HCl pH 7.6, 150 mM sodium chloride, was diluted from a 10x stock solution and and 0.01 % Tween 20 (v/v) added last.

Following the primary incubation, the membrane was washed vigorously in TBS-T pH 7.4 for 10 minutes. This was repeated three times with fresh changes of TBS-T pH 7.4. The membrane was then carefully transferred onto a 2 ml drop of secondary antibody (appropriate dilution in BLOTTO), incubated, and washed as before.
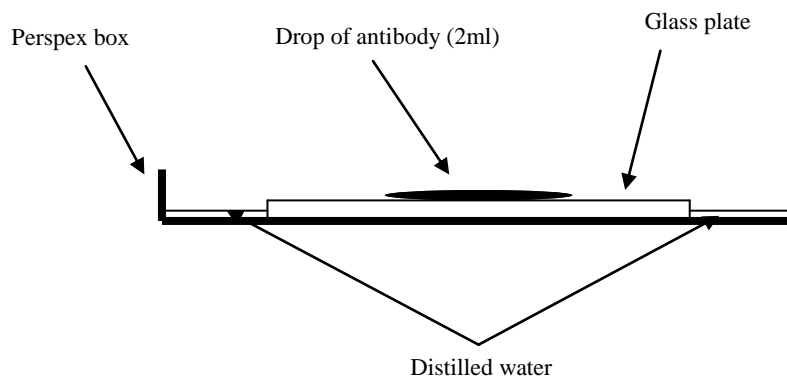
Figure 5: Antibody binding apparatus

2.7.4.3 **Immunodetection**

The secondary antibody was detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Product number 34095) purchased from ThermoScientific. SuperSignal® immunodetection was carried out according to the manufacturer's directions. The membrane was transferred protein-side up onto a glass plate. 500 µl of detection solution 1 was combined with 500 µl of detection solution 2 plus 1ml water, and poured over the membrane so that it was completely covered. The membrane was incubated at room temperature without agitation for one minute. Excess detection solution was removed using a pipette, and an OHT sheet placed over the membrane. The glass plate-membrane-OHT sandwich was exposed for an appropriate amount of time using a FUJIFILM Intelligent dark box II LAS-1000 system.

2.8   Statistical analysis

Comparison of the effects of each condition on malignant and normal cells were analysed using the student t test with Bonferroni correction for multiple analyses. Repeated measures ANOVA was used to evaluate changes of the means over time compared to baseline values in patient clinical samples. Two-tailed $p < 0.05$ was used as a threshold for statistical significance.

Chapter 3

In vitro investigations

3.1 Introduction

The dose-limiting toxicities of many current anticancer therapies treatments demonstrates the pressing importance of finding new agents that can ameliorate these toxicities and make treatments more tolerable, without compromising their effectiveness. Amifostine has been the most widely-investigated agent, but has had mixed and very limited success in clinical trials (Movsas, et al., 2005). Even more desirable would be compounds that both reduce normal tissue toxicity while enhancing the efficacy of anticancer therapies. Preclinical work has demonstrated that Se compounds act as multi-targeted modulators of the efficacy and toxicity of CT, resulting in improved tumour response rates and reduced organ-specific toxicity (S. Cao, F. A. Durrani, & Y. M. Rustum, 2004; M. Fakih, Cao, Durrani, & Rustum, 2005; Francescato, et al., 2001). Some aspects of these findings have been replicated in clinical trials with Se compounds, described in section 1.6.2, that suggest that they can both ameliorate the toxicity of CT and RT while enhancing treatment efficacy, though definitive clinical assessments of this have not yet been made.

Clinical trials, however, are expensive to run and answer a relatively limited number of questions so it is preferable to evaluate the mechanisms of action of Se using preclinical models wherever possible, as long as they are informative to the clinical situation. This approach was taken to help inform the analysis of patient samples taken in the course of the clinical trial described in Chapter 4, where patients with head and neck squamous cell carcinoma (HNSCC) were treated with a radical course of cisplatin and RT and were randomised to receive either SLM or placebo for 11 weeks, starting a week prior to treatment. Specifically, what

changes in PBMC taken at periodic intervals from these patients could be expected? Furthermore, could an appropriate *in vitro* model be established in which to replicate some of the intended analyses in PBMC as well as in malignant cells, in order to explore the differential impact of Se on malignant and normal tissues?

This chapter describes the approach undertaken to look at the effect of MSA, a Se compound that releases the active moiety methylselenol through non-enzymatic mechanisms *in vitro*, on cell viability, glutathione response, ER stress response and DNA damage and repair *in vitro*.

3.1.1 Cell viability

While the impact of Se on malignant cells can be readily studied *in vitro* with any number of malignant cell lines, it is more challenging to evaluate their effect on equivalent normal cells. A possible solution arose from the observation in 2008 that various aspects of the effects of Se on cells can be measured in human PBMC, both *in vitro* and *in vivo* (S Joel, unpublished observations). This offered the opportunity to compare the impact of Se compounds on normal and malignant cells *in vitro* by using fresh PBMC (derived from the buffy coats that are separated from red cells in blood donations) and malignant THP-1 human leukaemia cells. This in turn will help in investigating the biological impacts of high-dose Se supplementation in patients.

3.1.2 Glutathione

Cells posses a repertoire of enzymatic and non-enzymatic antioxidant defences. The various non-enzymatic defences include α-tocopherol (Vitamin E), ascorbic acid (Vitamin C), carotenoids, flavonoids and GSH, among others. Cellular redox homeostasis under normal conditions involves a balance between the intracellular levels and activities of these various antioxidants. GSH is the most abundant nonprotein cellular thiol (Arrick & Nathan, 1984). It is involved in the maintenance of the cellular redox state, removal of hydroperoxides, and the detoxification of many xenobiotics (Arrick & Nathan, 1984) It is present inside cells mainly in the reduced form (90 – 95% of total GSH). Intracellular GSH can

serve as an indicator of cellular health, and a cells ability to resist toxic challenges (Arrick & Nathan, 1984).

As discussed in section 1.11, acquired resistance against CT and RT significantly impairs the efficacy of current anticancer therapies. Increased intracellular GSH concentrations have been implicated in mediating resistance to both CT and RT.

3.1.3 Comet assay

The comet assay was used to assess the genotoxicity of MSA and cisplatin in PBMC. In addition, given that cisplatin is known to cross-link DNA strands and thus reduce RT-induced DNA fragmentation as assessed by comet assay an evaluation was made of whether cisplatin reduces the formation of a comet *in vitro* by the DNA-damaging agent H_2O_2.

3.1.4 ER stress response

ER stress is an important mechanism in cancer therapeutics, as demonstrated by the proteosome inhibitor bortezomib, which causes accumulation of aberrantly-folded proteins within the cell and induces ER stress, and thus apoptosis through induction of the unfolded protein response (UPR) (Szegezdi, et al., 2006). However, while inducing the UPR has been reported to sensitise malignant cells to cytotoxic drugs (C. C. Jiang, et al., 2007), others have reported ER stress provides resistance (Ranganathan, Zhang, Adam, & Aguirre-Ghiso, 2006). It has become clear that cell type and drug action can determine the differential response to ER stress.

Se supplementation has been shown to act through targeting mediators of the ER stress signal (Wu, et al., 2005), resulting in an increase in apoptosis. Additionally, Se appears to selectively sensitize tumour cells to a number of therapeutic drugs while at the same time increasing the resistance towards these drugs in normal cells (S. S. Cao, et al., 2004). This differential effect of Se administration on ER stress could, in part, account for the enhanced therapeutic efficacy of chemotherapy with Se.

The UPR can be activated by three different transmembrane receptors; PERK, IRE1, and ATF6. This study investigated the activation of UPR targets in response to MSA treatment in PBMC and THP-1 cells to evaluate whether MSA induces differential responses in the two cells types, i.e. pro-survival markers in PBMC and pro-apoptotic markers in THP-1 cells

3.1.5 Objectives

The overall objective of this chapter was to evaluate to evaluate whether PBMC and THP-1 cells could successfully be used as an *in vitro* model to determine differential effects of Se on normal and malignant mononuclear cells. The specific objectives were:

1. to evaluate the comparative effects of MSA on viability of PBMC and THP-1 cells and whether it differentially modulated the cytotoxicity of clinically-relevant concentrations of cisplatin or RT in these two cell populations;
2. to assess whether total intracellular GSH levels changed in response to MSA treatment in PBMC and THP-1 cells;
3. to investigate whether MSA alone or in combination with cisplatin and RT modified DNA damage and repair in these cells as assessed by the comet assay; and
4. to evaluate changes in ER stress response markers to MSA.

3.2 Methods

Methodology for ER stress response markers is described in section 2.7. Additional methodology is described below.

3.2.1 Cell viability

To study the cytotoxic effects of MSA or cisplatin on malignant and non-malignant cells, PBMC and THP-1 cells were incubated for 24 hrs in cell culture media as described in section 2.4. Cells were incubated with 0, 2.5, or 5 μM MSA

for 24 hours. In order to establish the cytotoxic effects, cell viability was measured using the tetrazolium salt MTT assay.

The differential response to cisplatin was investigated using mononuclear cells that differ in their phenotype (non-malignant vs. malignant). PBMC and THP-1 cells were incubated for 24 hours with two concentrations of cisplatin, and cell viability assessed using the MTT assay. The two concentrations of cisplatin used in this investigation were 8.33 μM, which can be considered clinically relevant (Johnsson, Bjork, Schutz, & Skarby, 1998; Johnsson, Hoglund, Grubb, & CavallinStahl, 1996), and a higher concentration of 20 μM.

When it became apparent that there was no difference in cell viability between the two cisplatin concentrations, subsequent experiments were carried out using only the lower, clinically-relevant, cisplatin concentration. In order to assess whether MSA enhanced the cytotoxicity of cisplatin, cells were pretreated with 0, 2.5 and 5 μM of MSA for 6 hours before cisplatin was added.

In order to assess whether THP-1 radiosensitivity was modulated by cisplatin and MSA, THP-1 cells were incubated for 24 hours with 8.33 μM cisplatin, and 2.5 or 5 μM MSA. Cells were irradiated with 10 Gy and viability assessed immediately.

3.2.1.1 MTT assay

Mosmann (1983) first described the conversion of a tetrazolium salt to the coloured product formazan as a measure of cell viability. The MTT (methyl-thiazol-tetrazolium) assay was used in order to assess the impact of various treatments on cell viability in THP-1 and PBMC cells. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from AppliChem (CAS-No: 298-93-1) and stored at 4 ° C. When required, it was dissolved in sterile PBS pH 7.4 at a concentration of 5 mg/ml, at room temperature. This solution was further sterilized by filtering through a 0.4 μm Whatman filter and stored in a dark bottle at 4 ° C for no longer than one month.

Cells were incubated in sterile greiner bio-one 24 well cell culture plates. Approximately 6 x 10^4 THP-1 cells were incubated in a volume of 1 ml with or without MSA or cisplatin. PBMC were incubated under the same conditions

except at a cell density of approximately 2 x 10^5. After 24 hours, 250 µl of the 5 mg/ml MTT stock solution was added to each well (final concentration 1mg/ml) and incubated for a further two hours. Following this two hour incubation, 1ml of extraction buffer (20% SDS (w/v), 50% dimethylformamide (v/v) pH 4.7) was added in order to lyse the cells, and solubilise the formazan crystals formed during the incubation with MTT. After an overnight incubation at 37 ˚C the optical densities were measured at 600 nm using a BMG Labtech FLUOstar OPTIMA plate reader, using the extraction buffer as a blank. It should be noted that the formazan production is proportional to the number of live cells.

3.2.2 Glutathione assay

The glutathione assay kit was purchased from Sigma-Aldrich (Catalog Number CS0260) and stored at 4 ˚C while not being used. The assay was carried out according to the manufacturer's directions. The assay measures total GSH by spectrophotometrically measuring the optical density of the yellow product 5-thio-2-nitrobenzoic acid (TNB) at 412 nm. TNB is formed by the reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). GSH reduces DTNB to TNB and the oxidized GSH (GSSG) formed is recycled by glutathione reductase and NADPH. Thus, using a kinetic assay, the total catalytic amounts of GSH can be measured.

PBMC and THP-1 cells were incubated with 0, 2.5 and 5 µM of MSA for six hours. Cells were then harvested and total GSH prepared as described below. Cells were cultured (see section 2.4) for six hours before being transferred to 1.7 ml Eppendorf tubes and pelleted at 1600 rpm for eight minutes. Cells were washed twice in sterile PBS, pH 7.4, before being pelleted a final time. Using a five percent 5-sulfosalicylic acid solution (SSA), a volume approximately three times that of the pellet was added into the same tube in order to deproteinize the sample. This preparation was vortexed and the suspension frozen and thawed twice (using liquid nitrogen to freeze and a 37 ºC bath to thaw). Following this, the suspension was placed at 4℃ for five minutes and then centrifuged at 10,000 x g for 10 minutes. After centrifugation the supernatant was removed with a pipette and aliquoted into a new 1.7 ml tube. The total volume of supernatant was measured for the calculation of total GSH.

Table 4: Glutathione reaction scheme

Sample measured	Sample volume	5% SSA	Working mixture	NADPH (0.16 mg/ml)
	Mix and incubate 5 minutes			Start
Reagent blank	n/a	10 μl	150 μl	50 μl
Standard curve (various concentrations)	10 μl	n/a	150 μl	50 μl
Unknown sample	X μl	10 μl	150 μl	50 μl

Using a 96-well greiner plate, the total GSH level was measured following the reaction scheme outlined in Table 4. The final concentration of the components in the reaction mixture were 95 mM potassium phosphate buffer, pH 7.0, 0.95 mM EDTA, 0.038 mg/ml (48 mM) NADPH, 0.031 mg/ml DTNB, 0.115 units/ml glutathione reductase, and 0.24% 5-sulfosalicylic acid. The BMG Labtech FLUOstar OPTIMA plate reader was set to 412 nm with kinetic read at 1 minute intervals for 5 minutes. Following the assay the values of the GSH standards were used to construct a standard curve. This was used to calculate the ΔA_{412}/min equivalent to 1 nmole of reduced glutathione per well. The following equation was then used to calculate the nmoles of GSH in the unknown sample using the following equation:

$$\text{nmoles of GSH per ml of sample} = \frac{\Delta A_{412}/\text{min (sample) x dilution}}{\Delta A_{412}/\text{min (1 nmole) x volume}}$$

where ΔA_{412}/min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction); ΔA_{412}/min (1 nmole) = slope calculated from standard curve for 1 nmole of GSH; dilution = dilution factor of original sample; volume = volume of sample in the reaction in ml.

3.2.3 Comet assay

The general methodology for the comet assay is described in section 2.6. The specific adaptation for this *in vitro* work is described here.

5×10^5 PBMC cells were incubated with 0, 2.5, or 5 µM MSA or 20 µM cisplatin for 24 hours in 1 ml of cell culture media (Chapter 2) in sterile greiner 24-well culture plates. After incubation cells were transferred to a 1.7 ml tube, and pelleted by centrifugation at 1600 rpm for 8 minutes at 4 °C. The supernatant was removed and cells were washed by resuspending them in 500 µl of sterile PBS pH 7.5. The cells were then pelleted by centrifugation at 1600 rpm for 8 minutes at 4 °C and washed again as before for a total of two washes. After the final wash step, PBMC were resuspended in 100 µl of sterile PBS pH 7.5. 10 µl of cell suspension (approximately 5×10^4 cells) was mixed with 70 µl of 0.5% low melting point agarose in PBS pH 7.4 and the preparation of slides carried out as described in section 2.6.

In order to assess whether cisplatin reduces the formation of a comet by H_2O_2 *in vitro*, PBMC conditioned as above with either media alone or cisplatin were exposed to H_2O_2 by immersing prepared slides in ice-cold 100 µM H_2O_2 for five minutes. Slides were then washed by immersing in ice-cold PBS pH 7.4 for five minutes. These steps were carried out on ice to ensure DNA repair was minimized. The comet assay methodology was then continued as described in section 2.6. Comparator slides were not exposed to H_2O_2.

3.2.4 ER stress response

In order to assess the *in vitro* effects of MSA on PBMC and THP-1 cells immunoblotting was carried out to asses protein levels of known UPR targets, as well as caspase-8. Both PBMC and THP-1 cells were plated at a cells density of 6×10^5 cells/ml in a total volume of 2 ml, in sterile greiner 6-well culture plates and incubated for six hours in the presence of 0, 2.5 and 5 µM MSA. The general methodology of protein isolation and immunoblotting is described in 2.7. Protein lysates were then probed for the pro-survival UPR proteins phospho-eIF2α and spliced XBP1, as well the pro-apoptotic UPR mediator CHOP and the pro-

apoptotic caspase-8. BLOTTO was used to block membranes and dilute antibodies in all westerns except phospho-eIF2α. Extremely high background was encountered when using BLOTTO with this antibody. Thus 3 % (w/v) bovine serum albumin was used to dilute the antibody to a working concentration, and block the membrane.

3.3 Results

3.3.1 Cell Viability

3.3.1.1 PBMC viability

Results of PBMC are generated from three separate buffy coats, each evaluated in triplicate. Figure 6 shows that PBMC viability was not significantly affected in response to the different concentrations of MSA used in this study.
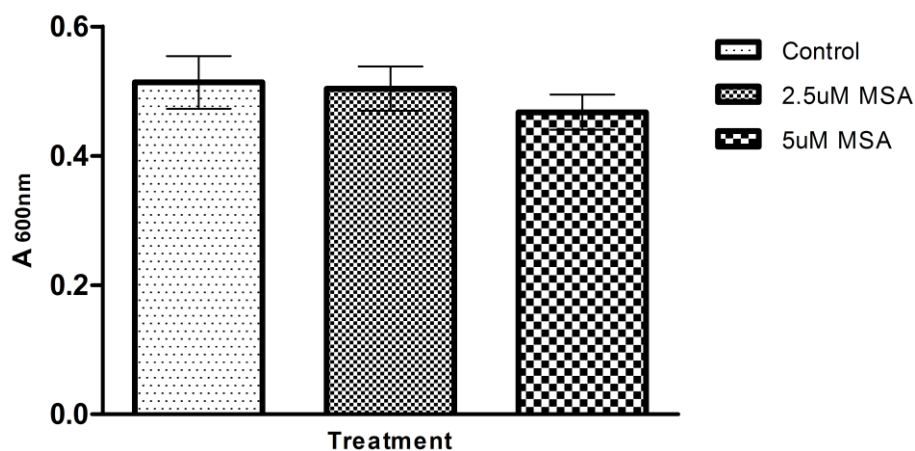
Figure 6: PBMC viability in response to 24hr MSA treatment (n=9).

Similarly, Figure 7 shows that concentrations of 8.33 or 20 µM cisplatin did not affect PBMC viability. Nor did pretreatment of PBMC with MSA for 6 hours before incubation with 8.33 µM cisplatin for 24 hours affect cell viability (Figure 8).
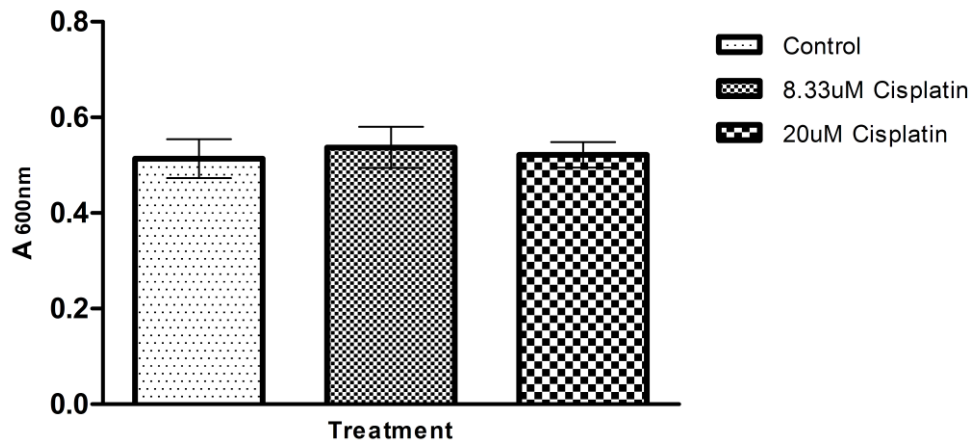
Figure 7: PBMC viability in response to 24hr cisplatin treatment (n=9).
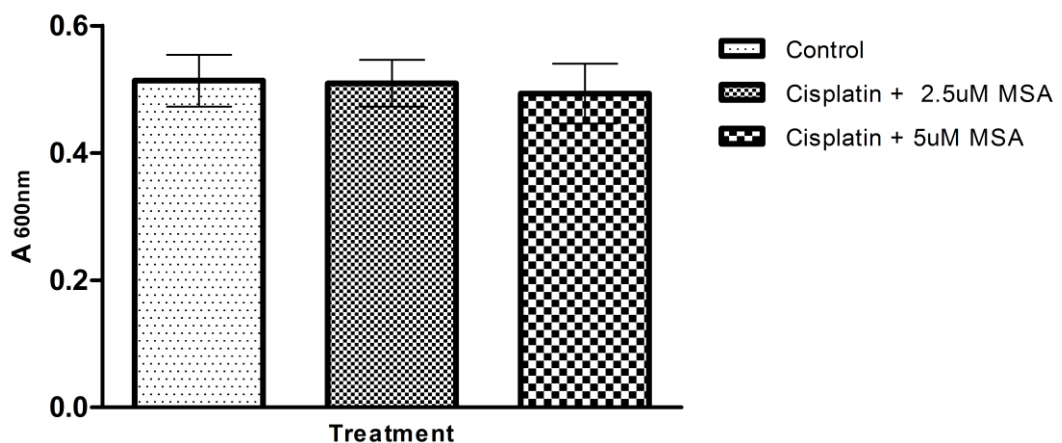
Figure 8: Effects of 6hr MSA pretreatment on PBMC viability in response to 24 hr treatment with 8.33 μM cisplatin (n=9).

In order to assess whether MSA provided protection for PBMC against RT, PBMC were incubated with 8.33 μM cisplatin or 2.5 or 5 μM MSA and irradiated with 10 Gy. Cell viability was immediately assessed after irradiation using the MTT assay. As shown in Figure 9, there was no significant difference in PBMC viability in any of the treatments directly after irradiation.
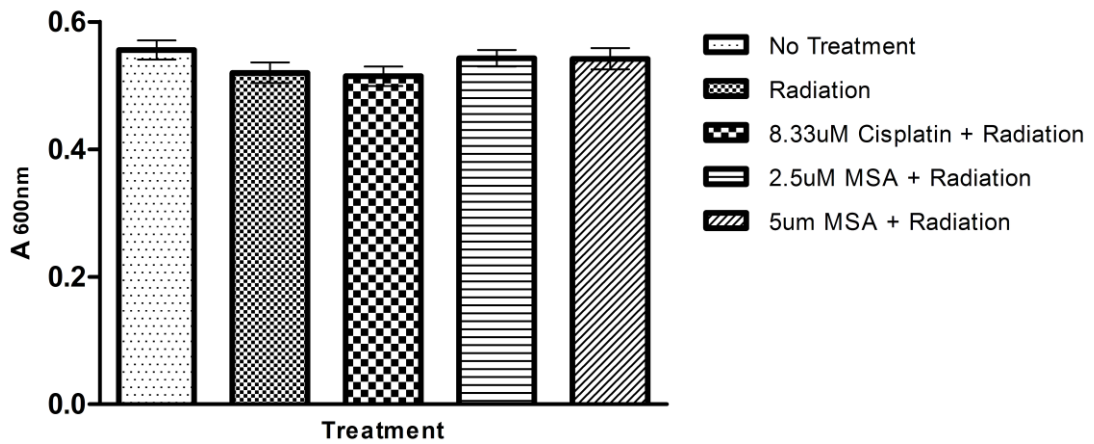
Figure 9: PBMC viability after 10 Gy irradiation (n=6)

3.3.1.2 THP-1 viability

The cytotoxicity of MSA on the human monocytic leukaemia THP-1 cell line was assessed using the MTT assay. Figure 10 shows that MSA reduced the viability of THP-1 cells in a concentration-dependent manner at 2.5 and 5 μM ($p = 0.0026$ and 0.0001 respectively).
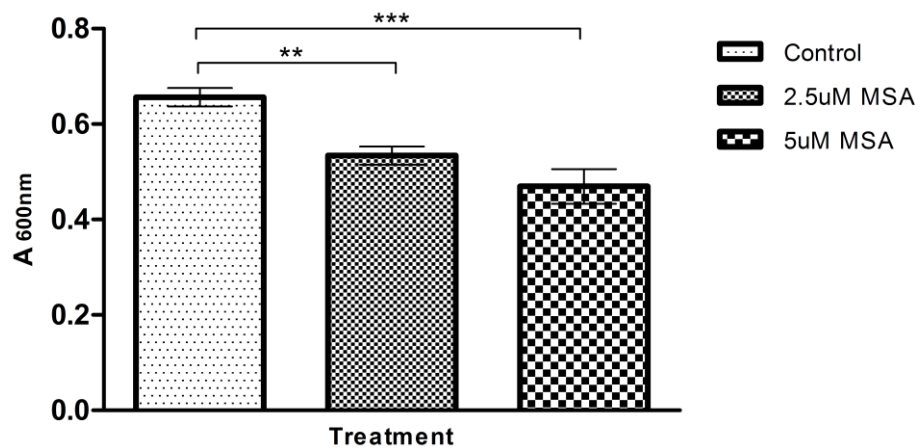
Figure 10: THP-1 cell viability in response to 24hr MSA treatment (results are obtained from two experiments; n=6; ** $p < 0.01$, *** $p < 0.001$)

Viability of THP-1 cells was also significantly reduced in response to 8.33 μM (p = 0.0005) and 20 μM (p = 0.0001) cisplatin. There was however no significant difference between 8.33 and 20 μM cisplatin on THP-1 cell viability (Figure 11).
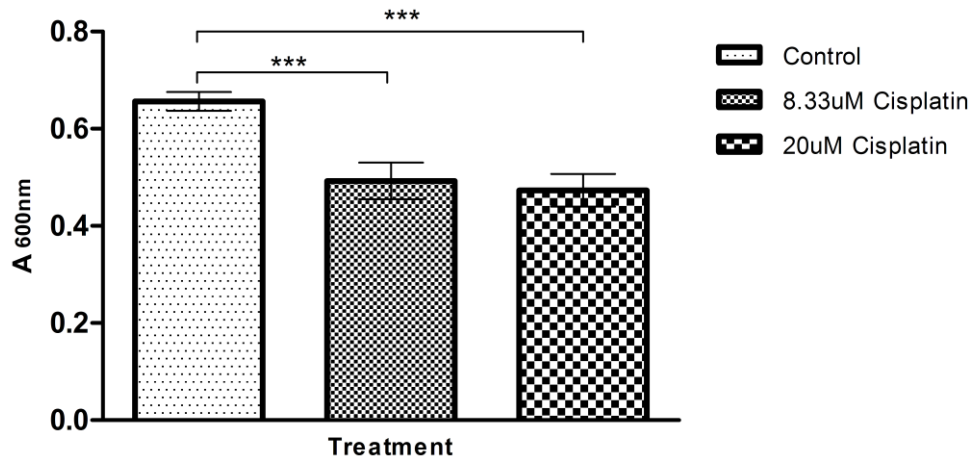
Figure 11: THP-1 cell viability in response to 24hr cisplatin treatment (results are obtained from two different buffy coats; n = 6, *** p <0.001).

Pretreatment with MSA for 6 hours enhanced the cytotoxicity of a 24 hour incubation with cisplatin in this cell line: 2.5 and 5 μM MSA with 8.33 μM cisplatin significantly reduced THP-1 cell viability when compared with cisplatin alone (p = 0.0067 and 0.0041 respectively, Figure 12).

Figure 13 shows that MSA increased radiosensitivity in a dose-dependent manner. MSA significantly affected THP-1 cell viability at both 2.5 μM (p = 0.0143) and 5 μM (p = 0.003). In contrast, the addition of 8.33 μM cisplatin did not significantly reduce THP-1 cell viability compared to radiation alone.
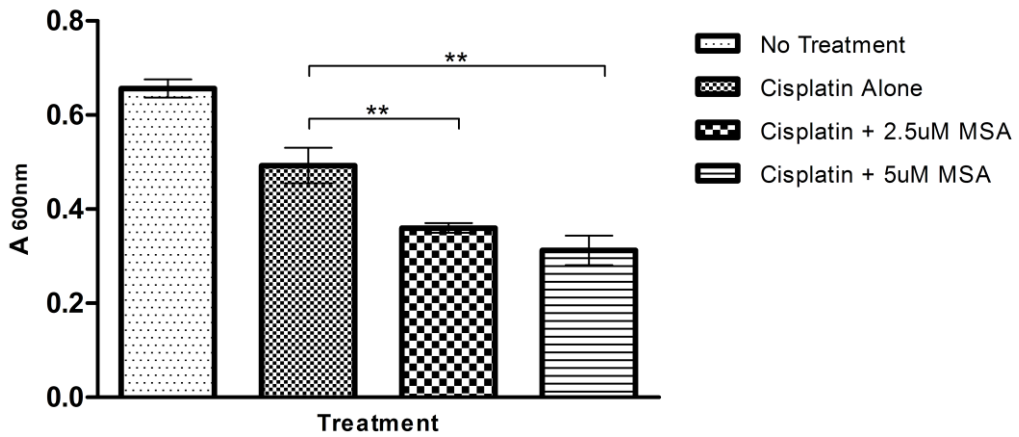
Figure 12: Effects of 6hr MSA pretreatment on THP-1 cell viability in response to 24hr cisplatin treatment (results are obtained from two experiments; n = 6, ** p <0.01).
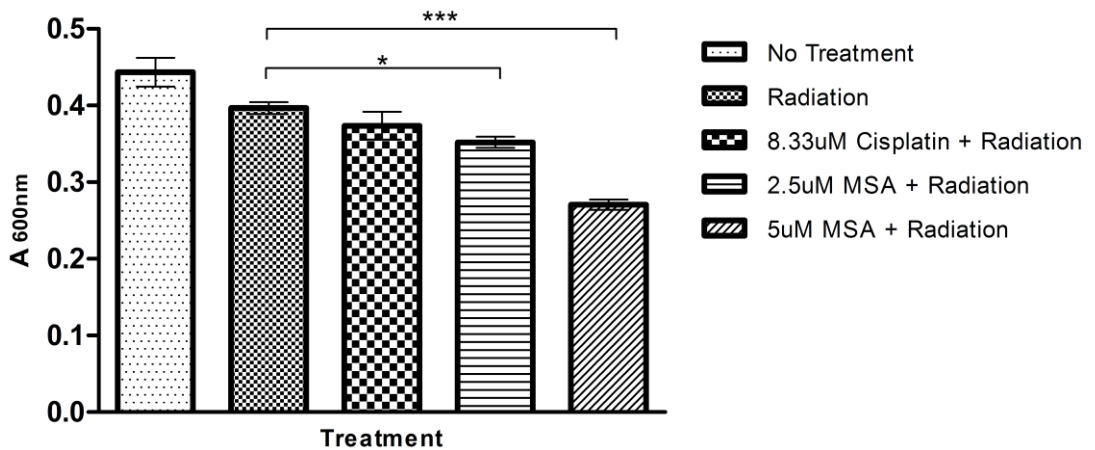
Figure 13: THP-1 cell viability after 10 Gy irradiation (results are obtained from two different buffy coats; n = 6, * p < 0.05, *** p <0.001).

3.3.2 Glutathione assay

Total GSH level in THP-1 cells was reduced significantly in a concentration-dependent manner as shown in Figure 14: 2.5 μM of MSA significantly reduced total GSH (p = 0.0050) as well as 5 μM of MSA (p = < 0.0001).
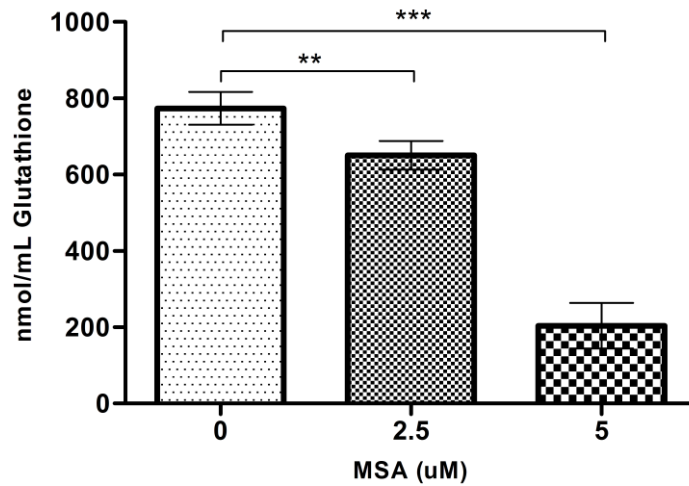
Figure 14: THP-1 glutathione levels in response to 6hr MSA treatment (results are obtained from two experiments; n = 6, ** p < 0.01, ** p <0.001).

A reversed impact of MSA on total GSH levels in non-malignant PBMC (as compared to malignant THP-1 cells) was observed (Figure 15), though again in a concentration-dependent manner: 2.5 µM of MSA significantly increased total GSH (p = 0.0080) as well as 5 µM of MSA (p = < 0.0001).
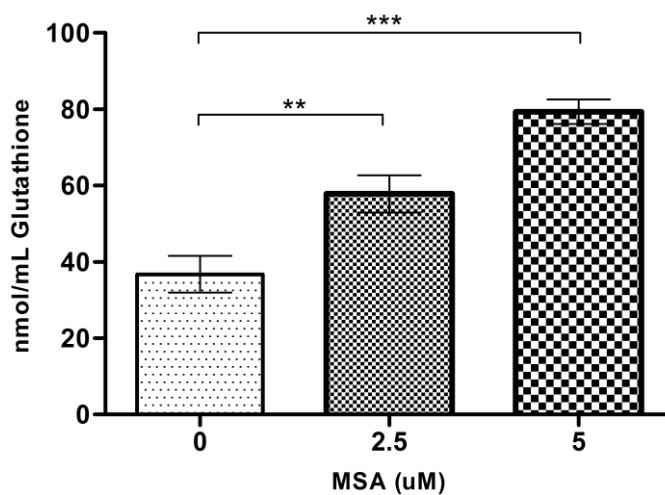
Figure 15: PBMC glutathione levels in response to 6hr MSA treatment (results are obtained from three different buffy coats; n = 10, ** p < 0.01, ** p <0.001).

3.3.3 Comet assay

The results are shown in Table 5. DNA damage was detectable in control PBMC at low levels and increased significantly with MSA 2.5 or 5 μM. H_2O_2 caused a greater degree of damage as expected but this was significantly reduced by cisplatin 20 μM (which modestly caused DNA damage in its own right). RT 10 Gy, on the other hand, caused such profound damage to PBMC that all PBMC samples had a maximum score.

Table 5. Comet assay results in PBMC

Treatment	Comet score (mean ± SD) (max. 400)
Control	7 ± 5.6
100 μM H_2O_2	149 ± 7.5
20 μM cisplatin	13 ± 4.1
20 μM cisplatin +100 H_2O_2	75 ± 7.1
2.5 μM MSA	17 ± 1.5
5 μM MSA	41 ± 3.5
RT 10 Gy	400

3.3.4 ER stress response

All experiments with PBMC obtained from buffy coats were carried out in duplicate using two separate buffy coats. THP-1 cell results are obtained from only one experiment. Results presented are representative of those experiments. B-actin was used as a loading control in all westerns. Equal loading of protein into each well is demonstrated in Figure 16.

PBMC   THP-1

β-actin

MSA   0 μM   2.5 μM   5 μM   0μM   2.5 μM   5 μM
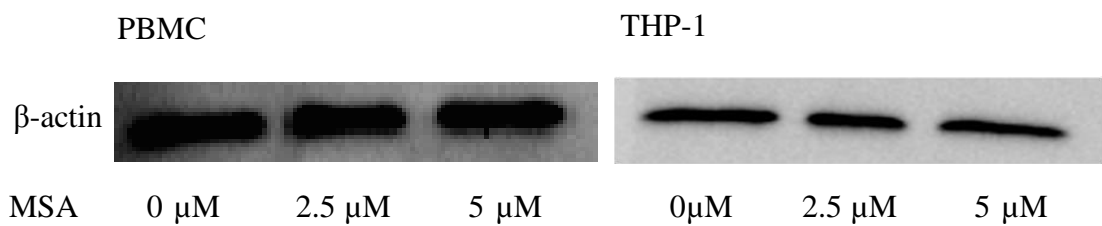
Figure 16: Loading control

As shown in Figure 17, MSA induced XBP1 splicing in PBMC in a concentration-dependent fashion. While THP-1 cells expressed spliced XBP1 at baseline, treatment with MSA increased XBP1 splicing, though 2.5 μM MSA produced higher levels of spliced XBP1 than 5 μM. After six hours, phospho-eIF2α was only slightly elevated in response to MSA treatment in both PBMC and THP-1 cells, though THP-1 cells exhibited higher levels of phospho-eIF2α at baseline compared to PBMC.

PBMC   THP-1

Spliced XBP1

Unspliced
XBP1

Phospho-eIF2α

GRP78

MSA   0 μM   2.5 μM   5 μM   0 μM   2.5 μM   5 μM
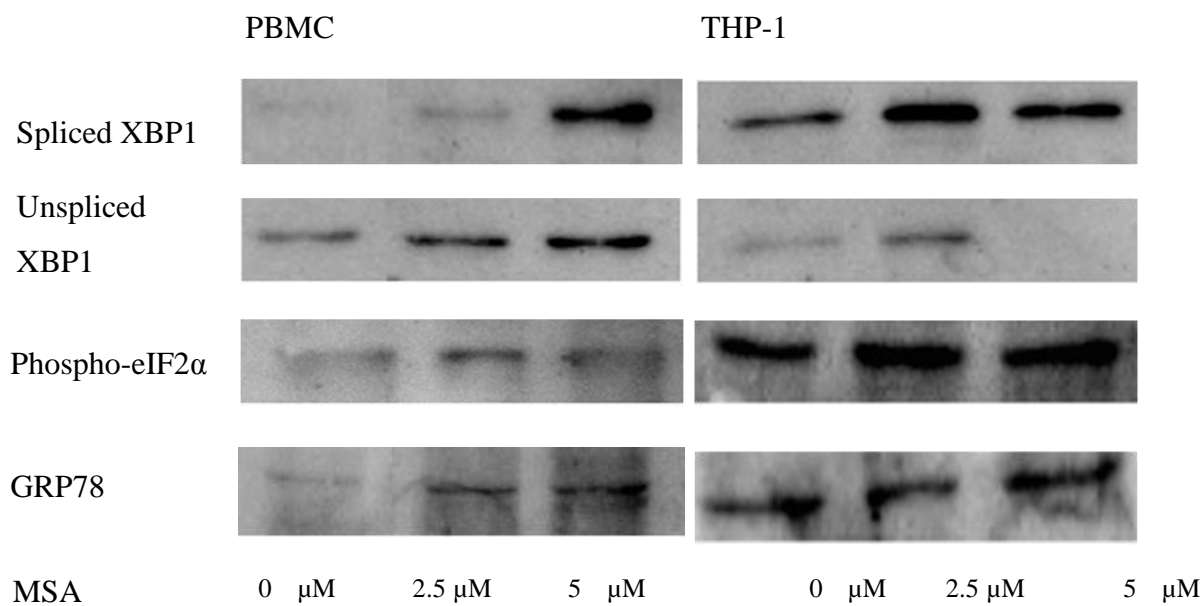
Figure 17: ER stress pro-survival markers

Expression of GRP78 was detected in both PBMC and THP-1 cells (**Figure 17**). In THP-1 cells it appears that GRP78 expression is unaltered in response to MSA treatment. Like phospho-eIF2α, GRP78 expression is elevated in the control.

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GRP78 is upregulated in response to MSA in PBMC. Expression of GRP78 appears to increase in a concentration-dependent manner as shown in **Figure 17**.

CHOP was shown to be expressed in PBMC at baseline, and was unchanged in response to MSA treatment (Figure 18). CHOP expression was unable to be detected in THP-1 cells. Caspase-8 expression was not induced in PBMC treated with MSA. In contrast, caspase-8 was slightly upregulated in THP-1 cells treated with MSA after six hours.

PBMC

CHOP			
MSA	0 μM	2.5 μM	5 μM

THP-1

Caspase 8			
MSA	0 μM	2.5 μM	5 μM
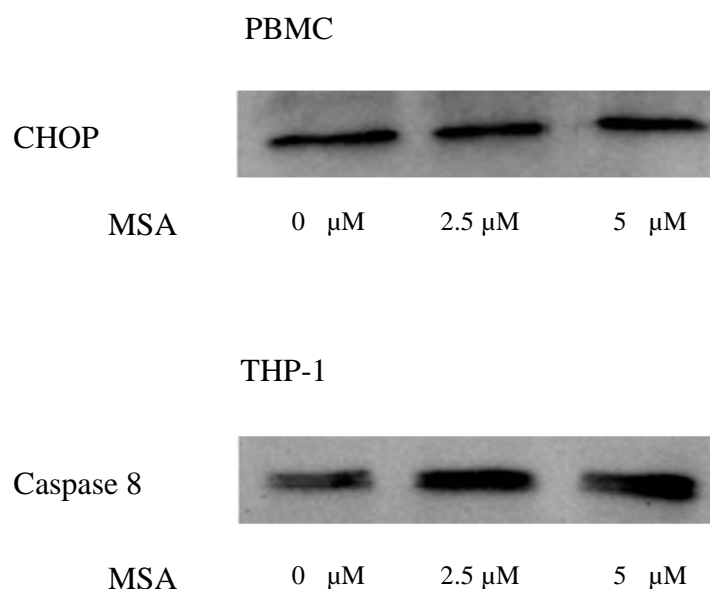
Figure 18: Pro-apoptotic markers

3.4 Discussion

3.4.1 Cell viability

This investigation was undertaken to evaluate whether PBMC and THP-1 cells could be used as a model to determine differential effects of Se on malignant and normal mononuclear cells. This was confirmed: MSA had no impact on PBMC viability, while it significantly reduced THP-1 cell viability. In addition this study showed differential effects of cisplatin in these cells, with no effect on viability of PBMC viability over 24 hours but marked reduction in viability of THP-1 cells in a concentration-dependent manner.

Furthermore, MSA increased the cytotoxicity of both cisplatin and radiation treatments in these malignant cells while no such effects were observed in PBMC. These concentration-dependent effects of MSA occurred at physiologically-relevant concentrations (Ip, et al., 2000). Given these effects of MSA on each cancer treatment, it would be interesting to see if it produced even better malignant cell kill when used in conjunction with both cisplatin and RT.

These data are in agreement with work by Vadgama et al. (2000) who demonstrated therapeutic synergy when Se derived from yeast was used in combination with various chemotherapeutic drugs in several malignant cell lines. This study showed that Se enhanced cell growth inhibition of CT and increased apoptosis in various malignant cell-lines whereas the non-malignant breast cell-line MCF-10 was insensitive to Se-induced growth inhibition and apoptosis induction.

Similarly previous studies showed that Se compounds increased the radiosensitivity of malignant cells. Using clonogeneic survival assays, Shin et al. (2007) showed that Se, in the form of SLM, enhanced the cytotoxicity of radiation on two malignant lung cell lines, while having little effect on normal diploid WI-38 cells. These authors further showed that SLM reduced both mRNA and protein levels of epidermal growth factor and DNAPK, which are involved in cell survival signalling and the DNA damage response respectively. In contrast, SLM had no impact on the expression profile of these elements in the non-malignant cell line.

PBMC from healthy blood donors were used during this study. Although no cell cycle assessment was undertaken, it can be largely assumed that isolated PBMC are post-mitotic in the G_0 phase. That is, PBMC were not actively progressing through various phases of the cell cycle. Given the cytotoxicity of cisplatin is maximal at the end of the G_1 phase, cells not progressing through the cell cycle could exhibit cellular resistance towards cisplatin (Gupta, et al., 2010). Using the MTT assay, PBMC were insensitive to MSA, cisplatin, and MSA + cisplatin treatments, as well as radiation. This resulted in no observed differences in cell viability between these treatments and controls (Figure 6,Figure 7,Figure 8 and Figure 9). However this is most likely due to the fact that PBMC were not actively dividing and senescent.

A number of studies investigating the impact of CT and RT on PBMC typically use a mitogen to activate cells into the cell cycle. Mitogens are chemical substances that trigger mitosis through signal transduction cascades involving mitogen-activated protein kinases. It would be recommended that further studies using PBMC specifically to investigate the cytotoxic effects and/or growth inhibitory effects of MSA, alone or in combination with radiation and chemotherapeutic drugs such as cisplatin, use a mitogen to activate mitosis.

Irradiation can be lethal at all stages of the cell cycle, though cellular resistance toward RT has been demonstrated in cells which undergo growth arrest (Waldman *et al.* 1997). While no significant difference in PBMC viability to 10Gy RT was detected, this was assessed immediately following irradiation. This does not necessarily indicate that cells were not suffering from a lethal dose of RT or even undergoing apoptosis, just that no effect was evident at a very early stage (in contrast to the THP-1 cells). Therefore in further experiments cell viability should be measured at later time points after irradiation in order to provide greater insight. Again, the use of a mitogen would also be useful.

There is a possibility that the observed difference between THP-1 cell\and PBMC viability is due to a different cell type (not just the difference in malignant and non-malignant phenotype). PBMC consist of lymphocytes, monocytes, and macrophages. Lymphocytes make up the majority of the population, with approximately 75% being T cells. THP-1 cells are an acute monocytic leukaemia cell line; therefore, in this study, the cell viability assays largely compared lymphocytes with monocytes, which could have had a significant impact on the response to MSA. Any future studies comparing the effects of MSA in PBMC and THP-1 cells would have to take this into account and perhaps aim to isolate monocytes preferentially from the buffy coats.

3.4.2 Glutathione

We investigated the change in total intracellular GSH in THP-1 cells and PBMC in response to MSA treatment *in vitro*. Interestingly, we observed differential effects of MSA in the two cell types: MSA decreased total GSH levels in THP-1

cells in a concentration-dependent manner, while in contrast MSA increased total GSH levels in PBMC, again in a concentration-dependent manner.

Interestingly THP-1 cells had significantly higher amounts of intracellular GSH than PBMC in the absence of MSA ($p < 0.0001$) (Figure 14 Figure 15). Malignant cells have acquired mutations that cause cellular adaptations favouring survival and this is one of them. GSH reacts with chemotherapeutic drugs, including cisplatin, forming inactive species (Berners-Price & Kuchel, 1990; Pendyala, Creaven, Perez, Zdanowicz, & Raghavan, 1995) and elevated GSH levels in response to cisplatin exposure has been implicated in resistance to platinum-based therapies (Agarwal & Kaye, 2003). Furthermore, in the nucleus elevated GSH levels have been implicated in resistance through the maintenance of particular protein redox states that are necessary for DNA repair (Lai, Ozols, Young, & Hamilton, 1989; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Clinically glutathione S-transferase levels in head and neck cancer patients have been associated with failure to respond to platinum-based therapy (Nishimura, et al., 1996). The cell cycle can be modulated by intracellular concentrations of GSH (Arrigo, 1999; Kern & Kehrer, 2005; Schafer & Buettner, 2001) and depletion of total intracellular GSH, causing the cellular environment to become more oxidized, has been associated with increased apoptosis and necrosis (Cai & Jones, 1998; Evens, et al., 2004; Voehringer & Meyn, 2000). Given this, drugs that target redox systems of cells are proposed to be useful in chemoprevention and chemotherapeutics (Evens, et al., 2004).

Section 3.3.1 showed that the MSA enhanced the reduction in THP-1 cell viability from cisplatin. Although no analysis of GSH changes in response to cisplatin alone was performed, nor of apoptosis, the reduction in GSH levels of THP-1 cells due to MSA exposure could well be responsible for this observed synergy. That is, reduced GSH levels would sensitize the cell to cisplatin, thus increasing the therapeutic efficacy of treatment. This is in agreement with previous studies that have shown that Se compounds enhance the therapeutic activity of CT in malignant cells. Moreover, in response to MSA treatment and total GSH depletion, THP-1 cells may accumulate at particular phases of the cell cycle; this in turn could lead to enhanced sensitivity towards treatments. However no analysis of cell cycle was done in this study. Therefore it would be interesting to

investigate whether cells accumulate at particular phases of the cell cycle in response to MSA treatment, as well as whether intracellular levels of GSH change in response to cisplatin or RT alone, and the effect of MSA on these changes.

The role of intracellular GSH in the cellular response to radiation has been evaluated in a number of studies. Radioprotection has been shown *in vitro* to be associated with increased levels of NPSH (Revesz & Bergstrand, 1963), while reduced GSH levels renders cells more sensitive to radiotherapy (Revesz, Bergstrand, & Modig, 1963). Radiation sensitivity of THP-1 cells was shown in section 3.3.1 to be enhanced by MSA in a concentration-dependent manner. Given these data, the reduction in intracellular GSH levels in THP-1 cells may account for this increased radiosensitivity.

The corollary is that the demonstrated ability of MSA to increase GSH levels in PBMC could therefore provide a protective effect, and may mediate, at least in part, the observed ability of Se compounds to reduce the toxicity of CT and RT in normal tissues (Muecke, et al., 2010; Sieja & Talerczyk, 2004). Further studies investigating whether this increase in GSH provides a reduction in toxicity in normal cells would therefore be warranted. As mentioned in earlier, use of a mitogen to provide actively mitotic normal cells may help to elucidate whether the increase in GSH levels in response to MSA in PBMC is responsible for the protective effects against CT and RT, or whether it is the lack of cell proliferation is important.

3.4.3 Comet assay

Some species of Se have been shown to be genotoxic, therefore, the comet assay was used to assess if MSA was capable of inducing DNA damage in PBMC *in vitro*, which it did significantly in a concentration-dependent manner at 2.5 and 5 µM of MSA. This is in contrast to the work carried out by Ip et al. (2000) that showed MSA did not induce DNA damage. Both this study and the study of Ip and colleagues used 24 hour cultures exposed to the same concentration of MSA and similar comet conditions. The only obvious difference is that this study used non-malignant PBMC, and Ip and colleagues used malignant TM2H and TM12 cells. This may reflect that the genotoxicity of MSA is dependent on cell type.

In order to confirm that cisplatin did indeed reduce the formation of comets in PBMC under the experimental conditions used in this assay; PBMC were incubated with 20 µM cisplatin for 24 hours. As shown in Table 5 cisplatin does indeed reduce the formation of comets induced by H_2O_2. This is in agreement with previous studies which have shown that cisplatin reduces the formation of comets (Merk & Speit, 1999; Pfuhler & Wolf, 1996). The dose of RT used in this study was overwhelming in terms of DNA damage detection by the comet assay, and it would be interesting to evaluate the impact of smaller RT doses such as 2 Gy, as used in the trial discussed in Chapter 4, on DNA damage to PBMC *in vitro*, and the ability of cisplatin to reduce the formation of comets in that setting.

3.4.4 ER stress response

MSA was shown to activate various UPR targets in both cell types (Figure 17 and Figure 18). The use of Se compounds, in particular MSA, has previously been shown to induce ER stress in a concentration- and time-dependent manner in a PC-3 human prostate cancer cell line (Wu, et al., 2005). The authors found that high concentrations of MSA induced pro-apoptotic ER stress-induced targets such as CHOP (GADD153). In contrast, low levels induced pro-survival proteins such as phosphorylated eukaryotic initiation factor-2 alpha (phospho-eIF2α) and glucose-regulated proteins 78 (GRP78) and 94 (GRP94).

In this study XBP1 splicing was induced by MSA in a concentration-dependent manner in PBMC, while in THP-1 cells 2.5 µM MSA appeared to induce higher expression of spliced XBP1 compared to 5 µM (**Figure 17**). XBP1 mRNA is induced by activated ATF6 and spliced into a highly active and stable transcription factor by activated IRE1, though a significant induction of XBP1 mRNA due to ATF6 was required to produce spliced XBP1 at a level sufficient for detection and transactivation (Yoshida et al. 2001). Furthermore, spliced XBP1 can keep activating transcription by autoregulating its own transcription as long as IRE1 is activated (Yoshida et al 2000). This study showed that spliced XBP1 was the dominant isoform in response to MSA in THP-1 cells (**Figure 17**). This is consistent with Yoshida et al. (2000) who showed only spliced XBP1 was detected at high levels in cell lysates in response to ER stress. Conversely, increased levels of unspliced XBP1 were observed in concert with increased

spliced XBP1 in PBMC (**Figure 17**). Treatment with 5 µM MSA induced higher levels of spliced XBP1 compared to baseline in PBMC than in THP-1 cells. This may reflect that 5 µM of MSA will afford PBMC a higher degree of survival against toxic challenges and further iterations of this experiment could be informative.

Induction of phospho-eIF2α was also shown in both PBMC and THP-1 cells (**Figure 17**). Phospho-eIF2α has been previously shown to be a pro-survival mechanism as it lessens the burden of aberrantly-folded proteins through translation inhibition. Phospho-eIF2α was already induced in control THP-1 cells, and only slightly upregulated in response to MSA. This baseline expression of phospho-eIF2α may reflect the malignant phenotype and survival strategy of THP-1 cells. However, since this experiment was only carried out once, this may reflect that these cells were stressed before the experiment started, that is, the levels of UPR targets in THP-1 cells were already upregulated and may reflect previous stresses, not induced by MSA. This experiment should be conducted again in order to confirm either way.

CHOP expression in PBMC was present in controls and MSA did not induce higher levels of expression (Figure 18). In contrast, CHOP was unable to be detected in THP-1 cells at the concentrations of MSA used. Wu et al. (2005) showed that CHOP induction was severely attenuated in GRP78 overexpressing cells (GRP78 allows cells to cope with ER stress). In the THP-1 cells GRP78 was upregulated in control conditions (**Figure 17**). Therefore, in this instance, the inability of MSA to induce CHOP expression may be the result of THP-1 cells expressing elevated levels of GRP78. This is in agreement with previous studies that have demonstrated resistance to various types of anticancer drugs in cells expressing enhanced levels of GRP78 (J. Li & Lee, 2006), and reducing GRP78 expression results in increased sensitivity towards treatments (Rahmani, et al., ; Y. Wang, et al., 2008). GRP78 was upregulated in response to MSA in PBMC. Therefore, PBMC exposed to MSA should have enhanced resistance to toxic challenges since GRP78 has been previously shown to provide cytoprotection to cells.

In this study MSA increased caspase-8 expression compared to baseline in THP-1 cells but not PBMC (Figure 18). This Se-induced caspase-8 activation is in agreement with previous studies that have shown Se compounds induce apoptosis through caspase-8 mediation (Li et al. 2008). The fact that Se does not induce caspase-8 in PBMC may reflect that Se compounds preferentially induce apoptosis in malignant cells, not normal cells. This has been demonstrated in numerous studies, and has been detailed in section 1.9.3.

3.5 Conclusions

1. This *in vitro* model using PBMC from healthy blood donors and THP-1 human leukaemia cells can successfully be used to evaluate the differential impact of MSA on these normal and malignant mononuclear white blood cells.

2. While PBMC do not suffer reduced viability from short-term exposure to MSA, cisplatin, RT or their combination, MSA alone is cytotoxic to THP-1 cells and enhances the cytotoxic effects of cisplatin and RT against THP-1 cells.

3. Total intracellular glutathione increases in PBMC in response to MSA whereas its levels fall in THP-1 cells, possibly mediating the differential effect of MSA on the cytotoxicity of cisplatin and RT in THP-1 cells.

4. MSA, cisplatin and RT are each genotoxic to PBMC under the conditions used, especially RT, and cisplatin can reduce the formation of comets induced by other agents.

5. MSA induces ER stress in both PBMC and THP-1 cells. While the response in PBMC is predominantly pro-survival, it is mixed in THP-1 cells, which have elevated levels of UPR markers, though caspase 8 expression is only induced from MSA in THP-1 cells.

Chapter 4

In vivo investigations

4.1 Introduction

This work towards an MSc was undertaken in conjunction with a clinical trial being conducted at the Regional Cancer Centre, Waikato Hospital. In this international, randomised, placebo-controlled, double-blind phase II trial patients with head and neck squamous cell carcinoma (HNSCC) were treated with a 7-week radical course of concurrent cisplatin chemotherapy and RT (CRT) and were randomised to receive either SLM or matching placebo for 11 weeks, starting a week prior to CRT treatment.

The primary objective of the trial was to assess whether SLM reduces the incidence of grade 3 or 4 mucositis in HNSCC patients treated with concurrent CRT over 7 weeks, with secondary endpoints including complete response rate, relapse-free survival, overall survival, quality of life, xerostomia, renal impairment, hearing loss, myelosuppression, chemoradiation dose delivery and safety.

In patients recruited in Hamilton, additional endpoints included plasma free cisplatin and plasma selenium pharmacokinetics and pharmacodynamic markers of biological activity of Se, as detailed below. The pharmacokinetic analyses will be conducted by a different department at the University of Waikato, but the pharmacodynamic biomarker analysis constitutes the subject of this chapter. Unfortunately because the trial is not yet complete, the treatment allocation code has not yet been broken and analysis of the pharmacodynamic results according to treatment with SLM or placebo cannot be made at the time of writing this thesis.

4.1.1 Objectives

The overall objective of this chapter was to evaluate to evaluate pharmacodynamic makers in HNSCC patients undergoing CRT. The specific objectives were:

1. to evaluate the DNA damage of PBMC obtained from patients before and during treatment using the comet assay;
2. to quantify VEGF-A and VEGF-R1 levels in plasma of patients before and during treatment;
3. to quantify GPX3 levels in plasma of patients before and during treatment; and
4. to evaluate changes in ER stress response markers to treatment.

4.2 Methods

4.2.1 Patient population

Patients with HNSCC planned for definitive treatment with a 7-week radical course of CRT were recruited for this study, which was approved by the Northern Y Regional Ethics Committee and the Institutional Review Board at Roswell Park Cancer Institute, Buffalo, NY, USA (the study sponsor). The inclusion criteria included: patients over 18 years of age; adequate liver, renal and bone marrow function; PBMC count ≥ 2.0 x 10^9/L; ECOG performance status ≥ 3, and life expectancy > 3 months. Patients were excluded if they had been treated with cytotoxic chemotherapy, anticancer biological therapy, hormone therapy or radiotherapy within the last 4 weeks; unable to swallow or absorb study tablets; concurrent Se supplements; or medical conditions which would compromise patients or data integrity.

4.2.2 Patient treatment

After screening, patients gave written informed consent and were randomly allocated to receive SLM 3200 µg/m^2 or matching placebo as capsules. In the first

week study capsules were taken twice daily to shorten the time to plasma steady state concentrations, then once daily for weeks 2–11. CRT started at the beginning of week 2.

CRT consisted of seven weeks of external beam RT given in 2 Gy fractions five days a week for 7 weeks, to a total dose of 70 Gy. Cisplatin 100 mg/m^2 was given intravenously on weeks 1, 4 and 7 with adequate hydration and anti-emetic measures.

4.2.3 Collection of samples for pharmacodynamic biomarker analysis

Blood for pharmacodynamic biomarker analysis was drawn from patients prior to starting study medication, at the end of week 1 (after SLM or placebo only), at the end of week 2 (after 1 week of CRT) and at the end of week 8 (after completion of CRT). 25 ml of whole blood was collected into EDTA tubes, 5 ml of which was analysed fresh in a comet assay and the other 20 ml was processed to extract PBMC for analysis of ER stress markers. 10 ml of blood was collected into a lithium heparin tube and plasma was separated in a refrigerated centrifuge. Five 1ml aliquots of plasma were stored at -80 ℃ until analyzed for VEGF-A, VEGFR-1 and glutathione peroxidase.

4.2.4 Comet assay

The alkaline comet assay developed by Singh *et al* (1988) was used to assess DNA damage in PBMC extracted from blood samples of HNSCC patients. The comet assay of PBMC extracted from buffy coats from healthy donors (see section 3.2.3) was used to confirm that conditions of the assay were working appropriately. The comet assay methodology is described in section 2.6.

4.2.5 Plasma VEGF-A ELISA

Plasma VEGF-A levels were measured with a human VEGF-A ELISA kit purchased from Abnova (catalogue number KA0166) and stored at 4 °C until required. The assay was run according to the manufacturer's directions. All

reagents were warmed to room temperature before use. Table 6 provides a list of the reagents provided and used in this assay. Standards were assayed in duplicate, and unknown samples were assayed in triplicate.

The wash buffer was prepared by diluting the 20x stock solution to 1x with MQ water. Microwell strips were then washed twice by pipetting 400 µl of wash buffer with an 8-channel micropipette, with thorough aspiration inbetween washes. Following the last wash step, the microwell strips were tapped on an absorbent paper towel to remove excess wash buffer.

Table 6. Reagents used in the plasma VEGF-A assay

Component	Amount
Microwell plate coated with polyclonal antibody to human VEGF-A	1 aluminium pouch
Biotin-conjugate anti-human VEGF-A polyclonal antibody	120 µl
Streptavidin-HRP	150 µl
Human VEGF-A standard lyophilized, 2 ng/ml upon reconstitution	2 vials
Sample diluent	12 ml
20x assay buffer (PBS with 1% Tween-20 and 10% BSA)	5 ml
20x wash buffer (PBS with 1% Tween-20)	50 ml
Substrate solution (tetramethyl-benzidine)	15 ml
Stop solution (1N sulfuric acid)	15 ml

100 µl of sample diluent was added to each of the standard wells, including the wells to be used as a blank. Wells which were going to contain the samples had 50 µl of sample diluent aliquoted into them. 100 µl of VEGF-A standard (which was reconstituted at least 10 minutes earlier with sterile MQ water) was added to duplicate wells and mixed through repeated aspiration (final concentration of standard one = 1000 pg/ml). Using serial dilution, 100 µl of standard one was added into each of the following 2 wells, mixed thoroughly through aspiration, of which 100 µl was then aliquoted into the following 2 wells and so on. 100 µl from

the last standard microwell was discarded so that all standards had 100 µl total volume. 50 µl of each plasma sample was then added in triplicate to sample wells. The microwell plate was then covered with an adhesive film and incubated at room temperature for two hours on a microplate shaker set at 100 rpm.

During the incubation the 20x assay buffer was diluted to 1x with MQ water. Approximately 10 minutes before the incubation was completed the biotin conjugate was prepared by diluting the stock 1:100 with assay buffer. After the incubation was complete, wells were emptied and washed six times with wash buffer as before. 100 µl of biotin conjugate was then added to all wells, before the plate was once again covered with an adhesive film and incubated at room temperature for one hour on a microplate shaker set at 100 rpm. Streptavidin-HRP stock solution was diluted 1:100 with assay buffer approximately 10 minutes before the second incubation was completed. After the second incubation was complete, wells were emptied and washed six times as before. Following the washing steps, 100 µl of streptavidin-HRP was added into all the wells. The plate was once again covered with an adhesive film and incubated at room temperature for one hour on a microplate shaker set at 100 rpm.

After the third incubation, the well were once again emptied and washed six times with wash buffer. 100 µl of TMB substrate solution was then added to all wells and incubated for an appropriate time in the dark. The colour development on the plate was monitored by using BMG Labtech FLUOstar OPTIMA plate reader set at 620 nm. The reaction was stopped when VEGF-A standard one (1000 pg/ml) reached an OD of 0.9, or as close to this value as possible, by adding 100 µl of stop solution (1N sulfuric acid) to all the wells. As with all additions of reagents in this assay, an 8-channel micropipette was used to uniformly distribute the stop solution to all microwells. Using the same plate reader, the absorbance of the wells was measured immediately using 450 nm as the primary wave length. A standard curve was constructed, which was used to determine the concentration of the samples. The concentrations of plasma samples were calculated by interpolation of the regression curve formula from the standard curve.

4.2.6 Plasma VEGFR-1 ELISA

The human sVEGFR-1 ELISA kit (catalogue number ALX-850-264-KI01, manufactured by Bender MedSystems) was stored at 4°C until use. The sVEGFR-1 assay is very similar to that of the VEGF-A assay in principle. A coloured product is formed in proportion to the amount of VEGFR-1 present in the wells and the OD_{450} is measured spectrophotometrically in order to quantify VEGFR-1. Standards and blanks were again measured in duplicate, samples in triplicate. Using an 8-channel micropipette, microwells were washed twice with 400 µl of 1x wash buffer (20x diluted with MQ water) with thorough aspiration of microwell contents between washes. After the final wash, excess wash buffer was removed by tapping the plate on an absorbent paper towel. 100 µl of 1x assay buffer (20x diluted with MQ water) was added to all standard wells and 50 µl of 1x assay buffer into sample wells. Reconstituted VEGFR-1 standard prepared at least 10 minutes earlier was added and diluted in the same fashion as the VEGF-A assay. This created two rows of human VEGFR-1 standard dilutions ranging from 10 – 0.16 ng/ml.

Table 7 illustrates the reagents provided with the assay kit.

Table 7. Reagents used in the plasma VEGFR-1 assay

Component	Amount
Microwell plate coated with monoclonal antibody to human VEGFR-1	1 aluminium pouch
Biotin-conjugate anti-human VEGFR-1 polyclonal antibody	100 µl
Streptavidin-HRP	150 µl
Human VEGFR-1 standard lyophilized, 20 ng/ml upon reconstitution	2 vials
Conjugate diluent	20 ml
20x assay buffer (PBS with 1% Tween-20 and 10% BSA)	5 ml
20x wash buffer (PBS with 1% Tween-20)	50 ml
Substrate solution (tetramethyl-benzidine)	15 ml
Stop solution (1M phosphoric acid)	15 ml

50 µl of plasma samples were then added in triplicate to sample wells. 50 µl of a 1:100 dilution of the stock biotin conjugate (diluted with conjugate diluent) was prepared and added to all wells. Following the addition of the biotin conjugate, the microplate was covered with an adhesive film and incubated at room temperature for two hours, shaking on a microplate shaker at 100 rpm. 10 minutes before the end of this incubation, the streptavidin-HRP solution was diluted 1:200 with the conjugate diluent. After the plate had been incubating for two hours, the wells were emptied and washed four times with wash buffer, with thorough aspiration inbetween washes. Excess wash buffer was tapped out on an absorbent paper towel. 100 µl of the diluted streptavidin-HRP solution was then added to all the wells. The plate was once again covered with an adhesive film, and incubated at room temperature for one hour on a microplate shaker at 100 rpm.

Finally, after incubating the wells with the streptavidin-HRP solution, wells were emptied and washed four times according to the previous wash step. 100 µl of TMB substrate solution was then added to all the wells, and colour development monitored at 620 nm using BMG Labtech FLUOstar OPTIMA plate reader. Stop solution was added when standard one (10 ng/ml) reached an OD of approximately 0.9. Once the reaction was stopped, the OD_{450} was measured immediately. A standard curve was constructed and used to determine the concentration of unknown plasma samples. The concentrations of plasma samples were calculated by interpolation of the regression curve formula from the standard curve.

4.2.7 Plasma glutathione peroxidase-3 ELISA

ELISA was used for the quantitative determination of glutathione peroxidase-3 (GPX3) in human plasma. The GPX3 (human) ELISA Kit (catalogue number AG-45A-0020EK-KI01) was purchased from AdipoGen ^{TM} and stored at 4 °C until use. All samples and kit components were equilibrated to room temperature before being used. Table 8 lists the components used in this assay. 5x wash and diluent concentrates were diluted to 1x with MQ water. The 100x detector was diluted to 1x with 1x diluent just prior to use. 1ml of MQ water was added to the

GPX3 standard to make a stock concentration of 64 ng/ml. Standards and samples were assayed in duplicate.

Table 8. Reagents used in the plasma glutathione peroxidase-3 assay

Component	Amount
Microwell plate coated with biotinylated polyclonal antibody to human GPX3	1 aluminium pouch
5x wash concentrate	100 ml
5x diluent	50 ml
Secondary antibody	12 ml
100x detector	150 µl
GPX3 standard	1 vial
Substrate I	6 ml
Substrate II	6 ml
Stop solution	12 ml

Using an 8-channel micropipette, 100 µl of 1x detector was pipetted into each standard well including the blanks. Using a micropipette 100 µl of the stock standard was pipetted in to the top two wells for a final concentration of 32 ng/ml (denoted standard one). Standard one was thoroughly mixed through repeated aspiration. Serial dilution was then used to prepare the rest of the standards by pipetting 100 µl of standard one into the below well and so on, until a total of seven standards were prepared in duplicate (32 ng/ml − 0.5 ng/ml). Plasma samples were diluted 200x by mixing 5 µl of sample with 995 µl of 1x diluent. 100 µl of each diluted sample were pipetted into wells. The plate was then covered with an adhesive film and incubated at 37˚C for one hour. Following this incubation the adhesive film was removed and the wells emptied. Using an 8-channel micropipette, all wells were washed 3 times with 300 µl of 1x wash solution, with aspiration of wash solution from wells between washes. After the final wash step, the wells were emptied, and the plate tapped on a dry absorbent paper towel to remove excess wash solution. 100 µl of secondary antibody was then added to each well and the plate was incubated at 37 ˚C for another hour.

After this incubation the wells were emptied, and washed three times as mentioned previously.

Substrate solution was prepared by adding one part substrate I with one part substrate II. 100 µl of substrate solution was added into all wells and incubated in the dark at room temperature. After 20 minutes, 100 µl of stop solution was added into all wells using an 8-channel micropipette. The OD_{450} was measured using BMG Labtech FLUOstar OPTIMA plate reader, and the standards used to prepare a standard curve. The concentrations of plasma samples were calculated by interpolation of the regression curve formula from the standard curve.

4.2.8 ER stress response markers in PBMC

The methodology for analysis of the ER stress response proteins GRP78, phosphorylated elF2α, CHOP, spliced XBP1, and caspase-8 is detailed in section 2.7. Additional details are described below.

4.2.8.1 Protein isolation from patient PBMC

PBMC were isolated as described in section 2.1. However after the final wash step the PBMC pellet was resuspended in 500 µl of sterile PBS pH 7.4. This cell suspension was transferred to a 1.7 ml tube where an equal volume of 2 x Laemmli buffer (0.25 M Tris pH 6.8, 5% SDS, 20% glycerol, 1% 2-mercaptoethanol (v/v), 0.02% bromophenol blue (w/v)) was added. The Laemmli protein suspension was vortexed thoroughly for 10 seconds before being incubated at 95 °C for 10 minutes. Following incubation the protein lysate was vortexed for another 10 seconds before being stored at -80 °C.

4.2.8.2 Protein quantification from Laemmli buffer

Laemmli buffer contains a number of substances that interfere with various protein quantification methods. Therefore, a number of methods were trialled in order to obtain accurate quantification of protein: protein was precipitated with 50% trichloroacetic acid or acetone, and quantified by measuring the 280nm absorbance; using the Bradford assay; or using the BCA assay previously described in Chapter 2. Both the 280nm and Bradford methods failed to accurately

quantify protein concentration (data not shown), however, the BCA method proved to be reliable and will be described below.

4.2.8.2.1 BCA method

Protein lysates in Laemmli buffer stored at -80 °C where incubated for 10 minutes at 95 °C. Tubes were vortexed for 10 seconds then placed on ice. 6.25 µl of the protein lysate was aliquoted to a new 0.6 ml tube. 43.75 µl of RIPA buffer was added and mixed thoroughly by repeated aspiration with a micropipette before 200 µl of -20 °C acetone was added. At the same time, 25 µl of BCA standards were pipetted into 0.6 ml tubes and 200 µl of -20 °C acetone was added. Unknown sample tube(s) and BCA standards were then incubated at -20 °C for 30 minutes before pelleting precipitated protein by centrifugation at 13,200 rpm for 10 minutes at 4 °C. The supernatant was subsequently removed with a micropipette and tubes were allowed to stand for 20 minutes in order to evaporate residual acetone. 25 µl of RIPA buffer was added into each tube which was then vortexed thoroughly. It should be noted that the pellet was unable to be resuspended. 200 µl of BCA (50:1) reagent was then added (the pellet became soluble at this point) and tubes were incubated at 37 °C for 30 minutes. Following the incubation, tubes were immediately placed on ice and the OD_{562} was measured by aliquoting 2 µl of standard, or sample, onto the pedestal of a NanoDrop ND-1000 Spectrophotometer. Standards were measured first in triplicate to prepare a standard curve. Sample values were then measured in triplicate, and protein concentrations calculated using the prepared standard curve.

4.3 Results

Recruitment into the clinical trial started later than anticipated and thus only eight patients were accrued over the period that this laboratory study was conducted, rather than the 16 anticipated. For various reasons not all patients had the full set of blood tests planned in the protocol, and one patient withdrew from the trial within 2 weeks of starting and only had a set of samples taken at baseline. The time points at which each patient had samples taken that could be analysed for each assay is shown in Table 9.

Table 9. Details of samples from each patient available for pharmacodynamic assays

Patient	Baseline	Week 1	Week 2	Week 8
A	●	●	●	●
B	●	●	●	●
C	●	●	●	●
D	●		●	●
E		●	●	
F	●	●	●	
G	●	●		
H	●			

• denotes obtained samples

4.3.1 Comet assay

The comet assay used in this study was unable to detect any DNA damage in PBMC from patients with HNSCC (all scores = 0). Figure 19 illustrates a patient comet as well as a positive control. These pictures are representative of all comet assays carried out on patient samples.
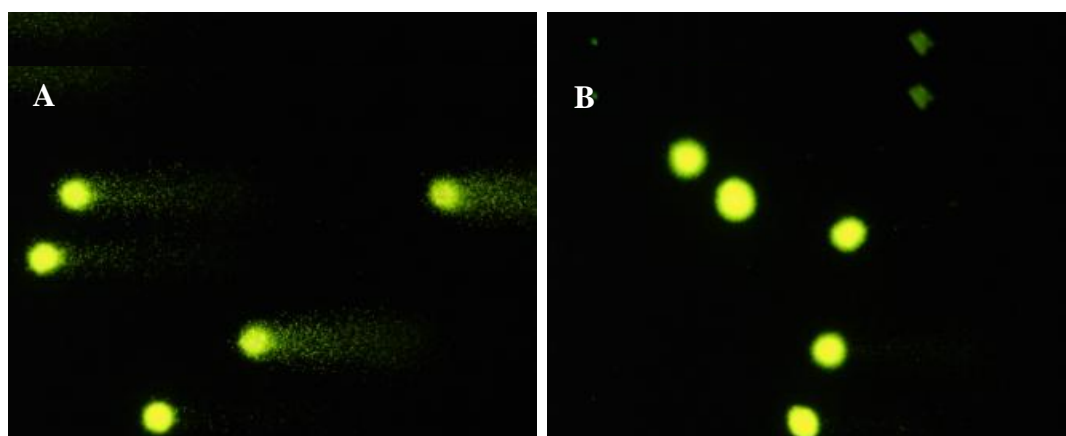
Figure 19. Comet assay examples: A: THP-1 positive control; B: PBMC from patient in the HNSCC trial.

4.3.2 Plasma VEGF-A

Plasma VEGF-A concentration-time profiles in Patients A – F are shown in Figure 20 (results of Patients G and H can be found in the Appendix B).
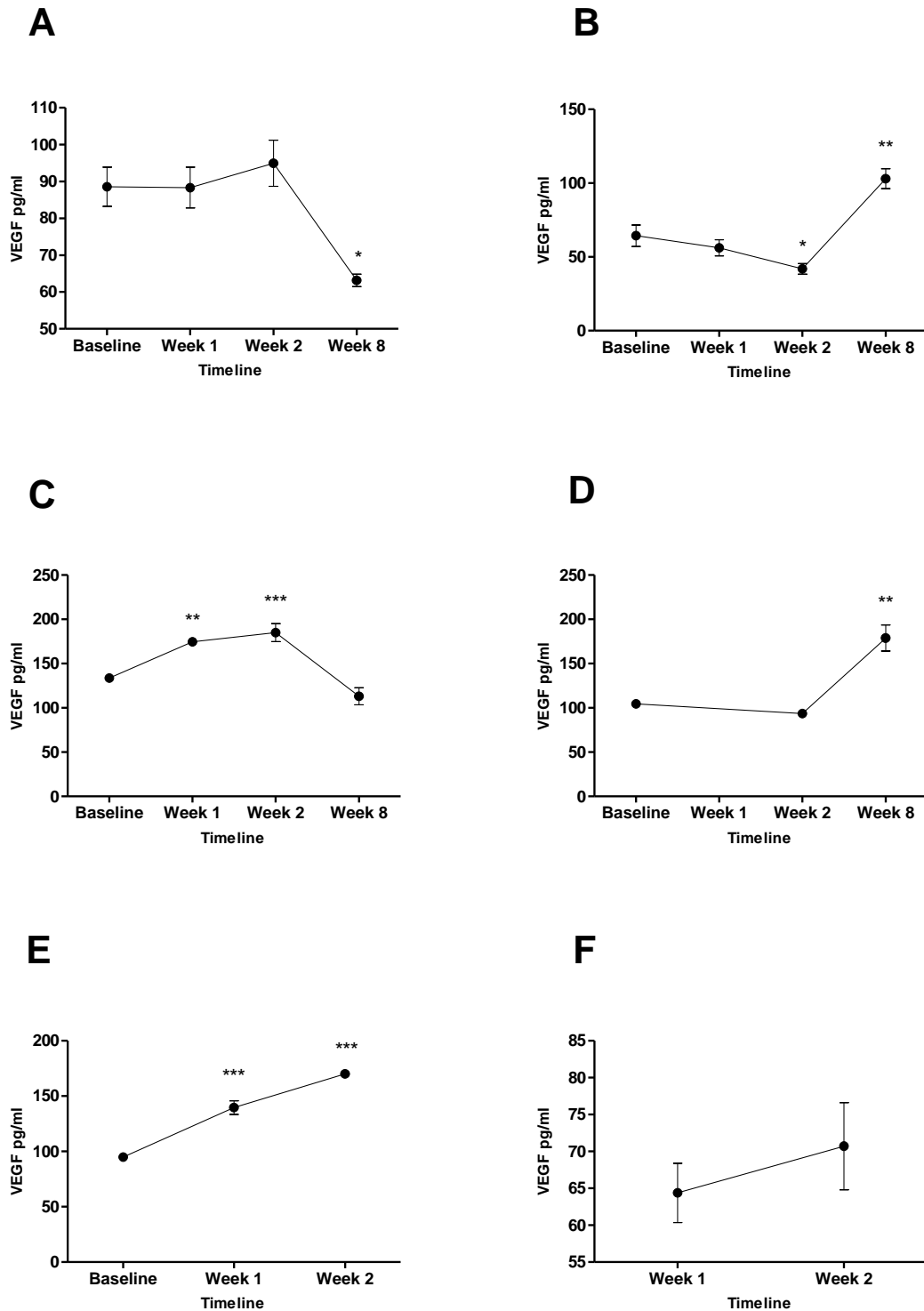
Figure 20. Plasma VEGF-A concentration-time profiles (* p < 0.05, ** p < 0.005, *** p < 0.0001).

4.3.3 Plasma VEGFR-1

Plasma VEGFR-1 concentration-time profiles in Patients A – F are shown in Figure 21 (results of Patients G and H can be found in the Appendix B).
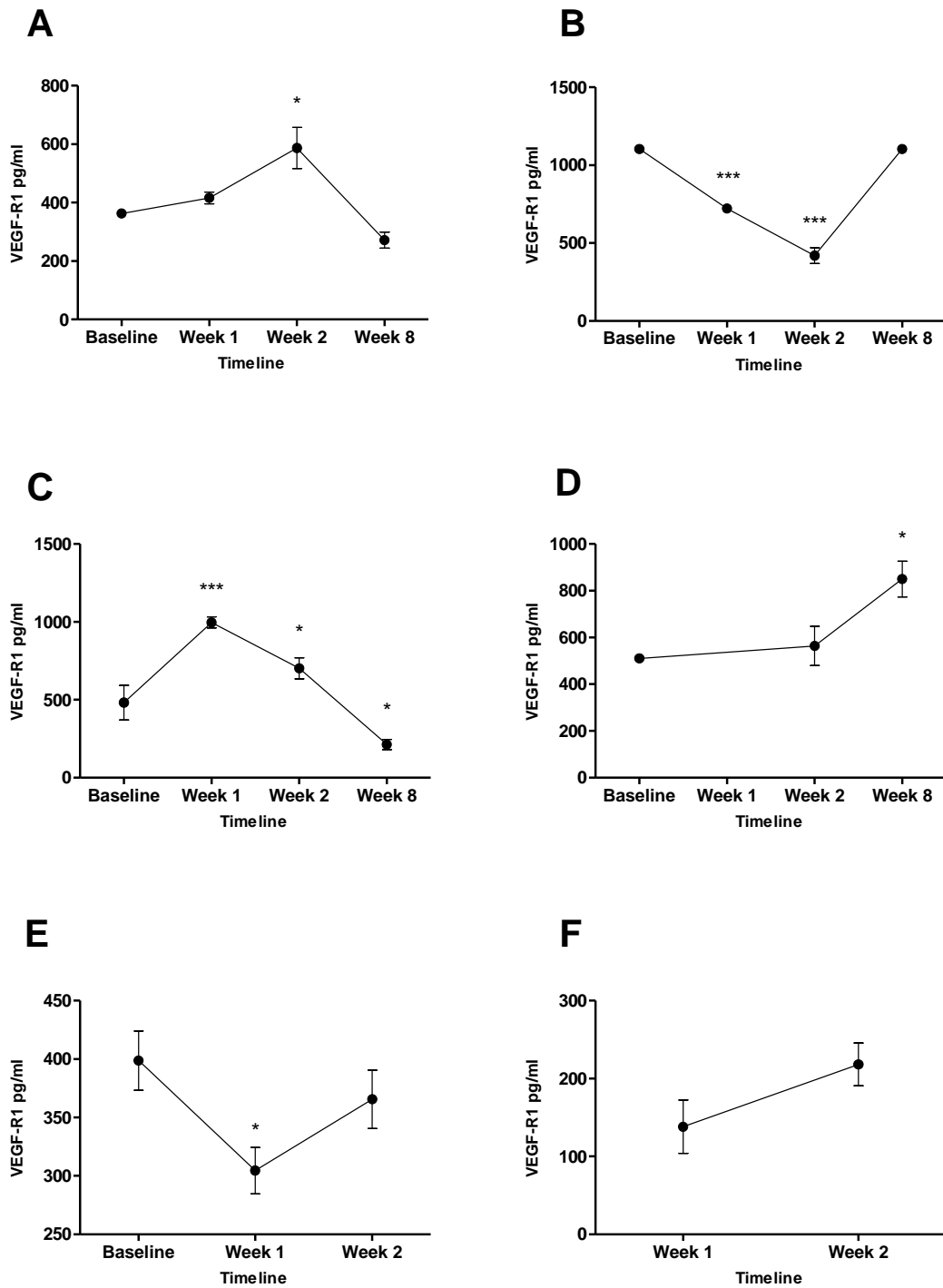
Figure 21. Plasma VEGFR-1 concentration-time profiles (* p < 0.05, ** p < 0.005, *** p < 0.0001).

4.3.4 Plasma GPX3

Plasma VEGFR-1 concentration-time profiles in Patients A – F are shown in Figure 22 (results of Patients G and H can be found in the Appendix B).
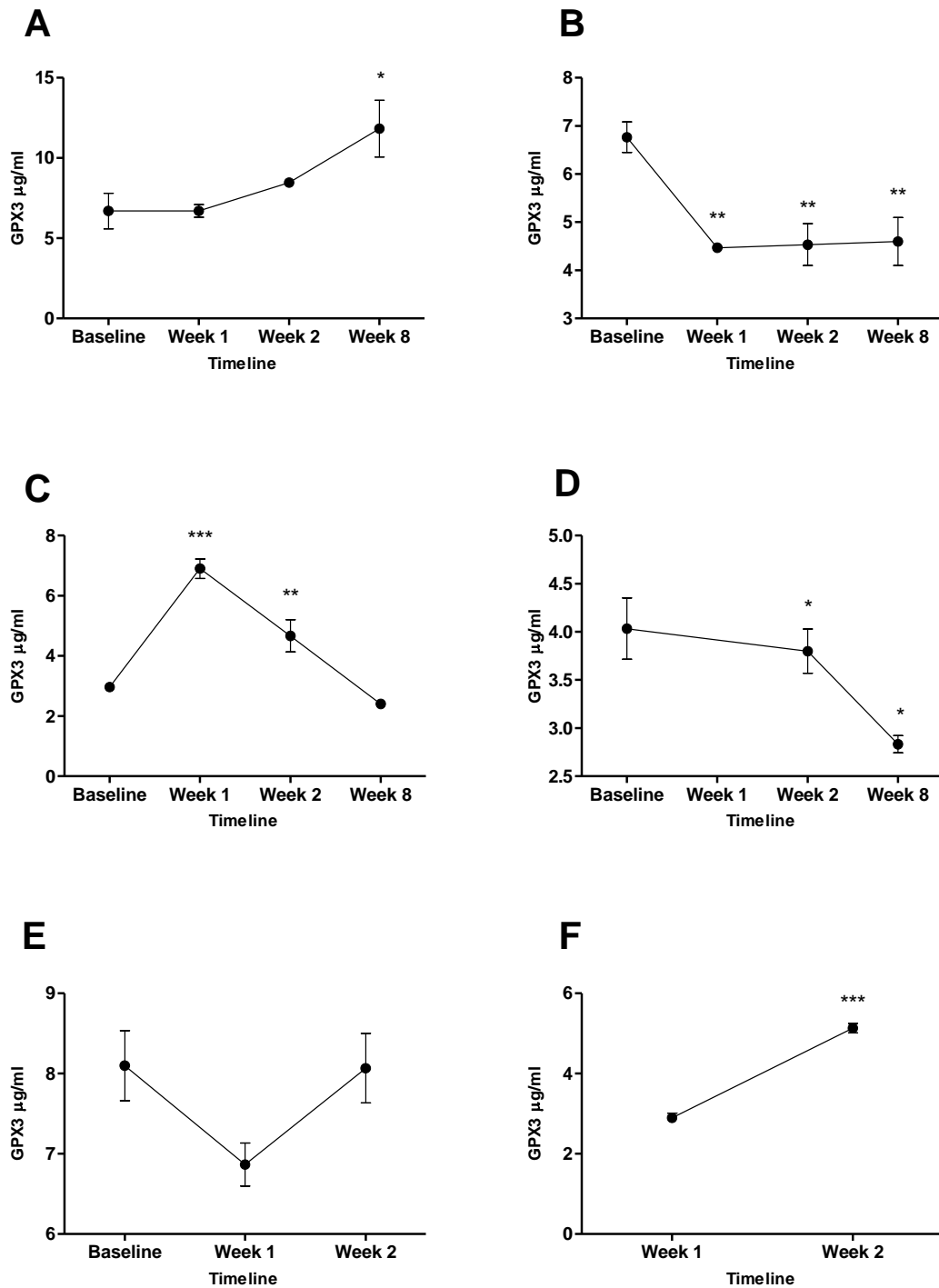
Figure 22. **Plasma GPX3 concentration-time profiles (* p < 0.05, ** p < 0.005, *** p < 0.0001).**

4.3.5 ER stress response

Unfortunately full data sets were unable to be obtained but available results of pro-survival and apoptotic ER stress proteins measured in trial patients are presented in Figure 23.
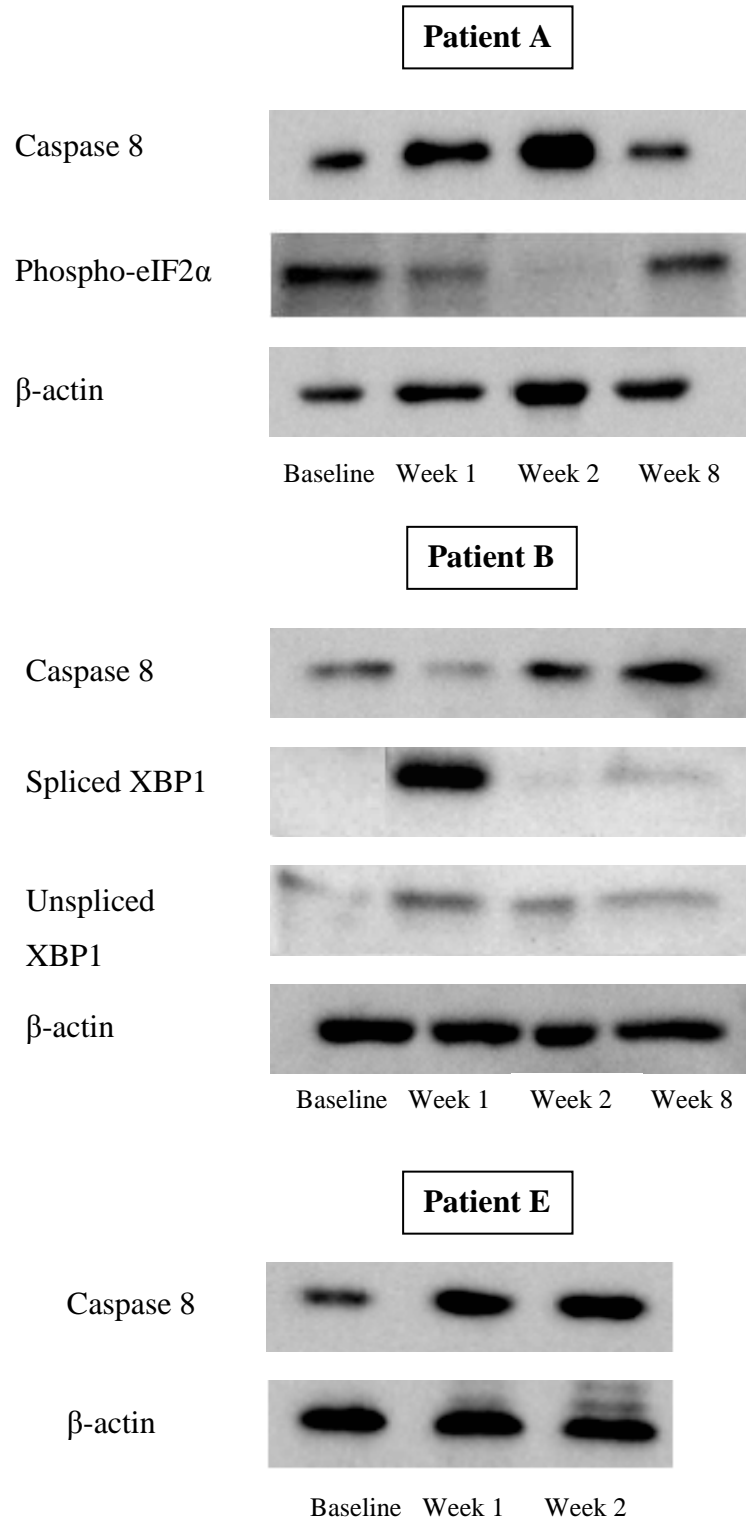
Figure 23: ER stress markers in HNSCC patients

4.4 Discussion

4.4.1 Comet assay

The comet assay has been used successfully in assessing endogenous or induced DNA damage of PBMC from human samples in numerous studies (Blasiak, Kowalik, Malecka-Panas, Drzewoski, & Wojewodzka, 2000; A. Collins, et al., 1997; Dusinska & Collins, 2008; Erkekoglu, et al., 2010; Kopjar, Garaj-Vrhovac, & Milas, 2002; Kotsopoulos, et al., 2010; Laffon, Valdiglesias, Pasaro, & Mendez, 2010; Santos, et al., 2009). In this study we assessed endogenous DNA damage of PBMC from HNSCC patients undergoing cisplatin and radiation treatment. The conditions of this assay were unable to detect any DNA damage, and conditions were shown to be working with other cells. However, this is not necessarily due to there being no damage present in cells from patients. RT is a potent inducer of DNA breaks, as shown in PBMC in the *in vitro* investigations detailed in section 3.3.3. Cisplatin is known to induce DNA inter- and intra-strand cross-links and previous work has demonstrated that cisplatin reduces the formation of comets *in vitro* (Merk & Speit, 1999; Pfuhler & Wolf, 1996). These authors concluded that the comet assay is a very sensitive tool for detecting the presence of cross-links through the reduction of comets. Therefore it would appear that the treatment of patients in this study with cisplatin has prevented the RT-induced DNA breaks in these patients' PBMC from resulting in DNA fragmentation that can be detected by the comet assay.

Since the aim of this investigation was to assess total DNA damage present in PBMC from patients being treated with both cisplatin and RT, and any effects of SLM on this, it would appear that the comet assay is not suitable at all for this purpose. If patients were undergoing RT treatment alone the comet assay may be more useful to detect total DNA damage in PBMC and an effect of SLM or other Se compounds could be investigated.

4.4.2 Markers of angiogenesis

4.4.2.1 VEGF-A

In this study, patients with HNSCC were randomized to either 3200 µg/m^2 SLM or placebo. While no indication of what study treatment patients received has been revealed at this point, there are intriguing results when examining individual patient responses during treatment.

In this study cohort inconsistent changes in plasma VEGF-A levels during therapy were seen. Two patients who continued with the trial until completion showed a significant increase in VEGF-A levels compared to baseline after eight weeks (B, D). Two other patients who participated in the trial for the full duration showed a significant decrease in VEGF-A from baseline at 8 weeks (A), and no change (C).

The significance of these changes is uncertain as longitudinal studies of changes in VEGF-A in patients during a course of treatment have not been described. However these changes might carry prognostic significance for the outcome of treatment given the importance of VEGF-A to tumour progression and metastasis. It is a well accepted that angiogenesis is essential for the growth and metastasis of solid tumours and there has been considerable research evaluating the association of angiogenic markers and the prognosis of various malignancies. Through examining the relationships between VEGF-A, microvessel density, epidermal growth factor, human papilloma virus, response to radiotherapy and clinical outcome in 85 tonsillar squamous cell carcinoma patients, Fie *et al.* (2009) showed that VEGF-A levels correlated with microvessel density and was associated with a poor prognosis. Similarly, Sullu and colleagues (2010) showed that VEGF-A expression may be important in the outcome of squamous cell carcinoma of the larynx. The authors found that the tumour grade was significantly associated with VEGF-A expression, and microvessel density was significantly higher in high-grade tumours. Furthermore, VEGF-A levels were correlated with the size of the tumour and lymph node metastasis. Another study involving 54 specimens of oral squamous cell carcinomas showed that tumour specimens, when compared to pre-neoplastic and normal tissues, had significantly elevated levels of VEGF-A (Margaritescu, et al., 2010). The poor prognosis associated with high VEGF-A expression and tumour metastasis has also been

confirmed in a number of other studies (Joo, Jung, Kim, & Sun, 2009; Onesto, et al., 2006; Tse, et al., 2007).

Given the poor prognosis associated with elevated levels of VEGF-A, as well as the reliance on VEGF-A expression for tumour progression, a number of studies have been carried out evaluating the effect of combining angiogenesis inhibitors with anticancer treatments. Examining the effects of bevacizumab, a monoclonal antibody that inhibits VEGF-A, Fujita et al. (2007) showed a dramatic inhibition of HNSCC xenografts in mice. When bevacizumab was combined with paclitaxel, another cytotoxic agent, there was a synergistic inhibition of tumour growth compared to using either agent separately. Furthermore, the authors showed that the microvessel density in tumours was reduced. Kruser et al. (2010) showed using a human HNSCC xenograft model that targeting the mediators of VEGF-A's action with motesanib, a potent inhibitor of VEGF receptors (including VEGF-R2), increased radiation sensitivity. These *in vitro* and *in vivo* data provide compelling evidence that VEGF-A, and the receptors which mediate the biological effects of VEGF-A, play an important role in the long-term response of HNSCC to treatment. The importance of these elevated levels of VEGF-A may be of clinical significance in later follow ups.

4.4.2.2 **VEGFR-1**

In this study VEGFR-1 levels changed inconsistently over the course of CT and RT. Only patient D had a significant increase in VEGFR-1 levels at eight weeks compared to baseline with other patients having very variable response during the treatment course. Patient C had a significantly reduced level of VEGFR-1 compared to baseline at eight weeks. These data indicate that angiogenesis mediators do indeed change during CT and RT in patients with HNSCC and that this response differs from patient to patient.

VEGFR-1 expression in tumours has been associated with poor survival in various cancers (Hirashima, et al., 2009; Okita, et al., 2009). Although the role of VEGFR-1 is not fully understood, it has been shown to be specifically involved in neoangiogenesis *in vivo* (Huusko, et al., 2010), in vascular branching (Kearney, et al., 2004) and activation of macrophage-lineage cell migration into tumour tissue

(Barleon, et al., 1996; Kerber, et al., 2008; Krysiak, et al., 2005; Muramatsu, Yamamoto, Osawa, & Shibuya).

As mentioned earlier, the same patient (D) shows a concurrent increase in VEGF-A and VEGFR-1 levels. This is interesting as increased levels of both VEGF-A and VEGFR-1 may reflect a poor survival outcome, which is consistent with previous studies (Fei, et al., 2009; Hirashima, et al., 2009; Joo, et al., 2009; Okita, et al., 2009; Onesto, et al., 2006; Sullu, et al., 2010).

4.4.3 Antioxidant capacity

This study investigated the levels of GPX3 in plasma of patients with HNSCC undergoing CT and RT. Plasma levels of GPX3 were shown to significantly change in relation to baseline in five of six patients examined, though inconsistently. Patient A showed no significant change in GPX3 levels during treatment, but at eight weeks GPX3 levels were significantly elevated. Patient C showed significantly elevated levels of GPX3 levels during treatment, but at eight weeks GPX3 had decreased to a level close to baseline. Patients B and D both showed decreased antioxidant capacity during treatment, which was maintained up to eight weeks.

It is difficult to interpret this study data without knowing whether each patient received SLM or not, but the results in the last two patients are consistent with previous studies that have demonstrated that cisplatin reduces antioxidant capacities of patients (Gupta, et al., 2010). Moreover, patients undergoing RT have been shown to have significantly reduced plasma Se levels after treatment compared to pretreatment levels (Franca, et al., 2010). There is little information regarding the expression of GPX3 during and after CT and RT.

Given that selenoprotein expression and activity is dependent on Se status, it is likely that the antioxidant capacity of patients would be reduced concurrently with lower plasma Se levels. Conversely, Se supplementation at high doses should increase GPX3 levels, unless patients had high baseline plasma Se that had already saturated GPX activity; indeed supplementation with as little as 200 µg of Se a day can saturate plasma GPX activity (Thomson, Robinson, Butler, &

Whanger, 1993). Thus the changes seen with several thousand micrograms of SLM are likely to be a lot less than might be expected.

Before treatment, cancer patients present with low-antioxidant levels and cancer therapies typically reduce the antioxidant capacity of the patient (Ladas, et al., 2004). Radiation damages cells directly by producing ROS which cause double-stranded DNA breaks, and altering other molecules in the cell. ROS formation is also one of the main mechanisms of many CT drugs, including cisplatin (Block, et al., 2007), and these ROS can often be the source of unwanted side-effects.

This raises an interesting debate as to the usefulness of antioxidant supplementation during anticancer therapies. Block et al. (2007) systematically reviewed the literature of antioxidant supplementation in combination with chemotherapy and found that antioxidant supplementation during chemotherapy resulted in fewer toxicities, allowing uninterrupted dosing. Reducing unwanted side-effects could lead to increasing continuation of treatment, as opposed to patients terminating treatment early due to unwanted toxicities. While none of the studies showed poorer outcomes in terms of survival, they were generally underpowered and poorly-designed to answer this question. Whether the activity of ROS can be safely blocked with antioxidants without compromising the anticancer efficacy of CT and RT remains to be determined.

It is questionable whether any improvement in antioxidant activity induced by Se supplementation significantly contributes to its modulation of the efficacy and toxicity of CT and RT, as the relative increase in antioxidant activity is likely to be modest, as discussed above, and there are several other mechanisms through which Se appears to mediate these modulatory effects. Nevertheless, the overall impact of Se in cancer trials appears to be beneficial. Most recently a phase III multicenter trial of RT $\pm$ CT for cervical and uterine cancer reported that Se supplementation reduced the proportion of patients experiencing severe diarrhoea from 46% to 21% (Muecke, et al., 2010).

4.4.3.1 Antioxidant capacity and angiogenesis

Interesting observations can be made when GPX3 levels are compared to VEGF-A levels. Both patients B and D, who had significantly reduced GPX3 levels at

eight weeks also had significantly elevated levels of VEGF-A. Conversely, elevated levels of GPX3 in patient A were accompanied by significantly reduced levels of VEGF-A.

This is consistent with previous observations that have shown that ROS play a critical role in not only normal, but also tumour angiogenesis (Maulik, 2002). Hydrogen peroxide treatment causes significant induction of VEGF expression which is blocked by antioxidants (Brauchle, Funk, Kind, & Werner, 1996). It has been shown that enhanced levels of hydrogen peroxide promote angiogenesis through the inhibition of PHD proteins leading to the accumulation of HIF (Komatsu, Kato, Nakayama, Miyagawa, & Kamata, 2008). As VEGF induction is dependent on HIF (Giaccia, et al., 2003), HIF accumulation leads to increased levels of VEGF-A. ROS are also involved in angiogenesis as down-stream signalling intermediates of VEGF-A (Colavitti, et al., 2002; Parenti, et al., 1998)

Various antioxidants have been shown to reduce angiogenesis. Antioxidants derived from Ginkgo biloba leaves were demonstrated to inhibit lymphocyte-induced angiogenesis (Monte, Davel, & de Lustig, 1994) and various other free radical scavengers, including thiol compounds, have been shown to inhibit the induction of macrophage-mediated angiogenesis (Fidelus, 1988; Koch, Cho, Burrows, Leibovich, & Polverini, 1988). T cell-induced angiogenesis is reliant on ROS (Fidelus, 1988) with hydrogen peroxide directly being involved in the angiogenic response (Monte, Davel, & Sacerdote de Lustig, 1997).

This inverse relationship between antioxidant activity and angiogenesis is consistent with data presented in this study, although admittedly the numbers of patients examined is too small to draw any conclusions in this regard. Similarly it is tempting to speculate that, given the decrease in antioxidant capacity of patients undergoing CT or RT from previous studies, patient A and C may well have received the SLM instead of placebo, as GPX3 levels at week eight were increased or maintained respectively. Conversely, patient B and D may have received placebo as both patients at week eight had significantly lower GPX3 levels and corresponding increase in angiogenic markers.

4.4.4 ER stress response

This study attempted to assess ER stress markers in HNSCC patients undergoing CRT. ER stress is an important mechanism in cancer therapeutics as demonstrated by the proteosome inhibitor bortezomib, which induces ER stress, and thus apoptosis, through increasing aberrantly-folded proteins (Szegezdi, et al., 2006). Se supplementation has been shown to act through targeting mediators of the ER stress signal (Wu, et al., 2005), resulting in an increase in apoptosis.

Additionally, Se appears to selectively sensitize tumour cells to a number of therapeutic drugs while at the same time increasing the resistance towards these drugs in normal cells (Cao, et al., 2004). This differential effect of Se administration on ER stress could, in part, account for the enhanced therapeutic efficacy of Se supplementation.

Unfortunately, full data sets of ER stress markers in patients were unable to be generated at this time. However, what is clear, in the three patients analysed, is that caspase-8 expression is modulated during CRT. What is interesting is that caspase-8 expression seems to be opposite that of pro-survival ER markers in PBMC, as expected. In patient A there were elevated levels of phospho-eIF2α at baseline. Expression of phospho-eIF2α decreased in week one and two compared to baseline, which coincided with increased levels of caspase-8 (Figure 23). At week 8, phospho-eIF2α levels were restored and caspase-8 levels were reduced. In patient B, spliced XBP1 was present at very high levels at week one. This also coincided with a slight decrease in caspase-8. When spliced XBP1 levels were reduced at weeks two and eight, caspase-8 levels were present at a higher abundance. A more complete data set would help to build a fuller picture, especially in the context of whether each patient had supplemental SLM or not.

4.5 Conclusions

1. The comet assay as used in this investigation is not a useful tool to use in assessing DNA damage in patients undergoing CRT, most likely due to DNA-cross-linking effects of cisplatin.

2. VEGF-A and VEGF-R1 levels are modulated in response to CRT but the absence of information on study treatment assignment means further interpretation is not feasible.

3. GPX3 levels are modulated in response to CRT and appear to be increased or maintained in patients that present more favourable levels of mediators of angiogenesis .

4. Insufficient data is available to adequately evaluate ER stress responses in PBMC from patients, though in general caspase-8 expression increases over time, as might be expected in cells exposed to CRT.

Chapter 5

General discussion

The overall goal of this work was to assess the biological effects of Se, cisplatin, RT and their combination on malignant and non-malignant cells, to provide insights into the biological markers which may be informative in clinical trials using Se compounds to modulate the efficacy and toxicity of CT and/or RT. Se compounds have been previously been shown to inhibit cell growth and inhibit apoptosis in a variety of tumour cell lines *in vitro* (Cho, Jung, & Chung, 1999; Jung, Zheng, Yoon, & Chung, 2001; Zeng, 2002). Furthermore, the effects of Se-induced apoptosis seem to be preferentially induced in malignant cells, not normal cells.

This study investigated the impact on cell viability of MSA, which provides a steady stream of methylselenol, the Se metabolite thought to mediate the therapeutic properties of Se. MSA was shown to inhibit THP-1 leukaemia cell growth and sensitize these cells to cisplatin and RT. Given these data, total GSH levels and various proteins known to be involved in ER stress and apoptosis were evaluated in both PBMC (from healthy donors) and THP-1 cells. GSH levels were shown to be dramatically reduced in response to MSA in THP-1 cells, while PBMC GSH levels increased under the same conditions. This is significant as GSH plays a pivotal role in a cell's ability to reduce toxic stress. These observations are consistent with previous studies that have shown that Se treatment modulates the sensitivity of tumours by reducing levels of total intracellular GSH.

ER stress markers were assessed and shown to be modulated in response to MSA. Pro-survival UPR proteins were upregulated in PBMC treated with MSA while no induction of the pro-apoptotic proteins CHOP or caspase-8 was observed. Conversely, caspase-8 in THP-1 cells was upregulated in response to MSA. The clinical trial also planned to evaluate the response to SLM of ER stress markers in

PBMC from HNSCC patients undergoing CRT. The exact pattern of ER stress in these clinical samples is still not clear, though the limited data available suggests that caspase-8 is increased in response to patient treatment and this modulation seems to be opposite to pro-survival UPR markers. This is consistent with the expected inhibition of protein translation due to phosphorylation of eIF2α, which would result in lower levels of pro-apoptotic protein translation, including caspase-8, and an increase in pro-survival proteins including spliced XBP1.

The importance of angiogenesis in tumour survival has been extensively documented (Fei, et al., 2009; Hirashima, et al., 2009; Joo, et al., 2009; Okita, et al., 2009; Onesto, et al., 2006; Sullu, et al., 2010). Patients that present with high levels of VEGF-A or VEGF-R1 typically have a poor prognosis. In this study the levels of both VEGF-A and VEGF-R1 were monitored in patients before and during treatment. Interestingly angiogenesis markers appeared to be upregulated at week 8 in patients that had significantly reduced GPX3 levels compared to baseline. Section 4.4.3.1 discussed the importance of antioxidant regulation in relation to angiogenesis. What may be occurring here is that high doses of Se modify tumour angiogenesis via an antioxidant mechanism, though this possibility has to be further evaluated.

Future clinical studies could usefully evaluate the effects of Se treatment on intracellular GSH in PBMC, as this appears to be a sensitive marker of Se effects, and determine the dose-response relationship for this effect. An increase in intracellular GSH in PBMC may parallel an increase in other normal tissues in response to Se supplementation that may, in part, mediate the observed benefits of Se in reducing toxicity of both CT and RT without compromising treatment efficacy.

There is insufficient data from this study to evaluate whether markers or angiogenesis or ER stress response may be useful indicators of Se effects in PBMC, and further evaluation of this in patients is warranted before any recommendation can be made. Similarly plasma GPX3 responses to Se cannot be adequately characterised from this study.

Overall this study has achieved part of its objectives, in defining the biological characteristics of the differential impact of Se on malignant and non-malignant cells in humans, with and without CT and RT. The results merit further investigation to better characterise the cellular responses and their potential clinical significance.

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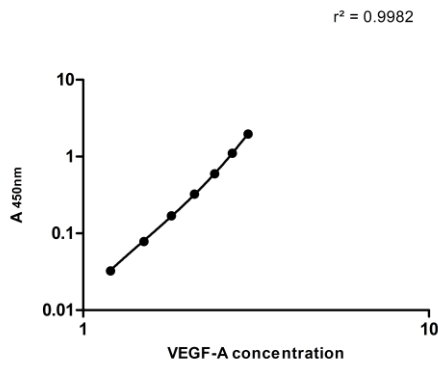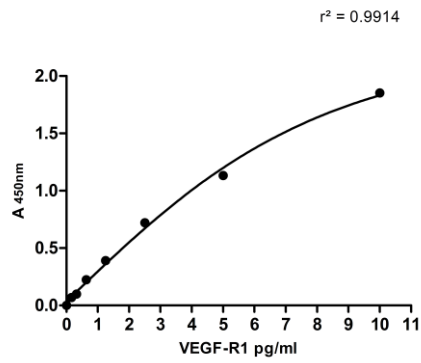
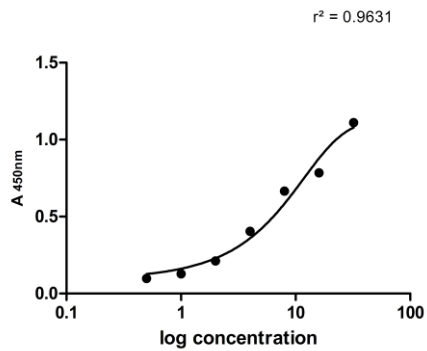# Appendix A
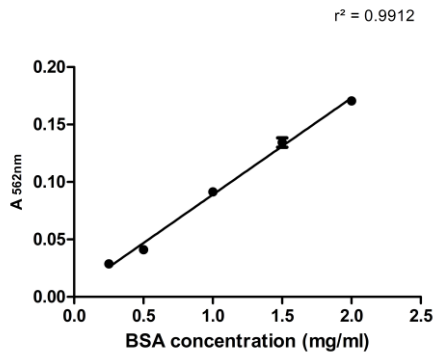
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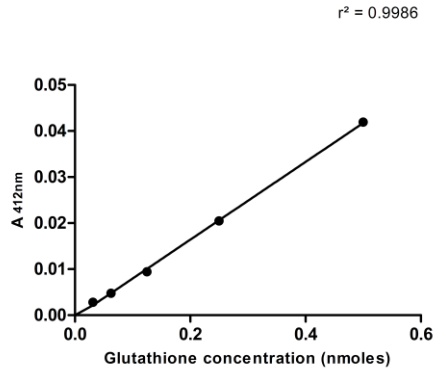
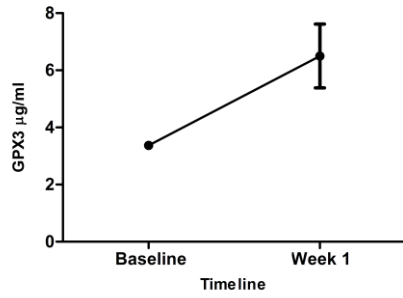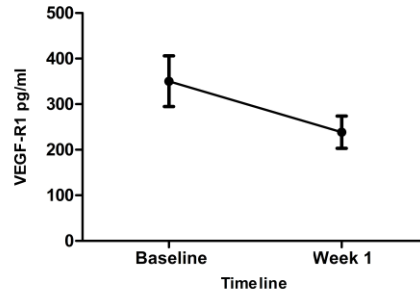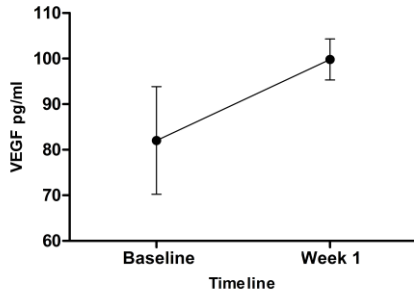Glutathione standard curve

VEGF-A standard curve

VEGF-R1 standard curve

GPX3 standard curve

Appendix B

Patient G

Patient H