Comparative Safety, Pharmacokinetic and Pharmacodynamic Evaluation of Three Oral Selenium Compounds in Cancer Patients

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Abstract

Selenium (Se) is an essential trace element involved in many biological processes in humans. Se compounds have demonstrated growth inhibition of malignant cells and tumours in many experimental models. Furthermore, combining Se with cancer therapies has yielded promising results in both preclinical studies and human trials, where reduced toxicity and enhanced anti-tumour efficacy is reported. However the optimal form and dose of Se in combination with anticancer therapies in terms of safety and efficacy has yet to be established, with particular concern about possible second malignancies due to DNA-damaging properties of both high-dose Se compounds and cancer therapies.

A key component of the toxicological study of new compounds is the assessment of their genotoxic potential. To achieve this a quantitative polymerase chain reaction-based method was developed that measured DNA damage within regions of nuclear and mitochondrial DNA relative to untreated controls, this assay was then validated in cancer cells and peripheral blood mononuclear cells (PBMCs). Subsequently, the method was optimised on a newly-acquired qPCR platform (Magnetic induction cycler) and used for measuring DNA damage in the clinical trial.

The primary research objective of determining the optimal form and dose of Se to be used alongside anticancer therapies was addressed through a randomised, double-blinded clinical trial. The safety, tolerability and pharmacokinetic (PK) and pharmacodynamic (PD) profiles of sodium selenite (SS), Se-methylselenocysteine (MSC) and seleno-l-methionine (SLM) were compared in patients with chronic lymphocytic leukaemia (CLL) and a cohort of patients with metastatic solid cancers. Patients received 400μg of elemental Se orally, as SS, MSC or SLM, for 8 weeks. Safety evaluations and bloods for PK and PD analyses were taken twice prior to treatment and at Days 2, 28 (week 4), 56 (week 8) and 84 (week 12) from commencing treatment. Each Se compound group (n=8) included four patients with CLL and four with solid cancers.

Twenty-four patients were treated and all Se compounds were well-tolerated, with no grade 3-4 toxicities attributed to the study drugs. The baseline-corrected area under the concentration-time curve for plasma Se was markedly higher with SLM than MSC or SS, consistent with previous reports. Assessment of DNA damage in normal and malignant PBMCs revealed negligible genotoxicity. In CLL patients, no fall in blood lymphocyte count was seen in those treated with SS, whereas the majority of those treated with SLM or MSC showed a reduction, with one patient achieving a partial response. Persistent reductions in plasma VEGF were observed in 7/12 patients with CLL and 4/12 with solid cancers. Minimal changes were observed in intracellular glutathione or markers of the endoplasmic reticulum stress response.

In conclusion, Se compounds are well-tolerated and non-genotoxic at this dose. A reduction in plasma VEGF occurred at lower doses than predicted whereas changes in ER stress markers and intracellular glutathione are consistent with the dose-response seen in tumour xenografts and cell lines. Evaluation of these compounds at the higher doses of 1600μg and 6400μg per day, where PD effects are predicted to be greater, is planned for a future study.

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3’-UTR	3’-untranslated region
$(CH_3)_2Se$	Dimethyl selenide
$(CH_3)_3Se$	Trimethylselenonium
2D	Two-dimensional
4PL	4-Parameter logistic
ABBCC1	ATP Binding Cassette C1
ALP	Alkaline phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
APPT	Activated partial thromboplastin time
Ara-C	Cytosine arabinoside
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATF4-6	Activating transcription factor 4-6
AUC	Area under the curve
BCA	Bicinochoninic acid
BCL2	B-cell Lymphoma 2
bFGF	Fibroblast growth factor
BID	Twice a day
BIP/GRP78	Endoplasmic chaperone BiP
BM	Bone marrow
bp	Base pair
BRCA1	Breast cancer 1 early onset
BSA	Bovine serum albumin

c-myc	v-myc avian myelocytomatosis viral oncogene homolog
CA-125	Cancer antigen 125
CBC	Complete blood count
CCRT	Concurrent chemo-radiation
cdk	cyclin-dependent kinase
CH_3SeCys	Se-methylselenocysteine
CH_3SeH	Methylselenol
CHOP	C/BEP homologous protein
CHOP (chemotherapy)	Cytoxan (cyclophosphamide), Hydroxyrubicin (Adriamycin, doxorubicin), Oncovin (vincristine), Prednisone
CLL	Chronic lymphocytic leukaemia
C_{MAX}	Maximum concentration
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
CR	Complete response
CT	Chemotherapy
DDR	DNA-damage response
DHB	District Health Board
DIO (1-3)	Iodothyronine Deiodinase Type I(1-3)
DLT	Dose-limiting toxicity
DMSO	Dimethyl sulfoxide
DNMT	DNA methylation transferase
dNTPS	Deoxynucleoside triphosphates
E2F1	E2F transcription factor 1
ECG	Electrocardiogram

ECL	Enhanced chemiluminescence
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
Efsec	Sec elongation factor
eIF2a	Eukaryotic translation initiation factor 2a
eIF4a3	Eukaryotic initiation factor 4a3
ELISA	Enzyme-linked immunosorbent assay
EORTC-QLQ-C30	European Organisation for Research and Treatment of Cancer quality of life questionnaire C30
EOS	End of study
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
F	Female
FCS	Foetal calf serum
FISH	Fluorescence in situ hybridisation
Flk1	Foetal liver kinase1
Flt1	Fms-like tyrosine kinase
G-C	Guanine-Cytosine
G-CSF	Granulocyte colony-stimulating factor
GADD34	Growth arrest and DNA damage-inducible protein 34
GAM	GSH assay mixture
Gd	Gadolinium
Ge	Germanium
GI	Gastrointestinal
GPx (1-4,6)	Glutathione peroxidase (1-4,6)

GRP78	Glucose regulated protein 78
GSH	Reduced glutathione
GSSeSG	Selenodiglutathione
Gy	Gray
H_2Se	Hydrogen selenide
HCL	Hydrochloride
HDAC	Histone deacetylase
HDSCC	Head and neck squamous cell carcinoma
HIF-1α	Hypoxia-inducible factor 1 alpha
HPLC	High performance liquid chromatography
HRP	Horseradish peroxide
HSePO3	Selenophosphate
HSP90	Heat shock protein 90
i p	Intraperitoneal
IARC	Agency for Research on Cancer
ICP-MS	Inductively-coupled plasma mass spectroscopy
iNOS	Inducible nitric oxide synthase
INR	International normalised ratio
IPA	Isopropyl Alcohol
ISIS	Integrated Sample Introduction System
K	Potassium
kb	Kilobase
kDa	Kilodaltons
LD50	Lethal concentration for 50% kill
LGC	Laboratory of the Government Chemist
LHRH	Luteinizing hormone-releasing hormone

LL	Leukaemia or lymphoma
LORD-Q	Long-run qPCR technique for DNA-damage quantification
M	Male
MAPK	Mitogen activated protein kinase
MIC	Magnetic Induction cycler
mJ	miljoules
MMP	Matrix metalloproteases
MOPS	3-(N-morpholino)propanesulfonic acid
MQ	MiliQ
mRNA	Messenger RNA
MRPs	Multidrug resistant proteins
MSA	Methylseleninic acid
MSC	Se-methylselenocysteine
MTD	Maximum tolerated dose
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MTT	Methyl-thiazol-tetrazolium cell viability assay
Na	Sodium
NCI-CTCAE	National Cancer Institute common terminology criteria for adverse events
nDNA	Nuclear DNA
NER	Nucleotide excision repair
NOAEL	No Observed Adverse Effect Level
NPC	Nutritional Prevention of Cancer trial
NSCLC	Non-small cell lung cancer
O_2	Oxygen

OM	Oral mucositis
OS	Overall survival
p53	Tumour protein p53
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
PD	Pharmacodynamic(s)
PDGFB	Platelet derived growth factor B
PERK	PRKR-like ER kinase
PHA-M	Phytohemagglutinin-M
PHD 2+3	Prolyl hydroxylase 2+3
PK	Pharmacokinetic(s)
PLGF	Placental growth factor
p.o.	Oral
Pro	Proline
PS	Performance status
PSA	Prostate-specific antigen
Q week	Weekly
QD	Once a day
qPCR	Quantitative Polymerase chain reaction
RBC	Red blood cell
RFS	Relapse-free survival
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Radiotherapy

SCLC	Squamous cell lung carcinoma
SCOTT	Standing Committee on Therapeutic Trials
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS -PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Se	Selenium
SE	Standard error
Sec	Selenocysteine
SECIS	Sec insertion sequence
SELECT	Se and Vitamin E Cancer Prevention Trial
SELENOF /Sep15	Selenoprotein F
SELENOH	Selenoprotein H
SELENOI	Selenoprotein I
SELENOK	Selenoprotein K
SELENOM	Selenoprotein M
SELENON	Selenoprotein N
SELENOO	Selenoprotein O
SELENOP/SEPP1	Selenoprotein P
SELENOS/SEPS1	Selenoprotein S
SELENOT	Selenoprotein T
SELENOV	Selenoprotein V
SELENOW	Selenoprotein W
SEM	Standard error of mean
SeO_2	Selenium dioxide
SLM	Seleno-L-methionine

SPS2	Selenophosphate synthase 2
SS	Sodium selenite
ST	Solid tumours
sXBP1	Spliced X-box binding protein 1
TBS	Tris buffered saline
TBS-T	Tris buffered saline- tween
TCA	Trichloroacetic acid
TE	Tris -EDTA
TGAM	Total glutathione assay mixture
TME	Tumour microenvironment
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
TrxR (1-3)	Thioredoxin reductase (1-3)
ULN	Upper limit of normal
UPA	Urokinase plasminogen activator
UPR	Unfolded protein response
UV(C)	Ultraviolet (C)
uXBP1	Xbp1 unspliced
VEGF (A)	Vascular endothelial growth factor (A)
WBC	White blood cell
XPC	Xeroderma pigmentosum complement group C
γ-GCS	γ- glutamylcysteine synthetase
γ-glutamyl-CH_3SeCys	γ-glutamyl-Se-methyl-selenocysteine

Chapter One: Introduction

1.1 Selenium and human health

The trace element selenium (Se) was discovered by the Swedish chemist Berzelius in 1817 and is named after the Greek moon goddess *Selene* [1]. Se was established as an essential element for animal and human health following on from work by Schwarz and Foltz [2] who showed that trace amounts of Se were effective in preventing liver necrosis in vitamin E-deficient rats. Since then 25 encoded Se-containing proteins (referred to as selenoproteins) have been identified in man, many of which play key roles in key physiological pathways [3].

1.1.1 Importance for normal health and deficiency diseases

The importance of Se to human health has been extensively studied across numerous medical disciplines including endocrinology, fertility, neurology and oncology [3]. Se performs a plethora of fundamental roles in the maintenance of human health. Selenoproteins containing one or more selenocysteine residues mediate effects on redox control, oxidative stress response, thyroid function, DNA synthesis, anti-inflammatory and immune responses with additional roles in sperm motility and viability [3]. Human diseases have been associated with severe Se deficiency such as Keshan disease, an endemic cardiomyopathy, and Kashin-Beck disease, an endemic osteoarthropathy, both of which were first identified in an area of China where soils were extremely low in Se [4].

Low Se intakes have been associated with increased cancer risk for certain tumour types [5]. Additionally, if the utilisation of Se is impaired then adverse consequences can precipitate, for example polymorphisms in selenoprotein genes have been associated with adverse health outcomes such as increases in bladder cancer in GPX-1-198Leu carriers [6]. More recently, single nucleotide polymorphisms in a number of selenoproteins including SEPP1 and GPX1 have been associated with breast cancer risk [7].

1.1.2 Intakes and geographical disparities

There is an enormous variation in dietary Se intakes worldwide, ranging from 7μg to 4990μg a day, with average intakes in Europe significantly lower than in the USA [8-10]. These huge differences in intake are multifactorial including the prevalence of nutritional supplementation, diet composition and the biogeography of Se.

Se is widespread in the Earth's crust at an average concentration of 0.09mg/kg [11]. The erosion of selenite and selenide-containing rocks accounts for Se occurrence in soils. However, in soils Se concentration varies considerably, being dependent on soil properties such as pH, redox status and microbiological activity. Shale soils are Se-rich whereas granite and volcanic soils are Se-poor [12]. Parts of the world with seleniferous (Se-rich) soils include the Great Plains of the USA and Canada, Hubei Province in China and parts of Columbia and Venezuela, whereas New Zealand, Denmark and Finland have on the whole non-seleniferous soils [13]. Importation of high-Se wheat from Australia has helped significantly increase Se intake in New Zealand from previously-low levels [8], though large geographical differences still remain with much lower intakes in the South Island due to sourcing locally-grown wheat [14].

1.1.3 Nutritional requirements

Recommendations for average daily Se intake are 60μg for men and 53μg for women [15] by the European Food Safety Authority or 55μg for both sexes by the Food and Nutritional Board of the National Academy of Medicine in the US [16]. Based on an analysis of all-cause mortality from adult participants in the US Third National and Nutritional Examination Survey with up to 18 years of follow-up, Se serum levels of 130 to 150μg/l are associated with the lowest hazard ratios for all-cause mortality [3]. However the idea of tailoring nutritional intakes to a target range of Se plasma levels and thus achieving optimal health outcomes is a vast over-simplification. The concept of Se status, as described by Combs [17], is comprised of four components, which include; intake, tissue Se (digestion,

absorption and transport), excretion and function. Despite this, a more accurate assessment of Se would be achieved by establishing suitable biomarkers for quantification of Se retention, of tissue Se levels and Se function to provide better information on the nutritional requirements for a given population.

1.1.4 Selenium biology including selenoproteins

The essential role of Se in human biology is due to its presence in proteins in the form of the amino acid selenocysteine (Sec).

1.1.4.1 Selenoprotein synthesis

1.1.4.1.1 Selenocysteine (Sec)

Selenocysteine or Sec is referred to as the 21st amino acid and is unique among the protein-forming amino acids. In contrast to the common amino acids the utilisation of Sec is fundamental to protein function, where Sec forms a key catalytic group in the active site [18]. Additionally it is the only naturally-occurring protein amino acid whose biosynthesis takes place with its own dedicated tRNA (Sec tRNA$^{(ser)sec}$) in all domains of life and is encoded by a UGA codon, which is a re-coding of a highly specific stop signal to Sec. Sec tRNA is synthesised by using serine as an intermediate, with selenophosphate synthase 2 (SPS2) directly involved in generating the monophosphate Se donor for the biosynthesis of Sec [19] and incorporation into the tRNA. Transcription of this codon is enabled by an RNA stem-loop structure referred to as SECIS (SEC insertion sequence). In eukaryotes the SECIS element is found within the 3'-untranslated region (3'-UTR) of the mRNAs, up to several kilobases from the UGA codon [20].

1.1.4.1.2 Sec insertion into protein

The incorporation of Sec into protein is a complicated and highly-regulated process involving a myriad of factors such as SECIS binding protein 2 (SBP2), bound to the SECIS element in the 3'-UTR of the selenoprotein mRNA and the Sec elongation factor (EFsec), bound with Sec tRNA$^{(ser)sec}$ with decoding occurring at the ribosomal acceptor site (Figure

1.1). Additional factors such as ribosomal protein L30, eukaryotic initiation factor eIF4a3, and nucleolin perform regulatory functions in the insertion process.

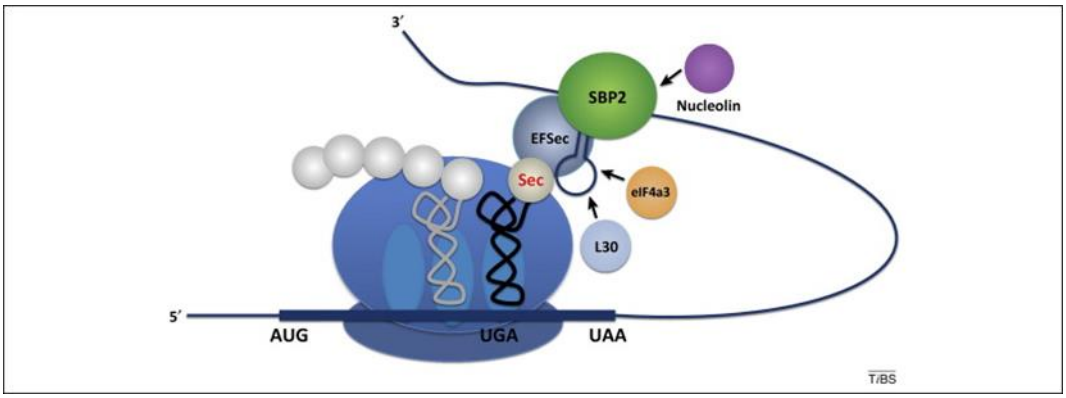

Figure 1.1 Incorporation of selenocysteine (Sec) into protein in mammals. Reproduced with permission (Elsevier) [21].

1.1.5 Human Selenoproteome

The human selenoproteome consists of 25 selenium-containing proteins, which are thought to mediate the physiological functions of selenium [21; 22]. To date only a portion of the selenocompounds have been characterised from a functional perspective [21].

1.1.5.1 Biological functions

The human selenoprotein family consists of five glutathione peroxidases, three thioredoxin reductases, three thyroid hormone deiodinases and a number of other proteins with varying functions and tissue/subcellular specificity. Table 1.1 summarises the Sec-containing proteins' known functions and location.

Table 1.1 Human selenoproteins, location and function. Reproduced with permission (Elsevier) [11; 21; 23; 24].

Selenoprotein	Location	Function
Glutathione peroxidase 1 (GPX1)	Cytoplasm	Reduces cellular hydrogen peroxide (H_2O_2)
Glutathione peroxidase 2 (GPX2)	Gastrointestinal	Reduces H_2O_2 in gut
Glutathione peroxidase 3 (GPX3)	Plasma	Reduces H_2O_2 in plasma
Glutathione peroxidase 4 (GPX4)	Widely distributed, often membrane-associated	Reduces phospholipid peroxidase, essential for sperm motility/viability
Glutathione peroxidase 6 (GPX6)	Olfactory epithelium	Unknown
Thioredoxin reductase Type 1 (TRXRD1)	Cytoplasm and nucleus	Regenerates reduced thioredoxin
Thioredoxin reductase Type 2 (TRXRD2)	Mitochondria	Regenerates reduced thioredoxin, essential for cardiomyocyte viability
Thioredoxin reductase Type 3 (TRXRD3)	Testes	Regenerates reduced thioredoxin
Iodothyronine deiodinase Type I (DIO1)	Liver, kidneys, thyroid and brown fat	Maintains systemic active thyroid hormone levels.
Iodothyronine deiodinase Type 2 (DIO2)	Central nervous system (CNS), brown adipose and skeletal muscle	Endoplasmic reticulum (ER) enzyme important for local active thyroid hormone

Selenoprotein	Location	Function
Iodothyronine deiodinase Type 3 (DIO3)	Placenta, foetus, uterus and CNS	Thyroid hormone inactivation
Methionine sulfoxide reductase B (MSRB1)	Cytoplasm and nucleus	Reduces sulfoxidated methionines on proteins
Selenoprotein F (SELENOF/Sep15)	ER-resident	A thiol-disulfide oxido-reductase with a putative role in protein folding
Selenoprotein H (SELENOH)	Nuclear, found in spleen and brain	Gene regulation of glutathione synthesis, redox sensing and transcription
Selenoprotein I (SELENOI)	Highest expression in brain and small intestine	Putative role in phospholipid synthesis
Selenoprotein K (SELENOK)	ER-resident	Calcium flux in immune cells, putative role in ER-associated degradation
Selenoprotein M (SELENOM)	ER-resident, neuronal cells	Possible role in regulation of body weight and energy metabolism
Selenoprotein N (SELENON)	ER-resident	Muscle development
Selenoprotein O (SELENOO)	Mitochondria	Possible redox role

Selenoprotein	Location	Function
Selenoprotein P (SEPP1)	Plasma (accounts for >50% of plasma Se reserves)	Homeostasis of tissue Se and transport
Selenoprotein S (SELENOS)	ER-resident	Role in ER-associated degradation
Selenoprotein T (SELENOT)	ER-resident	Calcium mobilisation, mechanism unclear
Selenoprotein V (SELENOV)	Testes-specific	Unknown
Selenoprotein W (SELENOW)	Prostate, brain, colon, heart and skeletal muscle	Putative antioxidant role, perhaps important in muscle growth
Selenophosphate synthase (SP2)	Kidney, liver, testes	Selenoprotein synthesis

1.1.5.1.1 Hierarchy

Dietary Se is necessary for selenoprotein biosynthesis, yet it has been observed that there is a hierarchy of selenoprotein synthesis with low levels of Se intake. In the case of the glutathione peroxidase family, synthesis of GPX1 protein and its corresponding mRNA are markedly reduced during Se deficiency, whereas GPX4 is largely maintained at low exposures [7]. It is important to note, however, that hierarchy is further complicated by tissue specificity, where in the case of GPX4 it ranks so high in the testes that it's hard for it to be depleted even at very low intakes, yet rapidly declines in non–privileged tissues such as the liver [25; 26].

1.1.5.1.2 Use as biomarkers of Se status

The plasma compartment-based selenoproteins GPX3 and selenoprotein P (SEPP1) are often used as measures of Se intake. Both

GPX3 and SEPP1 show plateaued expression at Se intakes that are deemed nutritionally adequate, with maximal expression observed at Se plasma concentrations of 50-70ng/ml [17; 27]. However, the plasma-abundant selenoproteins GPX3 and SEPP1 are of use in identifying nutritional deficiency and in monitoring responses of Se treatment in deficient individuals.

1.1.6 Se compounds

Foodstuffs contain diverse amounts and chemical forms of Se, including both organic and inorganic compounds (Figure 1.2 and Figure 1.3). Some of the most common Se compounds include: i) L-selenomethionine (SLM), the analogue of methionine in which Se has been incorporated in place of sulfur and is found mainly in plant sources, notably cereals; ii) Se-methylselenocysteine (MSC), which is found in plant sources such as garlic, Se-enriched yeast, onions and broccoli; iii) sodium selenite (SS) and selenate, which are components of dietary supplements, however selenate can also be found in plant sources (such as cabbage) and fish.

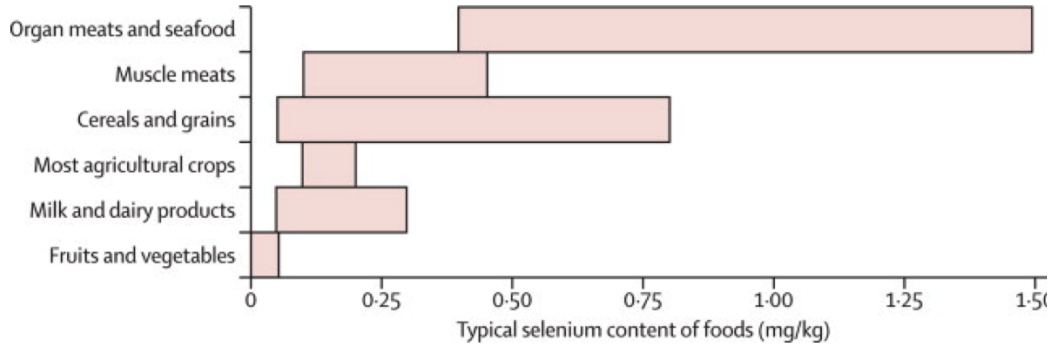

Figure 1.2 Typical Se content of common food sources. Reproduced with permission (Elsevier) [3].

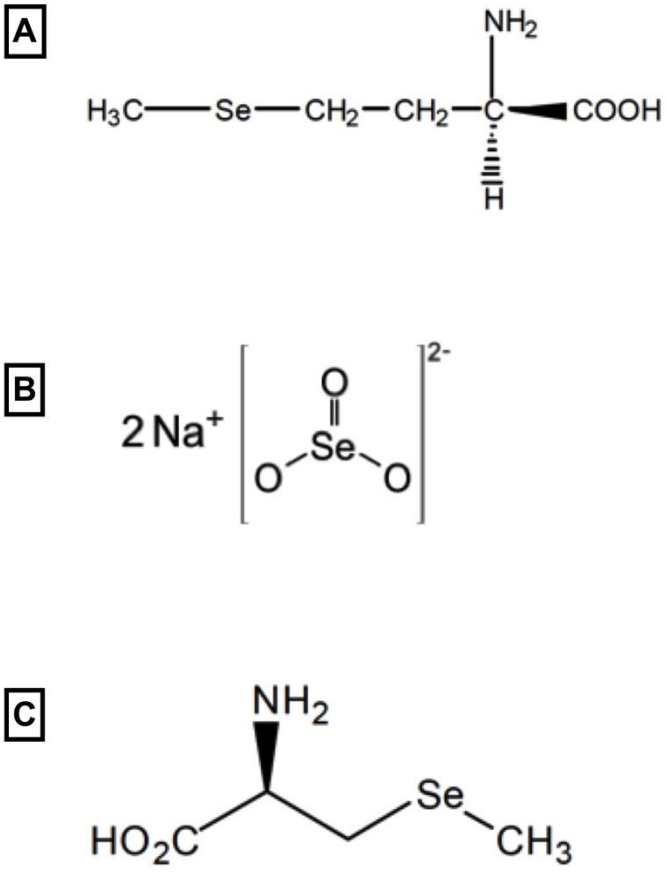

Figure 1.3 Selenium compounds. A) L-selenomethionine (SLM), B) sodium selenite (SS), C) Se-methylselenocysteine (MSC).

1.1.6.1 Pharmacokinetic (PK) characteristics

1.1.6.1.1 Absorption

1.1.6.1.1.1 L-selenomethionine (SLM)

Upon ingestion SLM is absorbed in the small intestine [28] via the same sodium ion-dependent carrier system used by methionine [29]. The bioavailability of SLM is in excess of 90% and has been shown in a study in four women using a radioisotope (^{75}Se–SLM) where >95% intestinal absorption was seen [30]. Additionally, more recent studies concur with these values in different cohorts of healthy volunteers [31; 32].

1.1.6.1.1.2 Sodium selenite (SS)

The absorption of SS via passive diffusion [33] has been found to be lower than that of the organic Se-containing compounds such as SLM [34]. Radioisotope studies in the 1970s demonstrated a range of absorption values from 44-77% at a low dose [35], whereas a single-dose study using 200μg of Se resulted in 84% bioavailability [36].

1.1.6.1.1.3 Se-methylselenocysteine (MSC)

Limited data exists on the oral bioavailability of MSC, however it has been reported that Se is readily absorbed from broccoli in which MSC is the predominant Se species [37]. Marshall *et al* compared MSC and SLM at two supra-nutritional daily doses of Se, 400 and 800μg, in Se-replete healthy volunteers [38]. The plasma Se maximum concentrations (C_{max}) obtained on day one were similar for both compounds, suggesting that MSC is comparable to SLM in terms of oral bioavailability.

1.1.6.1.2 Distribution

1.1.6.1.2.1 L-selenomethionine (SLM)

This selenoamino acid is unique in its distribution profile when compared with MSC and SS. SLM is non-specifically incorporated into body proteins such as albumin in place of methionine [39]. Animal studies have shown significant accumulation of Se as SLM in muscle, which is 10 times that of SS [40]. In human subjects levels of Se in skeletal muscle correlated directly with dietary intake of SLM [41].

1.1.6.1.2.2 Sodium selenite (SS)

Subsequent to oral intake and intestinal absorption of SS, Se distributes predominantly to the liver and kidneys of humans and animals [42].

1.1.6.1.2.3 Se-methylselenocysteine (MSC)

The tissue distribution of radioisotope-labelled MSC, methylseleninic acid (MSA) and SS was compared in Se-depleted rats following a single oral dose of 10μg of Se per kg of body weight [43]. The authors concluded

that MSC was more efficiently taken up in most organs, especially in the pancreas and duodenum, with similar uptake to the other compounds observed in the liver, spleen and kidney.

1.1.6.1.3 Metabolism and elimination/excretion

There are distinct differences in the metabolic processing and elimination of SLM, SS and MSC (Figure 1.4).

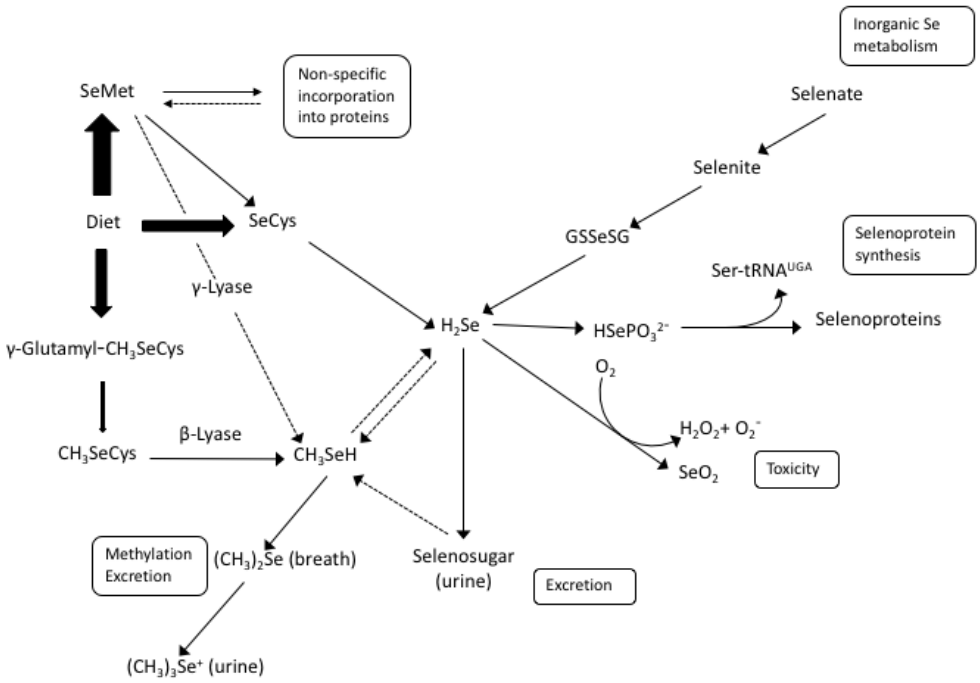

Figure 1.4 Metabolic pathway of dietary Se in humans. Selenomethionine (SeMet); selenocysteine (SeCys); selenodiglutathione (GSSeSG); γ-glutamyl-Se-methyl-selenocysteine (γ-glutamyl-CH_3SeCys); hydrogen selenide (H_2Se); selenophosphate ($HSePO_3$); Se-methylselenocysteine (CH_3SeCys); methylselenol (CH_3SeH); dimethyl selenide ($(CH_3)_2Se$); selenium dioxide (SeO_2); trimethylselenonium ion ($(CH_3)_3Se$). Reproduced with permission (Cambridge University Press)[44].

1.1.6.1.3.1 L-selenomethionine (SLM)

Once absorption takes place SLM is metabolised to hydrogen selenide via either selenocysteine or methylselenol. Subsequent incorporation into

selenoprotein via selenophosphate, methylation or conversion to selenosugars and excretion or oxidation yielding reactive oxygen species are three main metabolic fates of selenide [44]. Alternatively SLM may be incorporated non-specifically into protein in place of methionine resulting in total body accumulation of Se.

There is good evidence that the increased Se status attained after supplementation with organic forms of Se, such as MSC and SLM, is maintained for a longer period after their discontinuation compared to inorganic forms such as SS. Reported whole-body half-lives of SLM and SS in humans were 252 and 102 days respectively [45]. Accordingly, foods or supplements containing SLM can maintain the activities of selenoenzymes during periods of Se depletion for longer than those containing inorganic Se, owing to the recycling of SLM catabolised from protein stores.

1.1.6.1.3.2 Sodium selenite (SS)

Selenite is reduced to hydrogen selenide either directly by the thioredoxin system [46] or via the intermediate selenodiglutathione and glutathione-mediated glutathione reductase [47]. The absence of non-specific uptake into body protein results in lower retention compared to SLM [45].

1.1.6.1.3.3 Se-methylselenocysteine (MSC)

This monomethylated selenoamino acid is directly converted to methylselenol via the action of β-lyase found in many tissues. Methylselenol is then converted to selenide or undergoes methylation and excretion [44]. The urinary excretion of Se was measured in healthy patients taking SLM or MSC or placebo [38], with no significant differences in total urine excretion on day 84 observed between the patients taking MSC and SLM.

1.1.6.2 Safety

Se is thought to have a U-shaped toxicity profile in relation to Se status (Figure 1.5), with toxicity noted at both extremes of deprivation and excess [3].

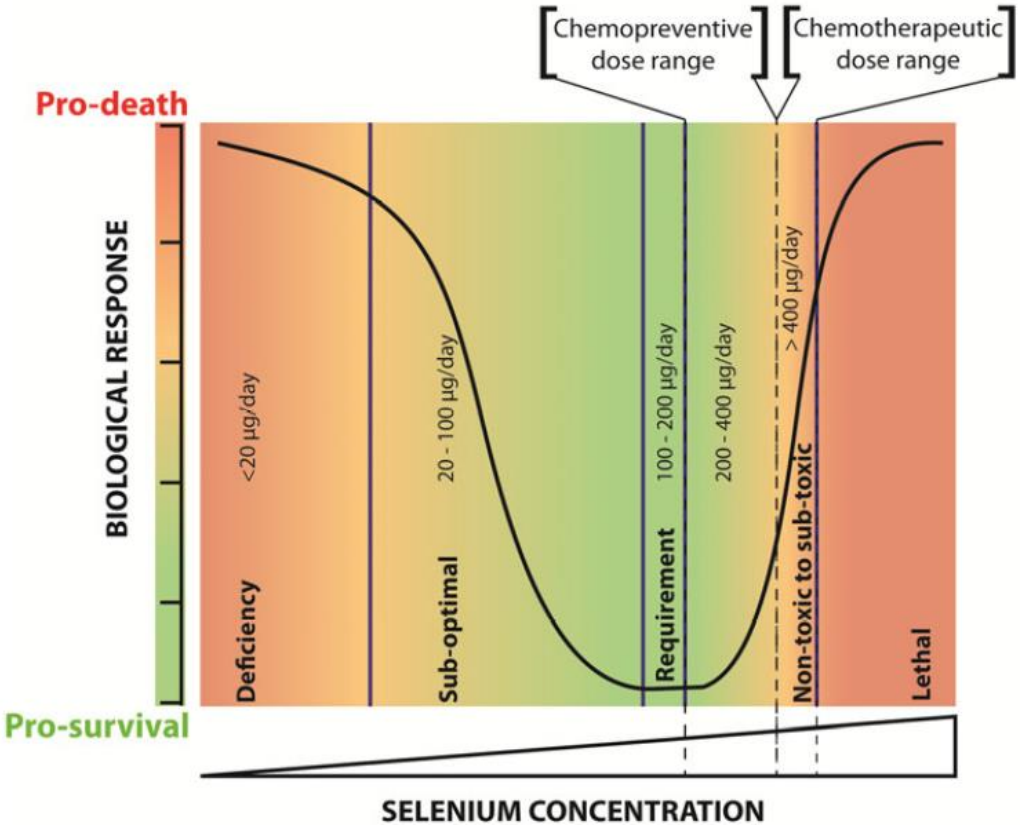

Figure 1.5 Selenium U-shaped toxicity profile. Reproduced with permission (John Wiley and Sons) [48].

Animal laboratory studies have shown that the organic forms of Se are both more effective and safer than the commonly-used inorganic forms such as SS, which are more genotoxic [49]. From 50% lethal dose (LD_{50}) determinations, SS was found to be four-fold more toxic than SLM when administered to mice intravenously and three-fold more toxic than Se-yeast when given orally to rats [8].

Unfortunately, human safety data is much more limited. The No Observed Adverse Effect Level (NOAEL) for Se in humans is variably reported as 400–850 µg/day [8; 50; 51]. Chronic Se toxicity would be expected after long-term consumption (over months to years) of more than 2,400–3,000 µg/day but is reversible [52]. Based on the available published literature, likely symptoms of toxicity (due to environmental exposure) include brittle hair and nails and their loss, gastrointestinal disturbances,

skin rash, garlic breath odour (caused by volatile selenium compounds) fatigue, irritability, and nervous system abnormalities. Impaired natural killer cell activity and endocrine disturbances may also occur [52].

1.1.6.2.1 Human studies at supranutritional doses

1.1.6.2.1.1 Sodium selenite (SS)

In a randomised controlled trial, SS was given orally to 20 patients with intermediate- and high-grade non-Hodgkin lymphoma at a dose of 200µg/kg/day on days 3 to 7 following their first course of chemotherapy [53]. This equated to a SS dose of 14,000µg daily for a 70kg patient. It was reported that 90% of patients experienced garlicky breath odour and some gastrointestinal upset (nausea and occasional vomiting) that was controlled with antiemetics. Additionally, a dose-escalation study evaluating intravenously-administered SS in cancer patients reported no dose-limiting toxicities below 10,200µg/m^2 per day (administered for 5 days per cycle), with the most common side effects observed being nausea, vomiting and fatigue [54].

1.1.6.2.1.2 L-selenomethionine (SLM)

A phase I study was conducted to determine the dose of SLM in combination with irinotecan required to achieve a plasma Se concentration greater than 15µM after 1 week of loading [55]. SLM was administered orally, twice a day for the first 7 days followed by a daily maintenance dose. Doses were escalated (Table 1.2) and SLM was observed to be well-tolerated in all patients, with attributable toxicity limited to mild garlic-like odour in breath and urine in about 50% of the patients [55]. This was seen more commonly during the initial period of SLM treatment and tended to ameliorate or disappear with prolonged treatment. No skin or nail toxicities secondary to SLM were documented. The non-tolerable dose of SLM was not defined in this study but the highest dose level administered in conjunction with irinotecan chemotherapy (7200µg twice a day for 1 week followed by 7200µg daily) was deemed to be the maximum tolerated dose (MTD), due to 1 of 6 patients developing grade 3 infection, neutropenia, diarrhoea, and hyponatraemia.

Table 1.2 Dosing schema within an SLM study. Reproduced with permission (Springer Nature)[56].

Phase I escalation schema			
Dose level	SLM loading (D-7–D-1)[a] (mcg PO BID)	SLM maintenance (D1 and on) (mcg PO QD)	Irinotecan (mg/m^2) Q week (start on D1)
1	3,200	2,800	125
2	3,200	3,200	125
3	4,000	3,200	125
4	4,000	4,000	125
5	4,800	4,800	125
6	5,600	5,600	125
7	7,200	7,200	125

[a] Day 1 follows day-1 (no day 0)

1.1.6.2.1.3 Se-methylselenocysteine (MSC)

A phase I, single dose, dose-escalation study was conducted administering MSC orally to fasted subjects at 3 dose levels: 400µg, 800µg and 1200µg [57] Serial blood and urine sampling was carried out along with formal toxicity evaluation using National Cancer Institute (NCI) Common Toxicity Criteria (CTC) v 3.0 [58]. No toxicities were reported that were deemed to be greater than grade 1. More recently, a multi-dose study compared a daily dose of elemental Se of 400µg or 800µg as MSC, SLM or placebo for 84 days in healthy volunteers [38]. The authors reported no toxicity over the course of the study as assessed by using the same formal toxicity evaluation as the previous study.

1.2 Se and cancer prevention

The initial hypothesis that Se may reduce cancer risk first surfaced in the 1960s after ecologic studies indicated an inverse relationship between Se content in forage crops and regional cancer rates in the US [59]. Schrauzer and colleagues [60] correlated age-corrected cancer mortality

data across a number of tumour types against apparent dietary Se intakes based on food consumption data from 27 countries. A significant inverse relationship was seen for large bowel, rectum, breast, ovary, prostate and lung tumours and for leukaemia.

Interesting results have also been obtained in animal models that have been used widely to investigate the potential cancer chemopreventive properties of Se supplementation [61]. Additionally, a review from 1986 [62] had estimated that a significant reduction in spontaneous, chemically-induced or virally-induced cancers with Se compound exposure could be seen in over half the studies. Subsequently, increased Se intake via diet or drinking water has been demonstrated to protect laboratory animals from developing mammary carcinomas [63]. However, epithelial cells from the prostate glands of Beagle dogs (which can develop spontaneous prostate cancer) showed increased apoptosis and a reduction in DNA damage when supplemented with either SLM or selenized yeast (SLM and MSC) in comparison to the controls [64].

1.2.1 Human studies

The Nutritional Prevention of Cancer (NPC) trial was the first double-blinded randomised chemoprevention study of Se in human subjects [65]. The trial recruited 1312 participants from south-eastern USA (an area known to have low Se intakes), who had a previous history of non-melanoma skin cancer. They were treated with 200μg of Se as selenized yeast (predominantly SLM) per day and follow-up after a mean of 4.5 years did not show benefit for the primary outcome of non-melanoma skin cancer. However, secondary analyses did reveal a significant reduction in cancer mortality for a number of common cancers including colon, prostate and lung, after a mean follow-up of 6.4 years. A later analysis of the data showed that only the reduction in prostate cancer mortality remained significant [66].

A more recent investigation, the Selenium and Vitamin E Cancer Trial (SELECT), used a 2x2 factorial double-blinded design to determine whether Se, in the form of 200μg of SLM, and/or vitamin E, could reduce the incidence of prostate cancer in 35,533 American men [67]. The study failed

to show a benefit for either or both interventions after a median follow-up of 5.5 years, and a recent update looking at the effect of the interventions according to baseline Se status (as reflected in toenail Se) also reported no benefit with SLM supplementation [68].

The negative results from the SELECT study has closed the door for some investigators on Se chemoprevention, while other groups point to the high baseline Se serum and toenail levels of study participants, the low percentage of smokers and the use of SLM instead of selenized yeast (SLM and MSC) as possible explanations for the disparity between these and the findings from the NPC study. This has led to significant controversy remaining in this area of research.

1.2.2 Mechanisms of prevention/ anti-tumorigenesis

Se affects various cellular processes and molecular pathways that may be involved in the anti-tumorigenic effects of Se (reviewed in [9]) which include:

- Seleno-enzymes involved in reducing DNA damage, oxidative stress and inflammation;
- Induction of phase II conjugating enzymes involved in detoxifying carcinogens and reducing DNA adduct formation;
- Enhancement of immune responses including cytotoxic lymphocyte and natural killer cell activity;
- Increase in tumour-suppressor protein p53, which inhibits proliferation, stimulates DNA repair and promotes apoptotic death by acting as a transcription factor for several genes, including the growth arrest and DNA damage-inducible (GADD) genes;
- Inactivation of protein kinase C, a signalling receptor that plays a crucial role in tumour promotion by oxidants;
- Alteration in DNA methylation, as abnormal methylation patterns are associated with neoplasia and inactivation of tumour-suppressor genes;
- Perturbation of the cell cycle resulting in growth inhibition, which may give time to allow DNA repair to take place;

- Induction of apoptosis of cancer cells, which generally involves the sequential activation of the caspases; and
- Inhibition of angiogenesis required for the growth and metastasis of tumours.

Reassuringly, there is a significant body of published work demonstrating these differential effects of Se compounds in normal malignant cells or tissues that would be favourable from both a chemoprevention and treatment perspective, as summarised in Table 1.3.

Table 1.3 Pharmacodynamic mechanisms of selenium supplementation in normal and malignant cells.

Pharmacodynamic properties	Effects in normal cells	Effects in malignant cells	Therapeutic significance
Antioxidant effects: a) Glutathione peroxidase (GPx)	Se-dependent GPx isoforms 1, 2, 3, 4 and 6 reduce oxidative damage; activity saturates with nutritional doses of Se [3]	Increased GPx1 activity in response to low dose Se (as SS and SLM) in LNCaP cells, resulting in reduced oxidative damage [69]	Mixed
b) Intracellular glutathione (GSH)	Increased GSH in PBMCs in response to MSA [70]	MSA reduced total GSH in leukaemia cells [70]	Favourable
c) Thioredoxin reductase (TrxR)	Essential selenoprotein [71], increased TrxR activity in red blood cells with selenized yeast [72]	At low dose SS and SLM increased TrxR activity and apoptosis in LNCaP cells (prostate adenocarcinoma) [69] as did SS in NSCLC cells [73], moreso in doxorubicin-resistant compared to doxorubicin-sensitive NSCLC cells [74]. Higher concentrations of SS reduced TrxR activity in mesothelioma [75], colorectal cancer [76] and NSCLC [73]. Differential effect with increase in TrxR activity and protein in doxorubicin-sensitive NSCLC but the opposite in doxorubicin-resistant NSCLC cells [74]	Mixed

Pharmacodynamic properties	Effects in normal cells	Effects in malignant cells	Therapeutic significance
DNA repair	Enhanced DNA repair with SLM in p53 wild-type mice and human fibroblasts [77; 78]	Increase in base excision repair in colorectal cancer lines by SLM is p53 wild type-dependent [79]. Unaltered DNA repair in SLM-treated p53-null human squamous cell carcinoma cell lines [80]. Low dose SS or SLM increased DNA repair in prostate cancer cell lines [69].	Mixed
Angiogenesis	In human umbilical vein endothelial cells MSA induced apoptosis *via* p38 MAPK activation whereas SS induced caspase-independent apoptosis [81]. MSA inhibited VEGF expression in mammary endothelial cells [82].	MSC and MSA reduced HIF-1α expression and thus angiogenesis in HNSCC and renal cell carcinoma models through inhibition of VEGF signalling mediated by COX-2, iNOS and degradation of prolyl hydroxylases under hypoxia [83-86]. This resulted in improved intra-tumoral vessel maturation, interstitial fluid pressure and reduced hypoxia [87].	Favourable
Invasion/Migration		Reduced cell invasion through inhibiting MMP-2, MMP-9 and UPA by MSA [88] and SS [89]	Favourable
Epigenetic		Inhibits DNMT and HDAC resulting in expression of silenced genes by SS [90] and MSA [91]	Favourable

Pharmacodynamic properties	Effects in normal cells	Effects in malignant cells	Therapeutic significance
Immunological	1mg/kg SS for 8 weeks increased antigen-specific $CD4^+$ T-cell responses [92]		Favourable
Drug efflux		Significant reduction in levels of the ABCC1 efflux pump in tumour cells [93]	Favourable

ATP Binding Cassette C1 (ABCC1); cyclooxygenase 2 (COX-2); DNA methyltransferase (DNMT); endoplasmic reticulum (ER); glutathione peroxidase (GPx); glutathione (GSH); histone deacetylase (HDAC); head and neck squamous cell carcinoma (HDSCC); hypoxia-inducible factor 1 alpha (HIF-1α); inducible nitric oxide synthase (iNOS); mitogen activated protein kinase (MAPK); matrix metalloproteases (MMP); methylseleninic acid (MSA); Se-methylselenocysteine (MSC); non-small cell lung cancer (NSCLC); tumour protein p53 (p53); peripheral blood mononuclear cells (PBMCs); selenium (Se); seleno-L-methionine (SLM); sodium selenite (SS); thioredoxin reductase (TrxR); urokinase plasminogen activator (UPA); vascular endothelial growth factor (VEGF).

1.3 Se use with cancer treatments

The challenge in using Se compounds in cancer patients in conjunction with chemotherapy and/or radiotherapy lies in being able to reduce normal tissue toxicities of these treatments without compromising their antitumour effects (or preferably enhancing them). However, despite numerous studies on the PD effects of Se in normal and malignant cells, it is still not known which form and dose of Se can be safely used and has the most favourable differential effect in normal and malignant tissues, especially in conjunction with chemotherapy and radiation. Current doses of Se are empirical or guided by PK, although the PK-PD relationship has not been established in humans, hence the optimal form and dose of Se to be used with chemotherapy or radiotherapy remains unclear. However, it has been demonstrated that PD biomarkers of Se effects can be measured in human white blood cells (WBC) *in vitro* and *in vivo* [94], thus enabling the relationship between Se PD and PK to be evaluated in clinical studies, which determine the optimal Se compound and dose to be incorporated into potentially-pivotal trials.

1.3.1 Efficacy (preclinical and clinical)

While Se is selectively cytotoxic to cancer cells at higher doses, it commonly augments the anticancer efficacy of chemotherapy and radiation in cell culture and tumour xenograft models (summarised in Table 1.3). Therapeutic synergy has been demonstrated between supranutritional doses of Se compounds and chemotherapy drugs, including cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin in human tumour xenografts of colorectal, ovarian, prostate and small cell and non-small cell lung carcinoma, head and neck squamous cell carcinoma and leukaemia [95-100].

However the strength of the interaction varied greatly, with differing xenograft models showing up to a 20-fold difference in Se dose potency [95]. Furthermore, the xenograft model and Se dose also influenced the apparent efficacy of the Se compounds. For example, at their MTD of 200µg/day

orally (approximately 8000µg/kg/day), MSC and SLM were both superior to SS in combination with irinotecan in head and neck squamous cell carcinoma xenografts (Figure 1.6) whereas SS and SLM (1000–1500µg/kg/day intraperitoneal (*i.p.*) injection) were equally dose-potent and effective when combined with cisplatin in an ovarian cancer xenograft model [100].

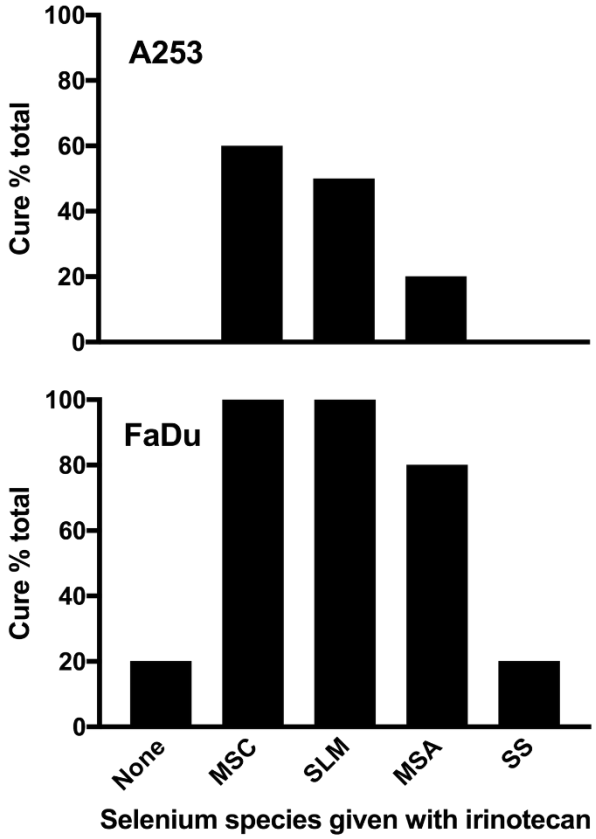

Figure 1.6 Tumour cure rates in nude mice with head and neck squamous cell carcinoma xenografts (5 mice per group). Se compounds differ in their ability to improve antitumour activity of irinotecan in xenografts. Se-methyl-selenocysteine (MSC); L-selenomethionine (SLM); methyl-seleninic acid (MSA); sodium selenite (SS). Courtesy of Dr Y Rustum. (Personal communication, reproduced with authors permission).

Attention should also be given to the schedule-dependency of Se in combination with other cancer treatments, where the effects of MSC or SLM have been seen to be maximal after 7 days pre-treatment, with little or no benefit found when co-administered without pre-incubation with Se in

human colorectal and head and neck squamous cell carcinoma xenograft models in mice [95; 101].

Very few clinical studies have been conducted to evaluate the impact of Se supplementation during chemotherapy or radiation on treatment efficacy. One randomised trial subjected 81 Se-deficient women to oral SS (500μg daily with radiotherapy and 300μg on non-radiotherapy days) or radiotherapy alone following surgery for gynaecological malignancies [102]. At a median follow-up of 67 months, disease-free survival was not found to be significantly different (log-rank p=0.65), although there was there a trend to improved overall survival in the Se-treated group (log-rank p=0.09) [103]. Another randomised, placebo-controlled phase 2 pilot study in 18 patients with locally-advanced head and neck squamous cell carcinoma receiving cisplatin concurrently with radiation administered oral placebo or SLM 3600μg/m^2 twice daily for 7 days prior to treatment then once daily until 3 weeks after chemoradiation completion [104]. This small study found there was no difference observed in disease-free or overall survival (log-rank p=0.39 and p=0.39 respectively). Lastly, a trial randomising 50 patients with non-Hodgkin lymphoma to either very high doses of SS (200μg/kg/day) with cyclophosphamide, vincristine, doxorubicin and prednisone chemotherapy or chemotherapy alone has been carried out [105]. A significantly greater tumour response rate was observed in the Se group compared to the controls (60% *vs.* 40% respectively), with correspondingly lower levels of the oncogene B-cell lymphoma-2 (*BCL2*) in bone marrow aspirates in Se-treated patients compared to controls.

1.3.2 Cancer treatment toxicity

Table 1.4 summarises extensive preclinical work that demonstrated the protective effects of various Se compounds against the toxicities of radiation [102; 106] and organ-specific toxicities of many cytotoxic drugs, including myelosuppression, mucositis, diarrhoea, alopecia, cystitis and nephrotoxicity [95; 96; 98; 107]. Specific toxicities of cisplatin in kidneys, bone marrow and intestine were improved without affecting antitumour efficacy [98; 108-111] or pharmacokinetics [112]. Impressive protection

from the lethal effects of six cytotoxic drugs with 200µg/day MSC was demonstrated in nude mice [96].

Similar protection by Se compounds from the normal tissue toxicities of chemotherapy has also been reported in several clinical trials (Table 1.5). In the setting of high-dose chemotherapy and allogeneic haematopoietic stem cell transplantation, a double-blinded randomised placebo-controlled study of oral selenized yeast in 77 patients reported a reduction in the incidence and duration of severe oral mucositis [113]. In a trial randomising 41 patients to 4,000µg Se (as seleno-kappacarrageenan) orally daily for 4 days before and after the first dose of cisplatin-based chemotherapy or to chemotherapy alone, significantly less myelosuppression and nephrotoxicity was seen in patients given Se [114]. A double-blind trial involving 62 women receiving cisplatin-based chemotherapy for ovarian cancer randomized patients to Se as selenized yeast, 200µg per day for 3 months, starting concurrently with chemotherapy [115]. Those patients randomized to Se experienced significantly less toxicity at 3 months of treatment, with a decrease in nausea, vomiting, abdominal pain, stomatitis, anorexia, alopecia, weakness and an increase in neutrophil counts. Another study, using high-dose SS in patients with non-Hodgkin lymphoma reported that the Se group had significantly fewer toxicities including a reduced infection rate and preservation of cardiac left ventricular ejection fraction in comparison to the control arm [116].

Another study reported significantly-increased blood Se with SS supplementation (the primary endpoint), and analysis of secondary endpoints showed a significant reduction in the actuarial incidence of grade 2 or worse diarrhoea (from 46.6% to 21.0%, p=0.039), without any Se-related side-effects [102]. Additionally, SS has also been assessed as a potential modulator of radiation-related toxicities in 39 head and neck cancer patients in a randomised controlled trial [106]. However, using the same SS dosing schedule as the study of Muecke *et al.* [102], there was no statistically-significant difference in the incidence of severe toxicities.

More recently SLM has been trialled alongside cisplatin-based chemoradiation for both head and neck squamous cell cancer (HNSCC) and

non-small cell lung cancer (NSCLC), with treatment toxicity as the primary outcome [104; 117]. In the HNSCC trial 18 patients were randomised to treatment with 3,600µg/m^2 SLM twice daily or placebo for 11 weeks, starting 1 week prior to chemoradiation. While no significant difference in grade 3 toxicities was observed, 50% of Se-treated patients had grades 0-1 mucositis compared to 25% in the placebo group [104]. In addition, a trend to reduced high-frequency hearing loss in SLM-treated patients was seen (Figure 1.7; unpublished data, M. Jameson). Lastly, a single-arm NSCLC study in 16 patients, administering 4,800µg/m^2 SLM twice daily for 7 days pre-radiotherapy then daily during chemoradiation, reported no Se-related toxicity but observed a lower-than-expected rate of grade 3 or worse toxicities, particularly for pneumonitis and anaemia [117].

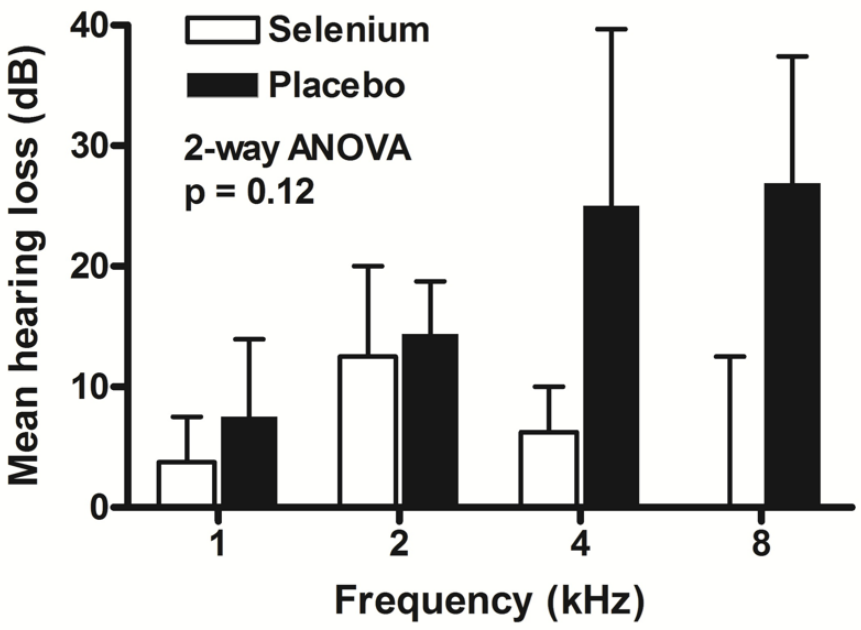

Figure 1.7 Hearing loss following chemoradiation in selenium-, and placebo-treated groups (n=6, 2 Selenium treated, 4 placebo, mean +/- SEM). Courtesy of M. Jameson.

Table 1.4 Combination of selenium with systemic therapies and radiation.

Pharmacodynamic properties	Effects in normal cells/tissues	Effects in malignant cells/tissues	Therapeutic significance
Antioxidant effects: Intracellular glutathione (GSH)	Protective increase in GSH in PBMCs in response to MSA, not compromised by combination with cisplatin, doxorubicin, Ara-C or radiation 2 Gy [70].	Pre-treatment of ovarian cancer xenograft-bearing mice with SLM or SS prevented an increase in intracellular GSH in response to cisplatin or melphalan [118]. MSA depletes intracellular GSH in THP-1 leukaemia cells despite combination with cisplatin, doxorubicin, Ara-C or radiation 2Gy [70].	Favourable
Cell death/ cytotoxicity	Protects against cisplatin-related renal proximal tubule injury [119]. Clinical trial evidence of less gastrointestinal, bone marrow, renal and cardiac toxicity with chemotherapy [113-116] and reduced diarrhoea after radiation [102; 106; 120].	Increased cytotoxicity in combination with chemotherapy, tamoxifen or radiation in several cell lines, including resistant cells *in vitro* [96; 121-125]. Therapeutic synergy between supra-nutritional doses of Se (as SLM, SS, MSA and MSC) and cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin in human tumour xenografts of small cell and non-small cell lung carcinoma, colorectal carcinoma, prostate carcinoma, head and neck squamous cell carcinoma and leukaemia [95-100]. Clinical trials show either uncompromised outcome or improved outcome [53; 102; 105; 115]	Favourable
Angiogenesis	MSC did not cause an increase in chemotherapy drug delivery to normal tissues [126]	MSC resulted in improved tumour blood vessel maturation and hypoxia with improved chemotherapy drug delivery to tumours [126; 127].	Favourable

Pharmacodynamic properties	Effects in normal cells/tissues	Effects in malignant cells/tissues	Therapeutic significance
Radio-sensitization	No radiosensitization by SLM in normal lung fibroblasts [128] MSA did not increase PBMC death from RT [70]	SLM caused radio-sensitization in two human lung cancer cell lines [128].	Favourable

Cytosine arabinoside (Ara-C); Gray (Gy); Methylseleninic acid (MSA); Se-methylselenocysteine (MSC); Peripheral blood mononuclear cells (PBMCs); Radiotherapy (RT); Selenium (Se); Seleno-L-methionine (SLM); Sodium selenite (SS).

Table 1.5 Clinical trials evaluating outcomes when selenium compounds were given with chemotherapy and/or radiotherapy.

Author, year published	Trial design [Patient number]	Cancer treatment [Cancer type]	Se form and dose	Outcomes: toxicity/efficacy
Hu *et al.* 1997 [114]	Randomised to Se with cycle 1 or 2 (not both) [n=41]	Cisplatin-based chemotherapy (60-80mg/m^2) [Lung, breast, gastric, colon, oesophagus, liver]	Oral kappa-seleno-carrageenan (4000µg Se/day for 4 days before and after chemotherapy)	• Significantly higher day 14 WBC 3.35±2.01 vs. 2.31±1.38 ×10^9/L ($p<0.05$) • Less G-CSF use, RBC transfusion and nephrotoxicity
Sieja *et al.* 2004 [115]	Randomised to Se or not [n=62]	Cisplatin (100mg/m^2) and cyclophosphamide (600mg/m^2) [Ovarian]	Oral selenized yeast 200µg/day for 3 months	• Significantly less nausea ($p<0.001$), vomiting ($p<0.001$), stomatitis ($p<0.0292$), abdominal pain ($p<0.0006$), decreased appetite ($p<0.001$) weakness ($p<0.001$), alopecia ($p<0.001$) • Significantly higher neutrophils • Trend to lower serum CA-125 (93.5 ± 200 *vs.* 228 ± 713 U/ml)
Weijl *et al.* 2004 [129]	Randomised to vitamins C, E and Se or placebo [n=48]	Cisplatin (60–100mg/m^2) [Testicular, bone, gastrointestinal, urogenital, head and neck, melanoma]	Oral SS 100µg twice daily in a milky beverage commencing 7 days before chemotherapy with cessation 3 weeks after completion of anticancer treatment	• No improvement in toxicity or response rate • 64% of supplement group non-compliant due to GI effects • Serum Se did not increase

Author, year published	Trial design [Patient number]	Cancer treatment [Cancer type]	Se form and dose	Outcomes: toxicity/efficacy
Asfour *et al.* 2006 [116]	Randomised to Se or not [n=30]	Day 1 cyclophosphamide 750mg/m^2, doxorubicin 50mg/m^2, vincristine 1.4mg/m^2 and prednisone 100mg daily x 5, Q3W [Non-Hodgkin lymphoma]	Oral SS 200µg/kg/day on days 3-7 of each chemotherapy cycle.	• Significantly less neutrophil apoptosis (67% *vs.* 20%, $p<0.05$) and reduced infection rate • Significantly better preservation of cardiac ejection fraction (62+/-4% *vs.* 69+/-5% $p<0.05$).
Asfour *et al.* 2007 [105]	Randomised to Se or not [n=50]	Same as Asfour 2006 [Non-Hodgkin lymphoma]	Oral SS 200µg/kg/day for first 30 days	• Significant decline of *Bcl-2* level in BM aspirate and increase in CD4:CD8 ratio in peripheral blood after 3 cycles (8.6 ± 6.9ng/ml vs. 36.9 ± 7.9ng/ml, P < 0.05) • Increased complete response rate in Se group (60% *vs.* 40%)
Fakih *et al.* 2006 [130]	Phase I dose escalation study [n=13]	Irinotecan 125–160mg/m^2 weekly [Colorectal, lung, pancreatic, gastric, mesothelioma]	Oral SLM 2200µg Se daily starting 1 week before chemotherapy continued for up to 10 cycles of treatment	• Irinotecan MTD was 125mg/m^2 • No diarrhoea >grade 2 at MTD • Responses seen in irinotecan-refractory population
Fakih *et al.* 2008 [56]	Phase I and PK study [n=31]	Irinotecan 125mg/m^2 weekly x 4 every 6 weeks [Metastatic or unresectable solid tumour]	Oral SLM; dose escalation from 2800–7200µg/day maintenance (twice daily loading for 1 week prior to starting irinotecan then continuing daily for up to 8 cycles)	• Stable disease >6 months in irinotecan-pre-treated patients • SLM well-tolerated at 7200µg dose level with irinotecan • No reduction in irinotecan toxicity

Author, year published	Trial design [Patient number]	Cancer treatment [Cancer type]	Se form and dose	Outcomes: toxicity/efficacy
Muecke *et al.* 2010, 2014 [102; 103]	Randomized to Se or not in patients with low blood Se (<84ng/ml) [n=81]	RT (external beam w/wo brachytherapy) [Endometrium or cervix]	Oral SS 500µg on days of RT and 300µg on days without treatment for up to 7 weeks.	• Significant increase in blood Se • Less ≥grade 2 diarrhoea (20.5% *vs.* 44.5%, *p*=0.04) • 5-Year survival 91.9% *vs.* 83.1% (*p*=NS) • 10-Year survival 55.3% *vs.* 42.1% (*p*=NS) • No Se side-effects observed
Buntzel *et al.* 2010 (2 papers with overlapping cohorts) [106; 120]	Randomised to Se or not in patients with low blood Se (not defined) [n=47/39]	RT (37/39 adjuvant) [Head and neck squamous carcinoma]	Oral SS 500µg on days of RT and 300µg on days without treatment for up to 7 weeks.	• Significantly less dysphagia in the last week of RT • Trend to less acute taste loss • No difference in xerostomia or mucositis • Significant transient increase in serum and whole blood Se
Corcoran *et al.* 2010 [131]	Phase I dose escalation [n=19]	Antiandrogens discontinued >4 weeks before trial; LHRH agonists were continued [Chemotherapy-naïve, castration-resistant prostate cancer]	Oral sodium selenate; dose escalation from 5000–90,000µg/day for 12 weeks (longer if responding)	• MTD was 60,000µg/day with linear PK • Fatigue, diarrhoea and muscle spasms dose-limiting at 90,000µg/day • One PSA response >50% and mean PSA doubling time increased

				(2.18 months before trial to 3.85 months)

Author, year published	Trial design [Patient number]	Cancer treatment [Cancer type]	Se form and dose	Outcomes: toxicity/efficacy
Vieira *et al.* 2015 [132]	Randomised, placebo-controlled crossover; both placebo periods were grouped for analysis (half had Se first) [n=39]	Various chemotherapy regimens (timing of Se supplementation not specified) [Paediatric LL or various ST]	Oral Se-glycine, age-dependent dosing (27–100 μg/day) for 30 days, 7 days washout then alternate medication for 30 days. Sustained dosing for 12 months in 16 patients.	• No significant change in EORTC QLQ-C30 scores for fatigue, nausea, appetite loss, physical function in LL group • Significant improvement in scores for nausea and appetite loss in ST group with both Se and placebo, for fatigue with placebo and for fatigue at 1 year with Se • Significant decrease in serum AST levels with Se (p=0.045) • General trend to improved scores
Mix *et al.* 2015 [104]	Randomised, placebo-controlled study of SLM [n=24]	Concurrent chemo-radiation (CCRT). RT 70Gy over 7 weeks; cisplatin 100mg/m^2 on days 1, 22 and 43 of RT [Stage III-IV head and neck squamous cell cancer]	Oral SLM at 3600μg/m^2 twice daily pre-treatment for 7 days then daily until 3 weeks after chemo-radiation completion or placebo (11 weeks from commencement)	• No significant change in mucositis or patient reported side effects • No difference in OS or RFS at 12 months

Author, year published	Trial design [Patient number]	Cancer treatment [Cancer type]	Se form and dose	Outcomes: toxicity/efficacy
Brodin *et al.* 2015 [54]	Phase I dose escalation study [n=34]	Retreated with first-line chemotherapy after SS [Cancer patients refractory to cytostatic drugs]	Intravenous SS given on 5 of 7 days for either 2 or 4 weeks; dose escalation to MTD	• MTD defined at 10.2mg/m^2 • DLTs reported to be acute, of short duration and reversible: fatigue, nausea, finger and leg cramps

Area under the curve (AUC); Bone marrow (BM); Dose-limiting toxicity (DLT); Aspartate aminotransferase (AST); The European Organisation for Research and Treatment of Cancer quality of life questionnaire C30 (EORTC-QLQ-C30); Concurrent chemo-radiation (CCRT); Granulocyte colony-stimulating factor (G-CSF); Gastrointestinal (GI); Gray (Gy); Luteinizing hormone-releasing hormone (LHRH); Leukaemia or lymphoma (LL); Solid tumours (ST); Maximum tolerated dose (MTD); Overall survival (OS); Pharmacokinetic (PK); Prostate-specific antigen (PSA); Red blood cell (RBC); Relapse-free survival (RFS); Radiotherapy (RT); Selenium (Se); Cancer antigen 125 (CA-125); Cytoxan, Hydroxyrubicin (Adriamycin), Oncovin (Vincristine), Prednisone (CHOP); Pharmacokinetic (PK); Se-methylselenocysteine (SLM); Sodium selenite (SS); Non-small-cell lung carcinoma (NSCLC).

1.3.3 Potential additive toxicity (genotoxicity and second malignancies)

The addition of treatments to existing therapies that are associated with a significant burden of toxicity must be done with great care and without the precipitation of further significant adverse effects. Most cancer treatments are DNA-damaging agents and exposure to these therapies is associated with a greater risk of developing secondary cancers [133]. In light of the above it is therefore imperative to ascertain the genotoxicity potential of the Se compounds if they are to be used in combination with chemotherapy or radiation-based treatments.

Se is not deemed to be carcinogenic by the International Agency for Research on Cancer (IARC) [134], yet there is evidence from *in vitro* work that Se exposure can be DNA-damaging [49]. In yeast models (often predictive for genotoxicity in humans), it has been shown that high doses of Se in the form of SS produced double-stranded DNA breaks (DSB), particularly in growing cells via generation of oxidative stress [135]. Despite this, SLM and MSC did not cause DNA breaks in the same model. In mammalian cell culture systems the inorganic Se compounds, at higher doses in particular, have demonstrated genotoxic potential in a number of different assays [49]. In the setting of human trials a study on a New Zealand population of healthy males showed no differences in basal DNA damage as assessed by the alkaline comet assay after exposure to 200μg of daily Se as selenized yeast [72]. However, the genotoxicity profile of Se compounds administered at supra-nutritional doses above 400μg has not been studied and certainly not in a comparative fashion, nor in cancer patients.

1.3.4 Evaluation of Se form and dose in current use

Previous preclinical data suggests that organic Se compounds (such as MSC and SLM) are safer and likely more effective than inorganic compounds such as SS, particularly at the higher doses that appear to be optimal for anticancer efficacy and beneficial interactions with

chemotherapy and radiation [96]. It remains unclear whether these high doses are needed to optimise the protective effect of Se in normal tissues, as clinical trials have suggested that significant reductions in toxicity can be achieved with more modest Se doses. Thus the appropriate doses of Se and the best compound to achieve this may differ depending on the desired effect, such as protection against toxicity, prevention of cancer or augmentation of efficacy of other anti-cancer therapies.

1.3.5 Evidence to determine optimal form and dose

There is a distinct lack of head-to-head data comparing the efficacy and safety of Se compounds in cancer patients and no consensus on the most appropriate dosing strategy to be employed in combination treatment protocols. Furthermore the relationship between pharmacokinetic and pharmacodynamics characteristics and anticancer efficacy of the Se compounds has not been established nor has the potential selectivity of effects in healthy and malignant cells been assessed *in vivo*.

1.4 Objectives of the thesis

1. To develop and validate a molecular biology-based method to measure DNA damage in genomic and mitochondrial DNA that can be used to assess the genotoxic potential of Se compounds within the context of a clinical trial.
2. To characterise the clinical and laboratory safety profile of three Se compounds (SS, SLM and MSC) dosed at 400μg per day in cancer patients.
3. To evaluate the pharmacokinetics (PK) of MSC, SLM and SS in cancer patients including the Se species formed in plasma and white blood cells (WBC).
4. To evaluate the pharmacodynamics (PD) of each type of Se including differential effects in normal and malignant WBC, where the malignant WBC will be drawn from patients with chronic lymphocytic leukaemia (CLL) and the normal WBC will be drawn

from metastatic cancer patients. PD pathways/processes to be assessed include:

- Endoplasmic reticulum (ER) stress
- Angiogenesis
- Intracellular glutathione metabolism
- GPX 3 in plasma (UK collaboration)
- Gene expression and DNA damage, repair and methylation studies (UK collaboration)

5. Make recommendations on amendments to the clinical trial protocol with respect to trial design and selection of suitable PD biomarker studies to accompany the safety and PK assessments in cohorts of patients to be treated at higher Se dose levels.

Chapter Two: Development of a qPCR-based DNA damage assay to assess genotoxic stress

2.1 Introduction

The integrity of genomic and mitochondrial DNA is under constant threat from endogenous reactive oxygen species produced by normal cellular metabolism and exogenous sources such as ultraviolet light and mutagens. To counter this, organisms use a complex network of pathways/processes that sense and repair defects, collectively referred to as the DNA damage response (DDR) [136]. However, loss-of-function mutations or epigenetic silencing of DDR genes, which are associated with human malignancies and inherited disorders such as Fanconi anaemia and Bloom's syndrome, can prevent repair of DNA [137]. A reliable method that detects and quantifies DNA lesions is invaluable in understanding disease-specific pathologies and screening chemical and environmental contaminants for genotoxic effects.

2.1.1 Genotoxicity assays available

A number of *in vitro* and *in vivo* techniques have been developed that assess the genotoxic potential of compounds/ chemical agents. The Ames test (also known as the reverse bacterial mutation assay) was first described in the 1970s and is used widely to measure *in vitro* mutagenicity [138]. The assay is based the ability of a substance to reverse mutations in certain bacterial strains carrying a defective gene that inhibits expression of an essential amino acid (such as tryptophan or histidine). Mutagens are able to reactivate the defective gene thus allowing the bacteria to synthesise their own amino acids and grow as a visible colony in minimal media conditions. Limitations of this assay include the extrapolation of data from bacterial models to humans and false positives observed with compounds containing histidine or histidine precursors [139].

The chromosomal aberration test is a cytogenetic technique evaluating effects on chromosomal integrity and morphology observed during metaphase arrest. The micronucleus assay is an alternative to the chromosomal aberration test with a more straightforward system of scoring, where during anaphase the accumulation of nuclear fragments can be observed as micronuclei residing in the cytoplasm of cells. These micronuclei are mis-incorporated chromosomes or chromosomal fragments in the daughter nuclei as a result of failure to correctly attach to the spindle assembly during segregation [140]. The micronucleus assay is used to measure DNA damage at a chromosomal level and can identify aneuploidy.

The comet assay is a commonly-used technique to assess DNA damage first described over 30 years ago [141]. This method quantifies migration of damaged DNA fragments from the nucleoid under electrophoresis and has become the gold standard for measuring cellular DNA damage. Initially the assay was run under neutral conditions, limiting the test to only the detection of double-stranded breaks. The alkaline comet assay, first described in 1988, allowed for the first time the quantification of additional single-stranded breaks and alkali-labile sites as a result of DNA unwinding upon treatment of the nucleoid at pH>13 [142]. Further adaptation of the assay has included the addition of enzyme digestion with specific endonucleases and DNA glycosylases that enable quantification of particular lesions such as oxidised purine bases [143]. The incorporation of fluorescence *in situ* hybridisation (FISH) probes into the COMET assay methodology has also enabled some gene-specific damage quantification to be determined [144]. However, FISH probes remain expensive and the technique has limited resolution and can only detect lesions in the vicinity of genes requiring probes in the region of 10kb [145].

The COMET assay and its various modifications, while still widely used, are not without some limitations. These include a large number of sources of experimental variability (small changes in buffer volumes/ slide depth, pH, low melting point agarose concentration, unwinding times, electrophoresis conditions) that can result in significant changes in outputs [146]. In addition, it requires a significant amount of person hours, due to the need for blinded

scoring of the cell images to avoid bias and the nature of the experimental setup, image capture and analysis. Lastly, the assay is unable to interrogate specific regions of genomic or mitochondrial DNA.

2.1.2 Molecular-based genotoxicity techniques

Toxicogenomic approaches are increasingly being utilised to both identify and categorise potential carcinogens such as microarray-based gene profiling [147]. Such methods can provide a global insight into biological responses to toxic compounds and comparisons can be made across libraries of compounds to give important qualitative data [148].

In the early 1990s, a ligation-mediated polymerase chain reaction (PCR) technique was developed to detect the distribution and repair of UV-induced DNA photoproducts [149]. This technique is dependent on the conversion of DNA adducts into strand breaks with a 5'-phosphate group. Subsequent ligation-mediated PCR involves a multistep process of primer extension, linker ligation, PCR, electrophoresis, electroblotting and hybridization with a sequence-specific probe. PCR-based assays have been further developed to provide a more direct quantitative measurement of DNA damage, where DNA damage results in the disruption of DNA polymerase activity and impaired DNA synthesis. Initial PCR-based methods, such as those developed by Jennerwin et al [150] and Kalinowski et al [151], measured cisplatin- and UVC-induced damage using radiolabelled deoxyribonucleotide triphosphates (dNTPs) to quantify PCR products of approximately 500-2600bp and compared their approaches to southern blotting-based techniques.

Subsequently, strand-specific PCR damage quantitation was developed to take into account the importance of transcription-coupled repair. This approach used a first round of PCR with biotinylated primers and streptavidin selection of the transcribed DNA strands and subsequent PCR with radiolabeled dNTPs and quantification [152]. More recently, following the advent of more robust and efficient TAQ polymerases and the use of improved fluorescence-based chemistries and imaging, semi-quantitative PCR DNA damage assays with target amplicons of between

~450bp and 25kb were validated [153; 154] and were updated to incorporate quantitative PCR (qPCR) [155]. However, for each approach multistep validation was required. Contemporaneously, the long-run qPCR technique for DNA damage quantification (LORD–Q) method was developed [156], where a high frequency of lesions in the DNA template corresponds to a lower concentration of full-length template and therefore a higher threshold value of the amplified product relative to the undamaged template. This technique utilized oligonucleotide primers designed towards 3-4kb target fragments and small internal amplicons of ~100bp, which were able to quantify DNA damage induced by a range of genotoxic stimuli and interrogate specific DNA loci from both mitochondrial and genomic DNA with reproducible sensitivity (Figure 2.1).

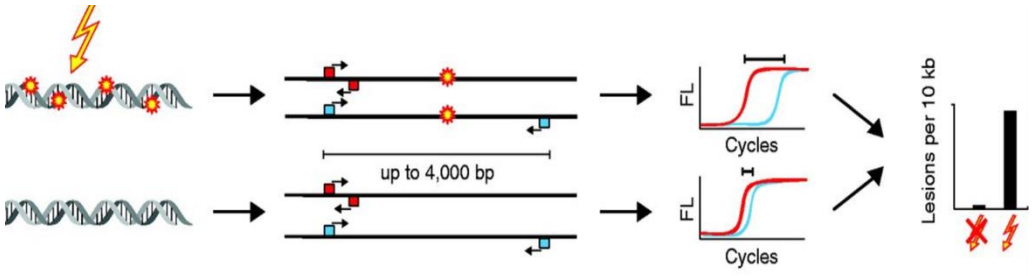

Figure 2.1 Damaged or intact DNA is extracted from cells, large 3-4kb sequences are successfully amplified from undamaged template DNA, whereas PCR elongation is impaired when DNA lesions are present, leading to elevated crossover threshold values. Short fragments of 50-100bp act as internal controls. Reproduced with permission (Oxford University Press) [156].

The LORD-Q method was validated by using the human T--lymphocyte Jurkat cell line following exposure to UVC radiation and a number of genotoxic agents including bleomycin, cisplatin and etoposide [156]. Comparisons of this technique were also made with the comet assay and to an already-established qPCR DNA damage method. DNA lesions rates were further validated by using DNA samples with an already-defined number of lesions following digestion with restriction endonuclease AleI. Due to the findings, the authors concluded that LORD-Q was superior to the

comet assay and previous qPCR technique in terms of damage detection sensitivity.

A limitation of this approach is that it has not been widely tested on different instruments (the published report used a Roche LightCycler 480 II instrument). Currently, a large number of qPCR platforms are commercially available, where rapid technological advances have improved ramp speeds and optical sensitivity, which provides improved performance and higher throughput [157]. Systems from Bio-Rad, Roche, Qiagen and Applied Biosystems are widely used, some are plate-based or rotor-based, with various multiplex and high-resolution melt capabilities with their own developed analysis software. Due to the significant differences between each platform, the application of a tailored approach to assay setup and validation in combination with appropriate reagents and plasticware is required.

Another disadvantage of this published approach is that it requires an expensive DNA polymerase and fluorophore. The performance of DNA binding dyes used in qPCR have previously been compared [158]. Initially, 15 dyes were tested, including SYBR green I, a range of SYTO dyes and SYTOX orange. Comparisons were made with respect to PCR inhibition, G-C binding preference and melt curve analysis. Interestingly, SYBR green I was shown to inhibit the PCR reaction in a concentration-dependent manner and exhibit preferential binding to G-C rich amplicons, which could become problematic in the context of multiplex reactions and melt curve analysis. The best-performing dye in the study was SYTO82, which demonstrated no PCR inhibition even at high concentrations and showed consistent melt curve characteristics in multiplex PCR. Because of this and the fact SYTO82 was least expensive, it was used in this study.

Here we report a modified long-run qPCR assay for use on a Corbett Rotor-Gene 6000 instrument with new primer sets for both nuclear and mitochondrial DNA loci. The method allows both small and large products to be amplified under the same PCR conditions using considerably cheaper consumables than in the published method. Our approach was validated by measuring DNA damage in: 1) cryopreserved and freshly-isolated

peripheral blood mononuclear cells (PMBCs) exposed to ultraviolet C (UVC) radiation (254nm), 2) suspension and adherent cancer cell lines exposed to UVC, and 3) cryopreserved PBMCs damaged by cytotoxic chemotherapy (cisplatin and bleomycin), and also by showing that the approach could be optimised for use on a different qPCR platform.

2.2 Methods

2.2.1 qPCR method optimisation on the Rotor-Gene platform

qPCR analysis was performed using a Corbett Rotor-Gene 6000 instrument (Qiagen, US). Each reaction was carried out in an Axygen® optically-clear thin-walled PCR tube (Corning, US) in a 36-well rotor. Reaction mixes were prepared in a laminar flow cabinet dedicated to PCR work. A considerable number of experimental conditions were tested and the path to the finalised approach is summarised below:

2.2.1.1 Primer selection and design

Initial testing of this technique was performed using primers that target a gene within the genomic DNA and a region within mitochondrial DNA that were utilised in the LORD-Q study [156]. Whilst the mitochondrial primers were able to amplify both large and small DNA fragments, as demonstrated by single bands on agarose gel electrophoresis and melt curve analysis, the p53 primers failed to amplify single products consistently.

Selection of an alternative nuclear amplicon was based on the principle that the suitable genomic region should be transcriptionally active in both human cancer cells and non-malignant cells such as peripheral blood mononuclear cells. Primers targeting a 3kb region within the E2F transcription factor-1 (E2F1) gene were chosen as, in addition to its role as a transcription factor in cell proliferation, it is also involved in the DNA damage response [159]. Four oligonucleotide primer sets were designed using Primer3 [160] for a long nuclear gene target of 3129bp in the E2F1 gene (Accession no: AF516106.1; 3427–6565bp) and a long mitochondrial target 3723bp (Accession no: NC_012920; 11492–15214bp). For each long

amplicon target, a matched reverse primer for an internal short amp50 and 150bp was also designed (Table 2.1). All primer sequences were tested using the primer blast web-based tool [161] to ensure primer specificity.

Table 2.1 Primers designed to amplify large and small products of the E2F1 gene and the mitochondrial target

Primer name	Tm (°C)	Sequence	Product Size (bp)
E2F1 large forward	65.2	GAGGCAGGACTCAGGACAAG	3129
E2F1 large reverse	65.2	CTCCTCACATGCAGCTACCA	
E2F1 small reverse	65.3	GGATGCCTCAGGGACCAG	164
Mitochondrial large forward	63.6	CGCCTCACACTCATTCTCAA	3723
Mitochondrial large reverse	62.6	AATGTATGGGATGGCGGATA	
Mitochondrial small reverse	62.9	CAAGGAAGGGGTAGGCTATG	55

2.2.1.2 Master mix optimisation

Selection of the most appropriate DNA polymerase was based on both performance and cost. Initial trials with kappa2G hot start, HiFi and robust enzymes (all in ready-mix forms trialled in units per 20uL reaction KAPA biosystems) displayed no improvements in PCR amplification parameters over HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia). Titration of magnesium concentration in the reaction mix (as $MgCl_2$) from 1mmol to 3mmol in 0.5mmol increments showed an optimal amplification at 2.5mmol. A range of template DNA concentrations from 1-50ng per 20μL were tested, with 25ng to 50ng per 20μL reaction chosen for the validation experiments.

The DNA-chelating fluorophore SYTO82 (Life technologies, US) was chosen on the basis of its reaction chemistry having no interference with DNA polymerase in long-run PCR in comparison to SYBR green [158]. The final master mix formula consisted of: 1X buffer B1, 2.5mM $MgCl_2$, 1U Hot FIREPol® DNA Polymerase (Solis Biodyne, Estonia), 200uM dNTPs (Genscript, US) and 2µM SYTO82 fluorophore (Life Technologies, US) in a 20µL reaction volume, which also contained primers at a concentration of 500nM each (IDT, US) and 25–50ng of DNA template diluted in MiliQ sterile water.

The PCR protocol was optimised through a series of steps. Annealing temperatures were first titrated from 60 to 62°C, then a touch-down protocol was incorporated into the run protocol to improve specificity of subsequent amplifications [162]. Annealing commenced at 65°C, reducing to 60°C over the first 10 cycles. Once it was decided to continue using Hot FIREPol® DNA polymerase the extension time was increased from 180 to 240 seconds to allow enough time for the enzyme to amplify the 3.7kb mitochondrial product (documented extension rate of 1kb/min). Acquisition of fluorescence of the large amplicons was collected at 82°C and small products at 72°C (Table 2.2).

Table 2.2 Optimized cycling conditions for qPCR on the Rotor-Gene 6000.

Cycle step	Incubation times
Initial denaturation (1 cycle)	95°C @ 15 mins
Hot start (10 cycles)	Step 1: 95°C @ 10s Step 2: 65°C (-0.5°C/cycle) @ 10s Step 3: 72°C @ 240s
Amplification (35 cycles)	Step 1: 95°C @ 15s Step 2: 60°C @ 15s Step 3: 72°C @ 240s acquiring to cycling A (yellow channel) Step 4: 82°C @ 10s acquiring to cycling B (yellow channel)
Melt curve	Ramp from 64°C to 95°C Hold for 90s on the 1st step Hold for 5s on the subsequent steps. Melt A (yellow channel)

2.2.1.3 qPCR DNA damage calculation

PCR amplification efficiencies for the target amplicons were calculated by comparative quantitation using the Corbett Rotor-Gene 6000 Application Software, version 1.7 (Qiagen, US). Average efficiencies for both large and small products across each experimental series were used for these calculations. Detected lesion rate per 10kb was determined using the following equation modified from the paper describing LORD-Q [156]:

$$\textbf{Lesions per 10kb} = \left[\left(E_L^{Cpl(\text{Sample})} \times E_S^{-Cps(\text{Sample})} / E_L^{Cpl(\text{Control})} \times E_S^{-Cps(\text{Control})}\right)^{1/a} - 1\right] \times 10{,}000$$

where E_L and E_S are the average amplification efficiencies of the large and short product, C_p values are the crossover (or threshold) values determined by the Rotor-Gene software (C_{pl}: long; C_{ps}: short) and a is the number of base pairs of the long fragment. All qPCR reactions for each sample were carried out in duplicate and the mean C_p values were used in the calculation of lesions in either genomic or mitochondrial DNA.

2.2.1.4 DNA extractions

Total cell DNA was isolated and purified, using Quick-gDNA™ Miniprep (Zymo Research, NZ) as per the manufacturer's instructions, from control and treated cells of interest. Prior to DNA extraction, the cell culture media was removed, the cells subsequently washed in PBS and then lysed immediately after by application of 400μL of the Quick-gDNA™ Miniprep lysis reagent to adherent cells directly onto the plate or to a suspension of cells after 5 min centrifugation at 200*g*. DNA was eluted into TE buffer (10mM Tris-HCl /1mM EDTA) and the DNA quantity and purity was determined on a NanoDrop 2000 (Thermo Scientific, US) by spectrometric analysis. An aliquot of the high quality DNA (A260:280 >1.8) was subsequently stored at 4°C, until required.

2.2.1.5 DNA gel electrophoresis

On completion of qPCR runs, 10μL of PCR reaction products were mixed with 2μL of 6X loading dye and loaded onto a 1.2% or 1.5% agarose gel (HyAgarose™ LE Agarose (Hydragene, US) in 1x TAE containing 0.2μg/mL ethidium bromide (1μL of 10mg/mL in 50mL). In addition, 5μL of 1kb ladder (New England Biolabs, US) was loaded into the first well. The gel was run at 90V at room temperature for approximately 30 minutes and then imaged as a TIFF file using the Omega Luma G (Aplegen®, US).

2.2.2 Cell culture and mycoplasma testing

2.2.2.1 Cell culture

THP1 and A549 human cell lines were obtained from ATCC and cultured in RPMI-1640 + GlutaMAX (Gibco, US), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, US), penicillin (100 units/mL) and streptomycin (100μg/mL) (Gibco, US). All cell culture experiments were carried out in an humidified incubator at 5% CO_2, 20% O_2 and 37°C. Adherent cells were harvested by trypsin incubation using 0.05% trypsin in EDTA (Gibco, US). Passaging of cells was carried out in accordance with ATCC guidelines [163; 164].

2.2.2.2 PBMC isolation

PBMC were extracted from buffy coats collected from healthy blood donors by the NZ Blood Service under ethical approval from the Northern B Health and Disability Ethics Committee (reference NTY/10/08/065/AM01) in a biohazard safety hood (HeraSafe, Heraeus, Hanau, Germany). Buffy coats were diluted with phosphate-buffered saline (PBS, pH7.4) and layered over approximately 15mL of Histopaque 1077 (Sigma-Aldrich, US) (previous equilibrated to room temperature) dropwise using a sterile Pasteur pipette (Sarstedt, Numbrecht, Germany). The diluted buffy coats layered over the Histopaque were then spun at room temperature in a balanced centrifuge (Multifuge 1, Heraeus, Osterode, Germany) at 400*g* for 30 mins with brake setting off. After centrifugation, the upper layer was carefully aspirated with a sterile Pasteur pipette to within 0.5cm of the opaque interface containing mononuclear cells, and the upper layer was discarded. The PBMC layer (Figure 2.2) was then transferred into sterile 15mL conical polypropylene centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and these cells were washed by adding approximately 8ml of sterile ice cold PBS. This was gently mixed then centrifuged for 10mins at 4°C at 250*g* (Eppendorf, Hamburg, Germany).

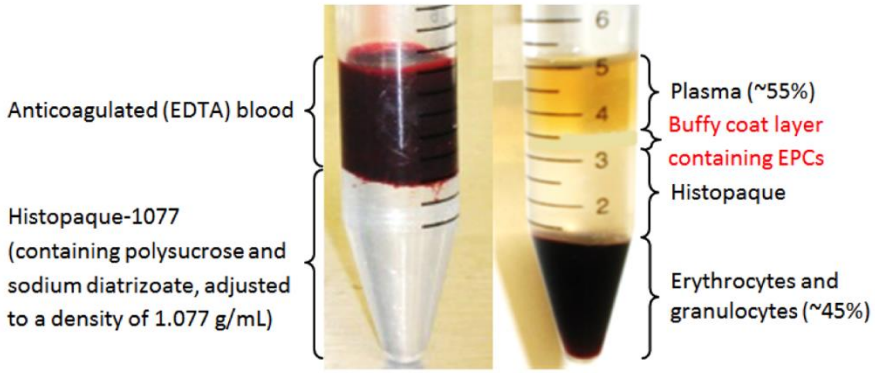

Figure 2.2 Whole blood sample before (left) and after (right) density centrifugation with Histopaque-1077 [165].

The supernatant was discarded and the cell pellet resuspended in 10mL cold PBS, a further two washes were performed to remove residual

platelets. as above then cells were finally resuspended in 5mL PBS for trypan blue exclusion staining and counting.

2.2.2.3 Trypan Blue exclusion (live /dead) staining and cell quantification

Washed PBMC's were diluted in cold PBS and then 1:1 with 0.4%w/v Trypan Blue (Sigma-Aldrich). Cell quantification was performed using a Neubauer haemocytometer (Fortuna, Germany). Approximately 10μL of cell mixture containing trypan blue was added to both sides of the haemocytometer two mins after initial mixing. In accordance with the standard counting protocol, cells were counted in the four larger corner squares on each side of the haemocytometer. Cells touching the left and bottom boundaries were excluded from the count. Cells that were stained blue were deemed to be dead (due to porous cell membrane). Cell viability was recorded as the percentage of unstained cells over the total number of cells. Samples were used in the experiments if viability exceeded 85%.

2.2.2.4 Mycoplasma testing

Cancer cells were tested for the presence of mycoplasma using a previously-developed endpoint PCR method [166]. This approach used mycoplasma-specific primers, generating an amplicon of ~500bp and an internal control fragment of DNA with an overlapping sequence complementary to the primer sequence. The internal control yields a larger amplicon of 986bp, providing a further positive control, where amplification failure alerts to the presence of PCR inhibitors. Positive control DNA was taken from a mouse fibroblast (NHI/3T3) cell line known to be contaminated with mycoplasma (kindly donated by AgResearch, Hamilton, NZ).

DNA extracted from A549 and THP1 cells (see Section 2.2.1.4) was added to a qPCR master mix (see Section 2.2.1.2) and run on the Rotor-Gene 6000 (cycling conditions as per Table 2.3). Positive and negative internal control DNA along with mycoplasma primers were run alongside

cell lines to be tested. Following the PCR reaction, 10µL samples were loaded and run on a 1.5% agarose gel (see Section 2.2.1.5).

Table 2.3 PCR cycling conditions for Mycoplasma assay.

Cycle step	Step parameters
Initial denaturation (1 cycle)	95°C @ 15min
Amplification (40 cycles)	Step 1: 95°C @ 10s
	Step 2: 60°C @ 15s
	Step 3: 72°C @ 60s, acquiring to Cycling A (Yellow channel)
Melt curve	Ramp from 65°C to 95°C
	Hold for 90s on the 1st step
	Hold for 5s on next steps. Melt A (Yellow channel)

2.2.3 Investigations used to validate qPCR method

2.2.3.1 UVC irradiation

UVC irradiation was carried out on THP1, A549 and fresh or cryopreserved PBMC cells. Cell concentrations were 2.5–5x10^5/mL for A549 cells, 5x10^5/mL for THP1 and 1x10^6/mL for PBMCs, aliquoted into identical 2mL media volumes in six-well Nunclon™ Delta plates (Thermo Scientific, US). UVC doses of either 20 or 100millijoules per cm^2 (mJ/cm^2) were given to each plate using the Bio-link BXL-254 cross-linker (Gibco, US). Cells were harvested immediately after UVC exposure, were washed in sterile PBS and centrifuged at 300*g* before being prepared for DNA extraction, except for some aliquots of fresh PBMCs that were used within the alkaline comet assay (see Section 2.2.3.2). Each experiment was performed in triplicate and results were obtained from two separate investigations.

2.2.3.2 Alkaline comet assay

Fronine microscope slides (ThermoFisher, Australia) were cleaned, coated with molten 1% HyAgarose™ LE Agarose (Hydragene, US) (diluted in distilled water) by immersion and air-dried. Experimentally-treated cells, at a concentration of $1x10^4$ cells per slide, were mixed into 0.5% low melting point (LMP) SeaPlaque™ GTG™ Agarose (Lonza, US) in PBS (pH 7.4) at 37°C and pipetted onto the agarose-coated slides. A 15x40mm coverslip was immediately placed on the LMP agarose/cell solution and the slides were left to gel at 4°C in the dark for 20 minutes. Next the slides were placed in comet lysis solution (pH 10; 2.5M sodium chloride, 100mM EDTA, 1.2mM Tris-HCl and 0.1% SDS), with 0.015% Triton X-100 added immediately prior to use, for 2 hours at 4°C.

Slides were then washed in cold PBS (pH 7.4) for 5 minutes and then placed in an Owl™ D3 Electrophoresis System chamber (Thermo Scientific, US) containing approximately 650mL of alkaline electrophoresis buffer (pH>13; 300mmol sodium hydroxide, 1mmol disodium EDTA) for 30 minutes in the dark. Then electrophoresis was carried out at 300mA and 20V for 20 minutes, after which slides were carefully removed from the chamber and washed three times in neutralisation buffer (Tris 0.4M; pH7.5) at 4°C. Cells were stained with SYBR gold fluorophore 10,000X concentrate (Life Technologies, US) diluted in distilled water for 10 minutes and excess stain washed off with two 5 min distilled water washes. Comet images were captured at 100X magnification using a Leica I3 filter block (excitation range; blue, excitation filter; Band pass 450-490nM dichromatic mirror; 510nM, suppression filter; Long pass 515nM) and Olympus DP70 camera (12.5 million-pixel digital colour). Slides were prepared in triplicate and 50 cells were scored per slide for each experimental condition. Olive tail moment was calculated for each cell using CometScore analysis software (Tritek Corp, US). Each experiment was performed in triplicate using cells from three different PBMC donors.

2.2.3.3 PBMC cryopreservation

PBMCs ($1x10^7$ cells per aliquot) were resuspended in sterile filtered 50% FCS (Gibco, US), 40% RPMI-1640 (Gibco, US) and 10% DMSO (Merck, Germany) in 1.8 mL Nunc™ conical sterile polypropylene cryo vials (Thermo Scientific, US) and placed overnight in a CoolCell® (Biocision, US) at -80°C for controlled cooling. These cells were then stored for 2, 4, 8 or 12 weeks at -80°C. When required, PBMC aliquots were thawed rapidly in a 37°C water bath, gradually equilibrated with pre-warmed culture media, diluted to 10X the original culture volume and the cells pelleted by centrifugation in a refrigerated centrifuge (Eppendorf, Germany) at 300*g* for 10 minutes. The residual storage media was discarded to ensure no carryover of DMSO and the cells were resuspended and maintained in RPMI-1640 + GlutaMAX (Gibco, US), supplemented with 10% heat-inactivated FCS (Gibco, US) and penicillin and streptomycin (Gibco, US) in standard culture conditions for 24 hours before UVC irradiation or treatment with cytotoxic compounds. Each experiment was performed in triplicate using cells from one PBMC donor.

2.2.3.4 Cytotoxic chemotherapy incubation

Bleomycin and cisplatin concentrations were chosen to match those used in a previous study [156]. A 10mg vial of bleomycin (Hospira, NZ) was reconstituted in sterile 0.9% w/v sodium chloride and stocks frozen at -20°C. Revived PBMCs were resuspended in serum-free media RPMI-1640 (Gibco, US) in six-well Nunclon™ Delta plates (Thermo Scientific, US) containing $2.5x10^6$ cells per well with bleomycin at concentrations of 10, 20, 30 or 40μM or vehicle (0.9% w/v sodium chloride). Plates were incubated for 30 minutes at 37°C in 5% CO_2 and 20% O_2, after which cells were washed in sterile PBS, centrifuged at 300*g* and prepared for DNA extraction (see Section 2.2.1.4). A 1mg/mL stock solution of cisplatin (Novartis, NZ) was diluted to the required concentration in complete media RPMI-1640 (Gibco, US). Revived PBMCs were resuspended in complete media RPMI-1640 (Gibco, US) in six-well Nunclon™ Delta plates (Thermo Scientific, US) containing $2.5x10^6$ cells per well with cisplatin at concentrations of 50 or

100μM or media control. Plates were incubated for 24 hours at 37°C in 5% CO_2 and 20% O_2, after which cells were washed in sterile PBS, centrifuged at 300*g* and prepared for DNA extraction (see Section 2.2.1.4). Each experiment was performed in triplicate using cells from two different PBMC donors.

2.2.4 Investigations to test the *in vitro* model

2.2.4.1 Cisplatin and phytohemaglutinin-M (PHA-M)

The development of a model to test potential genotoxins in a cell culture system using stimulated PBMCs rather than terminally-differentiated quiescent PMBCs was trialled. PHA-M is a potent mitogen used to stimulate cell proliferation in lymphocyte cultures and is isolated from the red kidney bean *Phaseolus vulgaris* [167].

Cryopreserved PBMCs previously isolated (see Section 2.2.2.2), thawed and revived by a method previously described (see Section 2.2.3.3), following re-quantification and viability checks (see Section 2.2.2.3), were diluted in complete media at a concentration of $1.66x10^6$/mL. This PBMC cell culture was split into 2 equal volumes and PHA-M (Gibco) was added to make a concentration of 1% v/v to one aliquot of cells, which was then further separated into 6 separate 1.5mL volumes in Nunclon™ Delta plates (Thermo Scientific, US). The other aliquot of PBMCs, the untreated control (PHA-M negative), was aliquoted into a second 6-well plate as 6 separate 1.5mL volumes. Both plates were placed at 37°C and 5% CO_2 in humidified incubator for 24 hours. After this incubation, cisplatin HCL was added to 3 wells in the PHA-M positive plate and 3 wells in the PHA-M negative plate to give a final concentration of cisplatin in each of those wells of 100μM. Sterile vehicle (cisplatin diluent was 0.9% sodium chloride) was added to the remaining wells, cell cultures were mixed then incubated for a further 24 hours. Cells were harvested, washed in ice-cold PBS, centrifuged at 300*g* for 10 minutes then DNA was isolated (see Section 2.2.1.4) for subsequent qPCR DNA damage assessment on the Rotor-Gene 6000 platform. Each experiment was performed in triplicate using cells from one PBMC donor.

2.2.4.2 Sodium selenite and methylseleninic acid preparation and treatment

Stock solutions containing 10mmol of elemental Se in PBS were prepared from methylseleninic acid (MSA) 95% w/w (Sigma-Aldrich, US) and sodium selenite (SS) 99% w/w (Sigma-Aldrich, US), filtered with a 0.20μm filter (Sartorius AG, Germany) and aliquots stored at -20°C.

PBMCs previously isolated from 3 different donors were thawed and revived from cryo-storage (see Section 2.2.3.3), followed by re-quantification and viability checks (see Section 2.2.2.3). Revived cells were diluted in complete media (see Section 2.2.3.3) and $2.5x10^6$/mL cells were plated in 2x6 well plates Nunclon™ Delta plates (Thermo Scientific, US) and incubated for 24 hours to allow the cells to recover. MSA and SS were made up to working solutions in complete media then were added to cells to give the desired Se concentrations and the cells were left to incubate for a further 24 hours, after which cells were harvested from the plates, washed in sterile PBS and centrifuged at 300*g* for 10 minutes before DNA was extracted (see Section 2.2.1.4). Each experiment was performed in triplicate using cells from each batch of donor derived PBMCs.

2.2.5 Data analysis and presentation

Graphs were constructed using Prism v7 (GraphPad Software, US), with data expressed as mean ± SE. Results of the qPCR and comet assays were tested using the unpaired t-test and two-sided p values <0.05 were considered significant. One-way ANOVA comparison was used to test differences between means at different time points or treatments.

2.2.6 Optimisation of qPCR assay on an additional platform

The following process can be applied to any new platform; in this investigation it was the magnetic induction cycler (MIC) qPCR instruments (Biomolecular Systems, Australia). This was acquired by our laboratory in 2016, in order to provide a more updated, fast and accurate platform for qPCR-based analyses. Further optimization of the qPCR cycling

parameters and master-mix composition was required for the generation of consistent results on this new platform. Fluorescence acquisition at two different cycle steps was not possible using the available software capabilities (MIC PCR v2.6). Furthermore, changes to annealing temperature were required to produce consistent amplification of both nuclear and mitochondrial large fragments. A minor change to the master mix composition with the inclusion of buffer B2 (Solis Biodyne), which contained detergent, in place in of buffer B1 marginally aided amplification efficiencies. Two separate MIC instruments were used, where the cycling conditions for the large product (Table 2.4) and the cycling conditions for the small products (Table 2.4) were run simultaneously.

Table 2.4 Cycling conditions for the large and small fragment MIC qPCR runs.

Parameters for large DNA fragments			***Parameters for small DNA fragments***		
Cycle step	**Temp (°C)**	**Time and iterations**	**Cycle step**	**Temp (°C)**	**Time and iterations**
Hold (hot start)	95	15 min	Hold (hot start)	95	15 min
Denature	95	10 sec	Denature	95	10 sec
Touchdown	69 to 64 (-0.5°C/ cycle)	12 sec (10 cycles)	Touchdown	65 to 60 (-0.5°C/ cycle)	10 sec (10 cycles)
Extension	72	240 sec	Extension	72	120
Acquisition	82	10 sec	Acquisition	72	120
Total cycles 45			Total cycles 35		
Melt	72 to 95	0.3°C/sec	Melt	64 to 95	0.225°C/sec

2.2.6.1 Validation of qPCR protocol on the MIC platform using UVC

An aliquot of cryopreserved PBMCs previously isolated from donor buffy coats (see Section 2.2.2.2) was thawed (see Section 2.2.3.3), cells were quantified and viability assessed (see Section 2.2.2.3). PBMCs were diluted further into complete media and 6 aliquots of $2.5x10^6$ cells were plated in 2x6 well plates Nunclon™ Delta plates (Thermo Scientific, US). One plate containing 3 wells of PBMCs was treated with $20mJ/cm^2$ of UVC (see Section 2.2.3.1) and the control plate remained untreated. Cells were harvested washed in PBS and DNA extracted and quantified (as per Section 2.2.1.4). The qPCR reactions were set up using 25ng of template DNA and master-mix and primers (see Section 2.2.1.2) with the substitution of buffer of B1 with buffer B2 (see Section 2.2.6). Reaction mixes were prepared in MIC qPCR tubes (Biomolecular Systems, Upper Coomera, QLD, Australia) in duplicate and run on the MIC instruments according to the optimized cycling conditions for both large and small amplicons (Table 2.4). DNA

lesion rates were calculated from 3 technical replicates as previously described (see Section 2.2.1.3).

2.3 Results

2.3.1 qPCR optimization on the Rotor-Gene 6000 instrument

Large nuclear and mitochondrial fragments amplified on the Rotor-Gene 6000 platform were run on a gel after 25 cycles post- touchdown (Figure 2.3). The signal from the mitochondrial fragments was already saturated at this point while E2F1 fragment amplification was approximately 4 cycles post crossover threshold. Melt curves support the amplification of a single product for both E2F1 and mitochondrial fragments.

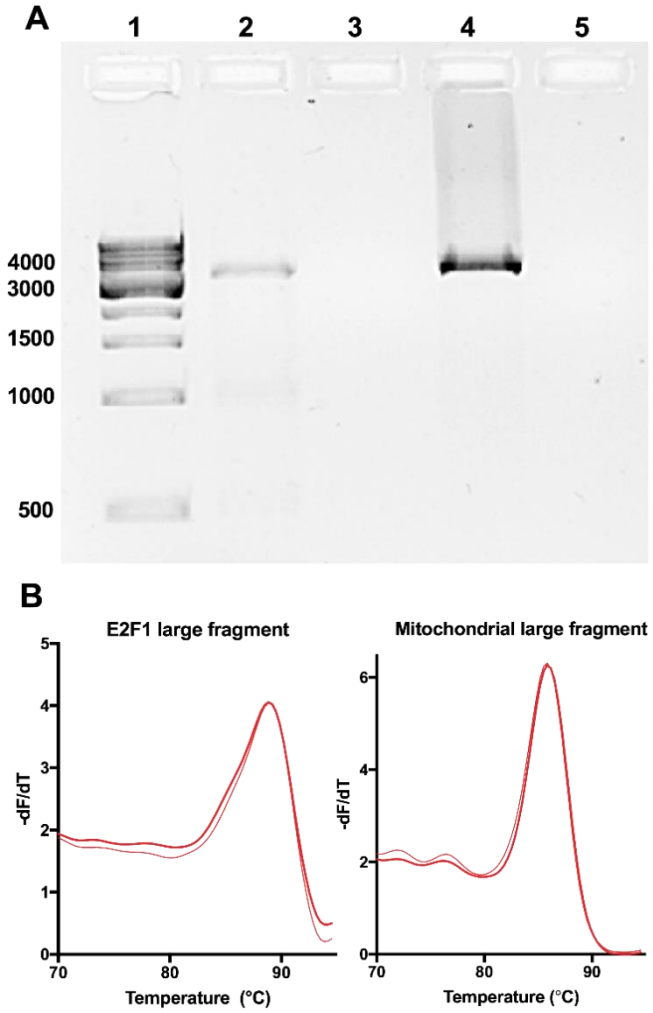

Figure 2.3 A) DNA agarose gel showing large PCR products at 25 cycles post-touchdown; lane 1: 1kb ladder; lane 2: nuclear fragment (E2F1); lane 4: mitochondrial fragments; lanes 3 and 5 contain no template controls. B) Melt curves of the E2F1 and mitochondrial large fragment PCR products on the Rotor-Gene.

2.3.2 Mycoplasma PCR

Using a PCR approach, it was clearly shown that no mycoplasma contamination was present in any of the A549 or THP1 cell lines (Figure 2.4).

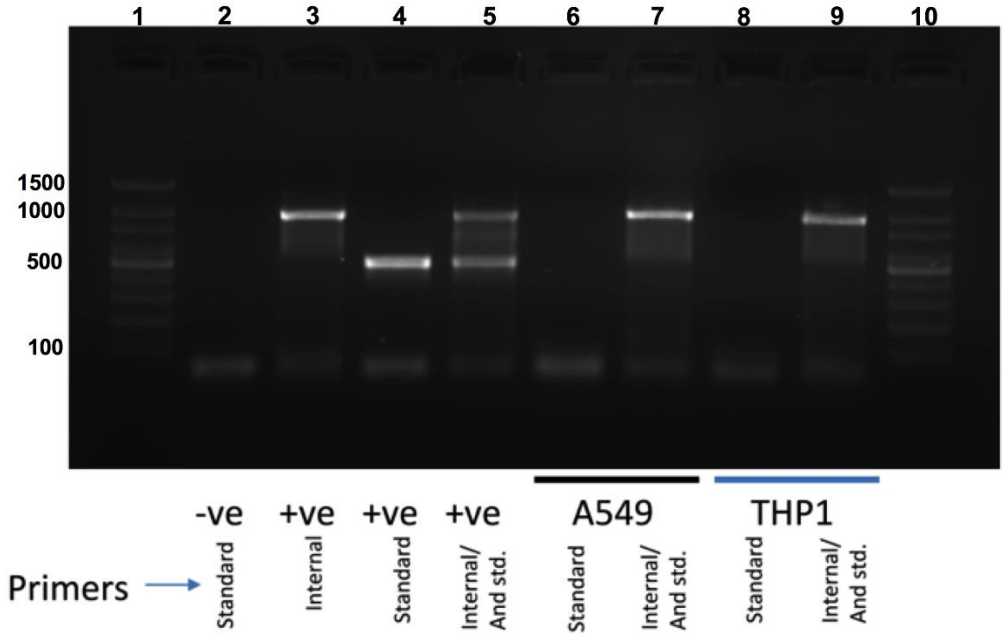

Figure 2.4 DNA agarose gel of mycoplasma PCR assay. Internal controls 986bp product and standard control of 500bp products are observed in lanes 3 and 4. DNA extracted from A549 and THP1 cells are shown to be PCR negative for mycoplasma contamination in lanes 6 and 8.

2.3.3 qPCR validation experiments on the Rotor-Gene 6000

2.3.3.1 Adherent and suspension cancer cell lines DNA damage quantitation

After 20 or 100mJ/cm^2 UVC (254nm) was applied to the A549 human lung cancer cell line and DNA extracted, qPCR was performed on both long and short fragments for both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) using our optimized touchdown protocol. Each experiment was performed in triplicate and results obtained from two independent investigations. DNA lesion rates were higher in the mitochondrial than nuclear DNA templates (n=5), with a clear dose response to UVC exposure in both target amplicons (Figures 2.5A and 2.5B). Possibly due to variation in Cp values for the E2F1 fragment between controls, there was no statistically-significant nDNA response at 20mJ/cm^2 for A549 cells (Figure 2.5A). A similar pattern of significant DNA damage was seen in the THP1 human monocytic leukaemia cell line (Figures 2.5C and 2.5D)

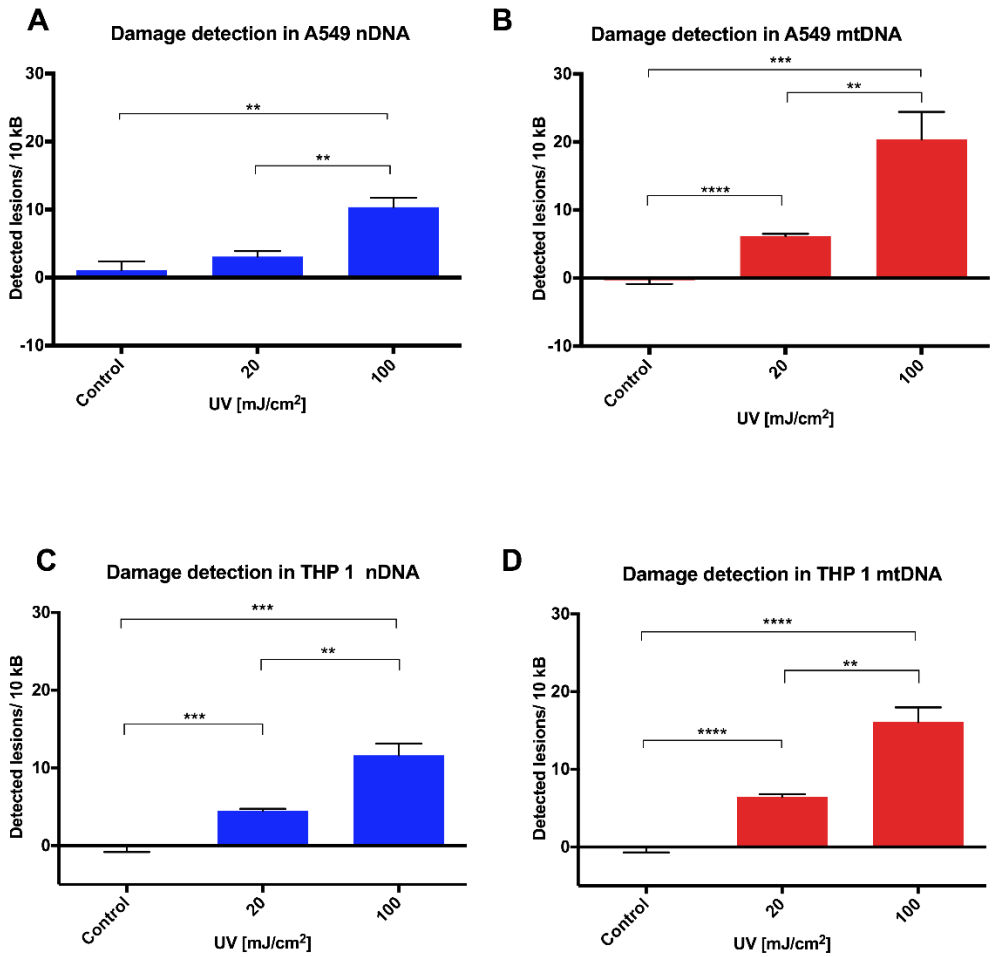

Figure 2.5 Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) damage quantitation in cancer cell lines. Genotoxic stimulation with UVC (20 or 100mJ/cm^2) was assessed in adherent A549 cells for nDNA (A) and mtDNA (B) and in the THP1 suspension cell line in nDNA (C) and mtDNA (D) using qPCR (n=5). Results are presented as mean ± SE. **P <0.01, ***P< 0.001, ****P <0.0001.

2.3.4 DNA damage quantitation in PBMC's using qPCR and the comet assay

PBMCs isolated from healthy donor buffy coats were equilibrated overnight in complete media then treated with UVC and aliquots taken for either the alkaline comet assay or DNA extraction for qPCR. Experiments were performed in triplicate from three different PBMC donors (n=9). qPCR revealed a UVC dose response in both nDNA (Figure 2.6A) and mtDNA (Figure 2.B). While the comet assay was able to distinguish between control and UVC-exposed cells, with significant variation in Olive tail moment

observed (Figures 2.6C and D), it failed to discriminate between 20 and 100mJ/cm^2 doses. The authors who first reported the LORD-Q approach also demonstrated the superiority of qPCR over the alkaline comet assay in detecting low-level DNA damage induced by exposure to bleomycin in Jurkat T cells [156].

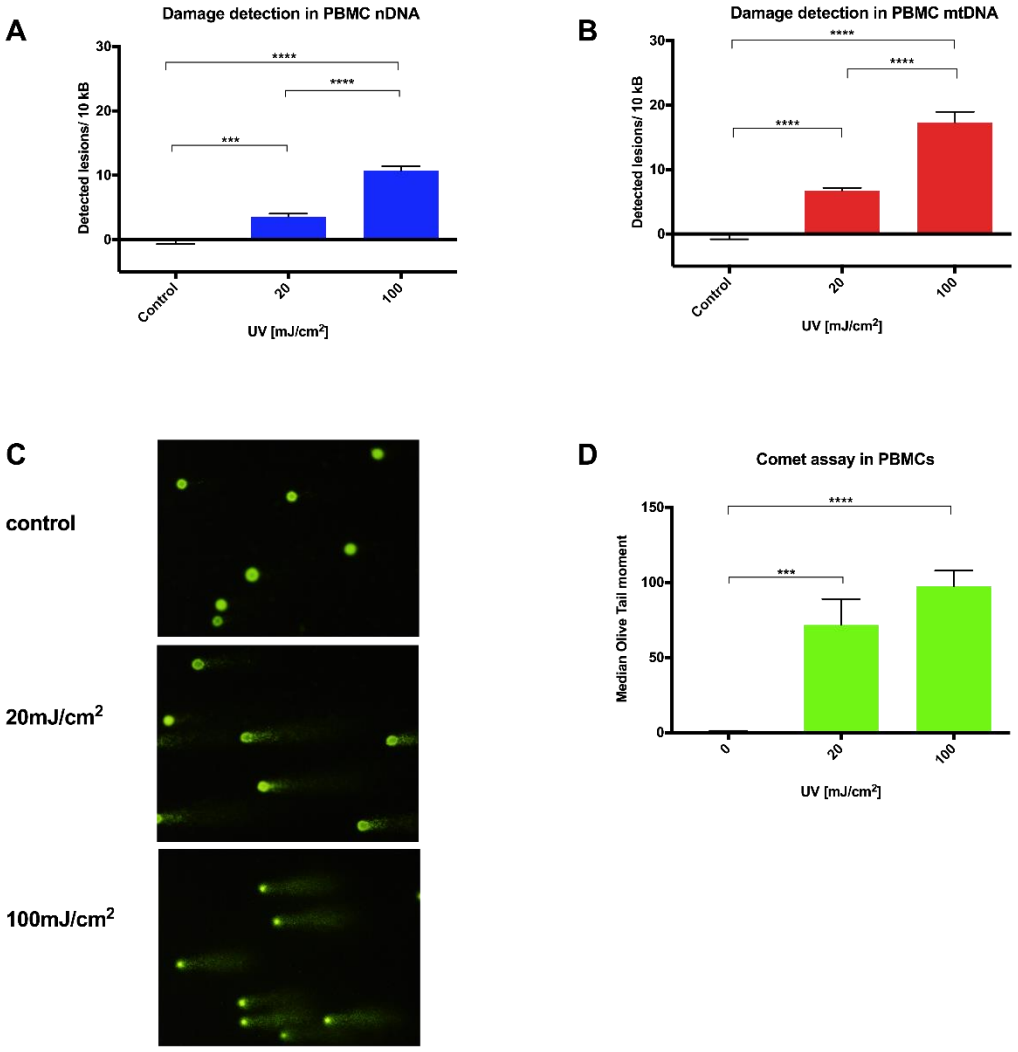

Figure 2.6 Comparison of the qPCR approach to the alkaline comet assay using PBMCs. Genotoxic stimulation with UVC (20 or 100mJ/cm^2) was assessed in freshly-isolated PBMCs in nDNA (A) and mtDNA (B) by qPCR (n=9). DNA damage was also assessed after the same treatments using the alkaline comet assay (C). Median Olive tail moments (D) were calculated for each slide (n=9). Results are presented as mean ± SE. ***P< 0.001, ****P <0.0001.

2.3.5 Effect of cryopreservation on DNA damage quantitation in PBMCs

The use of the qPCR assay in quantifying UVC-induced DNA damage in cryopreserved PBMCs was assessed (Figure 2.7). Experiments were performed in triplicate from one PBMC donor (n=3). For the nuclear DNA target the mean lesion rates observed across five time points, from t=0 weeks (fresh) to t=12 weeks at -80°C, after treatment with 20mJ/cm^2 of UVC were not significantly different (p=0.36). In contrast, induced DNA damage in the mitochondrial fragment was significantly greater at t=0 (mean 7.3 lesions/10kb) than at subsequent time points (p=0.034). However, we observed no further deterioration in DNA integrity over a 12 week period in either mitochondrial or nuclear DNA, with cells stored for different periods of time giving a similar DNA damage response to UVC challenge.

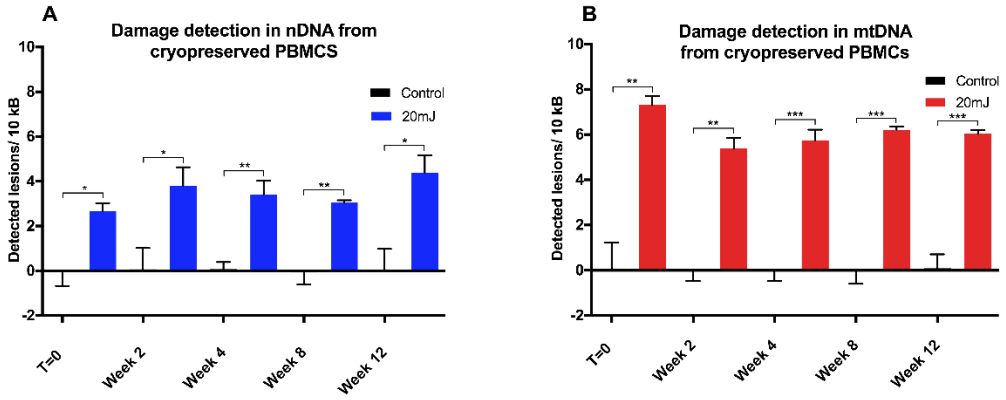

Figure 2.7 DNA damage assessment in cryopreserved PBMCs. qPCR measurement of UVC-induced nDNA (A) and mtDNA (B) damage in revived PBMCs following cryopreservation over a 12 week period (n=3). Results are presented as mean ± SE. *P<0.05, **P <0.01, ***P 0.001.

2.3.6 DNA damage quantitation in PBMC's exposed to cytotoxic drugs

PBMCs revived after cryopreservation (storage period <4 weeks for all PBMC aliquots) were treated with either the cytotoxic glycopeptide antibiotic bleomycin (Figures 2.8A and 2.8B), the platinum compound cisplatin (Figures 2.8C and 2.8D) or media as a control. Experiments were performed

in triplicate from two different PBMC donors (n=6). Initial experiments using bleomycin on PBMC's showed no significant nDNA damage (Figure 2.8A) and a slight significant increase in mtDNA damage (Figure 2.8B). Due to these findings, no more replicates were performed and another drug was tested for comparison.

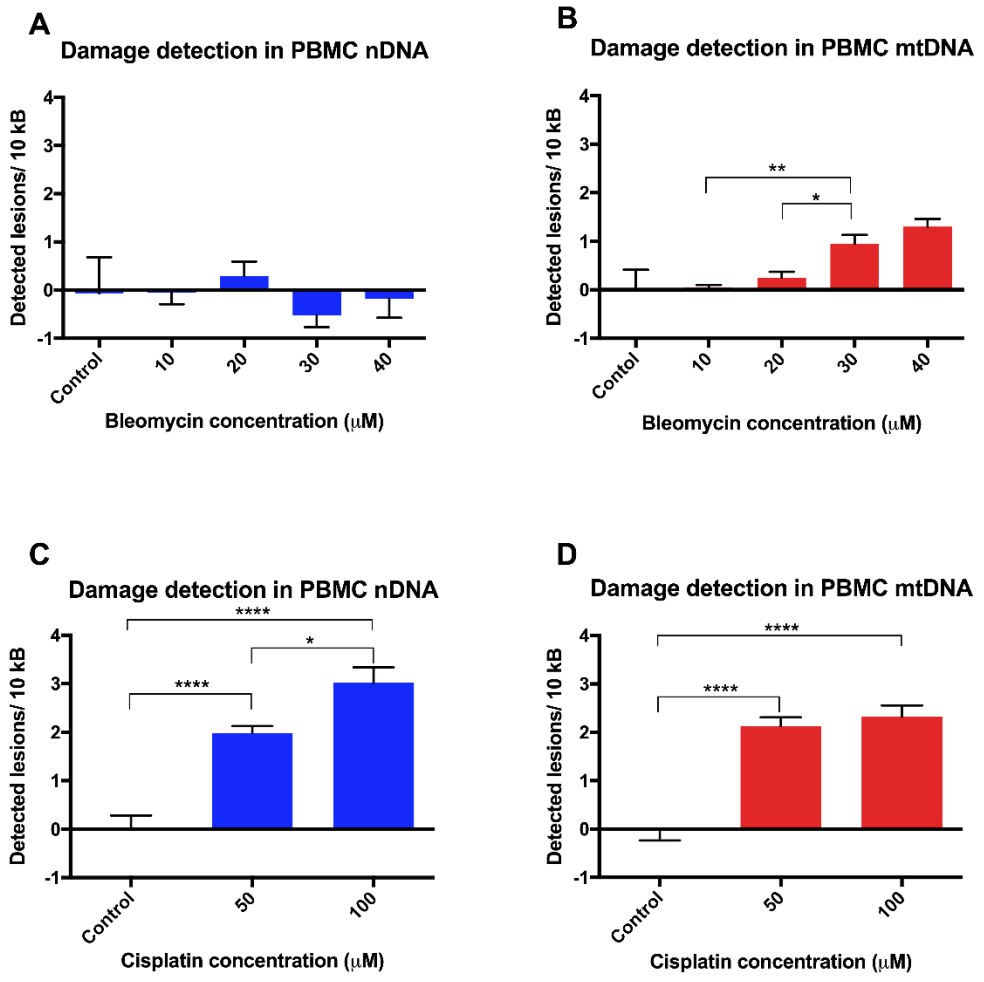

Figure 2.8 Cytotoxic chemotherapy-induced DNA damage in PBMCs. Quantitation of DNA lesion rates in revived PBMCs in nDNA (A) and mtDNA (B) following exposure to bleomycin in nDNA (C) and mtDNA (D) following exposure to cisplatin (n=6). Results are presented as mean ± SE. *P<0.05, **P <0.01, ***P 0.001, ****P <0.0001.

Interestingly, a higher frequency of DNA lesions was observed in nDNA than mtDNA in PBMCs treated with 100μM cisplatin (Figure 2.8C and 2.8D), both being greater than that seen for bleomycin treatment.

2.3.7 DNA damage quantification in PBMCs exposed to cisplatin, with or without prior treatment with PHA-M

Revived PBMCs were cultured for 24 hours with or without the addition of PHA-M, whereupon cisplatin or the vehicle control was added to the culture. After a further 24 hours of incubation, cells were harvested, washed and DNA damage quantified (Figure 2.9). Results suggest a slight increase in cisplatin-induced DNA damage in the cells pre-treated with PHA-M. However, the effects of PHA-M caused an increase in cell adherence to the plasticware (differentiating to a macrophage-like phenotype), resulting in incomplete harvesting of the cells. Therefore there were likely a higher proportion of damaged cells harvested from the PHA-M treated wells compared to control.

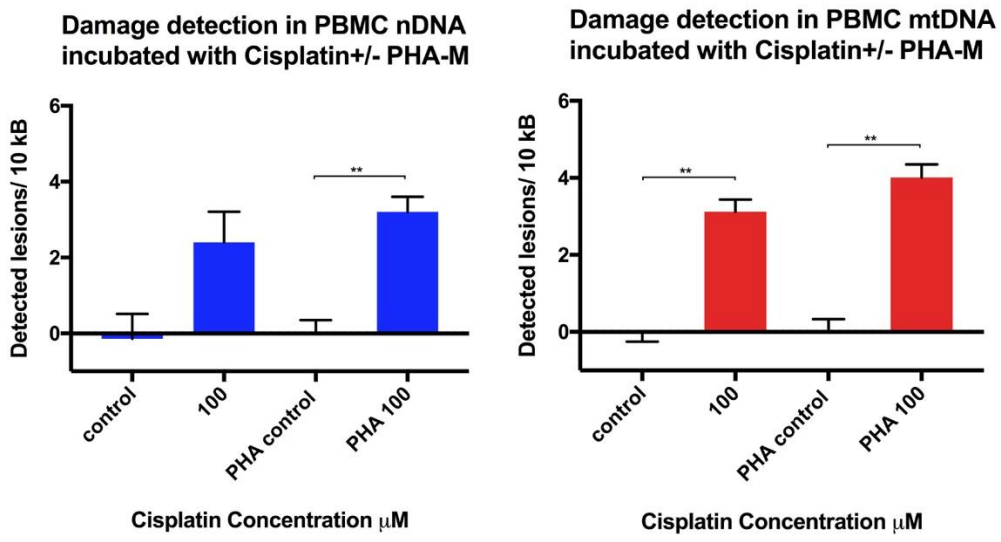

Figure 2.9 Quantitation of DNA lesion rates in revived PBMCs in nDNA and mtDNA following exposure to cisplatin, with or without prior treatment of PHA-M (n=3). Results are presented as mean ± SE. **P <0.01.

2.3.8 DNA damage quantification in PBMCs exposed to sodium selenite (SS) or methylseleninic acid (MSA)

The genotoxicity of SS and MSA was assessed in revived PBMCs after 24 hours exposure to selenium compounds or PBS control (Figure 2.10).

Experiments were performed in triplicate from three different PBMC donors (n=9).

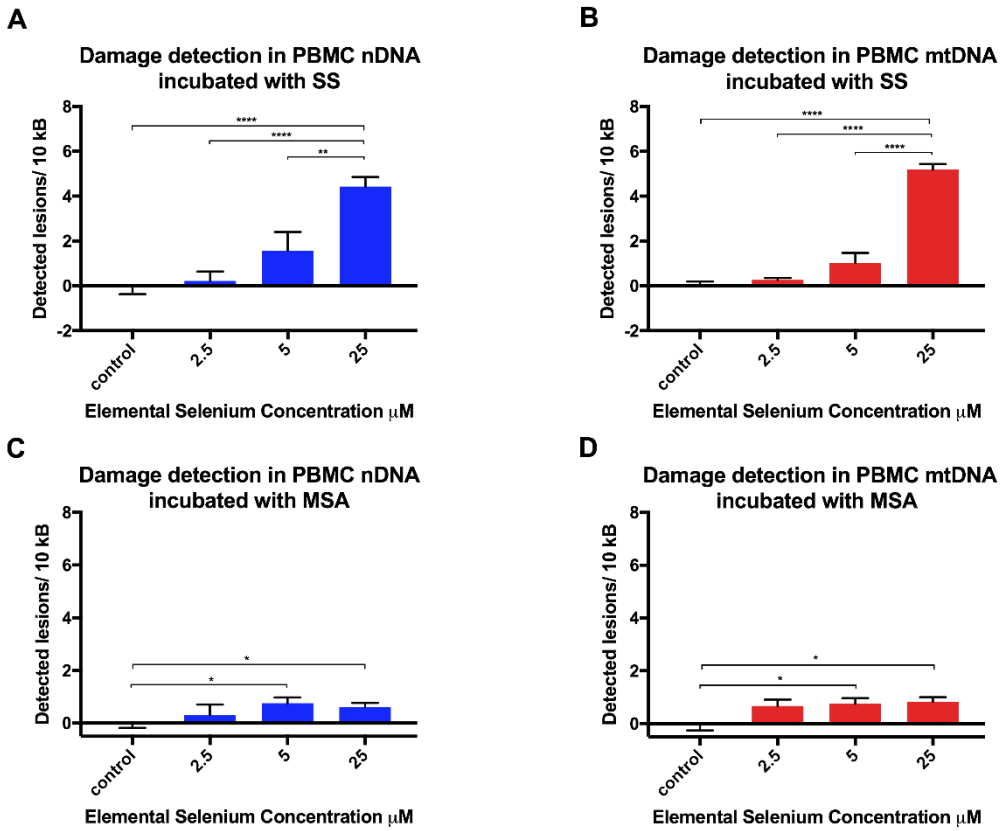

Figure 2.10 Selenium-induced DNA damage in PBMCs. Quantitation of DNA lesion rates in revived PBMCs in nDNA (A) and mtDNA (B) following exposure to SS, or in nDNA (C) and mtDNA (D) following exposure to MSA (n=9). Results are presented as mean ± SE. *P<0.05, **P <0.01, ****P <0.0001.

Organic MSA was seen to be less genotoxic to PBMCs at higher concentrations (5μM and 25μM) in both nuclear and mitochondrial DNA in comparison to SS. The highest DNA damage was observed in PBMCs treated with 25μM of SS, for both genomic and mitochondrial DNA.

2.3.9 qPCR optimisation on the MIC instrument

Consistent amplification of both mitochondrial and nuclear large fragments was achieved after a considerable number of trial conditions (Figure 2.11). Single fragments were shown to be amplified and the melt curves of the large DNA fragments supported this.

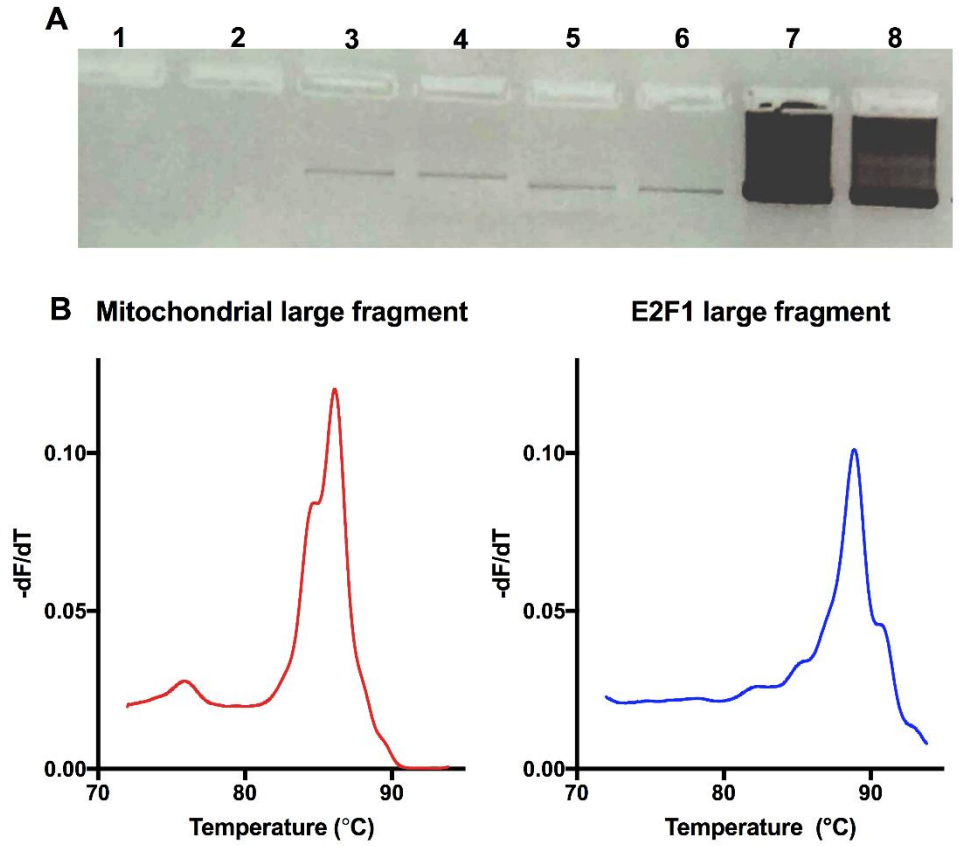

Figure 2.11 Optimisation of the qPCR assay on the MIC platform. Products from the large fragment amplification were run on an agarose gel (A), lanes 1 and 2 show E2F1 after 20 cycles, lanes 3 and 4 show mitochondrial fragment at 20 cycles, lanes 5 and 6 show E2F1 after 30 cycles, lanes 7 and 8 show mitochondrial fragment at 30 cycles. Melt curves were generated for both the nuclear and mitochondrial fragment (B).

Validation of the finalized PCR protocol was shown using UVC at the low dose of 20mJ/cm^2 (Figure 2.12), showing similar DNA lesion rates to those observed using the Rotor-Gene platform.

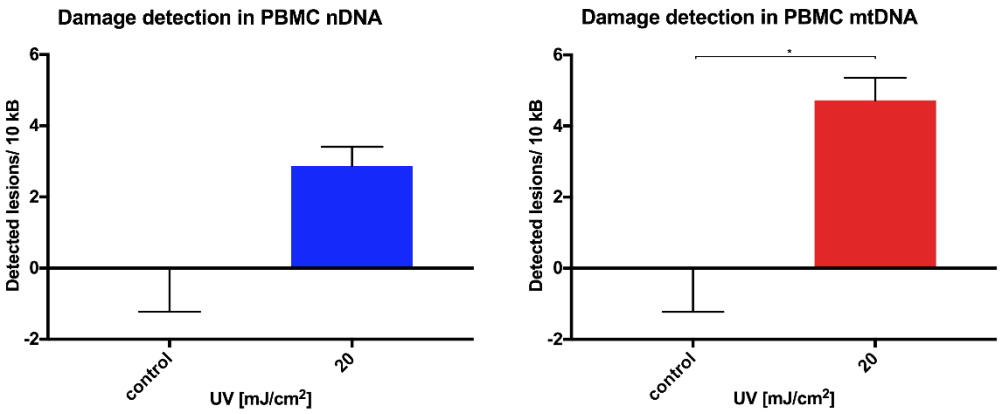

Figure 2.12 Quantitation of nDNA and mtDNA lesion rates in revived PBMCs following UVC (254nm) (n=3). Results are presented as mean ± SE. *P<0.05.

2.4 Discussion

Current techniques available to measure DNA damage, such as the comet assay, can be both time-consuming and laborious when applied to large sample numbers. There are increasing reports in the literature of methods that utilize molecular techniques like qPCR, which have the potential to reduce analysis time and offer a significantly-greater level of sensitivity and reproducibility. They also improve on the comet assay with sequence-specific DNA damage, identifying effects of potential toxicants separately on nuclear and mitochondrial DNA.

2.4.1 Development of the qPCR DNA damage assay

Initially, we evaluated the LORD–Q method using primer sets as previously described [8], testing for amplification of both the large nuclear gene, *p53* (3075bp) and a large mitochondrial (3723bp) product. qPCR was carried out using the recommended KAPAG2 (Peqlab) enzyme and cycling conditions with the fluorophore SYTO82 using a different platform to the original paper, the Corbett Rotor-Gene 6000 (Qiagen). We also tried substituting the Taq enzyme for the cheaper, Hot FIREPol® DNA Polymerase (Solis BioDyne) but the amplification gave variable results with

a number of non-specific products. This was particularly evident for the p53 product, which was confirmed by gel electrophoresis (data not shown).

Through a series of investigations, we eventually optimized this assay by targeting completely different DNA regions, using the less expensive Hot FIREPol® enzyme/SYTO82 fluorophore combination (approximately 6 times cheaper than the LORD-Q approach) and establishing the best cycling conditions for use on our own Corbett Rotor-Gene 6000. The assay work-up required a systematic approach to experimental design, with numerous test variables titrated in an iterative fashion to obtain consistent findings.

We eventually chose *E2F1* as a nuclear DNA target, as this gene plays a key role in cell cycle regulation and is likely to be in a transcriptionally-active region of the genome and therefore susceptible to experimentally-induced damage [159]. We anticipated that there would be a multitude of suitable targets throughout the genome and that some optimization would be required when using different qPCR instruments. The reproducibility of this modified qPCR assay was validated using UVC treatment (254nm) initially in adherent (A549) and suspension (THP1) cancer cells lines, then in PBMCs due to the forthcoming clinical investigation that would be involving such samples. In addition, a comparison between levels of induced DNA damage in cryopreserved versus freshly-isolated PBMCs helps to inform future experimental methodologies and clinical trial material handling protocols.

2.4.2 Contextualisation of validation assay results

Initial assay validation, using UVC to damage THP1 and A549 cell lines demonstrated reproducible damage quantification in both nDNA and mtDNA, which was very close to what had initially been shown in Jurkat T-cells in the LORD-Q study [156]. Alternative approaches to measuring DNA damage in THP1 cells have shown similar UVC-induced damage. As well as inducing hydrolytic and oxidative damage, UVC induces bipyrimidine sites in cellular DNA to form cyclobutane pyrimidine dimers, (6-4) photoproducts and their Dewar isomers [168]. Using high performance

liquid chromatography with electrospray ionisation tandem mass spectroscopy (HPLC-MS/MS), the UVC-induced bipyrimidine lesion rate in THP1 cells has been shown to be approximately 1 lesion per $1x10^4$ base pairs per 10mJ/cm^2 at a cell concentration of $6x10^6$/cm^2 [169].

Interestingly, in our study A549 and THP1 cells had much higher damage in mtDNA than nDNA, which was similar to the LORD-Q study where Jurkat T cells had been damaged by UVC [156]. Additionally, mtDNA has been shown to be more sensitive to oxidative stress than nDNA [170] , perhaps related to its proximity to a major source of endogenous reactive oxygen species, namely the electron transport chain located in the inner mitochondrial membrane. Furthermore, the nucleotide excision repair pathway responsible for repairing helix-distorting lesions, such as UVC-induced photoproducts, is absent in mitochondria [171] but active within the nucleus.

2.4.3 Comparison to comet assay

While the comet assay is still regarded as the standard genotoxicity assay, it does have a number of limitations. Firstly standardization of assay conditions is vital in reducing intra-assay variability [146]; even small changes in agarose concentration, alkaline unwinding times and electrophoresis conditions can significantly alter results. Additionally, even with the use of comet scoring software, bias can be introduced into the scoring process and, although the comet can be scaled up to deal with large sample numbers, significant time resources are required to perform the assay, then capture and score the images. In comparison, a qPCR -based technique provides a relative rather than absolute measurement of DNA damage. It allows differences in mitochondrial and nuclear DNA damage to be compared and can provide gene/region-specific information that may clarify understanding of certain pathological processes.

To our knowledge this report is the first comparison of a qPCR-based DNA damage assay with the comet assay in PBMCs. The results show our optimized protocol to be a suitable alternative technique to the comet assay

in quantifying genotoxicity from UVC exposure in PBMCs in a cost-efficient manner in both time and reagents.

2.4.4 Application to bio-monitoring studies

The use of human lymphocyte-based model systems for screening chemical compounds for cytotoxicity or cytoprotection is well-established. The cryopreservation of lymphocytes enables long-term storage and allows down-stream assays, like toxicity assays, on cells collected over an extended period of time, such as in a clinical study. The study of DNA damage and repair has been performed in cryopreserved PBMCs using the comet assay [172]. Whilst the isolation of PMBCs from whole blood is associated with some DNA damage, the additional cryopreservation step should not contribute to further damage, with responses following the induction of oxidative stress being similar between frozen and fresh lymphocytes [173]. Indeed we observed that over a 12-week period no further deterioration in DNA integrity was found in either mitochondrial or nuclear DNA, with cells stored for different periods of time giving a similar DNA damage response to UVC challenge. The utility of this approach has been demonstrated here in both fresh and in cryopreserved PBMCs and can be applied to the analysis of tissue-banked samples in a high-throughput qPCR format.

2.4.5 Assessment of cytotoxic chemotherapy-induced damage

The assay was also validated using cytotoxic chemotherapy agents, such as bleomycin and cisplatin, in revived PBMCs, which revealed variable susceptibility to their genotoxic effects. The observed lower levels of damage in nDNA in unstimulated PBMCs is not unexpected, given that previous studies on DNA damage following a 30 minute exposure of phytohemagglutinin-stimulated PBMCs to bleomycin 20μg/mL have shown a very low level of damage, as assessed by the alkaline comet assay, with <1% DNA in the tail [174] . A semi-quantitative PCR approach also showed nDNA from SV40-transformed human fibroblasts to be more sensitive than mtDNA to damage following incubation with cisplatin [175]. Other PCR-

based studies have been undertaken using cisplatin, where DNA adducts were measured in a p53 nuclear DNA target in donor PBMC cell lysates [176], and a lesion frequency of ~1.2/104 nucleotides was estimated after a 3 hour treatment with 100μg/mL cisplatin. However, this is not directly comparable to our data where cisplatin was used at much lower concentrations for 24 hour incubations and PCR was performed on high quality extracted DNA rather than directly on cell lysates.

2.4.6 Phytohemagglutinin-stimulated PBMCs

To test the impact of active cell proliferation on the genotoxicty of cisplatin against PBMCs, 1% v/v phytohemagglutin-M (PHA-M) was added to cell culture. The addition of mitogens such as PHA-M to PBMC culture is used routinely to study immunological responses [177]. Some cytotoxic chemotherapy agents are known to act specifically in certain phases of the cell cycle [178]. Cisplatin is a platinum alkylating agent that binds to DNA, generating adducts, cross-links and strand breaks and is not cell cycle-specific although cells display maximal sensitivity at G_1 phase just prior to DNA synthesis [179]. Cisplatin is therefore not the best cytotoxic agent to test in this model of stimulated PBMCs and our results should be interpreted with caution, as cells treated with PHA-M were incompletely harvested due to cell adherence following induction to a macrophage–like phenotype. In order to improve the accuracy of the assay, cells should be either trypsinised or cell lysis buffer added directly to the wells. Additionally, optimal periods for treatment with PHA-M should be established prior to treatment with a cell cycle-specific chemotherapy agent, with confirmatory cell cycle analysis performed using a suitable method.

2.4.7 Evaluating the genotoxicity of selenium exposure *in vitro*

Selenium compounds, particularly SS, have shown genotoxic potential in a number of experimental models [49]. It has been observed that both selenate and selenite can generate chromosomal aberrations in cultured PBMCs [180]. In our series of *in vitro* experiments SS induced DNA damage in a dose-dependent fashion in both nuclear and mitochondrial

amplicons relative to control, whereas MSA-exposed cells displayed much lower levels of DNA damage relative to control and no appreciable dose response was observed. These findings are in accordance with the existing literature where, in *Saccharomyces cerevisiae*, SS exposure resulted in double-stranded DNA breaks as assessed by pulse field-generated electrophoresis, while MSC and SLM did not demonstrate any genotoxicity in this model [135]. Indeed some *in vitro* models have shown that organic selenium compounds in particular may confer enhanced DNA repair properties [181].

2.4.8 Optimisation of the qPCR assay using the MIC platform

Whilst the Rotor-Gene platform produced acceptable outputs the opportunity to transfer the assay to the newer MIC platform offered a number of advantages. The rapid improvements in the field of qPCR have enabled the enhancement of instrument performance, such as faster ramp speeds, greater assay sensitivities and high-throughput capabilities with low inter-machine run variability in the case of the MIC qPCR instruments.

The process of assay adaptation to the new platform was not straightforward. Extensive trialling of experimental conditions was required before consistent and reliable outputs could be achieved. Most notably, the cycling and fluorescence acquisition parameters had to be aligned to the current software capabilities of the machine(s), running small internal controls simultaneously on one MIC instrument and the long products on another. Whilst the optimisation of the PCR conditions for the short fragments was easily performed, the run profile for successful amplification of the long 3-4kb fragments required a significant number of iterative changes in addition to modifications of the PCR master mix. Validation of the optimised protocol was shown using low doses of UVC on revived PBMCs, where similar lesion rates in the nuclear and mitochondrial DNA were observed to those found when using the Rotor-gene platform

Using the MIC qPCR platform, the assay has been optimised for use with clinical samples within the context of the selenium phase Ib study, enabling a more time-efficient analysis of patient samples with 6 patient

visits-worth of extracted DNA being tested simultaneously using two MIC qPCR instruments.

2.5 Conclusions

The successful implementation of a qPCR assay to measure nDNA and mtDNA damage requires optimization of primers from selected targets and qPCR cycling conditions for a given PCR platform and experimental system/cell line. Our comparison of induced DNA damage in fresh and cryopreserved PBMCs provides the basis for its future application in human bio-monitoring studies of bio-banked samples. This assay is recommended as a useful and versatile tool to assess DNA damage within genomic and mitochondrial DNA in any eukaryotic system, even in the same PCR run across different experimental groups, depending on instrument and software capabilities.

Chapter Three: Clinical Trial – Safety profile and clinical efficacy of selenium compounds in cancer patients

3.1 Introduction

A limited number of clinical trials in healthy participants or cancer patients undergoing systemic treatment with radiation or chemotherapy have assessed the effects of SS, SLM and MSC at supra-nutritional doses (see Table 1.5) [38; 182]. SS has been favoured by many European investigators whereas SLM has been extensively used in US-based studies. Prior to this investigation, MSC had not been studied in cancer patients, neither had there been a direct comparison of these Se compounds in cancer patients who are being treated concurrently or sequentially with chemotherapy or radiation. Additionally, it is still unclear which Se compounds offer the most potential to improve the efficacy and reduce the toxicities of anticancer therapies, without adding their own significant toxicities.

While there has been a thorough evaluation of the relationship between doses of SS or SLM and their acute toxicities, there is a lack of clarity about the relationship between dose and the PD effects of each compound in clinical trials. In principle it is preferable to determine the dose of each Se compound that optimises the PD effects mediating beneficial therapeutic interactions with chemotherapy and/or radiotherapy, and thus avoid using doses in excess of this. These Se compounds have dose-limiting acute toxicities [54; 130; 131] but, importantly, there has not yet been any evaluation of the influence of Se compounds on important late effects of these anticancer therapies in clinical trials conducted to date.

Treatment with genotoxic (DNA-damaging) cancer therapies, such as ionising radiation and certain cytotoxic chemotherapy drugs, is associated with an increased incidence of second malignancies after some years,

including solid tumours, myelodysplasia and acute leukaemia [133]. The genotoxic potential of Se compounds has been well-described, with SS being more potent than SLM and MSC appearing to have the lowest genotoxic potential of the three compounds [49]. Therefore, given the potential to increase toxicities, it is important to ascertain the genotoxic potential of Se compounds as part of an evaluation of safety with increasing dose. In addition, it will be important to include this information alongside data on the PK-PD relationship when choosing doses for further clinical study in combination with anticancer therapies.

Lastly, numerous *in vitro* and *in vivo* studies have shown Se to possess anti-tumorigenic properties [183] and a handful of human trials have highlighted some direct effects against established cancers [183]. Therefore, while the main focus of this research is to provide a clear rationale for the use of a specific type and dose of Se as an adjunctive therapy to cancer treatments, an exploratory endpoint of anti-tumour efficacy will also be included in the study protocol.

This chapter focuses on the overall design of the clinical trial and the evaluation of safety (including genotoxicity) and clinical efficacy of SS, SLM and MSC in cancer patients treated in the first dose cohort who received the equivalent of 400μg elemental Se per day. The recruited study population included patients with chronic lymphocytic leukaemia (CLL) or metastatic solid malignancies, in order to facilitate an understanding of the differential effects of the Se compounds in malignant and normal PBMC respectively. Baseline characteristics of recruited patients are reported for the three treatment groups in addition to treatment emergent adverse effects, selected tumour markers and lymphocyte counts. The genotoxicity of each of the Se compounds was evaluated using a qPCR assay with PBMCs, taken at six time points before, during and after exogenous Se exposure for each patient. Each of these measures alongside the PD effects (reported in chapters 5 and 6) will inform the rationale for selection of Se compounds and doses for future studies.

3.2 Methods and materials

3.2.1 Overall study objectives

3.2.1.1 Primary objective:

To determine the dose and form of Se that can be most safely and effectively used in clinical trials in combination with anticancer therapies.

3.2.1.2 Secondary objectives:

1. To characterise the clinical and laboratory safety of MSC, SLM and SS when taken orally by cancer (including CLL) patients at doses of 400, 1600 or 6400μg Se daily for eight weeks.
2. To evaluate the pharmacokinetics (PK) of MSC, SLM and SS in cancer patients at the different doses, including the Se species formed in plasma and WBC.
3. To evaluate PD of each dose and type of Se including differential effects in normal and malignant WBC. The malignant WBC will be drawn from patients with chronic lymphocytic leukaemia (CLL) and the normal WBC will be drawn from metastatic cancer patients. PD pathways assessed may include endoplasmic reticulum (ER) stress, angiogenesis, plasma antioxidant activity (glutathione peroxidase), intracellular glutathione metabolism, gene expression and DNA damage, repair and methylation.
4. To evaluate the relationship between dose, PK and PD of each Se compound in normal and malignant WBC.
5. As an exploratory endpoint, evaluate the impact of each Se compound and dose on tumour activity, as measured by serum relevant tumour markers in cancer patients and peripheral blood counts of lymphocytes in CLL patients.

3.2.1.3 Study endpoints

1. Safety including genotoxicity and tolerability of MSC, SLM and SS at the evaluated doses (See Chapter 3).

2. The dose response to each Se compound of tumour cells, as measured by relevant tumour markers in cancer patients and peripheral blood counts of lymphocytes in CLL patients (See Chapter 3).
3. Plasma Se PK and plasma and intracellular WBC Se species with MSC, SLM and SS at each dose level (See Chapter 4, plasma and intracellular WBC speciation data from collaborators was not available at the time of writing thesis).
4. Markers of PD mechanisms (ER stress, angiogenesis, apoptosis, gene expression and methylation, plasma GPX concentrations and intracellular glutathione metabolism) in plasma and malignant and normal WBC with each Se compound at each dose level (See Chapter 5 and 6, gene expression, methylation and plasma GPX data not available at the time of writing thesis).

3.2.2 Clinical study design

This phase Ib randomised, double-blinded, dose-escalation study plans to evaluate MSC, SLM or SS at three sequential dose levels: 400, 1600 and 6400µg/day of elemental Se. Each dose cohort will include 24 patients randomised to one of three study arms: MSC, SLM or SS. Each arm is balanced to include four patients with CLL and four with metastatic solid cancers (Figure 3.1). Each patient will take one capsule containing the allocated Se compound daily for 8 weeks.

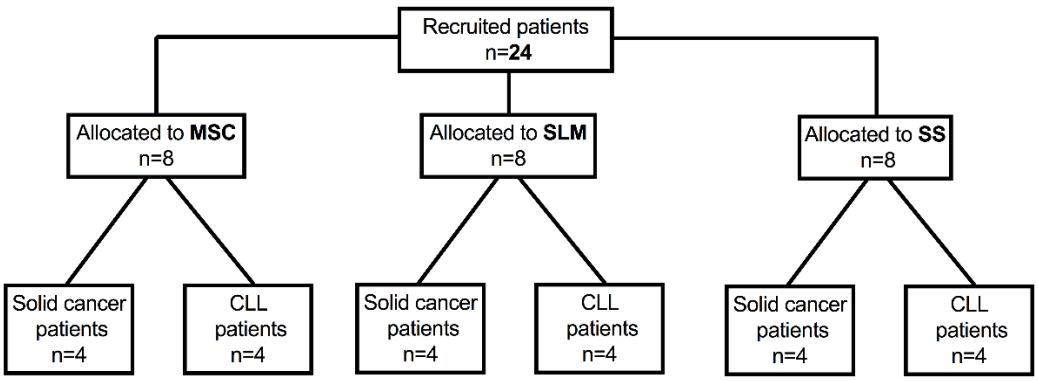

Figure 3.1 Treatment allocation of recruited patients in the clinical trial.

3.2.2.1 Dose escalation and dose-limiting toxicity

Once a dose cohort is filled, recruitment will be suspended until all patients have completed protocol assessments for at least 4 weeks after the start of study dosing in each patient. If dose-limiting toxicities, as defined below, are observed in no more than one of eight patients in each dose cohort for each Se compound, then recruitment of patients into the next dose cohort may commence. If dose-limiting toxicities are observed in two or more patients in a dose cohort for a specific Se compound then recruitment into that arm will be terminated but recruitment into arms involving other Se compounds may continue.

For this study, dose-limiting toxicity (DLT) is defined as any one of the following which is believed to be related to the study medication:

- Absolute neutrophil count (ANC) of $<1.0 \times 10^9$/L lasting at least 7 days or associated with fever (>38.5°C) or Grade 3-4 sepsis.
- Platelet count $<50 \times 10^9$/L lasting at least 7 days or accompanied by Grade 2 or greater haemorrhage/bleeding.
- Clinically significant non-haematological toxicity $\geq$ Grade 3 despite appropriate treatment (including nausea or vomiting not controlled with appropriate therapy).
- Grade 2 treatment-emergent neurotoxicity.
- Grade 2 allergic toxicity.

Adverse events meeting these criteria but not believed to be study medication-related (i.e. are believed to be related to underlying disease or a concomitant medication) will **NOT** qualify as a DLT for the purposes of this study.

3.2.3 Randomisation

Patients were stratified by diagnosis (CLL or solid cancer) then randomised to MSC, SLM or SS using permuted block randomisation (block size of 4) and a computer-based random allocation method. Randomisation was conducted by the clinical trials pharmacist at Waikato Hospital, with

patients, investigators, research and laboratory staff blinded to treatment allocation.

3.2.4 Study population

Eligibility criteria are list below:

3.2.4.1 Inclusion criteria

1. Patients with either proven CLL (peripheral blood lymphocyte count >10x10^9/l) or metastatic cancer, in whom the use of chemotherapy is not anticipated in the next 3 months.
2. Age > 18 years.
3. Adequate liver, renal and bone marrow function
   - ALP, ALT (or AST) < 5x upper limit of normal (ULN)
   - serum creatinine < 2x ULN
   - Hb > 90 g/l
   - WBC > 3.0 x 10^9/l
   - platelets > 100 x 10^9/l
   - Eastern Cooperative Oncology Group (ECOG) performance status $\leq$ 2
4. Life expectancy > 6 months.
5. Fertile patients (or fertile partners of male patients) must use effective contraception during, and for at least 12 weeks after completion of study treatment.

3.2.4.2 Exclusion criteria

1. Patients treated within the last 4 weeks with cytotoxic chemotherapy, systemic therapy that targets vascular endothelial growth factor (VEGF) signalling pathways or radiotherapy.
2. Unable to swallow or absorb study tablets.
3. Concurrent selenium supplements >100µg/day.

4. Allergy to any of the Se compounds being used in this study.
5. Concurrent medical conditions that, in the opinion of the investigators, would compromise either patient safety or the integrity of the data.

3.2.5 Study medication

All Se compounds were manufactured and supplied by Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520-5321, USA. Stated doses reflect micrograms of elemental Se in each capsule, not micrograms of the compound (See investigators brochure (IB) for more information in Appendix C).

3.2.6 Study assessments

Clinical and laboratory evaluations were undertaken twice at baseline, at least one week apart, then on Day 2 of dosing, at Weeks 4 and 8 of treatment then at Week 12, four weeks after the last Se dose (Figure 3.2). The first baseline assessment included a full medical history and physical examination, along with all the investigations are described below. All treatment-emergent adverse events, or those of greater severity than at baseline, were recorded and graded using the National Cancer Institute common toxicity criteria and adverse events (NCI CTCAE) version 4.03 [184]. Patients were given study diaries to record any changes in health state or in concomitant medication usage during trial participation and these were reviewed during each study visit.

At each study visit a number of tests were conducted for safety evaluation. These included urinalysis, electrocardiogram (ECG) and blood tests (complete blood count, renal and liver function, glucose, urate, calcium, phosphate and coagulation). Patients with solid cancers who had elevated serum tumour markers at baseline had estimations of these at Weeks 4 (day 28), 8 (day 56) and 12 (day 84). All patients with CLL had total lymphocyte counts recorded at each visit (inclusive of those with a malignant morphology, such as smear cells). Total lymphocyte counts were used

because of inconsistency in automated haemocytometer discrimination of CLL cells from abnormal or normal lymphocytes.

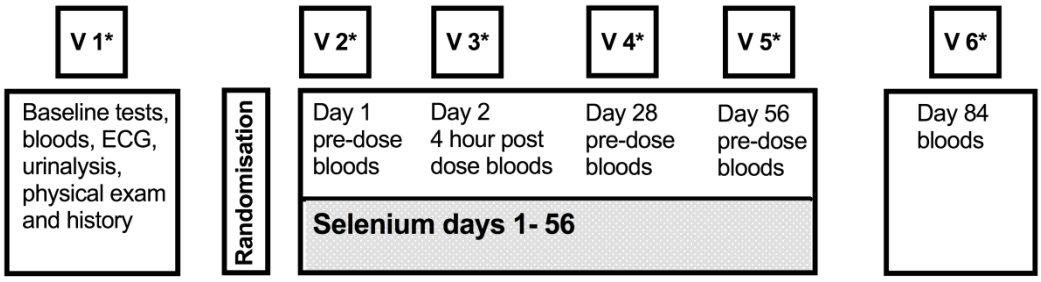

Figure 3.2 Study schedule (6 patient visits over a 14 week period).

A summary of the schedule of assessments and when they were carried out during the trial are shown in Table 3.1 and Figure 3.2.

Table 3.1 Schedule of assessments during the trial.

	Screening/ baseline[a]	Day 1	Day 2	+ 4 Weeks	+ 8 Weeks	+ 12 Weeks
Informed consent	X					
History	X					
Physical exam[b]	X	X	X	X	X	X
Concomitant medication	X	X	X	X	X	X
Adverse events	X	X	X	X	X	X
ECG	X	X	X	X	X	X
Urinalysis	X	X	X	X	X	X
Chemistry[c]	X	X	X	X	X	X
CBC[d]	X	X	X	X	X	X
INR/APTT [e]	X	X	X	X	X	X
Tumour markers[f]	X	X		X	X	X
PK[g]		X	X	X	X	X
PD[h]	X	X	X	X	X	X

a. No more than 28 days prior to commencing study treatment.
b. Comprehensive physical examination prior to study treatment then symptom-directed thereafter.
c. Na, K, creatinine, total bilirubin, alkaline phosphatase, AST, ALT, albumin, total protein, glucose, urate, calcium, phosphate.
d. Complete blood count including differential and platelet count.
e. Clotting parameters: International Normalised Ratio (INR) and Activated Partial Thromboplastin Time (APTT).
f. Tumour markers where relevant to each cancer patient.
g. Plasma PK (See Chapter 4).
h. PD assessments on plasma and PBMCs isolated from EDTA blood (See Chapters 5 and 6).

3.2.7 Pharmacodynamic and DNA damage assay sample collection

3.2.7.1 PBMC isolation

Venous blood collected in EDTA tubes (BD®, ON, Canada) was stored at room temperature prior to undergoing PBMC extraction using Histopaque 1077 (Sigma-Aldrich, St Louis, MO, USA) in a biohazard safety hood (HeraSafe, Heraeus, Hanau, Germany). PBMC extraction was carried out within 4 hours of collection (see Section 2.2.2.2). Quantities of venous blood collected for pharmacodynamics and genotoxicity assays were based on total white cell count obtained from hospital laboratory results using a Coulter counter (Table 3.2). Note all patients with CLL had a white cell count (WCC) >10 $x10^9$/L.

Table 3.2 Quantities of venous blood collected and white cell counts.

White cell count 1 $x10^9$/L	Approximate blood volume in EDTA tubes
<30	15mL
>30 - <100	8mL
>100	4mL

Washed PBMCs were diluted in cold PBS and mixed with 0.4%w/v trypan blue for live/dead staining and quantification as per section 2.2.2.3.

3.2.7.2 Cell suspension aliquots

All PBMCs were diluted to a final concentration of 5 $x10^6$ cells/mL in ice cold sterile PBS. Aliquots of cells were pipetted into sterile 1.5mL tubes (Neptune® Biotix, San Diego, CA, USA) as below:

- 1mL of cell suspension for cryopreservation and subsequent qPCR DNA damage assessment (see Section 3.2.8).
- 0.6mL of cell suspension split into a minimum of 3 tubes for western blotting experiments.(see chapter 5 and chapter 6)
- 0.25mL of cell suspension for intracellular glutathione assay (See chapter 6)
- 0.75mL of cell suspension for DNA repair analysis in 3 tubes (Sent to the University of Surrey, UK).
- 0.3mL of cell suspension in 3 aliquots at day 1 and week 4 for intracellular Se speciation (sent to the LGC group, UK).

All of the above were spun at 300*g* in a microcentrifuge (5415R, Eppendorf, Hamburg, Germany) for 10 minutes at 4°C. Cell pellets for DNA damage analysis were resuspended in freezing media (see Section 2.2.3.3) in Nunc cryotubes (Thermo Scientific, Waltham, MA, USA) and placed in a Coolcell® (Biocision, Larkspur, CA, USA) for controlled freezing and placed at -80° C. The supernatant was removed from all remaining aliquots of PBMCs and the cell pellets were then snap frozen in liquid nitrogen then stored at -80°C until use or transfer to the UK on dry ice.

3.2.7.3 DNA damage assay samples

Blood for DNA damage analysis was taken at each study visit. PBMCs isolated from whole blood on the same day and stored at -80°C until the day of analysis in two aliquots of 500μL of freezing media (see Section 2.2.3.3) containing $5x10^6$/mL cells per visit.

3.2.8 DNA damage assessment

DNA damage was measured using a qPCR-based technique that calculates nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) lesion rates relative to DNA extracted from pre-treatment blood samples (see Chapter 2) [185]. In brief, the PCR reactions included DNA extracted from PBMCs, primers designed to amplify >3000kb regions of nDNA and mtDNA and internal control amplicons of 50-100bp. Samples were assayed in

duplicate on Magnetic Induction cycler (MIC) instruments (BMS, Australia). Large and small amplicons reactions were run in parallel and lesion rates calculated as previously described [185]. The change in DNA damage was quantified relative to the first pre-treatment sample (or, where a PCR result was not available, the second pre-treatment sample) for both nDNA and mtDNA extracted from PBMCs isolated from blood taken at each assessment. Further details that include the mastermix composition, PCR cycling conditions and primer sequences can be found in Chapter 2.

3.2.9 Study conduct and regulatory approvals

The study was carried out following principles laid out in the Declaration of Helsinki and in accordance with the approved study protocol version 2.0 and 3.0 (see Appendix B for latest protocol). Ethical approval was obtained from the Northern A Health and Disability Ethics Committee (reference 13/NTA/172) and the University of Waikato ethics committee, including a protocol amendment to widen the eligibility criteria from patients with CLL or metastatic prostate cancer to those with CLL or metastatic solid cancer. All patients provided written informed consent. The study is registered with the Australian and New Zealand Clinical Trials Registry No: ACTRN12613000118707.

The Se compounds were approved for trial use following a successful application to the Health Research Council's Standing Committee on Therapeutic Trials (SCOTT), which included the preparation of an investigators brochure summarizing the pre-clinical and human study toxicology and pharmacokinetic data of SS, MSC and SLM (see Appendix C for IB).

Approval from Waikato DHB Research office was obtained following departmental sign off from finance, legal, oncology, pharmacy, laboratory department managers and final ratification by a member of the DHB executive. Maori consultation was carried out in association with Waikato DHB Te Puna Oranga Maori Health Research Committee.

3.2.10 Statistical analysis

All statistical analysis was conducted using Prism v. 7.0 and a two-sided $p<0.05$ was considered statistically significant. Descriptive statistics were used to summarise the safety, toxicity and PK data. Baseline characteristics were analysed using one-way ANOVA for continuous data and the chi-square test for categorical variables. One-way ANOVA followed by Dunnett's multiple comparison test was used to compare variance among group means from measurements at baseline and subsequent time points for all treatment arm/disease group combinations for both DNA damage rates and baseline-corrected total lymphocyte count. Repeated measures ANOVA was not used due to the presence of missing data. Pairwise comparisons were made using Tukey's multiple comparisons test post-ANOVA at different time points when baseline values were excluded. Estimations of baseline variation are plotted as 95% confidence intervals for both relative DNA damage rates and total lymphocyte counts calculated from samples obtained on the first two visits, prior to Se dosing.

3.2.11 Study funding

Funding for the clinical trial was obtained from Genesis Oncology Trust, Waikato Medical Research Foundation and Cycle for Life.

3.3 Study results

3.3.1 Recruitment

Between February 2015 and April 2017, 24 patients were recruited into the first dose cohort and randomised into the three study arms (Figure 3.3). Patient characteristics at baseline were balanced across Se compound groups, with no statistically-significant differences observed (Table 3.3). For age $p=0.88$, gender $p=0.85$ and ECOG performance status $p=0.99$. The metastatic cancers were well-distributed across treatment groups and included breast (n=3), melanoma (n=2), prostate (n=4) and one each of

follicular lymphoma, endometrial cancer and cutaneous squamous cell carcinoma.

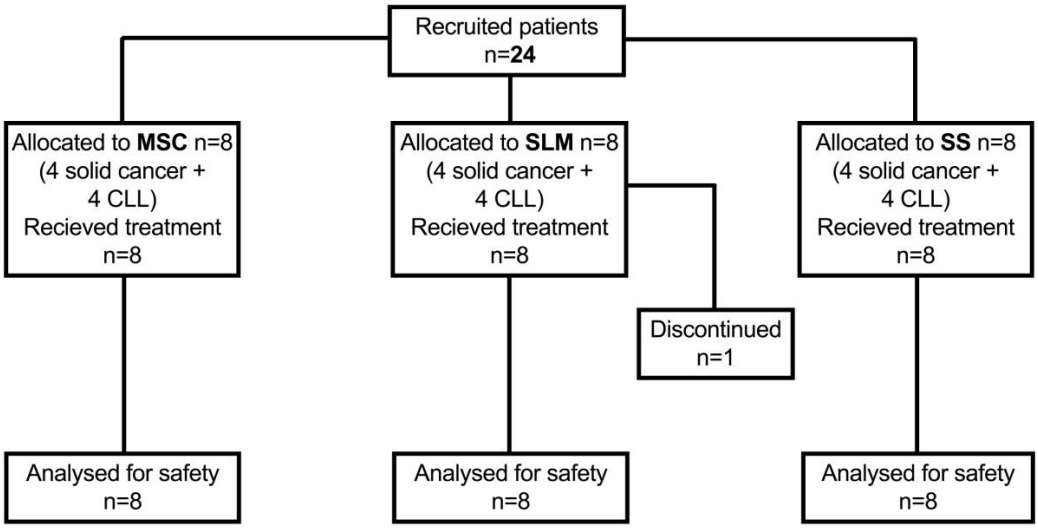

Figure 3.3 CONSORT diagram showing the allocation of patients into each study group.

Table 3.3 Baseline characteristics of recruited patients.

Characteristic	MSC (n=8)	SLM (n=8)	SS (n=8)
Median age, years (range)	71 (46-80)	70 (57-88)	69(55-82)
Gender			
M	5	4	4
F	3	4	4
ECOG PS			
0	7	7	7
1	1	1	1
Blood lymphocytes x 10^9/L, mean (SD)			
CLL	29.4 (23.8)	54.1 (37.2)	60.1 (55.0)
Solid cancer	1.5 (0.45)	1.4 (1.05)	1.8 (0.62)
Prior chemotherapy	4	1	3
Prior radiotherapy	2	2	2
Current targeted therapy*	3	3	3

ECOG PS: Eastern Cooperative Oncology Group Performance Status; * includes various hormonal therapies.

Targeted therapies included luteinising hormone releasing analogues for three patients and the androgen biosynthesis inhibitor abiraterone in one patient with prostate cancer. Endocrine treatments in patients with metastatic breast cancer consisted of anastrozole, letrozole and exemestane. Lastly, the monoclonal antibody, pembrolizumab was used to treat two patients with metastatic melanoma.

3.3.2 Safety Results

Of 24 randomised patients, 23 completed the 56 days treatment schedule. Only one patient discontinued on day 35 after an episode of grade 2 constipation, possibly attributable to Se. All treatment-emergent adverse events were reported by study participants, each of which were single (Table 3.4). The two episodes of at least grade 2 toxicity were attributable to causes other than Se, which were anaemia due to bleeding from an undiagnosed synchronous colon cancer in a patient with follicular non-Hodgkin lymphoma who was taking dabigatran for an atrial fibrillation and transient confusion associated with an undiagnosed brain metastasis in a patient with metastatic melanoma.

Table 3.4 Treatment-emergent adverse events.

Preferred term	**SS N=8**			**SLM N=8**			**MSC N=8**		
CTCAE grade	1	2	3	1	2	3	1	2	3
Rash	2	0	0	0	0	0	0	0	0
Proteinuria*	1	0	0	0	0	0	1	0	0
Haematuria*	0	0	0	1	0	0	0	0	0
Dyspepsia	0	0	0	1	0	0	0	0	0
Urinary frequency	0	0	0	0	0	0	1	0	0
QTc prolongation	0	0	0	1	0	0	0	0	0
Anaemia	0	0	0	0	0	1	0	0	0
Constipation	0	0	0	0	1	0	0	0	0
Confusion	0	1	0	0	0	0	0	0	0

***** Microscopic on dipstick

Two incidences of rash were observed, but were both of short duration of less than 3 days and self-limiting. The one incidence of QTc interval prolongation was 462ms with all subsequent ECGs showed a return to values <450ms. Increased urine frequency of one to two hourly micturation

was documented in one patient commencing MSC, which lasted for approximately 6 hours.

3.3.3 Genotoxicity evaluation using qPCR DNA damage assay

Levels of DNA damage, calculated as mtDNA and nDNA lesion rates relative to baseline, were observed to be low across all treatment groups and time points (Figure 3.4), in both disease cohort and DNA subtype (mtDNA or nDNA), with mean lesion rates in each patient/Se compound cohort being <1 per 10kb of DNA (Figure 3.5).

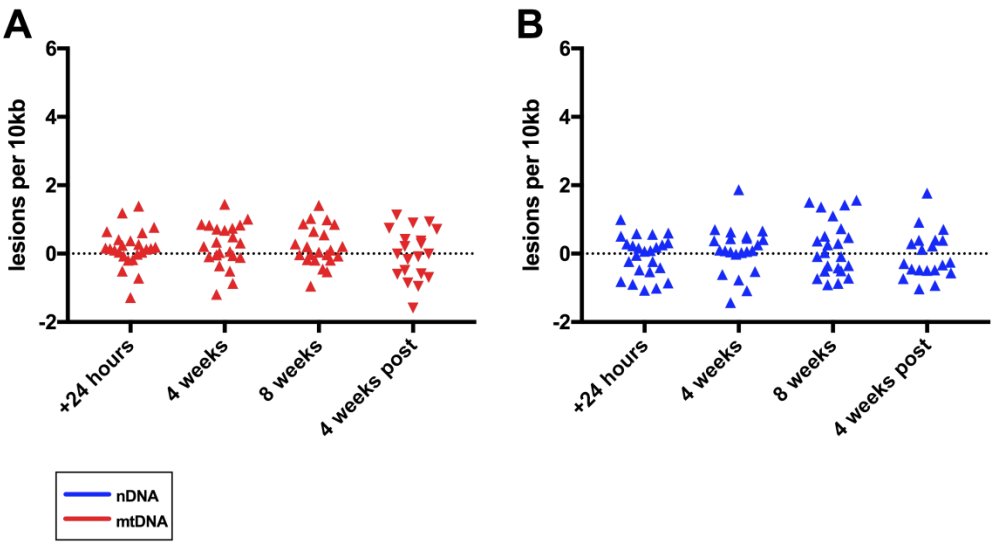

Figure 3.4 Change in mtDNA (A) and nDNA (B) DNA damage relative to pre-treatment baseline sample in all 24 patients post-exposure to Se compounds.

One way ANOVA showed no significant difference in group means for both mtDNA and nDNA when results from all 24 patients are compared. MtDNA gave $F(3,84)=0.706$, $P=0.551$ and nDNA gave $F(3,85)=0.668$, $P=0.5742$. In addition, post-hoc pairwise comparisons showed no significant differences between group means at any time using Tukey's multiple comparison test.

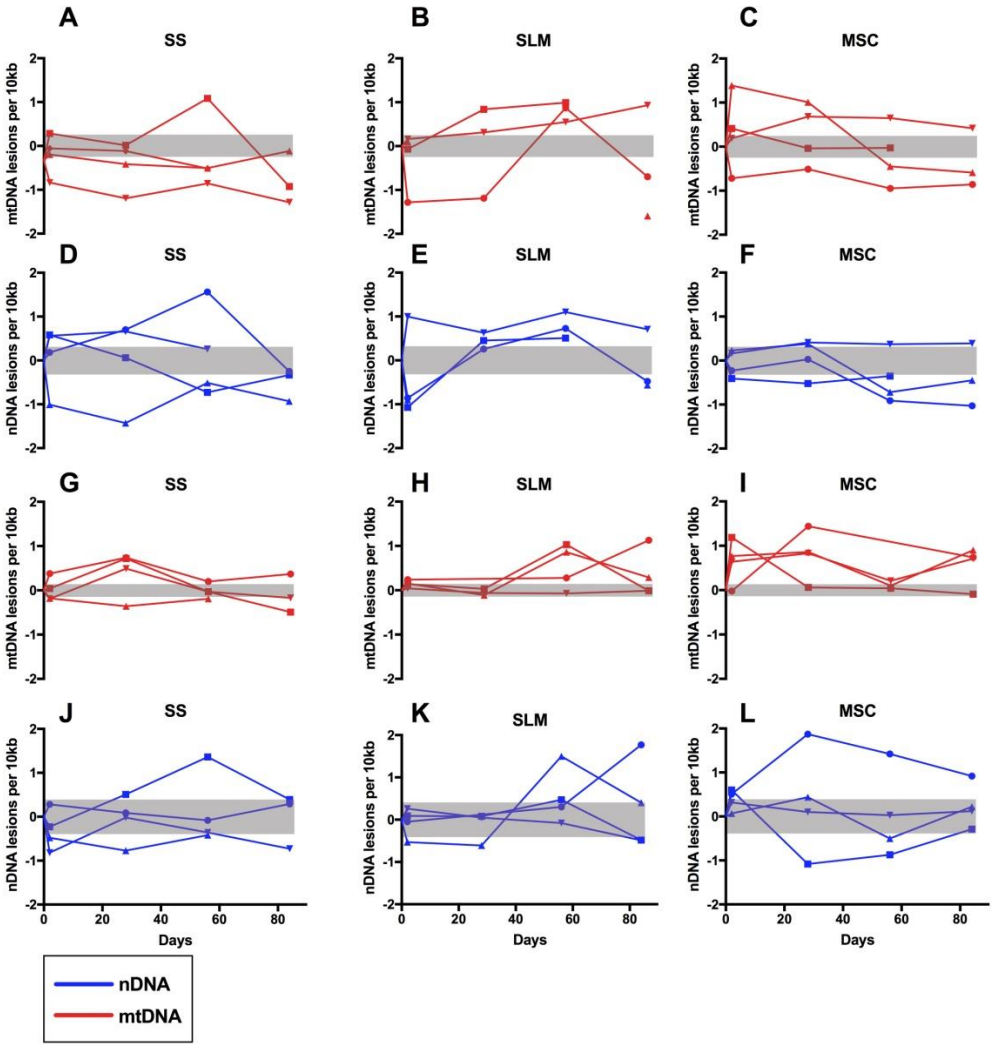

Figure 3.5 Relative DNA damage by disease group and Se compound showing DNA damage in the CLL patient cohort (A-F) and the solid cancer patient cohort (G-L). Grey bands represent the 95% confidence intervals of the relative DNA damage between visit 1 and 2 available for 8 patients in the CLL group and 8 patients in the solid cancer group. Each plot is labelled with the Se compound used.

Further statistical comparisons by disease and Se group yielded no significant differences between group means using one-way ANOVA (Figure 3.5). In addition the relative DNA damage was calculated between pre-Se exposure samples collected at visit 1 and visit 2 for 16 patients (8 in the solid cancer group and 8 in the CLL group). These values were compared to subsequent time points in each disease and Se compound group and still no significant differences by one-way ANOVA and post hoc Dunnett's multiple comparison test were observed.

3.3.4 Tumour markers

In two patients who had raised serum prostate-specific antigen (PSA) levels at baseline, no significant changes in PSA were observed across subsequent study visits. Two patients with metastatic breast cancer had raised serum CA15-3 levels at baseline; in one the levels remained stable, whereas in the other the levels rose significantly (>50%) by the end of dosing and in the final study visit (>100%), suggesting disease progression.

3.3.5 Total lymphocyte counts

Statistical analysis of these small groups (n=4 per Se compound per disease cohort) showed no significant changes in total lymphocyte counts over time in either the metastatic cancer or CLL cohorts for any Se compound by one-way ANOVA and post hoc Dunnett's test, compared to pre-treatment values (Figure 3.6). Only one patient in the MSC treated group met the International Working Group on CLL criteria for partial response [186], with a reduction of >50% from baseline observed at Week 12.

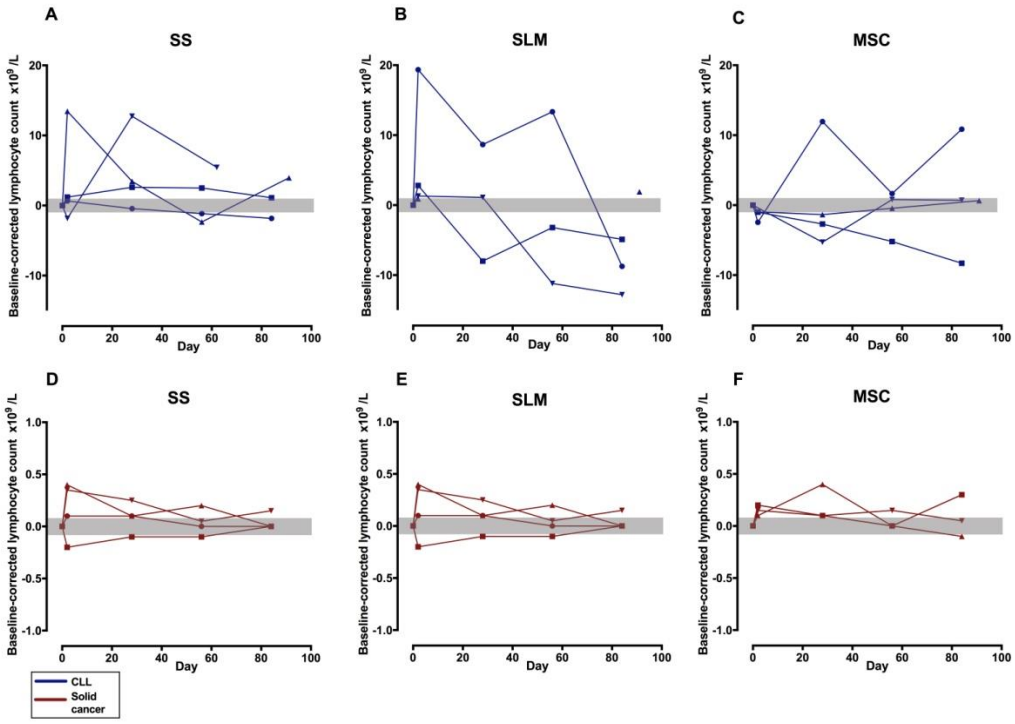

Figure 3.6 Change in baseline-corrected lymphocyte count over time for CLL patients (A-C) and solid cancer patients (D-F). Grey bands represent the 95% confidence intervals of the differences in lymphocyte count between visit 1 and 2 and average baseline value. Each plot is labelled with the Se compound used.

3.4 Discussion

These data represent the first comparison of three Se compounds, two of which have been the most commonly used in clinical trials with cancer patients. While a number of studies have shown individual Se compounds to be tolerated at much higher doses in cancer patients than those used in this dose cohort, often in combination with anti-cancer treatments [104; 105; 130; 131], none have directly compared these compounds in the same study population.

3.4.1 Safety

In this study all three compounds were well-tolerated and assessed as safe at the 400μg elemental Se per day dose level in this study population, with no clinically-significant treatment-emergent adverse events attributable

to Se at this low end of the supranutritional Se dosing spectrum. MSC is the least-studied of these Se compounds, but this study in cancer patients corroborates the reported safety of 400μg MSC per day in healthy adults [38].

3.4.2 Genotoxicity

A key component of our safety analysis included an assessment of genotoxicity of these compounds. For this we adopted a qPCR approach that enabled evaluation of both nuclear and mitochondrial DNA damage. Genotoxicity was insignificant at this 400μg per day dose level for all Se compounds studied, when compared to DNA damage observed before Se exposure began.

SS has been shown to generate single- and double-stranded DNA breaks [135; 187; 188] and other types of DNA damage at low levels of exposure (0.23μM) in cultured human lymphocytes [180]. In contrast the organic Se compounds used in this study have previously been shown to be safer. SLM is not genotoxic to human peripheral blood lymphocytes at Se concentrations of up to ~5.5μM as measured by the cytokinesis-block micronucleus assay [189] and an extensive *in vitro* and *in vivo* toxicological assessment of MSC in mice failed to demonstrate any mutagenic effects at oral doses of up to 4,630μg/kg [190].

Conversely, Se compounds have exhibited antigenotoxic properties, where both SLM and SS have enhanced DNA repair in cell lines and cultured lymphocytes [78; 174; 191], as has MSA (which is metabolised directly to methylselenol, comparable to MSC) in mouse mammary cells [181]. However, many of the previous studies that have evaluated Se in the setting of DNA repair and enhanced protection from genotoxins have done so at relatively low doses (200-400μg) in the context of chemoprevention [181]. The biological mechanisms mediating enhanced DNA repair or protection are yet to be fully understood and further questions on how these mechanisms relate to the different Se compounds and how they can be optimised *in vivo* still need to be addressed. This leads us to a conundrum

when using Se in cancer patients, as they have the potential to directly cause DNA damage yet conversely enhance repair of DNA lesions induced by anticancer therapies. The net benefit or harm will depend on the relative dose-potencies of each Se compound for each effect, and the current preclinical data available suggests that the organic Se compounds (SLM and MSC) have a better therapeutic ratio than the inorganic Se salts (such as SS) in this regard. However the most relevant test is *in vivo* dosing in humans, and this clinical trial offers the opportunity to evaluate DNA damage and effects on DNA repair pathways in subsequent cohorts of patients treated at much higher dose levels of these Se compounds, that are predicted to cause significant DNA damage, which is certain with SS.

3.4.3 Anti-cancer efficacy

The pre-trial assumption of minimal anti-cancer efficacy at the lowest dose level was confirmed, with no reductions in serum PSA or CA15-3 tumour markers observed. In a number of patients in the CLL cohorts, administration of organic Se compounds (SLM or MSC) was associated with a trend in reduction of lymphocyte counts, predominantly in CLL cells (Figure 3.6). However, sample sizes were small and no statistical significance was found. However no such trend was seen in lymphocytes in patients with solid malignancies.

Previous clinical trials have noted Se to have some modest anti-cancer activity (See Section 1.3.1). It appears that the majority of studies that describe these effects administered Se at the upper end of the reported supra-nutritional dosing spectrum with SS dosed at 500μg/day to 200μg/kg/day orally [102; 103; 105] or up to 10,200μg/m^2 intravenously [54], sodium selenate given orally at 60,000μg/day [131] or SLM being given orally at 2,200μg to 7200μg/day [55; 56]. Using SLM in human tumour xenograft models, a minimum plasma Se concentration of 15-20μM was required to elicit therapeutic augmentation with anti-cancer drugs in mice [192]. Consistent with this, Se concentrations <10μM were ineffective at reducing VEGF, HIF-1α and HIF-2α in renal cell carcinoma preclinical models [84]. It would therefore be reasonable to assume that the dose used

in this initial cohort of patients in this clinical trial is below the threshold required for achieving optimal anti- cancer effects *in vivo*. Data from the PK and PD analyses will shed further light on this.

3.5 Conclusions

All three compounds dosed at 400μg/day of elemental Se for 8 weeks are safe to use and well- tolerated in this cancer patient population. The results from the qPCR-based DNA damage assay revealed no significant nuclear or mitochondrial DNA-damaging effects, which included patients treated with inorganic SS. Anti-cancer efficacy was not expected at this dose level, though the small reductions observed in lymphocyte count in CLL patients are suggestive of some anti-tumour activity, which will be evaluated further at higher doses.

Chapter Four: Clinical Trial – Pharmacokinetic profile of selenium compounds in cancer patients

4.1 Introduction

Animal studies have shown marked differences between Se compounds in terms of pharmacokinetic (PK) properties including variations in their absorption, distribution, metabolism and excretion (see Section 1.1.6.1). The PK characteristics of a number of selenium compounds, administered at supranutritional doses have been studied in clinical trials as single compounds taken orally in healthy volunteers [182], in combination with cancer treatments (concomitant and sequential) orally [104; 117; 130], and intravenously [54], as well as direct comparisons between oral SLM and MSC [38]. However the relationships between PK and PD for organic Se compound effects, have only been partially characterized in tumour xenograft models [192], with no such relationship having been investigated in the clinical setting. There is a need for more PK and PD data from human trials, particularly in cancer patients, to better inform the PK-PD relationship and thus determine optimal dosing and choice of Se compound for future rational study design. Selenium speciation in both plasma and white cells, alongside its correlation with the PD data, will greater aid our understanding of Se biology and the role of small and large molecular weight Se species in both malignant and healthy cells.

The overall study objectives for PK-related aspects of the trial are to determine the plasma Se PK and Se species formed in plasma and WBC (separately in CLL cells and PBMC) with MSC, SLM and SS at each dose level. This chapter will present the plasma PK data at the 400μg dose level. The Se speciation data in plasma and WBC is being conducted by collaborators and was not available at the time of writing this thesis.

4.2 Methods and materials

4.2.1 Study design

See Section 3.2.2.

4.2.2 Se plasma PK profiling

4.2.2.1 Sampling scheduling and collection

Blood was collected in trace element tubes (K2 EDTA, BD®, ON, Canada) on Day 1 pre-dose (baseline), Day 2 four hours post-dose, Day 28 pre-dose (Week 4), Day 56 (Week 8, end of treatment) and Day 84 (Week 12, end of study), spun at 1000g for 10 minutes at 4°C (at Waikato DHB laboratory) and plasma stored at -80°C prior to frozen shipping to Canterbury Health Laboratories (Christchurch, NZ) for analysis. In patients who had plasma Se samples taken at baseline and Days 28, 56 and 84, the area under the plasma Se concentration-time curve (AUC) was calculated from baseline to end of dosing using the trapezoidal rule (Prism v. 7.0, GraphPad, La Jolla, CA., USA).

4.2.2.2 ICP-MS (Inductively-coupled plasma mass spectroscopy)

Plasma Se was analysed by ICP-MS (Canterbury Health Laboratories). Samples were diluted 1/25 in an ammonia/EDTA/IPA diluent and analysed on an Agilent 7700 ICP-MS (CA, US). An Agilent multielement standard was used for aqueous standardisation (range from 0.1 – 1000 μg/L). Se was routinely measured at isotope 78 using ^{72}Ge as the internal standard. Another isotope ^{82}Se was measured as an alternative to overcome any double charge interferences from gadolinium (^{156}Gd) seen in patients that have recently had contrast media. The assay used Seronorm™ Trace Element Serum Control L1 and L2 (Billingstad, Norway) as the accuracy control (% CV at 1.10 μmol/l is 3.1%; limit of detection is 0.009 μmol/l). The samples were aspirated using the High Matrix Introduction System into the argon plasma torch at 2700°C for ion formation. The ion stream passes through the sampler and skimmer cones and is focused by a series of lenses

into the Octopole Reaction System which consists of a temperature-controlled collision/reaction cell. Helium is used as the collision gas due to its high sensitivity and universality to aid in eliminating polyatomic ion interferences with the same mass as the elements of interest. The ions then pass into the quadrapole for separation and into the electron multiplier detector. The concentration is calculated using an aqueous standard calibration. The Integrated Sample Introduction System (ISIS) is added onto the sampling process of the analyser. It uses a high-speed pump and a 6 valve-port to allow separated sampling and probe washing, thereby decreasing time between analyses and reducing contamination between sampling and analysis (S. Grant, personal communication 2018, Canterbury Health Laboratories).

4.2.3 Statistical analysis

All statistical analysis was conducted using GraphPad Prism v. 7.0 and two-sided testing with $p<0.05$ considered statistically significant. One-way ANOVA was used to identify the statistical significance of variance among group means for plasma Se AUC values by treatment arm. Pairwise assessment of the treatment arms was carried out using Tukey's multiple comparison test post-one-way ANOVA.

4.3 Study results

4.3.1 Study population

24 patients recruited into the study at the 400μg dose cohort completed the eight-week treatment period, with the exception of one patient who discontinued taking SLM at day 35 (due to complaints of grade 2 constipation). All treatment groups were balanced in terms of patient demographics and other pre-treatment characteristics (see Table 3.3).

4.3.1.1 Plasma PK levels by Se compound group

Plasma PK levels were measured at all five timepoints (Table 4.1; Figure 4.1). The mean baseline plasma Se level was 1.29μM for all patients, with the mean for each subgroup within ~1.3% of this, and no significant difference (p=0.89, t-test) between males (1.29 μM) and females (1.30μM). In five patients taking Se supplements (≤100μg/day) before study enrolment, mean (SD) baseline Se plasma level was 1.52μM (0.26) compared to 1.23μM (0.16) for non-supplement takers (p=0.005, t-test).

The post-dose increment (t+4 hours) above baseline was greatest in the SLM-treated group where mean increase with SLM was 0.45μM, MSC was 0.26μM and SS was 0.20μM (p=0.008 for SLM v SS, p=0.36 for SS v MSC and p=0.24 for SLM v MSC). When adjusted for body weight as measured at Visit 1, differences in group means of post-dose increments per kilogram of body weight are significant for the SLM (6.55nM/kg) versus the SS (2.61nM/kg) group comparisons alone (p=0.01). Plasma levels of Se obtained from patients taking SLM continued to be elevated above those treated with MSC and SS at all subsequent time points, markedly so after the four-week washout period. For 3 patients, each of whom was in a different Se compound group, samples had been taken at day 91.

Table 4.1 Selenium plasma pharmacokinetic parameters.

Parameter	SS n=8	SLM n=8	MSC n=8
Plasma Se μM mean (SD)			
Baseline	1.29 (0.22)	1.28 (0.20)	1.31 (0.26)
Day 2 (4 h post-dose)	1.49 (0.23)	1.73 (0.26)***	1.57 (0.21)*
Day 28 (trough)	1.61 (0.1)***	2.72 (0.61)**	1.71 (0.27)***
Day 56†	1.49 (0.06)	3.01 (0.46)**	1.59 (0.12)
Day 84‡	1.28 (0.13)**	2.13 (0.19)***	1.3 (0.16)

*n=5; **n=6; ***n=7; †: samples taken on day 62 for 2 patients; ‡: samples taken on day 91 for 3 patients.

4.3.1.2 Area under the curve (AUC)

Patients allocated to SLM had a significantly-greater total Se exposure, as evidenced by the higher $AUC_{0\text{-}EOS}$ (Table 4.2) compared to those receiving either SS or MSC (p=<0.0001, Tukey's test; F(2, 14)=33.25). No significant differences in total AUC values were observed between the SS- and MSC-treated groups. Data was excluded from the analysis if sample levels were not available at all study visits or were taken post-dose instead of trough at week 4. Further analysis by disease group was not performed due to small numbers in each subgroup.

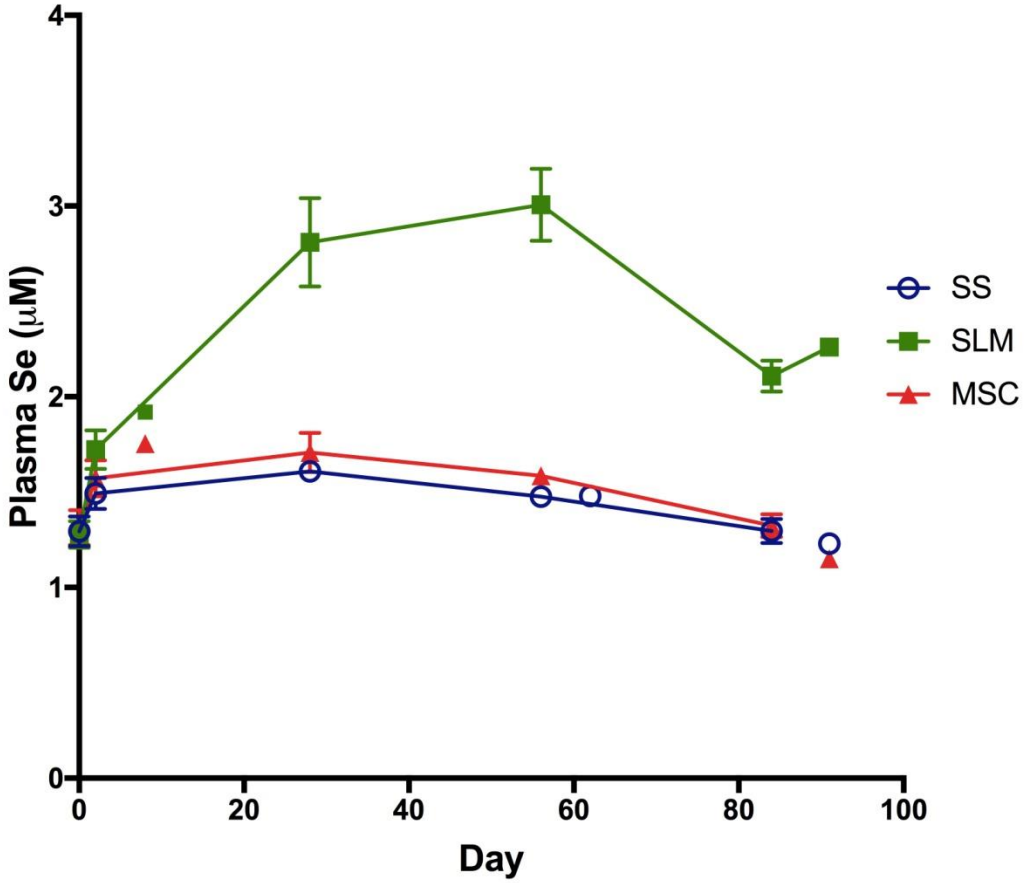

Figure 4.1 Plasma selenium concentrations measured over 56 days of treatment and 4-5 weeks after the last dose. Error bars are +/- SEM. Sodium selenite (SS); Seleno-L-methionine (SLM); Se-methylselenocysteine (MSC).

Table 4.2 Area under the curve (baseline to end of study)

	SS n=5	**SLM n=5**	**MSC n=7**
$AUC_{0\text{-}EOS}$ μM*h/L (SD)	426 (389)	2213 (480)	485 (355)

EOS: end of study.

4.4 Discussion

4.4.1 Baseline plasma Se levels

The baseline plasma Se levels of patients randomised into the study were well-balanced across the three treatment arms. The baseline average concentration across all groups was ~1.29μM, significantly below the range of 1.6-1.9μM associated with optimal longevity from large observational studies [3]. The five patients who were self -administering Se supplements prior to study registration had baseline levels (mean plasma Se 1.52μM) approaching the threshold of 1.6μM, supporting reports from other countries that standard dietary Se intakes in cancer patients may not achieve optimal levels without supplementation [193-195]. A comparison between baseline status and gender revealed no significant difference, compared to other larger-scale studies where males had higher levels than females [196].

4.4.2 Plasma Se profiles

The PK profile of the Se compounds highlights the greater systemic exposure that occurs with SLM compared to those receiving MSC or SS. The lower increment in plasma Se achieved with SS may be due to rapid plasma clearance of Se by red blood cells [197]. The much larger increases in plasma Se levels with SLM, compared to other Se compounds, has been widely observed [38; 44]. Explanations of this phenomenon relate to the non-specific incorporation of SLM into proteins, particularly albumin, in place of methionine, resulting in systemic accumulation and slower total body clearance [198]. While MSC does not compete with methionine and is not indiscriminately bound to protein. In single-dose studies, over a third of MSC is excreted via urine or faeces within 12 days, compared to only 15% of SLM [199]. In our study in both SS- and MSC-treated patients Se plasma levels returned almost to baseline after a four-week washout period, whereas in the SLM group mean plasma Se levels were still about 50% above baseline at the same time point. The difference in PK profiles suggests that control of systemic exposure is much more easily achieved with MSC and SS, whereas SLM presents challenges in terms of systemic

accumulation and clearance, which may be relevant to toxicities at higher doses and interactions with cancer therapies.

4.4.3 Implications for dosing strategy for Se combination studies

The relatively minimal differences between Se plasma levels at week 4 and week 8 for each compound were indicative that steady-state concentrations of plasma Se were achieved within 4 weeks of commencing oral Se administration for all three compounds in this study population. Maximum plasma levels recorded in this study were in patients treated with SLM; even when a patient had a post-dose level instead of a trough level, the maximal plasma concentration recorded remained under 4μM. The majority of pre-clinical Se studies suggest that the concentrations of Se should be in excess of 5μM and for certain PD effects to be elicited plasma levels towards 15-25μM should be targeted.

Intra-tumoral Se concentrations have been evaluated in head and neck tumour xenograft studies, where increase in the oral dosing of SLM and MSC did result in higher tumour Se concentrations [192]. To date, variable dosing strategies have been used in Se cancer trials to date [183] with flat dosing, dose per kg and dose per body surface area employed (See Table 1.5). While the relationship between supra-nutritional dosing of the Se compounds and their PK profile has been well-studied, the PK-PD relationship remains poorly understood. It would seem that Se compounds have a significantly wider therapeutic window than cytotoxic chemotherapy treatments, where dosing on body surface area is the standard approach. However the evidence to support dosing adults using such an approach is fairly limited [200] and would seem an overly complicated strategy when using potential adjunctive treatments such as Se compounds.

4.5 Conclusions

Baseline plasma Se levels in our study population were noted to be below the threshold recommended for optimal health. The plasma Se profile

for patients who received SLM differed significantly from both MSC and SS, in terms of the 4 hours post dose increment, steady state levels, levels at 4 weeks after dosing had been completed and for total $AUC_{0\text{-}eos}$. Minimal changes between plasma Se levels at day 28 and 56 suggest, steady state levels are achieved by day 28 in this study population dosed at 400μg of element Se per day. Planned dose escalation to the 1600μg/day dose level will form part of a future study currently being planned.

Chapter Five: Clinical Trial: Pharmacodynamic effects of Selenium compounds. Part 1: ER stress

5.1 Introduction

5.1.1 ER stress and the unfolded protein response

The ER is a key organelle in eukaryotic cells responsible for the storage and regulation of intracellular calcium, lipid biosynthesis and the trafficking and regulation of approximately one-third of all cellular proteins [201]. Changes in the ER environment such as redox state, fluctuations in calcium levels, nutrient status, rates of protein synthesis or inflammation can lead to the disruption of protein folding. ER stress is the term used to describe the cellular environment where misfolded or unfolded proteins accumulate. The collection of signalling cascades that results from this, and are responsible for maintenance of the ER protein folding environment, are referred to as the unfolded protein response (UPR). The UPR comprises three main arms (Figure 5.1) and is described below:

5.1.1.1 PRKR-like ER kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α) pathway

In non-stress conditions BIP/GRP78 and heat shock protein 90 (HSP90) bind to the ER luminal and cytoplasmic domains of PERK preventing action [202]. The presence of ER stress results in BIP/GRP78 binding to misfolded and unfolded proteins, enabling the release and activation of PERK and the phosphorylation of eIF2α at the serine 51 residue, results in an attenuation of protein translation [203]. Whilst PERK-eIF2α activation results in a global reduction in mRNA translation it also increases the translation of certain mRNAs such as ATF4 and ATF5 [204]. These transcription factors elicit a pro-survival response via genes responsible for antioxidant and amino acid biosynthesis pathway activation

[204]. Additionally C/BEP homologous protein (CHOP) is activated by ATF4 which, when sustained, can induce ER stress-mediated apoptosis [205]. Dephosphorylation of eIF2α is also mediated by ATF4 via growth arrest and DNA damage-inducible protein 34 (GADD34) [206].

5.1.1.2 Inositol-requiring protein 1α (IRE1α)-X-box binding protein 1 (XBP1) pathway

Under ER stress the transmembrane protein, IRE1α is activated following the binding of BIP/GRP78 to unfolded proteins, resulting in oligomerization and autophosphorylation [207]. The activated IRE1α cleaves XBP1 mRNA, resulting in the removal of a 26 base intron and producing a transcriptionally-active form (sXBP1), which activates genes that enhance protein folding, trafficking and increased protein degradation via ER-associated degradation (ERAD) [208]. Unspliced XBP1 (uXBP1*)* under chronic ER stress can act as a negative regulator of XBP1s and ATF6α promoting their degradation and attenuation of survival signalling [209; 210].

5.1.1.3 Activating transcription factor 6 (ATF6) pathway

The transmembrane protein ATF6α is activated on release from BIP/GRP78 under ER stress, whereupon ATF6α localises to the Golgi apparatus and undergoes processing to an active transcription factor. This cleaved form of ATF6α is responsible for transcription of genes that increase expression of *Xbp1* and ER capacity [210; 211].

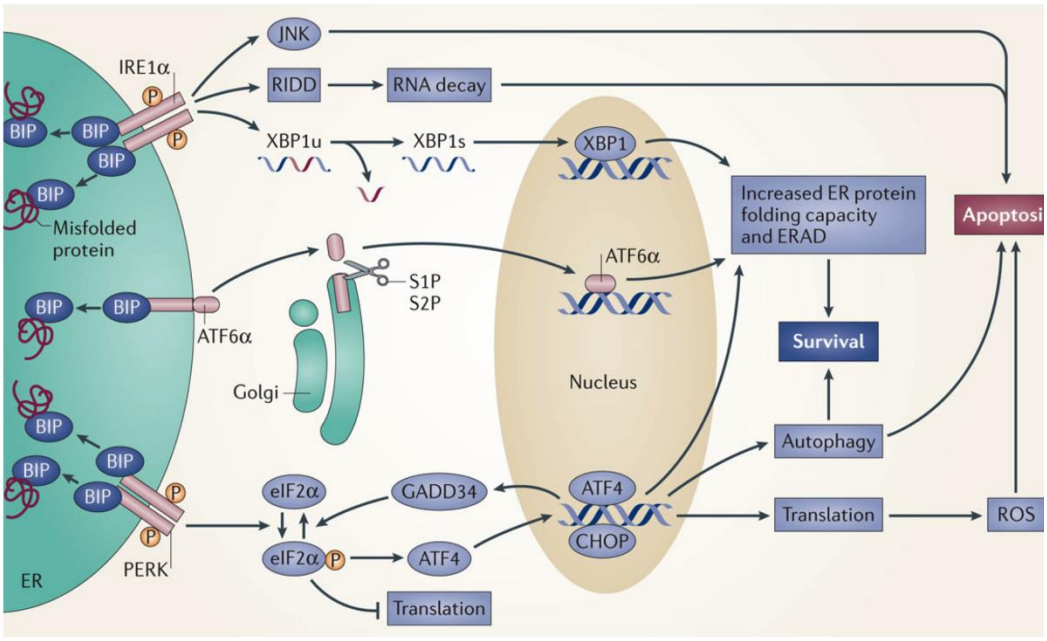

Figure 5.1 The unfolded protein response (UPR) signalling pathways. Reproduced with permission (Springer nature). [202]. The activation of three signalling cascades upon ER stress results in increased protein folding, transport, attenuation of protein synthesis and ER-associated protein degradation, with progression to apoptosis if protein misfolding is unresolved. Activating transcription factor 6α (ATF6α), immunoglobulin heavy-chain binding protein (BIP), C/EBP homologous protein (CHOP), eukaryotic translation initiation factor 2α (eIF2α), endoplasmic reticulum stress (ER), endoplasmic reticulum associated degradation (ERAD), growth arrest and DNA damage-inducible protein 34 (GADD34), inositol-requiring protein 1α (IRE1α), JUN N-terminal kinase (JNK), phosphorylation (P), PRKR-like kinase (PERK), regulated IRE1-dependent decay (RIDD), reactive oxygen species (ROS), proteases S1P and S2P (S1P, S2P), transcriptionally active spliced XBP1 (sXBP1), unspliced XBP1 (uXBP1).

5.1.2 The ER environment and cancer

ER stress and UPR activation is involved in the pathophysiology of many degenerative disease and is implicated in the development of malignant diseases [202]. Cancers arise and progress in stressful microenvironments, and the activation of the UPR forms a survival strategy in transformed cells, as well as contributing to tumour growth and chemo-resistance [212-214]. A number of major oncogenes and tumour suppressors have been identified as having key roles in altering Intracellular

Ca^{2+} signalling within the ER, promoting cancer cell survival and resistance to stress induced cell death [215].

As a result of poor vascularisation and rapid tumour growth the tumour microenvironment is both hypoxic and nutrient-deprived, leading to direct impacts on protein modification and the generation of severe ER stress. Post- translational folding or isomerisation of proteins in the ER has been shown to be oxygen-dependent [216]. Therefore tumour hypoxia can generate ER stress directly as well as increase the stability of selected UPR components, such as ATF4, which is degraded by a similar mechanism to HIF-1α via prolyl hydroxylation [217; 218]. It has also been observed that the UPR can be activated in an ER stress-independent manner, for example the angiogenic growth factor VEGF-A has been shown to activate the UPR in endothelial cells via phospholipase C-gamma and cross talk with mTOR complex 1 in the absence of ER stress [219]. The interplay between the UPR signalling cascades, immune defence, oncogenic and tumour suppressor pathways with the tumour microenvironment is both complex and highly interconnected [202].

The UPR is a target for therapeutic intervention in the clinic, with proteasome inhibitors such as bortezomib and carfilzomib part of the standard treatment of myeloma [220]. Many other approaches are at various stages of clinical development such as inhibitors of BIP/GRP78, HSP90 or XBP1 [202].

5.1.3 The ER and Selenium

Selenoproteins play an important but not fully-understood role within the ER environment, with seven of the 25 known mammalian selenoproteins residing in the ER [24]. To date these proteins have been implicated in protein folding, the chaperoning of misfolded proteins, roles in ER-associated protein degradation, calcium flux and homeostasis and in the response to oxidative and inflammatory stresses [221]. The physiological activity of these selenoproteins is yet to be fully characterised and their roles with respect to tumour biology remain poorly understood. Some

selenoproteins are thought to be tumour suppressors while others have been associated with an increase in malignant behaviour [222; 223].

5.1.4 Selenium supplementation and UPR

Se compounds cause global thiol redox modification of proteins, resulting in an accumulation of aberrantly-folded proteins in the ER that triggers the UPR [224]. It has been demonstrated that early phosphorylation of eIf2α (eIF2αP) at one hour of exposure to 10μM MSA in PC-3 cells and 2.5μM was sufficient to produce a time-dependent increase in GRP78 over 24 hours [225]. These authors report that the induction of caspase 12 and CHHOP/GADD153 pro-apoptotic markers by 5μM MSA is seen in PC-3 prostate cancer cells; and GRP94, GRP78 (survival inductor markers) and CHOP/GADD153 are up-regulated following exposure to 10μM MSA in a range of prostate cancer cell lines (LNCaP, LNCaP-LN3, DU145 and LAPC-4) [225]. In another *in vitro* study, PC-3 cells showed activation in all 3 UPR arms through western blotting and gene expression studies following exposure to 10μM MSA [226].

The effects of SS, SLM and MSC have also been compared. Only treatment with SS yield notable eIF2αP, with GRP78 and CHOP virtually unchanged in the oral carcinoma cell HSC-3 cell line, whilst SLM and MSC induced ER stress apoptosis via caspase 12 in A549 and HSC-3 cell lines respectively at differing concentrations and lengths of exposure [227]. However, variations across *in vitro* models can be considerable, with NB4 leukemic cells showing an increased sensitivity to SS, with 2μM sufficient to activate all 3 UPR pathways but concentrations of 5μM and above required to induce ATF6, CHOP and caspase 8 [228].

Differential ER stress responses have been observed following *in vitro* exposure to MSA between malignant THP1 and healthy donor PBMCs with pro-apoptotic vs. pro-survival UPR signatures observed [70] . This differential ER stress responses generated by Se exposure between malignant and healthy cells could be a major driver of improved therapeutic

selectivity when Se is administered alongside conventional anticancer treatments.

5.1.5 Rationale for selection of ER stress/ UPR biomarkers.

Candidate biomarkers were chosen on the basis of previous *in vitro* studies and proteins that have been identified as key components in growth arrest and in pro-apoptotic signalling cascades.

- GRP78: Major ER chaperone protein and master regulator of the UPR [229].
- CHOP: Transcription factor responsible for activation of apoptotic machinery under prolonged periods of induction [230].
- sXBP1: Corresponds to UPR activation resulting in enhanced ER capacity and ERAD [210].
- eIF2αP: Expression results in protein translation attenuation and survival response [231].
- Caspase 8: This is the initiator caspase in the death receptor pathway, also implicated in ER stress-induced apoptotic signalling [232].

This chapter will present the results from an assessment of the pharmacodynamic responses to the three Se compounds administered to cancer patients with a particular focus on key proteins that mediate cellular responses to ER stress.

5.2 Methods and Materials

5.2.1 Sample collection and processing

5.2.1.1 PMBC isolation

See sections 2.2.2.2 and 3.2.7.1.

5.2.1.2 PBMC viability and quantification

See section 2.2.2.3.

5.2.1.3 PBMC aliquoting

See section 3.2.7.2.

5.2.1.4 PBMC cryopreservation

See section 2.2.3.3.

5.2.2 Cell lysis

PBMC pellets (~1 x10^6 cells) were reconstituted in 75uL ice cold 1X radioimmunoprecipitation assay (RIPA) lysis buffer with protease and phosphatase inhibitors, which were added immediately before use (see Appendix A). This suspension was further mixed using a 200µL pipette every two minutes on ice over a 15 minute period. The mixture was then centrifuged at 13,300*g* for 15 minutes (5415R, Eppendorf, Hamburg, Germany) to pellet the cell debris, and the supernatant was transferred to a new tube. A 12.5µL aliquot of the cell lysate was taken for protein quantification and the remainder placed in storage at -80 °C.

5.2.3 Protein quantification of cell lysates

The Pierce™ BCA protein assay kit (Pierce Biotechnology Rockford, IL, USA. 23225) was used to estimate the protein concentrations of PBMC cell lysates. This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the selective colorimetric detection of the cuprous cation Cu^{1+} using a proprietary agent containing bicinochoninic acid (BCA). The purple-coloured water- soluble reaction product is formed by the chelation of two BCA molecules with one Cu^{1+} cation and exhibits strong absorbance at 562nm.

Firstly albumin (BSA) standards were diluted using RIPA buffer and stock albumin solution (Table 5.1) in 1.5mL microcentrifuge tubes (VWR, Radnor, PA, USA).

Table 5.1 Dilution of BSA standards for BCA protein quantitation.

Vial	Volume of diluent (μL)	Volume and Source of BSA (μL)	Final concentration of BSA in μg/L
A	0	150 of stock	2000
B	62.5	187.5 of stock	1500
C	162.5	162.5	1000
D	87.5	87.5 of B	750
E	162.5	162.5 of C	500
F	162.5	162.5 of E	250
G	162.5	162.5 of F	125
H	200	50 of G	25
I	200	0	0

Secondly, 12.5μL of RIPA buffer was mixed with 12.5μL of cell lysate, and 10μL of this diluted lysate was added in duplicate to a flat bottomed 96 well plate (Greiner-Bio-One 655101) along with duplicates of 10μL BSA standards from vials A-H (Table 5.2). A working BCA reagent was prepared by mixing 50 parts of reagent A with 1 part of BCA reagent B in a Falcon tube and mixed by vortexing to yield a clear green working reagent. 200μL of the working reagent was then added to all the wells containing BSA standards and diluted protein lysates. The plate was then covered with a plate lid and placed on a plate shaker-thermomixer (Thermomixer® comfort, Eppendorf, Hamburg, Germany) at 600rpm for 30 seconds and then incubated at 37°C for 30 minutes. Absorbance was measured at 562nm using a spectrophotometric plate reader (Multiskan Go, Thermo Fisher Scientific, Ratastie, Finland) and values corrected by subtraction of the OD

obtained from the blank replicates. Preparation of the standard curve of known BSA concentrations with curve fitting and calculations for unknown samples was performed using GraphPad prism v7.0 (GraphPad Software, La Jolla, CA, USA).

5.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sample tubes containing PBMC cells lysates (grouped by patients, 6 time points per patient) were removed from -80°C storage and placed on ice. For denaturation of protein secondary structure, a 1/4 of 4X Laemmli loading buffer (Appendix A) containing 5% β-mercaptoethanol (β-ME; added just before use) was dispensed into a boil-proof 1.5mL microcentrifuge tubes (VWR, NZ) and then 1X RIPA buffer (with protease inhibitors and phosphatase inhibitor cocktails (see Appendix A) was added to achieve a final 1X Laemmli buffer concentration after cell lysate addition giving a final concentration of 1.25%v/v β-ME. Next, cell lysate corresponding to 30μg of protein was added to each tube, then the tubes capped. All final volumes were between 8 and 15μL. The proteins in the cell lysate were then denatured by incubation at 70°C for 10 minutes in a thermomixer.

The cell lysate was split into two aliquots, each containing 15μg of protein and loaded onto two precast 10% GenScript ExpressPlus™ PAGE gels (Genscript, Piscataway, NJ, USA. M01010), which were placed on both sides of a Hoefer™ Mighty Small IISE250/SE260 electrophoresis system (Hoefer, Holliston, MA, USA). 3μL of protein molecular weight standards, WB Master (Genscript M00521), were loaded in well 1 and 3μL of Smart Multi Color Pre-Stained Protein Standard (Genscript M00443) were loaded into lane 7. Electrophoresis was carried out using 1X Tris- MOPS buffer (Express Gel running buffer powder – Genscript M00138) using the Atto Crosspower 1000 (Atto, Tokyo, Japan) at 140V for ~50 mins, until the dye fronts had reached the bottom 1/10th of the gels.

5.2.5 Protein transfer

Transfer of proteins from the SDS-PAGE gel onto WestClear™ nitrocellulose membranes (Genscript L00224A60) was carried out using the Genscript® eBlot® Protein Transfer System (Genscript L03010). Firstly, the anode pad of the eBlot® Transfer Pads apparatus was placed into the transfer system, this was followed by the nitrocellulose membrane (Genscript, pre-soaked in equilibration buffer – component of eBlot transfer pad kit L03011). The pre-run SDS-PAGE gel was then removed from the electrophoresis apparatus and placed onto the pre-wetted membrane. A plastic gel window was applied to prevent the anode and cathode pads (Genscript L03011) from becoming stuck to each other during the transfer. Lastly, a cathode pad was placed on the gel, the lid closed, and the transfer run for 7 minutes, as recommended by the manufacturer for proteins of interest ≤100kDa.

5.2.6 Ponceau S

The membrane were stained for 10 minutes using 0.1% w/v ponceau S in 5% acetic acid to check successful protein transfer, integrity of the protein, and equal protein loading of wells. Membranes were then destained with 1% acetic acid for 5 minutes.

5.2.7 Membrane cutting for WB targets and antibody incubation

The nitrocellulose membranes were carefully cut at approximately 50kDa and 46kDa respectively using the multicolour protein marker as a guide (Figure 5.2). The membranes were then blocked in 5% w/v solution of skim milk powder (Alpine, NZ) or 5% w/v BSA (Sigma) in 1X Tris-buffered saline with Tween 20 (TBS-T) (see Appendix A) for 1 hour at 4°C under agitation on an orbital shaker (Bellco Glass Inc. Vineland, NJ, USA). Primary antibodies were diluted in blocking buffer (Table 5.2) and incubated on the membranes at 4°C overnight under gentle agitation using an orbital shaker. The next morning the membranes were washed 3 x 5 minutes with TBS-T prior to incubation with the species-appropriate goat anti-mouse or goat anti-rabbit horseradish peroxide (HRP) secondary antibody (Table 5.2) for

1 hour at room temperature at low speed on the orbital shaker. The membranes were then washed another three times in TBS-T prior to treatment with the enhanced chemiluminescence (ECL) reagents. The Western Bright™ ECL spray (Advansta, Menlo Park, CA, USA) was used to visualise the protein bands. For this the two constituent reagents within the spray bottle were mixed on each actuation. Following complete coverage of membranes and incubation in the dark for two minutes, the ECL reagents were discarded and the ECL signal was then detected by placing the membranes in the Omega Lum™ G gel imaging system (Aplegen® Pleasanton, CA, USA. 81-12100-00) set to chemiluminescence (light emission at 428nm) and auto exposure. TIFF images were then quantified by densitometry.

Table 5.2 Primary and secondary antibodies.

Antibody type	Target protein	Species of origin	Clonality	Manufacturer	Cat no.	Dilution	Expected vs. observed size (kDa)
Primary	Beta Actin	Mouse	Monoclonal	Abcam	120-82260	1:3000 in BSA block	42
Primary	Caspase 8	Rabbit	Monoclonal	Abcam	000-07176	1:1000 in Milk block	55
Primary	CHOP	Mouse	Monoclonal	Enzo	ALX-804-551	1:1000 in milk block	31
Primary	eIF2αP (Serine 51).	Rabbit	Monoclonal	Abcam	120-321157	1:500 in milk block	36
Primary	GRP78	Rabbit	Polyclonal	Abcam	120-21685	1:1000 in milk block	75k
Primary	XBP1	Rabbit	Polyclonal	Abcam	120-37152	1:1000 in milk block	29 and 40(splice variants observed)
Secondary	Anti-Mouse	Goat	Polyclonal	Abcam	AB97023	1:5000 in milk block	NA
Secondary	Anti-Rabbit	Goat	Polyclonal	Abcam	AB9705	1:5000 in milk block	NA

5.2.8 Membrane Stripping

Nitrocellulose membranes previously probed and stored in TBS at 4°C, were incubated with Restore™ Western blotting stripping buffer (Thermo Scientific, Pierce Biotechnology, Waltham, MA, USA) for 15 minutes at RT. The membranes were then washed 3 times in TBS for 10 minutes each and re-blocked for an hour at RT using 5% low fat milk powder or BSA [Sigma, A9647]) before incubation with the appropriate primary and secondary antibodies in accordance with the processing plan (Figure 5.2). Membranes were stripped and reused a maximum of two times with washing and ECL-based visualisation. TIFF images were captured were quantified by densitometry.

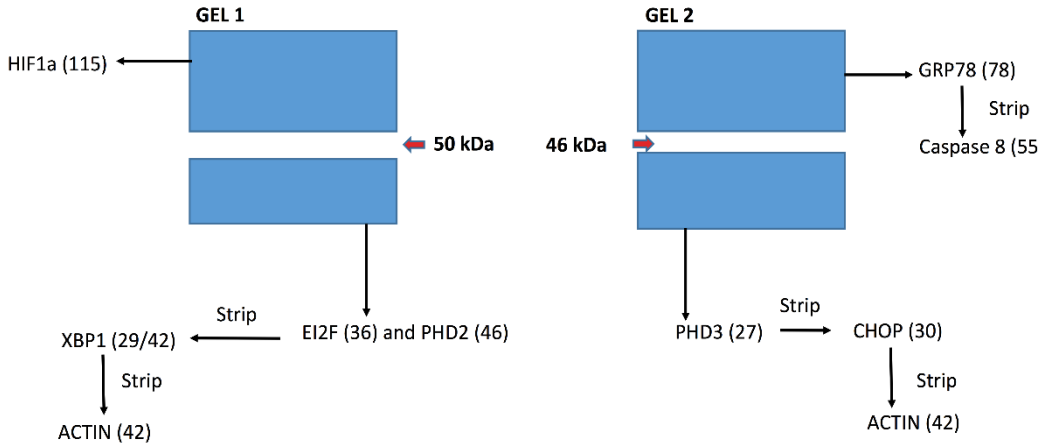

Figure 5.2 Nitrocellulose membrane probing and processing plan.

5.2.9 WB densitometry

Previously-captured images were imported into the Image Studio™ Lite software programme (LI-COR, Lincoln, NE, USA). Images were inverted and bands quantified using the analysis portion of the programme

which included background correction. Signal densitometry values were exported into Microsoft Excel for further analysis, including normalisation to the signal density of β-actin (loading control).

5.2.10 Analysis of WB

The background-corrected densitometric values for each target protein were determined by the Image Studio™ lite software and normalized to the corresponding β-actin band values for each sample (maximum 6 samples per patient). Relative expression of each target protein on visits 3–6 was calculated in relation to average β-actin-normalized values of visits 1 and 2 prior to Se administration [233]. Changes in relative expression over time for each target protein were analysed by disease group using one-way ANOVA and Tukey's multiple comparison test. Further analysis by disease and compound group was carried out as above with additional comparison made to the expression levels at baseline (visits 1 and 2 expression values calculated as 1+[V1-V2/mean]) and 1 –[V1-V2/mean]) using one-way ANOVA and Dunnett's multiple comparison test. All tests were performed in GraphPad Prism v7.0. The 95% confidence intervals for the difference in β-actin-normalized expression were plotted for each protein investigated.

5.3 Results

5.3.1 BCA quantification

Protein concentrations of the PBMC lysates were calculated in accordance with the manufacturer's guidelines. R^2 values for the albumin standard curves were greater than 0.99 using the polynomial quadratic curve fitting option. Protein lysate concentrations of each sample ranged from 0.33 to 1.88μg/μL.

5.3.2 Densitometry data using GRP78 (78kDa)

Band intensities were first normalised to β-actin expression and expression was then calculated relative to the average values of visits 1 and 2, prior to commencing Se (Figure 5.3).

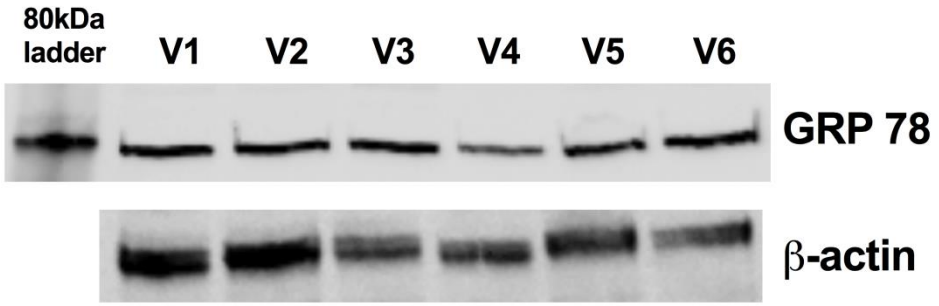

Figure 5.3 An example blot obtained for GRP78 from a patient involved in the study (patient 7).

5.3.2.1 GRP78 expression by disease group

No significant difference between time points were observed in either disease group by one-way ANOVA or post hoc Tukey's multiple comparison test (Figure 5.4). Solid cancer $F(3, 41)=1.5$, $p=0.2289$ and CLL $F(3,37)=02.269$, $p=0.0966$).

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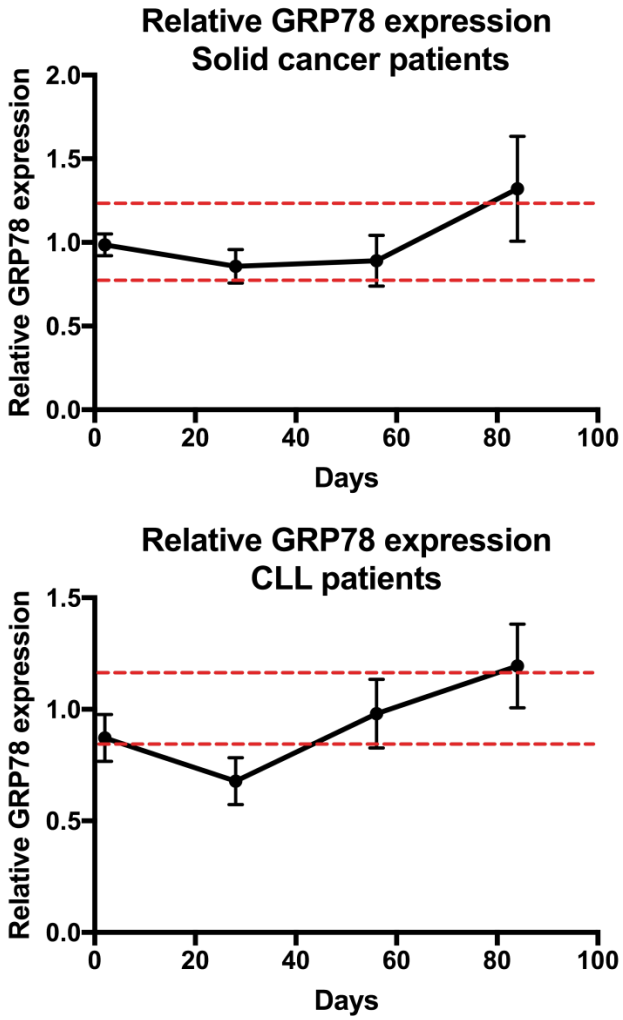

Figure 5.4 Relative expression of GRP78 (β-actin-normalised) compared to baseline values for all Se compounds per disease group (n=12 solid cancer group, n=11 CLL group). Error bars +/-SEM. Red dotted line represents the 95% confidence intervals of difference between each baseline visit and the average of the two visits.

5.3.2.2 GRP78 expression by disease and compound group.

No statistically significant difference in relative GRP78 expression was noted at different time points for any disease or compound group by one-way ANOVA and Tukey's multiple comparison tests, with the exception of the CLL patient group administered with MSC where ANOVA $F(3,8)=8.661$ $p=0.0068$ and $p=0.0136$ for day 2 to day 84 and $p=0.0089$ for day 28 to day 84 by Tukey's multiple comparison test (Figure 5.5). When comparing the variation in expression between the 2 baseline visits for each patient in this

group and expression at subsequent visits using Dunnett's multiple comparison test, no significant differences below the alpha threshold of 0.05 were observed.

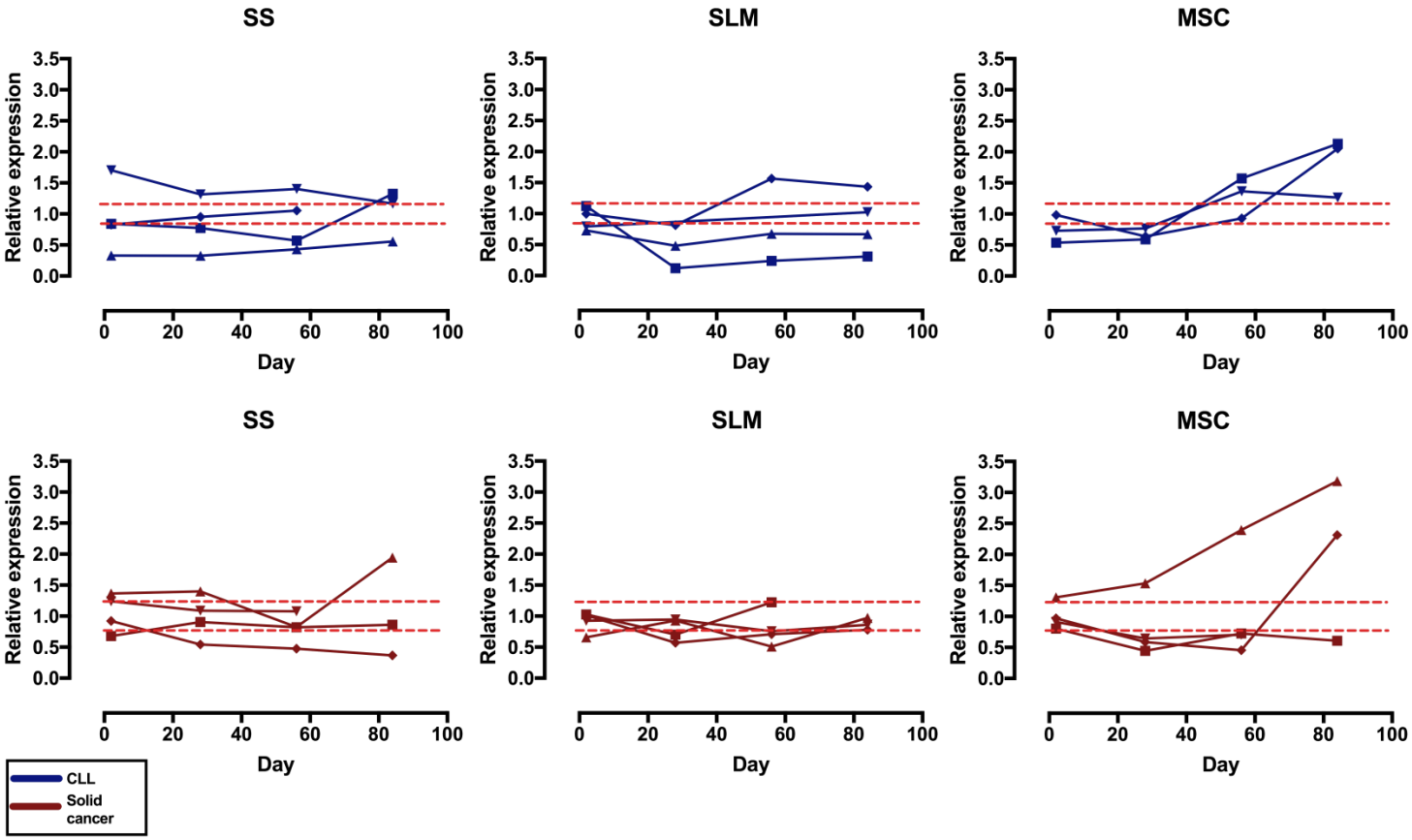

Figure 5.5 GRP78 expression by disease group and Se compound with 95% CI thresholds for differences at baseline visits marked in red. Each solid line represents one patient.

5.3.3 Densitometry data using eIF2αP (36kDa)

Band intensities were first normalised to β-actin expression and expression was then calculated relative to the average values of visits 1 and 2, prior to commencing Se (Figure 5.6).

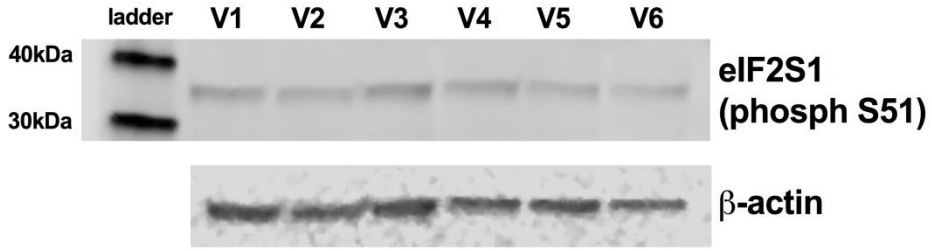

Figure 5.6 An example blot obtained for eIF2αP from a patient involved in the study (patient 3).

5.3.3.1 eIF2αP expression by disease group

No significant difference between time points was observed in either disease group by one-way ANOVA or post hoc Tukey's multiple comparison test (Figure 5.7). Solid cancer $F(3, 41)=0.09282$, $p=0.9636$ and CLL group $F(3,33)=0.04189$, $p=0.9884$).

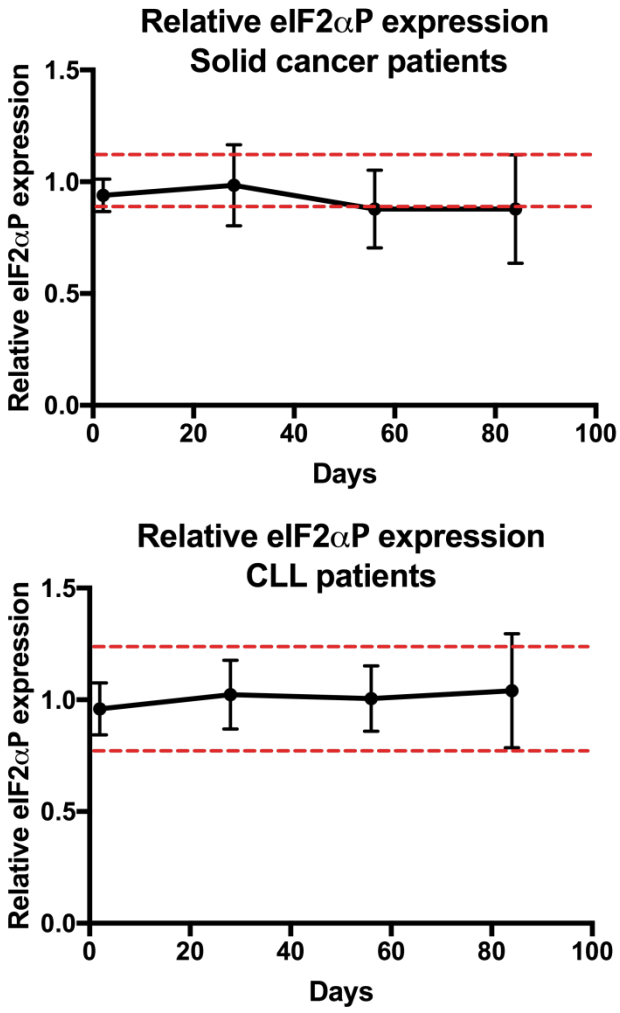

Figure 5.7 Relative expression of eIF2αP (β-actin-normalised) compared to baseline values for all Se compounds per disease group (n=12 solid cancer group, n=10 CLL group). Error bars +/-SEM. Red dotted line represents the 95% confidence intervals of difference between each baseline visit and the average of the two visits.

5.3.3.2 eIF2αP expression by disease and compound group.

No statistically-significant differences in relative eIF2αP expression was noted at different time points for any disease or compound group by one-way ANOVA and Tukey's multiple comparison tests with the exception of the solid cancer patient group administered with SLM, where (ANOVA $F(3,11)=5.743$ $p=0.0130$, and $p=0.0324$ for day 28 to day 56 and $p=0.0253$ for day 28 to day 84 by using Tukey's test (Figure 5.8). When comparing

the variation in expression between the 2 baseline visits for each patient in this group and expression at subsequent visits using Dunnett's multiple comparison test no significant differences below the alpha threshold of 0.05 were observed (baseline to day 28 p=0.8157,baseline to day 56 p=0.2771 and baseline to day 84 p=0.2230).

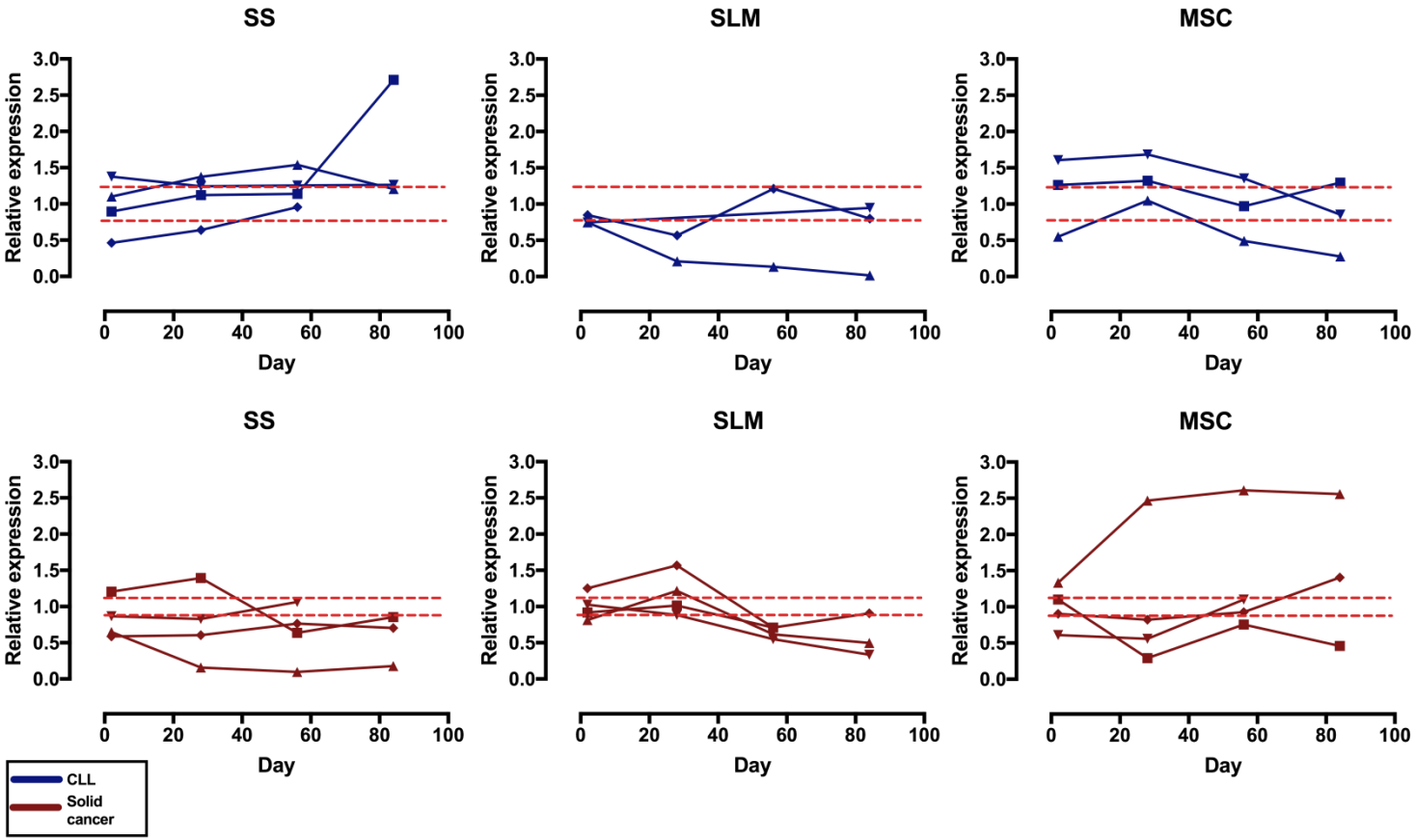

Figure 5.8 eIF2αP expression by disease group and Se compound with 95% CI thresholds for differences at baseline visits marked in red. Each solid line represents one patient.

5.3.4 Densitometry data using sXBP1 (37kDa)

Band intensities were first normalised to β-actin expression and expression was then calculated relative to the average values of visits 1 and 2, prior to commencing Se (Figure 5.9).

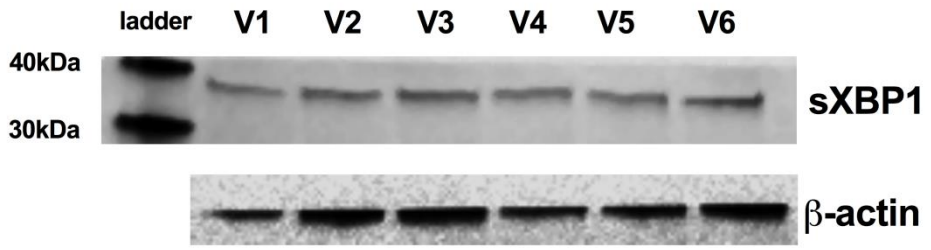

Figure 5.9 An example blot obtained for sXBP1 from a patient involved in the study (patient 4).

5.3.4.1 sXBP1 expression by disease group

No significant difference between time points was observed in either disease group by one-way ANOVA or post hoc Tukey's multiple comparison test (Figure 5.10). Solid cancer $F(3, 38)=0.4849$, $p=0.6948$ and CLL group $F(3,29)=0.4298$, $p=0.7332$).

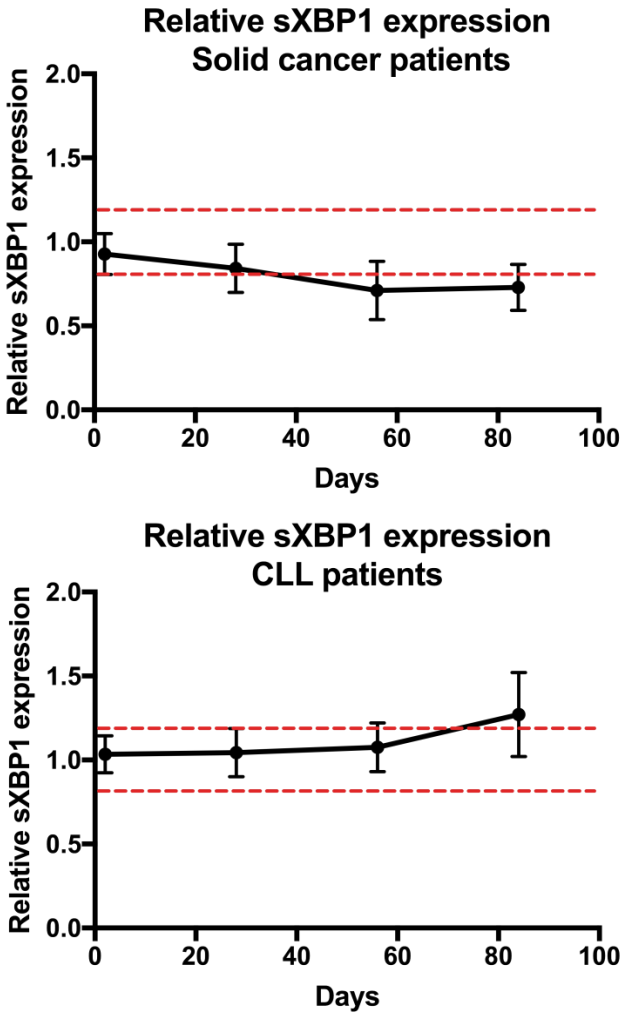

Figure 5.10 Relative expression of sXBP1 (β-actin-normalised) compared to baseline values for all Se compounds per disease group (n=11 solid cancer group, n=9 CLL group). Error bars +/-SEM. Red dotted line represents the 95% confidence intervals of difference between each baseline visit and the average of the two visits.

5.3.4.2 sXBP1 expression by disease and compound group.

No statistically-significant differences in relative sXBP1 expression was noted at different time points for any of the disease or compound groups by one-way ANOVA and Tukey's multiple comparison tests where $p > 0.05$ for all one-way ANOVA comparisons across the 6 disease/compound groups and the 6 comparisons for all time points using Tukey's for each group (Figure 5.11). When comparing the variation in

expression between the 2 baseline visits and expression at subsequent visits for all disease and Se compound groups using one-way ANOVA and Dunnett's multiple comparison no significant differences below the alpha threshold of 0.05 were noted in all 6 ANOVA and 24 pair-wise comparisons.

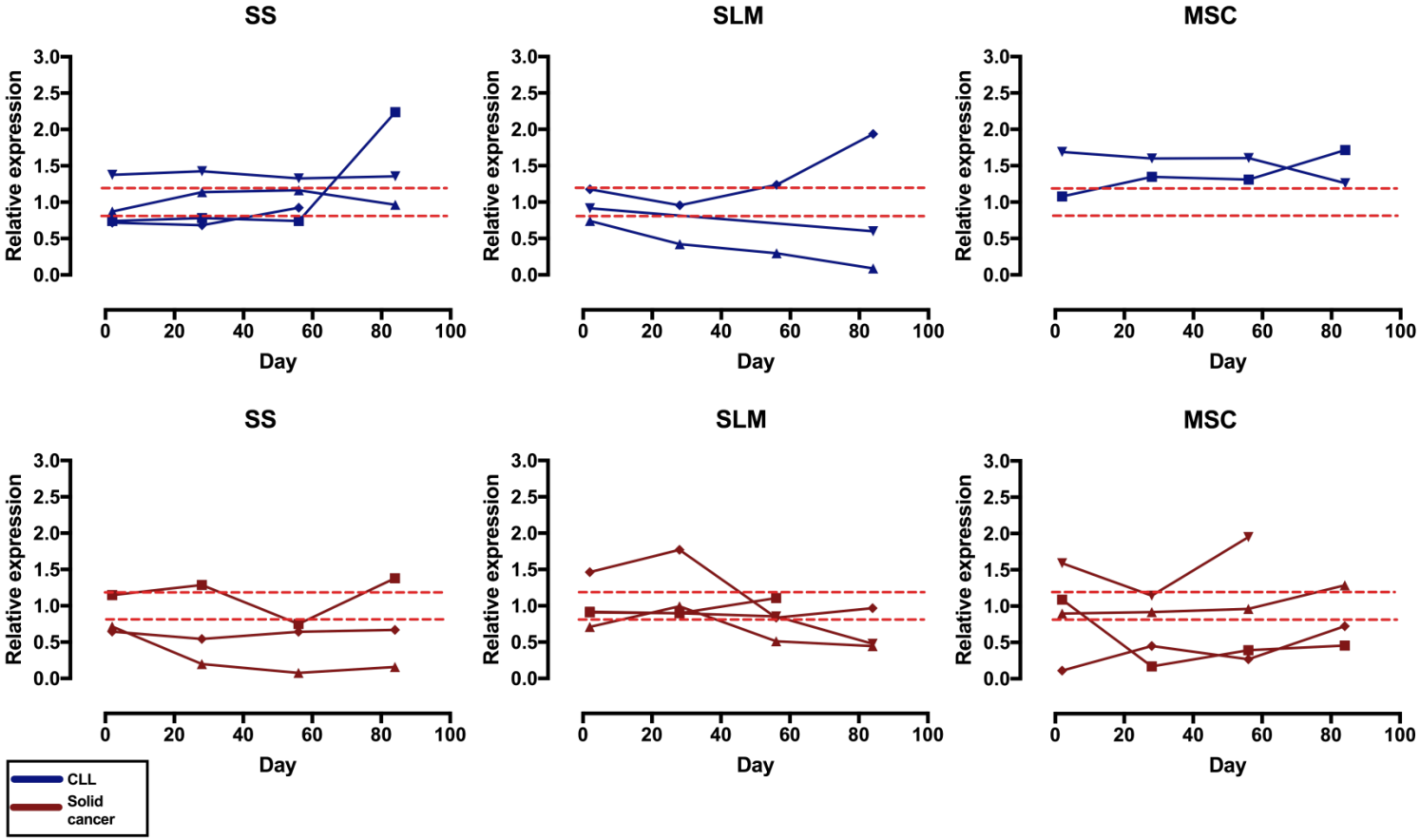

Figure 5.11 sXBP1 expression by disease group and Se compound with 95% CI thresholds for differences at baseline visits marked in red. Each solid line represents one patient.

5.3.5 Densitometry data using Caspase 8 (55kDa)

Band intensities were first normalised to β-actin expression and expression was then calculated relative to the average values of visits 1 and 2, prior to commencing Se (Figure 5.12).

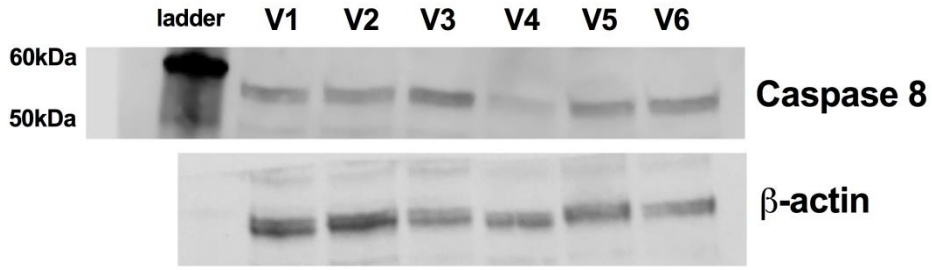

Figure 5.12 An example blot obtained for caspase 8 from a patient involved in the study (patient 7).

5.3.5.1 Caspase 8 expression by disease group

No significant difference between time points was observed in either disease group by one-way ANOVA or post hoc Tukey's multiple comparison test (Figure 5.13). Solid cancer $F(3, 41)=0.09282$, $p=0.9636$ and CLL group $F(3,33)=0.04189$, $p=0.9884$).

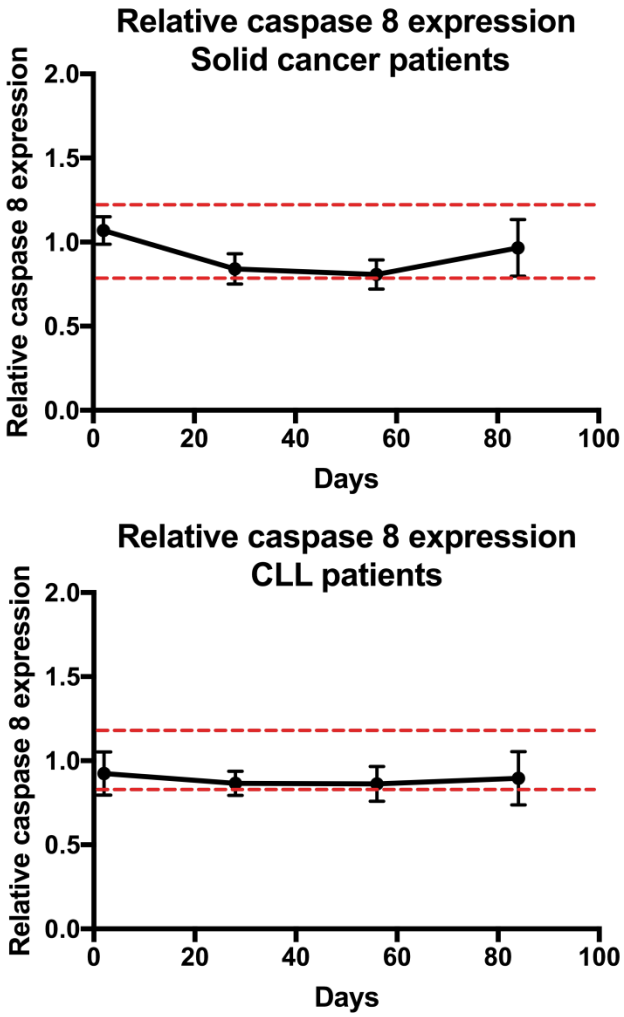

Figure 5.13 Relative expression of caspase 8 (β-actin-normalised) compared to baseline values for all Se compounds per disease group (n=12 solid cancer group, n=10 CLL group). Error bars +/-SEM. Red dotted line represents the 95% confidence intervals of difference between each baseline visit and the average of the two visits.

5.3.5.2 Caspase 8 expression by disease and compound group.

No statistically significant differences in relative expression of caspase 8 was noted at different time points for any disease or compound group by one-way ANOVA and Tukey's multiple comparison tests (Figure 5.14). When comparing the variation in expression between the 2 baseline visits and expression at subsequent visits for all disease and Se compound groups using one-way ANOVA and Dunnett's multiple comparison no

significant differences below the alpha threshold of 0.05 were noted in all 6 ANOVA and 24 pair-wise comparisons.

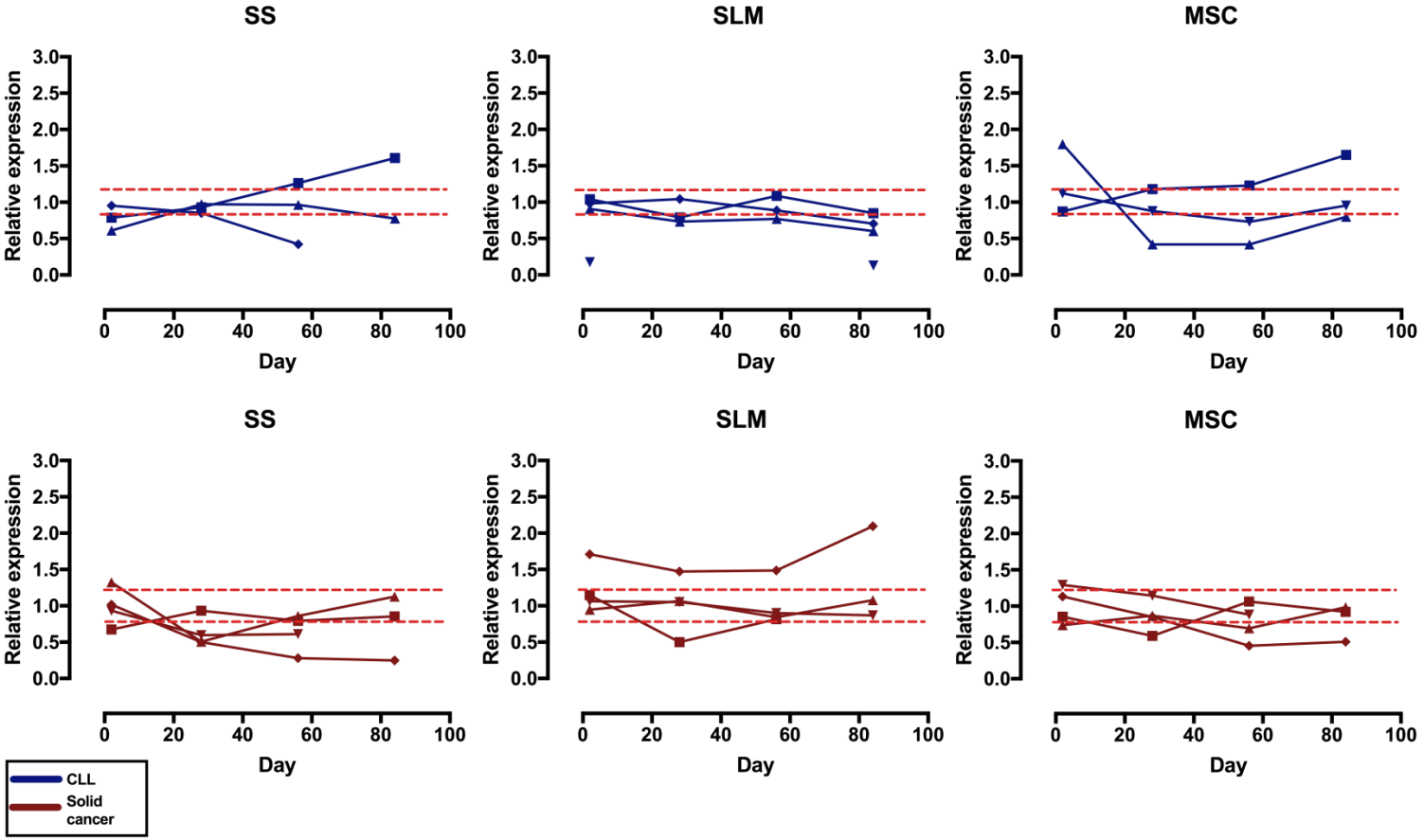

Figure 5.14 Caspase 8 expression by disease group and Se compound with 95% CI thresholds for differences at baseline visits marked in red. Each solid line represents one patient.

5.3.6 Densitometry data using CHOP expression (31kDa)

No band(s) could be detected that could correspond to expression of CHOP protein on the membrane (Figure 5.15).

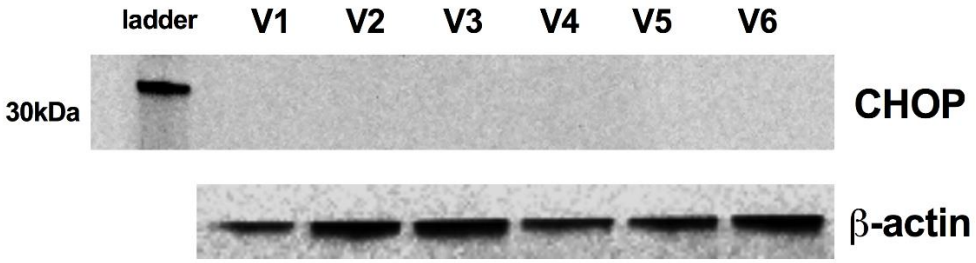

Figure 5.15 An example of a blot for CHOP from a patient involved in the study (patient 3) with no signal detected.

5.4 Discussion

Our results indicate minimal changes in the expression of proteins involved in the UPR during and after treatment/exposure of 400μg per day of elemental Se in the forms of SS, SLM or MSC when compared to baseline expression. This lack of change in expression of UPR target proteins in both malignant lymphocytes and PBMCs could be explained by the failure to reach the dose-response threshold required for an ER stress response with any of the Se compounds used at this dose. The highest steady state plasma Se level achieved with MSC or SS was 2.2μM, while those achieved with SLM were higher, due to non-specific incorporation of Se into the general protein pool and does not accurately reflect biologically-active Se. Increased production of GRP78 has been reported in prostate cancer cell lines treated with 2.5μM MSA [225], while another study found all three arms of the UPR were activated in prostate cancer cell lines following treatment with 10μM MSA [226]. However, a third study in HSC-3 cells, has shown that MSC at 100μM did not increase GRP78 or induce the pro-apoptotic protein CHOP [227].

This variation in ER stress protein expression may correspond to differences with the metabolism of MSA and MSC, whereby MSA is readily

reduced by non-enzyme and enzyme dependent processes to the putative anticancer metabolite that is methylselenol [234]. In contrast to this, MSC is dependent on the tissue abundance of key enzymes (such as β-lyase) for its conversion to methylselenol [234; 235]. In another study, a three-way comparison of the cytotoxicity of SS, SLM and MSC in a variety of human cell lines showed all three compounds could induce ER stress [227], though only 10μM SS was able to phosphorylate eIf2α in HSC-3 cells. Therefore activation of the UPR maybe a feature of the cytotoxic activity of both organic and inorganic selenocompounds in cancer cells.

The potential differential response between malignant and non-malignant cells to Se-induced ER stress has been suggested as a potential mechanism for enhancing the therapeutic selectivity of anti-cancer treatments [227]. The data from this study does not support this hypothesis at the Se dose used but it remains to be seen if significant differences will be observed between CLL lymphocytes and PBMCs when exposed to sustained levels above 5μM of elemental Se. A prior investigation has compared the effects of MSA on THP1 cells and donor PBMCs and while both cell types did show evidence of activation of ER stress response proteins (GRP78, sXBP1 and eIF2αP) in response to MSA concentrations ≤2.5μM, a differential response with respect to caspase 8 expression was seen, with a dose dependent increase in THP1s contrasting with a dose-dependent fall in PBMCs [70].

The results presented in this chapter must be interpreted in the context of using a pragmatic semi-quantitative western blotting approach. The number of target proteins, sample numbers and material available (number of cells) did result in compromises having to be made on optimal western blotting methodology, such as protein loading (15μg per lane), membrane cutting, and membrane stripping and re-probing. In future, the use of fluorescence-conjugated antibodies would allow a greater level of sensitivity [236] and remove the need for membrane cutting and stripping.

5.5 Conclusions

None of the Se compounds at the 400μg dose induced more than minimal changes in GRP78, eIF2αP, sXBP1 and caspase 8 protein expression compared to baseline expression, in either the CLL cohort or the solid cancer patient cohort. This is likely a dose-response threshold effect, with the majority of pre-clinical data showing ER stress modulation at doses of ≥ 5μM.

Chapter Six: Chapter Six: Clinical Trial Pharmacodynamic effects of Selenium compounds. Part 2: Angiogenesis and glutathione

6.1 Introduction

6.1.1 Angiogenesis and cancer

Angiogenesis is a term used to describe the process of new blood vessels sprouting from existing vasculature. The generation of new blood vessels is a fundamental requirement for tissue expansion and as early as the 1970s it had been proposed that no tumour could grow beyond $2mm^3$ without vascularization [237]. Over a decade later, important tumour-secreted pro-angiogenic growth factors were isolated, which included basic fibroblast growth factor (bFGF) and vascular endothelial growth factor A (VEGF-A) [238-240].

The differences between physiological and pathological angiogenesis are thought to be related to the regulation and balance of pro- and anti-angiogenic signalling [241]. A predominance of pro-angiogenic signalling factors (which includes VEGF isoforms, FGF isoforms, platelet-derived growth factor B [PDGF-B]) over angiogenesis inhibitors (such as thrombopsondin-1, angiostatin, endostatin, canastatin and tumstatin) is associated with malignant progression, resulting in formation of aberrant vascular structures, abnormal blood flow, increased blood vessel permeability and tumour hypoxia [241]. Complex interactions between tumour vasculature and the tumour microenvironment (TME) are mediated by a vast array of TME components, such as macrophages, myeloid and lymphoid cells, platelets, cancer-associated fibroblasts, adipocytes, the

extracellular matrix, ROS and metabolites such as lactate, that enable the maintenance and growth of malignant vasculature [242].

6.1.1.1 Role and regulation of hypoxia-inducible factor 1 (HIF-1)

The consumption and delivery of oxygen on a cellular level is tightly-regulated through the action of hypoxia-inducible factors (HIFs) [243]. In the hypoxic tumour microenvironment activated HIFs are responsible for the transcription of the VEGF gene, encoding a secreted endothelial growth factor that leads to stimulation of angiogenesis. The influence of HIFs are far-reaching, with over 800 HIF target genes identified [244; 245], and are responsible for many aspects of tumour biology in addition to angiogenesis, such as stem cell maintenance, autocrine growth factor signalling, invasion and metastasis.[246]

HIF-1 is a heterodimeric DNA-binding complex composed of HIF-1α and HIF-1β subunits. Oxygen regulation of HIF-1 activity is principally through the hydroxylation of its proline and asparagine (Figure 6.1) [247]. In summary, under normoxia, hydroxylation of HIF-1α Pro^{402} and Pro^{564} is catalyzed by the Fe(II)-, oxygen- and 2-oxoglutarate-dependent by prolyl hydroxylases (PHDs). The hydroxylated prolyl residues enable capture of HIF-1α by the von Hippel–Lindau protein (pVHL), resulting in its ubiquitination and subsequent proteolysis via the proteasome. Factor inhibiting HIF-1 (FIH-1) catalyses the hydroxylation of Asn^{803} preventing the recruitment and capability of the transcriptional co-activator p300. Under hypoxic conditions the O_2 and α-keto-glutarate-dependent hydroxylation reactions are inhibited, leading to HIF-1α stabilization, nuclear translocation, heterodimerisation with HIF-1β and binding to hypoxia-response elements (HREs) in the regulatory regions of target genes.

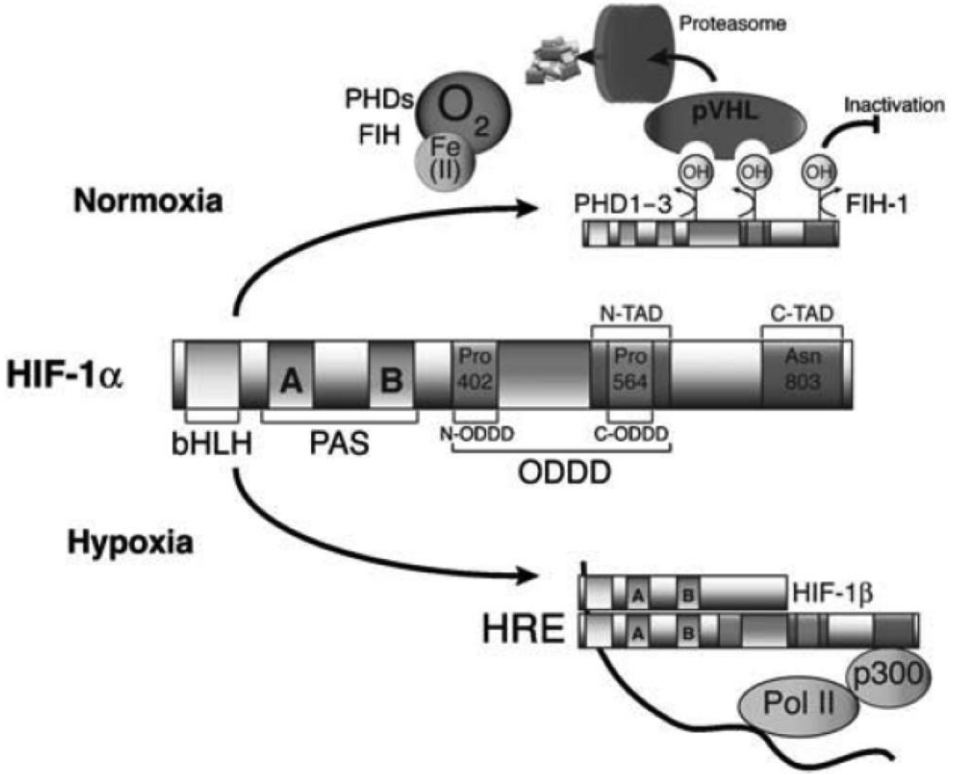

Figure 6.1 Regulation of HIF-1α protein by prolyl hydroxylation and proteasomal degradation Reproduced with permission (Springer Nature) [248].

6.1.1.2 Role of VEGF and tumour angiogenesis

The mammalian VEGF family consists of five secreted proteins, VEGF-A,B,C,D and placental growth factor (PLGF), interacting with different binding affinities to three VEGF tyrosine kinase receptors, namely VEGFR1, 2 and 3 [249]. The function of VEGF-A in the context of vascular endothelial cell (EC) signalling is perhaps the most understood of all the VEGFs. Regulation of vascular permeability and EC proliferation and migration stems from the interactions of VEGF-A with VEGFR1, encoded by the Fms-like tyrosine kinase 1 gene (Flt1) and VEGFR2, encoded by the foetal liver kinase1 gene (Flk1) [250]. VEGF-A is expressed in the majority of malignant tumours and is considered the predominant tumour angiogenesis factor [237; 251]. However VEGF-A exists in multiple isoforms due to variable splicing, resulting in another layer of biological complexity [252].

6.1.1.3 Targeting tumour angiogenesis

The development of anti-angiogenesis therapies has so far led to only modest improvements in clinical outcomes. Therapeutics approaches that have reached the clinic include monoclonal antibodies targeting circulating VEGF such as bevacizumab, and perturbation of VEGF receptor signalling via inhibition of intracellular tyrosine kinase domains with small molecules such as sorafenib and sunitinib [253]. The HIFs also remain targets for therapeutic intervention but no specific HIF inhibitors have progressed beyond clinical trials [254].

The combination of anti-angiogenic agents with conventional cytotoxicity chemotherapy and/or radiotherapy can result in improved treatment responses via improved tumour blood flow, reduced tumour hypoxia, improved drug delivery [255] and radiosensitisation [256]. However, many challenges remain concerning the successful implementation of this strategy, where discontinuation of the VEGF blockade has led to accelerated malignant progression, evidenced by local invasion and distant metastases due to rebound hypervascularisation [257]. Furthermore, combination therapy with VEGFR tyrosine kinase inhibitors and chemotherapy has not proven effective, and existing anti-angiogenesis treatments are associated with significant adverse effects and an unfavourable cost-benefit ratio [258].

6.1.2 Angiogenesis and Se

Se compounds have demonstrated anti-angiogenic properties both in cell lines and tumour xenograft pre-clinical models (see Table 1.3 and Table 1.4). In particular, the organoselenocompounds have shown promising inhibition of tumour angiogenesis, reducing tumour microvessel density and improving cytotoxic drug delivery [192]. This observed anti-angiogenic activity of Se compounds is thought to include HIF inhibition and reduced VEGFA expression [83; 84; 86; 259-261] with tumour-selective modes of action [192]. In previous preclinical studies using SLM in tumour xenograft models, a minimum plasma Se concentration of 15-20μM was required to elicit therapeutic augmentation with anti-cancer drugs in mice [192].

Consistent with this, Se concentrations <10μM were ineffective at reducing VEGF, HIF-1α and HIF-2α in renal cell carcinoma pre-clinical models [84].

6.1.3 Overview of glutathiones role in cellular homeostasis

The tripeptide glutathione, L-γ-glutamyl-L-cysteinyl-glycine (GSH) is essential for the maintenance of cellular redox homeostasis [262], and plays a key role in cellular defences against free radicals and in the regulation of carcinogenic processes [263; 264]. GSH is the most abundant free thiol in eukaryotic cells and helps maintain the intracellular redox environment, enabling the optimal functioning of cellular proteins. As the reduced form of glutathione, GSH is biologically-active, where during oxidative stress GSH is oxidized to glutathione disulfide (GSSG), and the GSH:GSSG ratio is often used as a measure of cellular oxidative stress [265]. Cells usually maintain a GSH:GSSG ratio with >90% of total glutathione in the reduced form, as a result of cytosolic GSH synthesis, exogenous GSH uptake and enzymatic reduction of GSSG [262]. Intracellular GSH is segregated into distinct subcellular pools within the cytosol, mitochondria, ER and nucleus, each with region-specific roles [266]. Nuclear GSH aids the preservation of nuclear proteins in a reducing environment and protection from ROS and/or ionizing radiation-induced DNA damage [267]. Within the ER GSH is involved in the regulation of protein disulfide isomerases (PDI) and protein folding in addition to buffering against ER- generated ROS [268; 269]. Changes in the ratio of GSH:GSSG are known triggers of the UPR and apoptosis [270].

6.1.4 Glutathione and Se and cancer

Increases in intracellular GSH levels corresponds with increased resistance to chemotherapeutic agents, including platinum compounds, anthracyclines, alkylating agents and arsenic [271]. High levels of GSH found in platinum-resistant cancer cells may result from an increase in expression of the gene for γ-glutamylcysteine synthetase (γ-GCS), a key enzyme in GSH synthesis [272-274]. In animal xenograft studies using A2780 human ovarian cancer cells, intraperitoneal (i.p.) treatment with SS

or SLM prior to administration of cisplatin prevented induction of cisplatin resistance and was also associated with a reduction in GSH [118]. In the same model SS was able to prevent the induction of carboplatin resistance, with the same anti-GSH effects described, with unpublished results suggesting a potential role of Se in reducing the expression of γ-GCS [275]. *In vitro* investigations using MSA in donor PBMCs and the THP1 monocytic leukaemia cell line showed a dose-dependent increase in intracellular GSH in PBMCs but with large GSH reductions observed in THP1 cells [70].

The interaction between GSH levels and SS cytotoxicity was studied in hepatoma cells, where the addition of reduced GSH extracellularly to SS resulted in the largest decrease in cell viability [276]. A possible explanation for this is that SS is activated in the reductive microenvironment, with increased uptake and cancer cell-specific cytotoxicity [277]. A high concentration of extracellular thiols is maintained in malignancy due to overexpression of the x_c- antiporter and multidrug-resistant proteins (MRPs), that increase cystine uptake and cysteine excretion [277].

This chapter will present the results from an assessment of the pharmacodynamic responses to the three Se compounds administered to cancer patients with a particular focus on angiogenesis and intracellular glutathione.

6.2 Methods and Materials

6.2.1 Sample collection and aliquoting

Aliquots of PBMCs from each study visit were obtained (see section 3.2.7.1) and snap-frozen in liquid nitrogen for subsequent storage at -80°C, until further processing for PD analyses.

6.2.2 Western blotting of angiogenic protein targets HIF-1α, PHD2 and PHD3 in PBMC's

6.2.2.1 Cell lysis

See section 5.2.2.

6.2.2.2 BCA quantification of cell lysates

See section 5.2.3.

6.2.2.3 SDS-PAGE

See section 5.2.4.

6.2.2.4 Protein Transfer

See section 5.2.5.

6.2.2.5 Ponceau S

See section 5.2.6.

6.2.2.6 Membrane cutting for WB targets and antibody incubation

See section 5.2.7. Primary and secondary antibodies used are described in Table 6.1.

6.2.2.7 Membrane stripping.

See section 5.2.8.

6.2.2.8 Western blotting densitometry

See section 5.2.9.

Table 6.1 Primary and secondary antibodies.

Primary/ Secondary	Target Protein	Species of Origin	Clonality	Manufacturer	Cat Number	Dilution	Expected versus observed size (kDa)
Primary	HIF-1α	Rabbit	polyclonal	Novus	NB100-479	1:1000 in milk block	115
Primary	PHD2	Rabbit	polyclonal	Abcam	Ab4561	1:1000 in milk block	46
Primary	PHD3	Rabbit	polyclonal	Abcam	Ab30782	1:1000 in milk block	27
Secondary	Anti-Rabbit	Goat	polyclonal	Abcam	AB9705	1:5000 in milk block	NA

6.2.3 Plasma VEGF measurement by ELISA

Plasma VEGF was measured from collect blood samples using the Human VEGF SimpleStep ELISA® Kit, Abcam, Cambridge, UK).

6.2.3.1 Blood samples for plasma measurement of VEGF by ELISA

Venous blood was collected in sodium heparin tubes (BD®, ON, Canada) from each patient during every study visit. The blood was spun at 1000g for 10 minutes at 4°C and plasma removed by a sterile Pasteur pipette, then 2 x 0.75mL aliquots were transferred to a pre-labelled sterile 1.5mL screw cap tubes (Neptune® Biotix, San Diego, CA, USA) and stored at -80°C.

6.2.3.2 Sample thawing and preparation for assay

Samples retrieved from -80°C storage were placed in ice buckets for controlled thawing over a 60 minute period and then spun at 2000*g* for 10 mins at 4°C (Eppendorf, Hamburg Germany). Following mixing, 75μL of each plasma sample was diluted with 75μL of sample diluent NS+ 2X Enhancer (see section 6.2.3.3) in a sterile 96 well setup plate (Nunc 167008, Thermo Fischer Scientific, Rosklide, Denmark).

6.2.3.3 ELISA reagent preparation

All kit reagents were equilibrated at room temperature prior to use and prepared on the day of the assay, according to manufacturer's instructions.

- 10mL of sample diluent NS+1X enhancer was made by combining 9.8mL of sample diluent NS and 200μL of cell extraction enhancer solution 50X followed by thorough but gentle mixing.
- 5mL of sample diluent NS+2X enhancer was prepared by combining 4.8mL of sample diluent NS with 200μL of cell extraction enhancer solution 50X followed by thorough but gentle mixing.

- 200mL of 1X Wash buffer PT was prepared by combining 20mL of wash buffer PT 10X with 180mL deionised water followed by thorough but gentle mixing.
- 5.5mL of antibody cocktail was prepared by combining 550μL of 10X capture antibody, 550μL of 10X detector antibody with 4.4mL antibody diluent 4Bl followed by thorough but gentle mixing.
- The VEGF lyophilized protein standard was reconstituted by adding 500μL of sample diluent NS + 1X Enhancer.

6.2.3.4 Preparation of standard curve

The VEGF standards were prepared in the same NUNC 96 well setup plate used to dilute the patient samples. VEGF stock solution was set up with a series of dilutions (Table 6.2).

Table 6.2 VEGF standards preparation.

Well	Volume of stock (4000pg/mL) per well	Volume of diluent (Diluent NS+ 1X enhancer)	VEGF concentration (pg/mL)
A	60μL	240μL	800
B	150μL of A	150μL	400
C	150μL of B	150μL	200
D	150μL of C	150μL	100
E	150μL of D	150μL	50
F	150μL of E	150μL	25
G	150μL of F	150μL	12.5
H	nil	240μL	0

6.2.3.5 Sample loading

50μL of each standard was added in duplicate to the assay wells with 50μL of each pre-diluted patient sample added in duplicate to the assay wells according to a pre-designed plate plan.

6.2.3.6 Antibody cocktail addition and incubation

50μL of the prepared antibody cocktail was added to all test wells (standards plus patient samples) using a multichannel pipette. The plate was sealed using the plate seal provided and placed on a plate shaker set to 400rpm for 1 hour at room temperature.

6.2.3.7 Plate washing

The assay plate was then washed with 3 x 350μL of wash buffer PT, using a microplate washer (Thermo Scientific Wellwash® Versa, Thermo Fisher Scientific, Ratastie, Finland) pre-primed with wash buffer and programmed to aspirate, then dispense 350μL of buffer and remove all liquid at each wash step.

6.2.3.8 TMB substrate incubation, stop solution addition and plate reading

100μL of TMB substrate was added to each well by multichannel pipette and the plate was protected from light using aluminium foil and placed on a plate shaker (400rpm) for 10 minutes at room temperature. 100μL of stop solution was added using a multichannel pipette and placed on a plate shaker for 1 minute prior to optical density (OD) measurement at 450nm using a spectrophotometric plate reader (Multiskan Go, Thermo Fisher Scientific, Ratastie, Finland).

6.2.3.9 Data handling and analysis

OD measurements were background-corrected and the standard curve was fitted using GraphPad Prism v7.0 (Graphpad Software, La Jolla, CA, USA) concentrations were $\log_{10}$-transformed and 4-parameter logistic regression used to fit the standard curve of known concentrations for the estimation of the unknown samples. Plasma VEGF values at each visit were expressed as percentage change from the average baseline values obtained from visits 1 and 2. 95% confidence intervals of the mean values from visits 1 and 2 were calculated to provide an estimated of variation prior to exposure to exogenous Se. Comparisons of values on visits 3-6 were made per disease cohort using one-way ANOVA and Tukey's multiple comparison test. Comparison to baseline values (visit 1 and 2) were made using Dunnett's multiple comparison test. Disease cohort and Se compound groups were assessed as above, with comparison to baseline values made

for the whole disease cohort and to the smaller (n=4) Se compound subgroups.

6.2.4 Glutathione assay

6.2.4.1 Cell lysis

Frozen PBMC pellets were retrieved from -80°C storage and placed on ice for controlled thawing. Any residual volume of PBS post-thaw was estimated visually and 100μL of cold lysis buffer (0.5% NP-40, Honeywell Fluka™ 56741, Seelze, Germany) in PBS with protease inhibitor cocktail was freshly added (Complete™ ULTRA mini, Roche Diagnostics, Mannheim, Germany) to all samples. Samples were then prepared and glutathione measured using the GSH/GSSG Ratio Detection Assay Kit II (Abcam, Cambridge, UK). Cells were homogenised with repeated pipetting according to the cell lysis protocol for the assay kit then samples were spun at 13,300*g* for 15 minutes at 4°C and the supernatants transferred to clean sterile tubes.

6.2.4.2 Protein quantification by BCA assay

The BCA assay was used to quantify the protein concentration of the PBMC cell lysates (see section 5.3.1). The resulting protein concentrations were used to normalise the GSH assay results to account for variable amounts of material from initial sampling, as material for the DNA damage assay and western blotting was prioritised over this assay.

6.2.4.3 Sample deproteinisation and neutralisation

Cell lysates were deproteinised using a Deproteinising Sample Preparation Kit (ab204708, Abcam, Cambridge, UK). In summary, 10μL of cold trichloroacetic acid (TCA) was added to the lysate and mixed to precipitate the protein. Following a 15 minute incubation on ice the samples were spun at 1200*g* for 5 minutes and the supernatant transferred to a clean pre-labelled tube. The deproteinised samples were then placed at -80°C storage until the day of the assay (within 2 weeks of initial storage). On the

morning of the fluorometric assay samples were thawed then neutralised with 7.5μL of cold commercial neutralisation solution, then tubes were placed on ice and allowed to vent CO_2 prior to use in the assay. Optimisation of the neutralisation process was performed previously using variable volumes of neutralisation solution and pH paper (Avantec MFS, Inc. Dublin, CA, USA) to ensure a final sample pH of between 4 and 6 in accordance with the manufacturer's instructions.

6.2.4.4 Total and reduced glutathione measurement by fluorometry

Total glutathione and reduced glutathione were measured in deproteinised and neutralised PBMC cell lysates using a fluorometric green-based assay detection kit (ab205811, Abcam, Cambridge, UK). Assay kits were removed from -20°C storage, and small vials were briefly spun at low speed (300*g*) prior to opening. The assay was equilibrated to room temperature before use and the Thiol Green indicator 100X was reconstituted with 100μL with MilliQ water and protected from light with aluminium foil and kept on ice during use. The lyophilized GSH standard was reconstituted with 200μL of Assay Buffer and protected from light with aluminium foil and kept on ice during use. The GSSG lyophilized standard was reconstituted with 200μL MilliQ water and protected from light with aluminium foil and kept on ice during use. The GSSG probe was reconstituted with 200μL MilliQ water to generate a 25X probe stock solution and protected from light with aluminium foil and kept on ice during use.

6.2.4.5 Standard curve preparation

The standards for GSH and GSSG were prepared in black plastic-walled clear-bottomed plates (Greinier Bio-one, 655906, Frickenhausen, Germany) for use in the final assay (Table 6.3 and Table 6.4) to generate the standard curves. One plate contained GSH standards and the other the GSSG standards (both pre-labelled).

Table 6.3 GSH standards preparation

Standard	Sample to dilute	Volume of standard (μL)	Volume of assay buffer (μL)	Final GSH concentration (μM)
1	10μM	300	0	10
2	std 1	115	115	5
3	std 2	115	115	2.5
4	std 3	115	115	1.25
5	std 4	115	115	0.625
6	std 5	115	115	0.3125
7	std 6	115	115	0.1563
8	none	0	230	0

Table 6.4 GSSG standards preparation

Standard	Sample to dilute	Volume of standard (μL)	Volume of assay buffer (μL)	Final GSSG concentration (μM)
1	10μM	200	200	5
2	std 1	115	115	2.5
3	std 2	115	115	1.25
4	std 3	115	115	0.625
5	std 4	115	115	0.3125
6	std 5	115	115	0.1563
7	std 6	115	115	0.0781
8	none	0	230	0

6.2.4.6 Sample loading and assay procedure and reagent incubation

10μL of diluted, deproteinised and neutralised lysate was added to wells on the assay plates with GSH and with GSSG standards in duplicate, sample wells were then made up to 50μL volume with lysis buffer (final sample dilution factor 1:5). GSH assay mixture (GAM) was freshly prepared by diluting the 100X Thiol Green stock solution in Assay Buffer by diluting 1:100 and mixed by vortexing, protected from light and kept on ice. Total glutathione assay mixture (TGAM) was prepared by diluting the 25X GSSG probe stock solution prepared at a 1:25 dilution with GAM and mixed by vortexing, protected from light and kept on ice. To the plate with GSH standards and patient samples, 50μL of GAM mixture was added by multichannel pipette to each standard and test well, the plate was protected from light and incubated for 30 minutes, then read by a fluorescence-enabled plate reader. To the plate with GSSG standards and patient samples, 50μL of TGAM mixture was added, the plate was protected from light and incubated for 30 minutes, then read by a fluorescence-enabled plate reader.

6.2.4.7 Fluorescence-enabled plate reading

Reduced GSH and total glutathione in patient samples was measured in a SpectraMax® i3 (Molecular Devices, Sunnyvale, CA, USA) fluorescence-enabled spectrophotometric plate reader with excitation set to 490nm and emission measured at 520nm.

6.2.5 Data analysis

Background subtraction was performed on the raw fluorescence values from each well. GraphPad Prism v7.0 (Graphpad Software, La Jolla, CA, USA) was used to fit the standard curves for both GSH and total glutathione, with 4-parameter logistic (4PL) regression providing R^2 values >0.99 being used for calculation of unknown sample values. Concentrations of total and reduced glutathione were adjusted for residual

volumes of PBS post-thaw, volumes of TCA and neutralisation solution and normalised to μg of protein lysate concentration.

Total glutathione values at each visit were expressed as percentage change from the average baseline values obtained from visits 1 and 2. 95% confidence intervals around the mean baseline values were calculated to provide an estimate of variation prior to exposure to exogenous Se. Comparisons of values on visits 3-6 were made per disease cohort using one-way ANOVA and Tukey's multiple comparison test. Comparison to baseline values (on visits 1 and visit 2) were made using Dunnett's multiple comparison test. Disease cohort and Se compound groups were assessed as above, with comparison to baseline values for the whole disease cohort.

6.3 Results

6.3.1 Western blotting

HIF-1α expression was only detected in 7 of the 24 patients tested and invariably the signal at around 110kDa was weak compared to background (Figure 6.2) and could not be quantified with any degree of precision. PHD2 and PHD3 were not detected in the blots performed and during optimisation phase in cancer cell lines.

Figure 6.2 HIF-1α blot with low signal on high background.

6.3.2 VEGF-A ELISA

Samples from 24 patients were analysed in duplicate over four 96-well assay plates. The standard curves for each plate were fitted using 4PL regression with R^2 values >0.998 for each set of standard dilution series. The coefficient of variability calculated for duplicate values across all 4

plates was 10.06%. Average levels from the two baseline (pre-treatment) sampling time points were similar between all patients with CLL and the cohort of patients with solid cancers with 309.6pg/mL (SD 106.3) for CLL and 301.9pg/mL (SD 51.1) for solid cancer patients, where p=0.726 was obtained using two-sided unpaired t-test.

6.3.2.1 Changes in VEGF-A levels by disease group.

Changes from the average baseline values of plasma VEGF-A at all subsequent visits are presented as percentage change from baseline. Figure 6.3 shows changes in plasma VEGF-A by disease cohort, with no significant changes seen between time points for either group where F(3,41)=0.2676 and p=0.8484 for the CLL cohort and F(3,43)=0.8755 and p=0.4613 for the solid cancer cohort, with no significant changes at each time point observed with Tukey's multiple comparison test. Further comparison with baseline values using one-way ANOVA and Dunnett's multiple comparison test also showed no significant change.

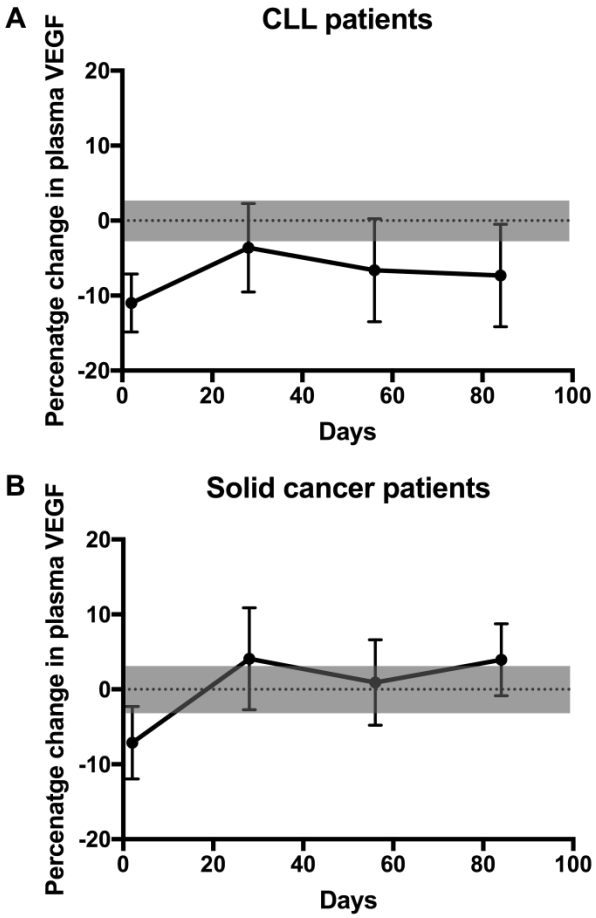

Figure 6.3 Change in plasma VEGF-A by disease cohort (A) CLL and (B) Solid cancer. Grey-shaded area represents 95% confidence intervals of change in baseline values at visit 1 and visit 2 compared to average.

6.3.2.2 Changes in plasma VEGF-A by disease and Se compound group

One-way ANOVA showed no significant differences in percentage change in VEGF-A from baseline levels over visits 3 to 6 for any disease/compound subgroup (Figure 6.4). However, comparing disease/compound groups to baseline variation for the whole disease cohort, did show the SLM-treated CLL patients had a significant drop of 19% in plasma VEGF-A at day 56 compared to baseline when using one-way ANOVA for all time points $F(4,33) = 3.414$, $p=0.0192$ and Dunnett's multiple comparison test for baseline versus day 56, where $p=0.0146$. In addition, in

SS-treated solid cancer patients a significant 14% drop of VEGF-A was observed at day 2 (p=0.0088). When the same comparisons were made to just the baseline values from each set of 4 patients per disease/compound group, only the latter comparison remained statistically significant (p=0.0412).

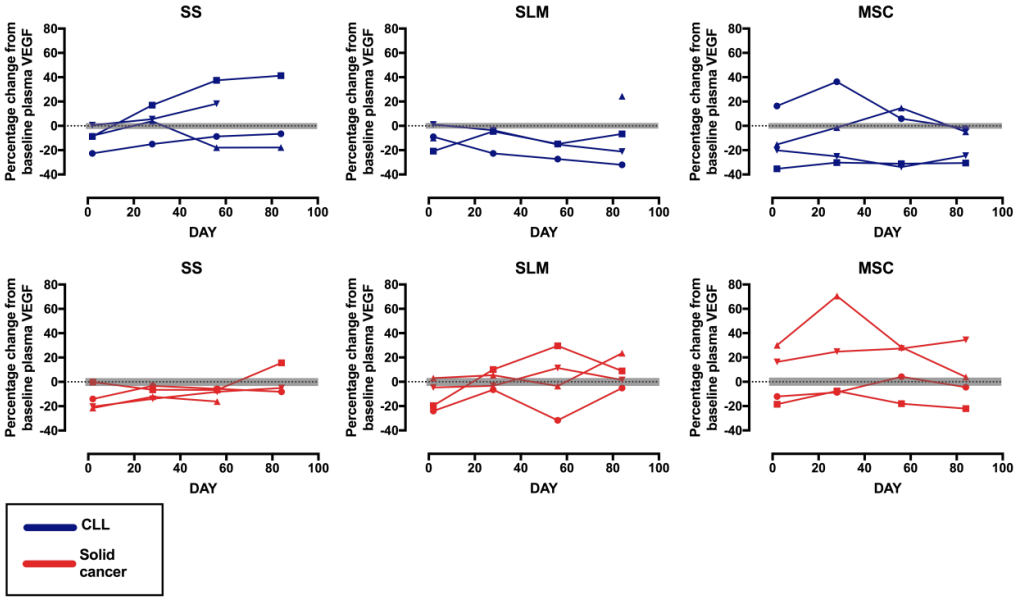

Figure 6.4 Change in plasma VEGF-A by disease and Se compound. Grey-shaded area represents 95% confidence intervals of change in baseline values at visit 1 and visit 2 compared to the average.

6.3.3 Glutathione assay

A proprietary Thiol Green-based fluorometric assay was used to quantity both total and reduced intracellular glutathione concentrations in PBMC lysates. Due to *ex vivo* oxidation of reduced glutathione, ratios of GSH:GSSG were seen to be significantly lower than what would normally be expected (average GSH:GSSG ratio of 0.86:1 in solid cancer patients at baseline rather than 100:1, which would be expected in quiescent non-malignant PBMCs [278]. Standard curves for total glutathione were produced using 4PL regression with R^2 values for all plates >0.998. The

coefficient of variation for duplicate values across all assay plates was 4.5% for total glutathione.

6.3.3.1 Total glutathione by disease group

The average baseline concentrations for total glutathione were significantly different from one another ($p=4x10^{-6}$) with 12.31 nM per mg of protein lysate (SD 2.11) for the CLL cohort and 7.14 nM per mg of protein lysate (SD 1.5) for the solid tumour cohort. However, changes at each time point following baseline visits were not significantly different from each other when using ANOVA or Tukey's test for either the CLL, where $F(3,40)=0.1495$, $p=0.9294$ or solid cancer groups, where $F(3,43)=8.755$, $p=0.4613$ (Figure 6.5).

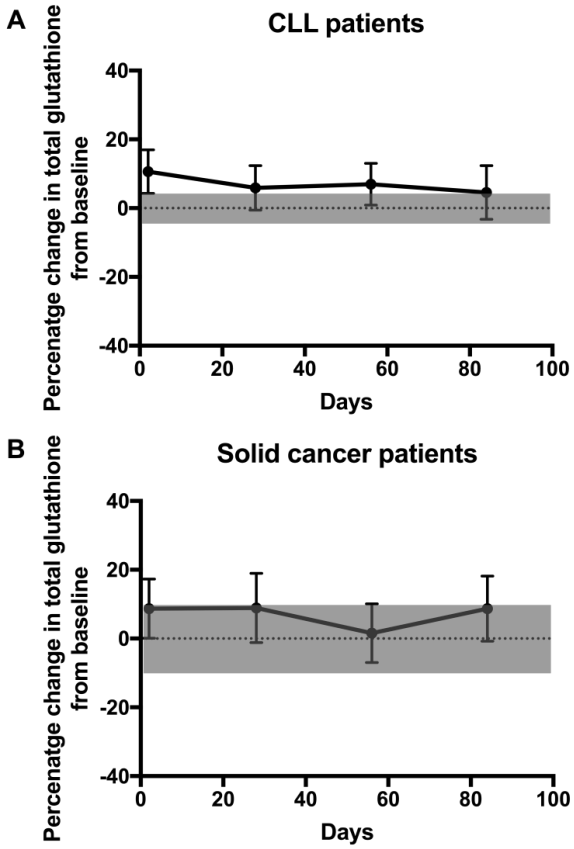

Figure 6.5 Change in total intracellular glutathione by disease cohort (A) CLL and (B) solid cancer. Grey-shaded area represents 95% confidence intervals of change in baseline values at visit 1 and visit 2 compared to average.

Further comparisons to baseline variation also yielded no significant changes in total glutathione in either disease cohort or Se compound group comparisons using one-way ANOVA and Dunnett's multiple comparisons test (Table 6.5).

Table 6.5 Comparison of change in total glutathione to baseline visits by disease group.

Disease Group	ANOVA	Dunnett's (adjusted *p* values)			
		V3	V4	V5	V6
CLL	F(4,55)=0.5227 *p*=0.7194	0.4468	0.8538	0.7748	0.9361
Solid cancer	F(4,58)=0.6427 *p*=0.6342	0.7436	0.9519	0.9998	0.9608

6.3.3.2 Change in glutathione by disease and Se compound groups

Changes in total glutathione over time (Table 6.6) or in comparison to baseline variation (Table 6.7) were not significantly different across all disease/Se compounds groups as per the analysis methodology, with the exception of one comparison to baseline at day 28 in the CLL group treated with MSC where an average increase in total glutathione of 23.5% (p=0.0481) was observed. Figure 6.6 shows the spider plots for each patient.

Table 6.6 One way ANOVA comparison of visits 3-6 by disease and compound groups.

Disease group and Se compound	ANOVA (F value)	ANOVA (*p* value)
CLL:		
SS	(3,10)=0.4856	0.4856
SLM	(3,10)=0.3379	0.7984
MSC	(3,12)=0.4101	0.7487
Solid cancer:		
SS	(3,11)=0.1635	0.9187
SLM	(3,10)=0.1244	0.9436
MSC	(3,12)=0.1654	0.9176

Table 6.7 Comparison of change in total glutathione to baseline visits by disease and Se compound subgroup. * indicates a significant difference p<0.5.

Disease Group and Se compound	ANOVA	Dunnett's (adjusted *p* values)			
		V3	V4	V5	V6
CLL:					
SS	F (4,25)=0.1729 *p*= 0.1751	0.7728	0.9898	0.1182	0.2674
SLM	F (4,25)=0.6723 *p*=0.6174	0.9414	0.6062	0.9344	0.8605
MSC	F (4,27)=2.312 *p*=0.0834	0.1651	0.0481*****	0.7382	0.6594
Solid cancer:					
SS	F (4,26)=0.6914 *p*=0.6046	0.4422	0.7453	0.9872	0.7864
SLM	F (4,25)=0.2038 *p*=0.9339	0.9787	0.9993	0.9994	0.8393
MSC	F (4,27)=0.6817 *p*=0.6107	0.5705	0.9431	0.4968	0.9309

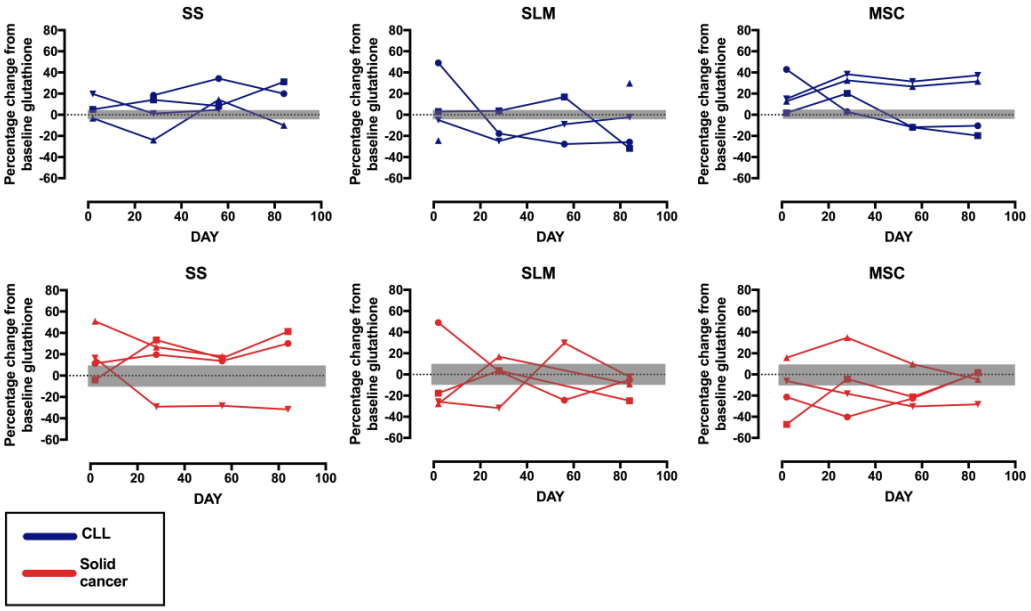

Figure 6.6 Change in intracellular glutathione by disease and Se compound group. Grey-shaded area represents 95% confidence intervals of change in baseline values at visit 1 and visit 2 relative to average baseline.

6.4 Discussion

6.4.1 HIF-1α, PHD2 and PHD3 expression

As previously mentioned (see section 5.4) limitations of the western blotting approach used resulted in failure to detect sufficient HIF-1α, PHD2 and PHD3 signal to quantify any differential expression over the course of the study. In future assays, increased protein loading to 25μg and increasing the primary antibody to 1:500 may allow the quantitation of expression by densitometry. Failure to detect sufficient signal for the HIF-1α protein may correspond to its low expression in normoxia [279]. While PHD2 is widely expressed in a variety of human tissues (including high levels in skeletal muscle and heart, with moderate levels in brain and pancreas) [280], data on expression levels in healthy PBMCs is absent from the literature. Furthermore PHD3 has a similar pattern of tissue expression with additional high levels in lung and placenta [281], but expression in normoxic PBMCs is not reported. The absence of signal for PHD2 and PHD3, could be due to

low tissue expression and or due to loss of target down to membrane stripping or failure of the primary antibodies themselves.

6.4.2 Plasma VEGF-A

Increased levels of circulating VEGF have been associated with numerous solid and haematological cancers [282], with plasma VEGF-A proposed as a potential biomarker for disease progression in diseases such as CLL where VEGF is secreted as an autocrine growth factor [283]. Average baseline levels of VEGF-A in the cohort of 12 CLL patients was 309.6pg/mL, which is greater than the 75th percentile value of 288pg/mL in CLL patients in a previous study [283]. The average baseline level in the solid cancer cohort of 301.9pg/mL is almost double the 161.9pg/mL reported in healthy volunteers from the CLL study [283], with similar magnitudes of difference of VEGF-A between healthy and cancer cohorts reported elsewhere [282].

Whilst reductions in VEGF-A plasma levels compared to baseline were observed in a number of patients, only the reductions seen in two disease/compound groups were of statistical significance (controlled for multiple testing using Dunnett's multiple comparison test) and only at a single visit per group. However, persistent reductions in plasma VEGF-A levels that were greater than the 95% confidence interval of the variation at baseline were observed in 7/12 patients with CLL and 4/12 in the solid cancer cohort. These reductions occurred in 3 out 4 CLL patients treated with SLM and 2 out of 4 patients treated with MSC. Furthermore, reductions in VEGF-A were observed in solid cancer patients treated with SS. A reduction in VEGF-A below the 95% CI thresholds of baseline variation was seen at 4 hours post-dose on day 2 in 16 out of 24 patients (7/12 in the solid cancer cohort and in 9/12 in the CLL cohort), suggestive of an acute PD effect that is not sustained in some groups. Whilst reductions as a percentage of baseline were relatively small they did persist through the treatment period for a number of patients and show some minor activity of these Se compounds at the relatively low dose tested in this study. The more statistically-robust method of repeated measures ANOVA likely

provides a greater degree of statistical precision than the 95% CI for baseline variation, especially when comparing multiple measures over time. While the former test is limited by requiring complete data sets, this approach is preferred for application to the higher dose cohorts planned.

A number of *in vitro* studies have shown Se compounds to reduce VEGF-A expression in various cell culture and animal models (see Table 1.3); for example MSA at 5-20μM significantly reduced secreted VEGF in PC3 and PC3-M cell lines in both normoxic and hypoxic conditions, along with decreased HIF-1α expression as a result of increased degradation to due to elevated levels of PHD2 [259]. The Se compounds SLM and MSC have, in a number of tumour xenograft models, been shown to target HIFs and perturb downstream pro-angiogenic signalling molecules such as VEGF-A [192]. Inorganic SS has also demonstrated *in vitro* inhibition of VEGF-A secretion in HSC-3 cells at 5μM, while MSC was more effective but was only tested at 50μM [227]. Based on this data the magnitude of effect is expected to be greater at higher doses that yield plasma Se levels >5μM. To date this is the first report on the effects of Se compounds on plasma VEGF-A levels in humans taking exogenous Se.

6.4.3 Intracellular glutathione

Higher levels of intracellular glutathione are often associated with a malignant cell phenotype [284]. The higher average levels of glutathione found at baseline in CLL cells (12.31nM/mg of protein) compared with those in PBMCs (7.14nM/mg of protein) is consistent with other studies where glutathione is almost twice as high in CLL cells compared to non-malignant controls [285]. These higher levels are associated with chemoresistant properties and are particularly associated with resistance to platinum chemotherapy drugs [118] as well as with alkylating agents used to treat CLL [286]. Therefore falls in glutathione are beneficial in terms of vulnerability to chemotherapy and RT, while an increase in non-malignant PBMCs protects them from these treatments [70].

The results of the fluorometric assessment of total glutathione from this study, when normalised to mg of protein lysate, are comparable to those

reported elsewhere in PMBCs using HPLC-based measurements (mean 23.7nM.mg^{-1} of protein [287], and 10 nM.mg^{-1} in monocytes [288]). Glutathione undergoes rapid auto-oxidation [289], and many protocols recommend the analysis of fresh samples for accurate determination of the GSH:GSSG ratio [288]. Therefore our results from the assay concerning GSH were omitted from further analysis as they would not be a reliable estimate of the GSH:GSSG ratio *in vivo*.

The results from the measurement of total glutathione in PBMCs and malignant lymphocytes indicate that 400μg of Se administered as MSC, SLM or SS was able to significantly alter basal expression of glutathione during treatment, an effect that persisted in some patients 4 weeks after cessation of treatment, in both disease cohorts. In xenograft studies with mice, tumour cells taken from animals pre-treated with 1.5mg/kg SS or SLM by intraperitoneal injection alongside cisplatin showed significant reductions in glutathione compared to control, and this observation was associated with reversal of resistance to cisplatin [118]. Therefore the failure to observe significant reductions in intracellular glutathione during exposure to Se compounds compared to baseline may be a function of dose, and gene expression studies of γ-GCS may help elucidate the mechanisms by which Se compounds might selectively impair glutathione synthesis at higher doses in malignant cells.

6.5 Conclusions

HIF-1α, PHD2 and PHD3 were not able to be assessed in samples from the study population, possibly due to methodological issues. There were, however, some promising signals of activity of Se compounds, with sustained reductions in plasma VEGF-A seen at much lower doses than predicted from animal models, particularly in the CLL disease cohort. Based on pre-clinical data we would expect the magnitude of reductions to be greater at the subsequent higher dose levels planned. The 400μg dose level does not appear to be sufficient to induce the differential response in total intracellular glutathione levels between malignant and normal cells that has

been noted in some *in vitro* studies. Due to logistical issues around sample storage and instability of GSH, total glutathione alone will be assayed in samples from the higher dose cohorts, in the future.

Chapter Seven: Summary and conclusions

7.1 Research outcomes

The introductory chapter outlines the key objectives for this research project and thesis. The overarching goal of this research is to improve the outcomes of cancer patients by using Se to reduce cancer treatment-related toxicities while not compromising (or preferably improving) their anticancer efficacy. This study seeks to contribute to this goal by identifying the Se compound, and dose, that can be optimally combined with systemic anticancer therapies to reduce toxicity and improve therapeutic selectivity. The work described in this thesis evaluates the first cohort of patients, dosed with 400μg of elemental Se per day for 8 weeks, as SS, MSC or SLM.

7.1.1 Develop and validate a molecular biology-based method to measure DNA damage in genomic and mitochondrial DNA that can be used to assess the genotoxic potential of Se compounds within the context of a clinical trial.

While Se has been shown in preclinical work to improve the efficacy of chemotherapy and radiation, it also has the potential to increase some important late effects of those treatments, namely second malignancies. This is because Se can be genotoxic, but the dose-potency of this varies greatly (see section 3.4.2). Of the three Se compounds selected for this trial, preclinical studies have shown that SS has the greatest genotoxic potential at lower, clinically-relevant doses, whereas MSC appears to be safest. Therefore the assessment of Se-induced DNA damage is a mandatory component of the safety analyses of these compounds, and is expected to inform the choice of Se compound and dose recommended for use in future trials of Se with cancer therapies.

As previously discussed, the comet assay remains a key method for the genotoxicity screening of new compounds by the main regulatory bodies

such as the FDA [290]. However it is both time-consuming and labour–intensive, outside of a validated high-throughput laboratory facility. A PCR-based approach, such as the one described in chapter 2 , provided an opportunity to assess genomic and mitochondrial DNA in a region-specific fashion, which can be scaled up to measure large numbers of samples simultaneously.

A critical part of the assay validation was also to check that our proposed method of PBMC cryopreservation and the duration of storage for our trial samples did not introduce significant amounts of *ex vivo* DNA damage. This was to make sure that any measured damage over baseline was due to experimental exposures rather than sample processing or storage. The results from our cryopreservation experiments were reassuring, with the amount of UVC-induced damage shown to be constant over time relative to baseline (see section 2.3.5).

In addition, the assay was also effective in quantifying DNA damage from cytotoxic compounds such as cisplatin and bleomycin in a dose-dependent and DNA location-specific manner. (see section 2.3.6). As expected the assay identified SS as being more genotoxic to PBMCs than MSA, following *in vitro* exposure (see section 2.3.8).

One limitation with the approach, was the requirement of optimising the conditions for use on a different qPCR instrument. Optimisation of the assay for use on the MIC-based platform required a considerable number of iterative changes using different cycling conditions and a non-critical minor change to the composition of the PCR reaction mastermix for consistent amplification of the long PCR products from DNA extracted from thawed PBMCs. However, once optimised and providing DNA extractions yielded sufficient DNA quantities (>15ng/μL), the assay performed well when applied to the clinical trial samples and our study objective was completed successfully.

7.1.2 To characterise the clinical and laboratory safety profile of three Se compounds (SS, SLM and MSC) dosed at 400μg per day in cancer patients.

Unsurprisingly 400μg of elemental Se daily for 8 weeks as SS, MSC or SLM was well tolerated by a group of patients with metastatic cancer or CLL. No safety signals were observed during the treatment and follow-up of the 24 patients recruited. A handful of grade 1 toxicities were recorded but were both transient and sporadic across the Se compound groups with no consistent pattern, with no grade 2 and 3 toxicities that were attributable to the Se compounds. Many of the patients were taking concomitant medicines for co-morbid non-cancer-related conditions, and are representative of the type of patients who might derive benefit from the combination of Se with anticancer treatments in the future.

Results from the genotoxicity assay showed no significant Se-induced DNA damage relative to the variation observed between visit 1 and visit 2 at baseline, with no significant differences between disease groups or Se compound. However, based on the existing literature, it is expected that significant genotoxicity could be detected at the higher doses of Se planned (that are expected to result in plasma Se concentrations >5μM), with those patients taking SS being at greatest risk. This should be taken into account when considering the relative safety and risk of dosing patients at higher doses, with one option to mitigate this by shortening the duration of Se administration at each dose.

7.1.3 To evaluate the pharmacokinetics of MSC, SLM and SS in cancer patients, including the Se species formed in plasma and WBC.

As expected from the literature, plasma levels of Se, quantified by ICP-MS, showed SLM to have a significantly greater $AUC_{0\text{-}EOS}$ than MSC or SS, with a larger post-dose increment than MSC or SS, and higher Se levels at

all subsequent dosing time points by some margin. Whilst plasma Se levels with both MSC and SS showed non-significant slight decreases with continued dosing beyond week 4, Se levels continued to increase in the SLM group and fell more slowly after discontinuation than in the MSC and SS cohorts. This underlined the ability of SLM to accumulate through non-specific incorporation into plasma proteins (see Figure 4.1). Review of the plasma Se concentration-time curves leads us to conclude that plasma Se has approximated steady state levels by ~ 4 weeks from the start of dosing at the 400μg.day^{-1} dose level for all Se compounds. Baseline levels in all groups were comparable and, as in most human studies from NZ, are below the plasma Se level (1.67μM) associated with optimal human health.[3]

Se speciation analysis in plasma, CLL cells and PBMCs is currently being undertaken by collaborators (Dr Heidi Goenaga-Infante, LGC Group, Teddington, UK). This will look at low molecular weight species in plasma such as the volatile methylated metabolites of Se, and high molecular weight species such as the plasma-abundant selenoproteins SEPP1 and GPX3. Intracellular Se species will include GPX3 and thioredoxin reductase.

7.1.4 To evaluate the pharmacodynamics (PD) of each Se compound, including differential effects in normal and malignant WBC.

7.1.4.1 ER stress

The ability of Se compounds to stimulate greater ER stress in cancer cells is well-documented in cancer cell lines. Despite seeing very little effects, previous preclinical studies showed that Se-precipitated ER stress pathway activation occurs at levels of Se exposure not achieved in our trial population treated at 400μg.day^{-1}, where the vast majority of our patients achieved C_{max} plasma levels <2.5μM. This does not take into account additional complexities, such as which Se species in plasma mediates the PD effect (free methylselenol vs. protein-bound Se vs. specific selenoproteins) and differences between *in vivo* and *in vitro* models.

7.1.4.2 Angiogenesis

Data from head and neck tumour xenograft models are particularly encouraging concerning the anti-angiogenic effects of Se, thought to be predominantly mediated by down-regulation of HIF-1α and VEGF-A, with MSC and MSA having the greatest effect [192]. In this study technical difficulties around antibody selection and optimisation, alongside limited amounts of available sample material per time point, led to the inability to adequately study the expression of HIF-1α and the hypoxia-sensing regulatory proteins PHD2 and PHD3.

However the results from the plasma VEGF-A assay are suggestive of some Se-induced anti-angiogenic activity at this lowest dose level, a much lower dose than predicted from previous animal models. Reductions observed, while relatively small, seemed consistent for a number of CLL patients, in particular those in the SLM treated group. Unfortunately, the very small numbers of patients in each disease and compound cohort severely limit the interpretation of these and other results. Recruitment of patients at the planned higher dose levels of Se may help to interpret the significance of these data. Furthermore, these higher doses are predicted to achieve plasma Se levels that correspond more closely to concentrations used in previous xenograft studies that produced meaningful antiangiogenic activity.[192] The underpinning mechanisms can be interrogated further in subsequent patients with PD studies that may include the quantification of oncogenic microRNA's such as miRNA-210, which have a known role in HIF-1α modulation, and can be inhibited by organic Se compounds.[192]

7.1.4.3 Intracellular glutathione

No significant changes in total intracellular glutathione were observed in CLL cells or PBMCs in response to any of the three Se compounds, compared to variations recorded prior to dosing. The observed difference in baseline glutathione concentrations between PBMCs and malignant lymphocytes corresponds to previously published data, with glutathione being significantly higher in malignant than in non-malignant cells. Pre-clinical data from tumour xenograft studies again is highly suggestive that

differential responses between normal and malignant cells in terms of Se modulation of intracellular glutathione occurs at the higher end of the Se compound dosing spectrum.[118]

The fluorometric assay employed appears sensitive and able to measure total glutathione levels consistently from cryopreserved PBMC lysates, once normalised to the amount of protein per lysate. It is not, however, useful for evaluation of oxidised vs. reduced glutathione when used with cryopreserved cells as glutathione levels fall during storage.

7.1.4.4 GPX3 in plasma (UK collaboration with Dr Heidi Goenaga-Infante, Laboratory of the Government Chemist (LGC))

Data outlining both the high and low molecular weight Se species present in the plasma samples is expected to be available in late 2018.

7.1.4.5 Gene expression and DNA damage, repair and methylation studies (UK collaboration, Dr Ruan Elliott and Dr Priti Chivers, University of Surrey)

Gene expression data on a selected panel of Se-responsive genes, including those involved in DNA repair, is also expected to be available shortly.

7.1.5 Recommendations on amendments to the clinical trial protocol with respect to trial design and selection of suitable biomarker studies to accompany the safety and PK assessments in cohorts of patients to be treated at higher Se dose levels.

As a result of our findings concerning the plasma PK profile of these compounds (reaching steady state by 4 weeks from start of dosing) we have

proposed a shortened duration of treatment for each Se compound at each dose level (Figure 7.1). Each recruited patient will still be randomised to take a single Se compound but, instead of a fixed dose being taken for 8 weeks, they will take the elemental Se equivalent of 1600μg per day for 4 weeks followed by escalation to 6400μg per day for the last 4 weeks, providing no dose-limiting toxicity is observed.

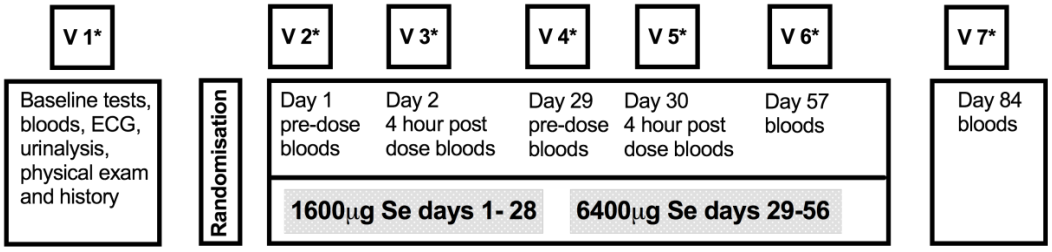

Figure 7.1 Revised study schedule, with two dose levels combined into one cohort.

In addition to the revised treatment schedule, the inclusion of qPCR analysis of miRNA-210 and miRNA-155 levels alongside the current list of PD markers is recommended. This will provide further information on the molecular signalling pathways that are implicated in Se effects on treatment resistance and angiogenesis. There may also be the potential to include global transcriptomic assessments comparing malignant and normal WBC pre-, peri- and post-Se if suitable funding is identified, or a more targeted analysis of candidate gene expression, depending on the findings from our collaborators at the University of Surrey.

The latest version of the trial protocol is near completion with planning underway for the next phase of the study to open to recruitment in late 2018.

7.2 Conclusion

Se compounds have pleiotropic effects on cancer cells and, in preclinical *in vivo* studies and some small clinical studies, have shown an

ability to differentially modulate the toxicity of cancer therapies without compromising treatment efficacy. However many questions remain concerning the optimal form and dose of Se and the appropriate clinical context in which to further evaluate its use as an adjunctive measure to improve cancer treatment outcomes. This body of work has provided a sound basis for proof-of-concept trials in terms of study design, including safety, PK and PD assessment in a mixed cancer patient population. Furthermore the safety data presented enables this research to be continued into doses that, according to experimental models, should elicit both anticancer and cytoprotective effects that may be of significant clinical benefit to patients undergoing cancer treatment.

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Appendix A: Buffers and Solutions

Cell lysis and SDS-PAGE

2X RIPA buffer

- 300 mM NaCl
- 10 mM EDTA pH8
- 100 mM Tris pH8
- 2% NP40
- 1% NaDeoxycholate
- 0.2% SDS

1X RIPA with protease inhibitor cocktail and phosphatase inhibitors

To make 1mL

- 500µL 2 X RIPA buffer
- 257µL sterile MiliQ water,
- 143µL 7X cOmplete™ ULTRA mini (Roche Diagnostics, Mannheim, Germany). Protease inhibitor cocktail (7X= 1 tablet dissolved in 1.5mL of sterile MiliQ water).
- 100µL 10X PhosStop™ (Roche Diagnostics, Mannheim, Germany) phosphatase inhibitor cocktail (10X = 1 tablet dissolved in 1mL of sterile MiliQ water).

4X Laemmli buffer

- 40% Glycerol
- 240mM Tris-HCL pH 6.8
- 8% SDS
- 0.04% Bromophenol blue
- 5% 2-Mercaptoethanol (added prior to loading sample)

DNA gel electrophoresis and PCR

TE (DNA elution)

To make 50mL of buffer

- 0.079g Tris
- 0.019 EDTA
- Make up to volume with MiliQ water
- Adjust pH to 8.0

50X TAE

To make 1L of 50X TAE buffer

- 242g Tris
- 57.1mL glacial acetic acid
- 100mL 0.5M EDTA
- Make up to volume with distilled water
- Adjust to pH 8.0

1X TAE

To make 2L of 1X TAE buffer

- 40mL of 50X TAE buffer
- Make up to volume with distilled water

6X DNA loading buffer

- 30% glycerol
- 0.25% bromophenol blue
- 0.25% xylene cyanol FF

Western blotting

10X TBS

To make 1L of 10X TBS

- 24.2g Tris base
- 80g Nacl
- Make up to 1L with distilled water

1X TBS

To make 1L of 1X TBS

- 100mL of 10X TBS
- 800mL of distilled water
- Adjust pH to 7.6 with HCL
- Make up to final volume with distilled water

1X TBST

To make 1L of 1X TBST

- 100mL 10XTBS
- 1mL Tween 20
- 800mL of distilled water
- Adjust pH to 7.6 with HCL
- Make up to final volume with distilled water

Ponceau S

To make 200mL OF 0.5% Ponceau S

- 1g Ponceau S
- 198mL distilled water
- 2mL glacial acetic acid

Ponceau S destain solution

To make up 250mL

- 240mL distilled water
- 2.5mL glacial acetic acid
- Make up to final volume with distilled water

Buffers other

10X PBS

To make up 1L of 10X PBS

- 25.6g Na_2HPO_2
- 80g NaCL
- 2g KCL
- 2gKH_2PO_2
- Dissolve in 800mL distilled water
- Make up to final volume with distilled water
- Autoclave

1X PBS

To make 1L of 1X PBS

- 100mL of 10X PBS
- 700mL of distilled water
- Adjust to pH 7.4
- Make up to final volume with distilled water
- Autoclave

Appendix B: Clinical Trial protocol

Clinical Study Protocol

Phase Ib, randomised, double-blind, dose-escalation study to identify the safest, most effective selenium compound for use in cancer patients

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Trial Registration: **ANZCTR Registration No: ACTRN12613000118707**

Amendments: Version 3 created 09 June 2015 to amend eligibility to include patients with cancer other than prostate cancer, and add the LORD-Q PCR method for assessing DNA damage.

Study Synopsis

Protocol #	Phase Ib randomised, double-blind, dose-escalation study to identify the safest, most effective selenium (Se) compound for use in cancer patients
Objectives	***Primary objective*** To determine the dose and form of Se that can be most safely and effectively used in clinical trials in combination with anticancer therapies. ***Secondary objectives*** • To characterise the clinical and laboratory safety of Se-methyl-selenocysteine (MSC), L-selenomethionine (SLM) and sodium selenite (SS) when taken orally by cancer patients at doses of 400, 1600 or 6400 □g Se daily for eight weeks. • To evaluate the pharmacokinetics (PK) of MSC, SLM and SS in cancer patients at these doses, including Se species formed in plasma and WBC. • To evaluate pharmacodynamics (PD) of each dose and type of Se including differential effects in normal and malignant white blood cells (WBC); the malignant WBC will be drawn from patients with chronic lymphocytic leukaemia (CLL) and the normal WBC will be drawn from metastatic cancer patients. PD pathways assessed may include endoplasmic reticulum (ER) stress, angiogenesis, plasma antioxidant activity (glutathione peroxidase), intracellular glutathione metabolism, gene expression and DNA damage, repair and methylation. • To evaluate the relationship between dose, PK and PD of each Se compound in normal and malignant WBC. • As an exploratory endpoint, to evaluate the impact of each Se compound and dose on tumour activity, as measured by serum PSA in cancer patients and blood lymphocyte counts in CLL patients.
Study design	This is a randomised phase 1b dose-escalation study with 3 arms: MSC, SLM or SS; each arm has 3 dose levels (400, 1600 and 6400 μg Se) in patients with CLL or advanced cancer stable on hormone therapy. Each dose level will be filled prior to escalation to the next dose level (if no dose-limiting toxicities are observed). There will be no intra-patient dose escalation.
Study duration	30 months
Study procedures	After signing of informed consent, patients with either CLL or cancer will be randomised to receive MSC, SLM or SS capsules daily for 8 weeks. Clinical and laboratory evaluations will be undertaken at baseline (twice for laboratory parameters: a week prior to dosing and on the day of dosing to establish intra-subject variance) then 1 day and 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing. Assessments will include history

	and symptom-directed physical examination, urinalysis, ECG and safety, PK and PD laboratory bloods.
Number of patients	A total of 72 eligible patients will be recruited, 36 with CLL and 36 with cancer. For each of the 3 Se compounds at each of 3 dose levels there will be 8 patients, 4 with CLL and 4 with cancer.
Type of patients	**Inclusion Criteria:** 1. Patients with either proven CLL (peripheral blood lymphocyte count > 10 x 10^9/l) or metastatic cancer, in whom the use of chemotherapy is not anticipated in the next 3 months 2. Age > 18 years 3. Adequate liver, renal and bone marrow function - ALP, ALT (or AST) < 5x upper limit of normal (ULN) - serum creatinine < 2x ULN - Hb > 90 g/l - WBC > 3.0 x 10^9/l - platelets > 100 x 10^9/l 4. ECOG performance status $\leq$ 2 5. Life expectancy > 6 months 6. Fertile patients must use effective contraception prior to, during, and for $\geq$ 12 weeks after completion of, study treatment. **Exclusion Criteria:** 1. Patients treated within the last 4 weeks with cytotoxic chemotherapy, systemic therapy that targets VEGF signalling pathways or radiotherapy 2. Unable to swallow or absorb study tablets 3. Concurrent selenium supplements > 100 μg/day 4. Allergy to any of the Se compounds being used in this study 5. Concurrent medical conditions that, in the opinion of the investigators, would compromise either patient safety or the integrity of the data
Treatments	**Arm A:** MSC 400 or 1600 or 6400 μg Se daily for 8 weeks **Arm B:** SLM 400 or 1600 or 6400 μg Se daily for 8 weeks **Arm C:** SS 400 or 1600 or 6400 μg Se daily for 8 weeks
Efficacy assessments	Laboratory evaluations (including antitumour effects) will be undertaken at baseline (a week prior to dosing and on the day of dosing to establish intra-subject variance) then 1 day and 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing. Samples for antitumour efficacy assessments will include: • serum tumour markers (where relevant) in cancer patients and peripheral blood counts of atypical lymphocytes in CLL patients. Samples for mechanistic PD assessments and PK will include:

	• Plasma for analysis of Se PK • Plasma and WBC for Se speciation (baseline and week 4 only) • Plasma for marker of tumour angiogenesis (VEGF-□) • Plasma for glutathione peroxidase (marker of antioxidant effects of Se) • Whole blood EDTA samples for assessment of DNA breaks using the alkaline and Bleomycin COMET assays • Whole blood EDTA samples for extraction of PBMC using Ficoll and analysed for: o Assessment of molecular markers of angiogenesis and ER stress; a protein lysate will be prepared from the PBMC and stored at -80 ºC until analysis by western blot for HIF-1□, prolyl hydroxylases 2 & 3, GRP78, spliced XBP1, CHOP, phosphorylated eIF2□, caspase 8; o Intracellular glutathione concentrations o Assessment of nuclear and mitochondrial DNA damage (using LORD-Q PCR), DNA repair (using NE-PER kit Thermo Scientific) and DNA methylation (bisulfite conversion, PCR amplification and pyrosequencing) – PBMC pellet snap frozen and stored at -80°C o Assessment of gene expression by mRNA analysis – 2.5ml blood collected in PAXgene tubes and stored at -80°C. The genetic pathways analysed may include those related to cell survival, DNA repair, selenoproteins and hypoxia. All blood for plasma samples will be spun down in a refrigerated centrifuge and plasma stored at -80°C. Plasma and WBC for Se speciation, DNA repair and methylation studies and gene arrays will be sent to the UK for analysis.
Safety assessments	Clinical and laboratory evaluations will be undertaken at baseline then 1 day and 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing. Assessments will include history and symptom-directed physical examination, urinalysis, ECG and safety laboratory bloods (CBC, renal and liver function, glucose, urate, calcium, phosphate, INR, APTT). All adverse events will be graded using the NCI CTCAE version 4.0.
Statistics	The primary analysis is for safety of each Se compound and dose, so patients will be grouped together (n=8) in each dose cohort for this and PK analyses. Frequency of adverse events will be compared using Fisher's exact test. An analysis of the relationships between dose, plasma Se concentration and change in PD markers in the different groups will be conducted using repeated measures analysis of variance methods or linear mixed modelling methods.
Number of centres	All patients will be recruited from Waikato Hospital, New Zealand

Time schedule	First patient enrolled: December 2013 Recruitment completed: December 2015

Schedule of Assessments

	Screening/ baseline [a]	Day 1	Day 2	+ 4 Weeks	+ 8 Weeks	+ 12 Weeks
Informed Consent	X					
History	X					
Physical Exam [b]	X	X	X	X	X	X
Con Meds	X	X	X	X	X	X
Adverse events	X	X	X	X	X	X
ECG	X	X	X	X	X	X
Urinalysis	X	X	X	X	X	X
Chemistry [c]	X	X	X	X	X	X
CBC [d]	X	X	X	X	X	X
INR/APTT [e]	X	X	X	X	X	X
Tumour Markers [f]	X	X		X	X	X
PK [g]		X	X	X	X	X
PD [h]	X	X	X	X	X	X

a. No more than 28 days prior to commencing study treatment.
b. Comprehensive physical examination prior to study treatment then symptom-directed thereafter.
c. Na, K, creatinine, total bilirubin, alkaline phosphatase, AST, ALT, albumin, total protein, glucose, urate, calcium, phosphate.
d. Complete blood count including differential and platelet count.
e. INR and APTT
f. Tumour markers where relevant to each cancer patient
g. Plasma for analysis of Se PK; plasma and WBC for Se speciation at Day 1 and Week 4 only.
h. Blood for PD markers at all time points

GLOSSARY

AE	Adverse Event
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CLL	Chronic lymphocytic leukaemia
CRF	Case Report Form
CTCAE	Common Terminology Criteria for Adverse Events
ECG	Electrocardiograph
ER	Endoplasmic reticulum
GCP	Good Clinical Practice
GPx	Glutathione Peroxidase
ICH	International Committee for Harmonisation
MSC	Se-methyl-selenocysteine
NCI	National Cancer Institute
PD	Pharmacodynamic
PK	Pharmacokinetic/s
PR	Partial Response
PSA	Prostate-Specific Antigen
QC	Quality Control
SAE	Serious Adverse Event
SD	Stable Disease
Se	Selenium
SLM	L-selenomethionine
SS	Sodium selenite
VEGF	Vascular endothelial growth factor

TAB LE OF CONTENTS

1. Background

1.1. Introduction

Se compounds can protect animals from the toxicities of chemotherapy or radiotherapy while enhancing their antitumour effects in a variety of human tumour xenograft models. The mechanisms that mediate these interactions include: a) provocation of the "unfolded protein response" through increasing endoplasmic reticulum stress, resulting in apoptosis in malignant cells but cell cycle arrest and repair in normal cells; b) inhibition of hypoxia-induced angiogenesis; c) enhanced DNA repair in normal cells but not malignant cells with p53 mutations, and d) reversal of cellular resistance to radiotherapy and some cytotoxic drugs. However these effects are highly dependent on the Se compound, dose and tumour. MSC was significantly more effective in these models than other forms of Se, including SLM and SS. While SLM and SS have been evaluated in relevant clinical trials, MSC has not. Genotoxicity appears to be more common with SS than MSC or SLM, which is especially relevant when these compounds are used in conjunction with other DNA-damaging agents such as cytotoxic drugs or ionising radiation, because of the increased risk of second malignancies long-term.

Clinical evaluation of Se compounds in conjunction with chemotherapy and/or radiotherapy has been conducted using varying doses and forms of Se, with significant reduction in toxicity being demonstrated in most trials. However antitumour efficacy has not been adequately evaluated in these trials, nor has there been any effort to compare the efficacy of these Se compounds. Current doses are empirical or guided by PK, though the relationship between PK and PD has not been established in humans, hence the optimal form and dose of Se to be used with chemotherapy or radiotherapy remains unclear. However recent work demonstrated that PD biomarkers of Se effects can be measured in human WBC *in vitro* and *in vivo*. Thus the relationship between Se PD and PK can now be evaluated *in vivo* in humans, allowing us to determine the optimal compound and dose to be taken forward into further trials.

1.1.1. Relevance

Chemotherapy and radiotherapy are cancer treatments that many patients dread due to their toxicities. If the results of laboratory research can be confirmed in clinical studies then Se compounds could significantly reduce the toxicity of these treatments without compromising their efficacy (or could significantly enhance outcomes in cancer patients). This would be a clinically important and exciting advance in cancer treatment that would be widely acceptable to patients.

1.2. Selenium and Chemotherapy or Radiotherapy

Preclinical *in vivo* studies have shown that Se compounds such as SLM and MSC, at supra-nutritional doses, act as multi-targeted modulators of both efficacy and toxicity of cytotoxic drugs and radiation (1). The net result is greater therapeutic selectivity through protecting against normal tissue toxicities while improving antitumour efficacy, thus improving the narrow therapeutic index that characterizes cytotoxic cancer therapy and radiotherapy.

However different Se compounds, at varying doses, are being investigated in different institutions (predominantly SS in Europe and SLM in the US) without any clinical comparison being undertaken. In contrast there is clear evidence from preclinical work that some Se compounds may be much more effective, and some are more toxic, than others. This toxicity includes DNA damage (genotoxicity) (2) which, when combined with other DNA-damaging agents, may have the potential to increase the risk of second malignancies. Furthermore, recent studies show overlapping and divergent effects of different Se compounds on gene and proteome expression, cell growth, PSA secretion and oxidative stress (3-5). Thus a comparison in the clinical setting to determine the optimal compound and dose for future clinical studies is clearly warranted. The mechanisms involved and details of earlier clinical trials with Se and chemotherapy and/or radiation are discussed below.

1.2.1. Reduction of Toxicity – Animal Studies

SLM, MSC and other Se compounds have been shown to significantly protect animals from organ-specific toxicities of many cytotoxic drugs, including myelosuppression, mucositis, diarrhoea, alopecia, cystitis and nephrotoxicity (1;6-8). Specific toxicities of cisplatin on kidneys, bone marrow and intestine were improved without affecting antitumour efficacy (7;9-12) or pharmacokinetics (13). Protection from lethal effects of several cytotoxic drugs was also demonstrated in nude mice (8) (**Figure 1**).

In a rat model of colon cancer, treatment with oxaliplatin 10 mg/kg achieved 50% complete responses (CR), but pre-treating the rats with MSC 750 µg/day for 14 days increased the tumour CR rate to 100% (7). Increasing the oxaliplatin dose to 20 mg/kg was lethal but this was completely prevented by MSC in the same schedule, with a 100% tumour CR rate and moreover leucopenia was also improved.

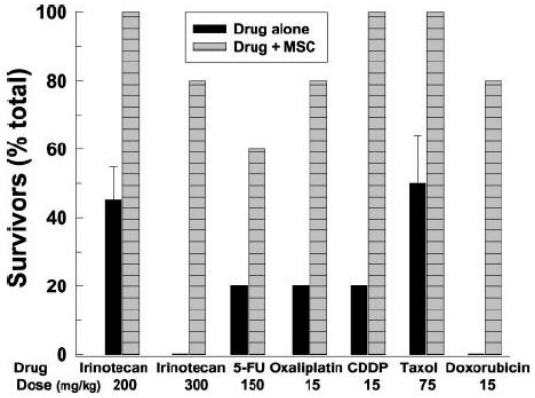

Fig. 1 Pre-treatment of nude mice with MSC reduces the lethality of cytotoxic drugs. From Cao et al, 2004.

1.2.2. Reduction of Toxicity – Human Studies

Few randomized clinical trials have evaluated the impact of Se compounds on chemotherapy toxicity. In one trial 41 patients were randomized to either Se (as seleno-kappacarrageenan) 4000 µg orally daily for 4 days before and after the first cycle of cisplatin-based chemotherapy (cisplatin dose 60–80 mg/m^2), or to chemotherapy alone. Addition of Se was associated with significantly higher white blood cell counts on day 14 than in the control group (3.35 vs. 2.31 x 10^9/l, $p < 0.05$), less need for G-CSF support (110 vs. 724 IU, $p < 0.05$), less red cell transfusion (0 vs. 62 ml, $p < 0.05$), and less nephrotoxicity as measured by urinary enzymes (14). No comment on antitumour efficacy was made.

In a double-blind trial involving 62 women receiving chemotherapy for ovarian cancer (cisplatin 100 mg/m^2 and cyclophosphamide 600 mg/m^2 every 3 weeks), patients were randomized to receive capsules of antioxidants with or without Se as selenized yeast, 200 µg per day for 3 months starting concurrently with chemotherapy (15). Those patients randomized to Se experienced significantly fewer toxicities at 3 months of treatment than the control group as measured by mean toxicity scores for specific toxicities (all p values < 0.05): nausea (0.97 vs. 2.03), vomiting (0.97 vs. 2.16), stomatitis (0.32 vs. 0.58), alopecia (2.12 vs. 2.55), abdominal pain (0.45 vs. 1.45), weakness (0.97 vs. 2.35) and loss of appetite (0.84 vs. 2.26). There was also better preservation of neutrophil counts at 3 months in the Se group (3.39 vs. 2.52 x 10^9/l, $p = 0.048$). No formal tumour response assessments were reported though the serum CA-125 tumour marker was non-significantly lower at 3 months in the Se group (93.5 ± 200 vs. 228 ± 713 U/ml).

A third trial randomised 50 patients receiving cisplatin-based chemotherapy to concurrent supplementation with SS 100 µg, vitamin C 1000 mg and vitamin E 400 mg daily in a milky beverage, or a placebo beverage without the vitamins and Se. Gastro-intestinal side effects led to 64% of the supplement group being non-compliant and, not surprisingly, their plasma Se levels did not change significantly during chemotherapy treatment. There was no improvement in nephrotoxicity, ototoxicity, myelosuppression or response rates (16).

A series of small randomised controlled trials (30–50 patients each) has been reported from one group using SS at 200µg/kg/day in conjunction with chemotherapy for patients with newly-diagnosed non-Hodgkin lymphoma (17-19). While the outcomes reported in each trial varied, overall the Se groups had significantly fewer toxicities (including infection and fall in cardiac ejection fraction), greater response rates and

improved overall survival. In addition there was enhanced apoptosis of lymphoma cells and reduced apoptosis of neutrophils in the Se groups.

A more recent randomised, controlled trial evaluated the ability of Se to reduce radiation-induced diarrhoea in 82 women receiving adjuvant RT (with or without chemotherapy) following surgery for gynaecological malignancies (20). Oral SS (500µg daily with RT and 300µg on non-RT days) significantly increased blood Se from 62.8 to 86.9 µg/l and reduced the actuarial incidence of $\geq$ grade 2 diarrhoea from 46.6% to 21.0% (p=0.039, log-rank), without any Se-related side effects. Actuarial overall survival at 5 years (with median follow-up of 38 months) was not significantly improved at 94.1% in the Se group and 86.1% in controls (p=0.308, log-rank).

In the setting of high dose chemotherapy and allogeneic haematopoietic stem cell transplantation, a double-blind randomised placebo-controlled study of oral selenized yeast in 77 patients reported a reduction in the incidence and duration of severe oral mucositis (21). Grade 3-4 mucositis was observed in 10.8% of the selenium-treated patients v 35.1% in the placebo group ($p<0.05$) and was of a shorter duration: 3.6 ± 1.84 days v 5.3 ± 2.2 days (p=0.014).

1.2.3. Antitumour Efficacy

Using preclinical *in vivo* models, therapeutic synergy was demonstrated between supra-nutritional doses of Se compounds and chemotherapy drugs. These included cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin in human tumour xenografts of small cell and non-small cell lung carcinoma, colorectal carcinoma, prostate carcinoma, head and neck squamous cell carcinoma (HNSCC) and leukaemia (1;7;8;22-24).

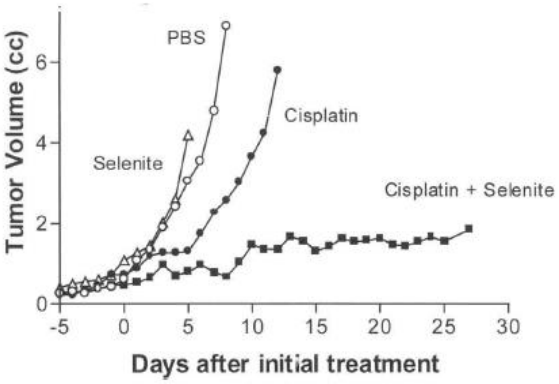

Fig. 2 Synergy between selenite and cisplatin in treating human ovarian cancer xenografts. From Frenkel and Caffrey, 2001.

Other workers have shown synergistic interactions between SS (1500 µg/kg/day i.p.) and cisplatin against human ovarian cancer xenografts in mice, shown in **Figure 2** (24). In addition pre-treatment with either SLM or selenite was effective in preventing human ovarian cancer cell lines and tumour xenografts from developing resistance to cisplatin or melphalan (25;26). A similar interaction of selenite with carboplatin in this model was reported (23).

The impact of dose, schedule and Se compound on the interaction with irinotecan has been studied in a human HNSCC xenograft model. The maximum effective dose varied from 10–200 µg/mouse/day, depending on the xenograft (1). Furthermore, the effects of MSC or SLM were schedule-dependent, with increasing protection from toxicity evident when administering these compounds for longer durations prior to initiation of irinotecan; the maximal benefit was evident after 7 days pre-treatment, shown in **Figure 3** (1;27)**.**

While SS and SLM (1000–1500 µg/kg/day i.p.) were equally dose-potent and effective when combined with cisplatin in an ovarian cancer xenograft model (24), MSC and SLM (200 µg/day orally) were both superior to SS in combination with irinotecan in HNSCC xenografts (**Figure 4)** (1).

1.2.4. Mechanisms

There are multiple mechanisms by which Se compounds achieve these beneficial interactions with chemotherapy, relating not only to pharmacokinetic, microenvironmental and cellular mechanisms of drug resistance, but also to differential effects on normal and malignant cells. While the active moiety is considered to be methylselenol (28-30), a discrete biochemical target or receptor has not been identified that mediates these effects but the microenvironmental, cellular and molecular sequelae of administration of Se compounds are being elaborated (1).

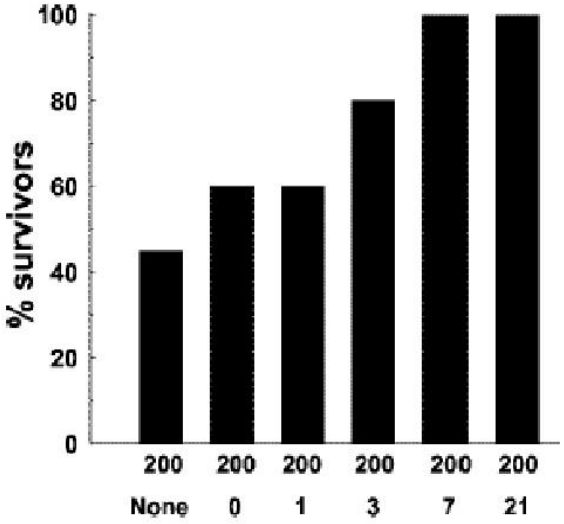

Fig. 3 Duration of MSC exposure prior to irinotecan determines the degree of protection. From Cao et al, 2004.

Angiogenesis

The disordered vasculature found in tumours, due to expression of factors that drive angiogenesis, such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (31), results in increased interstitial fluid pressure within tumours that restricts drug delivery (32). Hypoxia is another common consequence that drives further angiogenesis through induction of hypoxia-inducible factors such as HIF-1□ and induces cellular responses that restrict apoptosis in response to DNA damage from CT and RT (32-34). This interaction between microenvironmental and cellular factors contributes significantly to drug resistance (35). Targeting angiogenesis with antibodies to VEGF has been shown in preclinical and clinical settings to increase vessel maturation, reduce microvessel density, reduce interstitial fluid pressure, improve tumour oxygenation and improve responses to chemotherapy and radiation (32;36).

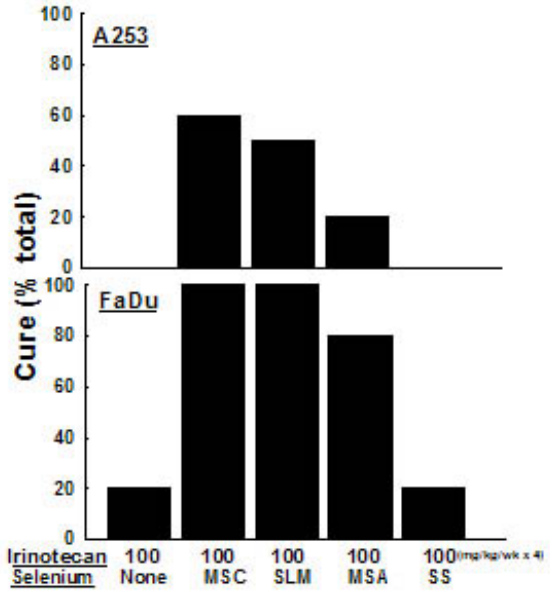

Fig. 4 Se compounds differ in their ability to improve antitumour activity of irinotecan in HNSCC xenografts; MSC: Se-methyl-selenocysteine; SLM: selenomethionine; MSA: methylseleninic acid; SS: sodium selenite. Y Rustum.

Se compounds have been shown by different workers to reduce HIF-1□ and VEGF expression in human prostate tumour cells (37) *in vitro* and to inhibit angiogenesis and reduce the expression of VEGF in mammary tumours (38) and human HNSCC xenografts (27;33). In the latter studies MSC inhibited neovascularisation, reduced microvessel density and induced vessel maturation, resulting in improved tumour perfusion, improved tumour (but not normal tissue) concentrations of irinotecan and its active metabolite, SN-38, and increased tumour response rates (39-42). Recent work has shown that MSC results in more than doubling of tumour concentrations of doxorubicin and capecitabine (43). The antiangiogenic effect was mediated through inhibition of COX-2, HIF-1□ and i-NOS and thus inhibition of VEGF induction (33).

Cellular Mechanisms of Drug Resistance and Selectivity of Selenium for Malignant Cells

There are multiple cellular mechanisms associated with development of resistance to chemotherapy, one of which is accumulation of compounds such as glutathione within cells that covalently bind and inactivate drugs (35). Pre-treatment of ovarian cancer xenograft-bearing mice with SLM or SS prevented an increase in intracellular glutathione in response to cisplatin or melphalan (25). These authors have shown that by

using this strategy they could prevent resistance developing to cisplatin, carboplatin or melphalan *in vivo* in the same model (23;25;26). A possible mechanism has been demonstrated by other authors, in that exposure of U-1285 human SCLC lines to SS *in vitro* increased the activity of glutathione reductase four-fold (44).

Activity of Se compounds in established cancer cell resistance has also been demonstrated. Resistance to irinotecan could be reversed *in vivo* with SLM and MSC (8), perhaps due in part to a significant reduction in levels of the ABCC1 efflux pump in tumour cells (39). However mechanisms other than effects on drug efflux are involved: drug- or radiation-resistant malignant cell lines were several-fold more sensitive to selenite-induced cytotoxicity *in vitro* than their non-resistant counterparts (44-46) without altering expression of the multidrug-resistance proteins MRP1, LRP and topoisomerase II-□ (44). At least part of this effect appeared to be mediated through induction of apoptosis (45;46), especially in P53-mutant cells (47;48), and differential modulation of redox status (44;45;47;49;50).

P53, which has a critical role in determining cell survival or death (through apoptosis) in response to DNA damage and other cell stresses (51), is mutated in many cancers (48). Se compounds have been shown to regulate P53 expression and activity (52;53). This resulted in enhanced DNA repair in normal tissues (which have wild-type P53 by definition) while malignant cell lines with mutant P53 or P53-null cell lines did not have altered DNA repair and remained sensitive to DNA damage from drugs or radiation (48;54). MSA was also shown to affect methylation of DNA through inhibiting histone deacetylase activity in lymphoma cell lines (55).

Se compounds cause global thiol redox modification of proteins, resulting in an accumulation of aberrantly folded proteins in the endoplasmic reticulum (ER) that triggers the "unfolded protein response" (56). This cellular response to ER stress induces cell cycle arrest while protein repair occurs or, if unsuccessful, commits the cell to apoptosis (57). The patterns of ER stress proteins are quite distinct for these two responses: the survival/rescue response involves molecules such as phosphorylated PERK and eIF2□, GRP78 and GRP94, whereas the apoptotic response involves GADD153 and activated caspases (56).

ER stress in tumours is also involved in control of cell proliferation and angiogenesis (58-61). Cells within tumours generally have more ER stress than normal tissues, in part due to higher rates of protein synthesis and hypoxia (59;60). The importance of ER stress as a target in cancer therapeutics is demonstrated by the activity of bortezomib, a proteosome inhibitor that acts through increasing protein misfolding and inducing ER stress, and thus apoptosis (62).

Higher levels of the ER stress regulator GRP78 are associated with resistance to CT (63), and modulation of the ER stress response by Se was recently reported to enhance the efficacy of chemotherapy with leukaemia cells (64). Further derivation of this work has shown that the ER stress response signatures and the changes after Se administration can be measured in human peripheral blood mononuclear cells (PBMC), both *in vitro* and *in vivo* (S Joel, unpublished results). Other investigators reported that an increase in ER stress due to Se administration preferentially provoked apoptosis in tumour cells but a survival response in normal tissues (56). This differential effect of Se on ER stress in normal versus malignant cells may account in part for the enhanced therapeutic outcomes from the combination of Se compounds with CT drugs and RT.

It is known that DNA methylation plays an important role in cancer initiation and progression by silencing or down-regulating the expression of some beneficial genes including selenoprotein genes. Studies have reported that a number of selenoprotein genes are methylated and down-regulated in cancer cells and there is a strong correlation between the DNA methylation and the silencing/down-regulation of these genes (65). A report by Xiang *et al* investigating the effect of high Se dose on cancer cells suggested that, at higher dose, Se interfered in DNA methylation by inhibiting the expression of DNA methyl-transferase genes (DNMT 1&3) (66).

Pilot Study of Selenium as a Modulator of Efficacy and Toxicity of Chemotherapy and Radiation on Normal and Malignant Cells – an informative model

This summer studentship project (unpublished work) evaluated the differential effect of the Se compound methylseleninic acid (MSA) on the response of normal peripheral blood mononuclear cells (PBMC) and THP-1 human monocytic leukaemia cells to cisplatin CT and RT. Using MSA concentrations of 2.5 and 5μM (that reflect plasma levels achievable with oral intake of Se approximating 400 and 1600 μg/day respectively) MSA did not compromise viability of PBMC but was cytotoxic to THP-1 cells, both as a single agent and in combination with cisplatin or RT 10 Gy (**Figure 5**). Intracellular glutathione concentrations increased substantially in PBMC (a protective response) with MSA and fell in THP-1 cells (**Figure 6**). Qualitatively different ER stress response protein expression was induced by MSA, with a pro-survival response in PBMC and a pro-apoptotic response in THP-1 cells (data not shown).

This pilot project clearly demonstrates that PD effects of Se compounds can be detected in WBC that can be easily and repeatedly accessed from patients. This model, in addition to offering an opportunity to examine such responses *in vivo*, also can be paired with detailed safety evaluation of each compound and clinical tolerability, with much greater relevance to subsequent clinical use. The inclusion of metastatic cancer and CLL patients in this study further offers the opportunity to evaluate the PD effects of Se compounds in both normal and malignant cells, with this pilot data and other work referenced earlier suggesting that significant differential PD differences can be detected between these groups.

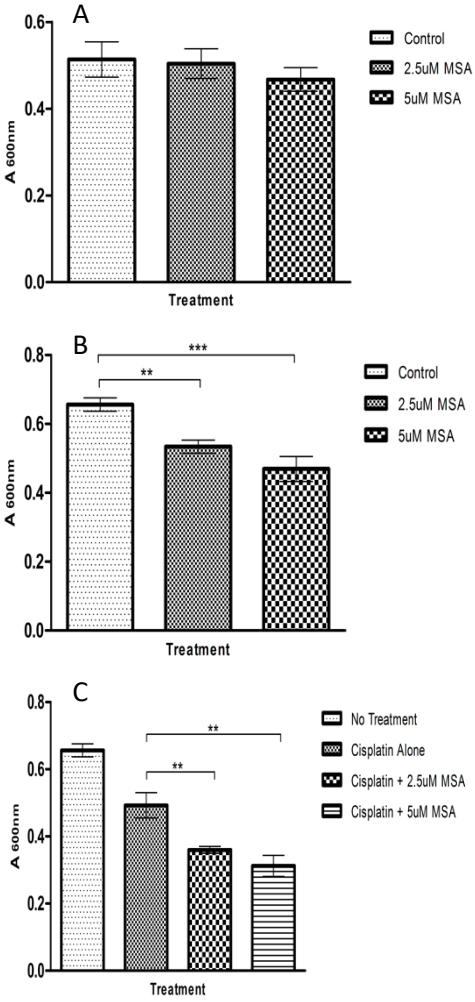

Fig 5. Viability of PBMC does not reduce with MSA (A) but does in THP-1 cells (B) with enhanced cytotoxicity of cisplatin in THP-1 cells (C).

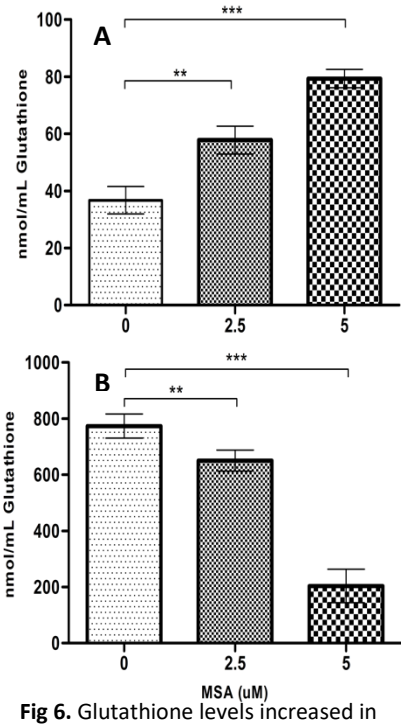

Fig 6. Glutathione levels increased in PBMC (A) and fell in THP-1 cells (B) with MSA.

1.3. Toxicity of Selenium in Clinical Trials

Se compounds have being used in various clinical trials, mainly in chemoprevention, at doses in the "nutritional" range (~50–200 μg/day) without evidence of toxicity (67). Chronic Se toxicity would be expected in humans after long-term

consumption (over months to years) of more than 2400-3000 g/day, but is reversible (68). Based on the published literature, likely symptoms of toxicity (due to environmental exposure) include brittle hair and nails and/or loss, gastrointestinal disturbances, skin rash, garlic breath odour (caused by volatile selenium compounds), fatigue, irritability, and nervous system abnormalities (68). Impaired natural killer cell activity and endocrine disturbances also occur.

However clinical trials using high doses of Se compounds have reported little significant toxicity with short-to-medium term administration. SS 20,000 μg (equivalent to 9,000 μg elemental Se) i.v. over 30 min daily x 5 produced no significant clinical or laboratory toxicity (Prof. K Schumacher, personal communication, 14 October 2005). Furthermore the clinical trials using SS at 200 μg/kg/day (equivalent to 14,000 μg/day [6400 μg Se] in a 70 kg adult) reported fewer toxicities when given with chemotherapy than in those patients who had chemotherapy alone for lymphoma (18;69;70). Selenized yeast (predominantly SLM) was given in doses of 3200 μg daily for up to 24 months in 16 patients with prostate cancer on "watchful waiting", with 1–5 patients reporting garlic breath, brittle nails or hair, stomach upset or dizziness, without any correlation with plasma Se levels (71). None of these patients reported symptoms of peripheral neuropathy. A single dose study of MSC given orally at 400, 800 or 1200 μg reported no significant clinical or laboratory toxicity (72). Finally, a recent phase I trial of sodium selenate (which is metabolised to selenite) established a maximum tolerated dose of 60,000 μg/day (25,000 μg Se] in metastatic castration-resistant cancer patients, with toxicities including nausea, diarrhoea, fatigue, muscle spasms, alopecia and nail disorders (73); slowing of PSA velocity was also observed, and one patient had > 50% reduction in serum PSA.

1.3.1. Toxicity of High-dose Selenium with Chemotherapy

A number of phase I trials using high-dose Se in conjunction with chemotherapy have been conducted, one of which is still in progress. In a dose escalation trial at RPCI, oral SLM (as 800 μg capsules) was started a week before irinotecan (125 mg/m^2 IV weekly x 4 every 6 weeks) and SLM was generally tolerated well (74). At the highest dose level tested, 7200 μg twice daily for 7 days then 7200 μg daily throughout treatment (scheduled to approximate steady-state plasma concentrations within 7 days), the majority of patients reported some nausea after taking SLM in the first week, less so if it was taken after meals. Prolonged stable disease (up to 9 months) has been seen in several patients with irinotecan-refractory metastatic colorectal cancer and other malignancies (75). Trough plasma Se concentrations after 7 days (18-29 μM) fell within the range at which beneficial interactions with chemotherapy occurred in mice (15-48 μM), so this dose schedule (with doses corrected for body surface area to 3600 μg/m^2) was recommended for phase II trials (76).

A trial at the Cancer Institute of New Jersey administered an IV infusion of SS over 5 hours daily for 3 days, starting the day before administration of carboplatin and paclitaxel chemotherapy every 3 weeks in patients with gynaecological malignancies. The highest SS dose administered, 1200 μg, has not caused any significant toxicity or alteration in expected carboplatin pharmacokinetics (77). A study evaluating the protective effects of Se 4000 μg daily (administered as seleno-kappacarrageenan) from 4 days before to 4 days after each dose of cisplatin-based chemotherapy reported no Se-related toxicity (14). Lastly, SS dosed at 200μg/kg/day for up to 7 days orally was well tolerated in a randomised clinical trial (78).

1.4. Selenium Speciation

The identification of intracellular Se species following exposure to MSA *in vitro* in lymphoma cell lines has been undertaken using novel mass spectrometry-based assays (79;80). The authors identified high levels of MSC and SLM to be the main intracellular Se species and, using solid phase microextraction fibres, trapped the volatile Se species dimethylselenide and diemethyldiselenide.

2. Rationale for study

The toxicities of chemotherapy and radiotherapy still limit their efficacy, utility and acceptability to patients and treating oncology staff. While growth factors have helped to limit the complications of low WBC counts with associated infection risk, other toxicities, including low platelet counts and non-haematological toxicities, still limit the doses and duration of treatment that can be safely used and result in poor quality of life for patients, treatment-related deaths and inadequate outcomes (1). There is a clear and pressing need to improve the tolerability of chemotherapy and radiotherapy without compromising their antitumour efficacy.

Se compounds at supranutritional doses may be able to achieve these aims, as evidenced by research summarised earlier. While Se is commonly taken as a supplement by people to help prevent or control cancer, there is insufficient clinical data to determine which Se compound is most effective and safe, and at what dose. Laboratory studies show that the organic forms of selenium are both more effective and safer than the commonly-used inorganic forms such as selenite. Much higher doses than usual appear to be needed for the best effect against established cancers, especially if they are being given with cancer treatments. However whether these high doses are needed to optimise the protective effect of Se in normal tissues is unclear; clinical trials suggest that significant reductions in toxicity can be achieved with more modest Se doses. Thus the appropriate doses of Se and the best compound to achieve these may differ depending on the desired effect: protection against toxicity, prevention of cancer or augmentation of efficacy of other anti-cancer therapies.

It is important to know which form of selenium can be most safely used at higher doses and have the best effect in normal and malignant cells. To study this, we will compare the effects of three different types of selenium: Se-methylselenocysteine (MSC), L-selenomethionine (SLM) and sodium selenite (SS) in people with either metastatic cancer or chronic lymphocytic leukaemia (CLL), looking at their safety, pharmacokinetic (PK) and pharmacodynamic (PD) characteristics and anticancer efficacy. Evaluation of comparative safety (including genotoxicity) and changes in PK and PD biomarkers in plasma and WBC drawn from cancer and CLL patients will also inform whether these Se compounds have differential protective effects in normal cells and cytotoxic effects in malignant cells. This will help researchers to avoid less effective forms and doses of Se that could also cause second malignancies or other serious toxicities.

The once daily dosing for 8 weeks is based on a previous PK studies showing this schedule will approximate steady state concentrations within 2 months (71).

Analysis and identification of the Se species including selenoprotein formation and low molecular weight metabolites following Se ingestion *in vivo* will help further our understanding of Se biology and its therapeutic utility as an adjunct to chemotherapy and/or radiotherapy.

3. Study Objectives

3.1. Study Hypothesis

These Se compounds have differential PD effects that confer protection against the toxic effects of chemotherapy and radiotherapy on normal WBC but enhance the antitumour efficacy against malignant WBC. The organic Se compounds, MSC and SLM, are superior to and safer than SS, as assessed by comparative safety, PK and PD studies. These latter studies will enable determination of an optimal dose and Se compound to take forward into further clinical evaluation in conjunction with chemotherapy and radiotherapy.

3.2. Study Objectives

3.2.1. Primary Objective

To determine the dose and form of Se that can be most safely and effectively used in clinical trials in combination with anticancer therapies.

3.2.2. Secondary Objectives

1. To characterise the clinical and laboratory safety of MSC, SLM and SS when taken orally by cancer (including CLL) patients at doses of 400, 1600 or 6400 □g Se daily for eight weeks.
2. To evaluate the pharmacokinetics (PK) of MSC, SLM and SS in cancer patients at these doses, including the Se species formed in plasma and WBC.
3. To evaluate PD of each dose and type of Se including differential effects in normal and malignant WBC; the malignant WBC will be drawn from patients with chronic lymphocytic leukaemia (CLL) and the normal WBC will be drawn from metastatic cancer patients. PD pathways assessed may include endoplasmic reticulum (ER) stress, angiogenesis, plasma antioxidant activity (glutathione peroxidase), intracellular glutathione metabolism, gene expression and DNA damage, repair and methylation..
4. To evaluate the relationship between dose, PK and PD of each Se compound in normal and malignant WBC.
5. As an exploratory endpoint, to evaluate the impact of each Se compound and dose on tumour activity, as measured by serum relevant tumour markers in cancer patients and peripheral blood counts of lymphocytes in CLL patients.

3.3. Study Endpoints

1. Safety and tolerability of MSC, SLM and SS at the evaluated doses
2. Plasma Se PK and plasma and intracellular WBC Se species with MSC, SLM and SS at each dose level
3. Markers of PD mechanisms (ER stress, angiogenesis, DNA breaks, apoptosis, DNA repair, gene expression and methylation, plasma GPX activity and intracellular glutathione metabolism) in plasma and malignant and normal WBC with each Se compound at each dose level
4. The relationship between Se PK and PD markers in normal and malignant WBC with each Se compound
5. The dose response to each Se compound of tumour cells, as measured by relevant tumour markers in cancer patients and peripheral blood counts of lymphocytes in CLL patients.

4. Study Design

4.1. Design

Randomised phase Ib double-blind, dose-escalation study with 3 arms: MSC, SLM or SS; each arm has 3 dose levels (400, 1600 and 6400 µg of elemental Se). A total of 72 eligible patients will be recruited, 36 with CLL and 36 with cancer. For each of the 3 Se compounds at each of 3 dose levels there will be 8 patients, 4 with CLL and 4 with cancer. Patients will receive the study drug as one capsule daily for 8 weeks. Clinical and laboratory evaluations (including bloods for PK and PD) will be undertaken twice at baseline (except plasma Se), on Days 1 and 2 then at 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing.

Patients will be randomised to MSC, SLM or SS starting at the lowest dose level. Once a dose cohort is full and safety evaluations out to 4 weeks from start of oral dosing in each patient have been completed without dose-limiting toxicity being observed, then the next dose cohort will be recruited.

4.2. Eligibility Criteria

4.2.1. Inclusion Criteria

1. Patients with either proven CLL (peripheral blood lymphocyte count > 10 x 10^9/l) or metastatic cancer, in whom the use of chemotherapy is not anticipated in the next 3 months
2. Age > 18 years
3. Adequate liver, renal and bone marrow function
   - ALP, ALT (or AST) < 5x upper limit of normal (ULN)
   - serum creatinine < 2x ULN
   - Hb > 90 g/l
   - WBC > 3.0 x 10^9/l
   - platelets > 100 x 10^9/l
4. ECOG performance status $\leq$ 2
5. Life expectancy > 6 months
6. Fertile patients (or fertile partners of male patients) must use effective contraception during, and for at least 12 weeks after completion of, study treatment

4.2.2. Exclusion Criteria

1. Patients treated within the last 4 weeks with cytotoxic chemotherapy, systemic therapy that targets VEGF signalling pathways or radiotherapy
2. Unable to swallow or absorb study tablets
3. Concurrent selenium supplements > 100 µg/day
4. Allergy to any of the Se compounds being used in this study
5. Concurrent medical conditions that, in the opinion of the investigators, would compromise either patient safety or the integrity of the data

4.3. Informed Consent and Registration

Each patient must willingly give written consent after being informed of the procedures to be followed, the experimental nature of the therapy, alternatives, potential benefits, side effects, risks, and discomforts.

Patients who have signed consent will be recorded on a screening log and will undergo screening procedures. Eligible patients will be registered and randomised as below.

4.4. Randomisation

Patients will be stratified by diagnosis (CLL or cancer) and gender to ensure equal representation of each gender at each dose level for each Se compound. Patients will then be randomised to MSC, SLM or SS at the current dose level using permuted block randomisation and a computer-based random allocation method. Randomisation will be conducted by the Clinical Trials Pharmacist at Waikato Hospital.

4.5. Dose Escalation

Once a dose cohort is filled, recruitment will be suspended until all patients have completed protocol assessments for at least 4 weeks after start of study dosing in each patient. If dose-limiting toxicities, as defined in the section following, are observed in no more than one of eight patients in each dose cohort for each Se compound, then recruitment of patients into the next dose cohort may commence.

If dose-limiting toxicities are observed in two or more patients in a dose cohort for a specific Se compound then recruitment into that arm will be terminated but recruitment into arms involving other Se compounds may continue.

4.5.1. Dose-limiting Toxicity

For this study, dose-limiting toxicity (DLT) is defined as any one of the following which is believed to be related to the study medication:

- Absolute neutrophil count (ANC) of $<1.0 \times 10^9$/L lasting at least 7 days or associated with fever (>38.5°C) or Grade 3-4 sepsis
- Platelet count $<50 \times 10^9$/L lasting at least 7 days or accompanied by Grade 2 or greater haemorrhage/bleeding
- Clinically significant non-haematological toxicity ≥ Grade 3 despite appropriate treatment (including nausea or vomiting not controlled with appropriate therapy)
- Grade 2 treatment-emergent neurotoxicity
- Grade 2 allergic toxicity

Adverse events meeting these criteria but not believed to be study medication-related (i.e. are believed to be related to underlying disease, a concomitant medication, etc.) will NOT qualify as a DLT for purposes of this study.

4.6. Study Termination

The study will be terminated when recruitment to all dose cohorts has been completed (as permitted above).

4.7. Study Duration

It is estimated that recruitment will take place over a period of 24 months. The study is planned to commence in December 2013.

5. Treatment Plan

5.1. Treatment Arms

The treatment arms for this study are:

- Arm A: MSC 400 or 1600 or 6400 µg Se daily for 8 weeks
- Arm B: SLM 400 or 1600 or 6400 µg Se daily for 8 weeks
- Arm C: SS 400 or 1600 or 6400 µg Se daily for 8 weeks

5.2. Dosing

Patients will be instructed to take their selenium capsule once a day for 8 weeks. The capsule should be taken by mouth in the morning, preferably with or after a meal (within 30 minutes).

5.2.1. Compliance with Study Medication

Compliance with the study drug dosing will be assessed at each visit, based on the patient diary. The medication bottle will be collected at the study visit 8 weeks after starting Se (when the patient is expected to have taken all 56 capsules) to assess overall compliance.

5.3. Anticipated Adverse Events

Se compounds have being used in various clinical trials, mainly in chemoprevention, at doses in the "nutritional" range (~50–200 µg/day) without evidence of toxicity (67). Chronic Se toxicity would be expected in humans after long-term consumption (over months to years) of more than 2400-3000 □g/day, but is reversible (68). Based on the published literature, likely symptoms of toxicity (due to environmental exposure) include brittle hair and nails and/or loss, gastrointestinal disturbances, skin rash, garlic breath odour (caused by volatile selenium compounds), fatigue, irritability, and nervous system abnormalities (68). Impaired natural killer cell activity and endocrine disturbances also occur.

However clinical trials using high doses of Se compounds have reported little significant toxicity with short-to-medium term administration. SS 20,000 µg (equivalent to 9,000 µg elemental Se) i.v. over 30 min daily x 5 produced no significant clinical or laboratory toxicity (Prof. K Schumacher, personal communication, 14 October 2005). Furthermore the clinical trials using SS at 200 µg/kg/day (equivalent to 14,000 µg/day [6400 µg Se] in a 70 kg adult) reported fewer toxicities when given with chemotherapy than in those patients who had chemotherapy alone for lymphoma (18;81;82). Selenized yeast (predominantly SLM) was given in doses of 3200 µg daily for up to 24 months in 16 patients with prostate cancer on "watchful waiting", with 1–5 patients reporting garlic breath, brittle nails or hair, stomach upset or dizziness, without any correlation with plasma Se levels (71). None of these patients reported symptoms of peripheral neuropathy. A single dose study of MSC given orally at 400, 800 or 1200 µg reported no significant clinical or laboratory toxicity (72). Finally, a recent phase I trial of sodium selenate (which is metabolised to selenite) established a maximum tolerated dose of 60,000 µg/day (25,000 µg Se] in metastatic castration-resistant prostate cancer patients, with toxicities including nausea, diarrhoea, fatigue, muscle spasms, alopecia and nail disorders (73); slowing of PSA velocity was also observed, and one patient had > 50% reduction in serum PSA.

5.4. Treatment Modifications

5.4.1. MSC, SLM, SS

No dose modification will be permitted for dermatological toxicities or garlic odour attributed to MSC, SLM or SS supplementation. In case of vomiting, no replacement will occur.

Patients who cannot tolerate the Se compounds with daily dosing may have a treatment break of up to 3 days on two occasions, but must then either recommence the study medication or come off study.

5.4.2. Ancillary Therapy

Patients should receive full supportive care, including anti-emetics, transfusions of blood and blood products, antibiotics, palliative radiotherapy etc, when appropriate.

During the period of participation in this study (including the 4 weeks between study medication completion and final study evaluation) patients may not receive any cytotoxic chemotherapy or systemic therapy that targets VEGF-signalling pathways (e.g. sunitinib, pazopanib). Hormonal therapies for control of cancer are permitted.

Se supplements in excess of 100 µg daily are not permitted.

5.5. Removal of Patients from the Study

The following are criteria for removal of patients from study:

- Study closure
- Unacceptable adverse event(s)
- Patient decision to withdraw
- In the judgment of the investigator, continued participation in the study would not be in the best interest of the patient.

In the event of patient withdrawal from the study, notify the Principal Investigator. Document the reason(s) for withdrawal. If withdrawal is due to adverse events, the patient should be followed up until these have resolved.

5.6. Replacement of Patients who Withdraw from the Study

Patients who withdraw from the study prior to taking any study medication may be replaced.

5.7. Unblinding of Study Medication During the Study

Unblinding of the medication that an individual patient is taking can be arranged by a request to the Trials Pharmacist at Waikato Hospital where:

- an adverse event has occurred that the patient or their responsible clinician believes is attributable to the study medication, and
- the event requires the withdrawal of the patient from the study, and
- knowledge of the Se compound taken is considered important to the current or subsequent management of the patient (such as an allergic reaction).

The principal investigator should be informed as soon as possible of such an occurrence.

6. DRUG FORMULATIONS AND PREPARATION

All Se compounds will be manufactured and supplied by Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520-5321, USA. Further details are available in the Investigator's Brochure. Doses stated reflect micrograms of elemental Se in each capsule, not micrograms of the compound.

6.1. Drug Formulation

6.1.1. Se-methylselenocysteine

Other names: MSC

Product description: Se-methylselenocysteine (MSC) will be supplied as 400, 1600 and 6400 µg capsules.

Storage requirements*:* Store at room temperature.

6.1.2. L-Selenomethionine

Other names: SLM

Product description: Se-methylselenocysteine (SLM) will be supplied as 400, 1600 and 6400 µg capsules.

Storage requirements*:* Store at room temperature.

6.1.3. Sodium Selenite

Other names: SS

Product description: Sodium selenite (SS) will be supplied as 400, 1600 and 6400 µg capsules.

Storage requirements*:* Store at room temperature.

6.2. Product Dispensing

The site pharmacist will be responsible for dispensing study medication to each patient with labelling as per institutional policy. It will be dispensed in sufficient quantities to last each patient 8 weeks (i.e. 56 capsules). The labelling of the containers will be coded so the patients, investigators and other site research staff remain blinded to treatment allocation.

7. Study Procedures

7.1. Assessments

Study procedures will be carried out as detailed in the Schedule of Assessments on page 212. Aspects of specific evaluations are discussed further below.

7.1.1. Clinical Evaluations

Comprehensive clinical evaluation (including physical examination) of each patient should be conducted by a consultant or registrar after informed consent is signed but prior to administration of study medications. Further clinical evaluations, including symptom-directed physical examination and vital signs, will be conducted on Days 1 and 2 and 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing.

7.1.2. Safety Laboratory Evaluations

Complete blood count (4 ml blood in EDTA tube), renal and liver function tests, urate, calcium, phosphate (4 ml blood in heparin tube), glucose (2 ml blood in fluoride tube) INR, APTT (4.5 ml blood in citrate tube) and urinalysis (dipstick) will be undertaken at baseline and on Days 1 and 2 then 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing. All these blood samples will be analysed at the Waikato Hospital Laboratory. Urine dipstick analysis (for pH, protein, blood and glucose) will be performed in the Oncology Dept.

7.1.3. Efficacy Laboratory Evaluations

The complete blood counts taken as above will enable assessment of peripheral blood lymphocyte count as an indirect reflection of efficacy in CLL patients. Serum tumour markers (where relevant) analysed on the biochemistry blood sample in cancer patients at all time points except Day 2 will allow assessment of response in cancer patients.

7.1.4. Electrocardiogram

A 12-lead ECG will be recorded at screening and Day 1 prior to dosing (concurrent with visits for pharmacodynamic endpoint blood sampling). The machine-calculated Bazett-corrected QT interval (QT_c) will be recorded on each occasion and a subject-specific average will be calculated from the baseline and Day 1 recordings. The ECG will be repeated on Day 2 then 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing.

If the QT_c is prolonged to > 450 ms on any occasion then two further ECGs will be recorded to calculate an average QT_c on that occasion. If the average of the QT_c is > 480 ms ($\geq$ Grade 2 toxicity) then the patient will be instructed to stop taking the study medication at that point and will come off study.

7.1.5. Pharmacokinetics

A 3 ml blood sample for plasma Se will be taken using a 6mL K2EDTA Trace Element tube prior to dosing on Day 1 and repeated on Day 2 about 4 hours after ingestion of the study medication. Further trough samples (i.e. taken prior to ingestion of study medication) will be collected 4 and 8 weeks after commencing Se and 4 weeks after end of Se dosing.

The blood sample will be sent chilled to the Waikato Hospital Laboratory where plasma will be separated and a 1 ml plasma aliquot will be stored at -80 ºC until analyzed at Canterbury Health Laboratories via the standard laboratory referral pathways.

7.1.6. Pharmacodynamic Markers

Laboratory evaluations for PD markers will be taken twice (at baseline visit and Day 1 prior to ingestion of study medication) to establish intra-subject variance and repeated on Day 2 and at 4

and 8 weeks after commencing Se and 4 weeks after end of Se dosing. All PD samples will be analysed at the Molecular Genetics Laboratory at the University of Waikato except where specified below.

Blood samples for PD assessments will include:

- 4 ml blood in heparin tube spun down in a refrigerated centrifuge and plasma stored at -80 °C for the following assays:
    - 0.5 ml aliquot of plasma for marker of tumour angiogenesis (VEGF-□) by ELISA
    - 1 ml plasma for glutathione peroxidase (selenoprotein and marker of antioxidant effects of Se) by colorimetric assay
- 6 ml blood in K2EDTA Trace Element tube spun down in a refrigerated centrifuge and 3 ml plasma stored at -80 °C (baseline and week 4 only) until analysis of Se speciation by Dr Heidi Goenaga-Infante at the LCG group, Teddington, UK*.
- 2.5ml gene expression into PAXgene tubes either frozen at -20 °C or RNA isolated and stored at –80°C until analysed at the University of Surrey*.
- up to 15 ml blood in EDTA tubes for the following assays *
    - 1ml whole blood for assessment of DNA breaks and repair using either the alkaline COMET assay with and without Bleomycin or using LORD-Q PCR.
    - Up to 14 ml blood for extraction of PBMC using Ficoll (conducted same day). The PBMC will be divided and used as follows:
        - 5 ml for assessment of molecular markers of hypoxia response regulation (HIF-1□, prolyl hydroxylases 2 & 3,) and ER stress (GRP78, spliced XBP1, CHOP, phosphorylated eIF2□, caspase 8); a protein lysate will be prepared from the PBMC and stored at -80 ºC until analysis by western blot.
        - up to 5ml for DNA repair and Methylation analysis at the University of Surrey**
        - 1.5 ml for intracellular glutathione concentrations by colorimetric assay
        - 1.5ml to provide 3 aliquots of 100 µl (each containing a lysate of 0.5 x 10^6 PBMC) for Se speciation (baseline and week 4 only) stored at -80 ºC.

* Less blood volume may be required for PBMC extraction from CLL patients due to lymphocytosis at presentation.

** Plasma and cell lysates for Se speciation, DNA repair and methylation and gene expression analysis will be batched and shipped on dry ice to the Department of Nutritional Sciences, University of Surrey, Guildford, UK and stored there for analysis by Dr Ruan Elliott and Dr Priti Chivers. Selected samples will be uplifted from there for analysis of selenium speciation by Dr Heidi Goenaga-Infante at the LCG Group, Teddington, UK.

8. Reporting requirements and Safety Monitoring

8.1. Reporting Requirements

8.1.1. Investigator Requirements and Responsibilities

The principal investigator (PI) of this study is ultimately responsible for every aspect of the study design, conduct, and final analysis.

8.1.2. Adverse Event (AE) Definition

An AE is any untoward medical event that occurs to a subject following the start of study drug administration, whether or not the event is considered drug-related. Pre-existing conditions are not

considered an AE unless the condition worsens by at least one grade following the start of study drug(s) administration. This study will utilize the NCI Common Toxicity Criteria for Adverse Events (CTCAE) version 4.0 to determine the severity (grade) of the adverse event. A copy of CTCAE version 4.0 can be downloaded from the CTEP homepage (http://ctep.info.nih.gov).

Death due to disease progression occurring 31 days or more from the day of last study drug administration will not be reported as an AE or SAE.

Any drug-related AE of grade 2 or higher should be followed for resolution until either 1) the start of subsequent anti-cancer therapy or 2) death.

8.1.3. Relationship of Adverse Event to Study Medication

The relationship of an AE or SAE to study medication will be classified using the following five categories:

- Definitely related
- Probably related
- Possibly related
- Unlikely related
- Definitely not related

An adverse event will be considered related to study medication if the attribution is definitely, probably or possibly related.

8.1.4. Serious Adverse Events

A SAE is any AE that results in any of the following outcomes:

- Death
- A life-threatening experience
- An inpatient hospitalization or prolongation of an existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly/birth defect

An event that is not listed above but that requires intervention to prevent one of the outcomes listed above is also considered a SAE. Elective hospitalizations that are not in response to an AE or are for blood product transfusions will not be considered a SAE.

Reporting Serious Adverse Events

All SAEs occurring during the course of the study or within 30 days of the last administration of the study medication must be reported to the study co-ordinator (or the PI) within 24 hours of the knowledge of the event. The study co-ordinator will forward SAEs to the Health and Disability Ethics Committee (HDEC) within the time-frames for expedited reporting set out in ICH guidelines (i.e., as soon as possible but within 7-15 days) that meet all of the following criteria:

- they are unexpected because they are not outlined in the investigator's brochure, and
- are not defined study end-points (e.g. death or hospitalisation), and
- occur in patients located in NZ, and
- if the study involves blinding, result in a decision to break the study code.

There is no requirement for the individual reporting of SAEs to the HDEC that do not meet all of these criteria.

SAE Follow Up information

All SAEs will be followed until resolution, stabilisation, death or until the subject receives additional cancer therapy.

8.1.5. Safety Committee

A Safety Committee will be formed that consists of the Safety Committee Chair, Principal Investigator, Coordinating Investigator and the Study Biostatistician. The Safety Committee will have the following responsibilities:

- during the dose-escalation phase of the study, review of safety data from the prior dose level in order to determine the next dose level
- review of safety issues as requested by the study investigators during the course of the study with the goal of recommending an appropriate course of action.

9. Data Analysis

The primary analysis is for safety of each Se compound and dose, so patients will be grouped together (n=8) in each dose cohort for this and plasma PK analyses. All data will be summarised by dose group and all subjects will be accounted for.

Descriptive statistics will be utilised for safety, efficacy, and PK parameters. Descriptive statistics for continuous variables will include the mean, standard deviation, median, and range (minimum/maximum); categorical variables will be presented as frequency counts and percentages.

Summary tables will present the number of subjects (per dose group) observed with AEs and corresponding percentages. The denominator used to calculate incidence percentages consists of subjects receiving at least one dose of study drug for each dose group. Within each table, the AEs will be categorized by MedDRA body system and preferred term. Additional subcategories will be based on event severity and relationship to study drug.

Fisher's exact test will be used to compare frequency of adverse events.

An analysis of the relationships between dose, plasma Se concentration and change in PD markers in the different groups will be conducted using repeated measures analysis of variance methods or linear mixed modelling methods.

10. ETHICAL and regulatory CONSIDERATIONS

10.1. Ethical Principles

The study will be conducted according to the principles of the Declaration of Helsinki (as Scotland 2000, as clarified in 2002), the International Conference on Harmonization Guidance on Good Clinical Practice and the requirements of all local regulatory authorities regarding the conduct of clinical trials and the protection of human subjects.

10.2. Informed Consent

Before recruitment and enrolment into the study, each prospective candidate will be given a full explanation of the study. The Patient Information Leaflet and consent form will be submitted for approval to the HDEC. Once this essential information has been provided to the subject and the investigators have been assured that an individual candidate understands the implications of participating in this study, the subject will be asked to give written consent to participate in the study.

10.3. Ethical Approval

The Principal Investigator must submit this protocol to the HDEC and is required to forward a copy of the written approval or advice signed by the Chairperson to the study co-ordinator. The date of the review, the study identifiers (protocol title and version) and the documents studied (protocol and informed consent material) should be clearly stated on the approval or advice sheet.

10.4. Confidentiality

All patient information must be treated in strict confidence. Data that identify any study subject must not be revealed to anyone not directly involved in the research project or the clinical care of that subject. An exception is where the patient has provided written consent for his/her records to be subject to source document verification. In this instance, the records may be inspected by (a) a study monitor for the purposes of source document verification or quality audit as stipulated in the Guidelines for Good Clinical Research Practice, or (b) a representative of a government regulatory authority for the purposes of official inspection. Records must be made available for inspection on the understanding that all information relating to study subjects will be treated in strict professional confidence.

The results of the study may be presented in reports, published in scientific journals or presented at medical meetings; however, subject names will never be used in any reports about the study.

10.5. Adherence to Protocol

Except for an emergency situation in which proper care for the protection, safety and wellbeing of the study subject requires alternative treatment, the study shall be conducted exactly as described in the approved protocol. Any deviation from the protocol must be recorded and explained.

10.6. Data Quality Assurance

Accurate, consistent, and reliable data will be ensured through the use Good Clinical Practices (GCP) guidelines regarding clinical data management practices and procedures.

10.7. Control of the Study Materials

The Principal Investigator or their representative will account for all study materials. All study medication will be kept under lock in an inaccessible location while in storage. The Principal Investigator shall maintain adequate records of the disposition of the study medication, including dates, quantity and use by subjects. Upon completion of the study, all remaining test products will be accounted for (including unused study medication returned by patients) and unused material will be destroyed by the Hospital Pharmacy.

10.8. Record Retention

The study site will be responsible for retention of all essential documents associated with the implementation of the study for a period of 10 years from the study termination date.

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Appendix 1 ECOG PERFORMANCE STATUS

SCORE	DESCRIPTION
0	Fully active, able to carry on all pre-disease performance without restriction.
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work.
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.

From Oken *et al* (83).

Appendix 2 Creatinine Clearance Calculation

Creatinine clearance will be calculated using the Cockcroft-Gault formula:

$$\text{Creatinine Clearance} = \frac{(140 - \text{age}) \times \text{weight (in kg)} \times 1.23 \times 0.85 \text{ (if female)}}{\text{Plasma creatinine } (\mu\text{mol/l})}$$

APPENDIX 3 PATIENT DIARY

Medications and symptoms in week # ____

Patient diary
Selenium trial
PI Dr. Michael Jameson

Patient Initials _____

Patient Study ID # _____

Medications

Place a tick in appropriate box each time you take a medication and write the name, dose and reason for taking it

Medication name	Dose	Reason	date	date	date	date	date	date	date	Comments
Example: Paracetamol	*500mg x 2 tablets*	*headache*		✓✓✓				✓		*Resolved 2 hours later*
Selenium study capsules	1 capsule	*on trial*								n/a

Symptoms and severity

Please enter symptom and severity (mild=1 moderate=2 severe=3)

Symptom name	date	date	date	date	date	date	date	Comments
Example: Headache		*3*				*1*		

ver 1 14/05/2012

APPENDIX 4 PATIENT INFORMATION LEAFLET AND CONSENT FORM

HEALTH WAIKATO
CARING FOR YOU
Waikato District Health Board

Participant Information Sheet

Study title:	***Phase I study of selenium in cancer patients***	
Locality:	**Waikato Hospital**	Ethics committee ref.: 13/NTA/172
Lead investigator:	**Dr Michael Jameson**	Contact phone number: 07 839 8899

You are invited to take part in a study looking at the effects of 3 different types of Selenium in cancer patients because your doctor has identified you as someone who may be eligible to take part due to the type of cancer you have. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep. This document is 8 pages long, including the Consent Form. Please make sure you have read and understood all the pages.

WHAT IS THE PURPOSE OF THE STUDY?

Background information

Selenium is an essential trace mineral and is commonly taken as a supplement by people to help prevent or control cancer. It is showing promise in being able to reduce side effects of radiotherapy or chemotherapy without reducing their effectiveness against cancers. However laboratory studies show that the forms of selenium found in plants are both more effective and safer than the commonly-used selenium salts such as selenite. Much higher doses than usual are needed for the best effect against cancers, especially if selenium is being given with cancer treatments, but it is not clear how much is enough, or too much.

What are the aims of the study?

In this clinical research study we want to know which form and dose of selenium can be most safely used and have the best protective effect in normal cells while still having

toxic effects in cancer cells so we can test this with other cancer treatments in future clinical trials.

To study this, we aim to compare the effects of three different types of selenium: Se-methylselenocysteine (MSC), L-selenomethionine (SLM) and sodium selenite (SS), each at 3 different doses. We will study these 3 types of selenium in people with either metastatic cancer (meaning it has spread to other parts of the body) or chronic lymphocytic leukaemia. Each patient in the study will take selenium capsules daily for 8 weeks, and we want to check them again 4 weeks afterwards. So if you take part in this study, your active participation will be for 3 months.

We will look closely at:

- any side effects including effects on standard blood and urine tests
- changes in levels and types of selenium in plasma and blood cells
- effects on normal white blood cells including gene activation and cell functions
- effects on leukaemia cells including gene activation and cell functions
- effects on cancer activity by measuring tumour markers (where relevant to you) in blood tests

Most of these tests will be done at Waikato Hospital or at the University of Waikato.

Samples to be sent overseas

More specialised tests (including gene activation and repair studies) will require blood samples to be sent to selenium experts at the University of Surrey and the Laboratory of the Government Chemist in the UK. If you do not want your samples sent overseas, you can indicate this on the consent form.

We will test 3 different doses of each selenium compound: 400 mcg (micrograms), 1600 mcg and 6400 mcg. The usual selenium intake in NZ is about 100 mcg per day.

The study will recruit 72 people overall and we will ask 24 patients (12 with chronic lymphocytic leukaemia and 12 with cancer) to take MSC or SLM or SS at the lowest dose (400 mcg) daily for 8 weeks. If there are no worrying side effects then the next 24 patients will take MSC or SLM or SS at 1600 mcg daily for 8 weeks. If that dose appears safe then the last group of 24 patients will take MSC or SLM or SS at 6400mcg daily for 8 weeks. That will complete the study.

WHAT WILL MY PARTICIPATION IN THE STUDY INVOLVE?

You have been invited to participate because your doctor has identified you as someone who may be eligible to take part in this study due to the type of cancer or leukaemia you have. If you agree to take part in this study, you must sign this consent form before any study procedures are done, and you will be given a copy of this form.

1) Screening for eligibility

A member of the study team will first need to confirm that you are eligible, and will ask you some questions about your health (including cancer diagnosis, prior treatments, previous and current health and medications) and any medication you take. A doctor will do a physical examination. The requirements for entering the study are:

- age 18 years and over
- have either chronic lymphocytic leukaemia or metastatic cancer, in whom the use of chemotherapy is not anticipated in the next 3 months
- not pregnant or breast-feeding
- no recent chemotherapy

- not taking more than 100 mcg of selenium supplements each day
- not allergic to any selenium compounds
- must be able to swallow capsules
- must have adequate liver, renal and bone marrow function

Blood samples (about 2 tablespoons) will be taken to check:

- that your kidneys, liver and bone marrow are working well enough
- that women going into the study are not pregnant
- tumour marker levels (if relevant to your cancer)
- the blood selenium level
- DNA damage, gene activity and cell functions in plasma and normal blood cells or leukaemia cells

You will also have an electrocardiogram (an ECG, to check the electrical conduction of the heart) and a urine test. Overall this visit will take about 1½ hours.

It is possible after these tests are reviewed that you will not be able to take part in this study (if you are not fully eligible).

2) During the Study

Second Visit

If you are eligible for the study you will have an appointment made about a week later to see a member of the study team again, who will ask you about any ongoing or new symptoms and current medication. If needed, a doctor will do a physical examination. You will also have another ECG, urine test and blood tests (about 2 tablespoons). This visit will take about an hour.

These two sets of tests a week apart will help us to see how much natural variation there is in the various tests we are doing, This helps us to tell if any changes that we measure when you are taking selenium are significant or not.

Study Medication

At the second visit you will be given a bottle of selenium capsules. We will use a computer programme to randomly decide (like flipping a coin) which type of selenium you will take. Neither you nor the doctors and nurses will know what type of selenium you are taking. This is because we could assess you differently if we know which medication you are taking. However, we can find out in the case of a life-threatening situation.

You should take one study capsule by mouth each day for 8 weeks (up to 30 minutes before or after a meal, preferably in the morning).

Please take the first dose on the day you receive the bottle of capsules.

Third Visit

This visit is the NEXT day after starting your study capsules. **Please DO take the study capsule first thing in the morning.** We will plan your outpatient clinic appointment so that we can take your blood tests (about 2 tablespoons) about **4 hours** after you took the capsule (to see the level selenium reaches in your blood). You will also have a urine test and ECG. This visit will take about an hour.

Fourth – Sixth Study Visits

You will be asked to come to an outpatient clinic visit for the study at the following times after starting the study medication:

- 4 weeks
- 8 weeks (please bring the bottle of study capsules with you)
- 12 weeks (i.e. 4 weeks after finishing the study medication)

Please do <u>not</u> take the study capsule on the morning of your clinic appointment until after you have had the blood tests.

At each visit you will have a short consultation with a doctor to enquire about any side effects and have a physical examination. You will also have another set of blood tests (about 2 tablespoons), urine test and ECG, as before. These visits will take about an hour.

After the 12 week visit you will have finished participation in the study.

<u>Diary</u>

We will give you a diary and will ask you to keep a record of any side effects you may get as well as writing down other medication you are taking each day (including the study medication). When you come to the outpatient clinic for your study appointments please bring the diary.

3) Using Other Medication During the Study

You are encouraged to keep taking your usual medications during the study, including any hormonal therapy for cancer. We do not want you to take any supplements that contain more than 100 mcg of selenium a day while you are on the study.

WHAT ARE THE POSSIBLE BENEFITS AND RISKS OF THIS STUDY?

What are the potential benefits of the study?

Clinical trials have shown that patients with metastatic cancer have fewer side effects from chemotherapy if they have selenium supplements. However these types and doses of selenium have not been carefully studied (or compared) in metastatic cancer or chronic lymphocytic leukaemia when patients are not having chemotherapy, so we do not know how likely it is that you will have an improvement. This study offers you the opportunity to see if the dose and type of selenium you would take has any effect on your illness..

You might also find it interesting or satisfying to be involved in research that may help other cancer patients in the future.

What are the potential risks of the study?

Most people have no side effects with selenium, even at high doses. However if you have unpleasant side effects you should discuss this with your study team. You might be advised to have a treatment break of up to 3 days. If the medication is still causing you unacceptable side effects then you can stop taking the capsules. We will keep following you up on the study as planned to see what impact taking the study capsules had.

Side effects can occur with doses over 2400-3000 mcg/day taken for months to years but they appear to be reversible on stopping therapy. Side effects at high doses include:

- brittle hair and nails (and occasionally loss of hair or nails)
- stomach or bowel upset
- skin rash
- “garlic” odour on breath or skin
- fatigue and irritability

- tingling in the hands and/or feet

Rarely allergic reactions, impaired immunity and hormone imbalance are seen.

Interactions of selenium with other medications

Selenium is a trace element and not known to interact with other medications.

Pregnancy and Breast-feeding

Because excessive selenium may be harmful to an unborn child, women must avoid becoming pregnant and men must avoid fathering children while receiving this study medication. For this reason, female patients with child-bearing potential will have a blood test performed prior to the start of the study to rule out pregnancy. Adequate birth control measures (i.e. oral, implanted or barrier methods) must be used (where there is a possibility of pregnancy) by participants while receiving selenium. Female patients should also not nurse their baby (breastfeed) while on this study. If you are female and become pregnant while participating in this study, you should notify your doctor immediately.

No method of birth control is 100% reliable except continuous abstinence. Relying on birth control to prevent pregnancy assumes extra risk as compared to continuous abstinence. There is no way for either you or the Investigator to know if your birth control has failed until after it has happened. An early pregnancy can be missed and you should not rely on the results of a home pregnancy test to be certain that you are not pregnant. Your participation in this study will be discontinued if it is determined that you are pregnant.

Who will look after my health problems during the study?

Your usual GP and specialists will still look after you while you are on this study. Any health problems that are not thought to be related to the study medication should be managed by them and the study team will keep them informed of any health problems.

Your doctor may stop the study medication, even without your approval, for a number of reasons, such as:

- you are allergic to the capsules
- you take other selenium supplements (more than 100 mcg per day)
- new information becomes available that indicates the study treatment is no longer in your best interests
- your doctor no longer feels this is the best treatment for you
- you become pregnant, start breastfeeding, or stop using contraceptives

WHO PAYS FOR THE STUDY?

This study is supported by grants from the Waikato Medical Research Foundation, Genesis Oncology Trust and the Cycle for Life charity in Thames. This funding will cover the direct costs of doing the laboratory tests and support the research work.

There are no charges to you or payments for participating in this study. You will be offered vouchers to cover petrol and parking costs for study visits to the hospital.

WHAT IF SOMETHING GOES WRONG?

If you were injured in this study, which is unlikely, you would be eligible for compensation from ACC just as you would be if you were injured in an accident at work or at home. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

WHAT ARE MY RIGHTS?

Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time without it affecting your ongoing medical care, but the information collected about you up to the point when you withdraw will continue to be processed if you agree.

You have the right to access information about you collected as part of the study. If you want to do so, please contact the investigators using the details below. This information will be kept confidential and any reports of this study will not contain information that could be used to identify you.

If, during the course of this study, significant new information (such as changes in the risks or benefits or new alternatives to treatment) becomes available that may affect your willingness to continue to participate, the study doctor and/or the study staff will let you know. The results of your blood counts and blood chemistry tests (and PSA if relevant) will be sent to your GP to keep them informed.

All details recorded for the study will remain confidential, and no information that could personally identify you will be given to anyone outside the team immediately responsible for your care both in and outside the hospital, except for auditors, Ethics Committees and regulatory authorities, who may have access to your medical records for the purpose of checking the accuracy of the information recorded by the research team.

No information about you, or provided by you during the research, will be disclosed to others without your written permission except if necessary to protect your rights or welfare (for example if you are injured and need emergency care) or if required by law. When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity.

WHAT HAPPENS AFTER THE STUDY?

If you wish to be informed of the results of the study your doctor can discuss these with you or you are welcome to request that a summary of the findings be sent to you. The results will eventually be presented at meetings and will be published in a reputable medical journal but this process often takes many months after a study is completed.

If you wish to have any remaining blood or urine samples returned to you after testing is complete (for cultural or other reasons), or earlier if you decide to withdraw from the study, you may request this on the consent form. Otherwise the samples will be destroyed according to standard laboratory protocols once the study is complete.

We will confidentially store the data from this study for 10 years in our Cancer Trials Unit at Waikato Hospital.

If you feel you benefited from taking the selenium study medication then, after you have finished your participation in the study, you are free to purchase a selenium supplement of the same strength.

WHO DO I CONTACT FOR MORE INFORMATION OR IF I HAVE CONCERNS?

If you have any questions, concerns or complaints about the study at any stage, you can contact the Lead Investigator:

Dr Michael Jameson

Consultant Medical Oncologist

Oncology Department

Waikato Hospital

Pembroke St

Hamilton 3240

Ph: (07) 839 8976

Email: michael.jameson@waikatodhb.health.nz

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

For Maori health support please contact:

Te Puna Oranga (Maori Health Services), Waikato Hospital

Ph: (07) 834 3628

Email: justine.solomon@waikatodhb.health.nz

You can also contact the Northern A Health and Disability Ethics Committee that approved this study on:

Phone: 0800 4 ETHICS

Email: hdecs@moh.govt.nz

HEALTH WAIKATO
CARING FOR YOU
Waikato District Health Board

Consent Form

If you need an INTERPRETER, please tell us.

Please tick to indicate you consent to the following:

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	Yes ☐	No ☐
I have been given sufficient time to consider whether or not to participate in this study.	Yes ☐	No ☐
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	Yes ☐	No ☐
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	Yes ☐	No ☐
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	Yes ☐	No ☐
I consent to the research staff collecting and processing my information, including information about my health.	Yes ☐	No ☐
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes ☐	No ☐
I consent to my GP or current provider being informed about my participation in the study and of any significant abnormal results obtained during the study.	Yes ☐	No ☐
I understand that there may be risks associated with the treatment in the event of myself or my partner becoming pregnant. I undertake to inform my partner of the risks and to take responsibility for the prevention of pregnancy.	Yes ☐	No ☐
I agree to my blood samples being sent overseas and I am aware that these samples will be disposed of using established guidelines for discarding biohazard waste.	Yes ☐	No ☐
I agree to an approved auditor appointed by the New Zealand Health and Disability Ethic Committees, or any relevant regulatory	Yes ☐	No ☐

authority or their approved representative reviewing my relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study.		
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.	Yes ☐	No ☐
I understand the compensation provisions in case of injury during the study.	Yes ☐	No ☐
I know who to contact if I have any questions about the study in general.	Yes ☐	No ☐
I understand my responsibilities as a study participant.	Yes ☐	No ☐
I wish to receive a summary of the results from the study.	Yes ☐	No ☐

Declaration by participant:

I hereby consent to take part in this study.

Participant's name:

Signature: Date:

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name:

Signature: Date:

Investigators Brochure

Se-methyl-L-selenocysteine

L-selenomethionine

Selenite

Prepared by:

Stephen Evans, Oncology Pharmacist, Waikato Hospital, Hamilton, New Zealand

Dr Michael Jameson, Consultant Medical Oncologist, Regional Cancer Centre, Waikato Hospital, Hamilton, New Zealand

List of abbreviations

GPX	Glutathione peroxidase
Met	Methionine
MSC	Se-methyl-L-selenocysteine, Se-methylselenocysteine
Se	Selenium
LSM	L-Selenomethionine
µg	Microgram (mcg)

Table of Contents

1 Introduction

This Investigators Brochure has been collated to provide background data on the three selenium (Se) compounds prepared for use in a clinical trial (Phase Ib, randomised, double-blind, dose-escalation trial to identify the safest, most effective selenium compound for use in cancer patients; ANZCTR Registration No: ACTRN12613000118707).

These compounds are:

- Se-methyl-L-selenocysteine (MSC)
- L-selenomethionine (LSM)
- Selenite (as the sodium salt)

Physicochemical data on each selenium compound supplied for the phase I clinical trial by Sabinsa Corporation, NJ, USA, is detailed below.

A comprehensive evaluation of each of these selenium compounds has been published by the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food in 2009 (1-3). Rather than duplicating the information detailed therein, each report has been attached as an appendix:

- **Appendix C1:** Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on Se-Methyl-L-Selenocysteine as a source of selenium added for nutritional purposes to food supplements following a request from the European Commission
- **Appendix C2:** Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on L-selenomethionine as a source of selenium added for nutritional purposes to food supplements, following a request from the European Commission
- **Appendix C3:** Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on a request from the Commission on selenious acid added for nutritional purposes to food supplements

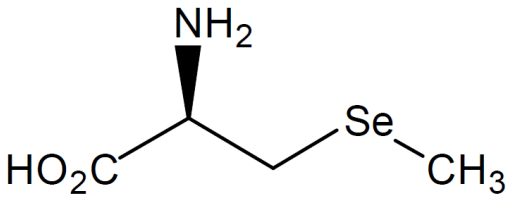
While L-selenomethionine and sodium selenite (or its acid form, selenious acid) are well characterized in clinical use, additional information has been published on Se-methyl-L-selenocysteine in a clinical trial subsequent to these EFSA reports, and is also detailed below.

Additionally the EFSA reports do not refer to recent studies investigating the pharmacokinetic and toxicity profiles of L-Selenomethionine and Sodium Selenite administered at supranutritional doses (i.e. > 400 µg of Selenium per day); these studies are also summarised below.

2 Chemical and pharmaceutical data

2.1 Se-methyl-L-selenocysteine (Methylselenocysteine, MSC)

2.1.1 Chemical Structure



Molecular weight: 182.08 g/mol

Molecular formula: $C_4H_9NO_2Se$

CAS Registry Number: 26046-90-2

2.1.2 Stereochemistry

Specific optical rotation: +6.5° to +8.5° (1% w/v soln in 1N HCl)

Assay by HPLC: NLT 98.0%

2.1.3 Physicochemical data

Loss on drying: Not more than 3.0% w/w (dried at 105°C)

Solubility: Soluble in water

pKa: 5.2

2.1.4 Formulation

Methylselenocysteine (containing 400 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: Methylselenocysteine = 972.05 mcg

(equivalent to elemental Selenium: 400 mcg)

Batch No of Methylselenocysteine used: **C130529**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Methylselenocysteine	400 mcg 972.05 mcg	 41.15% of Selenium	 5%	 1.021
2	Microcrystalline Cellulose				348.98
3	Size '0' Red/Red Hard Gelatin Capsule				

Methylselenocysteine (containing 1600 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: Methylselenocysteine = 3.89 mg

(equivalent to elemental Selenium: 1600 mcg)

Batch No of Methylselenocysteine used: **C130529**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Methylselenocysteine	1600 mcg 3.89 mg	 41.15% of Selenium	 5%	 4.083
2	Microcrystalline Cellulose				345.92
3	Size '0' Red/Red Hard Gelatin Capsule				

Methylselenocysteine (containing 6400 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: Methylselenocysteine = 15.553 mg

(equivalent to elemental Selenium: 6400 mcg)

Batch No of Methylselenocysteine used: **C130529**

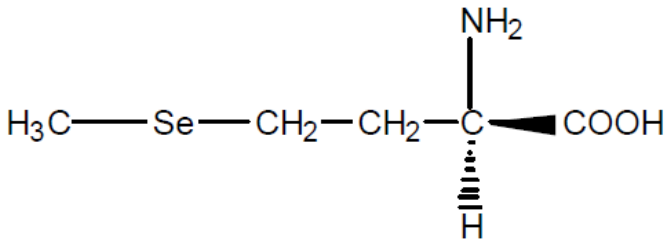
Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Methylselenocysteine	6400 mcg 15.553 mg	 41.15% of Selenium	 5%	 16.33
2	Microcrystalline Cellulose				333.67
3	Size '0' Red/Red Hard Gelatin Capsule				

2.1.5 Stability

Shelf life: 5 years

2.2 L-selenomethionine (LSM)

2.2.1 Chemical Structure



Molecular weight: 196.11 g/mol

Molecular formula: $C_5H_{11}NO_2Se$

CAS Registry Number: 3211-76-5

2.2.2 Stereochemistry

Specific optical rotation: +17° to +19.5° (1% w/v soln in 1N HCl)

Assay by HPLC: NLT 98.0%

2.2.3 Physicochemical data

Loss on drying: Not more than 1.0% w/w (dried at 105°C)

Solubility: Soluble in hot water, very slightly soluble in alcohol

pKa: Strongest acidic 1.56

Strongest basic 9.5

2.2.4 Formulation

L-Selenomethionine (containing 400 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: L-Selenomethionine = 1.004 mg

(equivalent to elemental Selenium: 400 mcg)

Batch No of L-Selenomethionine used**: G130148**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium L-Selenomethionine	400 mcg 1.004mg	 39.83% of Selenium	 5%	 1.054
2	Microcrystalline Cellulose				348.95
3	Size '0' Red/Red Hard Gelatin Capsule				

L-Selenomethionine (containing 1600 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: L-Selenomethionine = 4.017 mg

(equivalent to elemental Selenium: 1600 mcg)

Batch No of L-Selenomethionine used**: G130148**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium L-Selenomethionine	1600 mcg 4.017mg	 39.83% of Selenium	 5%	 4.218
2	Microcrystalline Cellulose				345.782
3	Size '0' Red/Red Hard Gelatin Capsule				

<u>L-Selenomethionine (containing 6400 mcg of elemental selenium) capsules</u>

Label Claim:

Each capsule contains: L-Selenomethionine = 16.07 mg

(equivalent to elemental Selenium: 6400 mcg)

Batch No of L-Selenomethionine used**: G130148**

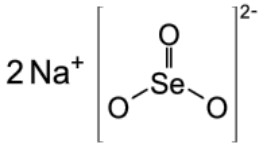
Si No	Ingredients	Label Claim	Grade/Assay	Over-ages	Input quantity (mg)
1	Selenium L-Selenomethionine	6400 mcg 16.07 mg	 39.83% of Selenium	 5%	 16.87
2	Microcrystalline Cellulose				333.13
3	Size '0' Red/Red Hard Gelatin Capsule				

2.2.4 Stability

Shelf life: 5 years

2.3 Sodium Selenite

2.3.1 Chemical Structure



Molecular weight: 172.94 g/mol

Molecular formula: Na_2O_3Se

CAS No. 10102-18-8

2.3.2 Stereochemistry

Specific optical rotation: Not Applicable

Assay by ICP-OES: 40.25 % w/w

2.3.3 Physicochemical data

Loss on drying: Not more than 1.0% w/w (dried at 105°C)

Solubility in water: 85 g/100 mL (20 °C)

pKa: Not Available

2.3.4 Formulation

Sodium Selenite (containing 400 mcg of elemental selenium) capsules

Label Claim:

Each capsule contains: Sodium selenite = 993.79 mcg

(equivalent to elemental Selenium: 400 mcg)

Lot No of Sodium Selenite used:**0000126420**

Manufacturer of Sodium Selenite: **HIMEDIA**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Sodium selenite	400 mcg 993.79mcg	 40.25% of Selenium	 5%	 1.043
2	Microcrystalline Cellulose				348.957
3	Size '0' Red/Red Hard Gelatin Capsule				

Sodium Selenite (containing 1600 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: Sodium selenite = 3.975 mg

(equivalent to elemental Selenium: 1600 mcg)

Lot No of Sodium Selenite used:**0000126420**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Sodium selenite	1600 mcg 3.975 mg	 40.25% of Selenium	 5%	 4.174
2	Microcrystalline Cellulose				345.83
3	Size '0' Red/Red Hard Gelatin Capsule				

Sodium Selenite (containing 6400 mcg of elemental Selenium) capsules

Label Claim:

Each capsule contains: Sodium selenite = 15.9 mg

(equivalent to elemental Selenium: 6400 mcg)

Lot No of Sodium Selenite used:**0000126420**

Si No	Ingredients	Label Claim	Grade/ Assay	Over-ages	Input quantity (mg)
1	Selenium Sodium selenite	6400 mcg 15.9 mg	 40.25% of Selenium	 5%	 16.695
2	Microcrystalline Cellulose				333.31
3	Size '0' Red/Red Hard Gelatin Capsule				

2.3.5 Stability

Since sodium selenite is being sourced by Sabinsa from another company, they do not have stability data for this formulation at present. However the formulation will be subjected to stability testing during the course of manufacture of the clinical trial capsules and the subsequent clinical investigation and the stability-indicating parameter will be content of selenium by ICP-OES.

3 Pharmacokinetics at Supranutritional Doses

3.1 Pharmacokinetics of Se-methyl-L-selenocysteine (MSC) in humans

A phase I, single dose, dose-escalation study was conducted administering MSC orally to fasted subjects at 3 dose levels: 400µg, 800µg and 1200µg (4). Serial blood and urine sampling was carried out along with formal toxicity evaluation using National Cancer Institute (NCI) Common Toxicity Criteria (CTC) v 3.0. Baseline characteristics are shown in Table 1.

Table 1. Comparison of placebo and selenium groups at baseline

	Study assignment (*n*)			
Characteristics	**Placebo (*n* = 3)**	**400 µg (*n* = 5)**	**800 µg (*n* = 5)**	**1,200 µg (*n* = 5)**
Age, y	39 (17)	28 (8.0)	33 (14.1)	33 (14.5)
Race (% European American)	100	80	100	100
Plasma selenium, ng/mL	134 (3.8)	136 (17.1)	127 (18.8)	114 (14.3)
Toenail selenium, µg/g	0.89 (0.122)	0.94 (0.063)	0.90 (0.050)	0.99 (0.146)
Height, m	1.89 (0.091)	1.83 (0.140)	1.80 (0.084)	1.78 (0.124)
Weight, kg	103 (12.9)	97 (14.9)	82 (15.1)	98.9 (19.1)
ECOG performance status (0–5); ref. 39	0	0	0	0

NOTE: All values are mean (SD).

There is little difference between the 400µg and 800µg plasma time curves, yet the increase C_{max} and AUC observed at the 1200µg dose suggests saturation in metabolism and/or excretion at higher doses (Figures 1 and 2). Table 2 shows the pharmacokinetic parameter estimates from the study and Figure 3 the urinary excretion profile.

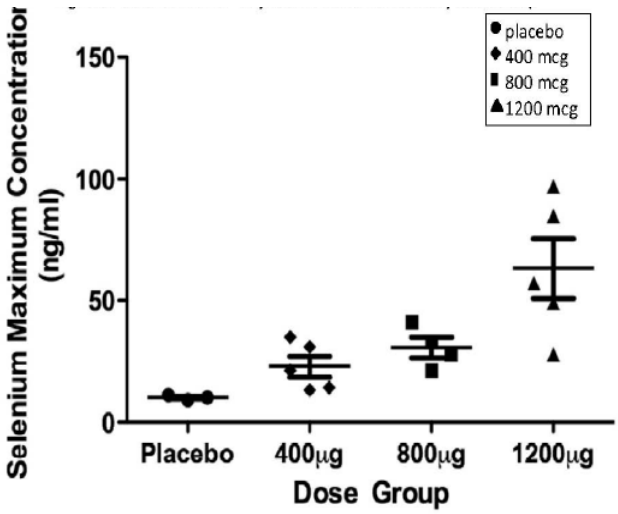


Figure 1. Mean plasma selenium concentration vs. time by dose

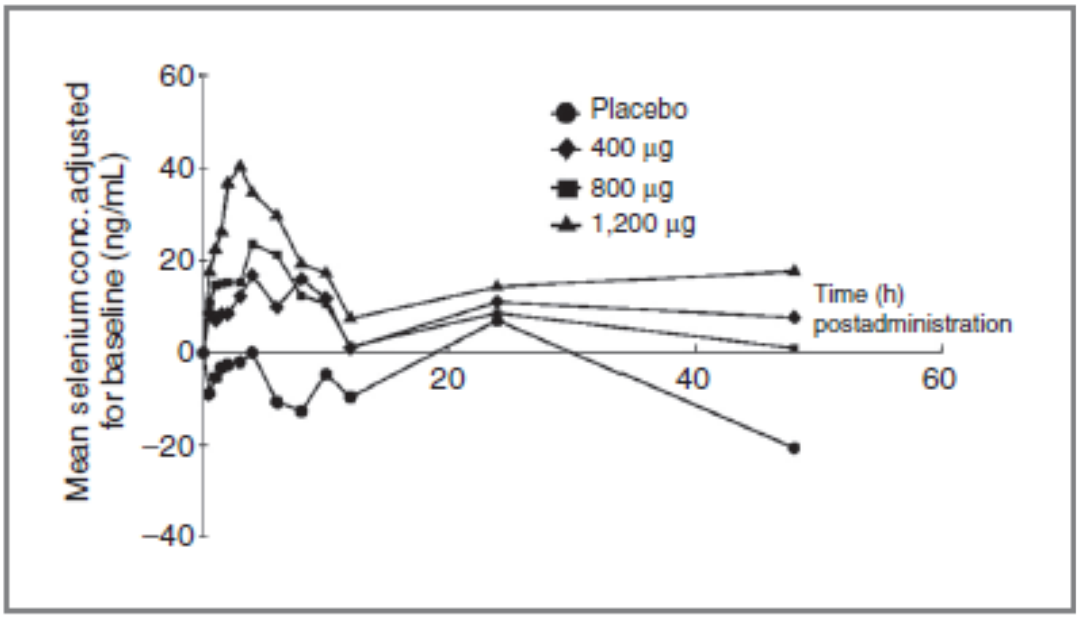


Figure 2. Selenium C_{max} adjusted for baseline levels by dose group

Table 2. Pharmacokinetic parameter estimates of MSC

	C_{max}, ng/mL	AUC, ng h/mL
Placebo		
Mean (SD)	10.0 (1.00)	<0 (*x*)
Median	10.0	<0
CV%	10.0	–
400 µg		
Mean (SD)	22.8 (9.9)	427.1 (276.5)
Median	21.0	435.5
95% CI	(−25.5 to 51.1)	(−79.6 to 934.0)
800 µg		
Mean (SD)	30.75 (8.4)	567.5 (243.0)[a]
Median	30.5	648.75
95% CI	(−19.3 to 60.8)	(37.6 to 1,100)
1,200 µg		
Mean (SD)	63.2 (27.8)[a]	1,077.9 (203.3)[a]
Median	57.0	1,055.3
95% CI	(14.9–91.5)	(571–1,580)

[a]$P < 0.05$; determined by comparison of each dose group (400, 800, and 1,200 µg) with placebo group.

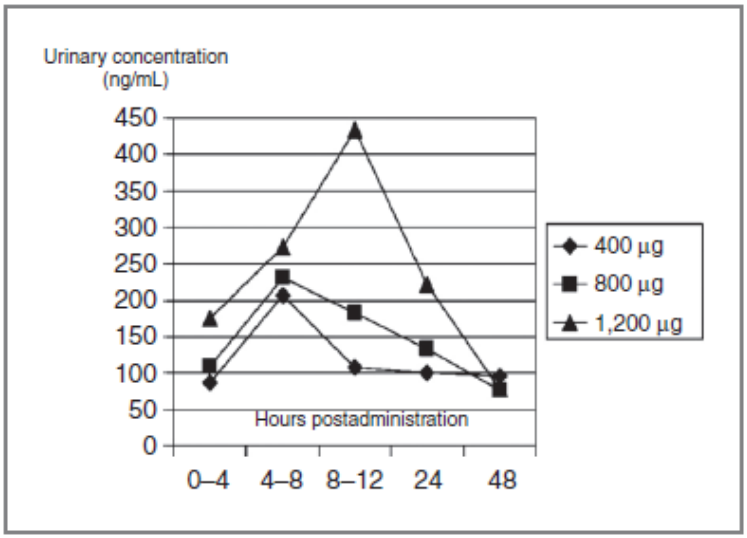


Figure 3. Mean urine selenium concentration both by selenium dose and by time post-administration

3.2 Pharmacokinetics of L-Selenomethionine (LSM) in humans

Fakih *et al* conducted a phase I study to determine the dose of LSM in combination with Irinotecan required to achieve a plasma Se concentration greater than 15µM after 1 week of loading (5). LSM was administered orally, twice a day for the first 7 days followed by a daily maintenance dose. Doses were escalated as per Table 3 and trough Se blood levels were sampled on days 1, 2, 8 and 28.

Table 3. Dosing schema from LSM (called SLM) study by Fakih *et al*

Phase I escalation schema			
Dose level	SLM loading (D-7–D-1)[a] (mcg PO BID)	SLM maintenance (D1 and on) (mcg PO QD)	Irinotecan (mg/m^2) Q week (start on D1)
1	3,200	2,800	125
2	3,200	3,200	125
3	4,000	3,200	125
4	4,000	4,000	125
5	4,800	4,800	125
6	5,600	5,600	125
7	7,200	7,200	125

Table 4 shows Se plasma levels on days 8 and 28 in patients from all dose cohorts. While there was significant inter-patient variability in Se levels at day 8, all patients from dose level 5 upwards achieved the target 15µM or more. There was no significant further

accumulation of Se observed at day 28 until dose level 7. Serum Se levels were measured every 6 weeks in patients who remained on study for more than 1 cycle (median number of cycles 2 (range1-8)), a plateau was reached at or around the levels achieved on day 28.

Table 4. Plasma selenium levels on days 8 and 28 in study patients at all dose cohorts.

Dose level (loading mcg BID/ maintenance mcg QD)	Pt no	Day 8 selenium level		Day 28 selenium level	
		ng/ml	µM	ng/ml	µM
DL1 (3,200/2,800)	1	785	9.94	866	10.96
DL1 (3,200/2,800)	2	1,647	20.85	1,598	20.23
DL1 (3,200/2,800)	3	1,199	15.18	No sample[a]	–
DL1 (3,200/2,800)	4	1,210	15.32	No sample[a]	–
DL1 (3,200/2,800)	5	900	11.39	1,237	15.66
DL1 (3,200/2,800)	6	1,225	15.51	1,216	15.39
DL2 (3,200/3,200)	7	633	8.01	721	9.13
DL2 (3,200/3,200)	8	1,110	14.05	No sample[a]	–
DL2 (3,200/3,200)	9	1,041	13.18	1,227	15.53
DL2 (3,200/3,200)	10	1,247	15.78	1,195	15.13
DL3 (4,000/3,200)	11	1,028	13.01	No sample[a]	–
DL3 (4,000/3,200)	12	1,298	16.43	No sample[a]	–
DL3 (4,000/3,200)	13	1,250	15.82	1,011	12.80
DL3 (4,000/3,200)	15	1,525	19.30	No sample[a]	–
DL4 (4,000/4,000)	17	1,563	19.78	No sample[a]	–
DL4 (4,000/4,000)	18	919	11.63	1,006	12.73
DL4 (4,000/4,000)	19	1,481	18.75	1,395	17.66
DL5 (4,800/4,800)	20	1,430	18.10	1,413	17.89
DL5 (4,800/4,800)	21	1,608	20.35	1,297	16.42
DL5 (4,800/4,800)	22	1,899	24.04	2,276	28.81
DL6 (5,600/5,600)	23	1,355	17.15	1,481	18.75
DL6 (5,600/5,600)	24	1,605	20.32	1,894	23.97
DL6 (5,600/5,600)	25	1,937	24.52	No sample[a]	–
DL6 (5,600/5,600)	26	1,245	15.76	1,528	19.34
DL7 (7,200/7,200)	27	NC[b]	–	1,662	21.04
DL7 (7,200/7,200)	28	2,089	26.44	2,608	33.01
DL7 (7,200/7,200)	29	2,280	28.86	No sample[a]	–
DL7 (7,200/7,200)	30	2,350	29.75	3,173	40.16
DL7 (7,200/7,200)	31	1,294	16.38	1,719	21.76
DL7 (7,200/7,200)	32	2,700	34.18	3,272	41.42
DL7 (7,200/7,200)	33	3,838	48.58	No sample[a]	–

[a] Day 29 sample was not collected

[b] Non-compliant

3.3 Pharmacokinetics of Selenite in humans

A phase I dose escalation of oral sodium selenate (which is metabolised predominantly to selenite) was conducted in 19 patients with prostate cancer by Corcoran et al (6). Doses escalated from 5,000µg a day to 30,000µg three times a day (t.d.s.) for up to 12 weeks Sodium selenate exhibited linear pharmacokinetics in terms of AUC vs. dose across the dose ranges studied (Table 5).

Table 5. Plasma selenate pharmacokinetics

	AUC_{0-24h} (ng h ml^{-1})	AUC_{0-168h} (ng h ml^{-1})	AUC_{0-336h} (ng h ml^{-1})	AUC_{last} (ng h ml^{-1})	$T_{1/2}$ (h)	$T_{max\ (0-last)}$ (h)	$C_{max\ (0-last)}$ (ng ml^{-1})	$C_{min\ (0-last)}$ (ng ml^{-1})
Single daily dose (mg)								
5	—	—	—	126.9	1.23	1	56	2.78
10	—	—	—	8311.9	1.61	1	112	30.6
15	—	—	—	440.8	1.38	0.7	154	40.3
30	—	—	—	666.4	2.9	2.2	166	65.3
Multi-dose								
30 mg (10 mg t.d.s.)								
n	3	3	3	3	3	3	3	3
Mean	502.39	1290.5	2049.3	2763.37	1.72	1.43	98.13	2.86
s.d.	161.92	293	426.7	972.46	0.25	0.6	56.36	0.91
CV %	32.23	22.7	20.8	35.19	14.8	42.05	57.43	31.85
45 mg (15 mg t.d.s.)								
n	2	2	2	2	2	2	2	2
Mean	549.1	2232.2	8437.1	15130.3	2.14	169.6	107.5	6.63
s.d.	17.5	92.8	6884.9	13931.5	1.34	237.1	19.2	1.99
CV %	3.2	4.2	81.6	92.1	62.6	139.8	17.8	29.99
60 mg (20 mg t.d.s.)								
n	6	6	6	6	6	6	6	6
Mean	1232.9	7201.1	11678.7	14354.7	3.09	2.6	165	11.23
s.d.	684	5892.2	9275.6	9632	1.04	1.2	72.3	5.57
CV %	55.5	81.8	79.4	67.1	32.5	46.1	43.9	49.61
90 mg (30 mg t.d.s.)								
n	4	4	3	3	4	4	4	4
Mean	1313.6	9783.1	15921.1	21521.4	2.26	13.4	218.8	18.63
s.d.	163.7	1862.6	3671.2	6843.7	0.58	23.5	90.7	8.73
CV %	12.5	19	23.1	31.8	25.7	174.7	41.5	46.87

Abbreviation: CV = coefficient of variance.

The major selenium metabolite identified in plasma was selenite. Selenite plasma concentrations showed signs of accumulation with T_{max} ranging from a mean of 90 hours for the 10,000µg t.d.s. cohort to 294 hours for the 30,000µg t.d.s. cohort. Plasma selenite was reported to reach steady state levels by 3 weeks. The ratio of selenite AUC_{504} vs. selenate AUC_{504} ranged from 1.3 x at 10,000µg t.d.s. to 7 x at the 30,000µg t.d.s. dose level. Selenomethionine, seleno-cyanate and methyl selenium species were other minor metabolites identified in plasma. For the t.d.s. dosing cohorts, 16–37% of total daily dose of selenium was recovered in the 24-h urine. Selenate was the major selenium species identified in the 24-h urine accounting for 10–24% (based on the mean for each t.d.s. dosing cohort) of total daily selenium administered. Selenomethionine and miscellaneous methyl selenium species were also major selenium compounds identified in 24-h urine; however, levels varied significantly across the patients in the t.d.s. dosing cohorts. Selenite was barely detectable in the urine, being <0.3% of total daily dose (6).

4 Toxicity at Supranutritional Doses

4.1 Toxicity of MSC in humans

Table 6 lists the adverse events (all listed as grade 1) observed in the Marshall study.

Table 6. Adverse events in a phase I MSC single dose study

	Adverse event		
MSC dose	Type	Grade[a]	Number of subjects
Placebo	Hypercholes -terolemia	1	1
	Hyperkalemia	1	1
	Elevated aspartate aminotransferase	1	1
400 µg	Anemia	1	1
	Hypernatremia	1	1
	Skin abrasion	1	1
	Blurry vision	1	1
	Hyperglycemia	1	1
	Headache	1	1
	Hyperkalemia	1	1
	Musculoskeletal pain	1	1
	Light headed (during blood draw)	1	1
800 µg	Dysgeusia	1	1
	Urinary frequency	1	1
	Hyperkalemia	1	1
	Diarrhea	1	1
	Sore throat	1	1
	Hypercholesterolemia	1	1
	Hyperglycemia	1	1
	Leukopenia	1	1
1,200 µg	Sore throat	1	1
	Bronchospasm	1	1
	Hypernatremia	1	1
	Hyperglycemia	1	1
	Headache	1	1

[a]NCI CTC version 3.0.

4.2 Toxicity of LSM in humans

In the Fakih study with LSM given in combination with Irinotecan, LSM was observed to be well-tolerated in all patients, with attributable toxicity limited to mild garlic-like odour in breath and urine in about 50% of the patients (5). This was seen more commonly during the induction LSM week and tended to ameliorate or disappear with prolonged treatment. No skin or nail toxicities secondary to LSM were documented. The non-tolerable dose of LSM was not defined in this study but the highest dose level administered (7200µg twice a day for 1 week followed by 7200µg daily) was deemed to be the MTD because 1 of 6 patients developed grade 3 infection, neutropenia, diarrhoea, and hyponatremia.

4.3 Toxicity of Selenite in humans

The toxicity of high dose oral sodium selenate (the main plasma metabolite being selenite) is listed in Table 7; two patients withdrew from the study due to unacceptable toxicity reported as grade 3 fatigue and grade 3 muscle cramps and diarrhoea, both patients in the 90,000µg per day cohort (6). The MTD was determined to be 60,000µg/day i.e. 20,000µg t.d.s.

Table 7. Toxicity experienced by ≥2 patients thought to be at least possibly related to the study drug

SOC	Preferred term	Grade 1/2 (%)	Grade 3 (%)
Gastrointestinal disorders	Constipation	4 (21)	0 (0)
	Diarrhoea	4 (21)	1 (5)
	Nausea	5 (26)	0 (0)
	Retching	2 (10)	0 (0)
	Vomiting	4 (21)	0 (0)
General disorders	Fatigue	8 (42)	1 (5)
Metabolism and nutrition	Decreased appetite	5 (26)	0 (0)
Musculoskeletal and connective tissue disorders	Arthralgia	2 (10)	0 (0)
	Muscle spasms	8 (42)	1 (5)
	Myalgia	3 (16)	0 (0)
Nervous system	Dizziness	4 (21)	0 (0)
	Headache	3 (16)	0 (0)
	Hypoaesthesia	2 (10)	0 (0)
	Lethargy	5 (26)	0 (0)
Skin	Alopecia	8 (42)	0 (0)
	Nail disorders	5 (26)	1 (5)
	Pain of skin	2 (10)	0 (0)

In a randomised controlled trial by Asfour *et al* sodium selenite was given orally to 20 patients with intermediate and high grade non-Hodgkin lymphoma at a dose of

200µg/kg/day on days 3 to 7 following their first course of CHOP chemotherapy (7). This equates to a dose of 14,000µg daily for a 70kg patient. The authors reported that 90% of patients experienced garlicky breath odour and some gastrointestinal upset (nausea and occasional vomiting that was controlled with antiemetics). Side effects deemed to be associated with high dose selenite are listed in Table 8 with no further elaboration of these in the published report.

Table 8. Sodium selenite side effects reported by Asfour *et al*.

	Acute (at initial therapy)	Delayed
General	Mild fever (40%)	None
GIT	Vomiting (85%)	Anorexia (30%)
	Loose motion (30%)	None
	Garlicky breath odor (90%)	None
Skin, nail and hair	Pruritis (20%)	Redness and blister formation 5%, brittle nail with white spots 20%, dry hair 30%
Respiratory	None	None
Cardiovascular	None	None
Nervous	None	Peripheral anesthesia 5%
Liver function	Elevated ALT (upper normal in 10% of patients)	None

5 Summary

The supranutritional doses of LSM and SS planned for use in the phase I clinical trial (400–6400µg daily) appear to be safe and well-tolerated. While safety and PK data for MSC has only been reported at doses up to 1200µg as a single dose, it is expected to be similarly well tolerated at comparable doses.

6 Reference List

(1) Aguilar F, Charrondiere UR, Dusemund B, Galtier P, Gilbert J, Gott DM et al. Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on a request from the Commission on selenious acid added for nutritional purposes to food supplements. The EFSA Journal 2009; 1009:1-17.

(2) Aguilar F, Charrondiere UR, Dusemund B, Galtier P, Gilbert J, Gott DM et al. Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on L-selenomethionine as a source of selenium added for nutritional purposes to food supplements, following a request from the European Commission. The EFSA Journal 2009; 1082:1-38.

(3) Aguilar F, Charrondiere UR, Dusemund B, Galtier P, Gilbert J, Gott DM et al. Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on Se-Methyl-L-Selenocysteine as a source of selenium added for nutritional purposes to food supplements following a request from the European Commission. The EFSA Journal 2009; 1067:1-23.

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(7) Asfour IA, El Tehewi MM, Ahmed MH, Abdel-Sattar MA, Moustafa NN, Hegab HM et al. High-dose sodium selenite can induce apoptosis of lymphoma cells in adult patients with non-Hodgkin's lymphoma. Biol Trace Elem Res 2009; 127(3):200-210.

Appendix C1

Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on Se-Methyl-L-Selenocysteine as a source of selenium added for nutritional purposes to food supplements following a request from the European Commission

Adobe Acrobat Document

Appendix C2
Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on L-selenomethionine as a source of selenium added for nutritional purposes to food supplements, following a request from the European Commission


Adobe Acrobat Document

Appendix C3
Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food on a request from the Commission on selenious acid added for nutritional purposes to food supplements

Adobe Acrobat Document

Appendix D: Published Manuscripts

ANTICANCER RESEARCH *37*: 6497-6509 (2017)
doi:10.21873/anticanres.12106

Review

Optimising Selenium for Modulation of Cancer Treatments

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[2]*Department of Oncology, Waikato Hospital, Hamilton, New Zealand*

Abstract. *Selenium is an essential trace element involved in many biological processes that are mediated through, at least, 25 selenoproteins expressed in humans. Extensive study of selenium compounds has demonstrated growth inhibition of malignant cells in a vast array of experimental models. Moreover combining selenium with conventional cancer therapy has yielded promising results in both pre-clinical studies and a cohort of human trials. The aim of this review is to highlight the current research evaluating the role of selenium compounds in combination with chemotherapy and radiation. Pharmacodymanic mechanisms responsible for the differential effects of the commonly studied compounds on healthy and malignant cells are presented and the pertinent in vitro and in vivo data summarised. The clinical utility of this approach is discussed both in terms of anti-tumour efficacy and toxicity prevention. Finally a case is made for novel trial designs to facilitate rapid progression into pivotal studies.*

Selenium (Se) is one of the most extensively studied trace elements, especially with regard to cancer. Most of this research relates to nutritional doses of Se in healthy people as primary prevention of cancer, with strong evidence that populations with low Se intakes are at higher risk of cancer (1-3); in these groups, supplementation may reduce cancer incidence and cancer-specific mortality (4). However it appears that there can be "too much of a good thing" with Se; not only does supplementing with supranutritional doses of Se in Se-replete populations not confer protection against cancer (5), laboratory and clinical studies suggest this may even increase the risk of cancer (6, 7).

Many patients extrapolate the prevention data and take Se (often at supranutritional doses) to try and control their existing cancer (8, 9). There is very little clinical research relevant to this practice but one study on prostate cancer showed no restraint of prostate-specific antigen (PSA) velocity and, of greater concern, in those with the highest baseline serum Se, supplementation with 800 μg daily of selenized yeast actually significantly increased PSA velocity (10).

While this generates concern about patients using Se as a sole treatment for cancer, especially in those who have adequate Se intakes, there is substantial preclinical research and some early clinical trials suggesting that Se compounds may significantly protect against the normal tissue toxicities of cancer therapies without compromising their anticancer efficacy (or even enhancing it) (11-13). The mechanisms that mediate this are being increasingly explored and understood and, in contrast to primary prevention, the beneficial interactions of Se with cancer treatments appear to be dose-dependent, with maximum efficacy at doses much greater than nutritional requirements (13).

While this is encouraging, considerable uncertainties slow the progress of research in this area. It is not clear what chemical form of Se is most effective, nor at what dose (which has varied from <100 to 90,000 μg/day). While the Se dose has sometimes been guided by preclinical pharmacokinetic (PK) studies, there has not been a clear evaluation of the pharmacokinetic-pharmacodynamic (PK-PD) relationship for various Se compounds, nor comparison of their differential effects on normal and malignant cells at different concentrations for each compound. Furthermore the toxicities of supranutritional doses of the different Se compounds in clinical use have not been systematically evaluated. Of particular concern is the genotoxicity of some forms of Se, particularly the inorganic salts (14), and the possibility that using these in conjunction with other

*These Authors contributed equally to this study.

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Key Words: Selenium, cancer, radiotherapy, chemotherapy, toxicity and efficacy, review.

genotoxic therapies (such as radiation and alkylating agents) could increase the risk of second malignancies and myelodysplasia. No clinical trials to date have reported on such end-points.

This review focuses on the preclinical and clinical evaluation of the interaction of Se compounds with chemotherapy and radiation, the PK-PD relationship of these compounds and differential effects and thresholds in normal and malignant cells. Ultimately we wish to use this information to inform the rational development of further clinical trials that can definitively assess whether these Se compounds can meaningfully reduce the burden of toxicities of cancer treatments for patients while maintaining or improving their efficacy, as suggested by preclinical studies.

Selenium – Role in Human Health

It is well known that Se intake varies hugely worldwide due to differences in diet and soil content of Se in different geographical locations. Although a recommended dietary intake of Se has been established, it is difficult to give a broad recommendation for a specific dose (6). 'Nutritional' doses ranging from 50-200 μg/day have been used mainly for primary prevention (15), and Se supplementation at these doses for Se-deplete individuals has been associated with lower overall mortality and incidence of certain cancer types (6). However, as alluded to earlier, it is well known that taking Se at supranutritional doses over a long duration can cause adverse effects, a reminder that Se is also recognised as a toxic element (6).

The role of Se in maintaining health is complex and it has many important biological functions including in redox signalling, thyroid metabolism, immune function, detoxification and antioxidant activity (6). These effects are primarily mediated by over 25 specific encoded selenoproteins, in which selenocysteine replaces cysteine. Among the more widely studied selenoproteins are the glutathione peroxidases (involved in antioxidant activity and sperm motility) and thioredoxin reductases (involved in intracellular redox regulation and signalling). The pronounced immunostimulant effects of Se can cause a wide array of effects, from increased proliferation of activated T-cells and natural killer cell activity to enhanced lymphocyte-mediated tumour cytotoxicity (6).

Selenium Compounds – Metabolism, Pharmacology and Toxicity

Se exists in different chemical forms and these compounds can be broadly categorized into organic and inorganic forms. The metabolic pathway of dietary Se is shown in Figure 1.

There is good evidence that the increased Se status attained after supplementation with organic forms of Se such as Se-methyl-selenocysteine (MSC) and L-selenomethionine (SLM), is maintained for a longer period after its discontinuation compared to inorganic forms such as sodium selenate reported whole-body half-lives of SLM and sodium selenate in humans were 252 and 102 days respectively (16). Accordingly, foods or supplements containing SLM can maintain the activities of selenoenzymes during periods of Se depletion for longer than those containing inorganic Se, owing to the recycling of SLM catabolised from protein stores.

Animal laboratory studies have shown that the organic forms of Se are both more effective and safer than the commonly-used inorganic forms such as sodium selenite (SS), which are more genotoxic (14). From 50% lethal dose (LD_{50}) determinations, SS was found to be fourfold more toxic than SLM when administered to mice intravenously and three-fold more toxic than Se-yeast when given orally to rats (17).

In humans safety data is more limited. The No Observed Adverse Effect Level (NOAEL) for Se in humans is variably reported as 400-850 μg/day (18,19). Chronic Se toxicity would be expected after long-term consumption (over months to years) of more than 2,400-3,000 μg/day but is reversible (20). Based on the published literature, likely symptoms of toxicity (due to environmental exposure) include brittle hair and nails and their loss, gastrointestinal disturbances, skin rash, garlic breath odour (caused by volatile selenium compounds) fatigue, irritability, and nervous system abnormalities. Impaired natural killer cell activity and endocrine disturbances may also occur (20).

Three major forms of Se (MSC, SLM and SS) are widely used in supplementation in both preclinical and clinical studies. The organic Se compound MSC is a water-soluble amino acid that is absorbed in mammals from the gastrointestinal tract and is well-tolerated (21). It is readily converted to the active moiety methylselenol through one-stage β-lyase conversion and remains more bioavailable for anticancer effects compared with the other forms (22). Methylselenol can be demethylated to yield selenide or methylated to yield dimethyl selenide, then released in the breath; methylated again, it yields trimethyl selenonium, which is excreted in urine (23). A phase 1, single-dose, dose-escalation study in 15 human subjects evaluated the toxicity and PK profile of MSC, administering 400, 800 and 1,200 μg of MSC orally and measuring Se plasma and urine levels. There was no significant clinical or laboratory toxicity and little difference was seen in PK parameters at the 400 and 800 μg dose levels (23).

The organic Se compound SLM is well-tolerated in humans at a dose of 7,200 μg (or comparable dosing at 3,600 $\mu g/m^2$) twice daily for 7 days followed by a once-daily maintenance dose for 10 weeks (24, 25). The only toxicity attribute to SLM was garlic-like odour in breath and urine that was seen more commonly during the induction SLM week and was found to disappear with prolonged treatment. All patients given 4,800 μg

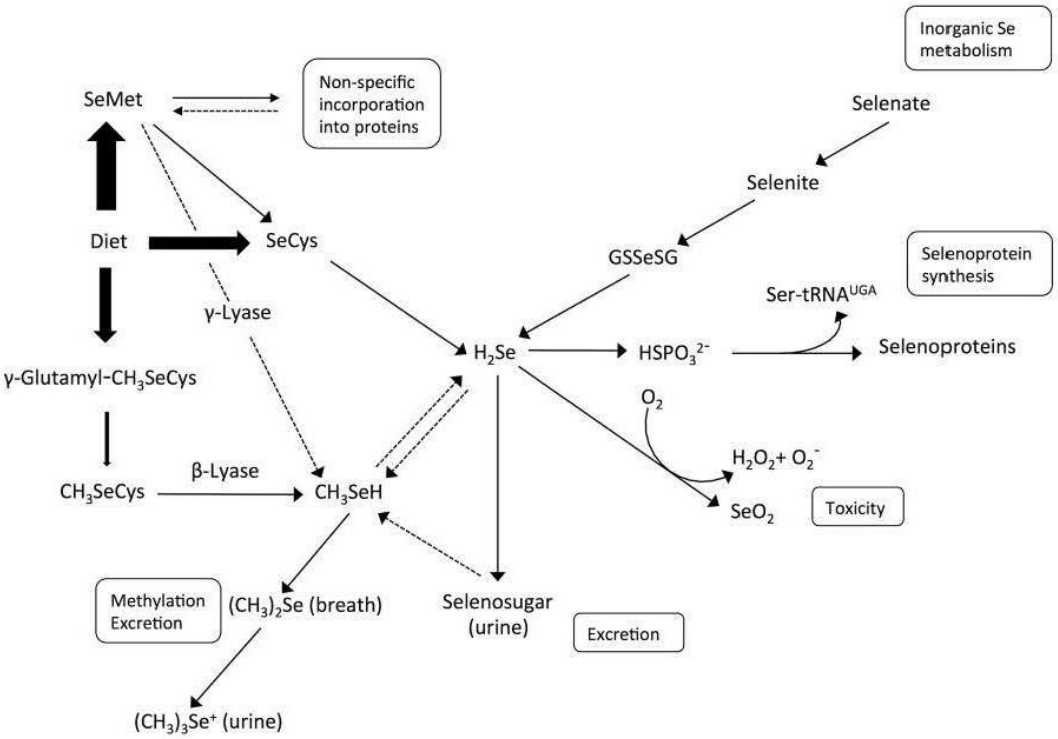

Figure 1. *Metabolic pathway of dietary Se in humans, from Rayman et al. (93). SeMet, Selenomethionine; SeCys, selenocysteine; GSSeSG, selenodiglutathione; γ-glutamyl-CH_3SeCys, γ-glutamyl-Se-methyl-selenocysteine; H_2Se, hydrogen selenide; $HSePO_3$, selenophosphate; CH_3SeCys, Se-methylselenocysteine;CH_3SeH, methylselenol; $(CH_3)2Se$, dimethyl selenide; SeO_2, selenium dioxide; $(CH_3)3Se$, trimethylselenonium ion.*

SLM twice daily achieved plasma Se levels >15 μM, the Se concentration required for reduced chemotherapy-induced toxicity and enhanced antitumor efficacy of chemotherapeutic drugs in preclinical animal models (13). The plasma Se dose levels exceeded 30 μM by day 28, thereby confirming the feasibility of using high-dose SLM in the clinic in this setting. In another study, selenized yeast (which predominantly contains SLM) was given in doses of 3,200 μg daily for up to 24 months in 16 patients with prostate cancer on 'watchful waiting', with five patients reporting minor toxicities, including garlic breath, brittle nails or hair, stomach upset or dizziness, without any correlation with plasma Se levels (18). None of these patients reported symptoms of peripheral neuropathy.

The inorganic compound sodium selenate (which is metabolised to selenite) was evaluated in a recent phase I trial, which established a maximum tolerated dose (MTD) of 60,000 μg daily orally in patients with metastatic castration-resistant prostate cancer (26). Dose-limiting toxicities of fatigue and diarrhoea were seen at 90,000 μg, with adverse events including nail disorders, muscle spasms, alopecia and nausea. Some of these were attributed to the accumulation of selenite, the more potent metabolite of selenate. In the same trial, slowing of PSA velocity was also observed, and one patient had >50% reduction in serum PSA. Another clinical trial adding SS 200 μg/kg/day (equivalent to 14,000 μg/day in a 70-kg adult) to chemotherapy in lymphoma patients reported less toxicity with combination therapy than with chemotherapy alone, including infections and reduction in cardiac function (27).

Pharmacodynamics of Selenium Compounds in Normal and Malignant Cells

Se affects varied cellular processes and molecular pathways that may be involved in the anti-cancer effect of Se [reviewed in (3)] and include:

- Seleno-enzymes involving the reduction of DNA damage, oxidative stress and inflammation;
- Induction of phase II conjugating enzymes involving detoxifying carcinogens and reducing DNA adduct formation;
- Enhancement of immune response including cytotoxic lymphocyte and natural killer cell activity;

• Increase in tumour-suppressor protein p53, which inhibits proliferation, stimulates DNA repair and promoting apoptotic death by acting as a transcription factor for several genes, including the growth arrest and DNA damage-inducible (GADD) genes;
• Inactivation of protein kinase C, a signalling receptor that plays a crucial role in tumour promotion by oxidants;
• Alteration in DNA methylation, as abnormal methylation patterns are associated with neoplasia and inactivation of tumour-suppressor genes;
• Perturbation of the cell cycle, resulting in growth inhibition and may allow DNA repair to take place;
• Induction of apoptosis of cancer cells, which generally involves the sequential activation of the caspases;
• Inhibition of angiogenesis required for the growth and metastasis of tumours.

Reassuringly, there is a significant body of published work demonstrating differential effects of Se compounds in normal *versus* malignant cells or tissues that would be favourable clinically, as summarised in Table I. However, while there is clear evidence from preclinical work that the efficacy and toxicity of Se compounds varies greatly (11,14), European clinical trials mostly use SS and those in the US use SLM, with no direct comparison of Se compounds or their PK-PD dose relationship.

Selenium with Chemotherapy and Radiotherapy

The challenge in using Se compounds in cancer patients in conjunction with chemotherapy and/or radiotherapy lies in being able to reduce normal tissue toxicities of these treatments without compromising their antitumour effects (or preferably enhancing those). However, despite numerous studies on the PD of Se in normal and malignant cells, it is still not known which form and dose of Se can be safely used and has the most favourable differential effect in normal and malignant tissues, especially in conjunction with chemotherapy and radiation. Current doses of Se are empirical or guided by PK, although the PK-PD relationship has not been established in humans, hence the optimal form and dose of Se to be used with chemotherapy or radiotherapy remains unclear. However Joel *et al.* have demonstrated that PD biomarkers of Se effects can be measured in human white blood cells (WBC) *in vitro* and *in vivo* (28), thus enabling the relationship between Se PD and PK to be evaluated in clinical studies and determine the optimal Se compound and dose to be incorporated into potentially pivotal trials.

Antitumour Efficacy

While Se is selectively cytotoxic to cancer cells at higher doses (summarised in Table I), it commonly augments the anticancer efficacy of chemotherapy and radiation in cell culture and tumour xenograft models (summarised in Table II). Therapeutic synergy has been demonstrated between supranutritional doses of Se compounds and chemotherapy drugs, including cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin in human tumour xenografts of colorectal, ovarian, prostate and small cell and non-small cell lung carcinoma, head and neck squamous cell carcinoma and leukaemia (11,13, 29-32).

However the strength of the interaction varied greatly, with differing xenograft models showing up to a 20-fold difference in Se dose potency) (11). Furthermore, the xenograft model and Se dose also influenced the apparent efficacy of the Se compounds. For example, at their MTD of 200 μg/day orally (approximately 8,000 μg/kg/day), MSC and SLM were both superior to SS in combination with irinotecan in head and neck squamous cell carcinoma xenografts (Figure 2) (11) whereas SS and SLM (1,000-1,500 μg/kg/day *i.p.*) were equally dose-potent and effective when combined with cisplatin in an ovarian cancer xenograft model (32).

Attention is also drawn to the schedule-dependency of Se in combination with other cancer treatments: the effects of MSC or SLM were maximal after 7 days pre-treatment, with little or no benefit seen when co-administered without pre-incubation with Se in human colorectal and head and neck squamous cell carcinoma xenograft models in mice (11, 33).

Few clinical studies have been conducted that evaluated the impact of Se supplementation during chemotherapy or radiation on treatment efficacy. Muecke *et al.* randomised 81 Se-deficient women to oral SS (500 μg daily with radiotherapy and 300 μg on non-radiotherapy days) or radiotherapy alone following surgery for gynaecological malignancies (34). At a median follow-up of 67 months, disease-free survival was not significantly different (log-rank p=0.65), though there was there a trend for improved overall survival in the selenium-treated group (log-rank p=0.09) (35).

Mix *et al.* conducted a randomised, placebo-controlled phase 2 pilot study in 18 patients with locally-advanced head and neck squamous cell carcinoma receiving cisplatin concurrently with radiation, administering placebo or 3,600 μg/m^2 SLM twice daily orally for 7 days prior to treatment then once daily until 3 weeks after chemoradiation completion. In this small study, there was no difference observed in disease-free or overall survival (log-rank p=0.39 and p=0.39 respectively) (24).

Asfour *et al.* reported a trial randomising 50 patients with non-Hodgkin lymphoma to either very high doses of SS (200 μg/kg/day) with cyclophosphamide, vincristine, doxorubicin and prednisone chemotherapy or chemotherapy alone (27). A significantly greater tumour response rate was observed in the Se group than controls (60% *vs.* 40% respectively), with correspondingly lower levels of the oncogene B-cell lymphoma 2 (BCL2) in bone marrow aspirates in Se-treated patients compared to controls.

Table I. *Pharmacodynamic mechanisms of selenium supplementation in normal and malignant cells.*

Pharmacodynamic properties	Effects in normal cells (Ref)	Effects in malignant cells (Ref)	Therapeutic significance
Antioxidant effects			
Glutathione peroxidase (GPx)	Se-dependent GPx isoforms 1, 2, 3, 4 and 6 reduce oxidative damage; activity saturates with nutritional doses of Se (6)	Increased GPx1 activity in response to low dose Se (as SS and SLM) in LNCaP cells, resulting in reduced oxidative damage (49)	Mixed
Intracellular glutathione (GSH)	Increased GSH in PBMCs in response to MSA (50)	MSA reduced total GSH in leukaemia cells (50)	Favourable
Thioredoxin reductase (TrxR)	Essential selenoprotein (51), increased TrxR activity in red blood cells with selenized yeast (52)	At low dose SS and SLM increased TrxR activity and apoptosis in LNCaP cells (prostate adenocarcinoma) (49) as did SS in NSCLC cells (53), more so in doxorubicin-resistant compared to doxorubicin-sensitive NSCLC cells (54) Higher concentrations of SS reduced TrxR activity in mesothelioma (55), colorectal cancer (56) and NSCLC (53). Differential effect with increase in TrxR activity and protein in doxorubicin-sensitive NSCLC but the opposite in doxorubicin-resistant NSCLC cells (54)	Mixed
Cell death/cytotoxicity	Minimal effects on normal cells (50, 51, 55)	Marked toxicity and cell death in varied malignant cell lines with dose-dependent and Se species-specific mechanisms of cell death or cell cycle arrest [reviewed in (57)] *e.g.* comparison of MSC, SS and SLM across multiple cancer cell lines showed: i) SLM-induced apoptosis is p53-dependent; ii) MSC instead induced p53-independent caspase activation; iii) ER stress-related signaling was associated with both SS and MSC (58) MSA-induced ER stress provoked a survival response at lower doses and apoptosis at higher doses in prostate cancer cell lines (59)	Favourable
DNA repair	Enhanced DNA repair with SLM in p53 wild-type mouse and human fibroblasts (60, 61)	Increase in base excision repair in colorectal cancer lines by SLM is p53 wild type-dependent (62) Unaltered DNA repair in SLM-treated p53-null human squamous cell carcinoma cell lines (63) Low dose SS or SLM increased DNA repair in prostate cancer cell lines (49)	Mixed
Angiogenesis	In human umbilical vein endothelial cells MSA induced apoptosis *via* p38 MAPK activation whereas SS induced caspase-independent apoptosis (64) MSA inhibited VEGF expression in mammary endothelial cells (65)	MSC and MSA reduced HIF-1α expression and thus angiogenesis in HNSCC and renal cell carcinoma models through inhibition of VEGF signaling mediated by COX-2, iNOS and degradation of prolyl hydroxylases under hypoxia (66-69). This resulted in improved intratumoral vessel maturation, interstitial fluid pressure and reduced hypoxia (70)	Favourable Favourable
Invasion/migration		Reduced cell invasion through inhibiting MMP-2, MMP-9 and UPA by MSA (71) and SS (72)	
Epigenetic		Inhibits DNMT and HDAC resulting in expression of silenced genes by SS (73) and MSA (74)	Favourable
Immunological	1 mg/kg SS for 8 weeks increased antigen-specific $CD4^+$ T-cell responses (75)		Favourable
Drug efflux		Significant reduction in levels of the ABCC1 efflux pump in tumour cells (76).	Favourable

ABCC1: ATP binding cassette C1; COX-2: cyclooxygenase 2; DNMT: DNA methyltransferase; ER: endoplasmic reticulum; GPx: glutathione peroxidase; GSH: glutathione; HDAC: histone deacetylase; HDSCC head and neck squamous cell carcinoma; HIF-1α: hypoxia-inducible factor 1 alpha; iNOS: inducible nitric oxide synthase; MAPK: mitogen activated protein kinase; MMP: matrix metalloproteases; MSA: methylseleninic acid; MSC: Se-methylselenocysteine; NSCLC: non-small cell lung cancer; p53: tumour protein p53; PBMCs: peripheral blood mononuclear cells; Se: selenium; SLM: seleno-L-methionine; SS: sodium selenite; TrxR: thioredoxin reductase; UPA: urokinase plasminogen activator; VEGF: vascular endothelial growth factor.

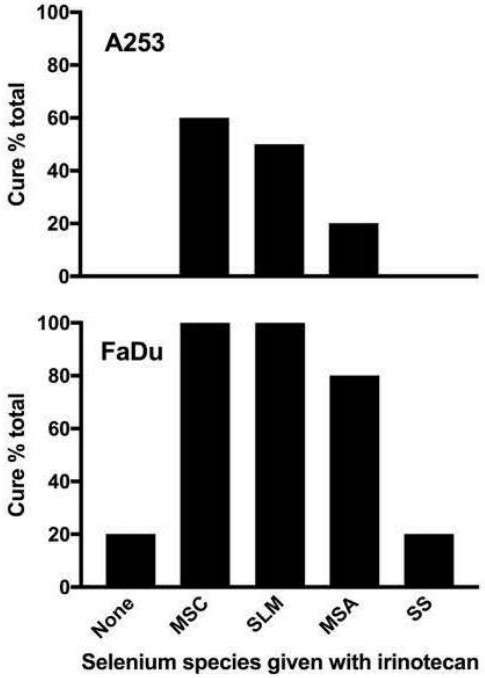

Figure 2. *Tumour cure rates in nude mice with head and neck squamous cell carcinoma xenografts (5 mice per group). Se compounds differ in their ability to improve antitumour activity of irinotecan in xenografts. MSC: Se-Methyl-selenocysteine; SLM: seleno-methionine; MSA: methyl-seleninic acid; SS: sodium selenite. (group means reported) Courtesy of Dr Y Rustum.*

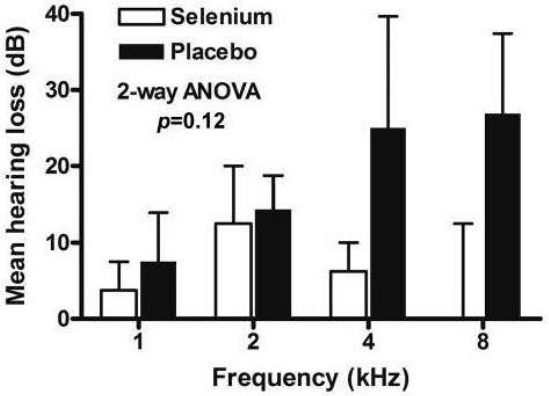

Figure 3. *Hearing loss following chemoradiation in selenium-, and placebo-treated groups. (n=6, 2 Selenium treated, 4 placebo, mean±SEM) Courtesy of M. Jameson.*

Reduction in Toxicity

Table II summarises extensive preclinical work that demonstrated the protective effects of various Se compounds against the toxicities of radiation (12, 36) and organ-specific toxicities of many cytotoxic drugs, including myelo-suppression, mucositis, diarrhoea, alopecia, cystitis and nephrotoxicity (11, 13, 30, 37). Specific toxicities of cisplatin in kidneys, bone marrow and intestine were improved without affecting antitumour efficacy (30, 38-41) or pharmacokinetics (42). Impressive protection from lethal effects of six cytotoxic drugs with 200 μg/day MSC was demonstrated in nude mice (13).

Similar protection by Se compounds from the normal tissue toxicities of chemotherapy has been reported in several clinical trials (Table III). In the setting of high-dose chemotherapy and allogeneic haematopoietic stem cell transplantation, a double-blind randomised placebo-controlled study of oral selenized yeast in 77 patients reported a reduction in the incidence and duration of severe oral mucositis (43). In a trial randomising 41 patients to 4,000 μg Se (as seleno-kappacarrageenan) orally daily for 4 days before and after the first dose of cisplatin-based chemotherapy or to chemotherapy alone, significantly less myelosuppression and nephrotoxicity was seen with Se (44). A double-blind trial involving 62 women receiving cisplatin-based chemotherapy for ovarian cancer randomized patients to Se as selenized yeast, 200 μg per day for 3 months, starting concurrently with chemotherapy (45). Those patients randomized to Se experienced significantly less toxicity at 3 months of treatment, including gastrointestinal, alopecia, weakness and neutropenia. The previously referenced four studies using high-dose SS in patients with non-Hodgkin lymphoma reported the Se group had significantly fewer toxicities (including infection and fall in cardiac left ventricular ejection fraction) and reduced apoptosis of neutrophils (46).

Data from the aforementioned study conducted by Muecke *et al.* reported significantly increased blood Se with SS supplementation (the primary endpoint) but analysis of secondary endpoints showed a significant reduction in the actuarial incidence of grade 2 or more diarrhoea (from 46.6% to 21.0%, p=0.039), without any Se-related side-effects (12). SS has also been assessed as a potential modulator of radiation-related toxicities in 39 head and neck cancer patients in a randomised controlled trial (36). Using the same dosing schedule of SS as the study of Muecke *et al.* (12), there was no statistically significant difference in the incidence of severe toxicities.

More recently SLM has been trialled alongside cisplatin-based chemoradiation for both head and neck squamous cell cancer and non-small cell lung cancer (NSCLC), with

Table II. *Combination of selenium with systemic therapies and radiation.*

Pharmacodynamic properties	Effects in normal cells/tissues (Ref)	Effects in malignant cells/tissues (Ref)	Therapeutic significance
Antioxidant effects - Intracellular glutathione (GSH)	Protective increase in GSH in PBMCs in response to MSA, not compromised by combination with cisplatin, doxorubicin, Ara-C or radiation 2 Gy (50)	Pre-treatment of ovarian cancer xenograft-bearing mice with SLM or SS prevented an increase in intracellular GSH in response to cisplatin or melphalan (77) MSA depletes intracellular GSH in THP-1 leukaemia cells despite combination with cisplatin, doxorubicin, Ara-C or radiation 2Gy (50)	Favourable
Cell death/cytotoxicity	Protects against cisplatin-related renal proximal tubule injury (78) Clinical trial evidence of less gastrointestinal, bone marrow, renal and cardiac toxicity with chemotherapy (43-45, 79) and reduced diarrhoea after radiation (12, 36, 80)	Increased cytotoxicity in combination with chemotherapy, tamoxifen or radiation in several cell lines, including resistant cells *in vitro* (13, 81-85) Therapeutic synergy between supra-nutritional doses of Se (as SLM, SS, MSA and MSC) and cisplatin, carboplatin, oxaliplatin, irinotecan, docetaxel, fluorouracil and doxorubicin in human tumour xenografts of small cell and non-small cell lung carcinoma, colorectal carcinoma, prostate carcinoma, head and neck squamous cell carcinoma and leukaemia (11, 13, 29-32) Clinical trials show either no compromised outcome or improved outcome (12, 27, 45, 46)	Favourable
Angiogenesis	MSC did not cause an increase in chemotherapy drug delivery to normal tissues (86)	MSC resulted in improved tumor blood vessel maturation and hypoxia with improved chemotherapy drug delivery (22, 86)	Favourable
Radiosensitization	No radiosensitization in normal lung fibroblasts by SLM (87). MSA did not increase PBMC death from RT (50).	SLM caused radiosensitization in two human lung cancer cell lines (87).	Favourable

Ara-C: cytosine arabinoside; Gy: gray; MSA: Methylseleninic acid; MSC: Se-methylselenocysteine; PBMCs: peripheral blood mononuclear cells; RT: radiotherapy Se: selenium; SLM: seleno-L-methionine; SS: sodium selenite.

treatment toxicity as the primary outcome (24, 47). In the head and neck trial 18 patients were randomised to pre-treatment with 3,600 μg/m^2 SLM twice daily or placebo before radiotherapy and then daily thereafter. While no significant difference in grade 3 toxicities was observed, 50% of Se-treated patients had grade 0-1 mucositis compared to 25% in the placebo group (24). In addition, a trend for reduced high-frequency hearing loss in Se-treated patients was seen (Figure 3; unpublished data, M. Jameson). The single-arm NSCLC study in 16 patients, administering 4,800 μg/m^2 SLM twice daily for 7 days pre-radiotherapy then daily during chemoradiation, reported no Se-related toxicity but a lower than expected rate of grade III or higher toxicities was observed, particularly for pneumonitis and anaemia (47).

Conclusion

While there have been major advances in cancer therapy in recent years, especially with the development of targeted-therapies, cytotoxic chemotherapy and radiation still remain a mainstay of treatment for many malignancies. The considerable toxicity of these treatments remains a major challenge in cancer management, one that has not been overcome despite extensive research into protective strategies (48).

In this context, Se appears to be unique in terms of its ability to protect against carcinogenesis as well as selectively target existing cancer cells and synergise with other cancer therapies, while protecting normal tissues from the cytotoxic effects of those treatments. These distinctive features enable, at least in preclinical models, administration of higher doses of cytotoxic agents than would be feasible otherwise, with improved cancer outcomes. If this widening of the notoriously narrow therapeutic index of cytotoxic treatments with Se is replicated in the clinical setting, this would represent a very significant advance in cancer management, and further clinical evaluation is clearly justified.

However, before large-scale clinical trials are undertaken to evaluate the ability of Se to modulate the efficacy and toxicity of anticancer therapies, more research is needed to determine which Se compound, and at what dose, can be

Table III. *Clinical trials evaluating outcomes when selenium compounds were given with chemotherapy and/or radiotherapy.*

Author & year of publication	N	Trial design	Cancer treatment	Cancer type	Selenium form & dose	Outcomes - toxicity/efficacy
Hu *et al.* 1997 (44)	41	Randomised to Se with cycle 1 or 2 (not both)	Cisplatin-based chemotherapy (60-80 mg/m^2)	Lung, breast, gastric, colon, oesophagus, liver	Oral kappa-seleno-carrageenan (4000 μg Se/day for 4 days before and after chemotherapy)	• Significantly higher day 14 WBC 3.35±2.01 *vs.* 2.31±1.38×10^9/l (p<0.05) • Less G-CSF and RBC transfusion • less nephrotoxicity
Sieja *et al.* 2004 (45)	62	Randomised to Se or not	Cisplatin (100 mg/m^2) and cyclophosphamide (600 mg/m^2)	Ovarian	Oral selenized yeast 200 μg/day for 3 months	• Significantly less nausea (p<0.001), vomiting (p<0.001), stomatitis (p<0.0292), abdominal pain (p<0.0006), anorexia, weakness (p<0.001), alopecia (p<0.001) • Significantly higher neutrophils • Trend to lower serum CA-125 (93.5±200 *vs.* 228±713 U/ml)
Weijl *et al.* 2004 (88)	48	Randomised to vitamins C, E and Se or placebo	Cisplatin dose intensity (60-100 mg/m^2)	Testicular, bone, gastrointestinal, urogenital, head and neck, melanoma	Oral sodium selenite 100 μg daily	• No improvement in toxicity or response rate • 64% Of supplement group non-compliant due to GI effects • Serum Se did not increase
Gounder *et al.* 2005 (89)	11	Phase I dose escalation	Paclitaxel (175 mg/m^2) and Carboplatin AUC 5 cycle 1 then AUC6	Gynaecologic malignancies	IV sodium selenite 50 μg, 100 μg or 200 μg over 5 hours two days prior to chemotherapy	• Selenite can be safely administered with this chemotherapy • No significant alteration of predicted carboplatin AUC
Asfour *et al.* 2006 (79)	30	Randomised to Se or not	Cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2, vincristine 1.4 mg/m^2 (all day 1) and prednisone 100 mg daily x 5, (CHOP) every 4 weeks	Non-Hodgkin lymphoma	Oral sodium selenite 200 μg/kg/day	• Significantly less apoptosis of neutrophils (67% *vs.* 20%, p<0.05) and reduced infection rate • Significantly better preservation of cardiac ejection fraction (62+/-4% *vs.* 69±5% p<0.05).
Asfour *et al.* 2007 (27)	50	Randomised to Se or not	CHOP (as for Asfour 2006)	Non-Hodgkin lymphoma	Oral sodium selenite 200 μg/kg/day for first 30 days	• Significant decline of *Bcl-2* level in BM aspirate and increase in CD4/CD8 ratio in peripheral blood after 3 cycles (8.6±6.9 ng/ml *vs.* 3 6.9±7.9 ng/ml, p<0.05) • Increased complete response rate in Se group (60% *vs.* 40%)
Fakih *et al.* 2006 (90)	13	Phase I dose escalation study	Irinotecan 125-160 mg/m^2 weekly	Colorectal, lung, pancreatic, gastric, mesothelioma	Oral seleno-methionine 2200 μg Se daily starting 1 week before chemotherapy	• Irinotecan MTD 125 mg/m^2 • No diarrhoea > grade 2 at MTD • Responses seen in irinotecan-refractory population
Fakih *et al.* 2008 (25)	31	Phase I and PK study	Irinotecan 125 mg/m^2 weekly x 4 every 6 weeks	Metastatic or unresectable solid tumour	Oral seleno-methionine; dose escalation from 2800-7200 μg/day maintenance (twice daily loading for 1 week prior to starting irinotecan)	• Prolonged stable disease >6 months in irinotecan-pre-treated patients • SLM well-tolerated at 7200 μg twice daily for 1 week followed by 7,200 μg daily with irinotecan • No reduction in irinotecan toxicity
Asfour *et al.* 2009 (46)	40	Randomised to Se or not	CHOP (as for Asfour 2006)	Non-Hodgkin's lymphoma	Oral sodium selenite 200 μg/kg/day for 5 days	• Significantly greater increase in apoptotic marrow cells on day 8 of cycle 1 in Se group (49.2% *vs.* 29.7%) • Significant increase in tumour reduction • Preservation of cardiac ejection fraction in Se group but not controls (p=0.04)

Table III. *Continued*

Table III. *Continued*

Author & year of publication	N	Trial design	Cancer treatment	Cancer type	Selenium form & dose	Outcomes - toxicity/efficacy
Muecke *et al*. 2010. 2014 (12, 35)	81	Randomized to Se or not in patients with low blood Se (<84 ng/ml)	Radiotherapy (RT) (external beam w/wo brachy-therapy)	Endometrium or cervix	Oral sodium selenite 500 μg on days of RT and 300 μg on days without treatment	• Significant increase in blood Se • Less ≥grade 2 diarrhoea (20.5% *vs*. 44.5%, p=0.04) • 5-Year survival 91.9% *vs*. 83.1% (p=NS) • 10-Year survival 55.3% *vs*. 42.1% (p=NS) • No Se side-effects observed
Buntzel *et al*. 2010 (2 papers with overlapping cohorts) (36, 80)	47 or 39	Randomised to Se or not in patients with low blood Se (not defined)	RT (37/39 adjuvant)	Head and neck squamous carcinoma	Oral sodium selenite 500 μg on days of RT and 300 μg on days without treatment.	• Significantly less dysphagia in the last week of RT • Trend to less acute taste loss • No difference in xerostomia or mucositis • Significant transient increase in serum and whole blood Se
Corcoran *et al*. 2010 (26)	19	Phase I dose escalation	Antiandrogens discontinued >4 weeks before trial; LHRH agonists were continued	Chemotherapy-naïve, castration-resistant prostate cancer	Oral sodium selenate; dose escalation from 5,000-90.000 μg/day for 12 weeks (longer if responding)	• MTD was 60,000 μg/day with linear PK • fatigue, diarrhoea and muscle spasms dose-limiting at 90.000 μg/day • One PSA response >50% and mean PSA doubling time increased (2.18 months before trial to 3.85 months)
Jahangard-Rafsanjani *et al*. (43)	77	Randomised to Se or placebo	High-dose busulfan + cyclophosphamide then allogeneic haematopoietic stem cell transplantation	Acute leukaemia	Oral selenized yeast (200 μg) *vs*. placebo twice daily from day 0 to 14 days after transplant	• Significant reduction in incidence of grades 3-4 oral mucositis (OM) (10.8% *vs*. 35.1%) • Significantly shorter • Significantly shorter duration of grades 2-4 OM (3.6±1.8 days *vs*. 5.2±2.2 days)
Vieira *et al*. 2015 (91)	39	Randomised, placebo-controlled crossover; both placebo periods were grouped for analysis (half had Se first)	Various chemotherapy regimens (timing of Se supplementation not specified)	Paediatric leukaemia or lymphoma (LL) or various solid tumours (ST)	Selenium-glycine age-dependent dosing (27-100 μg/day) for 30 days, 7 days washout then alternate medication for 30 days. Sustained dosing for 12 months in 16 patients.	• No significant change in EORTC QLQ-C30 scores for fatigue, nausea, appetite loss, physical function in LL group • Significant improvement in scores for nausea and appetite loss in ST group with both Se and placebo, for fatigue with placebo and for fatigue at 1 year with Se • Significant decrease in serum AST levels with Se (p=0.045) • General trend to improvement in scores
Mix *et al*. 2015 (24)	18	Randomised, placebo-controlled Study of SLM.	Concurrent chemoradiation (CCRT). RT 70 Gy over 7 weeks; cisplatin 100 mg/m^2 on days 1, 22 and 43 of RT	Stage III-IV head and neck squamous cell cancer	SLM at 3600 μg/m^2 twice daily pre-treatment for 7 days and daily until 3 weeks after chemoradiation completion or placebo.	• No significant change in mucositis or patient reported side effects • No difference in OS or RFS at 12 months
Mix *et al*. 2015 (47)	16	Single arm phase II study of SLM	CCRT with RT 60-66 Gy and weekly paclitaxel 50 mg/m^2 and carboplatin AUC 2 over 6 weeks	Stage III NSCLC	SLM at 4800 μg/m^2 twice daily pre-treatment for 7 days and daily for 6 weeks or completion of chemoradiation.	• No Se-related toxicity observed • Lower than expected rates of grade III or higher toxicities observed particularly for pneumonitis and anaemia

Table III. *Continued*

Table III. *Continued*

Author & year of publication	N	Trial design	Cancer treatment	Cancer type	Selenium form & dose	Outcomes - toxicity/efficacy
Brodin *et al.* 2015 (92)	34	Phase I dose escalation study	Chemotherapy given after intravenous SS (retreated with first-line chemotherapy)	Cancer patients refractory to cytostatic drugs	Intravenous SS given on 5 of 7 days for either 2 or 4 weeks; dose escalation to MTD	• Protocol defined MTD of 10.2 mg/m^2 • DLTs reported to be acute, of short duration and reversible, namely fatigue, nausea, finger and leg cramps

AUC: Area under the curve; BM: bone marrow; DLT: dose-limiting toxicity; AST: aspartate aminotransferase; EORTC-QLQ-C30 The European Organisation for Research and Treatment of Cancer quality of life questionnaire C30; CCRT: Concurrent chemoradiation G-CSF: granulocyte colony-stimulating factor; GI: gastrointestinal; Gy: gray; LHRH: luteinizing hormone-releasing hormone; LL: leukaemia or lymphoma; MTD: maxium tolerated dose; OM: oral mucositis OS: overall survival; PK: pharmacokinetic: PSA: prostate-specific antigen; RBC: red blood cell; RFS: relapse-free survival; RT: radiotherapy; Se: selenium; SLM: seleno-L-methionine; SS: sodium selenite; ST: solid tumours; WBC: white blood cell count.

most safely and effectively used in such trials in patients with cancer. The preclinical data suggest that organic Se compounds (such as MSC and SLM) are safer and likely more effective than inorganic compounds such as SS, particularly at the higher doses that appear to be optimal for anticancer efficacy and beneficial interactions with chemotherapy and radiation.

More rigorous evaluation in clinical trials is needed to characterise the relationship between PK and PD endpoints and comparative safety (including genotoxicity) for the Se compounds in clinical use. Innovative trial designs could include patients selected with malignancies that enable the evaluation of differential effects of Se compounds in normal cells and malignant cells. These trials are critical to inform the selection of the optimal Se compound and dose for future clinical trials and equally to avoid less effective or more toxic forms and doses of Se.

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Development of a qPCR Method to Measure Mitochondrial and Genomic DNA Damage with Application to Chemotherapy-Induced DNA Damage and Cryopreserved Cells

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Abstract: DNA damage quantitation assays such as the comet assay have focused on the measurement of total nuclear damage per cell. The adoption of PCR-based techniques to quantify DNA damage has enabled sequence- and organelle-specific assessment of DNA lesions. Here we report on an adaptation of a qPCR technique to assess DNA damage in nuclear and mitochondrial targets relative to control. Novel aspects of this assay include application of the assay to the Rotor-Gene platform with optimized DNA polymerase/fluorophore/primer set combination in a touchdown PCR protocol. Assay validation was performed using ultraviolet C radiation in A549 and THP1 cancer cell lines. A comparison was made to the comet assay applied to peripheral blood mononuclear cells, and an estimation of the effects of cryopreservation on ultraviolet C-induced DNA damage was carried out. Finally, dose responses for DNA damage were measured in peripheral blood mononuclear cells following exposure to the cytotoxic agents bleomycin and cisplatin. We show reproducible experimental outputs across the tested conditions and concordance with published findings with respect to mitochondrial and nuclear genotoxic susceptibilities. The application of this DNA damage assay to a wide range of clinical and laboratory-derived samples is both feasible and resource-efficient.

Keywords: LORD-Q; qPCR; comet assay; DNA damage; PBMC; THP1; A549; bleomycin; cisplatin

1. Introduction

The integrity of genomic and mitochondrial DNA is under constant threat from endogenous reactive oxygen species produced by normal cellular metabolism and exogenous sources such as ultraviolet light and mutagens. To counter this, organisms use a complex network of pathways/processes that sense and repair defects, collectively referred to as the DNA damage response (DDR) [1]. However, loss of function mutations or epigenetic silencing of DDR genes—which are associated with human malignancies and inherited disorders such as Fanconi anaemia and Bloom's syndrome—can prevent repair of DNA [2]. A reliable method that detects and quantifies DNA lesions is valuable in understanding disease-specific pathologies and screening chemical and environmental contaminants for genotoxic effects.

Quantification of mutagenic effects originated with the micronucleus assay [3], which was largely superseded by the single-cell gel electrophoresis or comet assay [4]. This method quantifies the

migration of damaged DNA fragments from the nucleoid under electrophoresis and has become the gold standard for measuring cellular DNA damage. However, researchers are exploring PCR-based approaches, where DNA damage results in the disruption of DNA polymerase activity and impaired DNA synthesis. Early investigations used semi-quantitative PCR approaches with target amplicons of between ~450 bp and 25 kb [5,6], which has been updated to incorporate quantitative PCR (qPCR) [7]; however, due to the multistep validation required, its use is not straightforward. Contemporaneously, the long-run qPCR technique for DNA-damage quantification (LORD-Q) method was developed [8], in which a high frequency of lesions in the DNA template corresponds to a lower concentration of full-length template and therefore a higher threshold value of the amplified product relative to the undamaged template. This technique utilized oligonucleotide primers designed for 3–4 kb target fragments and small internal amplicons of ~100 bp, and was able to quantify DNA damage induced by a range of genotoxic stimuli and interrogate specific DNA loci in both mitochondrial and genomic DNA with reproducible sensitivity. However, this method has not been widely tested on different instruments (the published report used Roche LightCycler 480 II instrument) and has the disadvantage of requiring an expensive DNA polymerase and fluorophore.

Here we report a modified long-run qPCR assay for use on a Corbett Rotor-Gene 6000 instrument, with alternative primer sets for both nuclear and mitochondrial DNA loci. The method allows both small and large products to be amplified under the same PCR conditions using considerably cheaper consumables than the published method. Our approach was validated by measuring DNA damage in: (1) cryopreserved and freshly-isolated peripheral blood mononuclear cells (PMBCs) exposed to ultraviolet C (UVC) radiation (254 nm); (2) suspension and adherent cancer cell lines exposed to UVC; and (3) cryopreserved PBMCs damaged by cytotoxic chemotherapy (cisplatin and bleomycin).

2. Materials and Methods

2.1. Cell Culture

THP1 and A549 human cell lines were obtained from ATCC and cultured in RPMI-1640 + GlutaMAX (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, USA), penicillin, and streptomycin (Gibco, USA). All cell culture experiments were carried out in a humidified incubator at 5% CO_2 and 37 °C. Adherent cells were harvested by trypsin incubation using 0.05% trypsin in EDTA (Gibco, USA). PBMCs were extracted from buffy coats collected from healthy blood donors by the NZ Blood Service under ethical approval from the Northern B Health and Disability Ethics Committee (reference NTY/10/08/065/AM01). Buffy coats were diluted with phosphate-buffered saline (PBS, pH 7.4) and layered over Histopaque 1077 (Sigma-Aldrich, St Louis, MO, USA) in 15 mL conical sterile polypropylene centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany, The cells were centrifuged at 400 *g* for 30 min, the interphase removed and washed three times in 10 mL of sterile PBS and centrifuged at 250 *g* to remove residual platelets. Finally, the cells were quantified in a Neubauer chamber (Fortuna, Wertheim, Germany) and viability was assessed by 0.4% *w*/*v* trypan blue exclusion (Sigma-Aldrich) in PBS. Samples were used in the experiments if viability exceeded 85%.

2.2. Cryopreservation of PBMCs

PBMCs were resuspended in sterile filtered 50% FCS (Gibco, USA), 40% RPMI (Gibco, USA) and 10% DMSO (Merck, Darmstadt, Germany) in 1.8 mL Nunc™ conical sterile polypropylene cryo vials (Thermo Scientific, Waltham, MA, USA) and placed overnight in a CoolCell® (Biocision, Larkspur, CA, USA) at −80 °C for controlled cooling. These cells were then stored for 2, 4, 8, or 12 weeks at −80 °C. When required, PBMC aliquots were thawed rapidly in a 37 °C water bath, gradually equilibrated with pre-warmed culture media, diluted to 10× the original culture volume, and the cells were pelleted by centrifugation in a refrigerated centrifuge (Eppendorf, Hamburg, Germany) at 300 *g* for 10 min. The residual storage media was discarded to ensure no carryover of DMSO, and the cells

were resuspended and maintained in RPMI-1640 + GlutaMAX (Gibco, USA) and supplemented with 10% heat-inactivated FCS (Gibco, USA) in standard culture conditions for 24 h before UVC irradiation or treatment with cytotoxic compounds.

2.3. UVC Irradiation of Cells

UVC irradiation was carried out on THP1, A549, and fresh or cryopreserved PBMC cells. Cell concentrations were 2.5–5 $\times$ 10^5/mL for A549 cells, 5 $\times$ 10^5/mL for THP1, and 1 $\times$ 10^6/mL for PBMCs, aliquoted into identical 2 mL media volumes in six-well Nunclon™ Delta plates (Thermo Scientific, USA). UVC doses of either 20 or 100 millijoules per cm^2 (mJ/cm^2) were given to each plate using the Bio-link BXL-254 cross-linker (Gibco, USA). Cells were harvested immediately after UVC exposure, and were prepared for DNA extraction, except for aliquots of fresh PBMCs that were used within the alkaline comet assay.

2.4. Cytotoxic Chemotherapy Incubation

Bleomycin and cisplatin concentrations were chosen to match those used in a previous study [8]. A 10 mg vial of bleomycin (Hospira, Lake Forest, IL, USA) was reconstituted in sterile 0.9% *w*/*v* sodium chloride and stocks frozen at −20 °C. Revived PBMCs were resuspended in serum-free media RPMI-1640 (Gibco, USA) in six-well Nunclon™ Delta plates (Thermo Scientific, USA) containing 2.5 $\times$ 10^6 cells per well with bleomycin at concentrations of 10, 20, 30, or 40 μM or vehicle (0.9% *w*/*v* sodium chloride). Plates were incubated for 30 min at 37 °C in 5% CO_2, after which cells were washed in PBS and prepared for DNA extraction. A 1 mg/mL stock solution of cisplatin (Novartis, Auckland, New Zealand) was diluted to the required concentration in complete media RPMI-1640 (Gibco, USA). Revived PBMCs were resuspended in complete media RPMI-1640 (Gibco, USA) in six-well Nunclon™ Delta plates (Thermo Scientific, USA) containing 2.5 $\times$ 10^6 cells per well with cisplatin at concentrations of 50 or 100 μM or media control. Plates were incubated for 24 h at 37 °C in 5% CO_2, after which cells were washed in PBS and prepared for DNA extraction.

2.5. DNA Extraction

Total cell DNA was isolated and purified using Quick-gDNA^{TM} Miniprep (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions from control and treated cells. The cells were washed in PBS and then lysed immediately after UV or chemotherapy treatment by application of 400 μL of the Quick-gDNA^{TM} Miniprep lysis reagent to adherent cells directly on the plate or to suspension cells after 5 min centrifugation at 200 *g*. DNA eluted in TE buffer (10 mM Tris-HCl/1 mM EDTA) was quantified, and an assessment of DNA purity was determined on a NanoDrop 2000 (Thermo Scientific, USA) by spectrometric analysis. An aliquot of the high quality DNA (A260:280 > 1.8) was subsequently stored at 4 °C.

2.6. Alkaline Comet Assay

Fronine microscope slides (Thermo Fisher, Taren Point, Australia) were cleaned, coated with molten 1% HyAgarose™ LE Agarose (Hydragene, Xiamen, China) by emersion, and air-dried. Experimentally-treated cells at a concentration of 1 $\times$ 10^4 cells per slide were mixed into 37 °C 0.5% low melting point (LMP) SeaPlaque™ GTG™ Agarose (FMC Bioproducts, Rockland, ME, USA) in PBS and pipetted onto the agarose-coated slides. A 15 $\times$ 40 mm coverslip was immediately placed on the LMP agarose/cell solution, and the slides were left to gel at 4 °C in the dark for 20 min. Next, the slides were placed in comet lysis solution (pH 10; 2.5 M sodium chloride, 100 mM EDTA, 1.2 mM Tris-HCl, and 0.1% SDS), with 0.015% Triton X-100 added immediately prior to use, for 2 h at 4 °C. Slides were then washed in cold PBS for 5 min and then placed in an Owl™ D3 Electrophoresis System chamber (Thermo Scientific, USA) containing approximately 650 mL of alkaline electrophoresis buffer (pH > 13; 300 mmol sodium hydroxide, 1 mmol disodium EDTA) for 30 min in the dark. Then, electrophoresis was carried out at 300 mA and 20 V for 20 min, after which slides were carefully

removed from the chamber and washed three times in neutralization buffer (Tris 0.4 M; pH 7.5) at 4 °C. Cells were stained with SYBR gold fluorophore 10,000× concentrate (Life technologies, USA) diluted in distilled water for 10 min, and excess stain was washed off with two 5 min distilled water washes. Comet images were captured at 100× magnification using a Leica I3 filter block and Olympus DP70 camera. Slides were prepared in triplicate, and 50 cells were scored per slide for each experimental condition. Olive tail moment was calculated for each cell using CometScore analysis software (Tritek Corp, Sumerduck, VA, USA).

2.7. Modified Long-Run qPCR Technique for DNA-Damage Quantification

Four oligonucleotide primer sets were designed using Primer3 [9] for a long nuclear gene target of 3129 bp in the E2F transcription factor 1 (E2F1) gene (accession no: AF516106.1; 3427–6565 bp) and a long mitochondrial target 3723 bp (accession no: NC_012920; 11492–15214 bp). For each long amplicon target, a matched reverse primer for an internal short amplicon of between 50 and 150 bp was also designed (Table 1). All primer sequences were tested using the primer blast web tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure primer specificity.

Table 1. Primers designed to amplify large and small products for E2F1 and the mitochondrial targets.

Primer Name	Temp. (°C)	Sequence	Product Size (bp)
E2F1 large forward	65.2	GAGGCAGGACTCAGGACAAG	3129
E2F1 large reverse	65.2	CTCCTCACATGCAGCTACCA	
E2F1 small reverse	65.3	GGATGCCTCAGGGACCAG	164
Mitochondrial large forward	63.6	CGCCTCACACTCATTCTCAA	3723
Mitochondrial large reverse	62.6	AATGTATGGGATGGCGGATA	
Mitochondrial small reverse	62.9	CAAGGAAGGGGTAGGCTATG	55

qPCR analysis was performed using a Corbett Rotor-Gene 6000 instrument (Corbett Research, Mortlake, Australia). All reaction components were optimized through extensive trialing. Final conditions were: 1× buffer B1, 2.5 mM $MgCl_2$, 1 U Hot FIREPol® DNA Polymerase (Solis Biodyne, Tartu, Estonia), 200 µM dNTPs (Genscript, Piscataway, NJ, USA) and 2 µM SYTO82 fluorophore (Life Technologies, USA) in a 20 µL reaction volume, which also contained primer at a concentration of 500 nM and 25–50 ng of DNA template (diluted in MQ sterile water). Each reaction was carried out in an Axygen® optically-clear thin-walled PCR tube (Corning, Tewksbury, MA, USA) in a 36-well rotor. The optimized cycling conditions (Table 2) included a Hotstart step (10 cycles with a 0.5 °C decrease per cycle) and an extension of 240 s to ensure amplification of the long product.

Table 2. Optimized cycling conditions for quantitative polymerase chain reaction (qPCR).

Cycle Step	Incubation Times
Initial denaturation (1 cycle)	95 °C at 15 min
Hot start (10 cycles)	Step 1: 95 °C at 10 s Step 2: 65 °C (−0.5 °C/cycle) at 10 s Step 3: 72 °C at 240 s
Amplification (35 cycles)	Step 1: 95 °C at 15 s Step 2: 60 °C at 15 s Step 3: 72 °C at 240 s acquiring to cycling A (yellow channel) Step 4: 82 °C at 10 s acquiring to cycling B (yellow channel)
Melt curve	Ramp from 64 °C to 95 °C Hold for 90 s on the 1st step Hold for 5 s on the subsequent steps. Melt A (yellow channel)

PCR amplification efficiencies for the target amplicons were calculated by comparative quantitation using the Corbett Rotor-Gene 6000 Application Software, version 1.7 (Qiagen,Valencia, CA USA). Average efficiencies for both large and small products across each experimental series were used for these calculations. Detected lesion rate per 10 kb was determined using the following equation modified from Lehle et al. [8]:

$$\text{Lesions per 10 kb} = [(E_L{}^{Cpl(\text{Sample})} \times E_S{}^{-Cps(\text{Sample})} / E_L{}^{Cpl(\text{Control})} \times E_S{}^{-Cps(\text{Control})})^{1/a} - 1] \times 10{,}000$$

where E_L and E_S are the average amplification efficiencies of the large and short product, C_p values are the crossover (or threshold) values determined by the Rotor-Gene software (C_{pl}: long; C_{ps}: short), and a is the number of base pairs of the long fragment. All qPCR reactions for each sample were carried out in duplicate, and the mean C_p values were used in the calculation of lesions in either genomic or mitochondrial DNA. An Excel spreadsheet for the above calculation is included in the supplementary materials (Table S1).

2.8. Data Analysis

Graphs were constructed using Prism v7 (GraphPad Software, La Jolla, CA., USA), with data expressed as mean ± SE. Results of the qPCR and comet assays were tested using the unpaired *t*-test and two-sided p values < 0.05 were considered significant. One-way ANOVA comparison was used to test differences between means at different time points.

3. Results

3.1. Adherent and Suspension Cancer Cell Lines DNA Damage Quantitation

After 20 or 100 mJ/cm^2 UVC (254 nm) was applied to the A549 human lung cancer cell line and DNA extracted; qPCR was performed on both long and short fragments for both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) using our optimized touchdown protocol. Each experiment was performed in triplicate, and results obtained from two independent investigations. DNA lesion rates were higher in the mitochondrial than in nuclear DNA templates, with a clear dose response to UVC exposure in both target amplicons (Figure 1A,B). Due perhaps to variation in C_p values for the E2F1 fragment between controls, there was no statistically significant nDNA response at 20 mJ/cm^2. A similar pattern of significant DNA damage from both 20 and 100 mJ/cm^2 UVC was seen in the THP1 human monocytic leukemia cell line (Figure 1C,D).

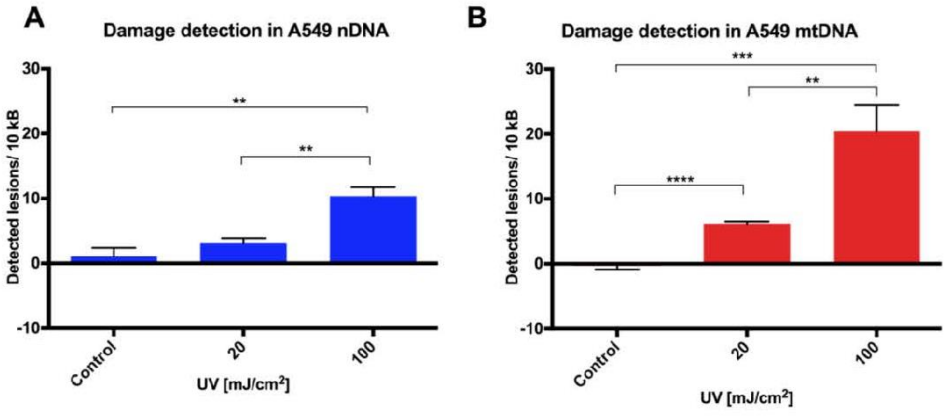

Figure 1. *Cont.*

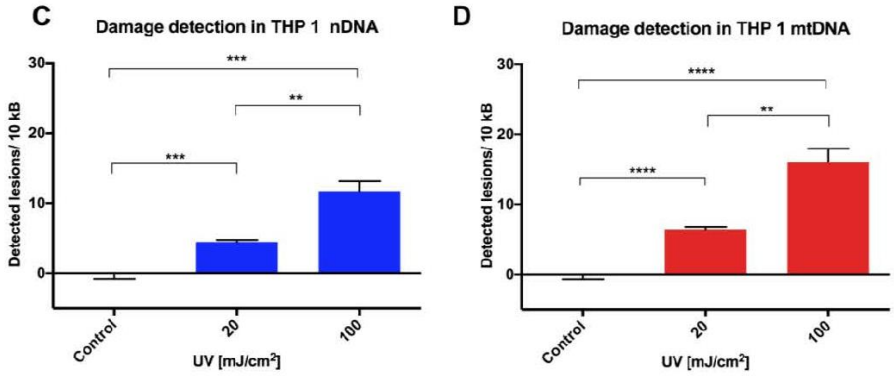

Figure 1. Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) damage quantitation in cancer cell lines. Genotoxic stimulation with UVC (20 or 100 mJ/cm^2) was assessed in adherent A549 cells in (**A**) nDNA and (**B**) mtDNA, and in the THP1 suspension cell line in (**C**) nDNA and (**D**) mtDNA using qPCR ($n = 5$). Results are presented as mean ± SE. ** p <0.01, *** p < 0.001, **** p <0.0001.

3.2. DNA Damage Quantitation in PBMCs Using qPCR and the Comet Assay

PBMCs isolated from healthy donor buffy coats were equilibrated overnight in complete media and then treated with UVC. Aliquots were then taken for either the alkaline comet assay or DNA extraction for qPCR. Experiments were performed in triplicate from three different PBMC donors ($n = 9$). qPCR revealed a UVC dose response in both nDNA (Figure 2A) and mtDNA (Figure 2B) across the nine replicates. While the comet assay was able to distinguish between control and UVC-exposed cells (Figure 2C), it failed to discriminate between 20 and 100 mJ/cm^2 doses, with significant variation in Olive tail moment observed (Figure 2D). The authors who first reported the LORD-Q approach also demonstrated the superiority of qPCR over the alkaline comet assay in detecting low-level DNA damage induced by exposure to bleomycin in Jurkat T cells [8].

3.3. Effect of Cryopreservation on DNA Damage Quantitation in PBMC's

The use of the qPCR assay in quantifying UVC-induced DNA damage in cryopreserved PBMCs was assessed (Figure 3). Experiments were performed in triplicate from one PBMC donor ($n = 3$). For the nuclear DNA target (*E2F1*), the mean lesion rates observed across five time points from $t = 0$ (fresh) to $t = 12$ weeks at −80 °C after treatment with 20 mJ of UVC were not significantly different ($p = 0.36$). In contrast, induced DNA damage in the mitochondrial fragment was significantly greater at $t = 0$ (mean 7.3 lesions/10 kb) than at subsequent time points ($p = 0.034$). We observed that no further deterioration in DNA integrity was found over a 12 week period in either mitochondrial or nuclear DNA, with cells stored for different periods of time giving similar DNA damage response to UVC challenge.

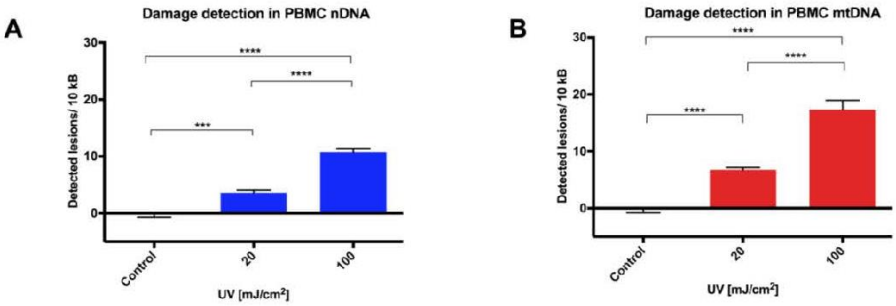

Figure 2. *Cont.*

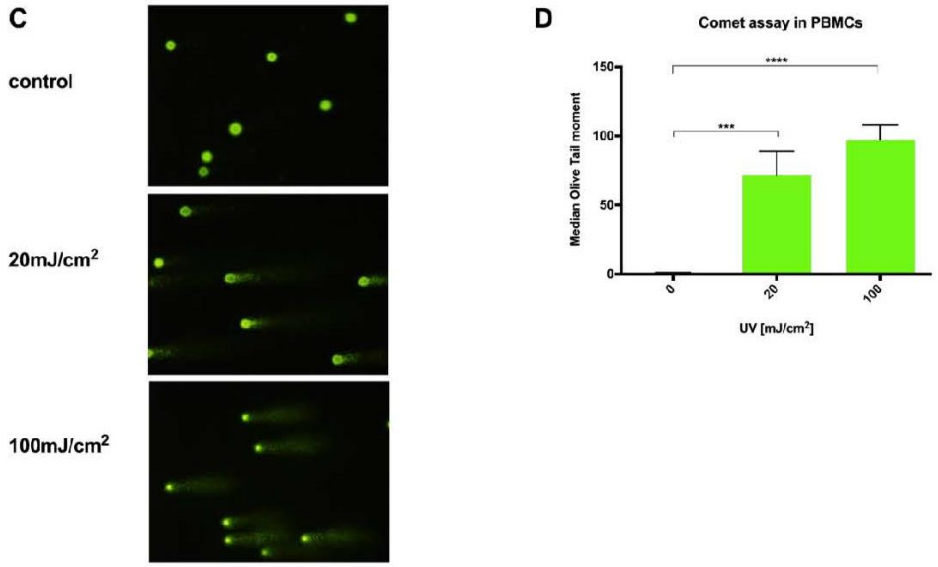

Figure 2. Comparison of the qPCR approach to the alkaline comet assay using peripheral blood mononuclear cells (PBMCs). Genotoxic stimulation with ultraviolet C (UVC; 20 or 100 mJ/cm^2) was assessed in freshly isolated PBMCs in (**A**) nDNA and (**B**) mtDNA by qPCR ($n = 9$). DNA damage was also assessed after the same treatments using (**C**) the alkaline comet assay, and (**D**) median Olive tail moments were calculated for each slide ($n = 9$). Results are presented as mean ± SE. *** $p < 0.001$, **** $p < 0.0001$.

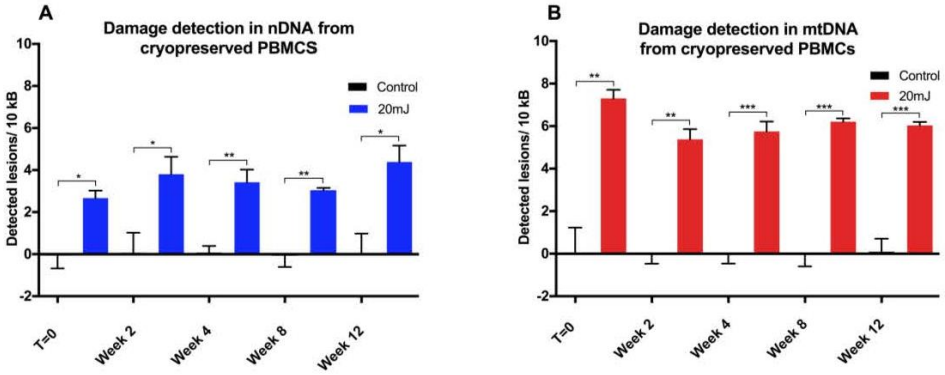

Figure 3. DNA damage assessment in cryopreserved PBMCs. qPCR measurement of UVC-induced (**A**) nDNA and(**B**) mtDNA damage in revived PBMCs following cryopreservation over 12 weeks ($n = 3$). Results are presented as mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. DNA Damage Quantitation in PBMCs Exposed to Cytotoxic Drugs

PBMCs revived after cryopreservation (storage period <4 weeks for all PBMC aliquots) were treated with either the cytotoxic glycopeptide antibiotic bleomycin (Figure 4A,B), the platinum compound cisplatin (Figure 4C,D), or media as a control. Experiments were performed in triplicate from two different PBMC donors ($n = 6$). Initial experiments using bleomycin on PBMCs showed no significant nDNA damage, and a slight significant increase in mtDNA damage. Due to these findings, no more replicates were performed, and another drug was tested for comparison. Interestingly, a higher frequency of DNA lesions was observed in nDNA than mtDNA in PBMCs treated with 100 μM cisplatin (Figure 4C,D), both being greater than for bleomycin treatment.

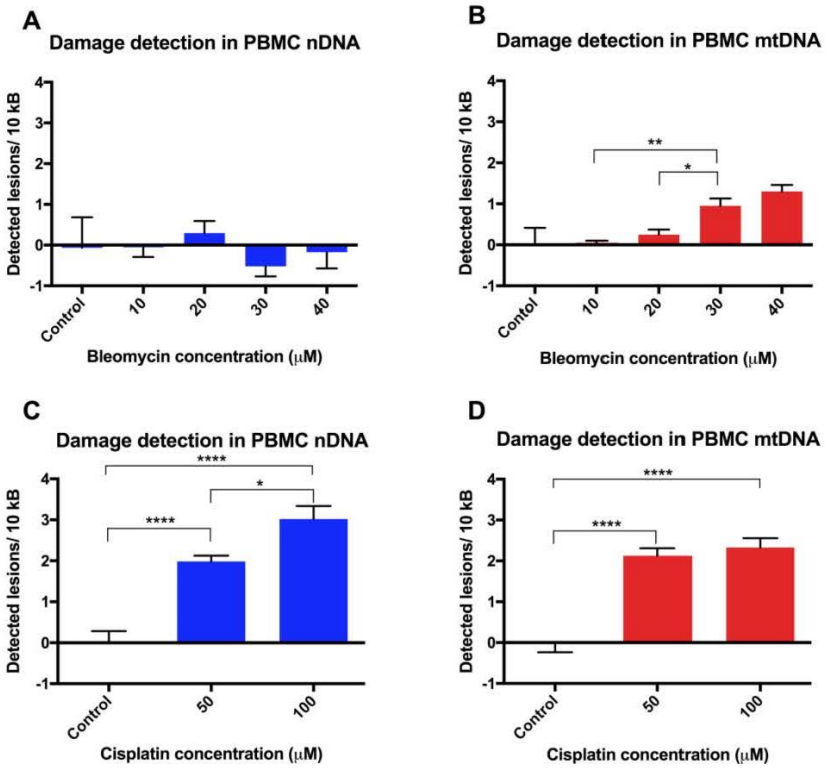

Figure 4. Cytotoxic chemotherapy-induced DNA damage in PBMCs. Quantitation of DNA lesion rates in revived PBMCs in (**A**) nDNA and (**B**) mtDNA following exposure to bleomycin or in (**C**) nDNA and(**D**) mtDNA following exposure to cisplatin ($n = 6$). Results are presented as mean ± SE. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

4. Discussion

Current techniques available to measure DNA damage (such as the comet assay) can be both time-consuming and laborious when applied to large sample numbers. There are increasing reports in the literature of methods that utilize molecular techniques like qPCR, which have the potential to reduce analysis time and offer a significant level of sensitivity and reproducibility. They also improve on the comet assay with sequence-specific DNA damage, quantifying the effects of potential toxicants separately on nuclear and mitochondrial DNA.

Initially, we evaluated the LORD-Q method using primer sets as previously described [8], testing for amplification of both the large nuclear gene (*p53*) (3075 bp) and large mitochondrial (3723 bp) products. qPCR was carried out using the recommended KAPAG2 (Peqlab) enzyme and cycling conditions with the SYTO82 fluorophore using a Corbett Rotor-Gene 6000 (Qiagen). We also tried substituting the enzyme for the FIREPol® DNA Polymerase (Solis BioDyne), but the amplification gave variable results with a number of non-specific products. This was particularly evident for the p53 product, as confirmed by gel electrophoresis. Through a series of investigations, we eventually optimized this assay by targeting different DNA regions, using a less expensive HotFirePOL enzyme/SYTO82 fluorophore combination (approximately six times cheaper than the LORD-Q approach) and establishing the best cycling conditions for use on our own qPCR system. We chose *E2F1* as a nuclear DNA target, as this gene plays a key role in cell cycle regulation, is likely to be in a transcriptionally-active region of the genome, and is therefore susceptible to experimentally-induced damage [10]. We anticipate that there would be a multitude of suitable targets throughout the genome, and that some optimization would be required when using different qPCR

instruments. The reproducibility of this modified qPCR assay was validated using UVC treatment (254 nm) initially in adherent (A549) and suspension (THP1) cancer cells lines, then in PBMCs due to a forthcoming clinical investigation involving such samples. Moreover, the comparison between levels of induced DNA damage in cryopreserved versus freshly-isolated PBMCs informs future experimental methodologies and clinical trial material handling protocols.

Initial assay validation using UVC to damage THP1 and A549 cell lines demonstrated reproducible damage quantification in both nDNA and mtDNA, which was very close to what had initially been shown in Jurkat T-cells in the LORD-Q study [8]. Alternative approaches to measuring DNA damage in THP1 cells have shown similar UVC-induced damage. In addition to inducing hydrolytic and oxidative damage, UVC induces bipyrimidine sites in cellular DNA to form cyclobutane pyrimidine dimers, (6–4) photoproducts and their Dewar isomers [11]. Using high performance liquid chromatography with electrospray ionization tandem mass spectroscopy (HPLC-MS/MS), the UVC-induced bipyrimidine lesion rate in THP1 cells was approximately 1 lesion per 1×10^4 base pairs per 10 mJ/cm^2 at a cell concentration of 6×10^6/cm^2 [12]. Interestingly, A549 and THP1 cells had much higher damage in mtDNA than nDNA, which was similar to another study, where Jurkat T cells had been damaged by UVC [8]. Mitochondrial DNA has been shown to be more sensitive to oxidative stress than nDNA [13], perhaps related to its proximity to a major source of endogenous reactive oxygen species, namely the electron transport chain located in the inner mitochondrial membrane. Furthermore, the nucleotide excision repair pathway responsible for repairing helix-distorting lesions (such as UVC-induced photoproducts) is absent in mitochondria [14].

While the comet assay is still regarded as the standard genotoxicity assay, it does have a number of limitations. Firstly, standardization of assay conditions is vital in reducing intra-assay variability [15]; even small changes in agarose concentration, alkaline unwinding times, and electrophoresis conditions can significantly alter the results. Additionally, even with the use of comet scoring software, bias can be introduced into the scoring process and, although the comet can be scaled up to deal with large sample numbers, significant time resources are required to perform the assay, then capture and score the images. In comparison, a qPCR-based technique provides a relative rather than absolute measurement of DNA damage. It allows differences in mitochondrial and nuclear damage to be compared, and can provide gene/region-specific information that may clarify our understanding of certain pathological processes. To our knowledge, this report is the first comparison of a qPCR-based DNA damage assay with the comet assay in PBMCs. The results show our optimized protocol to be a suitable alternative technique to the comet assay in quantifying genotoxicity from UVC exposure in PBMCs in a time and reagent cost-efficient manner.

The use of human lymphocyte-based model systems for screening chemical compounds for cytotoxicity or cytoprotection is well-established. The cryopreservation of lymphocytes enables long-term storage and allows down-stream assays (such as toxicity assays) on cells collected over an extended period of time, such as in a clinical study. The study of DNA damage and repair has been performed in cryopreserved PBMCs using the comet assay [16]. Whilst the isolation of PMBCs from whole blood is associated with some DNA damage, the additional cryopreservation step should not contribute to further damage, with responses following the induction of oxidative stress being similar between frozen and fresh lymphocytes [17]. Indeed, we observed that no further deterioration in DNA integrity was found in either mitochondrial or nuclear DNA over a 12 week period, with cells stored for different periods of time giving similar DNA damage response to UVC challenge. The utility of this approach has been demonstrated here in both fresh and in cryopreserved PBMCs, and can be applied to the analysis of tissue-banked samples in a high throughput qPCR format.

Finally, assay validation using the cytotoxic chemotherapy agents bleomycin and cisplatin in revived PBMCs revealed variable susceptibility to their genotoxic effects. The observed lower levels of damage in nDNA in unstimulated PBMCs is not unexpected, given that previous studies on DNA damage following a 30 min exposure of phytohemagglutinin-stimulated PBMCs to bleomycin 20 μg/mL have shown a very low level of damage, as assessed by the alkaline comet assay,

with <1% DNA in the tail [18]. A semi-quantitative PCR approach also showed nDNA from SV40-transformed human fibroblasts to be more sensitive than mtDNA to damage following incubation with cisplatin [19]. Other PCR-based studies have been undertaken using cisplatin, where DNA adducts were measured in a p53 nuclear DNA target in donor PBMC cell lysates [20], and a lesion frequency of ~1.2/104 nucleotides was estimated after a 3 h treatment with 100 μg/mL cisplatin. However, this is not directly comparable to our data, where cisplatin was used at lower concentrations for 24 h incubations and PCR was performed on high quality extracted DNA rather than directly on cell lysates.

5. Conclusions

The successful assay implementation of a qPCR assay to measure nDNA and mtDNA damage requires optimization of primers from selected targets and qPCR cycling conditions for a given PCR platform and experimental system/cell line. Our comparison of induced DNA damage in fresh and cryopreserved PBMCs provides the basis for future applications, such as human bio-monitoring studies of bio-banked samples. This assay is recommended as a useful and versatile tool to assess DNA damage within genomic and mitochondrial DNA in any eukaryotic system, even in the same PCR run across different experimental groups.

Supplementary Materials: The following are available online at www.mdpi.com/2079-7737/5/4/39/s1, Table S1: qPCR DNA damage calculator.

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Conflicts of Interest: The authors declare no conflict of interest.

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Comparative Safety and Pharmacokinetic Evaluation of Three Oral Selenium Compounds in Cancer Patients

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Abstract
Selenium (Se) compounds have demonstrated anticancer properties in both preclinical and clinical studies, with particular promise in combination therapy where the optimal form and dose of selenium has yet to be established. In a phase I randomised double-blinded study, the safety, tolerability and pharmacokinetic (PK) profiles of sodium selenite (SS), Se-methylselenocysteine (MSC) and seleno-l-methionine (SLM) were compared in patients with chronic lymphocytic leukaemia and a cohort of patients with solid malignancies. Twenty-four patients received 400 μg of elemental Se as either SS, MSC or SLM for 8 weeks. None of the Se compounds were associated with any significant toxicities, and the total plasma Se AUC of SLM was markedly raised in comparison to MSC and SS. DNA damage assessment revealed negligible genotoxicity, and some minor reductions in lymphocyte counts were observed. At the dose level used, all three Se compounds are well-tolerated and non-genotoxic. Further analyses of the pharmacodynamic effects of Se on healthy and malignant peripheral blood mononuclear cells will inform the future evaluation of higher doses of these Se compounds. The study is registered under the Australian and New Zealand Clinical Trials Registry No: ACTRN12613000118707.

Keywords Selenium · Pharmacokinetics · Clinical trial · Methylselenocysteine · Cancer · Chronic lymphocytic leukaemia · Double-blinded

Background

The essential trace element selenium (Se) is widely used by cancer patients as a nutritional supplement [1]. Interest in Se as a chemoprevention agent peaked following the report of a reduced incidence of colon, lung and prostate cancers as a secondary endpoint in the Nutritional Prevention of Cancer (NPC) study, in which patients with a previous history of non-melanoma skin cancer took 200 μg daily of elemental Se (as selenized yeast) [2]. Preclinical models supported this, demonstrating several mechanisms that explained the chemopreventive effect of Se compounds [3]. However, the much larger SELECT study of 35,000 men randomised to 200 μg of Se (as seleno-L-methionine) or vitamin E, alone or in combination, showed no reduction in incidence of prostate or other cancers [4]. A possible explanation for the negative findings from the SELECT study is that the study population was to a large extent Se-replete with baseline mean serum Se levels of approximately 135 μg/L. In terms of long-term supplementation, there is no strong argument for administering Se to patients already well-supplied [5].

However, this applies to cancer prevention studies, whereas the pharmacodynamic (PD) effects of Se in conjunction with cancer therapies occur in a higher dose range, likely independent of baseline serum concentration. While this has tempered enthusiasm for using Se compounds in chemoprevention studies, there has been ongoing interest in using them to favourably modulate the toxicity and efficacy of cancer therapies.

The PD mechanisms that mediate the interaction between Se compounds and cancer therapies such as cytotoxic chemotherapy and radiation overlap with those that mediate cancer prevention [3, 6], but it is generally observed that higher doses of Se are required to activate these optimally than those doses used in chemoprevention studies [7]. Preclinical work in vitro and

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in vivo models has shown that adding Se compounds to a variety of chemotherapy or hormonal drugs or radiation augments their anticancer effects while reducing some of their toxicities [8–11]. The underlying mechanisms responsible for the favourable interactions between cancer treatments and Se include impairment of tumour angiogenesis, modulation of the endoplasmic reticulum (ER) and oxidative stress responses and enhanced DNA repair [6, 12, 13]. Human studies of Se supplementation during cancer therapy have reported significant reduction in toxicity with cytotoxic drugs including cisplatin, doxorubicin, cyclophosphamide and busulphan [14–16], without compromising treatment efficacy [17]. However, phase 3 trials adequately powered to measure important endpoints such as cancer outcomes, survival and long-term safety have not yet been performed. In addition, questions remain around the optimal form and dose of Se to be used in this setting, and the impact of baseline Se status.

The main forms of Se used in clinical trials with cancer therapies in recent years are the inorganic compound sodium selenite (SS) and the organic compound seleno-L-methionine (SLM) [8]. However, comparative xenograft studies of Se compounds in combination with chemotherapy demonstrated considerable therapeutic differences between these compounds, with SLM being more effective than SS [18] and another organic Se compound, Se-methylselenocysteine (MSC), being the most effective overall [6, 18]. MSC was the most efficient of the three compounds at generating methylselenol, the metabolite thought to mediate most of the interactions with cancer therapies [19]. However, SS is favoured by some investigators due to its reported reactive oxygen species-driven cytotoxicity being highly selective in inducing cell death in malignant cells over healthy cells [12, 20].

There are also significant differences in toxicity profiles between these compounds, with SS having the disadvantage of causing more genotoxicity than SLM or MSC at comparable doses, which is therapeutically significant when using these compounds with DNA-damaging cancer therapies [21]. Late toxicities such as myelodysplasia, acute leukaemia or other malignancies could be the consequence of such combinations, which argues for using the Se compound with the least genotoxicity in such studies.

Other unresolved issues complicate the design of future studies combining Se compounds with cancer therapies, the most problematic of which is a lack of clarity in understanding the basis for dose selection. Due to concerns about poorer tolerance of radiation (RT) in Se-deficient patients, some studies administered SS at a dose (500 μg/day with RT and 300 μg/day on non-RT days) calculated to increase plasma Se to normal levels, with variable results [17]. In gynaecological cancer patients being treated with radical RT, those randomised to SS had significantly reduced acute bowel toxicity but comparable survival to the control group [22], whereas other studies using a similar dose in head and neck cancer patients receiving RT have shown no benefit [23]. Other trials dose-escalated SLM to achieve plasma Se concentrations comparable to those seen in mice dosed at their maximum tolerated dose (MTD), which was also the most effective dose in the xenograft models [24]. This resulted in dosing humans orally with SLM up to 3600 μg/m^2/day, which was generally well-tolerated [25–28]. Yet, others have taken a traditional approach of dosing at the MTD in humans, determined as 60,000 μg/day for oral sodium selenate (which is metabolised to SS) [29] and 10,200 μg/m^2/day for intravenous SS [20] another group used oral SS at 200 μg/kg/day [30]. Conversely MSC, which appears optimal for efficacy and safety in laboratory studies when compared to SS or SLM, has only been evaluated at single oral doses up to 1200 μg [31] or repeated oral daily doses up to 800 μg/day [32]. While it is reassuring that these Se compounds appear to be safe (at least in the short term) across this huge variation in doses, it is not clear from these clinical studies that higher doses or particular Se compounds have greater efficacy in humans, nor the basis on which this should be determined (PD, toxicity or anticancer efficacy endpoints).

The preclinical and clinical work to date supports the hypothesis that Se compounds can reduce toxicities of chemotherapy and RT and improve cancer outcomes but larger well-designed and adequately powered trials are required to confirm these important benefits. In order to maximise the chance of identifying these important therapeutic interactions of Se compounds with chemotherapy and/or RT in future clinical trials, it is important to evaluate further the relationship between Se compounds, dose, PK, PD and safety in humans.

Here, we report on the results from a cohort of cancer patients dosed at 400 μg of elemental Se per day in a phase Ib randomised, double-blinded clinical trial evaluating oral administration of SS, SLM and MSC. The primary objective was to determine the dose and form of Se that can be most safely and effectively used in clinical trials in combination with anticancer therapies. Secondary objectives include characterisation of the clinical and laboratory safety profile of the Se compounds, determination of plasma PK, evaluation of DNA damage in peripheral blood mononuclear cells (PBMCs) and exploration of PD markers in plasma and PBMC. The study population included patients with chronic lymphocytic leukaemia (CLL) or metastatic solid malignancies, in order to facilitate an understanding of the differential effects of the Se compounds in malignant and normal PBMC [33]. This report includes safety and PK data. Evaluation of PD mechanisms will be reported separately.

Methods

Patient Eligibility

Inclusion criteria consisted of patients with either proven CLL (and peripheral blood lymphocyte count > 10×10^9/l) or metastatic solid cancers, in whom the use of chemotherapy was not anticipated in the next 3 months. Additional inclusion criteria

included: age > 18 years; ECOG performance status ≤ 2; adequate renal, liver and bone marrow function; and life expectancy > 6 months. Exclusion criteria included patients currently taking more than 100 μg of elemental Se daily, or those who had received chemotherapy, RT or anti-VEGF treatments in the preceding 4 weeks. Ethical approval was obtained through Northern A Health and Disability Ethics Committee (13/NTA/172), and all patients provided written informed consent. The study is registered with the Australian and New Zealand Clinical Trials Registry No: ACTRN12613000118707.

Study Design

This phase Ib double-blinded study randomised patients to one of three arms: MSC, SLM or SS; each arm was balanced to include four patients with CLL and four with metastatic solid cancers (Supplementary Fig. 1). Patients included in this report took one capsule containing the equivalent of 400 μg/day of elemental Se daily for 8 weeks.

Randomisation

Patients were stratified by diagnosis (CLL or solid cancer) then randomised to MSC, SLM or SS using permuted block randomisation and a computer-based random allocation method. Randomisation was conducted by the clinical trials pharmacist at Waikato Hospital but patients, investigators, research and laboratory staff were blinded to treatment allocation.

Study Medication

All Se compounds were manufactured and supplied by Sabinsa Corporation, 20 Lake Drive, East Windsor, NJ 08520-5321, USA. Stated doses reflect micrograms of elemental Se in each capsule, not micrograms of the compound.

Study Assessments

Clinical and laboratory evaluations were undertaken twice at baseline at least 1 week apart, then on day 2 of dosing, at weeks 4 and 8 of treatment then 4 weeks after the last Se dose (week 12). The first baseline assessment included a full medical history and physical examination. All treatment-emergent adverse events, or of greater severity than at baseline, were recorded and graded using the NCI CTCAE version 4.03. Patients were given study diaries to record any changes in health state or in concomitant medication usage during trial participation, and these were reviewed at each study visit.

At each study visit, the following tests were conducted for safety evaluation: urinalysis, ECG and blood tests (complete blood count, renal and liver function, glucose, urate, calcium, phosphate and coagulation). Plasma Se samples were taken once at baseline, 4 h post-dose on day 2, then trough levels were taken on weeks 4 and 8, and finally at week 12. Patients with solid cancers who had elevated serum tumour markers at baseline had estimations of these at weeks 4, 8 and 12. All patients with CLL had total lymphocyte counts recorded at each visit (inclusive of those with a malignant morphology, such as smear cells).

Blood for PD and DNA damage analyses was taken at each study visit and processed to produce plasma (spun at 1000×*g* at 4 °C for 10 min) and PBMCs (as previously described [34]), which were stored at − 80 °C until analysed.

DNA Damage Assessment

DNA damage was measured using a qPCR-based technique that calculates nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) lesion rates relative to DNA extracted from pretreatment blood samples, as previously described [34]. In brief, the PCR reactions included DNA extracted from PBMCs, primers designed to amplify > 3000 kb regions of nDNA and mtDNA and internal control amplicons of 50–100 bp. Samples were assayed in duplicate on magnetic induction cycler (MIC) instruments (BMS, Australia). Large and small amplicon reactions were run in parallel, and lesion rates were calculated as previously described [34] (see supplementary materials for further details). The change in DNA damage was quantified relative to the first pretreatment sample (or, where a PCR result was not available, the second pretreatment sample) for both nDNA and mtDNA extracted from PBMCs isolated from blood taken at each assessment.

Pharmacokinetic Evaluation

Venous blood for Se measurements was collected in trace element tubes (K2 EDTA BD), and separated plasma was analysed by Canterbury Health Laboratories using inductively-coupled plasma mass spectroscopy on an Agilent 7700 ICP-MS instrument (Agilent Technologies, CA, USA). In patients who had plasma Se samples taken at baseline, weeks 4 and 8 and end of study, the area under the plasma Se concentration-time curve (AUC) was calculated from baseline to end of dosing using the trapezoidal rule (Prism v. 7.0, GraphPad, La Jolla, CA, USA).

Statistical Analysis

All statistical analysis was conducted using Prism v. 7.0; two-sided $p < 0.05$ was considered statistically significant. Descriptive statistics were used to summarise the safety, toxicity and pharmacokinetic data. Baseline characteristics were analysed using one-way ANOVA for continuous data and the chi-square test for categorical variables. One-way ANOVA was used to identify the statistical significance of variance among group means for plasma Se AUC values by treatment arm. Pairwise assessment of the treatment arms was carried out using

Tukey's multiple comparison test. One-way ANOVA and Dunnett's multiple comparison test was used to compare variance among group means from measurements at baseline and subsequent time points for all treatment arm/disease group combinations for both DNA damage rates and baseline-corrected total lymphocyte count. Estimations of baseline variation are plotted as 95% confidence intervals for both relative DNA damage rates and total lymphocyte counts calculated from two baseline samples obtained prior to Se dosing.

Results

Recruitment

Between February 2015 and April 2017, 24 patients were recruited and randomised into the three study arms. Patient characteristics at baseline were balanced across Se compound groups, with no statistically-significant differences observed (Table 1). The metastatic cancers were well-distributed across treatment groups and included breast ($n = 3$), melanoma ($n = 2$), prostate ($n = 4$) and one each of follicular lymphoma, endometrial cancer and cutaneous squamous cell carcinoma.

Safety Results

Of 24 randomised patients, 23 completed the 56-day treatment schedule; one patient discontinued on day 35 after an episode of grade 2 constipation, possibly attributable to Se. Table 2 lists the single episodes all treatment-emergent adverse events reported by study participants. The two episodes of ≥ grade 2 toxicity were attributable to other causes: anaemia due to bleeding from an undiagnosed colon cancer while on dabigatran, and transient confusion associated with an undiagnosed brain metastasis.

DNA Damage Assay

Levels of DNA damage, calculated as mtDNA and nDNA lesion rates relative to baseline, were observed to be low across all treatment groups and time points, by both disease cohort and DNA subtype (mtDNA or nDNA), with mean lesion rates in each patient/Se compound cohort being < 1 per 10 kb of DNA (Fig. 1).

Pharmacokinetic Results

PK results summarised in Table 3 and Fig. 2 show Se plasma levels over the course of the study. In five patients taking Se supplements before study enrolment, mean (SD) baseline Se plasma level was 1.52 μM (0.26) compared to 1.23 μM (0.16) for non-supplement takers ($p = 0.005$). Patients allocated to SLM had a significantly greater total Se exposure as evidenced by the higher AUC ($p < 0.0001$ compared to either SS or MSC). Additionally the increase in plasma Se levels from baseline at 4 h post-dose was greatest in this group (mean increase with SLM was 0.45 μM, 0.31 μM with MSC and 0.20 μM with SS ($p = 0.008$ for SLM vs SS, $p = 0.36$ for SS vs MSC, and $p = 0.24$ for SLM vs MSC).

Table 1 Baseline characteristics of recruited patients ($n = 24$)

Characteristic	MSC ($n = 8$)	SLM ($n = 8$)	SS ($n = 8$)
Median age, years (range)	71 (46–80)	70 (57–88)	69 (55–82)
Sex			
M	5	4	4
F	3	4	4
ECOG PS			
0	7	7	7
1	1	1	1
Blood lymphocytes × 10^9/L, mean (SD)			
CLL	29.4 (23.8)	54.1 (37.2)	60.1 (55.0)
Solid cancer	1.5 (0.45)	1.4 (1.05)	1.8 (0.62)
Prior chemotherapy	4	1	3
Prior radiotherapy	2	2	2
Current targeted therapy[a]	3	3	3

ECOG PS: Eastern Cooperative Oncology Group performance status

[a] Includes hormonal therapies

Tumour Markers

In two patients who had raised serum prostate-specific antigen (PSA) levels at baseline, no significant changes in PSA were observed across subsequent study visits. Two patients with metastatic breast cancer had raised serum CA15-3 levels at baseline; in one, the levels remained stable, whereas in the other the levels were rising significantly (> 50%) by the end of dosing and at the end of study visit (> 100%), suggesting disease progression.

Table 2 Treatment-emergent adverse events

	SS, $n = 8$			SLM, $n = 8$			MSC, $n = 8$		
CTCAE grade	I	II	III	I	II	III	I	II	III
Rash	2	0	0	0	0	0	0	0	0
Proteinuria[a]	1	0	0	0	0	0	1	0	0
Haematuria[a]	0	0	0	1	0	0	0	0	0
Dyspepsia	0	0	0	1	0	0	0	0	0
Urinary frequency	0	0	0	0	0	0	1	0	0
QTc prolongation	0	0	0	1	0	0	0	0	0
Anaemia	0	0	0	0	0	1	0	0	0
Constipation	0	0	0	0	1	0	0	0	0
Confusion	0	1	0	0	0	0	0	0	0

[a] Microscopic on dipstick

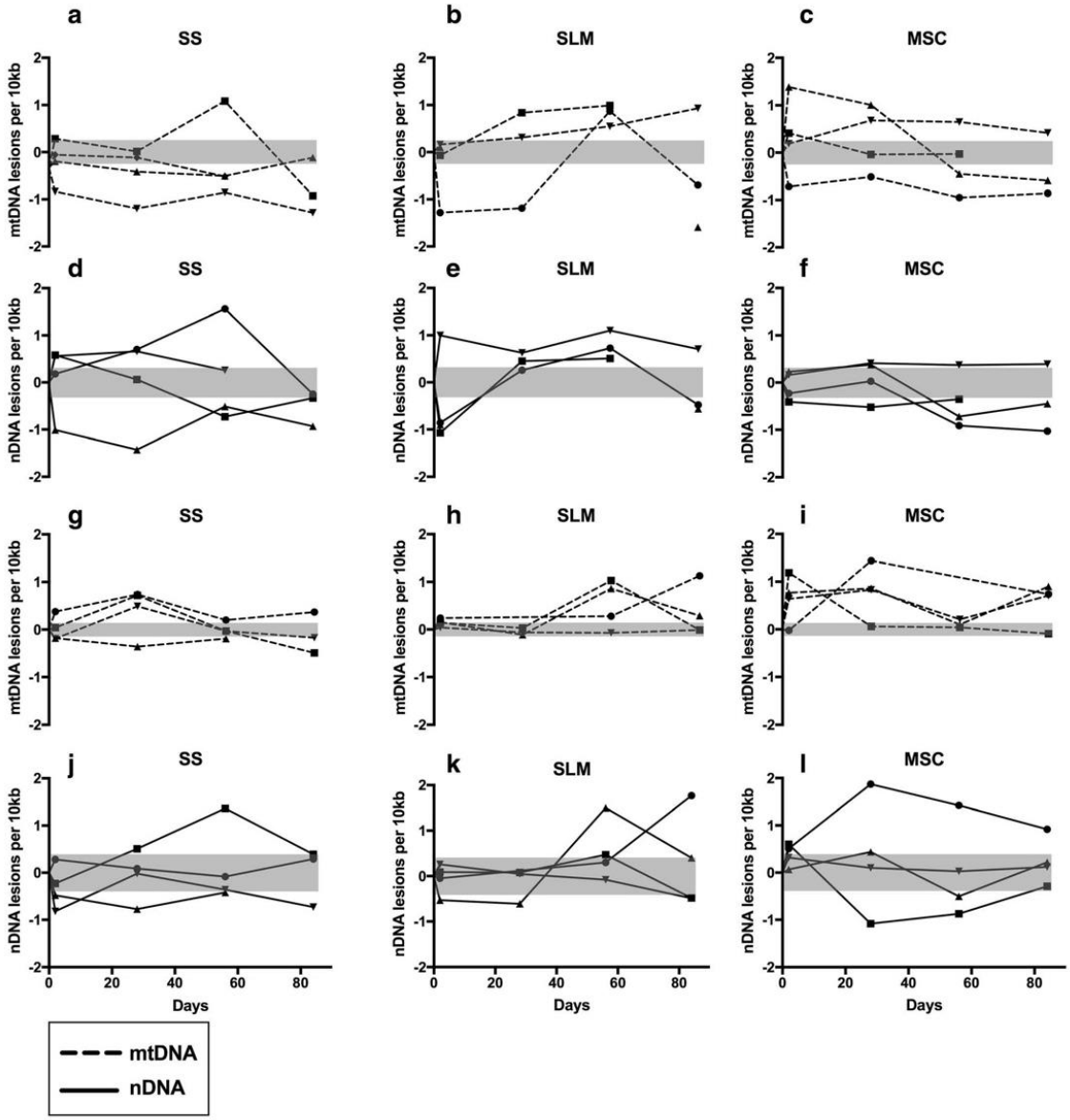

Fig. 1 DNA damage assay evaluating change in lesion rates per 10 kilobases in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) relative to pretreatment baseline in individual patients. The grey bars represent the 95% confidence interval for variance in mtDNA or nDNA lesions rates across two pretreatment samples taken 1–2 weeks apart in each disease cohort ($n = 8$ each cohort). **a**–**f** DNA damage in CLL patient cohort. **g**–**l** Solid cancer patient cohort. SS sodium selenite, SLM seleno-L-methionine, MSC Se-methylselenocysteine

Total Lymphocyte Counts

Statistical analysis of these small groups ($n = 4$ per Se compound per disease cohort) showed no significant changes in total lymphocyte counts over time in either the metastatic cancer or CLL cohorts for any Se compound (Fig. 3). Total lymphocyte counts were used because of inconsistency in automated haemocytometer discrimination of CLL cells from abnormal or normal lymphocytes. Only one patient met the International Working Group on CLL criteria for partial response [35], with a reduction of > 50% from baseline observed at week 12.

Table 3 Selenium plasma pharmacokinetic parameters

Parameter	SS, $n=8$	SLM, $n=8$	MSC, $n=8$
Plasma Se (μM), mean (SD)			
Baseline	1.29 (0.22)	1.28 (0.20)	1.31 (0.26)
Day 2 (4 h post-dose)	1.49 (0.23)	1.75 (0.26)[c]	1.57 (0.21)[e]
Day 28 (trough)	1.61 (0.1)[c]	2.72 (0.61)[d]	1.71 (0.27)[c]
Day 56[a]	1.49 (0.06)	3.01 (0.46)[d]	1.59 (0.12)
Day 84[b]	1.28 (0.13)[d]	2.13 (0.19)[c]	1.3 (0.16)
$AUC_{0\text{-}EOS}$ (μM h/L) (SD)	426 (389)[e]	2213 (480)[e]	485 (355)[c]

EOS end of study

[a] Samples taken on day 62 for 2 patients

[b] Samples taken on day 91 for 3 patients (equally split between compounds)

[c] $n=7$

[d] $n=6$

[e] $n=5$

Discussion

These data represent the first three-way comparison of these Se compounds, two of which are those most commonly used in clinical trials with cancer patients. While a number of studies have shown individual Se compounds to be tolerated at much higher doses in cancer patients, often in combination with anticancer treatments [25, 27, 36, 37], none have directly compared these compounds in the same study population. In this study, all three compounds were well-tolerated and assessed as safe to use at 400 μg elemental Se per day in this study population, with no clinically-significant treatment-emergent adverse events attributable to Se at this low end of the supranutritional Se dosing spectrum. MSC is the least studied of these Se compounds, but this study in cancer patients corroborates the reported safety of 400 μg MSC per day in healthy adults [32].

A key component of our safety analysis included an assessment of genotoxicity of these compounds. For this, we adopted a qPCR approach that enabled evaluation of both nuclear and mitochondrial DNA damage. Genotoxicity was insignificant at 400 μg per day for all Se compounds studied, compared to the variation seen without Se exposure. SS has been shown to generate single- and double-stranded DNA breaks [38–40] and other types of DNA damage at low levels of exposure (0.23 μM) in cultured human lymphocytes [41]. In contrast, the organic Se compounds used in this study appear to be safer: SLM was not genotoxic to human peripheral blood lymphocytes at Se concentrations of up to ~5.5 μM as measured by the cytokinesis-block micronucleus assay [42], and an extensive in vitro and in vivo toxicological assessment of MSC in mice failed to demonstrate any mutagenic effects at oral doses of up to 4630 μg/kg [43].

Conversely, Se compounds have been seen to exhibit antigenotoxic properties: both SLM and SS enhanced DNA repair in cell lines and cultured lymphocytes [44–46], as did methylseleninic acid (which is metabolised directly to methylselenol, comparable to MSC) in mouse mammary cells [47]. Thus, there is a conundrum in using Se in cancer patients: they have the potential to directly cause DNA damage

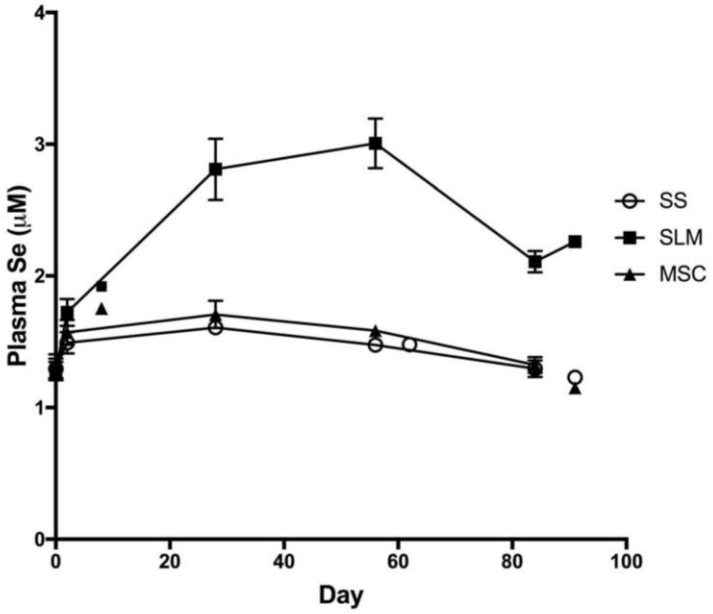

Fig. 2 Plasma selenium concentrations measured over 56 days of treatment and 4–5 weeks after the last dose. Error bars are ± SEM. SS sodium selenite, SLM seleno-L-methionine, MSC Se-methylselenocysteine

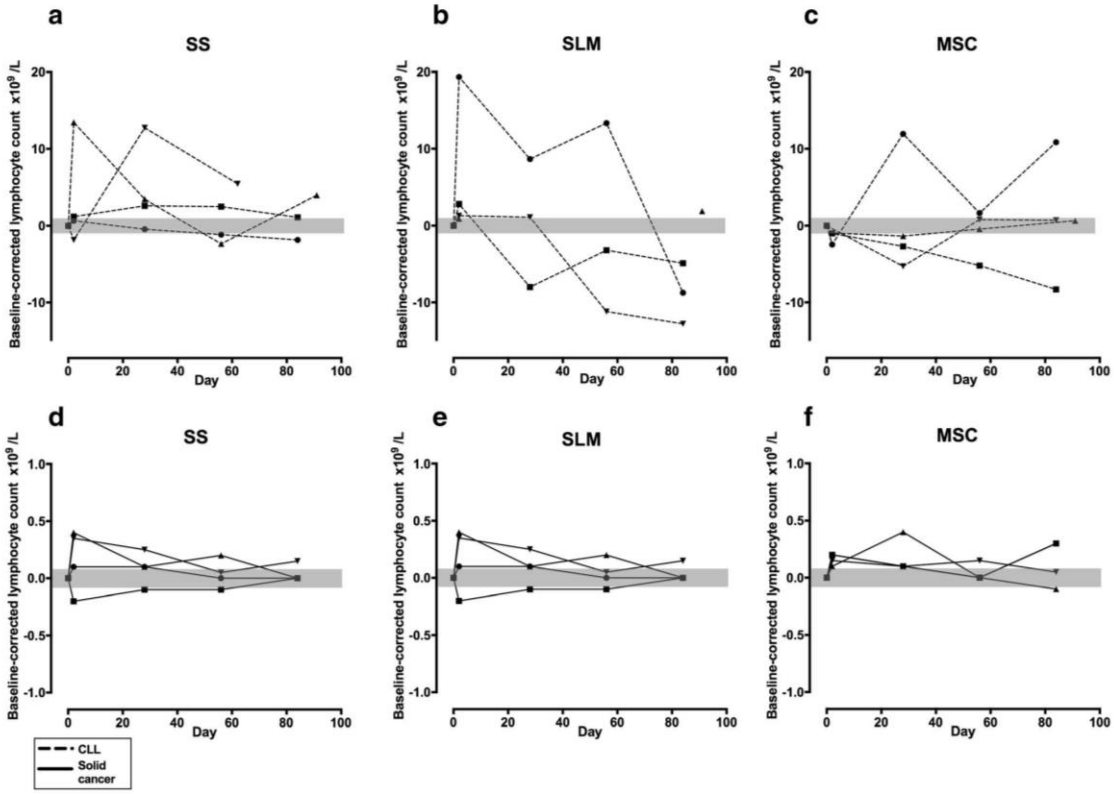

Fig. 3 Changes in baseline corrected total blood lymphocyte count per patient (including CLL cells) during the study. **a**–**c** CLL cohort. **d**–**f** Solid cancer cohort. SS sodium selenite, SLM seleno-L-methionine, MSC Se-methylselenocysteine

yet conversely enhance repair of DNA lesions induced by anticancer therapies. The net benefit or harm will depend on the relative dose potencies of each Se compound for each effect, and the preclinical data to date suggests that the organic Se compounds have a better therapeutic ratio than the Se salts in this regard. However, the most relevant test is in vivo dosing in humans, and this clinical trial offers the opportunity to evaluate DNA damage and effects on DNA repair pathways in subsequent cohorts of patients treated at higher dose levels of these Se compounds, that are predicted to cause significant DNA damage, especially with SS.

The PK profile of the Se compounds highlights the greater systemic exposure that occurs with SLM compared to those receiving MSC or SS. The lower plasma Se levels achieved with SS may be due to rapid plasma clearance of Se by red blood cells [48]. The much larger increases in plasma Se levels with SLM, compared to other Se compounds, has been widely observed [32, 49]. Explanations of this phenomenon relate to the non-specific incorporation of SLM into proteins, particularly albumin, in place of methionine, resulting in systemic accumulation and slower total body clearance [50]. In single-dose studies, over a third of MSC is excreted via urine or faeces within 12 days, compared to only 15% of SLM [51]. In our study in both SS- and MSC-treated patients Se plasma levels returned almost to baseline after a 4-week washout period, whereas in the SLM group, mean plasma Se levels were still about 50% above baseline at the same time point. The difference in PK profiles suggests that control of systemic exposure is much more easily achieved with MSC and SS, whereas SLM presents challenges in terms of systemic accumulation and clearance, which may be relevant to toxicities at higher doses and interactions with cancer therapies.

The pretrial assumption of limited anti-cancer efficacy at the lowest dose level was confirmed, with no reductions in serum PSA or CA15-3 tumour markers observed. In a number of patients in the CLL cohorts, administration of organic Se compounds (SLM or MSC) was associated with a reduction in lymphocyte counts (predominantly CLL cells) but sample sizes were small and no statistical significance was found. However, no such trend was seen in lymphocytes in patients with solid malignancies.

In previous preclinical studies using SLM in tumour xenograft models, a minimum plasma Se concentration of 15–20 μM was required to elicit therapeutic augmentation with anticancer drugs in mice [7]. Consistent with this, Se concentrations < 10 μM were ineffective at reducing VEGF, HIF-1α and HIF-2α in renal cell carcinoma preclinical models [52]. Inorganic forms of Se have shown some potential anticancer activity in human studies at high doses; for example, high-dose oral sodium selenate (60,000 μg/day) in patients with castration-resistant prostate cancer showed a modest increase in mean PSA doubling time during treatment [36]. Intravenous SS was administered to 34 patients with various therapy-resistant tumours; while the focus of the study was safety, treatment with IV SS was able to re-sensitise some patients to chemotherapy on which their tumours had previously progressed [20].

Further analysis is underway looking at PD markers of Se activity in both malignant and non-malignant white blood cells from patient samples and will be reported separately. Particular focus will be on markers of angiogenesis, endoplasmic reticulum stress response and gene expression profiling of the DNA damage response apparatus. Analysis of this data will inform an updated panel of PD biomarkers that, subject to additional funding grant applications being successful, may be evaluated in patients treated at higher doses of these three Se compounds (1600 μg/day and 6400 μg/day of elemental Se), aimed to achieve plasma Se levels at which PD mechanisms are activated in preclinical models.

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Authors' Contributions SOE contributed to study design, sample collection, performed the experiments, analysed the data and wrote the manuscript. GMJ contributed to experimental design, laboratory experiments and contributed to the manuscript. SB contributed to experimental design and contributed to the manuscript. HJBG contributed to study conduct, patient assessment and to the manuscript. MBJ, as study PI, conceived the project, wrote the trial protocol, oversaw the clinical and experimental aspects of the study (including patient assessment), assisted with data collection, analysis and interpretation and co-wrote the study manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Ethics Approval Ethical approval was obtained through Northern A Health and Disability Ethics Committee (HDEC) (13/NTA/172). All procedures performed in this study were in accordance with the ethical standards of the Waikato DHB research committee and HDEC and with the 1964 Declaration of Helsinki and its later amendments.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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