School of Biomedical Sciences

Novel Strategies for Inhibiting Cancer Growth

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

Signature: 

Date: 6. 08. 2010

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ABSTRACT

At present, most cancers are treated with surgery, radiotherapy and chemotherapy, used alone or in combination. Surgery and radiotherapy are the primary treatment modalities after early detection of cancers and they significantly increase the patient survival time. However, the value of cytotoxic chemotherapy has recently been questioned (Morgan, Ward and Barton 2004. *Clinical Oncology* 16: 549-560) as it became apparent that single cytotoxic chemotherapy barely contributes to the five-year survival rate in cancer. However, different chemotherapeutic strategies may be combined improving their efficacy dramatically.

In this study, ninety two different compounds having anti-cancer potential were tested in various combinations against four human cancer cell lines (MCF-7 breast; HepG2 liver; Caco-2 colon and JU77 mesothelioma) in an attempt to find compounds that effectively eradicate cancer cell lines within a therapeutic range. The cancer cell lines have been chosen to illustrate the diversity in cancer cell signaling. The diversity of the signalling patterns in different cancer cell lines becomes apparent when monitoring the viability of these cell lines during different chemotherapeutic treatments. Several potent combinations of narrow and broad-spectrum inhibitors were discovered that combined well in killing cell lines. Most of these combinations were specific against single cell lines. For example, PD98058, LY294002, bromocriptine and 120nM staurosporine promoted cell death in JU77 mesothelioma cells, but this combination was not fully effective in killing MCF-7 cells. On the other hand, bromocriptine or oxytocin and 120nM staurosporine effectively killed MCF-7 cells but not JU77 cells. However, some combinations worked effectively to kill multiple cell lines. Two such examples include geftinib and 120nM staurosporine or selenomethionine and selenite.

Broad-spectrum inhibitors alone effectively killed cell populations, but at concentrations too high to be applied *in vivo*, due to their toxicity. However, the concentration of the broad-spectrum inhibitors needed in the combinations, to ensure eradication of a cell line, could be substantially reduced (in some cases by several orders of magnitude) by using appropriate combinations of narrow-spectrum inhibitors. For example, 6μM staurosporine alone was required to kill JU77 cells. However, 6nM staurosporine was effective in killing JU77 cells when combined with PD98058, LY294002 and rapamycin. Other successful combinations included: 1. vitamin C, suramin and letrozole; 2. flavine adenine nucleotide or selenotrisulphides and β-lapachone; 3. a most promising combination, because of its potency and bioavailability, was selenite combined with selenomethionine and lithium chloride.

Whilst the culture system using banks of cancer cell lines can be a useful tool for selecting effective cytotoxic compounds and their combinations, its translation to effective *in vivo* strategies is limited. In most such studies fetal calf serum is included as a growth supplement for cells in culture. However, this study demonstrates that the inclusion of adult human albumin is also important as the adsorption of compounds to it differ significantly to fetal albumin (Brodersen and Honore 1989 *Acta Paediatr.Scand.* May, 78(3): 342-6). However, of the combinations reported above combination 2. and 3. remained effective in adult human albumin within a therapeutic range.

A tumour model was then established by injecting JU77 mesothelioma cells into nude balb/c mice. JU77 cells are a “non-tumourigenic” human cell line. However, the cells became “tumourigenic” when insulin-like growth factor-1 was also co-injected on a daily basis. In this manner, a subcutaneous tumour of JU77 mesothelioma cells rapidly grew approaching maximum

size over nine days. Nude balb/c mice bearing JU77 tumours were treated with selenite, selenomethionine and lithium chloride combination while supplementing insulin-like growth factor 1 continuously. Tumours rapidly decreased in size and became invisible after five days. In contrast, tumours treated with carrier and insulin-like growth factor 1 continued to grow. Low side effects of the treatment with the selenite combination were observed. The most notable observation was a moderate loss in weight of the mice, only following the first day of treatment. The inclusion of insulin-like growth factor 1 to maintain the tumours altered some of the haematological and biochemical parameters but the selenite combination did not further change these parameters. The histology of the tumours illustrated thick, closely packed layers of malignant cells that surrounded a central necrosis and rapidly decreased in thickness as the selenite combination was applied leaving behind a few clusters of necrotic cells and an inflammatory infiltration. In normal tissues, only a few abnormalities were discovered mainly due to the inherited defect of the nude mice. These abnormalities were exaggerated by treatment with insulin-like growth factor 1 but did not further change with the selenite combination. These results indicated that the selenite combination affected the tumour tissues highly selective with little side effects to the normal tissues.

In future, it will be interesting to know whether this compound combination is similar effective for tumours that grow without supplementation of insulin-like growth factor-1. In other tumour models, similar high concentrations of insulin-like growth factor-1 have been reported without supplementation due to existing “autocrine loops” and this might be a reason why the immune responses in these models is also disabled against aberrant cancerous signalling. This interesting new aspect which focuses on the endocrine response due to aberrant cancerous signaling is a promising path for inhibiting cancer growth.

1.0 LITERATURE REVIEW

In 1971, Richard Nixon declared a “war on cancer”. But despite all good intentions, the incidence of cancer continues to rise at an alarming rate. In 2007, over 7 million people worldwide died from cancer. By the year 2050, it is estimated that the human toll will exceed 17 million people worldwide. The impact on our lives is staggering, from both a human and financial perspective.

The primary modalities for treating cancer include surgery, radiotherapy and chemotherapy, used alone or in combination. The value of some of these strategies, in particular conventional chemotherapy, has been questioned because it has been illustrated repeatedly in large studies that chemotherapy contributes very little to the five-year survival rate in cancer.

In 2004 the Australian oncologists Morgan, Ward and Barton published an article, “The Contribution of Cytotoxic Chemotherapy to 5-year Survival in Adult Malignancies”. The authors conducted an evidence-based analysis of cancer treatments. They found an overall 5-year survival rate in adult malignancies in Australia of 60%. Cytotoxic chemotherapy increased the survival rate by only 2.3% in Australia and 2.1% in USA. The authors emphasized that these figures “should be regarded as the upper limit of effectiveness” (i.e., they are an optimistic rather than a pessimistic estimate) and concluded that the funding and availability of chemotherapeutic drugs should undergo rigorous evaluation in terms of cost-effectiveness and impact on quality of life.

Interestingly, this is not the first time that efficacy of chemotherapy has been reviewed. In 1992, the German epidemiologist Abel reviewed the chemotherapeutic success rates of more than 350 medical centres around

the world and estimated the success rate as 3%. He concluded that there is simply no scientific evidence available that chemotherapy can “extend in any appreciable way the lives of patients suffering from the most common organic cancers”. Obviously, despite a huge progress in drug development over the years the success rate of chemotherapy has stayed the same. Scientists and clinicians all over the world are looking for possible reasons.

Analysing the reasons for treatment failures, experiments were undertaken to illustrate how cancer cells are able to counteract the lethal effects of chemotherapeutics. Insulin-like growth factor-1 receptor was recognised as an important survival receptor on mammalian cells and has been the focus of intense research. Its pathway interactions in cancer have been reported by Navarro and Baserga in 2001. They observed that insulin-like growth factor-1 receptor activates three separate signals through insulin-receptor substrate 1, Shc and the mitochondrial Raf location. While the activity of the receptor did not cease when inhibiting a single pathway, it ceased when inactivating any two of the three pathways or all of them. Navarro and Baserga described this survival signalling as “limited redundant”. Still, inhibiting one pathway alone was not enough to induce cell death which explains why some compounds with narrow target structures fail to kill the cancer cells.

A second important observation was made by Thimmaiah *et.al.* in 2003 who reported that a downstream molecule in the signalling cascade of the insulin-like growth factor-1 receptor interacted with molecules of other signalling systems. The signalling systems together determined cell death. This explains that single receptors inhibited alone rarely promote cell death. In most cases, a combined inhibition of different receptor systems is necessary to achieve the desired effect.

To date, only a few reports of chemotherapeutic combinations targeting simultaneously different receptor systems are published. In investigating this complex network of receptor systems in terms of cellular function, especially in terms of survival, many unknown interactions have yet to be examined.

However, compared to the complexity of the signalling network and the task to investigate it, most of the metabolic interactions already seem to be characterised: The changes in the synthesis and breakdown of glucose, fats and proteins as well as energy creation and consumption along membrane structures have been well described (Nelson and Cox 2000). It seems as if the cellular "hardware" (the structure of molecules and molecular interactions) is known quite well while the cellular "software" (how molecular reactions are regulated) remains a challenge. Treatment with chemotherapeutic agents is described either by a change in signalling pathways or by a change in metabolism. Obviously, signalling creates metabolic consequences and metabolism induces signalling changes. A single metabolic change inside a cancer cell has the potential to create a snowball effect and change the total metabolism of these cells as well as their signalling structure.

The following review describes some relevant metabolic changes in the malignant cells and their hosts. Like a jigsaw puzzle, pieces of information will be assembled, describing a mechanism that has the potential to explain the initiation and perpetuation of cancer growth. Out of several clinical options, chronic renal failure has been chosen as an example to demonstrate how the metabolism shifts from the carbon cycle to the nitrogen cycle, and the potentially disastrous consequences of this kind of precancerous situation. In chronic renal failure (CFR), the incidence of cancer is increased (Stopper *et.al.* 2003). This increase in cancer incidence can be

explained by the simultaneous elevation of levels of advanced glycosylated end products (AGEs) (for example methylglyoxal-BSA), which are genotoxic and change metabolism. Experiments infusing advanced glycosylated end products, for example methylglyoxal-BSA, into healthy animals leads to increased cancer incidences similar to chronic renal failure (Stopper *et.al.* 2003).

The origin of methylglyoxal synthesis was first described by Freedberg, Kistler and Lin in 1971, who discovered that in Escherichia coli the conversion from glycerol to triose phosphate had two control mechanisms: the rate of synthesis of glycerol kinase and the feedback inhibition of its activity by fructose-1,6-bisphosphate. A strain that had lost both control mechanisms by double mutation, resulting in excessive synthesis of glycerol-3-phosphate, produces a bactericidal factor from glycerol in a two step reaction: the excess glycerol-3-phosphate is converted to dihydroxyacetone phosphate, which is further metabolized to methylglyoxal. In the mammalian cell, glycerol is mainly produced from triglycerides. This is illustrated in Fig 1.

Regulation of methylglyoxal-toxicity was discovered by Zhu and Lin in 1987. They observed that in bacteria like Escherichia coli accumulating glyceraldehyde and lactaldehyde attenuate the toxicity of methylglyoxal. Glyceraldehyde and dihydroxyacetone phosphate are produced when growing Escherichia coli on L-galactose (a structural analogue of L-fucose); lactaldehyde and dihydroxyacetone phosphate are formed when growing E.coli on L-fucose. However, normally the aldehydes are further metabolized and loose their attenuating effect. Zhu inhibited the metabolising enzymes (aldehyde dehydrogenase or lactaldehyde dehydrogenase), enhancing thereby the concentration of these aldehydes and observed an attenuating effect.

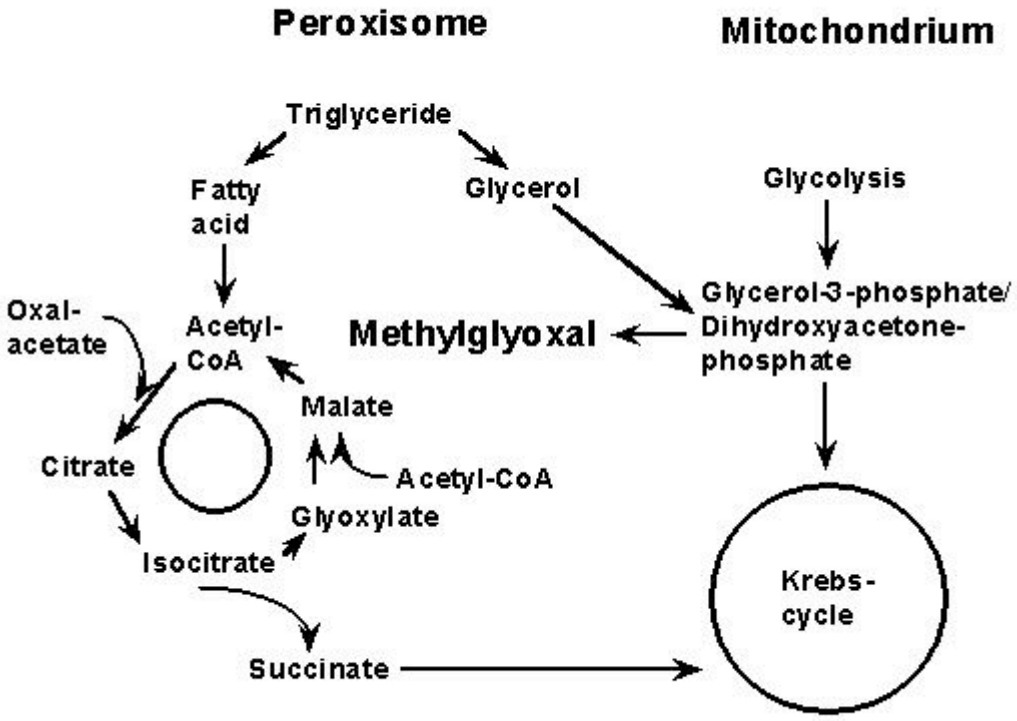

Figure 1 Relationship between the glyoxylate cycle (fatty acid cycle) in the peroxisomes and glycolysis in the mitochondria

Similar to the situation in bacteria, both metabolites L-glyceraldehyde and L-lactaldehyde inhibit the substrate methylglyoxal in mammalian cells and exert a protective effect against its toxicity (Ray *et.al.* 1994; Guerra *et.al.* 2006). Inhibiting the dehydrogenases also enhances the aldehydes in mammalian cells. Biswas *et.al.* in 1996 realised that these aldehyde metabolising enzymes are totally absent in rapidly growing, highly malignant cells.

Methylglyoxal is metabolized in two different pathways: normally it is broken down by glyoxylase I and II (Brandt and Siegel 1978): glyoxylase I (S-lactoyl-glutathione methylglyoxal-lyase) and glyoxylase II (S-2 hydroxyacylglutathione hydroxylase) transform methylglyoxal to S-D-lactoylglutathione and to lactate/glutathione (Creighton *et.al.* 1988). Alternatively, methylglyoxal can be metabolized by methylglyoxal reductase (Murata *et.al.* 1985), non-specific aldose reductase (Van der Jagt *et.al.* 1992), aldehyde dehydrogenase and/or alcohol dehydrogenase (Misra *et.al.* 1996), which transform it to acetol/lactaldehyde and later to 1,2-propanediol/lactate (Van der Jagt *et.al.* 1992), as shown in Fig 2.

Excess methylglyoxal and/or acetol react with albumin *in vivo* forming advanced glycosylated end products (AGEs) (Van der Jagt *et.al.* 1992). However, the direct measurement of AGE`s in blood is problematic (Brandt and Siegel 1978). Inhibiting aldose reductase reverses the effects of advanced glycosylated end products (Nakamura *et.al.* 2000) and switches glucose toxicity back to hyperglycemia (Sun *et.al.* 2006). Remarkably, AGEs change metabolism in cancer cells: complex I of the electron chain is inhibited in malignant cells and no effect is observed on the respiration of normal cells (Ray *et.al.* 1991). AGEs inhibit the glycerol-3-phosphate dehydrogenase complex (Ray, Basu and Ray 1997) and the glycolysis (Nicolay *et.al.* 2006) of tumour cells, thereby decreasing their ATP and

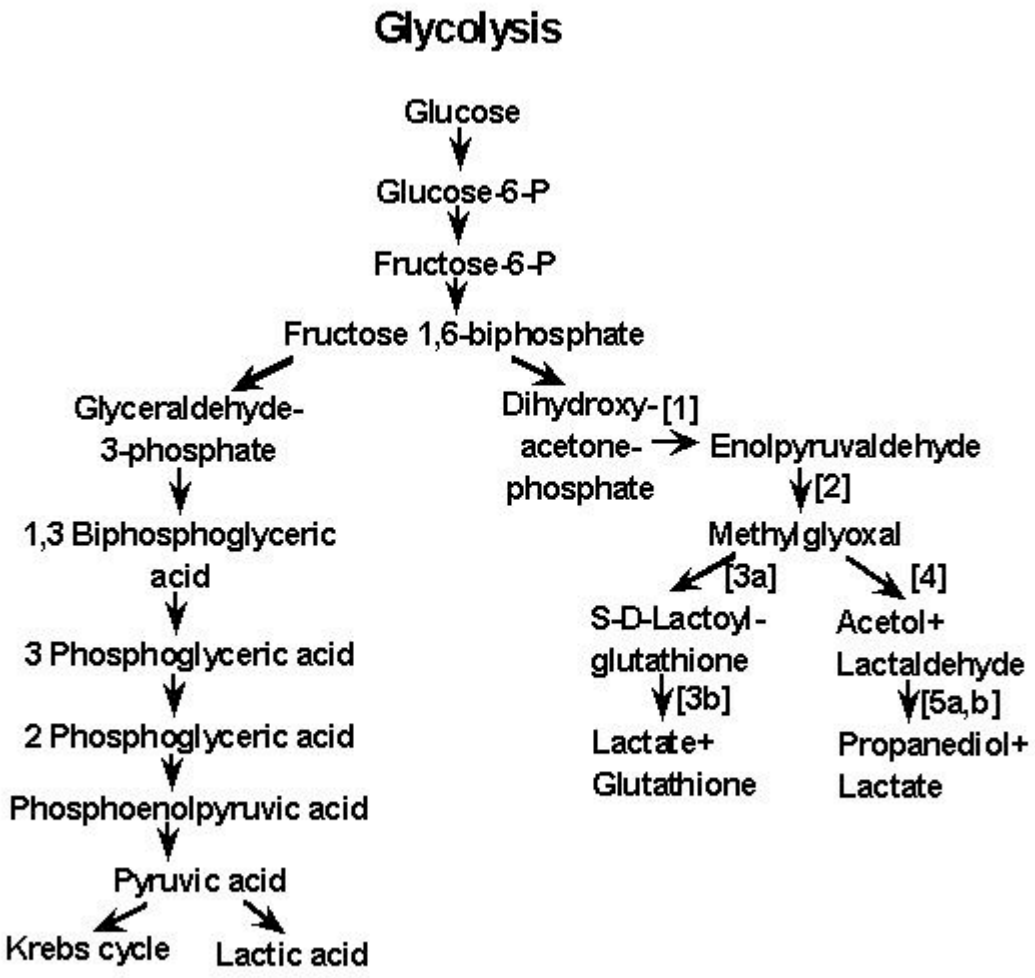

Figure 2 Glycolysis and methylglyoxal production:

[1] Methylglyoxal synthetase, [2] Tautomerization, [3a] Glyoxylase I, [3b] Glyoxylase II, [4] Aldose reductase, [5a] Acetol reductase, [5b] Lactaldehyde dehydrogenase

glutathione. They also strongly inhibit alpha-ketoglutarate-, malate- and pyruvate-dependent respiration in cancer cells (Ray *et.al.* 1994). Finally, by inhibiting alpha-ketoglutarate dependent respiration in cancer cells, AGEs cause nitrosative stress (Obrosava *et.al.* 2005). This is crucial for the development of cancer as explained below.

Schreibmayer *et.al.* in 1980 discovered that the absolute concentration of alpha-ketoglutarate is highest in tumours, while the absolute concentrations of citrate, succinate, malate and glutamate are highest in host livers. A possible explanation of this observation is that AGEs are also formed in tumour-bearing diseases, inhibiting alpha-ketoglutarate dehydrogenase. However, another option is that tumour bearers synthesize alpha-ketoglutarate in excess. Schreibmayer stated only that tumours were the primary source of increased alpha-ketoglutarate in blood of tumour bearers and that alpha-ketoglutarate and glutamate did not correlate in livers or in tumours. The regulation of alpha-ketoglutarate metabolism is of great interest as a potential target for cancer therapy, in particular because methylglyoxal influences it.

Rakhamanova and Popova in 2006 indicated that synthesis of alpha-ketoglutarate is mainly determined by cytoplasmic NADPH-isocitrate dehydrogenase (86%), whereas utilization is dependent on cytoplasmic aspartate aminotransferase (78%). Alpha-ketoglutarate links the carbon and nitrogen cycles: inhibiting metabolism of alpha-ketoglutarate inside the carbon cycle, for example with methylglyoxal promotes its accumulation and enhances transamination from amino acids through alpha-ketoglutarate to glutamate. The ammonium group of glutamate can either be passed on to oxalacetate forming aspartate or can be part of carbamoyl phosphate synthesis which is used for the urea cycle (Nelson and Cox 2000, 634).

Overall, excess alpha-ketoglutarate will lead to increased catabolism of amino acids and nitrogen flux into the urea cycle (see Fig 3).

Consequently, inside the urea cycle arginine will be elevated. It either forms ornithine and urea, or it reacts with calcium ion-dependent nitric oxide synthetase to produce nitric oxide (NO) (Nelson and Cox 2000, 449) and citrulline. While urea excretion will not increase due to renal impairment, the metabolic urge to produce nitric oxide increases steadily in a vicious circle. Nitric oxide will partially act as a dissolved gas, but it can also form instant reactions with albumin, immunoglobulin and sulphydryl-group containing enzymes, thereby forming S-nitrosothiols. Excess S-nitrosylation of all kinds of intra- and extracellular enzymes is called "nitrosative stress".

Surprisingly, Christen *et.al.* in 2007 observed that until birth foetuses produce high amounts of S-nitrosylated albumin. Foetal calf serum is a mandatory supplement in cancer cell experiments: cancer cells do not grow in the absence of S-nitrosylated albumin: for example when supplemented with adult (non-nitrosylated) albumin only. However, the amount of nitrosylated albumin can vary, so part of the foetal calf serum may be replaced by adult albumin. With S-nitrosylated albumin sufficiently available, transnitrosylation of other sulphydryl-groups containing molecules takes place immediately (Scharfstein *et.al.* 1994).

Transnitrosylation across the cell membrane is mediated by L-cysteine which is taken up into the cell and leads to accumulation of intracellular S-nitrothiols of all kinds (Li and Whorton 2007). However, transnitrosylation may also happen as a reverse process from other sulphydryl-group containing molecules back to albumin (Tsikas *et.al.* 2001).

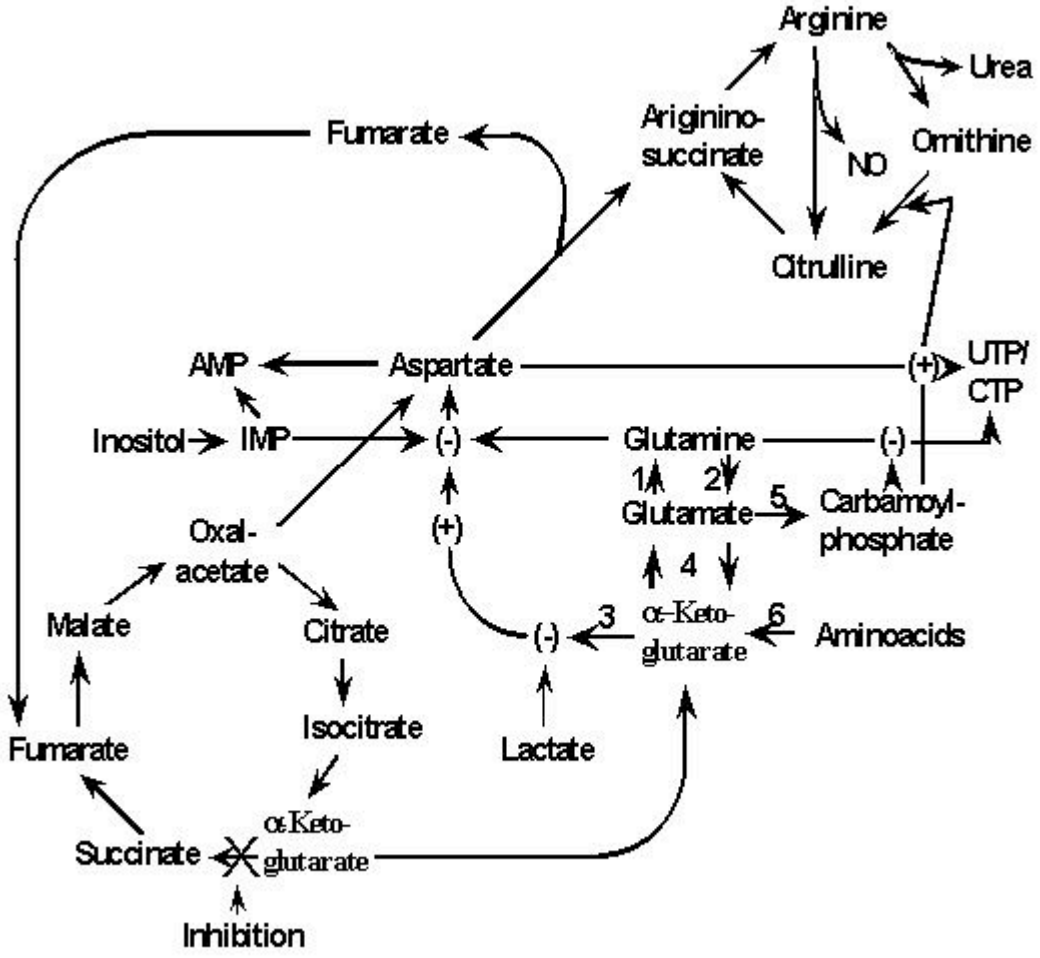

Figure 3 The regulation of metabolic flux from the carbon cycle (lower cycle) to the nitrogen cycle (upper cycle).

Raising concentrations of α-keto-glutarate influence the synthesis of aspartate, purines and pyrimidines. Enzymes which are involved are: 1 glutamine synthetase, 2 glutaminase, 3 aspartate transaminase, 4 glutamate dehydrogenase, 5 carbamoyl synthetase, 6 trans-amination from amino acids

The effects of S-nitrothiols are extensive. Some of these effects induce self-perpetuation of cancer cell growth. The following key observations are noteworthy:

1. Burzynski, Wang and Hnatowitch in 1995 observe that S-nitrosylation of albumin decreases palmitate adherence to albumin and that uptake of palmitate by hepatocytes increases 27%. More glycerol is synthesized, promoting more advanced glycosylated end products.
2. Mitsumoto *et.al.* in 2001 note that S-nitrosoglutathione interacts with glyoxylase I and inhibits methylglyoxal metabolism.
3. Freedman *et.al.* in 1995 and Naseem *et.al.* in 1996 describe S-nitrosoglutathione as inhibiting platelet function. Glutathione peroxidase or H_2O_2 potentiate this effect.
4. Padgett and Whorton in 1997 state that S-nitrosylation inhibits glyceraldehyde-3-phosphate dehydrogenase, thereby increasing dihydroxyacetone phosphate. This adds to the formation of advanced glycosylated end products induced by methylglyoxal, and starts a feedback loop in the metabolic cycles. The reaction is reversed by low-molecular weight thiols.
5. Jaiswal, LaRusso and Gores in 2001 illustrate the inactivation of mitochondrial aldehyde-dehydrogenase by S-nitrosylation which can act as a nitrate reductase.
6. Dahm, Moore and Murphy in 2006 observe that S-nitrosylation of complex I of the mitochondrial electron chain correlates with a significant loss of its activity, reversed by thiol reductants. This seems to be a similar mode of inhibition to methylglyoxal, regarding the mitochondrial complex I.

In addition, it has been noted that S-nitrosylation:

7. Changes signalling molecules like insulin receptor, insulin receptor substrate-1 and AKT (protein kinase B) (Kaneki *et.al.* 2007).

8. Inhibits caspases, the main effectors of apoptosis (Dall`Agnol *et.al.* 2006).
9. Promotes synthesis of nitrosylcobolamin from cobalamin (vitamin B12) inhibiting methylmalonyl-CoA mutase: the formation of succinyl CoA from methionine, threonine, isoleucine and/or valine ceases, and causes cell death in many cancer cell lines (Tang *et.al.* 2006; Nelson and Cox 2000, 650).
10. Inhibits ubiquitin-proteasomal degradation of Bcl-2. Bcl-2 is a key apoptosis regulatory protein of the mitochondrial death pathway (Azad *et.al.* 2006).
11. Targets hypoxia inducible factor-1 alpha, a key regulator sensing oxygen partial pressure (Sumbayev *et.al.* 2003).
12. Reversibly inactivates argininosuccinate synthetase (Hao, Xie and Gross, 2004).
13. Affects thioredoxin inducing apoptosis signal-regulating kinase 1 (Sumbayev 2003).
14. Inactivates methionine adenosyltransferase which converts methionine into S-adenosyl-methionine (Ruiz *et.al.* 1998).

This incomplete list demonstrates that S-nitrosylation switches metabolism, affecting all kinds of important cellular enzymes. Taken together, these findings suggest that S-nitrosylation is involved in onset and perpetuation of cancer. However, its specific role in various cancers is yet to be fully elucidated.

However, S-nitrosylation maybe reversed. Five different observations are reported: first, Stubauer, Giuffre and Sarti in 1999 emphasize that the redox state of copper determines if thiol groups scavenge or release nitric oxide: the two-valent copper ion (Cu 2+) promotes S-nitrosylation; the one-valent copper ion (Cu +) releases nitric oxide from thiol groups. A compound like penicillamine, which reduces the copper ions, releases nitric oxide from

thiol groups and reduces S-nitrosylation. Second, simultaneous dimerization of glutathione competes with and reverses S-nitrosylation in the presence of Cu (2+). Third, serum concentrations of ceruloplasmin determine the rate of glutathione nitrosylation (Inoue *et.al.* 1999). Tetrathiomolybdate decreases the transcription rate of this multi-copper-containing plasma protein with ferroxidase activity, slowing down S-nitrosylation. Fourth, Vanin, Malenkova and Serezhenkov in 1997 discovered that a concentration level of more than 10μM ferric iron ion concentration (Fe 2+) destabilizes S-nitrosylation. The ratio of oxidized to reduced iron (Fe 2+/ Fe 3+) can increase due to chelation or reduction of oxidized iron. Coves and Fontecave in 1987 characterised an enzyme called NAD(P)H:flavin oxidoreductase (or flavin reductase) catalysing the reduction of free flavins, flavin mono-nucleotides, flavin adenosine dinucleotides or riboflavins, and rapidly transferring electrons to free iron or ferric complexes like ferrisiderophores, ferritin and ferric citrate (Sirivech, Frieden and Osaki 1974). Finally, reducing S-nitrosylation is also possible by inhibiting the uptake of S-nitrosylated L-cysteine with large neutral amino acids (Li and Whorton 2007): this reduces incorporation of S-nitrosylated cysteine and the following transnitrosylation from S-nitrosylated cysteine to other sulphydryl-group containing molecules.
In summary, a vast increase in protein metabolism and a shift from the Krebs cycle to the urea cycle can be observed in situations with enhanced intracellular α-ketoglutarate concentrations. Additional ammonium flux into the urea cycle results in extensive nitrosylation and nitrosation of proteins. This creates a procarcinogenic situation. Strategies to inhibit nitric oxide generation during chronic inflammation or to scavenge reactive nitrogen species may prove useful in decreasing the risk of cancer (Jaiswal, La Rousso and Gores 2001). Another important aspect of excess ammonium flux is uncontrolled pyrimidine synthesis, which is an important requirement for unlimited cancer cell growth. Usually, pyrimidine synthesis

is regulated by the concentration of glutamine. This can be bypassed by large amounts of ammonium ions metabolized through the carbamoyl phosphate pathway (Monks, Chisena and Cysyk 1985) as illustrated in Fig 3. Under these circumstances increased pyrimidine synthesis is solely dependent on the availability of aspartate (Pausch *et.al.* 1985). Availability of aspartate is dependent upon its origin: inhibition of aspartate synthesis from oxalacetate in cancer cells is caused by malate and results in the formation of citrate and alanine (Moreadith and Lehninger 1984). However, in normal cells malate increases aspartate production. Inhibition of synthesis from glutamine is caused by inositol or lactate in either cell type and lactate is derived from the ribose moiety of inosine (Kovacevic *et.al.* 1987; Kovacevic, Brkljac and Jerance 1991).

Availability of aspartate is also dependent on its metabolism: aspartate and inositol form purines: adenosine monophosphate (AMP) and guanidine monophosphate (GMP). Purines inhibit cancer growth (Hugo *et.al.* 1992). However, aspartate and carbamoylphosphate also form the precursors for pyrimidine synthesis. Therefore, aspartate which may be consumed in other synthetic pathways can be a limiting factor in a situation where excess, uncontrolled pyrimidine is synthesised due to elevated ammonium flux. This review of the literature dealing with interconnections between the fatty acid cycle, the Krebs cycle and the urea cycle is not a complete picture of the pathways, but does provide the necessary information to understand the inhibiting effects of compound combinations and their effect on the cellular metabolism. The literature illustrating interconnections between signalling pathways is more complex: Lodish *et.al.* describe in 2003 a huge diversity of receptors on the cell membrane. Some important receptor families are receptor tyrosine kinases, G-protein coupled receptors, cytokine receptors, TGF-beta receptors, hedgehog receptors, Wnt receptors, and Notch receptors. They regulate the transcription of genes, the translation of

messenger RNA and most protein interactions. Each family is further subdivided. As an example, the first group of receptors mentioned above, the receptor tyrosine kinase family, comprise an insulin-like growth factor-1 and -2 receptor, insulin receptor, epidermal growth factor receptor, platelet derived growth factor, vascular endothelial growth factor, fibroblast growth factor, nerve growth factor and others. The receptors of each family can interconnect with members of other families: the tyrosine-kinase receptor family can connect with the G-protein-coupled receptor family but it may also connect with any other receptor family. An overview of the connections between only two of the families, the tyrosine kinase receptors and the G-protein coupled receptors, is illustrated in Fig 4 (Alberts *et.al.* 2002). But many interconnections are still unknown: In principle, Fig 4 illustrates that each receptor has at least one, but usually several, signalling pathways. The pathways of different receptors either share signalling molecules or interconnect at certain signalling levels.

Additionally, an orchestra of intra-cellular receptors exists, located in either the cytosol or the nucleus. Nuclear receptors include hormone receptors (oestrogen-, corticoid-, androgen-, parathormone etc.), nuclear factors (nuclear factor-kappa B, hepatic nuclear factor) and others (Alberts *et.al.* 2002).

Complete inhibition of the overlapping receptor signalling pathways depends on two factors: first, all the possible diversions and connections of the combined receptors have to be taken into account. As a general rule, this is most easily achieved by inhibiting targets closest to the receptors. As more signalling molecules in a cascade are activated, it becomes more difficult to achieve total inhibition of a pathway. Second, the limited redundancy of each signalling combination must be considered. Not all possible inhibitions have to be in place at the same time, because inhibition

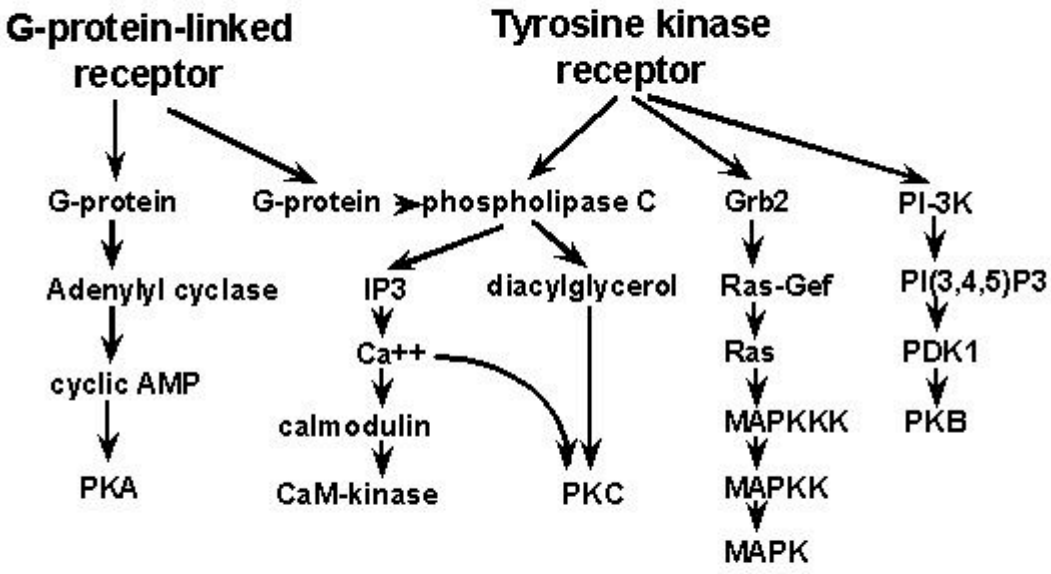

Figure 4 Signalling connections between the G-protein-linked receptors and the tyrosine kinase receptors.

(Adapted from Molecular Biology of the cell, B.Alberts et.al. 2002.)

of a critical share of them can impair signalling function (Navarro and Baserga 2001).

As a result, redundancy can be different in cancer cells compared to normal cells due to the differences in the signaling pathways. Ahmad et al. in 2004 observe that insulin-like growth factor-1 in normal cells requires the epidermal growth factor receptor tyrosine kinase to exert a mitogenic effect. However, in cancer cells it does not. There are many other examples illustrating how cancer signalling varies. These crucial cancer-specific differences emerge as possible targets for treatment. Usually, treatments target only a single cancer-specific structure. However, the simultaneous inhibition of several cancer-specific targets could be more effective.

This review focuses on compounds that have been chosen primarily because, used singularly, they kill cancer cells selectively. The toxic side-effects of many of these compounds *in vivo* observed at the doses killing complete cell populations *in vitro* are invariably problematic. However, combining these compounds reduces the concentration needed to kill cancer cells but does not decrease their selective action between cancer and normal cells. Quite often, a therapeutic range can be achieved. It is not intended to combine these compounds with commonly used chemotherapeutic agents. The aim is to target the limited redundant, constitutively activated signalling pathways of cancer cells inducing minimal side effects in normal cells.

The broad-spectrum inhibitors that will be investigated are staurosporine, genistein, mifepristone, vitamin C, selenite and trisulphides, β-lapachone, glycolysis inhibitors, antibiotics, diethylmaleate and hydrogen peroxide. These compounds have been chosen as most of them are known to be

cancer-selective and/or cancer-preventive. However, the chosen compounds are only a small selection in the huge variety of compounds that could have been chosen for investigation. Day by day, the number of natural broad-inhibiting compounds that undergo biochemical investigations expands rapidly. Pharmaceutical companies are more and more interested in developing some of these compounds for cancer therapy. Examples are staurosporine, genistein or β-lapachone. These broad-inhibiting compounds have the great advantage that they already target multiple structures in a prefixed fashion. When combining them with only a few additional compounds, they induce a powerful, cancer-selective treatment response. The complexity of their action is under intense investigation as many aspects of their broad-inhibiting action are still unknown. When listing their action, it sometimes seems as if there is little connection between different aspects of their actions. The following part of the literature review intends to list and to connect the effects of each of these compounds on cell surface receptors and signalling pathways. Where possible their mechanism of action is described. Finally, the therapeutic implications of using these compounds *in vivo* are described.

Staurosporine is a cell-permeable, potent compound, isolated from Streptomyces staurosporeus. It inhibits multiple cellular protein tyrosine kinases. Around a thousand protein tyrosine kinases are known to exist, of which only 91 have been identified. There are 59 receptor tyrosine kinases and 32 non-receptor tyrosine kinases (Sigma-Aldrich 2007). These enzymes catalyse phosphorylation of tyrosine residues of receptor and non-receptor types. Receptor protein tyrosine kinases are located in the membrane with an extracellular, transmembrane and intracellular domain. Their signalling can be aberrant in diseases like diabetes, in inflammatory diseases and cancer (Sigma-Aldrich 2007). Non-receptor tyrosine kinases are located in the cytoplasm or nucleus, or anchored to the inner leaflet of the plasma

membrane. These non-receptor tyrosine kinases are grouped into eight families: SRC, JAK, ABL, FAK, FPS, CSK, SYK and BTK. Most families are involved in cell growth and some of them are involved in cancer cell growth (Sigma-Aldrich 2007).

Staurosporine binds to the adenosine triphosphate binding (ATP) domain of tyrosine kinases and thereby inhibits them. It has a broad specificity. The concentration of stauropsorine needed to inhibit various kinases differs markedly (see Table 1).

At 6μM staurosporine induces apoptosis in all cancer cell lines. Apoptosis induced by staurosporine is positively regulated by translocating protein kinase C. Simultaneously, the induction of hepatocyte growth factor synthesis can be observed without protein kinase inhibition (Yagi *et.al.* 2003). This in turn induces angiogenesis independently of any vascular endothelial growth factor (Sengupta *et.al.* 2003). Staurosporine augments ornithine decarboxylase and activates Bcl-2 (Stressgen Bioreagents 2006). Apoptosis induced by staurosporine is negatively regulated by p38 and p44/42 mitogen-activated protein kinases as well as phosphatidyl-inositol-3- kinase involving the caspase cascade; it is also negatively regulated by protein kinase A without caspase involvement (Yamaki *et.al.* 2002). It inhibits topoisomerase II (Lassota, Singh and Kramer 1996), reversibly arrests human lung epithelial cells in cell cycle phase G 1, and is used to synchronize cell populations (Lin, Chrest and Gabrielson 1992). Staurosporine inhibits the insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters in adipose cells (Nishimura and Simpson 1994).

Table 1: Cellular kinases inhibited by staurosporine

CK1 (100µM)	CK = Cyclin kinase
CK2 (19.5µM)	
IGF-1R (6.2µM)	IGF-1R = Insulin-like growth factor receptor
CSK (2µM)	CSK = C-terminal SRC-kinase
ERK (1.5µM)	ERK = Extracellular-regulated kinase
EGFR (600nM)	EGFR = Epidermal growth factor receptor
IR (60nM)	IR = Insulin receptor
PKA (15nM)	PKA = Protein kinase A
PKG (18nM)	PKG = Protein kinase G
PKC a,b,g,d,e (58, 65, 49, 325, 160nM)	PKC = Protein kinase C
MLCK (21nM)	MLCK = Myosin light chain kinase
CaMK II (20nM)	CaMK =Calmodulin kinase
LYN (20nM)	LYN = LYN-kinase
SYK (16nM)	SYK = SYK-kinase
SRC (6nM)	SRC = SRC-kinase
S6K (5nM)	S6K = S6 Kinase
FGR (2nM)	FGR = FGR-kinase

In vivo, staurosporine is weakly and reversibly adsorbed to the red blood cells of rats. The endothelium of lung and heart acts as a sink for staurosporine. The plasma range after bolus injection of staurosporine is in the range of 1-10ng/ml for 2.7hr, which indicates that it has to be delivered by continuous infusion to stop cell proliferation (Gurley *et.al.* 1998). Its half-life in plasma is 51.6 minutes. In clinical settings, staurosporine and its combinations are too toxic for use, and are replaced by 7-hydroxy-staurosporine (UCN-01) (Fuse *et.al.* 2005).

Genistein is a soybean-derived protein tyrosine kinase inhibitor. The expression of the insulin-like growth factor-1 receptor is inconsistently up- or down-regulated depending on the cell line (Kim, Shin and Park 2005; Chen and Wong 2004). However, the expression, internalization and degradation of the epidermal growth factor receptor and its closely related ErbB2/Neu receptor are consistently down-regulated (Yang *et.al.* 1996) independent of the cell line and without any changes in autophosphorylation (Peterson and Barnes 1993). The transcription and translation of c-erbB-2 and c-raf-1 and their down-stream molecules c-jun and c-fos are decreased (Dalu *et.al.* 1998; Li et.al 2004). Genistein inhibits the glycine receptor (Huang and Dillon 2000). It decreases vascular endothelial growth factor (Ravindranath *et.al.* 2004), glutathione reductase (Elliot *et.al.* 1992), aromatase (Whitehead and Lacey 2003), topoisomerase I and II (Novogen 2007) and the signal transduction by inositol-3-phosphate (Shen and Weber 1997). Genistein increases basal cAMP levels and potentiates forskolin-induced cAMP accumulation (Burvall, Palmberg and Larsson 2002). It alters the cAMP-dependent chloride transport of the cystic fibrosis transmembrane conductance regulator, first activating it at low concentrations but inhibiting it at higher concentrations (Moran and Zegarra-Moran 2005). It also directly affects the transmembrane regulator (Illek *et.al.* 1995). Together with its metabolites equol and p-ethyl phenol, it

up-regulates prostaglandin F2 alpha (PGF2 alpha) (Woclawek-Potocka *et.al.* 2005). It up-regulates lipopolysaccharide-sensitive inducible nitric oxide synthetase through estrogen receptors (Nakaya, Tachibana and Yamada 2005). It increases metallothionine expression (Kuo and Leavitt 1999).

Genistein renders volume-sensitive chloride channels inactive (Shi *et.al.* 2002), disables the regulation of active Na+/K+-transport by dopaminergic agonists (Nakai *et.al.* 1999) and acts with insulin on the Na+/K+/2Cl-cotransporter (Ueda-Nishimura *et.al.* 2005). It binds to thromboxane A2 receptors and inhibits platelets (Guerrero *et.al.* 2005). It retards hepatic growth factors promoting wound healing (Watanabe *et.al.* 1994) as well as myoblast proliferation, fusion and myotube protein synthesis (Ji *et.al.* 1999). Below 60µM, genistein induces cellular differentiation (Constantinou, Krygier and Mehta 1998), dendrite-like structures and tyrosinase activity (Kiguchi, Constantinou and Hubermann 1990). Above 60µM , cell viability changes independently of topoisomerase II inhibition (Salti *et.al.* 2000). Genistein arrests the cell cycle in phase G2/M (Novogen 2007) and combines with quercetin to arrest the cell cycle in phase (G1/S) (Shen and Weber 1997) or with vitamin D to arrest the cycle in phase (G1/0) (Rao *et.al.* 2002).

In vivo, genistein adheres to subdomain IIa of human serum albumin and is displaced by other compounds fitting into this pocket, like warfarin, diazepam or similar isoflavonoids (Mahesha *et.al.* 2006). This changes the distribution, elimination and potency of the compound. Genistein is rapidly absorbed, glucuronidated in the gut wall with a significant entero-hepatic circulation (Sfakianos *et.al.* 1997). Not all humans metabolize genistein well, and are classified as high or low metabolizers. The half-live of genistein in plasma is estimated as eight hours (Watanabe *et.al.* 1998), but

in renal insufficiency it accumulates and may not return to the baseline for more than seven days (Walker *et.al.* 2001). Administered intravenously into the forearm, genistein produces a nitric oxide-dependent dilatation of the vasculature (Fanti *et.al.* 1999). In young, healthy females it changes the length of menstruation due to hormonal effects (Watanabe *et.al.* 2000).

Mifepristone (RU486) inhibits progestin-induced cell growth. However, in the absence of progestins RU486 exerts intrinsic activity and stimulates cell growth (Bowden, Hissom and Moore 1989). Mifepristone self-oxidizes with nitroso species catalysed by cytochrome P450 (CYP) 3A4 thereby complexing the heme of the enzyme and inactivating it (Jang and Benet 1998). It inhibits 17-hydroxylase activity in human testicular, ovarian and adrenal steroidgenesis (Sanchey *et.al.* 1989). In pregnant women, it sensitizes the myometrium to the contraction-inducing activity of prostaglandins. With increasing doses, mifepristone progressively inhibits the progesterone-, the glukokortikoid- and finally the androgen-receptor (RxList 2006). It competes with cyproterone acetate at the androgen-receptor binding site, forming an overlapping steroid scaffold in the binding pocket (Honer *et.al.* 2003). Mifepristone also inhibits the alpha folate receptor (Tran *et.al.* 2005).

Mifepristone decreases progestin-induced phosphorylation of insulin receptor substrate 2, phosphadityl inositol 3 kinase and protein kinase B (AKT) (Cui *et.al.* 2003). It suppresses transcription of the vascular endothelial growth factor and messenger molecule bcl-2 (Zhang *et.al.* 2006). However, it enhances signalling through phosphorylation of p38, p42/p44, and cyclin D1 and enables S-phase progression (Skildum, Faivre and Lange 2005). It stimulates the binding activity of nuclear factor-kappa B (Han and Sidell 2003), and the expression of cyclooxygenase 2 (Hardy *et.al.* 2006), and the phosphorylation of signalling molecule bax (Zhang

et.al. 2006). Mifepristone up-regulates nitric oxide production (Martin, Alalami and van den Berg 1999) and induces apoptosis in a dose- and time-dependent manner (Li *et.al.* 2003). It arrests cells in G0/G1 phase after completing the cell cycle (Li *et.al.* 2003).

In vivo, 70% of mifepristone is absorbed with 40% bioavailability after the first pass effect in the liver (Sarkar 2002). It is bound to albumin and alpha 1-acid glycoprotein, the latter being quickly saturated. After a single administration, mifepristone is eliminated non-linearly and can be detected in serum up to 11 days later. Mifepristone is metabolized by N-demethylation and terminal hydroxylation of the 17-propynyl chain, and finally excreted into the faeces (RxList 2006). Treatment with mifepristone enlarges the pituitary and the ovaries which can be explained as prolactin-induced estrogen effects that are not opposed by progesterone (Di Mattina *et.al.* 1987). The clinical picture is very similar to the polycystic ovary syndrome (Ruiz *et.al.* 1995). During a combined treatment with mifepristone and bromocriptin (a prolactin inhibitor), growth of the pituitary and the ovaries is significantly suppressed (van der Schoot, Uilenbroek and Slappendel 1990). These hormonal changes are beneficial in treatment of benign unresectable and/or recurrent meningiomas. Anti-neoplastic activity has been noticed in tamoxifen-resistant breast cancer and hormone- insensitive prostate cancer (Sartor and Figg 1996), and long-term treatment is well tolerated (Grunberg *et.al.* 2006).

Vitamin C and ascorbate exert antineoplastic activity and are used in combination with arsenic trioxide for the treatment of lymphoid malignancies such as multiple myeloma and some lymphomas (Campbell et.al. 2007). As antioxidants they prevent lipid peroxidation and protein oxidation in the presence of iron ions, copper ions and hydrogen peroxide (Suh, Zhu and Frei 2003). Vitamin C is oxidized to semidehydro-ascorbate

and dehydro-ascorbate (Linster and Schaftingen 2007), enters the mitochondria via the glucose transporter (Glut 1), accumulates there as mitochondrial ascorbic acid (mtAA) and protects mitochondrial DNA (KC, Carcamo and Golde 2005). It activates the p38 mitogen-activated protein kinase (Bowie and O`Neill 2000), induces circulating insulin-like growth factor binding proteins 1 and 2 (Peterkofsky *et.al.* 1994), expresses collagen IV and promotes retinoblastoma (Rb) phosphorylation. (Saeed, Peng and Mety 2003). It enhances concentration-dependent glucose synthesis (Braun *et.al.* 1996) and generates hydrogen peroxide, depending on the glutathione levels (Han *et.al.* 2004). Vitamin C increases synthesis of high density lipoproteins, decreases low density lipoproteins (Mehl-Madrona 2004) and inhibits enzymes that degrade the extracellular matrix. Vitamin C inhibits interleukin 1 and 8 (Bowie and O`Neill 2000), cyclo-oxygenase 2 (Han *et.al.* 2004), nuclear factor kappa B (Carcamo *et.al.* 2004), lactate dehydrogenase (Russel *et.al.* 2004) and steroid hydroxylation (Pintauro and Bergan 1982).

Alone or in combination with selenite, vitamin C causes growth inhibition, redifferentiation of cancer cells (Zheng, Sun and Wang 2002) and caspase independent apoptosis (Verrax *et.al.* 2004). In combination with vitamin K3 (menadione) it decreases the formation of lactate, adenosine triphosphate and nicotinamide adenine dinucleotide by up to 80% (Verrax *et.al.* 2007) and promotes “autoschizis”, a special form of cell death (Verrax *et.al.* 2003).

Therapeutic plasma concentrations above 400mg/dL are needed *in vivo* to treat cancer (CIHF 2007). However, 3g of vitamin C given four-hourly as a maximum tolerated oral dose, results only in plasma levels of 4.3mg/dL and a single intravenous dose of 50g vitamin C results only in plasma levels of 265mg/dL. Vitamin C is, therefore, used in combination with radiotherapy

or chemotherapy (Dettman 2003).

Selenium has been used to treat cancer in physiological and supra-physiological doses, as inorganic and organic compounds and in its four different states of oxidation, elemental selenium, selenide, selenite and selenate with oxidation states of 0, -2, +4 and +6.

Selenium uptake and insertion is unique. It has its own codon in mRNA that specifies its insertion into selenoproteins as selenocysteine (SeCys) (Rayman, 2005). Selenocysteine, the 21st amino acid, is then incorporated into selenoproteins. The mode of incorporation is significantly different from other amino acids (Hatfield and Gladyshev, 2002). Selenoproteins that may be particularly relevant to cancer risk include the glutathione peroxidases, 15kDa selenoprotein, selenoprotein P and the thioredoxin reductases (Rayman, 2005).

Selenium is a nutritional essential trace element with anti-carcinogenic properties (Nomura et al. 2000) and it has been reported that daily selenium supplementation in the diet reduces the overall development of cancers. In 1996, Clark et al. published a study in which he stated that a 10 year supplementation with a moderate daily dose of 200μg day^{-1} selenium-enriched yeast significantly reduced total cancer incidence, total cancer deaths and incidence of carcinomas at sites other than the skin. However, it was later pointed out by Coombs Jr. (2004) that the chemical specification of selenium in food is important to achieve this reduction in cancer risk. A number of mechanisms have been suggested to explain the anti-cancer effects of selenium. This list can only be incomplete. However, the main effects are listed by Rayman in 2005. The effects describe the differences between a low versus a normal selenium concentration in serum: a reduction of DNA damage; a reduction of oxidative stress; a reduction of

inflammation; an induction of phase II conjugating enzymes so that carcinogens can be detoxified and DNA adduct formation can be reduced; an enhancement of the immune response; a stimulation of DNA repair; an inactivation of protein kinase C; an alteration in DNA methylation; a blockage of the cell cycle to enhance DNA repair; an induction of apoptosis due to sequential activation of caspases; an inhibition of angiogenesis and other mechanisms. In most of these chemopreventive investigations, selenomethionine or high selenium-enriched yeast was used as the chemopreventive supplement.

However, as outlined by Coombs Jr. (2004) it seems to be important to specify the chemical form of selenium that has been applied. Anti-cancer properties are published for several selenium-containing compounds e.g. for metabolites of selenoproteins, for selenodiglutathione, for hydrogen selenide and for methylated selenides. However, their modes of action differ. Coombs Jr. (2004) illustrates the metabolic relationship between these compounds and specifies their generally accepted effects on mammalian cells.

Another important aspect of supplementing selenium compounds is the nutritional requirement for them. A dietary intake of 70µg/day selenium for men and 60µg selenium for women is recommended by the Medical Research Council in Australia (Position Statement Aug. 2005). An upper intake limit of 400µg/day selenium intake seems to be accepted as a safe measure. However, in supra-nutritional doses selenium intake in its different chemical specification can also be toxic. The speed of selenium accumulation which ultimately culminates in the appearance of a disease called selenosis appears to be different for inorganic or organic compounds with organic selenium agents accumulating faster than their inorganic counterparts. In contrast, it also has been reported that sub-nutritional

uptake leading to selenium deficiency causes the so-called Keeshan disease (Liu *et al.* 2002).

It is of note that cell cycle-related effects of selenium compounds are concentration-dependent. Zeng (2002) illustrates that low concentrations of selenite and selenomethionine up-regulate multiple key cell-cycle-related mRNA- and total phosphorylated protein-levels. In contrast, high concentrations of these compounds inhibit cell growth and induce apoptosis. The different sensitivity of cancer versus normal cells to some selenium-containing chemotherapeutics is quite remarkable and warrants further investigations in particular of the following observations: selenium-containing compounds with different chemical specification have been combined to increase the overall cytotoxicity to cancer cells (e.g. selenite and selenomethionine); selenium compounds have also been combined with cytotoxic chemotherapy increasing cytotoxicity to cancer cells whilst minimising toxicity of cytotoxic chemotherapeutics to normal tissues in a compound-dependent manner (Zhang 2007).

The following paragraphs further details the plethora of biochemical effects attributed to various selenium-based inorganic and organic compounds. These two groups differ significantly in their *in vitro* and *in vivo* properties. Research has been reported on the cancer-selectivity of elemental grey and nano red selenium, selenide, selenite, selenate, selenic- and methylselenic acid, selenomethionine, selenocysteine and synthesized (organic) compounds. The list of newly developed or discovered compounds is continuously growing and not all of them are described here.

Selenite is a potent inorganic anti-cancer selective agent (Birringer, Pilawa and Flohe 2002). It induces synthesis of glutathione peroxidase (GPX-1) and membrane-bound phospholipid hydroperoxide glutathione peroxidase (GPX-4) (Miller *et.al.* 2001) and protects cellular membranes from

oxidative attack (Hawkes and Tappel 1983). Usually, fatty acid hydroperoxide, a substrate of the above-mentioned phospholipid hydroperoxide glutathione peroxidase (GPX-4), induces interleukin 1 (Brigelius-Flohe *et.al.* 1997) and activates the binding of nuclear factor kappa B. Alternatively, interleukin 6 can induce binding as well (Renard *et.al.* 1997). Nuclear factor kappa B controls the expression of membrane type 1 matrix metalloproteinase (but not the tissue type), of matrix metalloproteinases 2 and 9 (Park *et.al.* 2007), of cytokines, of cell adhesion molecules and of inducible nitric oxide synthetase (Kim and Stadtman 1997). Selenite inhibits the binding of two nuclear factors, the activating protein 1 and the nuclear factor kappa B (Handel *et.al.* 1995; Kim and Stadtman 1997). Selenite oxidizes insoluble protease-resistant, fibrin-like polymers of cell membranes and changes their polythiols to disulphides (Lipinski 2005).

Selenite affects signalling pathways: It binds to Sp-1 in the androgen receptor promoter region and downregulates androgen receptor expression (Husbeck *et.al.* 2006). Selenite reduces the serum levels of growth hormone and somatomedin C (Thorlacius-Usiing, Flyvbjerg and Orskov 1988), decreasing the amount of circulating insulin-like growth factor-1 (Gronback *et.al.* 1995). It irreversibly inhibits glucocorticoid binding (Tashima *et.al.* 1989). It inhibits cJUN N-terminal kinase, stress-activated protein kinase and p38 mitogen-activated protein kinase (Park *et.al.* 2000). It inhibits caspase-9 and caspase-3 through a redox mechanism (Park *et.al.* 2000; Yoon *et.al.* 2002) and sensitises cells resistant to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to undergo caspase-mediated apoptosis (Hu *et.al.* 2006; Yamaguchi *et.al.* 2005). It decreases the ratio between type I and type II isozymes of cyclo-adenosine mono phosphate kinase (cAMP), which explains that selenite is cancer-selective (Liu *et.al.* 1986).

Selenite exerts metabolic changes: Intracellularly, it increases oxygen consumption and oxidizes glutathione as well as nicotinamide adenine dinucleotide phosphate (NADPH) (Anundi, Stahl and Hoegberg 1984; Bahmre *et.al.* 2000); it non-competitively, dose-dependently inhibits liver-DNA-methylase (the DNA-methylating protein) (Cox 1985): generally, inhibiting DNA-methylase promotes apoptosis in all cells (Vanyushin 2006). However, due to the differences in DNA-methylation patterns between malignant and normal cells, selenite exhibits selectivity for cancer cells (Vanyushin 2006). Selenite decreases serum glucose and the precursors for gluconeogenesis including lactate, alanine and glycerol (Iizuka *et.al.* 1993), and inhibits activity of gluconeogenic enzymes including pyruvate carboxylase and glucose-6-phosphatase. It promotes the activity of glycolytic enzymes, in particular glycokinase and phospho-fructokinase (Iizuka *et.al.* 1993). Lactate dehydrogenase and NADP isocitric dehydrogenase are enhanced; succinic dehydrogenase is decreased (Nebbia, Gremmels and Soffietti 1990). Selenite promotes the opening of the mitochondrial permeability transition pore, releases cytochrome c from the membrane and enhances mitochondrial aspartate transaminase activity (Yhu, Woo and Huang 2002). It promotes calcium ion release from the mitochondrial matrix and uncouples respiration (Shilo *et.al.* 2003).

In microorganisms, selenite is incorporated into several compounds that depend on the selenium for their activity: selenocysteine, selenophosphate, formate dehydrogenase H, seleno tRNA and deoxyribose-phosphate aldolase (Larcouciere, Levine and Stadtman 2002). As part of protein synthesis, the incorporation of selenium into selenocysteine is encoded by a stop codon UGA in the mRNA and involves a unique tRNA (Daniels 1996). Selenocysteine is the precursor of a minimum of thirteen different selenoproteins (Daniels 1996).

In vivo, selenite is mainly bound to haemoglobin, and in approximately the following proportions to three binding-proteins in plasma: selenoprotein P 52%, glutathione peroxidase 39%, albumin 9% (Sheehan and Halls 1999). Selenium is rapidly internalised and incorporated into erythrocytes (Mas, Jiang and Sarkar 1988). Its uptake, metabolism to selenide and release as selenium depends on the amount of intracellular reduced glutathione. The gamma glutamyl transferase inhibitor serine-borate and the glutathione depleter diethylmaleate both reduce the cellular uptake of selenite (Anunadi, Hogberg and Stahl 1984). Inhibiting intracellular glutathione reductase, for example with chromate, decreases the release of selenium from cells (Gasiewicz and Smith 1978).

Selenite is efficiently absorbed when taken by mouth (Janghorbani et.al 1990), with a steady state in blood achieved after 12-17 days (Hansen and Kristensen 1979). However, this has been rejected by Patterson *et.al*. (1989) who illustrates this to happen after several months. After injection, 88% selenite is taken up by the anion exchange carrier into red blood cells (Styblo, Kalouskova and Pavlik 1988; Suzuki *et al.* 1998). With the uptake of 66µg selenite, full expression of glutathione peroxidase is achieved (Xia *et.al.* 2005). Stopping selenite supplementation results in a half-life of decay of glutathione peroxidase of 4.2 days (Ip and Hayes 1989). Most of the selenite consumed is excreted as selenium in the faeces or urine. However, about one third of the ingested selenium is volatilised forming dimethylselenide (Janghorbani *et.al.* 1990). Volatilisation of selenite is delayed with cumene hydroperoxide and completely stopped with tert-butylbenzoic acid (Stahl, Anundi and Hogberg 1984).

There are other clinical observations regarding exposure to selenium: selenium deficiency, for example in Keeshan disease, leads to a decrease in

hepatic 5`deiodinase- and thyroxin-3 syntheses (Jianhua, Ohtsuka and Hayashi 2000). Applying selenite alone causes cataracts as it induces oxidative stress (Gupta *et.al.* 2002) which can be prevented by simultaneous administration of green tea or vitamin C (Gupta *et.al.* 2002; Devamanoharan *et.al.* 1991). Selenite inhibits complement activation through the alternative pathway at the level of complement factor C3 (Hou, Jiang and He 1993) and induces an antithrombotic effect by stimulation of glutathione peroxidase (Ricetti *et.al.* 2000). Without a certain blood concentration of selenoprotein P, selenite administered intravenously will lead to nervous system disorders (Hill *et.al.* 2004): the striatum in the brain is affected by raising levels of dihydroxyphenylacetic acid and homovanillic acid (Tsunoda, Johnson and Sharma 2000). Selenite reduces lymphoedema after oral surgery (Zimmermann *et.al.* 2005) and attenuates arginine-induced pancreatitis (Hardman *et.al.* 2005). As selenite induces glutathione peroxidase, an inverse correlation between the severity of lymphoedema or pancreatitis and glutathione peroxidase-activity has been observed. Selenite inhibits progression of hormone refractory prostate carcinoma (Corcoran, Najdovska and Costello 2004) and slows growth of crocidolite-induced mesothelioma (Yang, Luo and Liu 1994). This has been explained by a decrease in angiogenesis. For cancer treatment “normal” selenium concentrations (usually below 2µM) have to be greatly exceeded (Husbeck *et.al.* 2006); serum levels of over 7µM are required to achieve complete inhibition of the nuclear factor kappa B *in vivo* (Kim and Stadtman 1997). Resistance to selenite treatment has been observed in different settings (Pinson, Sagot and Daignan-Fornier 2000).

Selenite and sulphydryl group-containing compounds form cancer-selective **selenotrisulphides** (Abdullaev, MacVicar and Frenkel 1992). Selenite reacts with the following sulphydryl group-containing compounds: cysteine, cysteamine, methionine, reduced glutathione, mercaptoethanol,

oxidized and reduced alpha lipoic acid and penicillamine. Selective reactivity to cancer cells seems to be due to the formation of selenotrisulphides, not to selenite itself (Ip and Hayes 1989).

The sequence of compound application is crucial: when intracellular sulphydryl groups are elevated or reduced prior to the application of selenite, a loss in cell-killing efficacy can be observed. As an example, selenite does not inhibit growth or induce apoptosis if reduced glutathione has been applied twenty-four hours prior to its administration. However, the simultaneous administration of reduced glutathione with selenite has a dramatic effect (Kuchan and Milner 1991). Withholding reduced glutathione and other sulphydryl group-containing compounds with diethylmaleate twenty-four hours prior to applying selenite inhibits growth and apoptosis; that can be reversed with trisulphides (Frenkel and Falvey 1988).

There are multiple mechanisms of trisulphide action: trisulphides inhibit amino acid incorporation into proteins including the branched chain amino acid leucine (Vernie et.al 1979), reduce elongation factor 2 (Vernie *et.al.* 1975) and inhibit DNA/RNA-polymerases (Frenkel, Walcott and Middleton 1987). Trisulfides target RNA polymerase II; they do not affect the initiation-stage of forming an enzyme binary complex, but do affect the elongation stage (Frenkel and Falvey 1989). They generate five to twenty-five-fold more active oxygen species in tumour cells than in normal cells (Terada *et.al.* 1999; Yan and Spallholz 1993) and they inhibit colony formation by cancer cells (Caffrey and Frenkel 1991).

In contrast to selenite, organic **selenomethionine** dose-dependently increases plasma selenium levels and accumulates intracellularly (Burk *et.al.* 2006). It affects normal cells but requires a 100-fold higher

concentration than cancer cells do (Redman *et.al.* 1998). Selenomethionine synergises with selenite, topoisomerase I inhibitors (Rustum *et.al.* 2005), taxanes, platinum, 5-FU and anthracyclines (Fakih *et.al.* 2005). Selenomethionine is well absorbed orally (Shen *et.al.* 1997) and further catalysed to methylselenol (CH3SeH) by cystathionine-gamma-lyase (Okuno *et.al.* 2005). More recent literature is pointing out that methylselenol has anti-carcinogenic function.

Differences between **Methylselenic acid** and selenite have been observed: Methylselenic acid does not affect the androgen receptor promoter Sp-1 (Husbeck *et.al.* 2006) and induces no measurable superoxide radical levels (Shen, Ding and Ong 2002). Differences in the phosphorylation status of protein kinase B, extracellular regulated kinases 1 and 2, JNK 1 and 2 and p38 mitogen activated protein kinase have been observed (Jiang *et.al.* 2002). Methylselenic acid is taken up into the liver, reduced to methylselenol, and transformed into selenide, selenosugar A and selenosugar B.

Red elemental selenium or red nanoselenium has only 10% of the potency of selenite in synergy with glutathione and is seven times less toxic (Gao, Zhang and Zhang 2000).

Other well-known synthetic organic selenium compounds are **Ebselen** and **pXSC. Ebselen** inhibits electron transfer from reduced nicotinamide adenine dinucleotide phosphate cytochrome P450 to flavin adenine mono- and dinucleotide by flavin reductase (Nagi *et.al.* 1989). It also inhibits fatty acid chain elongation, beta-ketoacyl CoA reductase and dehydrase/enoyl CoA reductase (Laguna *et.al.* 1989). It depletes intracellular sulphydryl-group containing compounds and induces apoptosis (Yang, Shen and Ong 2000). **pXSC** decreases COX-2 specific activity without affecting protein

levels (Rao *et.al.* 2001).

β-Lapachone is an antineoplastic compound discovered in the bark of the Amazonian tree *bignoniaceae tabebuja avellanedae* (Choi, Cheong and Choi 2003). At concentration of 5-10μM *in vitro* it competitively inhibits glucocorticoid binding to the cell membrane, targeting antigenic determinants which seem to be shared between the cytoplasmatic gluco-corticoid receptor and DNA polymerase. However, it does not affect the mineralocorticoid-, estrogen-, androgen- or progesterone-binding site (Schmidt, Miller-Diener and Litwack 1984). It abrogates reverse transcriptase (Schmidt, Bollum and Litwack 1982), interleukin 6, vascular endothelial growth factor, nuclear factor kappa B and intracellular adhesion molecule 1(Gupta *et.al.* 2002; Choi, Cheong and Choi 2003). It inhibits NADPH-dependent cytochrome P450 destruction and NADPH-dependent iron-catalysed microsomal lipid peroxidation (Dubin, Fernandez Villamil and Stoppani 1990). However, β-lapachone increases caspase-9 and caspase-3 activity without affecting caspase-8 (Choi, Cheong and Choi 2003), and interacts with topoisomerase 1 (Oliveira-Brett, Goulart and Abreu 2002). It induces an elongation factor E_2F_1 and a Ca-ion-dependent cysteine protease (Pink *et.al.* 2000), promotes synthesis of hydrogen peroxide (Cruz, Docampo and Boveris 1978) and activates the S-phase checkpoint (Li *et.al.* 2003). Its mechanism is thought to be a two-step oxidation: it reduces NAD(P)H:quinone oxidoreductase 1 (NQO1) to an unstable hydroquinone which then reforms back into the parent compound, reducing FE(III)ADP and diverting reducing equivalents from NADPH to dioxygen (Reinicke *et.al.* 2005). The first early apoptotic events are cytochrome c release with mandatory caspase-3 activation (Li *et.al.* 1999). However, β-lapachone also induces necrosis and is inhibited by dicoumarol (Pink *et.al.* 2000). β-lapachone synergises with vitamin C (Dubin, Fernandez Villamil and Stoppani 1990) and genistein (Kumi-Diaka *et.al.*

2004).

In vivo, β-lapachone is applied as an equimolar complex with α- or β-cyclodextrins to increase the solubility of the compound (Nasongkla *et.al.* 2003). Administration of β-lapachone is strongly abortive (Guerra *et.al.* 2001) and combines well with taxol and radiation (Li *et.al.* 1999; Park *et.al.* 2005).

The following section of this review focuses on the use of **glycolysis** inhibition, **antibiotic** administration, **diethylmaleate-** and **H_2O_2-**addition in cancer treatment: Disturbing the lactate-to-pyruvate ratio of **glycolysis**, the three metabolites fructose-1,6-biphosphate, glyceraldehyde-3-phosphate and dihydroxyacetonephosphate accumulate (Tilton *et.al.* 1991), forming advanced glycosylation end products and additional lactate. T-lymphocytes are suppressed at high lactic acid levels: a 95% reduction of their cytokine synthesis and proliferative activity and 50% reduction of their cytotoxic activity can be observed (Fischer *et.al.* 2007). Inhibiting glycolysis under aerobic conditions is cancer-selective and enhances the efficacy of any chemotherapy (Kim *et.al.* 2002).

Oxamate competes with pyruvate and inhibits the rate of reduction to lactate. The effects of oxamate are concentration-dependent: below 0.4mM it inhibits gluconeogenesis, but above 0.4mM has no apparent influence it (de Arriba *et.al.* 2003). Another glycolysis inhibitor, **iodoacetate,** targets nonspecific sulphydryl group-containing enzymes like glyceraldehyde-3-phophate dehydrogenase and glucose-6-phosphate dehydrogenase. Synthesis of glutamate decreases in the presence of iodoacetate. This can be reversed by administration of citrate (Birnbaum and Demain 1969). Combined with dimethylsulphoxide, iodoacetate inhibits tumour progression (Fahim *et.al.* 2003). Finally, iodoacetate influences tumour

immunity (Apffel, Arnason and Peters 1966).

The rationale for using **antibiotics** in tumour treatment is twofold: First, some antibiotics like tetracyclines or sulphonamides inhibit isozymes of glutathione-S-transferases (GST M3-3 and GST P1-1) in some circumstances inducing apoptosis (Mukanganyama *et.al.* 2002). Tetracyclines inhibit the cleavage of matrixmetalloproteinases (Saikali and Singh 2003), sulphonamides reduce the synthesis of uric acid (Chertow *et.al.* 1996). Second and equally importantly, they affect the multiplication and cellular interference of mycoplasma, chlamydia, streptococci, heliobacter and other intracellular parasites: there is a high correlation between mycoplasma infection and human carcinomas (Huang *et.al.* 2001). Mycoplasma-mediated malignant transformation is associated with high H-ras and c-myc levels which induce the nuclear factor kappa B (Zhang *et.al.* 1997) and enhance inducible nitric oxide synthetase independently of cytokines (Kagemann *et.al.* 2005). In the presence of the urokinase-type plasminogen activator, mycoplasma adheres to plasminogen receptors promoting intracellular invasion and potently reducing cellular antioxidant activity (Yavlovich *et.al.* 2004 and 2006, Yavlovich, Tarshis and Rottem 2004). Trypsin treatment of cells detaches mycoplasma from human cells (Razin *et.al.* 1981).

Diethylmaleate is a glutathione depleting agent binding to glutathione-S-transferase. It enhances blood lactate levels similar to advanced glycosylation end products (Jimenez *et.al.* 1986). Diethylmaleate increases L-cysteine transport across the cell membrane (Ruiz *et.al.* 2003). At high concentrations it causes the immediate and sustained loss of intracellular glutathione (Casey *et.al.* 2002); however, in low concentrations (0.025-0.1mM) it increases intracellular glutathione levels (Bannai 1984). It is excreted into the bile.

H_2O_2 is an important mediator of cellular signalling and induces dose-dependent apoptosis. A remarkable inhibition in the phosphorylation of insulin-like growth factor-1 can be observed. However, it increases phosphorylation of insulin- and epidermal growth factor receptors (Schmitt, Klein and Droge 2006; Zhung and Schnellmann 2004). It stimulates glucose transport across the membrane, up-regulates the insulin-induced vascular endothelial growth factor and the hypoxia-induced factor 1 alpha through p70S6K1-kinase (Zhou *et.al.* 2007) and mediates cell adhesion (Chiarugi *et.al.* 2003). It promotes phosphorylation of stress-activated protein kinase and JNK-kinase, extracellular regulated kinase 1 and 2, proteinkinase B (AKT) and src-kinase (Inanami *et.al.* 1999; Zhung and Schnellmann 2004), releasing mitochondrial Ca-ions and arachidonate. H_2O_2 up-regulates polyADP-ribosylation activity and pyruvate dehydrogenase and decreases adenosine triphophate synthesis (Ramasarma 1990). Finally, it is inactivated by NAD(P)H-dependent catalases (Calderon *et.al.* 2006) and antioxidants.

In vivo treatment with H_2O_2 inhibits tumour cell attachment to extracellular matrix proteins and enhances metastasis (Kundu, Zhang and Fulton 1995).

This review of the literature details the mechanisms of how the metabolic flux in many cancer cells is shifted from the carbon cycle to the nitrogen cycle resulting in excessive nitrosylation of proteins. The effects of excessive nitrosylation of proteins are deleterious and impact on cell growth, survival, migration and angiogenesis. Indeed, the role of nitric oxide in cancer development is a highly active area of current research worldwide. Preventing excessive nitrosylation of proteins may be a

mandatory requirement to combat cancer. In addition, the actions of several broad-spectrum inhibitors that show promise for cancer therapy by targeting various metabolic signaling pathways in cancer cells are reviewed. Some of these inhibitors target nitric oxide production and/or nitrosylation. Others impact on the cellular synthesis of hydrogen peroxide. The mode of action of each compound has been described in detail. However, this review of the literature also highlights the need of improvement for most current chemotherapeutic approaches. More effective strategies require that combined chemotherapeutic approaches are used to overcome the redundant nature of signaling processes in cancer cells.

1.1 The Present Study

Cancer cell biology is extremely complex and invariably involves multiple constitutively activated steps in overlapping networks of intracellular signaling pathways. Thus, it is perhaps not surprising that individual narrow-spectrum inhibitors often have limited efficacy in the treatment of cancer. Unfortunately, clinical trials using individual, broad-spectrum inhibitors also have yielded disappointing results. Consequently, there has been a plethora of studies evaluating combinations of chemotherapeutic agents.

The aim of this study was to evaluate a selection of combinations of chemotherapy agents. Many of the individual compounds chosen in this study were selected as they either are currently in various stages of development within various pharmaceutical companies (eg staurosporine developed as its derivates UCN-01 or CGP41251 by Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co, Ltd Shizuoka-ken, Japan; or genistein developed as GCP by Pharmaceutical Company Amin Up Chemicals Sappiro Japan) or are being trialed under the special access

scheme (SAS) provisions. These SAS provisions allow developmental therapies to be tested in end-stage cancer patients for whom conventional cancer therapies have failed.

The hypothesis to be tested in this study was that some combinations of broad-spectrum inhibitors together with narrow-spectrum inhibitors would be more effective in killing cancer cells than either the broad- or the narrow-spectrum inhibitors used alone. This approach was based on the rational that inhibition of multiple aberrant signalling pathways was essential to eradicate cancer cell populations. Broad-inhibiting compounds target multiple aberrant signaling steps. However, within a therapeutic range, most of these compounds are only partially effective. Narrow spectrum inhibitors were then added to the combination in an attempt to completely eradicate cancer cell populations.

The first objective of this study was to establish continual and optimal growth of a series of cancerous cell lines including: a) JU-77, a human mesothelioma cell line b) MCF-7, a human breast cancer cell line; c) Caeco-2, a human colon cancer cell line; d) HepG2, a human hepatoblastoma cell line. This thesis reports predominantly on the results from the JU77 and MCF-7 cell lines. Human albumin was included in many of the cell culture tests because it is known that it can change the potency of chemotherapeutic agents. High levels of IGF-1 were also included in many of the cell culture tests to simulate the autocrine loops of tumours *in vivo*, which also attenuates the action of many chemotherapeutic agents.

The second objective was to identify specific and generalized inhibitors against various receptor tyrosine kinases, which are involved in signalling pathways of the: a) insulin receptor; b) insulin like growth factor receptor 1 (IGF-1R); c) epidermal growth factor receptor (EGFR); d) growth hormone (GH)/ prolactin receptor (PRL); e) oestrogen receptor (ER) and f) progesterone receptor (PR). The third objective was to determine the

minimum dose of agents required to effectively kill cancer cell populations.

The final objective of this study was to establish an animal model (nude mice growing human xenografts of cancer cells) of cancer to enable the most promising candidate combination(s) to be tested *in vivo.* This entailed histopathology reporting on the xenograft tumours to enable the efficacy of the control and treated animals to be more fully assessed and clinical biochemistry blood reports to enable animal well-being to be more fully monitored.

2. 0 MATERIALS AND METHODS

2.1 Materials

Various inhibitors were obtained from three companies (Sigma, NSW, Australia; Merck, UK; Biaffin, Germany) listed in Appendix 1, general pharmacies or Dr.W.Barnes, General Practitioner and Nutritional Specialist in 246 South Terrace/Fremantle. The four cancer cell lines (JU77, MCF-7, Caco-2 and HepG2) were a kind gift of Dr.Simon Fox, Curtin University of Technology who obtained the JU77 cell line from the team of Manning *et.al.*1991 and the other three cell lines from American Tissue Cell Cultures, USA. Materials and medium for cell cultures were ordered from Invitrogen, Victoria, Australia. Foetal calf serum was ordered from SAFC Biosciences, Victoria, Australia. Growth factors were ordered from GroPep, South Australia. Nude balb/c mice were ordered from the Animal Resources Centre, Murdoch University, Western Australia.

2.2 Methods

2.2.1 Establishing cell cultures of human cancer cell lines

2.2.1.1 Description of the four cell lines

Mesothelioma cell line JU77 (ATCC No. none):

Establishment and characterization of the mesothelioma cell line JU77 was performed by Manning *et.al.* in 1991. The cells were derived from the serosal cavities of untreated male patients with known crocidolite asbestos exposure. Diagnosis was made by cytology, histology and electron microscopy. The cells appear in spindle form and have a minimum doubling time of 18 hours. They carry the epithelial membrane antigen (EMA) and cytokeratin. They lack carcinoembryonic antigen (CEA) and epithelial mucin. Cell junctions, glycogen and numerous long, thin branching microvilli are typical. An abnormal karyotype exists, with between 40 and 80 chromosomes. The mesothelioma cell line JU77 was

initially established in RPMI supplemented with 5% foetal calf serum (FBS), 2mM L-glutamine, penicillin (100IU/ml), streptomycin (100ug/ml) and neomycin (100ug/ml) in a humidified atmosphere of 95% air and 5% CO2.

Breast cancer cell line MCF-7 (ATCC No. HTB-22):

The cells were derived from the pleural effusion of a 69 year old Caucasian female with adenocarcinoma and were characterised by Soule *et.al.* in 1973. The cells have a minimal doubling time of 29 hours and are able to process estradiol via cytoplasmatic estrogen receptors; the secretion of insulin-like growth factor binding proteins 2, 4 and 5 can be modulated by treatment with antiestrogens. Growth is inhibited by tumour necrosis factor alpha. The cells express the WNT7B oncogene and contain the Tx-4 oncogene. Stem line chromosome numbers ranges from hypertriploid to hypotetra-ploid, with the chromosomes numbering between 66 and 87. The cells are tumourigenic in mice. The breast cancer cell line MCF-7 was initially grown in RPMI supplemented with 5% foetal calf serum (FBS), 2mM L-glutamine, penicillin (100IU/ml), streptomycin (100ug/ml) and neomycin (100ug/ml) in a humidified atmosphere of 95% air and 5% CO2.

Colon cancer cell line Caco-2 (ATCC No. HTB-37):

This cell line was derived from a 72 year old Caucasian male with moderately well differentiated adenocarcinoma consistent with colonic primary (grade II) (Rousset 1986). Cells are epithelial-like, and upon confluence they express characteristics of enterocytic differentiation. The cells have a minimal doubling time of 62 hours. They express heat stable enterotoxin and epidermal growth factor. They produce keratin, retinoic acid binding protein 1 and retinol binding protein 2. The stemline modal chromosome number is 96, occurring at 16%, with polyploidy at 3.2%. The cells are tumourigenic in mice. Caco-2 was initially grown in DMEM supplemented with 10% foetal calf serum (FBS), L-glutamine, penicillin

(100IU/ml), streptomycin (100ug/ml) and neomycin (100ug/ml) in a humidified atmosphere of 95% air and 5% CO2.

Hepatocellular liver carcinoma cancer cell HepG2 (ATCC No. HB-8065): This cell line was derived from the liver tissue of a 15 year old Caucasian male and was characterised by Darlington, Kelly and Buffone in 1987. These cells are epithelial in morphology and have a minimal doubling time of 59 hours. They express receptors for insulin and insulin-like growth factor-2. In addition, they express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activity. Under oxidative stress (gramoxon) they decrease the expression of apoA-1 mRNA and increase the expression of catalase mRNA. They secrete alpha fetoprotein, albumin, alpha2 macroglobulin, alpha1 antitrypsin, transferrin, alpha1 antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, complement (C4), C3 activator, fibrinogen, alpha1 acid glycoprotein, alpha2 HS glycoprotein, beta lipoprotein and retinol binding protein. There is no evidence of a Hepatitis B virus genome in this cell line. The chromosomal modal number is 55, ranging between 50 and 60. Chromosome 1 is rearranged. The cells are not tumourigenic in mice. The hepatocellular cell line HepG2 was first established in DMEM and 1mM sodium pyruvate, supplemented with 10% foetal calf serum (FBS), 2mM L-glutamine, penicillin (100IU/ml), streptomycin (100ug/ml) and neomycin (100ug/ml) in a humidified atmosphere of 95% air and 5%CO2.

2.2.1.2 Medium conditions for optimal cancer growth

Once cells were established as described above in section 2.2.1.1 they were grown in various media in an attempt to find a common medium that could support exponential growth for each cell line. As detailed in the results section of this thesis, the medium of choice, supporting exponential growth of each cell line, was DMEM supplemented with 10% foetal calf serum (FBS), 110 mg/L (=1mmol) of sodium pyruvate, 2mM L-glutamine and

100nM IGF-1. The inclusion of L-glutamine is crucial to the growth of most cancer cell lines.

2.2.1.3 Selection of antibiotics

Antibiotics are added to cell cultures to reduce the risk of contamination, but some antibiotics, especially when used in combination with other agents, may inhibit growth or induce cell death. An example is neomycin, which is often used in cell cultures and heavily influences the effect of other compounds. In this research, several antibiotics were tested in cultures with other inhibiting agents; details are given in section 3.1.9. In this study a mixture of penicillin 100IU/ml and streptomycin 100ug/ml was chosen as this antibiotic mixture did not appear to influence cell growth or cell death when combined with various other agents.

2.2.1.4 Creating a tumour-like environment in vivo

It is well documented that the effects of compounds on cell inhibition or cell death can be attenuated by treatment with high insulin-like growth factor-1 (Tao *et.al.* 2007). So-called "autocrine loops" inside tumours often create an environment high in these growth factors. In an attempt to mimic a tumour-like environment in cancer cell cultures, insulin-like growth factor-1 was included in the media at 100nM. At this concentration, all cells grew at their maximum doubling rates.

2.2.1.5 Treatment with trypsin

All four cancer cell lines grew as monolayers and adhered to the flat bottoms of standard 25cm^2 culture flasks. The medium was changed every third day. After cell lines reached 90% confluency, they were detached by treatment with 0.016% trypsin for 5 minutes. Great care was taken in using trypsin to detach cells because it has been shown that concentration and time of exposure of cells to trypsin influences the rates of cell death: Furthermore it has been shown that exposing cells to trypsin activates the insulin receptor and other cell surface receptors (Internal communication

and Clark *et.al.* 1991).

No additional compound combination was applied for 24 hours following trypsin treatments, to minimize the influence of trypsin on treatment. In addition, the lowest possible trypsin concentration was used to detach the cells (0.016%) at the shortest possible exposure time (5 min). After removing the cells from the flasks, cells were centrifuged with a force of 100g for 5min, resuspended in medium, counted and fourfould diluted. A quarter was reseeded into the flasks. The rest was used for experiments in 24 well plates at a density of 10^4 cells/ml.

2.2.1.6 Centralising cells towards the middle of 24 wells

Seeding cancer cells in suspension led to an even spread of cells across a well. This made it very difficult to judge cell morphology and cell staining along the walls of the wells, because the outer area of a well reflects light irregularly and cells in this area appeared only as silhouettes. To focus the cells in the middle of wells, they were pipetted into wells already containing some medium. Cancer cells in 125µl were added to wells containing 375µl medium: in the next 15 minutes, the cells assembled and adhered in the middle of the well. Assembling the cells in the middle of the well was necessary to ensure that the cells were actually in contact with each other.

2.2.1.7 Determining cell density

The usual procedure to determine cell density is to remove the cells from their flasks and count them in a "Neugebauer" chamber. This chamber contains a determined volume of fluid between a bottom glass cover slip and slide. The chamber has engraved lines that define a highly accurate area. Counting the cells in this area determines the cell density in a given volume.

2.2.2 Methylene blue-staining of cell lines

After cells had been treated with compound combinations most of the methylene blue-stained cells literally disintegrated if treated with trypsin. This made it impossible to count them in a "Neugebauer" chamber. False low readings were recorded. The same observation was made treating cells first with trypsin and then staining them with methylene blue. To avoid this problem, the cells were stained in situ in the wells. However, the lack of a precisely engraved area on the bottom of the wells, made it impossible to determine the absolute number of stained cells per volume in the wells. Nevertheless, it was still possible to count the relative number of stained to non-stained cells in a given area which was the approach taken in this study. For this purpose, a hole was created by puncturing thin aluminium foil with a 23-gauge needle. The size of the hole was equivalent to four large squares in the "Neugebauer" chamber, determined by putting the punched aluminium over the chamber to check that the hole was equivalent to the size of the four large squares of the Neugebauer chamber. The foil was then fixed to the underside of the well so that the hole was centred.

2.2.3 Regrowth of compound treated cell lines

To verify the viability of cancer cells after treatment with compound combinations, the cells were split into two populations which were treated identically. After 24 hours, the number of methylene blue-stained cells was counted in the first population, whilst the medium was changed in an attempt to regrow the second population. After 24 hours the regrown population was stained and the differences in the two ratios of stained to non-stained cells was determined.

2.2.4 Preparation of compounds for *in vitro* treatments

2.2.4.1 Dissolving compounds

Most of the compounds were dissolved in medium as stock solutions at

concentrations tenfold higher than the concentrations reported to inhibit 50% of their target structure (IC50). However, due to a wide range in solubility some of the compounds had to be dissolved in medium on lower stock concentration. Some drugs could not even be dissolved on concentrations needed for 50% inhibition of their target structure (IC50). Compounds that were not directly soluble in medium were first dissolved in DMSO or ethanol prior to dilution into medium. Dissolving a compound in dimethyl sulphoxide or ethanol before dissolving it in water is a standard procedure in the management and combination of drugs.

Compounds which are insoluble in both, polar protic and aprotic solvents cannot be handled using standard laboratory protocols. These substances are not bioavailable, and treatment of cell cultures or animals with these compounds is extremely difficult. A drug that dissolves transiently in dimethyl sulphoxide or ethanol and can then be mixed with medium successfully retains a certain end concentration of dimethyl sulphoxide and ethanol, together with the drug compounds, all of which will be applied to the cell culture. As both solvents, dimethyl sulphoxide and ethanol, themselves have an effect on cancer cells, the final concentration of these solvents was kept below 0.1% if they were used. This concentration does not affect the growth of cancer cells and has no known effect in combination with other compounds.
Appendix 2 illustrates the molecular weights, the solubility and the applied range of the used compounds.

2.2.4.2 Preparing compounds at their IC50 concentrations

All compounds were tested at concentrations based on their known IC50 for their target molecules. Compound with demonstrated effectiveness at their IC50 were further tested over a range of concentrations prepared by serial dilutions of the drug solution. The effect of the inclusion of heat-treated human albumin (56 degrees for 25min) was also determined with

compounds as it is known that albumin binds many drug molecules and in doing so, may alter their properties. To predict if this level of combined compounds could be achieved *in vivo* the maximum tolerated dose of each drug was compared with the levels needed in culture.

2.2.5 Compound preparation for *in vivo* studies

2.2.5.1 Minimal compound concentrations

When trialling compounds *in vivo*, the speed of uptake, metabolism and excretion of each compound fluctuates. In addition, the uptake, metabolism and excretion of a compound vary depending on the administration route. Furthermore, some compounds are "recycled" in the entero-hepatic circulation, which makes it very hard to determine how much is available in the blood stream at any point of time. In an attempt to take these issues into account when selecting compound concentrations for *in vivo* trials, the pharmacodynamic profile of compounds in serum were firstly evaluated by plotting concentrations of drugs in serum against time. The area below the plot demonstrates the absolute amount of drug available at each time in the serum, as long as the starting concentration is known. By overlapping the areas of the pharmacodynamic plots of the compounds, so that after peaking each drug just exceeded the minimal amount of drug needed to induce cell death, a "window" was created which guaranteed a minimal level for each compound in blood similar to that found in culture. It has to be kept in mind, however, that overlapping the minimal levels of the compounds after peaking, may create toxic levels during the phase of peaking.

2.2.5.2 The maximum tolerated dose of combinations

When determining the maximum tolerated dose of a compound combination, accumulation of some compounds has to be considered. The pharmacodynamic parameters of accumulating compounds change with every repeated administration. The tissue concentration and, eventually, the

serum concentration increase with each administration. There are two main scenarios of accumulation: intra- and extracellular accumulation of compounds. Extracellular accumulation or re-entry of compounds is caused by the excretion of compounds through the bile into the gut and their reabsorption into the blood stream: this entero-hepatic circulation is a mechanism that leads to accumulation of compounds administered repeatedly. Any compound excreted through the bile can be part of this process. Intracellular accumulation is a result of a compound`s ability to pass membranes and incorporate into cellular structures. Intracellular accumulation can take place after repeated administration. Organic compounds usually accumulate better than inorganic compounds.

A combination of accumulating and non-accumulating compounds was chosen to be trialled. For a period of nine days a fixed concentration of 75µg lithium chloride once daily with 70µg selenite and 70µg selenomethionine twice daily was chosen to be applied *in vivo*. This treatment was not toxic to normal tissues due to the slow intracellular accumulation of selenomethionine but had great impact on cancer cells. Administration longer than nine days requires a reduction in concentration of selenomethionine.

2.2.6 Creating tumours in nude mice

2.2.6.1 Animal Handling and Ethics

Approval of this study (N58-06) was provided by the Animal Experimentation and Ethics Committee of Curtin University of Technology. Animal well-being was carefully monitored daily and tumour growth determined using micro callipers. Treatments with chemo-therapeutic commenced as soon as the tumour diameter reached 3mm in diameter. This generally took between 10 – 15 days of tumour cell growth. A small tumour size (3mm) was chosen for this study to avoid advanced tumour pathology and to avoid spreading of the tumour. This approach

avoided the animals feeling sickness or pain or loss in body weight, which can occur when very large tumours (>50mm) are grown in these animals.

All the chemotherapeutic compounds that were administered to mice in this study have been previously characterised in biochemical, pharmaceutical and drug metabolism studies. Indeed, most of the compounds used in this study are presently in use in humans. The doses of compounds to be given to mice in this study were well within these guidelines and in most instances were delivered at much lower levels.

Test animals (n=2) for each treatment were sacrificed daily by i.p. injection of 170mg/kg pentobarbitone (Nembutal, Merial, Australia). The tumour in each mouse as well as other tissues (gut, liver, lung, spleen etc.) was collected into 10% formalin. Control animals (for each route of delivery), were treated only with the compound carrier. Tumour growth in control animals was never allowed to exceed 10mm in diameter and as noted above, great care was taken to ensure that the animals were not unwell.

2.2.6.2 Dietary regime of the mice

The rats in this study were fed *ad libitum* with a fixed formulation diet for laboratory rats and mice fortified with vitamins and minerals to meet the requirements of breeding animals (Specialty Feeds, Glen Forrest, Western Australia [www.specialtyfeeds.com]). The total fat content was deliberately kept low at around 5%, to maximise the long term breeding performance of most strains. All nutritional parameters of this diet met or exceeded the National Research Council (NRC) guidelines for rats and mice. Mammalian meals were excluded from the diet, however the diet did contain fish meal. The diet included wheat, barley, lupins, soya meal, fish meal, mixed vegetable oils, canola oil, salt, calcium carbonate, dicalcium

phosphate, magnesium oxide, and a vitamin and trace mineral premix. The diet comprised 20% protein, 4.8% total fat, 4.8% crude fibre, 7.6% acid detergent fibre, 16.4% neutral detergent fibre and 59.4% total carbohydrates.

2.2.6.3 Marking the mice and randomizing

Six week-old male nude balb/c mice were individually identified by making cuts to their ears. First, the mice were exposed to a 100% CO2 atmosphere in a small container with a glass top until they lost consciousness. The ears of the mice were then cut. A generator for random numbers on the following internet website created the order of treated animals: www.cognitive-tools.de/Easy-Mapping/Wissensmanagement/Mapping_Software/Zufallszahlen_erzeugen/zufallszahlen-erzeugen.html.

The numbers and how often they were used was typed into the generator and a random list of numbers was presented. The first three numbers chosen by the generator determined the animals to be used as controls (9 days without treatment but with 9 days of insulin-like growth factor-1 application). The next three numbers determined the animals of day 4 (before start of treatment but with 4 days of insulin-like growth factor-1 application), followed by the animals of day 6.5 (2.5days of treatment and 6.5 days of insulin-like growth factor-1 application), finally followed by the animals of day 9 (5 days of treatment and 9 days of insulin-like growth factor-1 application). The order of the numbers created by the generator was 3,7,9,1,10,12,2,6,11,5,4 and 8. Mice regained consciousness within 30-40 seconds by breathing normal air.

2.2.6.4 Preparation of cells grown in vitro for injection in vivo

The mesothelioma cell line JU77 was chosen to create a xenograft in nude mice. Cells were grown exponentially in a 25cm^2 flask, then passaged into three 75cm^2 flasks and thereafter cultivated in six 225cm^2 flasks. The cells were detached from the flasks with trypsin 0.016%, washed twice in 0.9%

NaCl solution, counted in the Neugebauer chamber and resuspended in PBS. The JU77 cells were kept on ice and 10^7 cells were injected into the left groin of the mice. The timespan between detaching the cells and injecting them into the groin of the mice did not exceed fifteen minutes. There was no need to anaesthetise the mice for the subcutaneous injection of cells.

2.2.6.5 Maintaining tumour growth with growth factors in vivo

The non-transformed cancer cell line JU77 alone did not immediately create xenografts in nude balb/c mice when injected into the groin. This cell line JU77 is one of several human cancer cell lines which do not grow as a xenograft in nude mice. Human cancer cell lines which do not grow in nude mice are generally labelled "non-tumourigenic" in mice. However, injected tumour cells of the "non-tumourigenic" cell line JU77 did grow under the skin of the mice, if 10nM insulin-like growth factor-1 was injected subcutaneously into the opposite groin of the nude mice. The injections of insulin-like growth factor-1 into the opposite side ensured that the fluid injected with it did not influence measurement of tumour size on the side of cell injection and these injections had to be continued daily to maintain tumour growth. A volume of 100µl was applied for this purpose.

2.2.6.6 Measuring tumour size

Tumour size was measured with a digital caliper in two orthogonal dimensions. The smallest reliably measured span with this digital caliper was 0.01mm. Repeated measurement of the same tumours showed that there was a risk that tumours were compressed from the sides with the instrument, changing their diameters; to avoid this, no direct contact with the skin was made with the caliper. When tumours were very small, the transparency of the skin was determined by diaphany. The cells of these very small subcutaneous tumours reflected the light differently to normal skin. If these white "spots" under the pink could not be seen any more, the

remaining tumour-free skin was excised for histology.

2.2.6.7 Observation of tumour size, weight and side-effects

First, tumour cells were injected subcutaneously into the groin and tumours were grown for 4 days with administration of insulin-like growth factor-1 subcutaneously into the opposite groin. At the start of treatment mice were injected twice a day into the peritoneum with 100µl compound mixture; this continued for 5 days. Tumour diameters, weight of each mouse and side-effects were recorded on a daily basis. No anaesthetic was needed for the injection of insulin-like growth factor-1 below the skin or injection of compound combinations into the peritoneum.

2.2.6.8 Haematology and Biochemistry

The following list shows the day and the animal when haematological and biochemical parameters were taken:

day	animal
4	1, 10, 12
6.5	2, 6, 11
9	4, 5, 8
9(controls)	3, 7, 9

To determine the haematological and biochemical parameters, mice were anaesthetised with ether and exsanguinated by cardiac puncture using a 23G needle and a 5ml syringe. Every care was taken that cardiac puncture did not cause haemolysis by creating too much negative pressure on the syringe. The blood was separated into two commercial pediatric 0.5ml sample containers: One was mixed with EDTA powder for blood count (lilac), the other one was mixed with Lithium/Heparin (green) to measure electrolytes, liver enzymes, creatinine and albumin. Anaesthetised animals were euthanised with 4.5mg (170mg/kg) pentobarbital intraperitoneally.

All these experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purpose.

2.2.6.9 Histology

After euthanasing the mice, a skin sample at the tumor location was taken from each mouse. In addition, a sample of gut, liver, spleen and lung of each animal was kept in 10% formalin for 24 hours. All samples were processed automatically in a Hypercenter Tissue Processor (Shendon), inserted into wax-blocks and cut to 4μm thick histoslides according to standard pathological procedures. They were stained in hematoxylin and eosin.

Tumour samples of animals as well as the samples of their normal tissues (gut, liver, spleen and lung) were collected at day 4, day 6.5 and day 9. Great care was taken to evaluate whether the tumours had spread locally or from the injection site to other tissues. Several hundred serial sections of the tumours and the surrounding tissues were taken every 4μm to search for local and systemic metastasis. Particular attention was directed towards finding some remaining dead cells following treatment.

Serial sections of tumours were evaluated by Emeritus Professor John Papadimitriou (OSJ BA MBBS MD PhD FRC Path FRCPA) (University of Western Australia). Prof. Papadimitrou evaluated each tissue section and provided guidance and advice regarding tumour morphology.

2.2.7 Statistical calculations

2.2.7.1 Estimating LD_{100} values

Probit analysis is the preferred method for giving the most closely fitting results when biological responses are plotted against their causal stimuli (or logarithms thereof) (Finney, 1971, 1978). The SPSS version 16.0.2 for Macintosh was used to undertake the Probit analyses in this study. This procedure provides an estimate of the strength of a stimulus required to induce a certain proportion of responses and was used in this study to provide estimates of the lethal dose of compounds inducing the death of 100% of cells in culture (LD_{100}). Regression coefficients, intercept and

standard error, Pearson goodness-of-fit chi-square, observed and expected frequencies, and confidence intervals were evaluated as part of the Probit analysis.

2.2.7.2 Comparing LD_{100} values

The t test was used to compare two means from independent samples. The ordinary one-way analysis of variance (ANOVA) was used to compare values when more than two independent samples were compared. These statistical tests assume that the data is sampled from populations following a Gaussian bell-shaped distribution but, generally, are reliable even if the distribution is only approximately Gaussian. ANOVA and t tests were performed using the GraphPad InStat® for Macintosh (Version 3.0b).

"Post" tests were also used when comparing three or more groups (as it is not appropriate to repeatedly use a t test to compare various pairs of columns). The Bonferroni test was used to compare selected pairs of columns whereas the Tukey-Kramer Multiple Comparison tests was used when comparing all pairs of columns. The Dunnett test was used when comparing means relative to the control treatment. These tests apply "corrections" that are designed to offset the advantage of post hoc selection of the most extreme comparisons.

2.2.7.3 Selecting the number of mice

It is unethical to use too many or too few animals in research protocols. Therefore, the number of animals used in the study were selected to ensure they were sufficient to ensure that the objectives and endpoints of this study could be appropriately realised. Reduction of tumour size was the key measure of success in this study. Treatments were commenced on tumours that reached 3 mm in diameter. Given that only treatments achieving a minimum reduction of 80% in tumour size (to less than 0.6 mm in diameter) were of interest in this study, and that a minimum 5% change in

tumour size (0.15 mm) could be microscopically detected, it was estimated that one animal per treatment and day were needed to achieve 95% confidence of prediction. Four animals were used for each treatement group and the experiment repeated to ensure a high level of confidence in the results.

2.2.7.4 Comparing tumour size and weight trends in animals following various treatments.

Animals were treated as described in section 2.2.6. The slope of each data set was used to evaluate whether tumour size or weight altered as a function of time following the initiation of a treatment on day 4. Slopes of lines were first calculated and compared using the Prism software package (GraphPad Software, Inc, La Jolla, CA 92037 USA). Prism was then utilized to test the null hypothesis that the overall slope of a line is zero. In essence, if there were no linear relationship between X and Y overall, Prism calculated the probability that randomly selected points would differ from a horizontal regression line. The P values were calculated from an F test and its degrees of freedom. You would get exactly the same P value from the t ratio computed by dividing the slope by its standard error.

A P value (two-tailed) testing the null hypothesis that the slopes are all identical (the lines are parallel) was calculated to determine the chance that randomly selected data points would have slopes different to that observed. For P values less than 0.05, it was concluded that the lines were significantly different. For P values greater than 0.05, it was concluded that the slopes were not significantly different. This method is equivalent to an Analysis of Covariance (ANCOVA), although ANCOVA can be extended to more complicated situations. It also is equivalent to using Prism's nonlinear regression analysis with a straight-line model, and using an F test to compare a global model where slope is shared among the data sets with a model where each dataset gets its own slope.

3.0 RESULTS

3.1 Compound combinations in cell culture

The viability of cancer cell lines was tested in the presence of a wide range of inhibitors selected because they either targeted the key receptors of intracellular signalling pathways or other factors important for cell survival. Over 99 compounds were evaluated in various combinations against JU77, MCF-7, Caco-2 and HepG2 human cancer cell lines (Table 2). In general, inhibitors used singularly within their known therapeutic human concentration range failed to kill the entire cell population of a cancer cell line. When used in combination they increased the cell killing potency to a varying extent.

3.1.1 Combinations with staurosporine

Staurosporine is a broad range tyrosine kinase inhibitor binding to the adenosine triphosphate binding (ATP) domain of tyrosine kinases. Used alone, it affects cell viability dose-dependently, and in micromolar concentrations kills cell populations. However, in combination with narrow-spectrum inhibitors, far less staurosporine exerts the same potent effects. Fig 5 highlights the differences in the viability of two cancer cell lines, JU77 and MCF-7, in the presence of narrow-spectrum inhibitors (geftinib, PD98058, LY294002, bromocriptine and oxytocin), alone or in combination with staurosporine. Narrow-spectrum inhibitors themselves had little effect in changing cellular viability. This changed substantially in the presence of staurosporine (Table 3, $p<0.001$). The choice of narrow-spectrum inhibitors in combination with staurosporine determined if only a proportion of cells were killed or if the cell line was eradicated. Most of the combinations that eradicated a cell line were only effective in one cell line: Fig 5 illustrates that JU77 cells were killed by PD98058, LY294002 (two pathway inhibitors of the insulin-like growth factor-1 receptor) and bromocriptine (a dopamine receptor agonist). MCF-7 cells were eradicated

Table 2 Summary of combinations of compounds tested in JU77 cells.

Over 30,000 compounds and combinations were tested for their ability to cause death in JU77 cells.

(i) Single Compounds	**A**	**B**
Compounds 1-99		
(ii) Double combinations		
Each of compounds 1-99 with each compound in column A	12, 13, 25, 27, 39,46, 50, 51, 62, 66, 72, 82, 88 *or* 97	
(iii) Triple combinations		
Each of compounds 1-99 with each compound in column A, and each compound in column B	3, 6, 9, 17, 35, 36, 37, 38, 48, 53, 55, 58, 68, 71, 89 *or* 96	12, 13, 25, 27, 39, 46, 50, 51, 62, 66, 72, 82/83*, 88 *or* 97
(iv) Quadruple combinations		
Each of compounds 1-99 with each combination in column A and each compound in column B	9 *and* 37, 9 *and* 55, 9 *and* 71, 17 *and* 89, 35 *and* 48 or 55 *and* 71	12, 13, 25, 27, 39, 46, 50, 51, 62, 66, 72, 82/83*, 88 *or* 97
(v) Quintuple combinations		
Each of compounds 1-99 with each combination in column A and each compound in column B	9, 55 *and* 71 *or* 37, 55 *and* 71	12, 13, 25, 27, 39, 46, 50, 51, 62, 66, 72, 82/83*, 88 *or* 97
(vi) Sextuple combinations		
Each of compounds 1-99 with the combination in column A and each compound in column B	9, 37, 55 *and* 71	12, 13, 25, 27, 39, 46, 50, 51, 62, 66, 72, 82/83*, 88 *or* 97

- Compounds 82 and 83 pre-incubated together

1. 17-Aageldanamycin
2. Adrenaline
3. 8Cl-cAMP
4. Ascorbate
5. Aspirin
6. ATP
7. Avandia
8. Bromelin
9. Bromocriptin
10. t-Butyl benzoic acid
11. Caesium chloride
12. Chloramphenicol
13. Ciprobay
14. Citrate
15. Clindamycin
16. Curcumin
17. Cyproterone acetate
18. 2-Deoxyglucose
19. Dexamethasone
20. DHEA
21. DHLA(Trisulphide)
22. Dichloroacetate
23. Dicumarol
24. Diphenyleneiodonium
25. Diethylmaleate
26. Digoxin
27. Doxycyclin
28. DMSO
29. EGCG
30. Emodin
31. Epichlorohydrin
32. Equol
33. Estradiol
34. p-Ethylphenol
35. Forskolin
36. Gadolinium chloride
37. Geftinib
38. Geldanamycin
39. Genistein
40. Glibenclamide
41. Glutathione red.
42. Glycerol
43. Glyceryltrinitrate
44. Haloperidol
45. Hesperidin/Hesperitin
46. H_2O_2
47. 1,6Hexabromocriptin
48. IBMX
49. Ibuprufen
50. Iodoacetate
51. β-Lapachone
52. Letrozole
53. Lithium chloride
54. α-Lipoic acid
55. LY294002
56. Magnesium chloride
57. Menadione
58. Mercaptoethanol
59. Metformin
60. Methylene blue
61. Metoprolol
62. Mifepristone
63. NADH
64. NADPH
65. Naphtalene
66. Oxamate
67. Oxythiamine
68. Oxytocin
69. Parthenolide
70. PD153035
71. PD98058
72. Penicillin/Streptomycin
73. Picropodophyllin
74. PP2 Src kinase inhibitor
75. Progesterone
76. Quercetin
77. Rapamycin
78. Red Clover
79. Salbutamol
80. Salicylic acid
81. Selenate
82. Selenite
83. Selenomethionine
84. Se-piccolinate
85. Suphamethoxazole
86. Sulphosalazine
87. Suramin
88. Staurosporine
89. Tamoxifen
90. Tautomycin
91. Tetrathiomolybdate
92. Theophylline
93. Thiosulphate
94. Trimethoprime
95. Trypsin
96. Verapamil
97. Vitamin C
98. Warfarin
99. Wortmannin

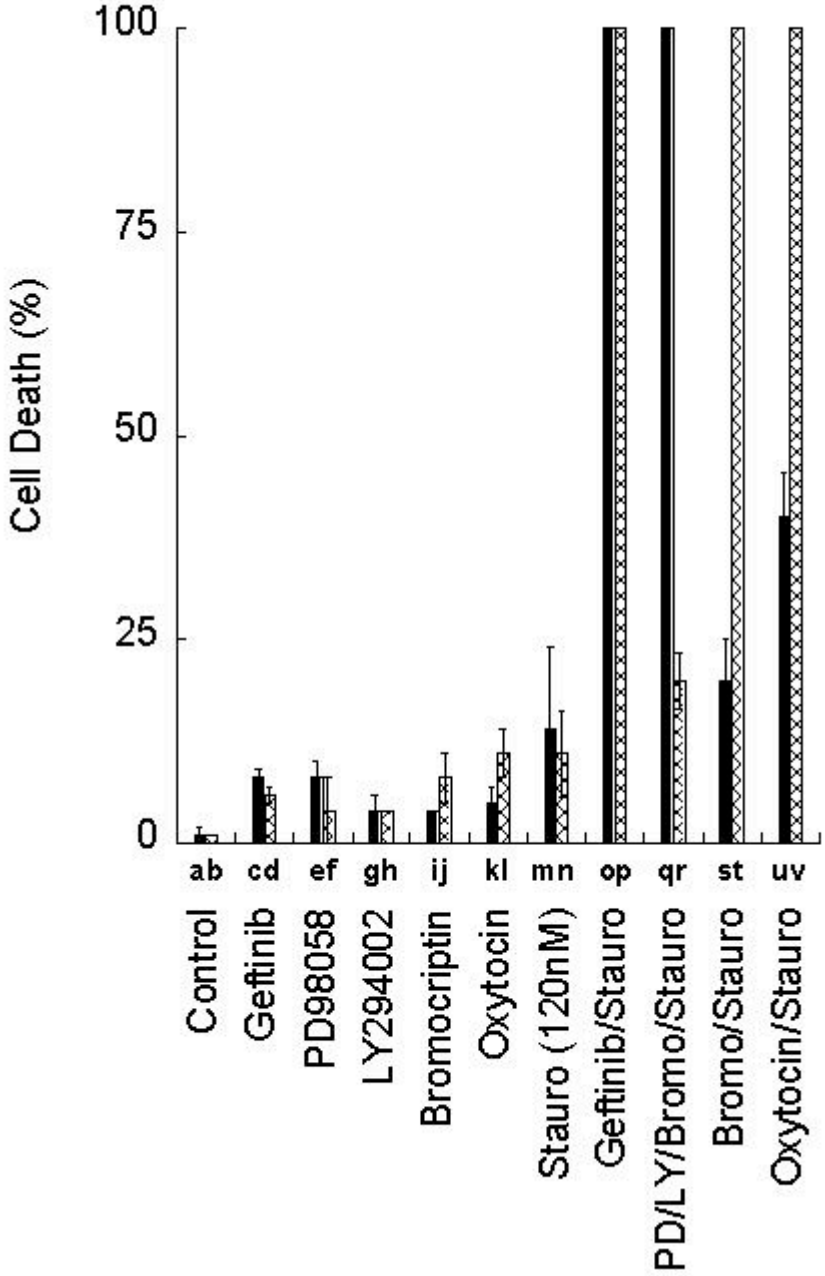

Figure 5 The viability of JU77 and MCF-7 cells in the presence of various narrow-spectrum compounds alone or in combination with staurosporine.

The viability of JU77 cells (■) and MCF-7 cells (⊠) was measured in the presence of 120 nM staurosporine, 1mM geftinib, 20mM PD98058, 20mM LY294002, 0.75nM bromocriptin, 100nM oxytocin, either singularly or in various combinations. Viability of cells was determined as described in the Methods section 3.2. The bars represent the mean + SD of three determinations.

Table 3 **A statistical analysis of the lethal effects of various narrow-spectrum compounds alone or in combination with staurosporine in JU77 and MCF-7 cells.**

Description	LE %CD	SEM	Ref. Fig	Level of Significance Comparison to [Fig x]
Stauro Geft	100	0	**5o**	[**5p**] P > 0.05 (NS)
Stauro Geft PD LY Br	100	0	**5q**	[**5r**] P < 0.001***
Stauro Bromo	20	2.88	**5s**	[**5t**] P < 0.001***
Stauro Oxytocin	40	3.22	**5u**	[**5v**] P < 0.001***
Geft	8	0.57	**5c**	[**5o**] P < 0.001***
	6	0.57	**5d**	[**5p**] P < 0.001***
PD	8	1.16	**5e**	[**5q**] P < 0.001***
	4	2.31	**5f**	[**5r**] P < 0.001***
LY	4	1.16	**5g**	[**5q**] P < 0.001***
	4	0	**5h**	[**5r**] P < 0.001***
Bromo	4	0	**5i**	[**5q**] P < 0.001*** [**5s**] P < 0.001***
	8	1.73	**5j**	[**5r**] P < 0.05 * [**5t**] P < 0.001***
Oxytocin	5	1.16	**5k**	[**5u**] P < 0.001***
	11	1.73	**5l**	[**5v**] P< 0.001***
Stauro	14	5.77	**5m**	[**5o**],[**5q**],[**5u**] P<0.001 [**5s**] P > 0.05 (NS)
	11	3.05	**5n**	[**5p**],[**5t**],[**5v**] P<0.001 [**5r**] P > 0.05 (NS)

Lethal effects of compounds and their combinations were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Stauro, Staurosporine]; [Geft, Geftinib]; [PD, PD98058]; [LY, LY294002]; [Br, Bromocriptin]; [Oxy, Oxytocin], [LE %CD, Lethal effect in % cell death]; [Ref. Fig , Reference Figure].

in the presence of bromocriptine and staurosporine, or oxytocin and staurosporine. Table 3 illustrates that these differences between the cell lines are extremely significant (all $p<0.001$). Remarkably, one combination containing geftinib (an inhibitor of the epidermal growth factor receptor) and staurosporine killed both cell lines. Eradication of cell lines was confirmed by lack of success in regrowing the cell lines.

The minimal concentration of staurosporine required to eradicate a cell population was dependent on the choice of narrow-spectrum inhibitor. The minimal concentration decreased when staurosporine was combined with narrow-spectrum inhibitors in the following order (illustrated in Fig 6): epidermal growth factor receptor inhibitor (geftinib), insulin-like growth factor-1 receptor pathway inhibitors (PD98058, LY294002), dopamine receptor agonist (bromocriptine). A combined treatment using an inhibitor of the mammalian target of rapamycin (rapamycin) was equally effective than using bromocriptin (Table 4, $p>0.05$ (NS)).

Following the removal of the epidermal growth factor receptor inhibitor from the combinations it became apparent that geftinib had no influence on the percentage of cell death induced by a combination containing staurosporine and the rest of the narrow-spectrum inhibitors (Table 5, $p>0.05$ (NS)). The narrow-spectrum inhibitors themselves and staurosporine reduced the final concentration of staurosporine into a therapeutic range of estimated 11nM. Again, the optimal combination was PD98058, LY249002, staurosporine either including bromocriptine or including rapamycin, as illustrated in Fig 7, with no statistic difference between the two of them (Table 5, $p>0.05$ (NS)).

In an attempt to replace staurosporine completely, many other combinations

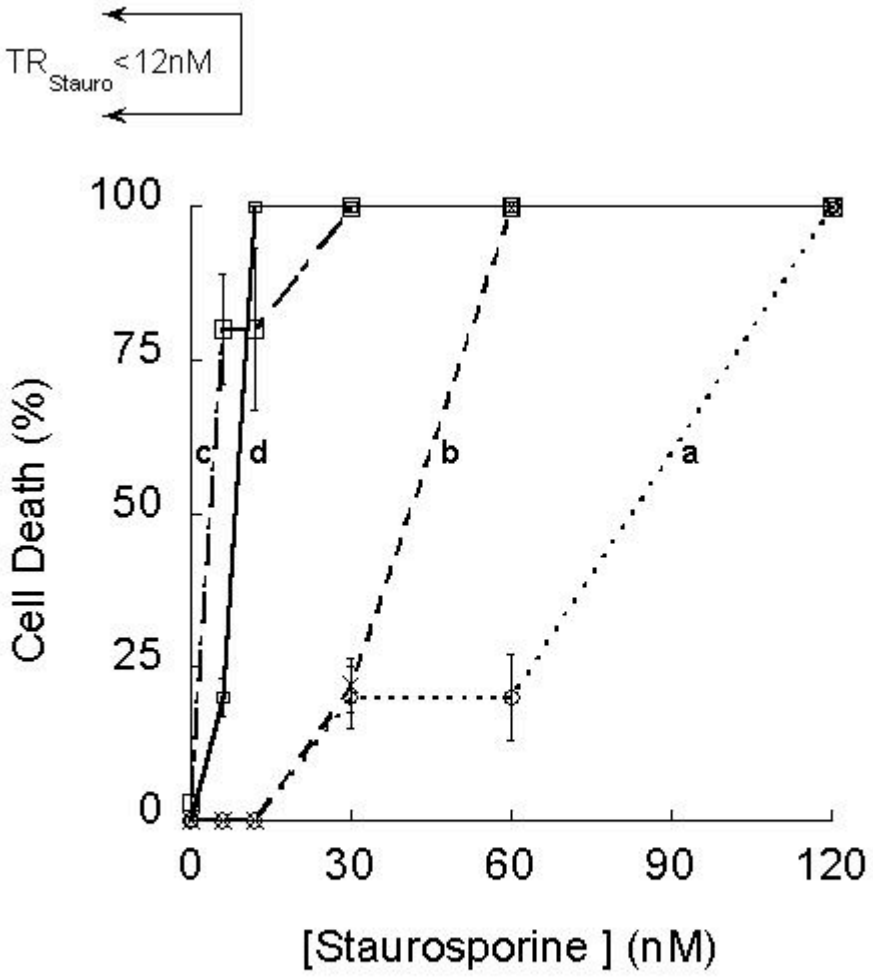

Figure 6 The minimal concentration of staurosporine promoting complete death of JU77 cells in the presence of narrow-spectrum inhibitors including the epidermal growth factor receptor inhibitor.

JU77 cells in their exponential growth phase were treated with varying concentrations of staurosporine in the presence of: 1mM geftinib (¨O¨), 1mM geftinib, 20mM PD98058, 20mM LY294002 (-- x --), 1mM geftinib, 20mM PD98058, 20mM LY294002, 0.75nM bromocriptin (—□ –), 1mM geftinib, 20mM PD98058, 20mM LY294002, 20nM rapamycin (—▫—). The therapeutic range (TR) in which staurosporine can be used in vivo is illustrated at the top left of the figure. Data points are the mean + SD of three determinations.

Table 4 A statistical analysis of the lethal dose estimates of various narrow-spectrum compounds alone or in combination with staurosporine in JU77 and MCF-7 cells.

Description	LD_{100} (nM)	SEM (nM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Stauro	>500		**5**	
Stauro Geft	131	13	**6a**	
Stauro Geft PD LY	51	5	**6b**	[**6a**] P < 0.001 ***
Stauro Geft PD LY Br	11	1	**6c**	[**6b**] P < 0.001 ***
Stauro Geft PD LY Ra	11	1	**6d**	[**6b**] P <0.001 *** [**6c**] P =1 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 6 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Stauro, Staurosporine]; [Geft, Geftinib]; [PD, PD98058]; [LY, LY294002]; [Br, Bromocriptin]; [Ra, Rapamycin]; [Ref. Fig , Reference Figure].

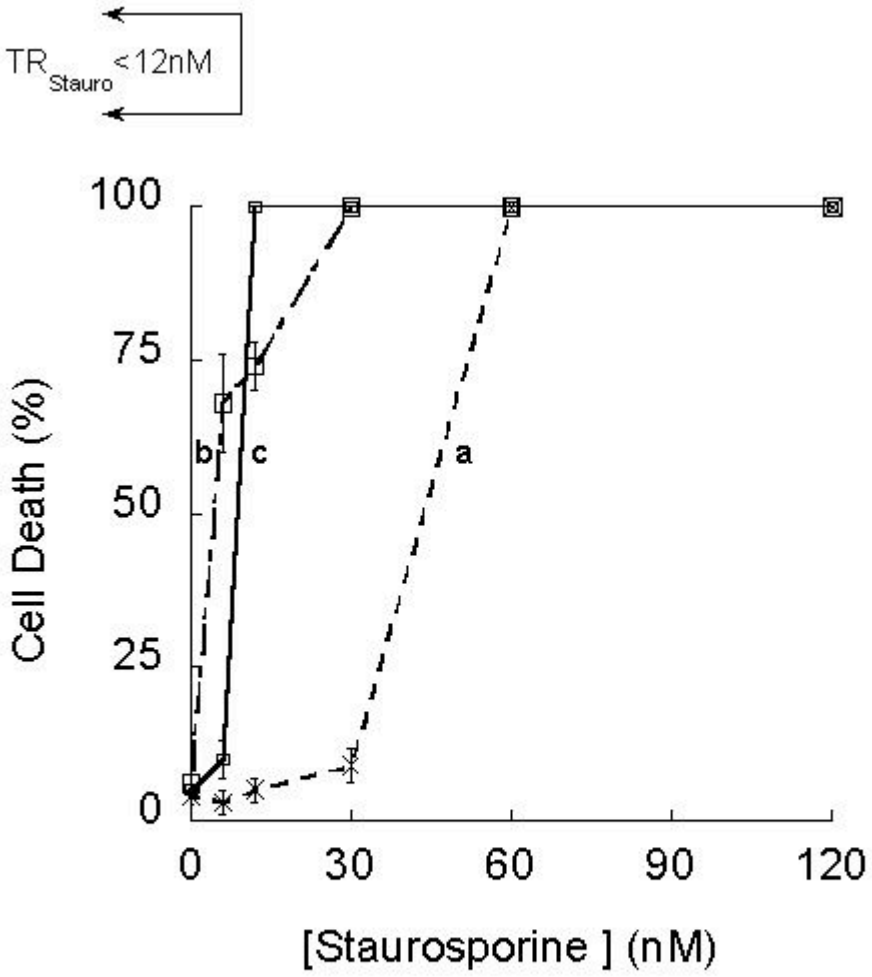

Figure 7 The minimal concentration of staurosporine promoting complete death of JU77 cells in the presence of narrow-spectrum inhibitors excluding the epidermal growth factor receptor.

JU77 cells in their exponential growth phase were treated with varying concentrations of staurosporine in the presence of: 20mM PD98058, 20mM LY294002 (--x--), 20mM PD98058, 20mM LY294002, 0.75nM bromocriptin (—□—), 20mM PD98058, 20mM LY294002, 20mM rapamycin (—▫—). The therapeutic range (TR) in which staurosporine can be used in vivo is illustrated at the top left of the figure. Data points are the mean + SD of three determinations.

Table 5 A statistical analysis of the lethal dose estimates of staurosporine combinations excluding geftinib but including other narrow spectrum inhibitors in JU77 cells.

Description	LD_{100} (nM)	SEM (nM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Stauro PD LY	68	6	**7a**	[**6b**] P > 0.05 (NS)
Stauro PD LY Br	20	2	**7b**	[**7a**] P < 0.001*** [**6c**] P > 0.05 (NS)
Stauro PD LY Ra	15	2	**7c**	[**7a**] P < 0.001*** [**7b**] P > 0.05 (NS) [**6d**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 7 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Stauro, Staurosporine]; [PD, PD98058]; [LY, LY294002]; [Br, Bromocriptin]; [Ra, Rapamycin]; [Ref. Fig , Reference Figure].

were trialled. One combination comprised the two inhibitors of insulin-like growth factor-1 receptor, an agonist for the dopamine receptor, geldanamycin and 6nM staurosporine; this is illustrated in Fig 8. However, including geldanamycin did not significantly decrease the concentration of staurosporine (Table 6, $p>0.05$ (NS)). Geldanamycin is a benzoquinone ansamycin antibiotic that binds to heat shock protein 90 and alters its function. It induces the degradation of proteins that are mutated in tumour cells preferentially over their normal counterparts (Bedin *et.al.* 2004). In contrast, the combination of geldanamycin and 6nM staurosporine killed only a proportion of the JU77 cells.

In MCF-7 cells, the minimal concentration of staurosporine required to eradicate this cell line was estimated as 42nM or greater, irrespective of the narrow-spectrum inhibitors combined with it (Table 7, all $p>0.05$ (NS)). The concentration of staurosporine needed in the presence of geftinib (inhibitor of epidermal growth factor receptor) or a combination of PD98058, LY294002 (inhibitors of insulin-like growth factor-1 receptor pathways) and rapamycin (inhibitor of mammalian target of rapamycin) was equally high in the JU77 cell line than in the MCF-7 cell line (Table 7, both $p>0.05$ (NS)). This is illustrated in Figure 6, 7 and 9, respectively. However, a combined treatment of geldanamycin and staurosporine exerted an extreme significant difference (Table 7, $p<0.001$), as illustrated in Figure 8 and 9.

3.1.2 Combinations with genistein

The flavonoid genistein is derived from the plant family *leguminosae*, which includes the soyabean. Genistein possesses a wide spectrum of physiological and pharmacological functions due to its protein tyrosine kinase inhibition. The binding of genistein to human serum albumin in a cell-free system has been studied by Mahesha *et.al.* in 2005 who reported

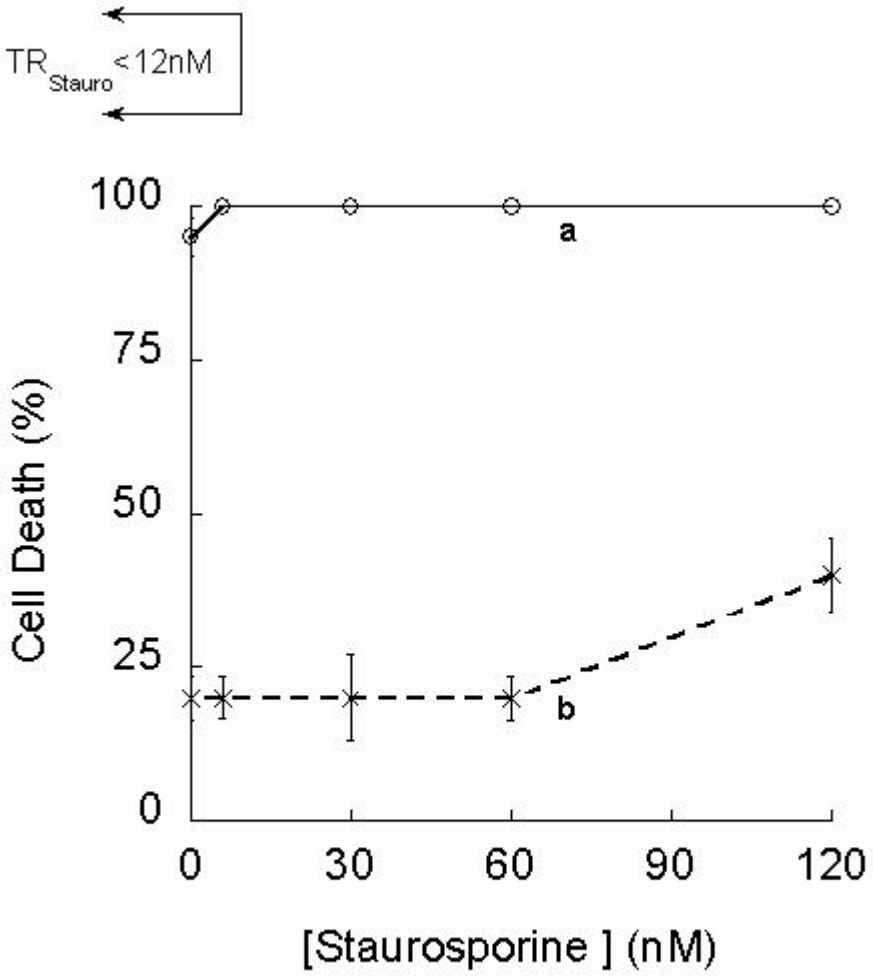

Figure 8 The minimal concentration of staurosporine promoting complete death of JU77 cells in the presence of narrow-spectrum inhibitors and geldanamycin.

JU77 cells in their exponential growth phase were treated with varying concentrations of staurosporine in the presence of: 20μM PD98058, 20mM LY294002, 0.75nM bromocriptin, 20nM geldanamycin (—o—) and 20nM geldanamycin (-- x --). The therapeutic range (TR) in which staurosporine can be used in vivo is illustrated at the top left of the Figureure. Data points are the mean + SD of three determinations.

Table 6 A statistical analysis of the lethal dose estimates of staurosporine combinations including geldanamycin and narrow spectrum inhibitors in JU77 cells.

Description	LD_{100} (nM)	SEM (nM)	Ref. Fig	Level of Significance Comparison to [Fig x]]
Stauro Geld	863	222	**8b**	[**8a**] P < 0.001 ***
Stauro Geld PD LY Br	2	1	**8a**	[**7b**] P > 0.05 (NS) [**7a**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 8 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Stauro, Staurosporine]; [PD, PD98058]; [LY, LY294002]; [Br, Bromocriptin]; [Geld, Geldanamycin]; [Ref. Fig , Reference Figure].

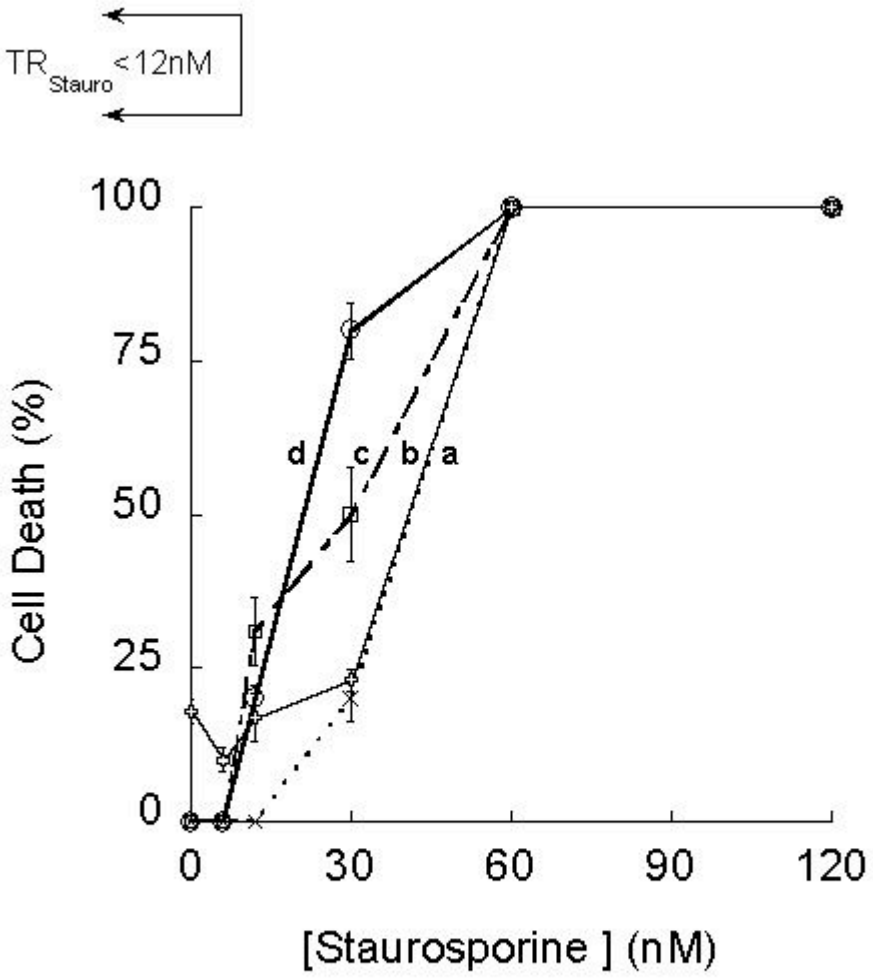

Figure 9 The minimal concentration of staurosporine promoting complete death of MCF-7 cells in the presence of narrow-spectrum inhibitors, mifepristone and geldanamycin.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of staurosporine in the presence of: 1μM geftinib (····· x ·····), 20μM PD98058, 20μM LY294002, 20nM rapamycin (—o—), 100nM oxytocin, 20μM mifepristone (—□ -), 20nM geldanamycin (—✞—). The therapeutic range (TR) in which staurosporine can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 7 A statistical analysis of the lethal dose estimates of staurosporine combinations including mifepristone, geldanamycin and narrow spectrum inhibitors in MCF-7 cells.

Description	LD_{100} (nM)	SEM (nM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Stauro Mif Oxy	59	6	**9c**	
Stauro Geld	82	11	**9b**	[**9c**] P > 0.05 (NS) [**8a**] P < 0.001 ***
Stauro Geft	51	5	**9a**	[**9b**] P > 0.05 (NS) [**9c**] P > 0.05 (NS) [**9d**] P > 0.05 (NS) [**6a**] P > 0.05 (NS)
Stauro PD LY Ra	42	2	**9d**	[**9b**] P > 0.05 (NS) [**9c**] P > 0.05 (NS) [**7c**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 9 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Stauro, Staurosporine]; [Mif, Mifepristone]; [Oxy, Oxytocin]; [Geld, Geldanamycin]; [Geft, Geftinib]; [PD, PD98058]; [LY, LY294002]; [Ra, Rapamycin]; [Ref. Fig , Reference Figure].

that the activity of genistein in a cell culture changes after contact with human serum albumin. Figure 10 illustrates the influence of human serum albumin on the cell killing potency of genistein. Supplementing JU77 cell cultures with human serum albumin significantly increased the concentration of genistein needed to eradicate the JU77 cell line (Table 8, $p<0.05$). Genistein presumably engaged into the subdomain IIa of human serum albumin as detailed in Mahesha`s study, altering its availability. Figure 10 illustrates that genistein recovered its full potency when displaced from this pocket by an equimolar concentration of warfarin (Table 8, $p<0.001$).

Genistein was hydrolysed to genistein chalcone to change its bioavailability. This enhanced the solubility enormously. Both compounds displayed a marked cell death in the presence of letrozole (an inhibitor of aromatase) and suramin (a broad-range inhibitor of growth factor receptors). However, equimolar genistein was twice as potent as genistein chalcone in killing JU77 cells, as illustrated in Figure 11 and Table 9, $p<0.05$).

The potency of genistein was substantially increased in JU77 cells treated with a combination of letrozole and inhibitors of insulin-like growth factor-1 receptor (Figure 12 and Table 10, $p<0.001$). A combination of PD98058, LY294002, letrozole and genistein was equipotent in JU77 cells, compared to suramin, letrozole and genistein (Table 10, $p>0.05$ (NS)).

The effectiveness of all these compound combinations was cell line-dependent. Similar to their effects on JU77 cells, the combination of genistein with suramin and letrozole used on MCF-7 cells was no more potent than genistein with letrozole (Table 11, $p>0.05$ (NS) and Table 9, $p>0.05$ (NS)). However, the potency of genistein with PD98058,

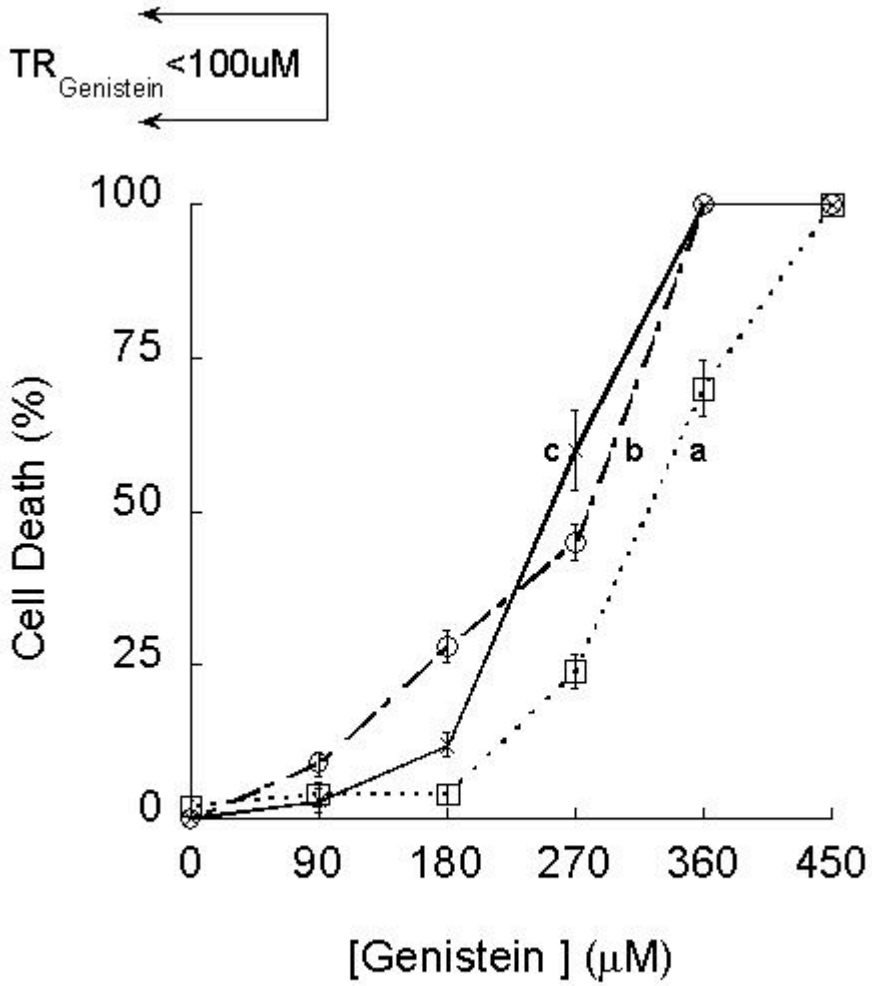

Figure 10 The effect of genistein on the death of JU77 cells in the presence of human serum albumin and warfarin.

JU77 cells in their exponential growth phase were treated with varying concentrations of genistein on its own and in the presence of albumin and warfarin: No additives (—o–), 40g/L albumin (···□···), 40g/L albumin, 100μM warfarin (—x —). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 8 A statistical analysis of the lethal dose estimates of genistein including human serum albumin and warfarin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]]
Gen	444	26	**10a**	
Gen Alb	542	25	**10b**	[**10a**] P < 0.05 *
Gen Alb Warf	382	29	**10c**	[**10a**] P < 0.001*** [**10b**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 10 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [Alb, Albumin]; [Warf, Warfarin]; [Ref. Fig , Reference Figure].

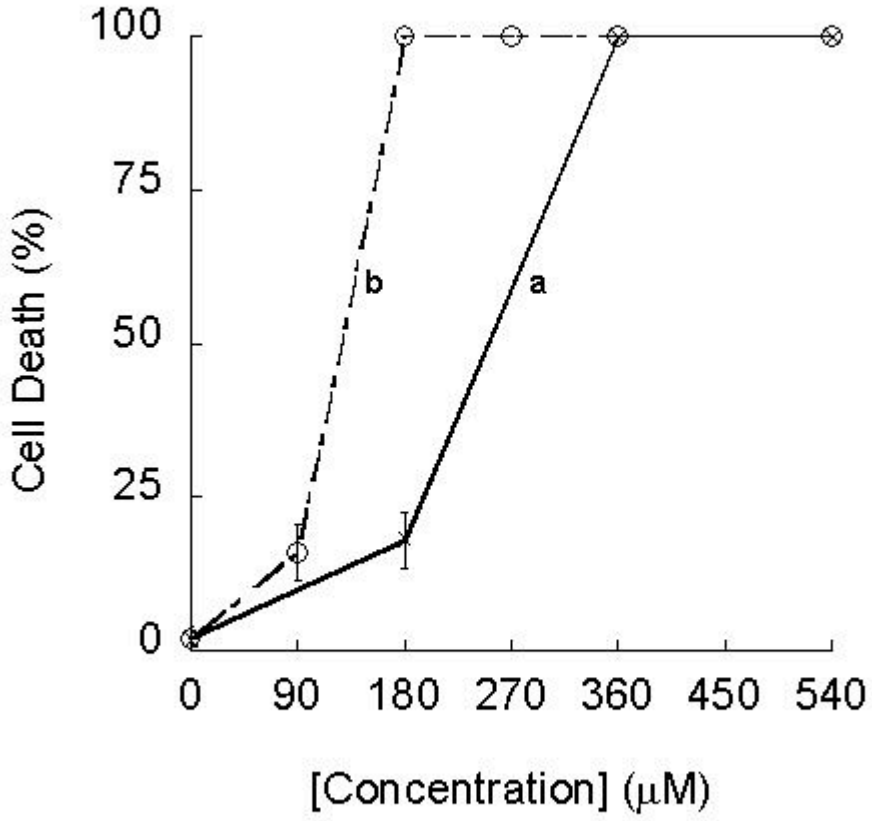

Figure 11 The effect of genistein and genistein chalcone on the death of JU77 cells in the presence of letrozole and suramin.

JU77 cells in their exponential growth phase were treated with varying concentrations of either genistein or genistein chalcone in the presence of letrozole and suramin: genistein with 1μM letrozole and 180mM suramin (—o –), genistein chalcone with 1μM letrozole and 180mM suramin (—x —). Data points are the mean ± SD of three determinations.

Table 9 A statistical analysis of the lethal dose estimates of genistein and genistein chalcone including letrozole and suramin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]]
Gen/L/Sur	195	35	**11b**	
GenCh/L/Sur	390	93	**11a**	[**11b**] $P < 0.05$ *

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 11 as described in the Methods. Data were compared the students t-test as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [GenCh, Genistein Chalcone]; [L, Letrozole]; [Sur, Suramin]; [Ref. Fig , Reference Figure].

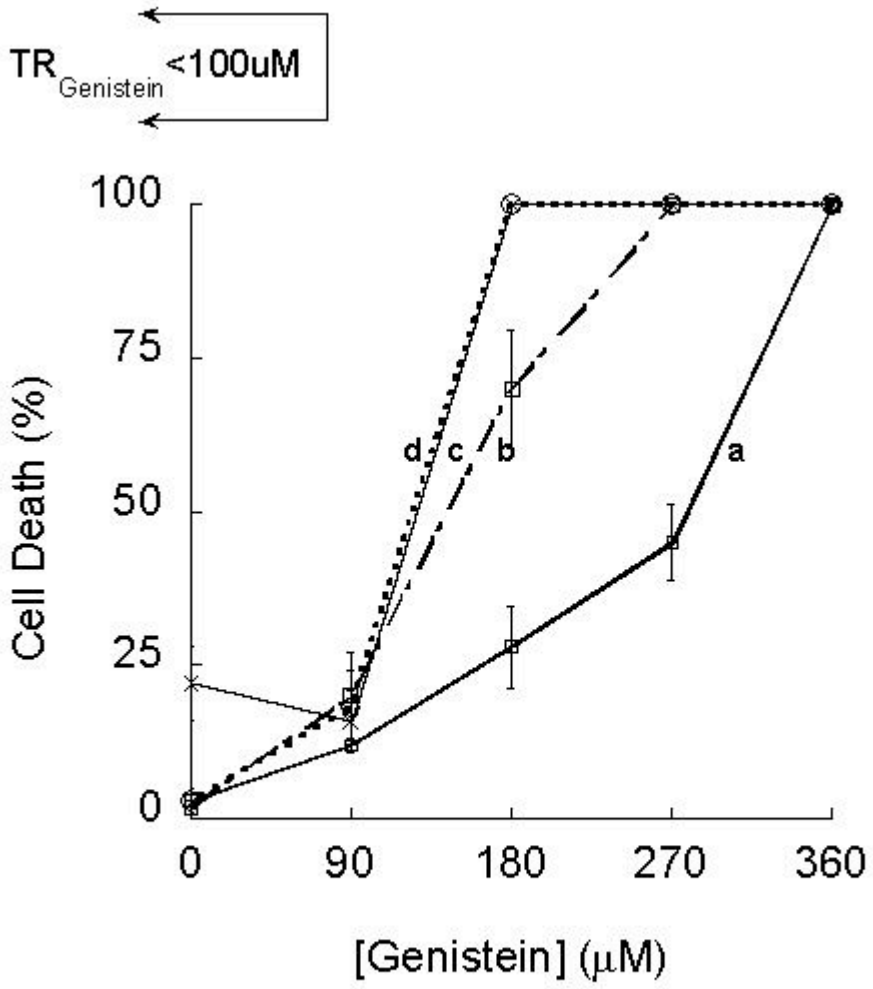

Figure 12 The minimal concentration of genistein promoting complete death of JU77 cells in the presence of PD98058, LY294002, letrozole and suramin.

JU77 cells in their exponential growth phase were treated with varying concentrations of genistein singularly or in the presence of: 20μM PD98058, 20μM LY294002, 1μM letrozole (···o···), 180mM suramin, 1μM letrozole (—x —), 1μM letrozole (—□ -), no additives (—▫—). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 10 A statistical analysis of the lethal dose estimates of genistein including letrozole, suramin, PD98058 and LY294002 in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gen	481	38	**12a**	
Gen/L	283	11	**12b**	[**12a**] P < 0.001***
Gen/L/Sur	260	45	**12c**	[**12a**] P < 0.001*** [**12b**] P > 0.05 (NS)
Gen/L/PD/LY	202	30	**12d**	[**12a**] P < 0.001*** [**12b**] P > 0.05 (NS) [**12c**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 12 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [L, Letrozole]; [Sur, Suramin]; [PD, PD98058]; [LY, LY294002]; [Ref. Fig , Reference Figure].

LY294002 and letrozole did change in MCF-7 cells and presented as the most effective (Figure 13, table 11, $p<0.001$). Supplementing MCF-7 cell cultures with human serum albumin changed the situation again: now suramin was the most potent compound in the presence of genistein (Figure 14, table 12, $p<0.001$). However, as illustrated in Figure 13 and 14, none of the combinations reduced the concentration of genistein to within a therapeutic range of 100µM.

Combinations with gadolinium chloride, a lanthanide, were investigated, as they are known to suppress nitric oxide overproduction and to recover glutathione-synthesis during carcinogenic challenge (Abdel-Zaher *et.al.* 2007). Gadolinium chloride combined with letrozole increased cell death in the presence of genistein (or genistein chalcone) (Figure 15, table 12, $p<0.001$). However, a therapeutic range of gadolinium chloride was only achieved in the absence of human serum albumin.

Another pair of compounds also combined well in the presence of genistein. The combination of forskolin (an inhibitor of protein kinase C) and 3-isobutyl-1-methylxanthine (IBMX) (a broad phosphodiesterase inhibitor) decreased phosphorylation of elongation factor 2, an essential factor for cell replication (Feschenko *et.al.* 2002). The observed increase in cell killing potency of forkolin/IBMX in the presence of genistein correlated with concentrations of IBMX, as illustrated in Figure 16. However, this was statistically not significant (table 14, $p>0.05$ (NS)).

3.1.3 Combinations with mifepristone

The anti-progesterone mifepristone or RU486 was originally developed as an abortion drug. Later, the compound was also recognized as having a significant anti-neoplastic activity due to its effect on cytochrome P450 and the steroidgenesis. The description of the overlapping steroid scaffold in the binding pocket of the receptor by C.Honer in 2003 is crucial in

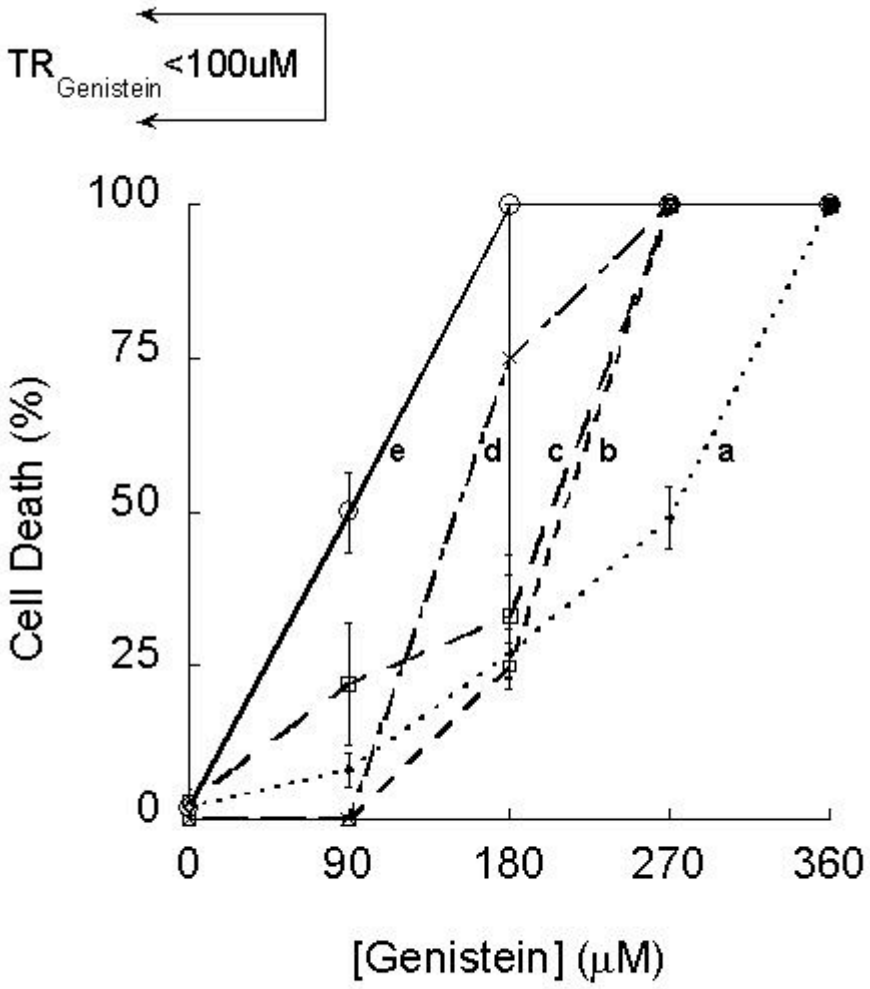

Figure 13 The minimum concentration of genestein promoting the death of MCF-7 cells in the presence of geftinib, PD980580, LY294002, letrozole and suramin.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of genistein in the presence: 1μM geftinib (—x -), 20μM PD98058, 20μM LY294002, 1μM letrozole (—o—), 180mM suramin, 1μM letrozole (- ▫ -), 1μM letrozole (—☐—), no additives (- - ✞ - -). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 11 A statistical analysis of the lethal dose estimates of genistein including geftinib, letrozole, suramin, PD98058 and LY194002 in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gen	455	31	**13a**	
Gen L	345	32	**13c**	[**13b**] P > 0.05 (NS)
Gen Geft	218	0	**13d**	[**13a**] P < 0.001*** [**13b**] P > 0.05 (NS) [**13c**] P < 0.05 *
Gen Sur L	293	10	**13b**	[**13a**] P < 0.001*** [**13c**] P > 0.05 (NS) [**12c**] P > 0.05 (NS)
Gen PD LY L	177	6	**13e**	[**12d**] P > 0.05 (NS) [**13a**] P < 0.001*** [**13b**] P < 0.05 * [**13c**] P < 0.001*** [**13d**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 13 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [L, Letrozole], [Geft, Geftinib], [Sur, Suramin]; [PD, PD98058], [LY, LY294008]; [Ref.Fig , Reference Figure].

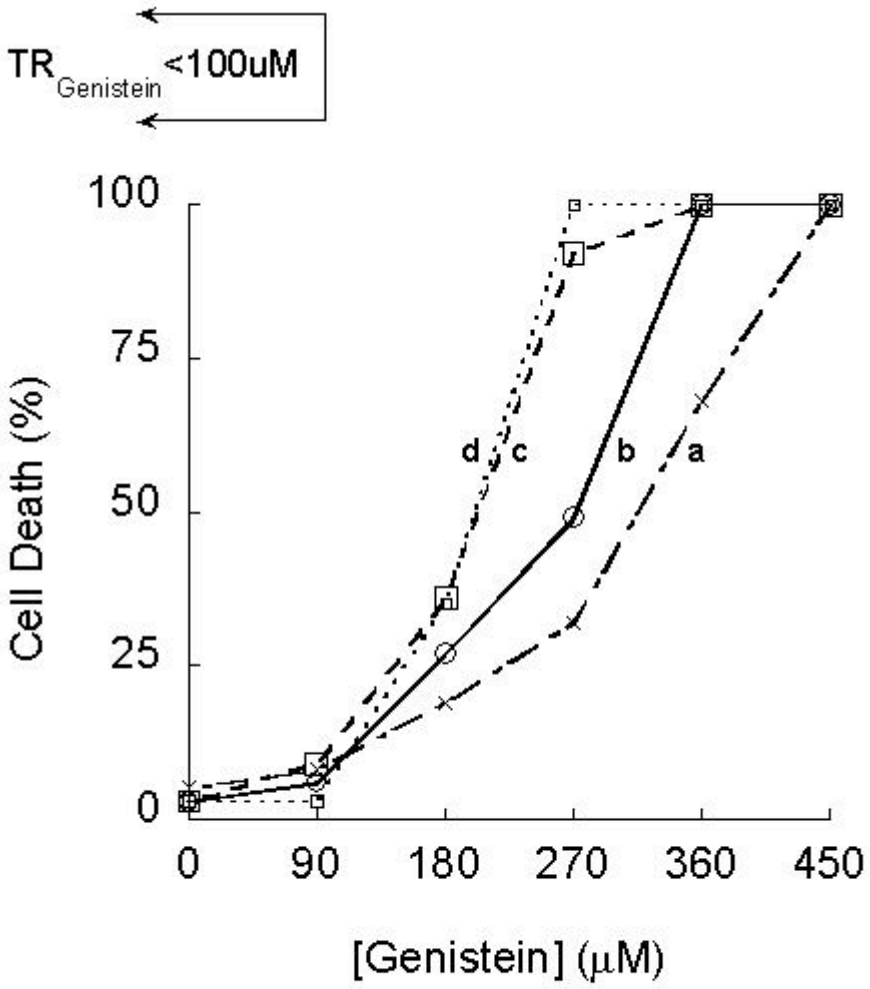

Figure 14 The minimal concentration of genistein promoting complete death of MCF-7 cells grown in the presence of human serum albumin, suramin and PD98058.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of genistein in the presence of: 40g/L albumin, 180mM suramin (—o—), 40g/L albumin, 20μM PD98058 (—x –), 40g/L albumin (····· □ ·····), no additives (– ☐ –). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left of the figure. The results are representative of two experiments.

Table 12 A statistical analysis of the lethal dose estimates of genistein including suramin and PD98058 in the presence of human serum albumin in MCF-7 cells

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gen	447	33	**14b**	[**14c**] P < 0.01** [**14d**] P < 0.01**
Gen Alb	583	44	**14a**	[**14b**] P >0.05 (NS) [**14c**] P >0.05 (NS) [**14d**] P >0.05 (NS)
Gen Alb Sur	319	71	**14d**	[**14c**] P >0.05 (NS)
Gen Alb PD	356	20	**14c**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 14 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [L, Letrozole]; [Sur, Suramin]; [PD, PD98058]; [Ref. Fig , Reference Figure].

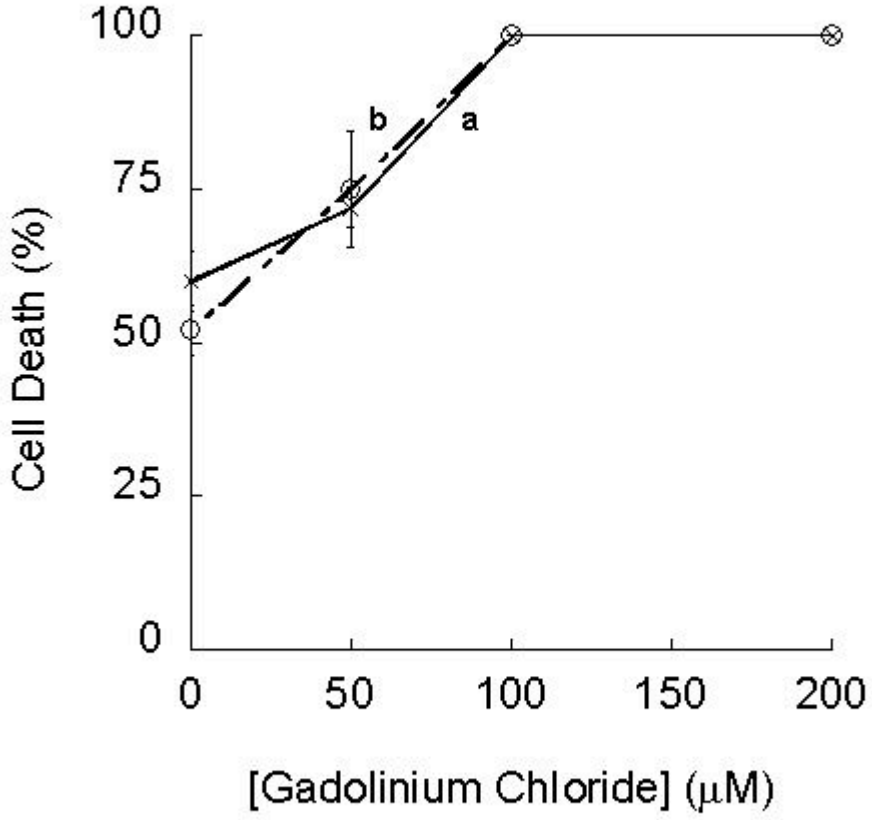

Figure 15 The minimal concentration of gadolinium chloride promoting complete death of JU77 cells in the presence of genistein or genistein chalcone with letrozole.

JU77 cells in their exponential growth phase were treated with varying concentrations of gadolinium chloride in the presence of: 135μM genistein, 1μM letrozole (—x —), 270μM genistein chalcone, 1μM letrozole (—o –). Data points are the mean ± SD of three determinations.

Table 13 A statistical analysis of the lethal dose estimates of gadolinium chloride including letrozole and genistein or genistein chalcone in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gd/Gen/L	121	16	**15a**	[**15b**] P > 0.05 (NS) [**11b**] P < 0.01 ** [**12b**] P <0.001***
Gd/GenCh/L	135	23	**15b**	[**11a**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 15 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [GenCh, Genistein chalcone], [L, Letrozole], [Gd, Gadolinium chloride]; [Ref.Fig , Reference Figure].

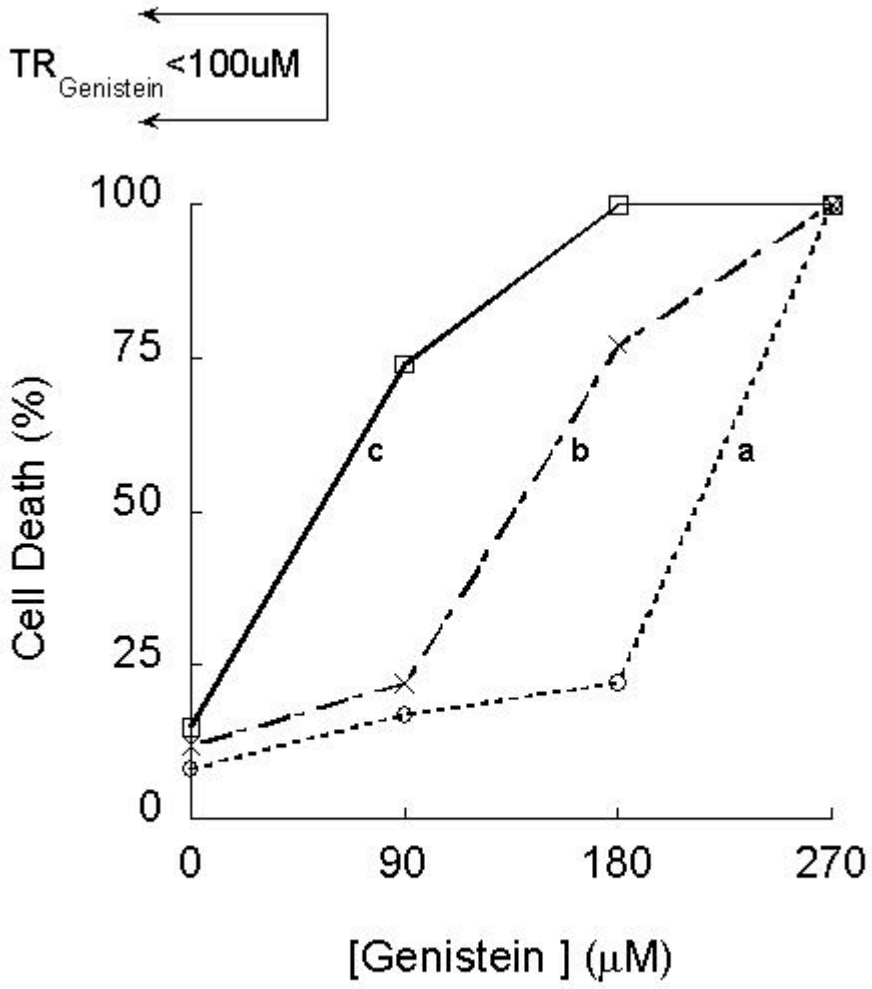

Figure 16 The effect of genistein on the death of JU77 cells in the presence of forskolin and 3-isobutyl-1-methylxanthine (IBMX).

JU77 cells in their exponential growth phase were treated with varying concentrations of genistein in the presence of: 100μM/500μM forskolin/IBMX (···o···), 100μM/750μM forskolin/IBMX (—x –), 100 μM/1000 μM forskolin/IBMX (—□—). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left of the figure. The results are representative of two experiments.

Table 14 A statistical analysis of the lethal dose estimates of genistein including forskolin and varying concentrations of 3-isobutyl-1-methyl-xanthine (IBMX)

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gen Forsk IBMX500	391	132	**14a**	[**14b**] P > 0.05 (NS) [**14c**] P > 0.05 (NS)
Gen Forsk IBMX750	302	31	**14b**	[**14c**] P > 0.05 (NS)
Gen Forsk IBMX1000	171	8	**14c**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 16 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [Forsk, Forskolin]; [IBMX, 3-isobutyl-1-methyl-xanthine]; [Ref. Fig , Reference Figure].

understanding the interactions of mifepristone with other anti-steroids.

The combination of mifepristone with cyproterone acetate (an inhibitor of the androgen receptor) promoted cell death in JU77 as illustrated in Figure 17. The cell killing potency of mifepristone correlated with the concentration of cyproterone acetate (table 15, $p<0.001$). As an example, 100μM cyproterone acetate increased the eradicating potency of mifepristone by a third. However, a therapeutic level could not be achieved using this dual combination.

In comparison to the JU77 cell line, the MCF-7 cell line did not seem to be homogenous. MCF-7 cells could not be eradicated with mifepristone only. Interestingly, 75% of cells died in the presence of more than 30μM mifepristone. However, further increases in mifepristone concentration did not further enhance cell death. This could be explained by the presence of two subpopulations of cells, one resistant to the action of mifepristone. Nevertheless, all cells died in the presence of mifepristone and just 12nM staurosporine (Figure 18 and table 16).

To understand the kinases potentially involved in the survival of the "mifepristone-resistant" MCF-7 cell population, staurosporine was replaced with several compounds (illustrated in Figure 19 and table 17) which targeted the following molecular structures: the epidermal growth factor receptor (geftinib), the dopamine receptor (bromocriptine), the insulin-like growth factor-1 receptor (PD98058, LY294002), the oxytocin receptor (oxytocin), heat shock protein 90 (geldanamycin), elongation factor 2 (forskolin, IBMX), growth factor receptors (suramin) and estrogen receptor

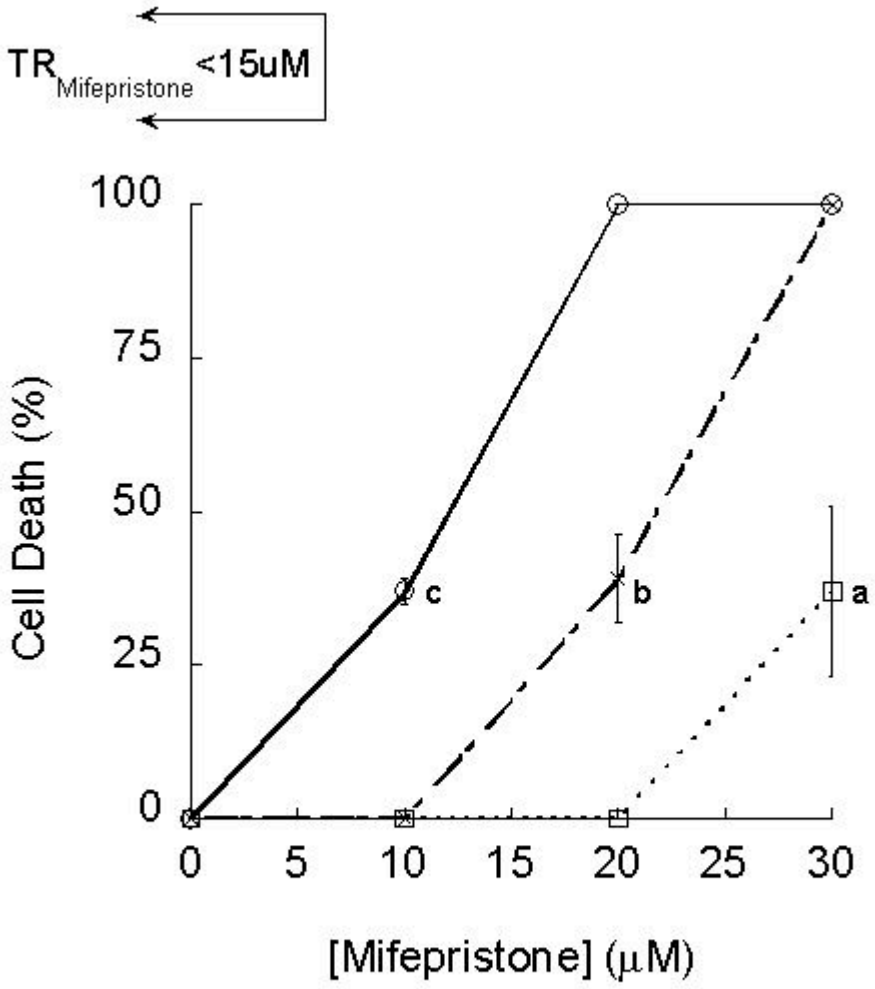

Figure 17 The effect of mifepristone on the death of JU77 cells in the presence of cyproterone acetate.

JU77 cells in their exponential growth phase were treated with varying concentrations of mifepristone in the presence of: 100mM cyproterone acetate (—o—), 50mM cyproterone acetate (—x‑), 20mM cyproterone acetate (···□···). The therapeutic range (TR) in which mifepristone can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 15 A statistical analysis of the lethal dose estimates of mifepristone including varying concentrations of cyproterone acetate in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Mif/CA 20	40	3	**17a**	[**17c**] $P < 0.001$***
Mif/CA 50	28	1	**17b**	[**17a**] $P < 0.001$***
Mif/CA 100	16	0	**17c**	[**17b**] $P < 0.001$***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 17 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Mif, Mifepristone]; [CA, Cyproterone acetate]; [Ref.Fig , Reference Figure].

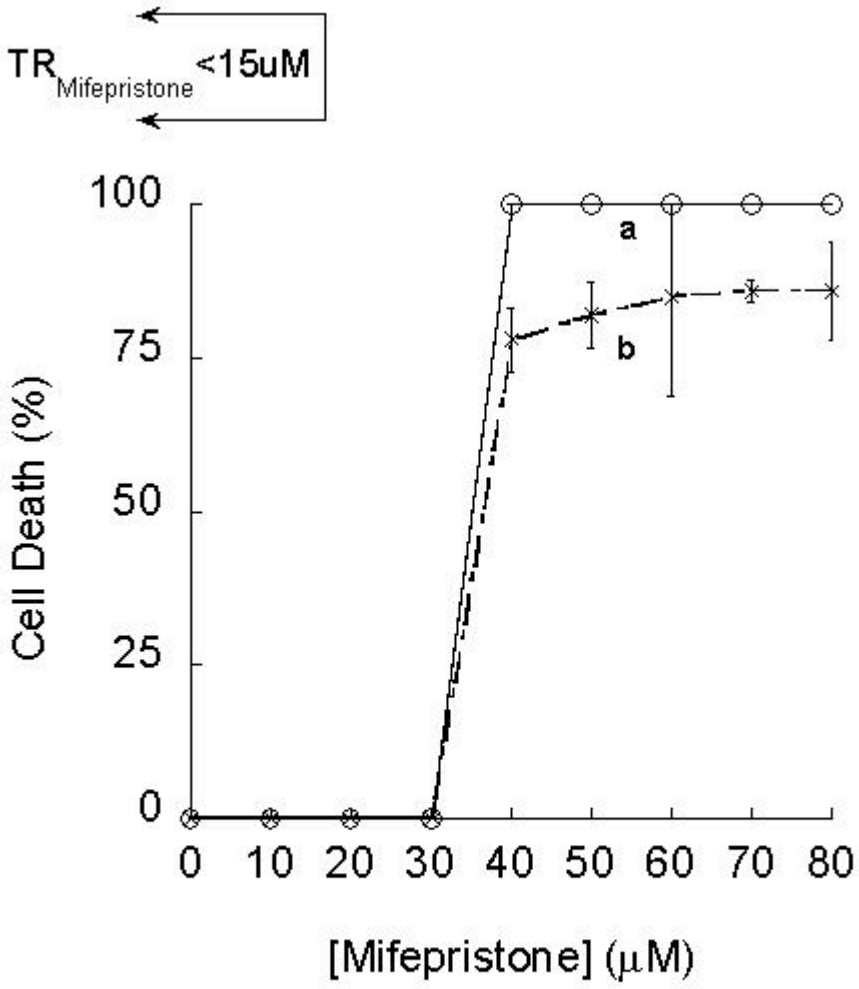

Figure 18 The effect of mifepristone on the death of MCF-7 cells in the presence or absence of staurosporine.

MCF-7 cells in their exponential growth phase were treated with mifepristone singularly or in the presence of staurosporine: No additive (—x –), 12nM staurosporine (—o—). The therapeutic range (TR) in which mifepristone can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 16 A statistical analysis of the lethal dose estimates of mifepristone including staurosporine in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Mif	87	8	**18a**	[**18b**] P is N/A
Mif / Stauro	21	0	**18b**	Standarddeviation=0

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 18 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Mif, Mifepristone]; [Stauro, Staurosporine]; [Ref.Fig , Reference Figure].

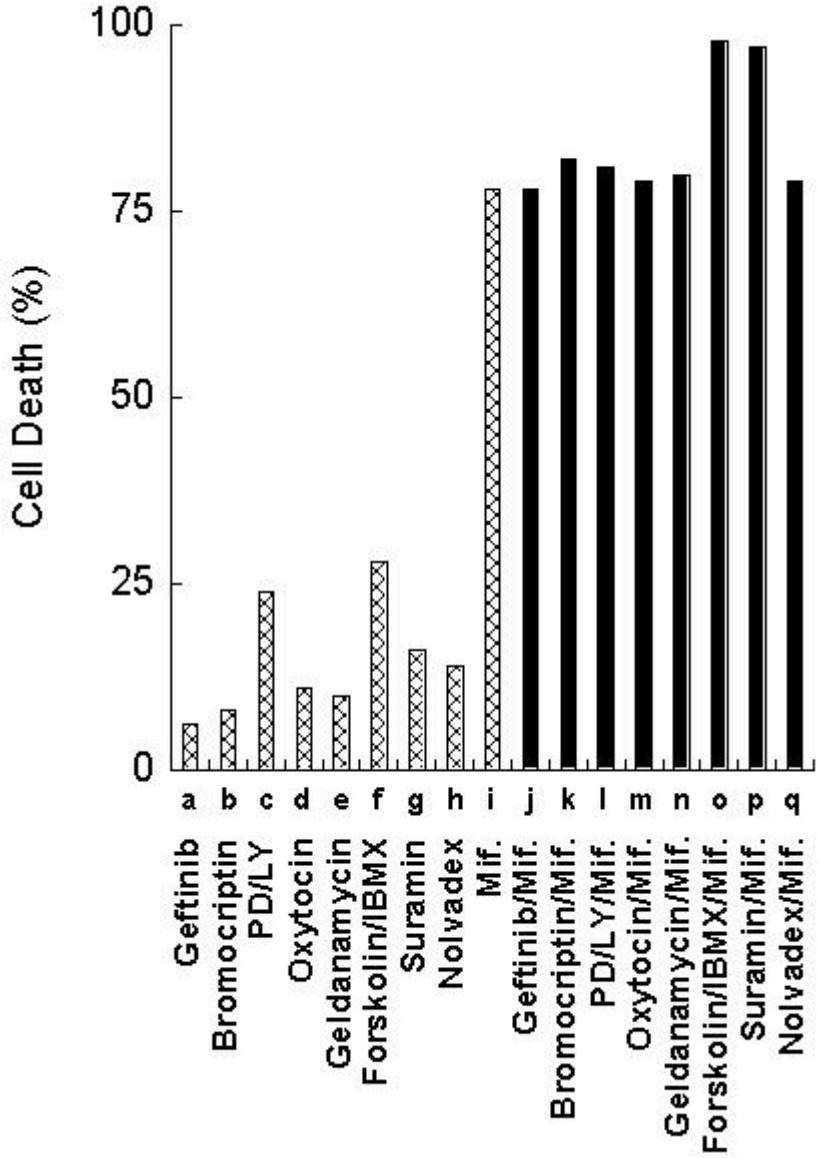

Figure 19 The effect of mifepristone on the death of MCF-7 cells in the presence of narrow and broad-spectrum inhibitors.

MCF-7 cells in their exponential growth phase were treated with no additive, 1μM geftinib, 0.75nM bromocriptin, 20μM/20μM PD98058/LY294002, 100nM oxytocin, 20nM geldanamycin, 75μM/750μM forskolin/IBMX, 180mM suramin or 5μM nolvadex in the absence (⊠) or in the presence (■) of 40μM mifepristone (Mif.). The results are representative of two experiments.

Table 17 A statistical analysis of the lethal effects of mifepristone including narrow- and broad-spectrum inhibitors in MCF-7 cells.

Description	LE %CD	Ref. Fig	Level of Significance Comparison to [Fig x]
Geft Mif	78	**17j**	[**17a**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
Bromo Mif	82	**17k**	[**17b**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
PD LY Mif	81	**17l**	[**17c**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
Oxy Mif	79	**17m**	[**17d**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
Geld Mif	80	**17n**	[**17e**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
Forsk IBMX Mif	98	**17o**	[**17f**],[**17i**] 2P<0.001***
			[**17p**] P < 0.001***
Sur Mif	97	**17p**	[**17g**],[**17i**]2P<0.001***
Nolv Mif	79	**17q**	[**17h**] P < 0.001***
			[**17o**] P < 0.001***
			[**17p**] P < 0.001***
Geft	6	**17a**	
Bromo	8	**17b**	
PD LY	24	**17c**	
Oxy	11	**17d**	
Geld	10	**17e**	
Forsk IBMX	28	**17f**	
Sur	16	**17g**	
Nolv	14	**17h**	
Mif	78	**17i**	

Lethal effects of compounds and their combinations were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Geft, Geftinib]; [Bromo, Bromocriptin]; [PD, PD98058]; [LY, LY294002]; [Oxy, Oxytocin]; [Geld, Geldanamycin]; [Forsk/IBMX, Forskolin/3-isobutyl-methylxanthine]; [Sur, Suramin]; [Nolv, Nolvadex]; [Mif, Mifepristone]; [LE %CD, Lethal effect in % cell death]; [Ref. Fig , Reference Figure].

(nolvadex). Forskolin and IBMX or suramin displayed a strong effect on all MCF-7 cells in the presence of 30μM mifepristone, but none of the compounds eradicated the cell line as staurosporine did. Applying mifepristone singularly at concentrations less than 30μM, staurosporine affected neither MCF-7 nor JU77 cells. However, at these low concentrations mifepristone combined well with cyproterone acetate in both cell lines: increasing concentrations of cyproterone acetate enhanced the percentage of cell death, as illustrated in Figure 20 and table 18

3.1.4 Combinations with vitamin C

Vitamin C is an antineoplastic compound regulating the synthesis of insulin-like growth factor binding proteins 1 and 2, thereby affecting collagen gene expression and proteoglycan synthesis (Peterkofsky *et.al.* 1994). It prevents the degradation of the extracellular matrix. In JU77 cells, cell viability and vitamin C (ascorbic acid) concentrations were inversely correlated. In the absence of human serum albumin JU77 cells died with increasing concentrations of vitamin C and were eradicated with 2mM vitamin C. However, in the presence of human serum albumin, a "lag-phase" was observed with no change in viability up to 3.5mM vitamin C (Figure 21). Above a 3.5mM concentration of vitamin C, cell viability in the presence of human serum albumin correlated with increasing concentrations of vitamin C. Vitamin C concentrations above an estimated concentration of 11mM eradicated the JU77 cell population. However, this concentration exceeded a therapeutic level. In comparison, vitamin C significantly lost its cell killing potency when added to JU77 cells in the presence of human serum albumin , as illustrated in table 19, $p<0.001$.

In MCF-7 cells, human serum albumin also changed the cell killing potency of vitamin C, as illustrated in Figure 22 and table 20, $p<0.001$. However,

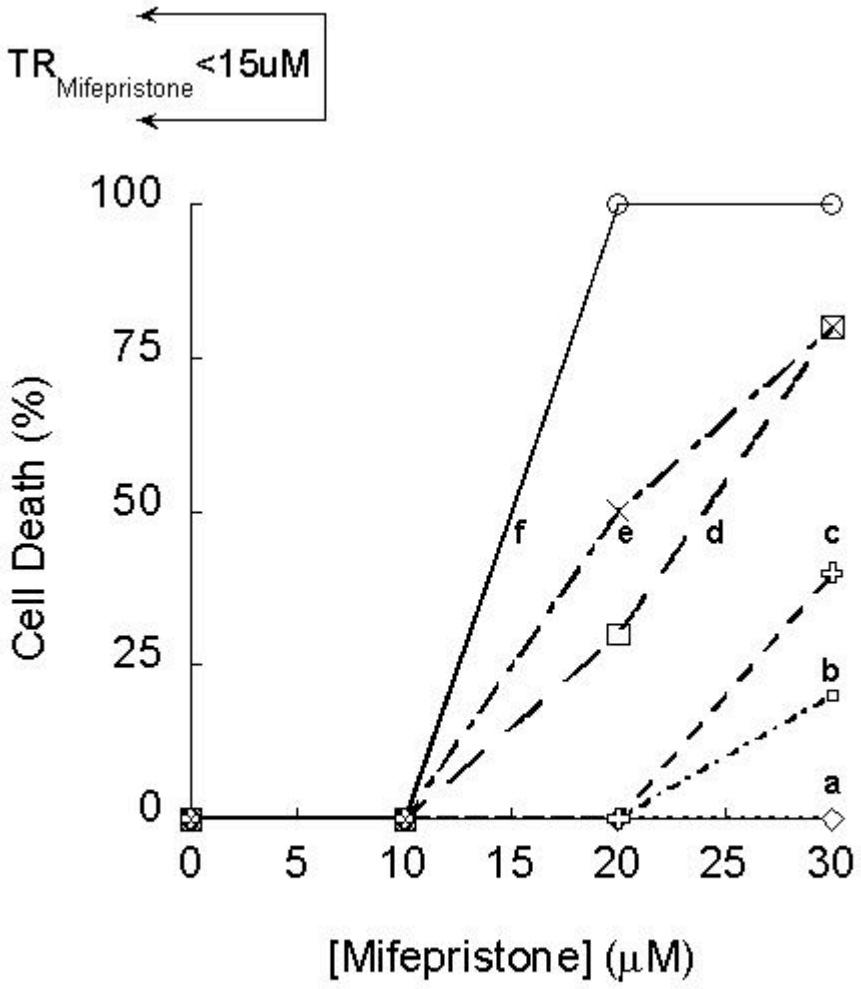

Figure 20 The effect of mifepristone on the death of MCF-7 cells in the presence of cyproterone acetate.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of mifepristone in the presence of cyproterone acetate: 0μM cyproterone acetate (···◊···), 5μM cyproterone acetate (- ▫ -), 10μM cyproterone acetate (– – ✞ – –), 20μM cyproterone acetate (– ☐ –), 50μM cyproterone acetate (—x –), 100μM cyproterone acetate (—o—). The therapeutic range (TR) in which mifepristone can be used *in vivo* is illustrated at the top left of the figure. The results are representative of two experiments.

Table 18 A statistical analysis of the lethal dose estimates of mifepristone including varying concentrations of cyproterone acetate in MCF- cells

Description	LD_{100} (μM)	SEM (μM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Mif CA 0	N/A	N/A	**20a**	Couldn`t be determined Slope of data is zero
Mif CA 5	43	8	**20b**	[**20c**] P > 0.05 (NS) [**20d**] P > 0.05 (NS) [**20e**] P > 0.05 (NS) [**20f**] P < 0.001***
Mif CA 10	40	2	**20c**	[**20d**] P > 0.05 (NS) [**20e**] P > 0.05 (NS) [**20f**] P < 0.01**
Mif CA 20	39	1	**20d**	[**20e**] P > 0.05 (NS) [**20f**] P < 0.01**
Mif CA 50	39	1	**20e**	[**20f**] P < 0.01**
Mif CA 100	19	1	**20f**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 20 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Mif, Mifepristone]; [CA, Cyproterone acetate]; [Ref. Fig , Reference Figure].

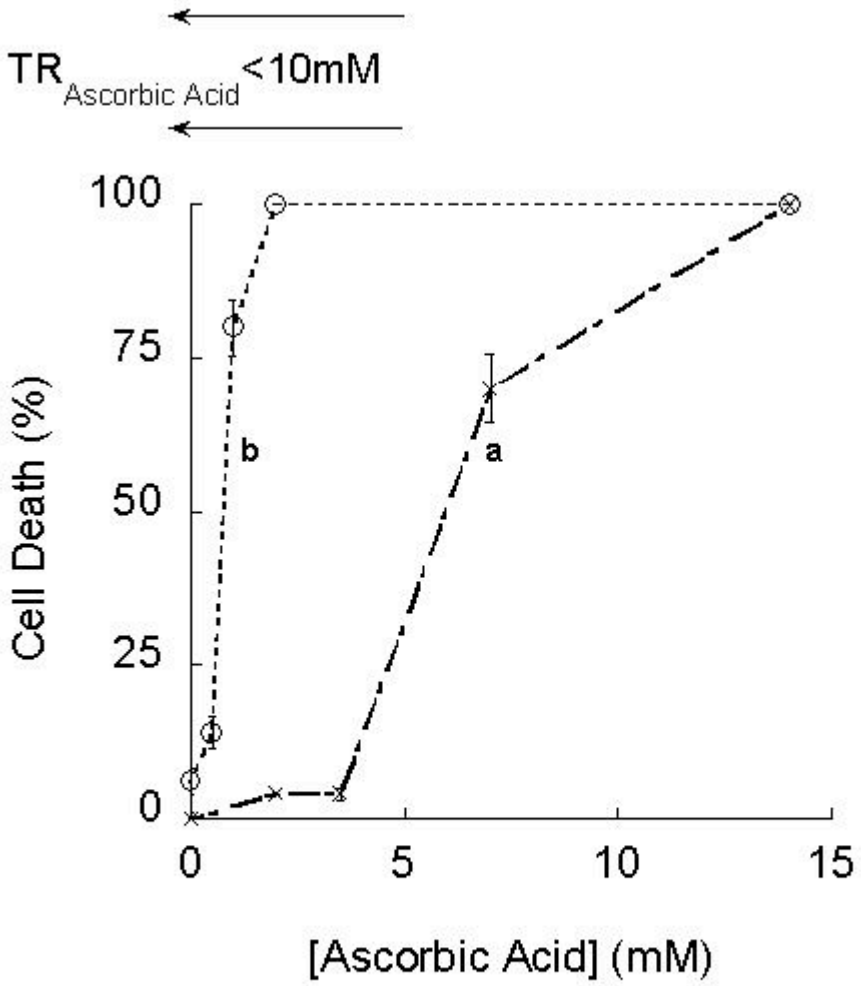

Figure 21 The effect of vitamin C on the death of JU77 cells in the presence or absence of human serum albumin.

JU77 cells in their exponential growth phase were treated with varying concentrations of vitamin C singularly and in the presence of albumin: No additives (····o····), 40g/L albumin (—x –). The therapeutic range (TR) in which vitamin C can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 19 A statistical analysis of the lethal dose estimates of vitamin C including human serum albumin in JU77 cells.

Description	LD_{100} (mM)	SEM (mM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Vit.C	2	0	**21b**	[**21a**] P <0.001***
Vit.C /Alb	11	1	**21a**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 21 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Vit.C, Vitamin C]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

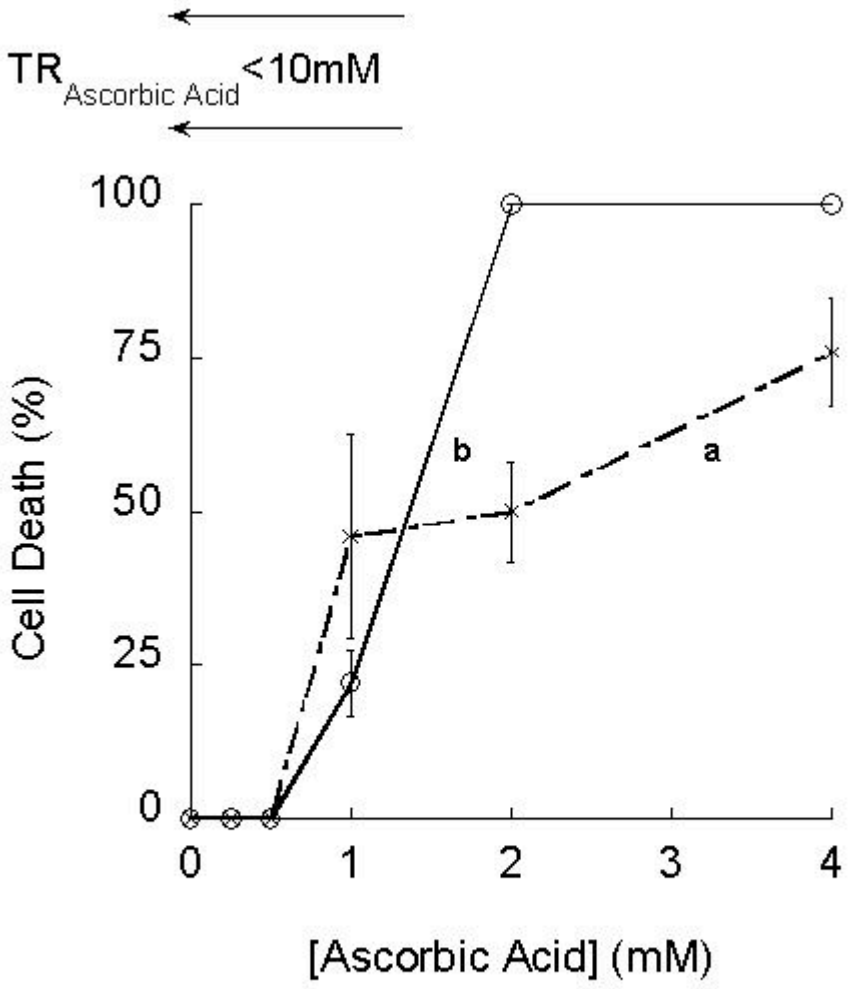

Figure 22 The effect of vitamin C on the death of MCF-7 cells in the presence or absence of human serum albumin.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of vitamin C on its own and in the presence of albumin: No additives (—o—), 40g/L albumin (—x -). The therapeutic range (TR) in which vitamin C can be used *in vivo* is illustrated at the top left of the figure. Data points are the mean ± SD of three determinations.

Table 20 A statistical analysis of the lethal dose estimates of vitamin C including human serum albumin in MCF-7 cells.

Description	LD_{100} (mM)	SEM (mM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Vit.C	2	0	**22b**	[**21b**] P > 0.05 (NS) [**22a**] P < 0.001***
Vit.C /Alb	11	1	**22a**	[**21a**] P <0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 22 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Vit.C, Vitamin C]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

there was also a significant difference comparing the two treatments with vitamin C and albumin in JU77 or MCF-7 cells (table 20, $p<0.001$).

A very effective combination with vitamin C was observed in the absence of human serum albumin: A mitogen-activated protein kinase inhibitor (PD98058), genistein and vitamin C promoted cell death in JU77 cells (Figure 23). However, the presence of human serum albumin attenuated the effects of all this combination on cell death of JU77 cells (table 21, $p<0.001$). Other effective combinations were letrozole (an inhibitor of aromatase), suramin (a broad inhibitor of receptor tyrosine kinases) and vitamin C or mifepristone (an inhibitor of the progesterone receptor), suramin and vitamin C inducing complete cell death in JU77 cells (Figure 24) in the absence of human serum albumin. The addition of letrozole and suramin or mifepristone and suramin to JU77 cells treated with vitamin C significantly changed the cell killing potency (table 22, $p< 0.001$). However, the presence of human serum albumin attenuated the effects of both combinations on cell death of JU77 cells.

3.1.5 Combinations with selenite

Selenite is an inorganic selenium compound that oxidizes fibrin-polymers of cell membranes (Lipinski 2005) and induces the expression of membrane-anchored peroxidases (GPX-4) and serum peroxidases (GPX-1) protecting cell membranes against the lipid peroxidizing effects of fatty acid hydroperoxides and hydrogen peroxide (Brigelius-Flohe, R., 1997). Other compounds containing selenium are inorganic selenate and organic selenomethionine. Their activity is distinct from selenite`s effects.

Increasing selenite concentrations decreased the viability of JU77 cells as illustrated in Figure 25. Adding human serum albumin changed the threshold of selenite-dependent cell death from an estimated concentration

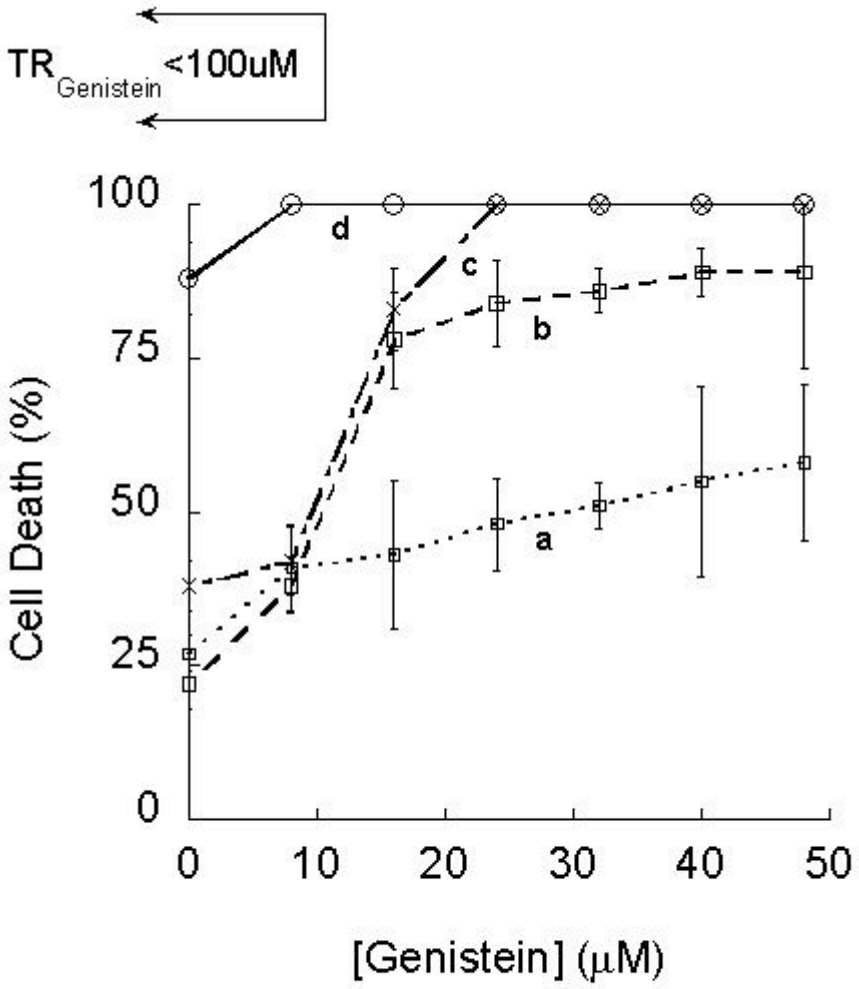

Figure 23 The effect of genistein on the death of JU77 cells in the presence vitamin C and PD98058 with or without human serum albumin.

JU77 cells in their exponential growth phase were treated with varying concentrations of genistein in the presence of: 1mM vitamin C, 20μM PD98058 (—**o**—), 1mM vitamin C (—**x**–), 1mM vitamin C, 20μM PD98058, 40g/L albumin (–□–), 1mM vitamin C, 40g/L albumin (····▫····). The therapeutic range (TR) in which genistein can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 21 A statistical analysis of the lethal dose estimates of genistein including vitamin C and PD98058 in the presence or absence of human serum albumin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Gen/Vit.C	29	2	**23c**	[**10b**] P < 0.001*** [**21b**] P > 0.05 (NS) [**23a**] P < 0.001*** [**23d**] P > 0.05 (NS)
Gen/PD/Vit.C	4	4	**23d**	[**23b**] P > 0.05 (NS)
Gen/PD/Vit.C/Alb	62	9	**23b**	[**23a**] P < 0.001*** [**23d**] P < 0.001***
Gen/Vit.C/Alb	188	45	**23a**	[**10a**] P < 0.001*** [**21a**] P < 0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 23 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gen, Genistein]; [PD, PD98058]; [Vit.C, Vitamin C]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

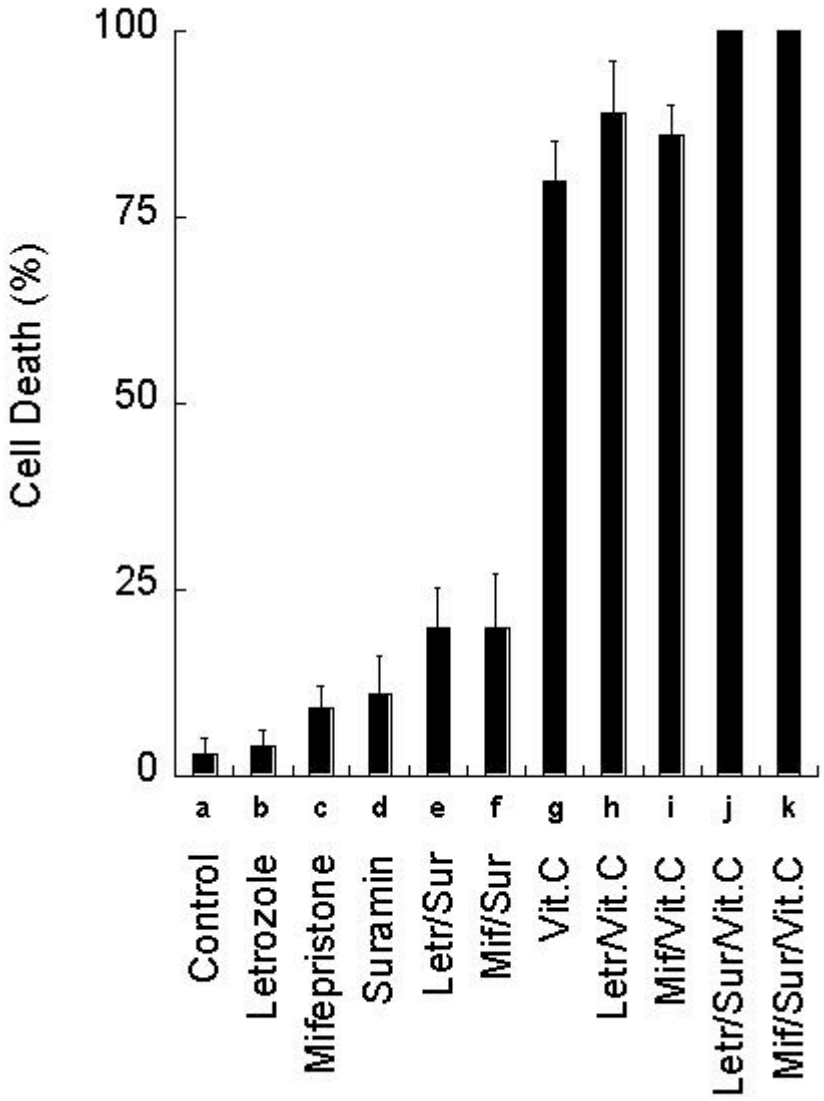

Figure 24 The effect of suramin, letrozole and mifepristone combinations with vitamin C on the death of JU77 cells.

JU77 cells growing exponentially were treated with 1mM vitamin C, 1μM letrozole and 180mM suramin or 1mM vitamin C, 20μM mifepristone and 180mM suramin. Data points are the mean $\pm$ SD of three determinations.

Table 22 A statistical analysis of the lethal effects of vitamin C including letrozole, mifepristone and suramin in JU77 cells.

Description	LE %CD	SEM	Ref. Fig	Level of Significance Comparison to [Fig x]
L Sur Vit.C	100	0	**22j**	[**22h**] P > 0.05 (NS) [**22g**] P < 0.001*** [**22d**] P < 0.001*** [**22b**] P < 0.001***
Mif Sur Vit.C	100	0	**22k**	[**22i**] P < 0.05 * [**22g**] P < 0.001*** [**22d**] P < 0.001*** [**22c**] P < 0.001***
L Vit.C	89	4.04	**22h**	[**22g**] P > 0.05 (NS)
Mif Vit.C	86	2.39	**22i**	[**22g**] P > 0.05 (NS)
Vit.C	80	3.05	**22g**	
L Sur	20	2.98	**22e**	
Mif Sur	20	4.16	**22f**	
L	4	1.15	**22b**	
Mif	9	1.73	**22c**	
Sur	11	2.88	**22d**	

Lethal effects of compounds and their combinations were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [L, Letrozole]; [Sur, Suramin]; [Mif, Mifepristone]; [Vit.C, Vitamin C]; [LE %CD, Lethal effect in % cell death]; [Ref. Fig , Reference Figure].

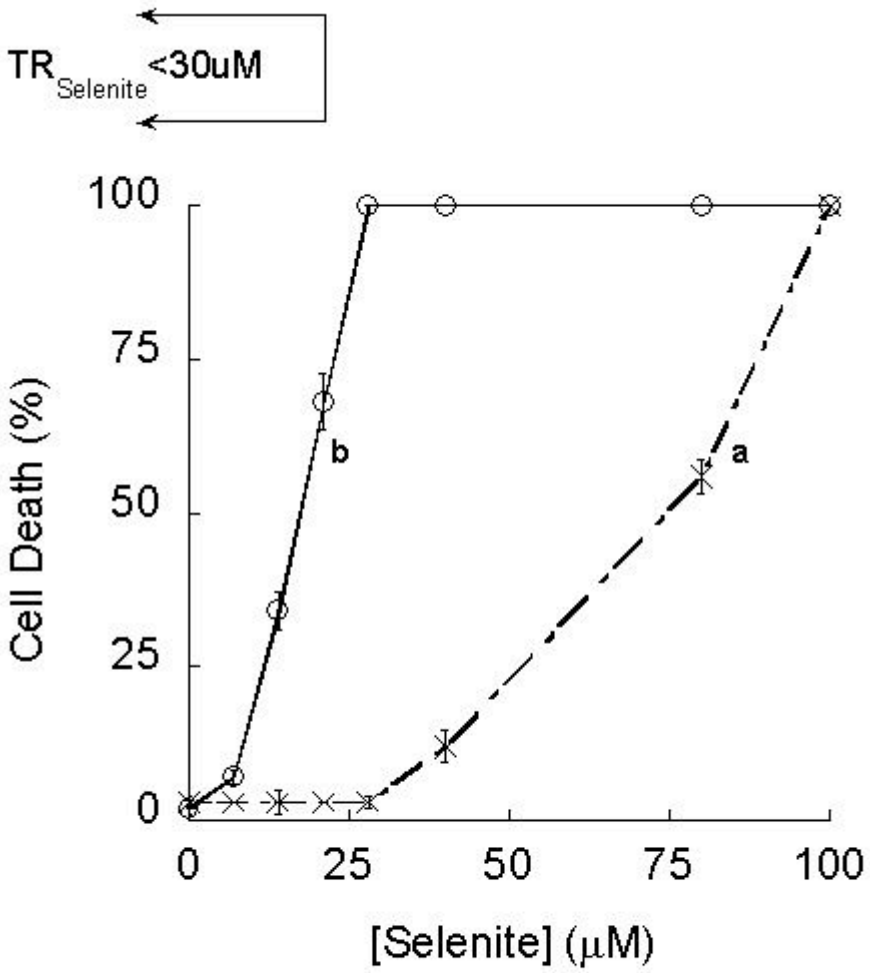

Figure 25 The effect of selenite on the death of JU77 cells in the presence or absence of human serum albumin.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite singularly or in the presence of albumin: No additives (—o—), 40g/L albumin (···· x ····). The therapeutic range (TR) in which selenite can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 23 A statistical analysis of the lethal dose estimates of selenite including human serum albumin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Sele	32	1	**25b**	[**25a**] P <0.001***
Sele/Alb	128	7	**25a**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 25 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Sele, Selenite]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

of 7μM to 28μM (table 23, $p<0.001$) and increased the concentration-dependent cell death for eradication of JU77 cells from an estimated concentration of 32μM to 128μM. Other selenium containing compounds like selenate and selenomethionine used singularly did not influence the viability of JU77 cells as illustrated in Figure 26. Lithium chloride and geldanamycin seemed to increase the cell-killing potency of selenite in JU77 cells in the absence of albumin (Figure 26 and 27, respectively). However, this was not statistically significant (table 24 and 25, $p>0.05$ (NS)). Both compounds seemed equally potent in combination with selenite (table 27, $p>0.05$ (NS)), and only a very small increase could be observed with either selenate or selenomethionine (table 24, $p<0.001$ and table 25, $p<0.001$). 17-Allyl-amino-geldanamycin (17-AAG), a derivate replacing geldanamycin in clinical settings, had to be applied at a tenfold higher concentration relative to geldanamycin to induce a similar cell killing effect (Figure 28 and table 26, $p>0.05$).

Of other compounds tested on JU77 cells, gadolinium chloride strongly increased cell death induced by selenite. The combination was only a third as potent when tested in the presence of human serum albumin (table 27, $p<0.001$). However, this was still within a therapeutic concentration range (Figure 29).

As in JU77 cells, the viability of MCF-7 cells decreased with increasing selenite concentrations. The inclusion of albumin in the culture medium had the same effect on selenite action in both cell lines (Figure 25 and 30, table 28, $p>0.05$ (NS)), and lithium chloride increased the potency of selenite in MCF-7 cells (table 29, $p<0.01$). Two additional compounds (metformin and 8Cl-cAMP) combined well with selenite in MCF-7 cells (Figure 31 and table 29, $p<0.05$ and $p<0.001$), but thiosulfate did not (Figure 31 and table 29, $p>0.05$ (NS)).

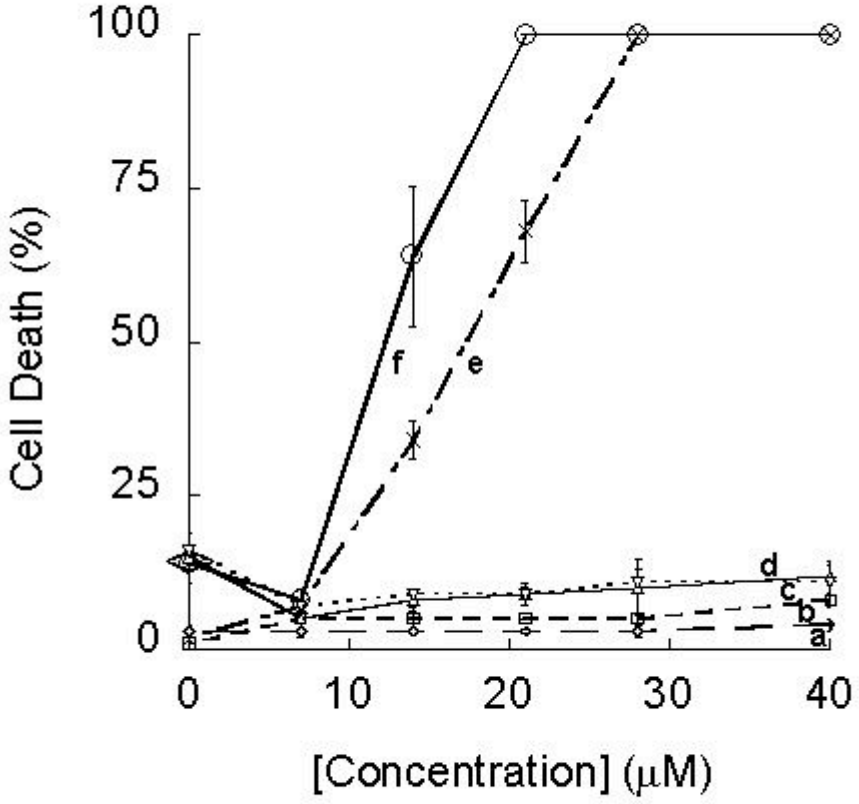

Figure 26 The effect of three selenium compounds - selenite, selenate and selenomethionine - on the death of JU77 cells in the presence or absence of lithium chloride.

JU77 cells in their exponential growth phase were treated with varying concentrations of: selenite and 0.5mM lithium chloride (—**o**—), selenite only (—**x**–), selenate and 0.5mM lithium chloride (—△—), selenate only (– – ✞ – –), selenomethionine and 0.5mM lithium chloride (—▽—), selenomethionine only (- ▫ -). Lithium chloride (0.5mM) used alone causes 14% cell death and is marked on the X-axis as (◊). Data points are the mean ± SD of three determinations.

Table 24 A statistical analysis of the lethal dose estimates of three selenium compounds – selenite, selenate and selenomethionine – including lithium chloride in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Li	26	2	**26f**	
Senate/Li	4779	0	**26c**	
Seme/Li	-1609	0	**26d**	
Senite	32	1	**26e**	[**26a**] P < 0.001*** [**26b**] P < 0.01 ** [**26c**] P < 0.01 ** [**26d**] P < 0.001*** [**26f**] P > 0.05 (NS)
Senate	1489	0	**26a**	[**26b**] P < 0.001*** [**26c**] P < 0.001*** [**26d**] P < 0.001*** [**26f**] P < 0.001***
Seme	324	122	**26b**	[**26c**] P < 0.001*** [**26d**] P < 0.001*** [**26f**] P < 0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 26 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Senate, Selenate]; [Seme, Selenomethionine]; [Li, Lithium chloride]; [Ref.Fig , Reference Figure].

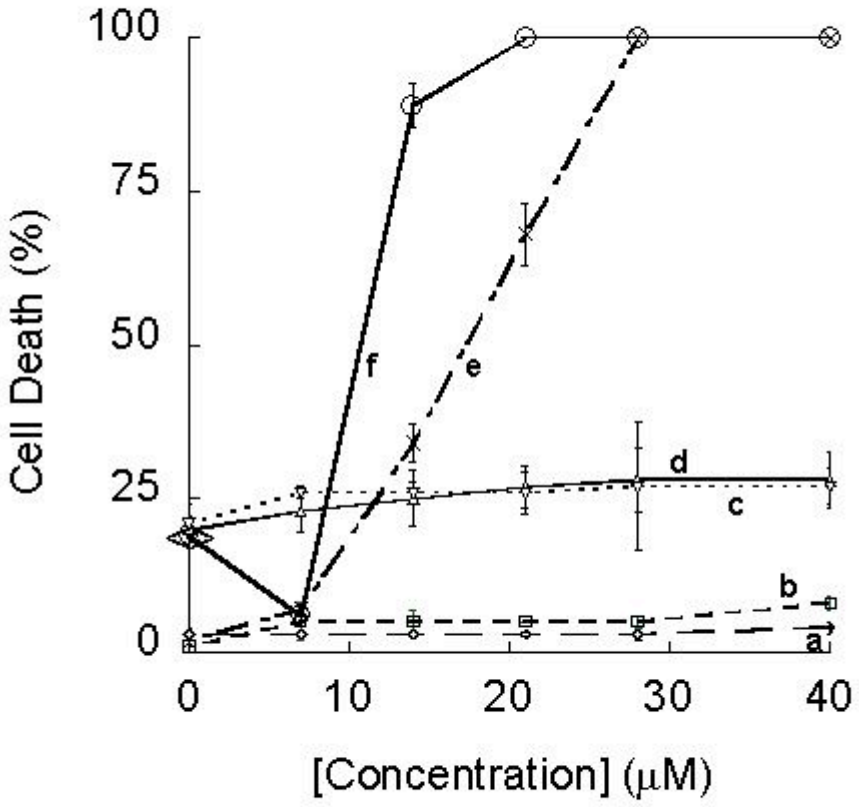

Figure 27 The effect of three selenium compounds - selenite, selenate and selenomethionine - on the death of JU77 cells in the presence or absence of geldanamycin.

JU77 cells in their exponential growth phase were treated with varying concentrations of: selenite and 20nM geldanamycin (—o—), selenite only (—x –), selenate and 20nM geldanamycin (—△—), selenate only (– – ✞ – –), selenomethionine and 20nM geldanamycin (–▽—), selenomethionine only (- □ -). Geldanamycin sy 20nM used singularly caused 20% cell death and is marked on the X-axis as (◊). Data points are the mean ± SD of three determinations.

Table 25 A statistical analysis of the lethal dose estimates of three selenium compounds – selenite, selenate and selenomethionine – including geldanamycin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Geld	23	3	**27f**	[**26f**] P > 0.05 (NS)
Senate/Geld	489	372	**27c**	[**26c**] P < 0.001***
Seme/Geld	838	0	**27d**	[**26d**] P < 0.001***
Senite	32	1	**27e**	[**27a**] P < 0.001*** [**27b**] P > 0.05 (NS) [**27c**] P > 0.05 (NS) [**27d**] P < 0.001*** [**27f**] P > 0.05 (NS)
Senate	1489	0	**27a**	[**27b**] P < 0.001*** [**27c**] P < 0.001*** [**27d**] P < 0.05 * [**27f**] P < 0.001***
Seme	324	122	**27b**	[**27c**] P > 0.05 (NS) [**27d**] P > 0.05 (NS) [**27f**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 27 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Senate, Selenate]; [Seme, Selenomethionine]; [Geld, Geldanamycin]; [Ref.Fig , Reference Figure].

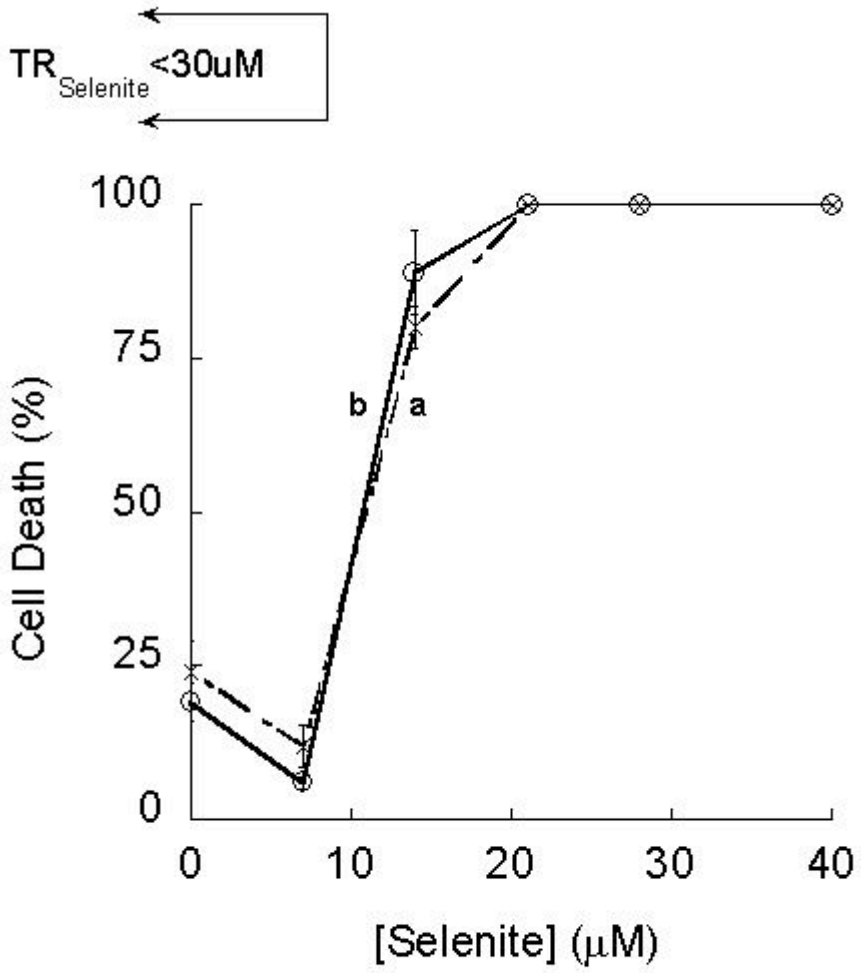

Figure 28 The effect of selenite on the death of JU77 cells in the presence of human serum albumin and geldanamycin or 17-allylamino-geldanamycin.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite in the presence of: 20nM geldanamycin and 40g/L albumin (—o—), 200nM 17-allylamino-geldanamycin and 40g/L albumin (—x –). The therapeutic range (TR) in which selenite can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 26 A statistical analysis of the lethal dose estimates of selenite including human serum albumin and geldanamycin or 17-allylamino-geldanamycin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Geld/Alb	23	3	**28b**	
Senite/17-AAG/Alb	25	3	**28a**	[**28b**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 28 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Geld, Geldanamycin]; [17-AAG, 17-Allylamino-geldanamycin]; [Alb, Albumin]; [Ref. Fig , Reference Figure].

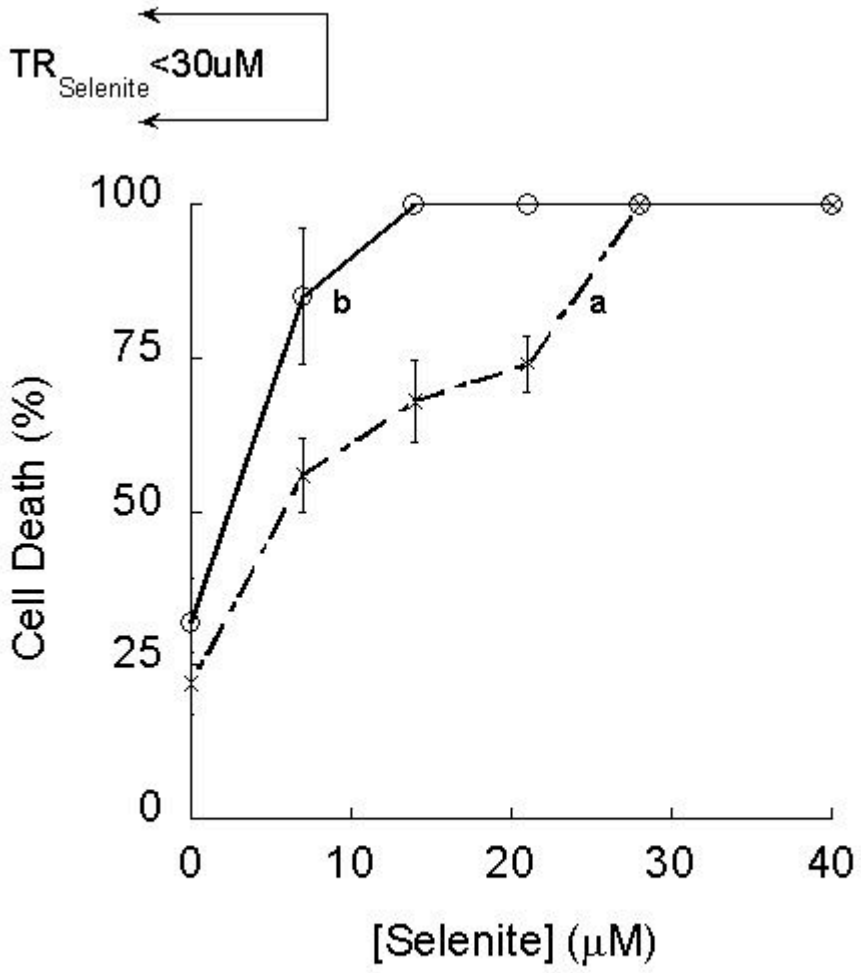

Figure 29 The effect of selenite on the death of JU77 cells in the presence of gadolinium chloride with or without human serum albumin.

JU77 cell in their exponential growth phase were treated with varying concentrations of selenite in the presence of: 200μM gadolinium chloride (—o—), 200μM gadolinium chloride, 40g/L albumin (—x -). The therapeutic range (TR) in which selenite can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 27 A statistical analysis of the lethal dose estimates of selenite and gadolinium chloride including human serum albumin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Gd	13	1	**29b**	
Senite/Gd/Alb	36	3	**29a**	[**29b**] $P < 0.001$*** [**25a**] $P < 0.001$***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 29 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Gd, Gadolinium chloride]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

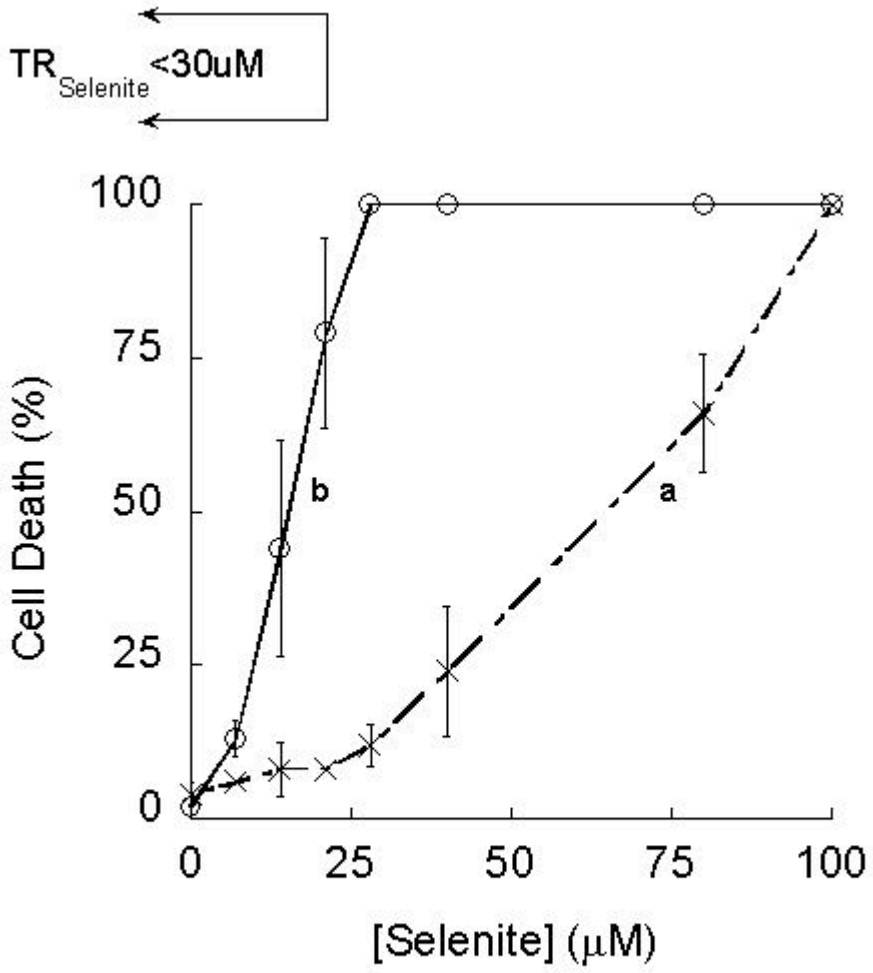

Figure 30 The effect of selenite on the death of MCF-7 cells in the presence or absence of human serum albumin.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of selenite in the presence or absence of albumin: No additives (—o—), 40g/L albumin (—x –). The therapeutic range (TR) in which selenite can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 28 A statistical analysis of the lethal dose estimates of selenite including human serum albumin in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite	30	2	**30b**	[**25b**] P > 0.05 (NS)
Senite/Alb	128	7	**30a**	[**25a**] P > 0.05 (NS) [**30b**] P < 0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 30 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

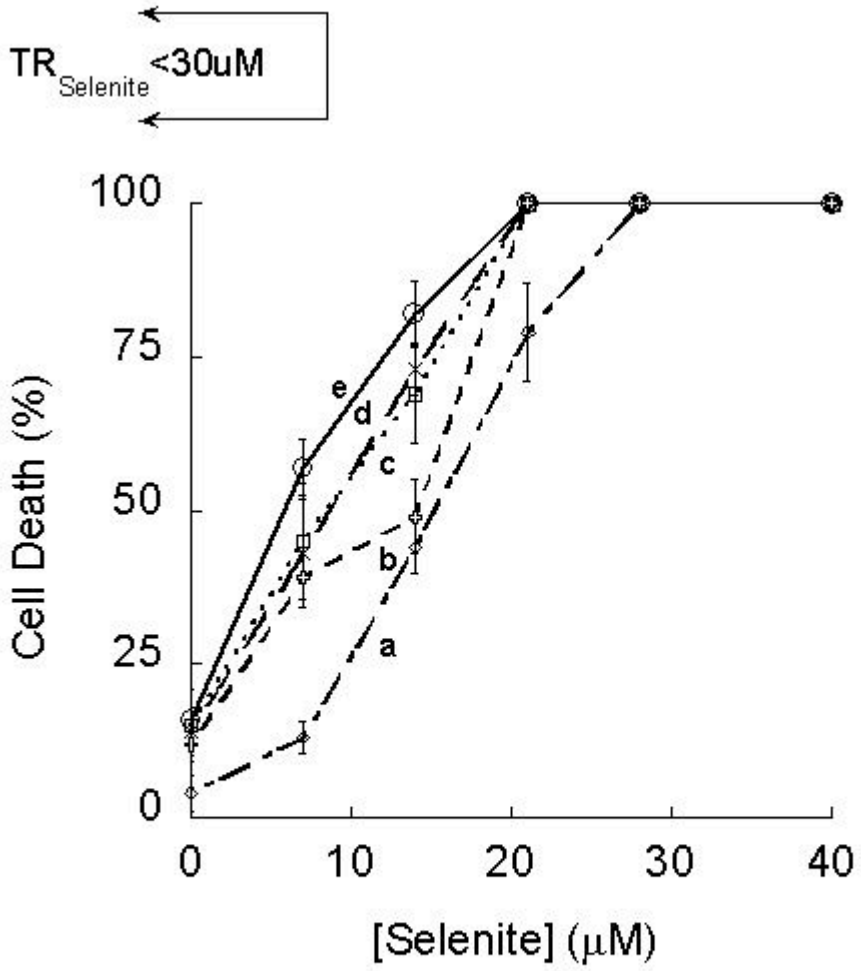

Figure 31 The effect of selenite on the death of MCF-7 cells in the presence of metformin or lithium chloride or thiosulfate or 8Cl-cAMP.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of selenite in the presence of: No additive (—◊ -), 50μM metformin (···□···), 0.5mM lithium chloride (—x —), 0.5mM thiosulfate (- - ✞ - -), 20μM 8Cl-cAMP (—o—). The therapeutic range (TR) in which selenite can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 29 A statistical analysis of the lethal dose estimates of selenite including metformin, 8Cl-cAMP, thiosulfate or lithium chloride in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite	31	1	**31a**	
Senite/Metf	25	2	**31c**	[**31a**] P < 0.05 * [**31b**] P > 0.05 (NS) [**31d**] P > 0.05 (NS) [**31e**] P > 0.05 (NS)
Senite/8Cl-cAMP	22	0	**31e**	[**31a**] P < 0.001***
Senite/Thio	28	2	**31b**	[**31a**] P > 0.05 (NS) [**31e**] P < 0.05 *
Senite/Li	24	1	**31d**	[**31a**] P < 0.01 ** [**31b**] P > 0.05 (NS) [**31e**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 31 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Metf, Metformin]; [8Cl-cAMP, 8Chloro-cyclic Adenosinmonophosphate]; [Thio, Thiosulfate]; [Li, Lithium chloride] [Ref. Fig , Reference Figure].

3.1.6 Combinations with trisulphides

Selenite and sulphydryl group-containing compounds form cancer-selective selenotrisulphides (Abdullaev, Mac Vicar and Frenkel 1992) that target the elongation stage of RNA-polymerase 2 (Frenkel, Walcott and Middleton 1987). Many trisulphides were synthesised with different non sulphydryl group-containing compounds.

Figure 32 illustrates how increasing concentrations of trisulphides directly correlated with a loss in viability of JU77 cells in the presence of human serum albumin. Four different sulphydryl group-containing compounds were compared: selenomethionine, dihydrolipoic acid, cysteine and glutathione. An equimolar concentration of each sulphydryl group-containing compound was combined with selenite. The potency of all the trisulphides appeared to be identical (table 30, $p>0.05$ (NS)). The minimal trisulphide-forming selenite concentration that eradicated JU77 cells decreased to an estimated 48μM in the presence of human serum albumin. None of the sulphydryl group- containing compounds alone completely killed the JU77 cell population in the presence of human serum albumin (Figure 33 and table 31).

Selenodimethionine is the trisulphide synthesised with inorganic selenite and organic selenomethionine. In JU77 cells, selenodimethionine substantially increased cell death in combination with lithium chloride or geldanamycin in the presence of human serum albumin (table 32, $p<0.05$ and $p<0.01$) reducing the concentration of both selenite and seleno-methionine to an estimated 35μM and 38μM, respectively, which is well in the therapeutic range (Figure 34). In MCF-7 cells, no statistic significant increase in cell death was observed using selenodimethionine and lithium chloride in the presence of albumin (Figure 35 and table 33, $p>0.05$ (NS)).

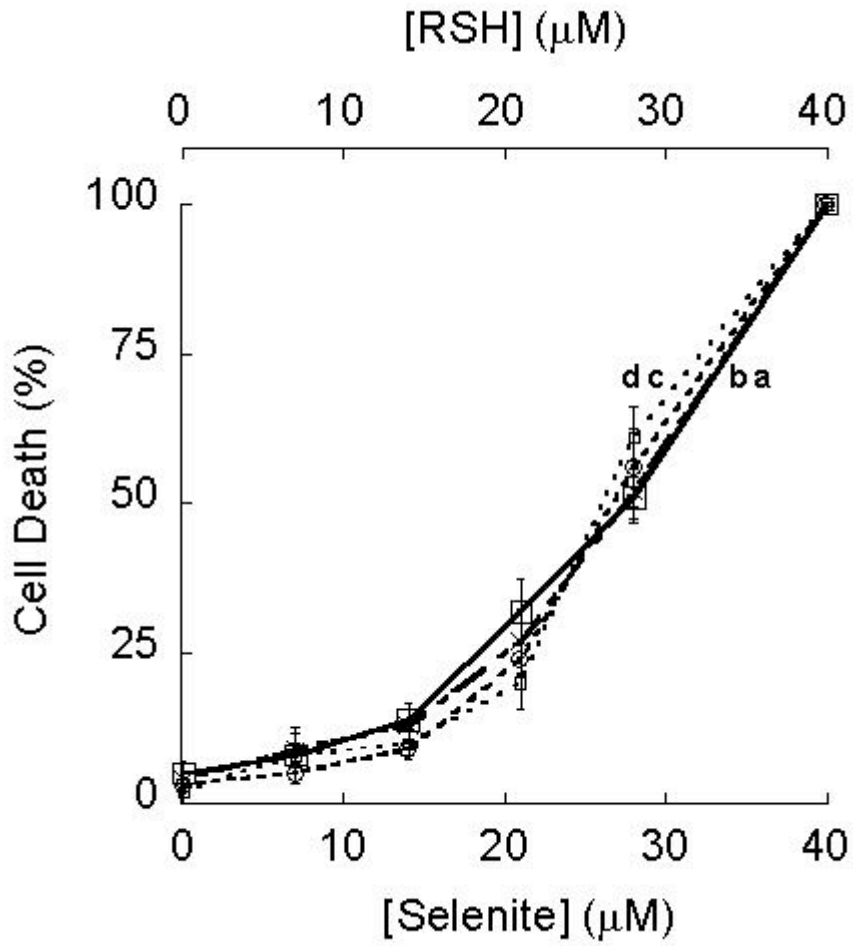

Figure 32 The effect of simultaneous application of selenite and sulphydryl group-containing compounds (RSH) on the death of JU77 cells in the presence of human serum albumin.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite and the following sulphydryl group-containing compounds in 40g/L albumin: selenite/dihydrolipoic acid in 40g/L albumin (—□—), selenite/cysteine in 40g/L albumin (—**x** –), selenite/selenomethionine in 40g/L albumin (**-- o--**), selenite/glutathione in 40g/L albumin (**-- ▫ --**). Data points are the mean ± SD of three determinations.

Table 30 A statistical analysis of the lethal dose estimates of selenite and sulphydryl group-containing compounds (RSH) in the presence of human serum albumin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Glut/Alb	48	3	**32d**	
Senite/Seme/Alb	48	3	**32a**	[**32d**] P > 0.05 (NS) [**25a**] P < 0.001***
Senite/Cyst/Alb	51	3	**32b**	[**32a**] P > 0.05 (NS) [**32d**] P > 0.05 (NS)
Senite/DHLA/Alb	51	3	**32c**	[**32a**] P > 0.05 (NS) [**32b**] P > 0.05 (NS) [**32d**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 32 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [DHLA, Dihydrolipoic acid]; [Cyst, Cysteine]; [Seme, Selenomethionine]; [Alb, Albumin]; [Ref. Fig , Reference Figure].

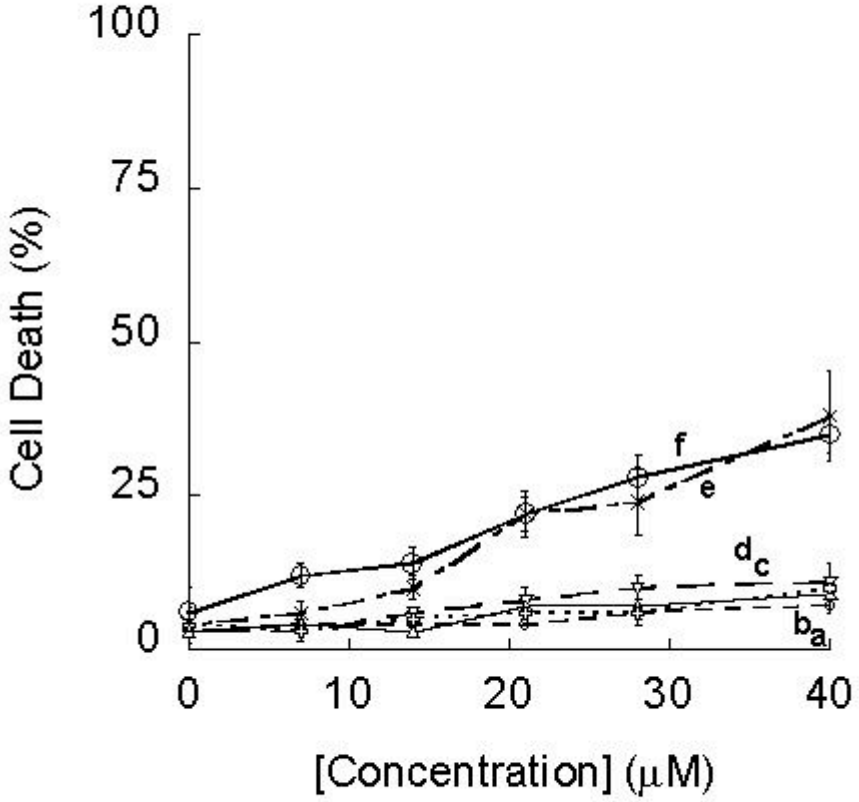

Figure 33 The effect of sulphydryl group-containing compounds on the death of JU77 cells in the presence of human serum albumin.

JU77 cells in their exponential growth phase were treated with varying concentrations
of dihydrolipoic acid (—o—), lipoic acid (—x -), cysteine (—▽—), selenomethionine (-- o--), glutathione (—△—) and mercaptoethanol (- - ✞ - -) in the presence of 40g/L albumin. Data points are the mean ± SD of three determinations.

Table 31 A statistical analysis of the lethal dose estimates of sulphydryl group-containing compounds in the presence of human serum albumin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Seme/Alb	427	349	**33a**	[**33b**] P > 0.05 (NS) [**33c**] P > 0.05 (NS) [**33d**] P > 0.05 (NS) [**32a**] P > 0.05 (NS)
Cyst/Alb	237	46	**33d**	[**32b**] P < 0.01 **
Lipoic/Alb	110	7	**33f**	[**33a**] P > 0.05 (NS) [**33b**] P > 0.05 (NS) [**33c**] P > 0.05 (NS) [**33d**] P > 0.05 (NS)
DHLA/Alb	135	11	**33e**	[**33a**] P > 0.05 (NS) [**33b**] P > 0.05 (NS) [**33c**] P > 0.05 (NS) [**33d**] P > 0.05 (NS) [**33f**] P > 0.05 (NS) [**32c**] P < 0.001***
Merc/Alb	316	104	**33c**	[**33d**] P > 0.05 (NS)
Glut/Alb	296	88	**33b**	[**33c**] P > 0.05 (NS) [**33d**] P > 0.05 (NS) [**32d**] P < 0.01 **

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 33 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Seme, Selenomethionine]; [Cyst, Cysteine]; [Lipoic, Lipoic acid]; [DHLA, Dihydrolipoic acid]; [Merc, Mercaptoethanol]; [Glut, Glutathione]; [Ref.Fig , Reference Figure].

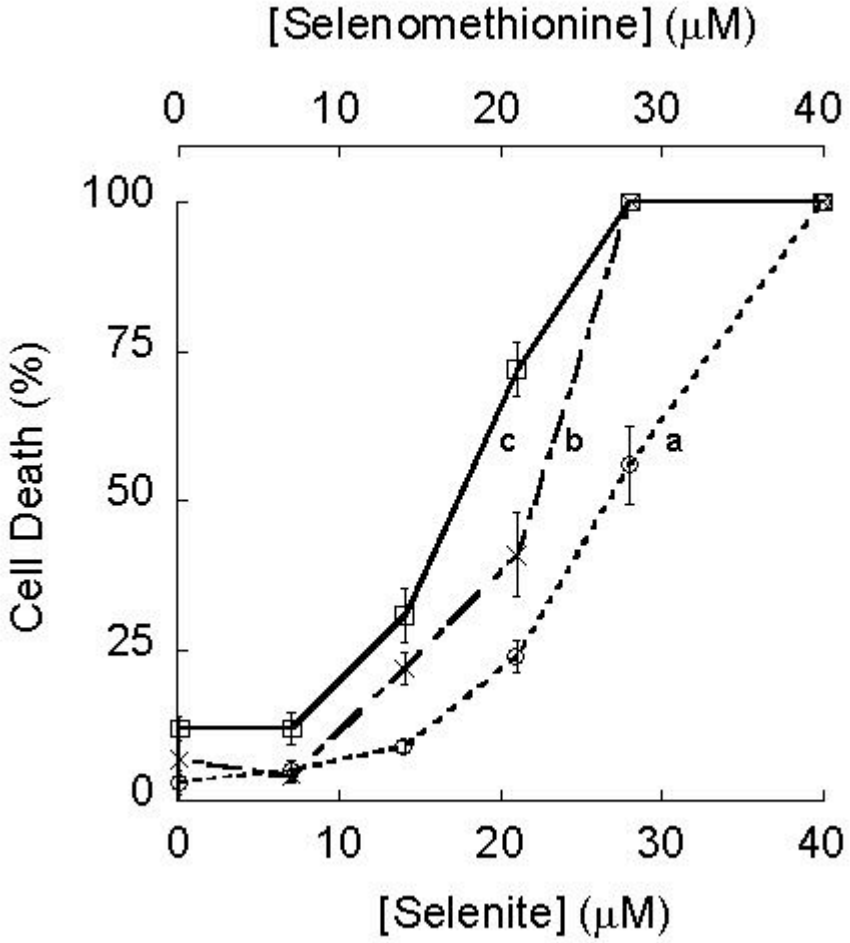

Figure 34 The effect of a combination of selenite and selenomethionine on the death of JU77 cells in the presence of human serum albumin, lithium chloride or geldanamycin.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite and selenomethionine in the presence of: 40g/L albumin (-- o--), 40g/L albumin, 0.5mM lithium chloride (—x —), 40g/L albumin, 20nM geldanamycin (—□ –). Data points are the mean ± SD of three determinations.

Table 32 A statistical analysis of the lethal dose estimates of selenite and selenomethionine in the presence of human serum albumin including lithium chloride or geldanamycin in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Seme/Alb	48	3	**34a**	
Senite/Seme/Li/Alb	38	3	**34b**	[**34a**] $P < 0.05$ *
Senite/Seme/Geld/Alb	35	2	**34c**	[**34a**] $P < 0.01$ ** [**34b**] $P > 0.05$ (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 34 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Seme, Selenomethionine]; [Li, Lithium chloride]; [Geld, Geldanamycin]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

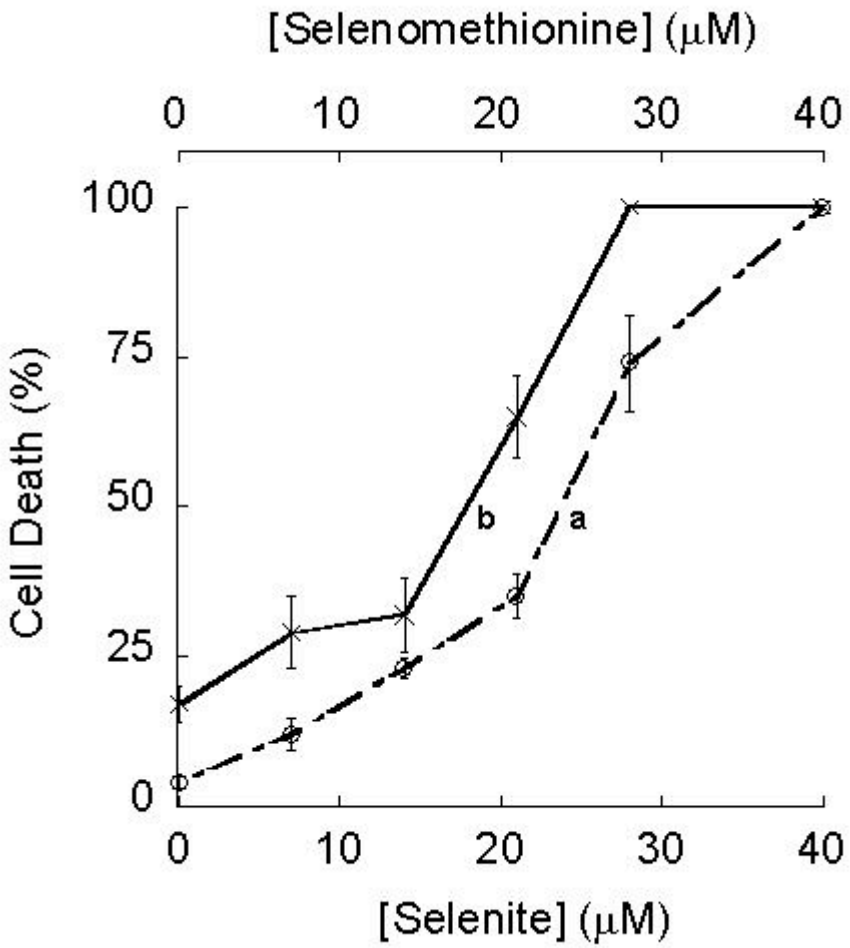

Figure 35 The effect of a combination of selenite and selenomethionine on the death of MCF-7 cells in the presence of human serum albumin and lithium chloride.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of selenite and selenomethionine in the presence of: 40g/L albumin, 0.5mM lithium chloride (—o –) 40g/L albumin (—x —). Data points are the mean ± SD of three determinations.

Table 33 A statistical analysis of the lethal dose estimates of selenite and selenomethionine in the presence of human serum albumin including lithium chloride in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Seme/Alb	46	2	**35a**	[**34a**] P > 0.05 (NS)
Senite/Seme/Li/Alb	40	3	**35b**	[**34b**] P > 0.05 (NS) [**35a**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 35 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Seme, Selenomethionine]; [Li, Lithium chloride]; [Alb,Albumin]; [Ref.Fig , Reference Figure].

In JU77 cells, selenodimethionine also increased cell death in combination with mercaptoethanol. Mercaptoethanol reduced the minimal concentration of selenodimethionine that promoted complete death of JU77 cells from an estimated 49μM to 14μM (Figure 36 and table 34, $p<0.001$). However, other RSH-group containing compounds like cysteine and glutathione did not change the activity of selenodimethionine significantly (table 34, both $p>0.05$ (NS)).

A combination of selenite and dihydrolipoic acid (DHLA-trisulphide) was prepared in an attempt to reduce the total concentration of selenium needed to promote death of MCF-7 and JU77 cells. The DHLA-trisulphide was then combined with a low concentration of selenomethionine, mercaptoethanol and gadolinium chloride. Figure 37 illustrates that in the presence of gadolinium chloride the total concentration of selenite needed to promote complete death of a JU77 cell population decreased to an estimated concentration of 8μM (table 35, $p<0.001$). This was the lowest concentration of trisulphide (and total selenium) observed to be effective in the presence of human serum albumin.

The four cell lines were equally susceptible to a combination of selenite and dihydrolipoic acid with low concentrations of selenomethionine and mercaptoethanol in the presence of human serum albumin, as highlighted in Figure 38. The mesothelioma cell line JU77, the breast cancer cell line MCF-7, the colon cancer cell line Caco-2 and the hepatocellular carcinoma cell line HepG2 were all killed with the same concentration using this combination (table 36, $p>0.05$ (NS)).

3.1.7 Combinations with β-lapachone

β-lapachone inhibits antigenic determinants that seem to be shared between

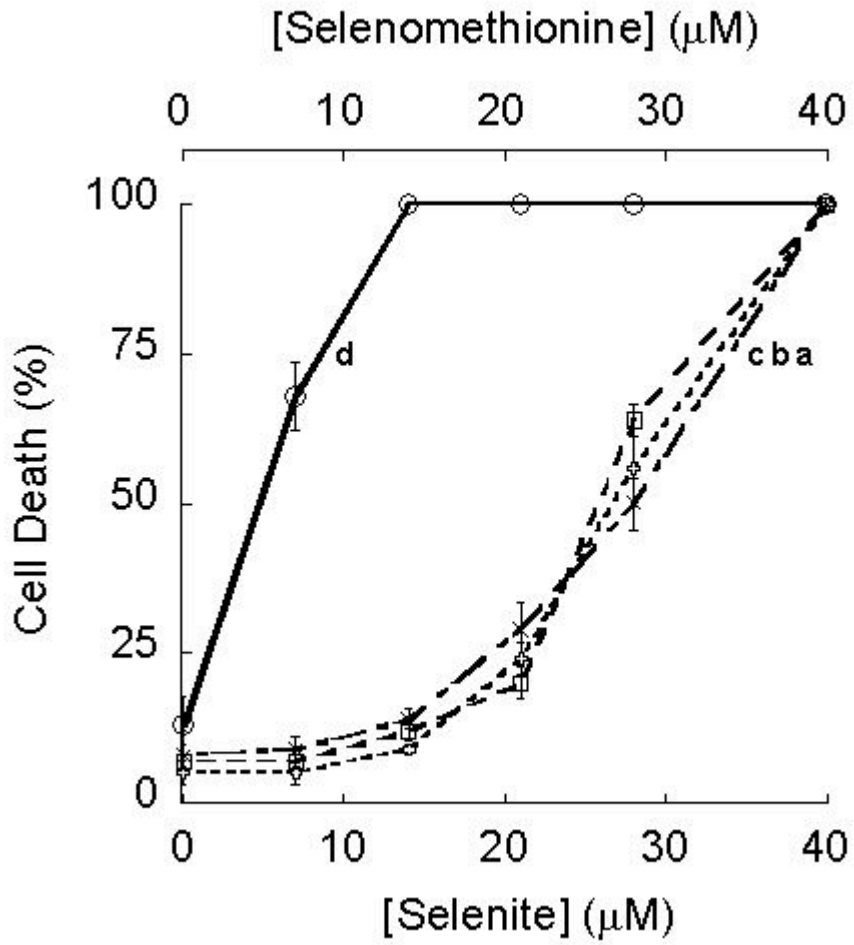

Figure 36 The effect of a combination of selenite and selenomethionine on the death of JU77 cells in the presence of human serum albumin and various other sulphydryl group-containing compounds.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite and selenomethionine in the presence of: 40g/L albumin (⁻ ⁻ ✞ ⁻ ⁻), 40g/L albumin and 40μM cysteine (—x ⁻), 40g/L albumin and 40μM glutathione (⁻ ☐ ⁻), 40g/L albumin and 40μM mercaptoethanol (—o—). Data points are the mean ± SD of three determinations.

Table 34 A statistical analysis of the lethal dose estimates of selenite and selenomethionine in the presence of human serum albumin including various other sulphydryl group-containing compounds in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Senite/Seme/Alb	49	4	**36b**	[**36a**] P > 0.05 (NS) [**36c**] P > 0.05 (NS)
Senite/Seme/Merc/Alb	14	1	**36d**	[**36b**] P < 0.001***
Senite/Seme/Cyst/Alb	54	4	**36a**	[**36c**] P > 0.05 (NS) [**36d**] P < 0.001*** [**32b**] P > 0.05 (NS)
Senite/Seme/Glut/Alb	50	4	**36c**	[**36d**] P < 0.001*** [**32d**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 36 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Senite, Selenite]; [Seme, Selenomethionine]; [Cyst, Cysteine]; [Merc, Mercaptoethanol]; [Glut, Glutathione]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

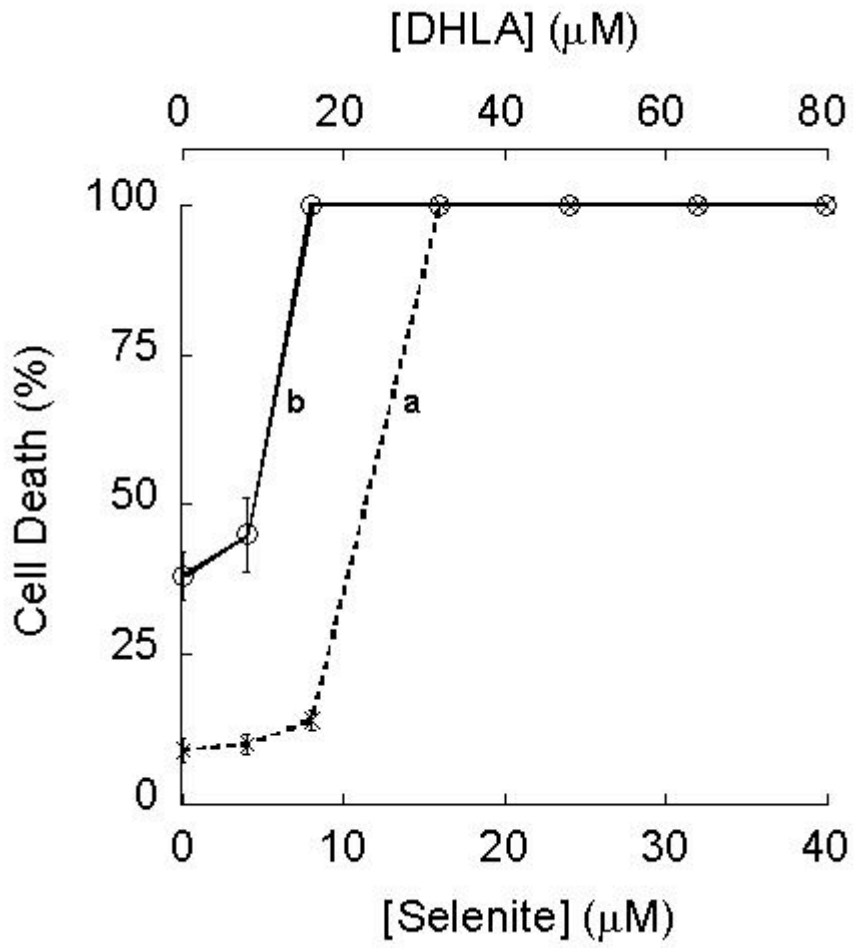

Figure 37 The effect of a combination of selenite and dihydrolipoic acid on the death of JU77 cells in the presence of human serum albumin, selenomethionine and mercaptoethanol with or without gadolinium chloride.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite and dihydrolipoic acid in the presence of 40g/L albumin, 3μM selenomethionine and 40μM mercaptoethanol (-- x --) or 40g/L albumin, 3μM selenomethionine, 40μM mercaptoethanol and 100μM gadolinium chloride (—o—). Data points are the mean ± SD of three determinations.

Table 35 A statistical analysis of the lethal dose estimates of selenite, dihydrolipoic acid, selenomethionine and mercaptoethanol in the presence of human serum albumin including gadolinium chloride in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Merc Seme DHLA Senite Alb	20	2	**37a**	[**37b**] P < 0.001***
Gd Merc Seme DHLA Senite Alb	8	4	**37b**	[**32c**] P < 0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 37 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Gd, Gadolinium chloride]; [Merc, Mercaptoethanol]; [Seme, Seleno-methionine]; [DHLA, Dihydrolipoic acid]; [Senite, Selenite]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

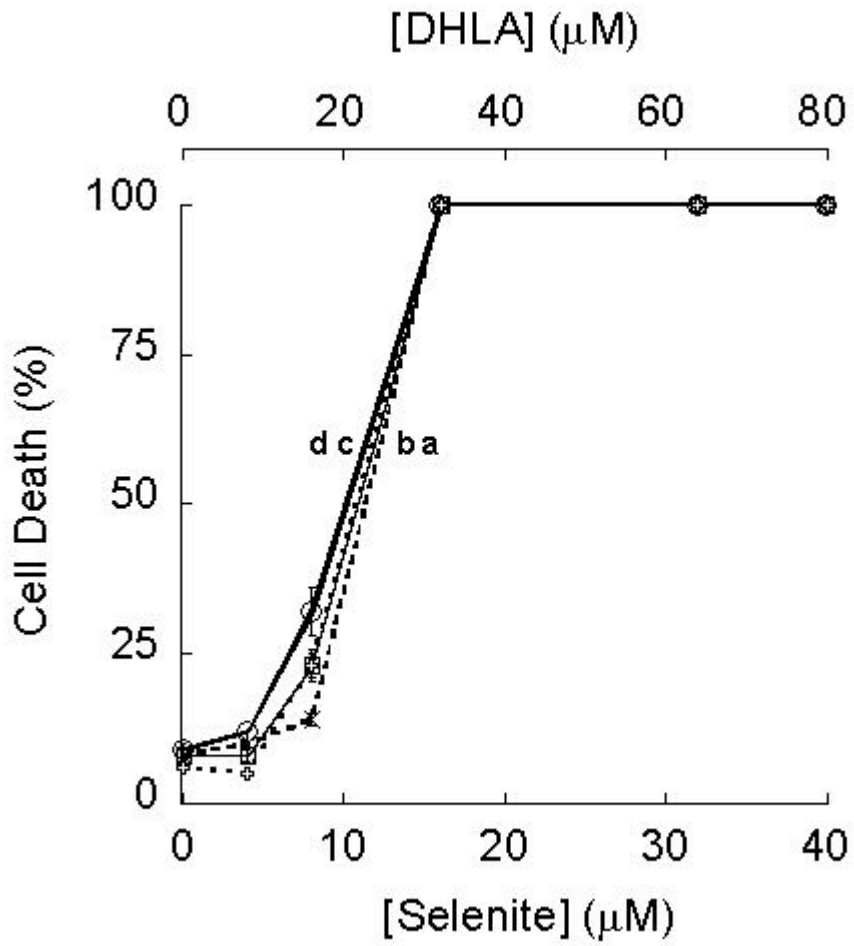

Figure 38 The effect of a combination of selenite and dihydrolipoic acid on the death of four different cell lines in the presence of human serum albumin, selenomethionine and mercaptoethanol.

JU77 cells (-- x --), MCF-7 cells (—o—), Caco-2 cells (—□—) and HepG2 cells (– – ✞ – –) in their exponential growth phases were treated with varying concentrations of selenite and dihydrolipoic acid (DHLA) in the presence of 40g/L albumin, 3μM selenomethionine and 40μM mercaptoethanol. Data points are the mean ± SD of three determinations.

Table 36 A statistical analysis of the lethal dose estimates of selenite, dihydrolipoic acid selenomethionine and mercaptoethanol in the presence of human serum albumin in fourdifferent cell lines.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Merc Seme DHLA Senite Alb (HepG)	20	2	**38d**	
Merc Seme DHLA Senite Alb (JU77)	20	3	**38a**	[**38b**] P > 0.05 (NS) [**38c**] P > 0.05 (NS) [**38d**] P > 0.05 (NS)
Merc Seme DHLA Senite Alb (MCF-7)	19	2	**38b**	[**38c**] P > 0.05 (NS) [**38d**] P > 0.05 (NS)
Merc Seme DHLA Senite Alb (Caco-2)	19	2	**38c**	[**38d**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 38 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Merc, Mercaptoethanol]; [Seme, Selenomethionine]; [DHLA, Dihydrolipoic acid]; [Senite, Selenite]; [Alb, Albumin]; [Ref. Fig , Reference Figure].

the cytoplasmatic glucocorticoid receptor and DNA polymerase alpha (Schmidt, Miller-Diener and Litwack 1984). There is some evidence that its cell death-inducing potency is increased with compounds that reduce NADPH-dependent lipid peroxidation and/or inhibit DNA or RNA-polymerases.

β-lapachone alone eradicated JU77 cell populations between an estimated 31μM to 40μM in the presence of human serum albumin, a concentration that exceeds a therapeutic range (Figure 39). Combinations with trisulphides or flavin adenine dinucleotide (FAD), as well as with menadione, geftinib and thiosulfate did not significantly increase the potency of β-lapachone (table 37, all $p>0.05$ (NS)).

In MCF-7 cells, β-lapachone exerted its maximal cell killing potency between an estimated 29μM to 33μM in the presence of human serum albumin. As occurred in the JU77 cells, combinations with trisulphides or flavin adenine dinucleotide did not significantly reduced the concentration of β-lapachone in MCF-7 cells, as highlighted in Figure 40 and table 38, $p>0.05$.

3.1.8 Combinations with glycolysis inhibitors

A fundamental difference between normal and cancerous cells was reported in 1924 by Warburg, Posener and Negelein, who discovered that the ratio between glycolysis and respiration differs in normal and cancerous cells. He demonstrated that glycolytic flux under aerobic conditions is greater in cancer cells than in their normal counterparts. In general, the potency of chemotherapeutic combinations increases with the simultaneous inhibition of glycolysis (Kim *et.al.* 2002).

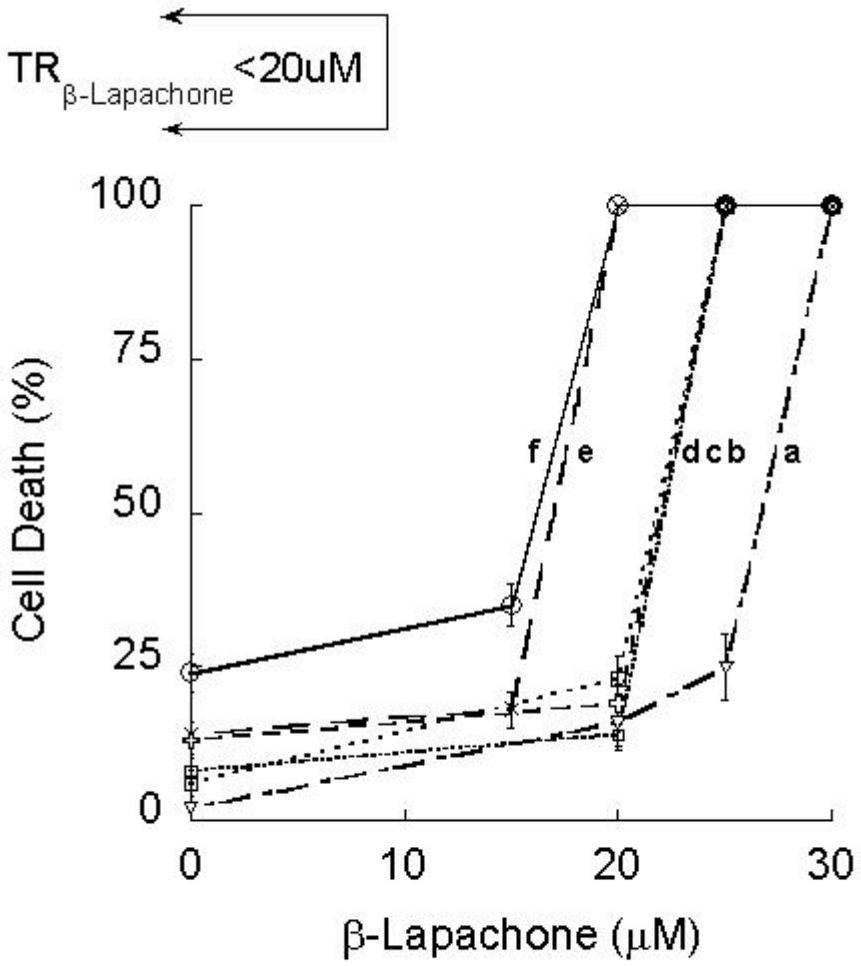

Figure 39 The effect of β-lapachone on the death of JU77 cells in the presence of human serum albumin and menadione or geftinib or thiosulfate or a combination of selenite and dihydrolipoic acid or flavin adenine dinucleotide.

JU77 cells in their exponential growth phase were treated with varying concentrations of β-lapachone in the presence of 40g/L albumin (—▽—), 40g/L albumin and 25μM menadione (– – ✞ – –), 40g/L albumin and 1μM geftinib (– □ –), 40g/L albumin and 0.5mM thiosulfate (····· ▫ ·····), 40g/L albumin and 16μM/32μM selenite/dihydrolipoic acid (—o—) or 40g/L albumin and 100μM flavin adenine dinucleotide (—x —). The therapeutic range (TR) in which β-lapachone can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 37 A statistical analysis of the lethal dose estimates of β-lapachone in the presence of human serum albumin including menadione or geftinib or thiosulfate or a combination of selenite and dihydrolipoic acid or flavin adenine dinucleotide in JU77 cells.

Description	LD_{100} (μM)	SEM (μM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Lap Alb	39	0	**39a**	
Lap Menad Alb	40	12	**39c**	[**39a**] P > 0.05 (NS) [**39b**] P > 0.05 (NS) [**39d**] P > 0.05 (NS)
Lap Thio Alb	38	14	**39b**	[**39a**] P > 0.05 (NS)
Lap Geft Alb	36	9	**39d**	[**39a**] P > 0.05 (NS) [**39b**] P > 0.05 (NS)
Lap FAD Alb	31	5	**39e**	[**39a**] P > 0.05 (NS) [**39b**] P > 0.05 (NS) [**39c**] P > 0.05 (NS) [**39d**] P > 0.05 (NS)
Lap DHLA Senite Alb	31	5	**39f**	[**39a**] P > 0.05 (NS) [**39b**] P > 0.05 (NS) [**39c**] P > 0.05 (NS) [**39d**] P > 0.05 (NS) [**39e**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 39 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Lap, β-Lapachone]; [Alb, Albumin]; [Menad, Menadione]; [Thio, Thiosulfate]; [Geft, Geftinib]; [FAD, Flavine adenine dinucleotide]; [DHLA, Dihydrolipoic acid]; [Senite, Selenite]; [Ref.Fig , Reference Figure].

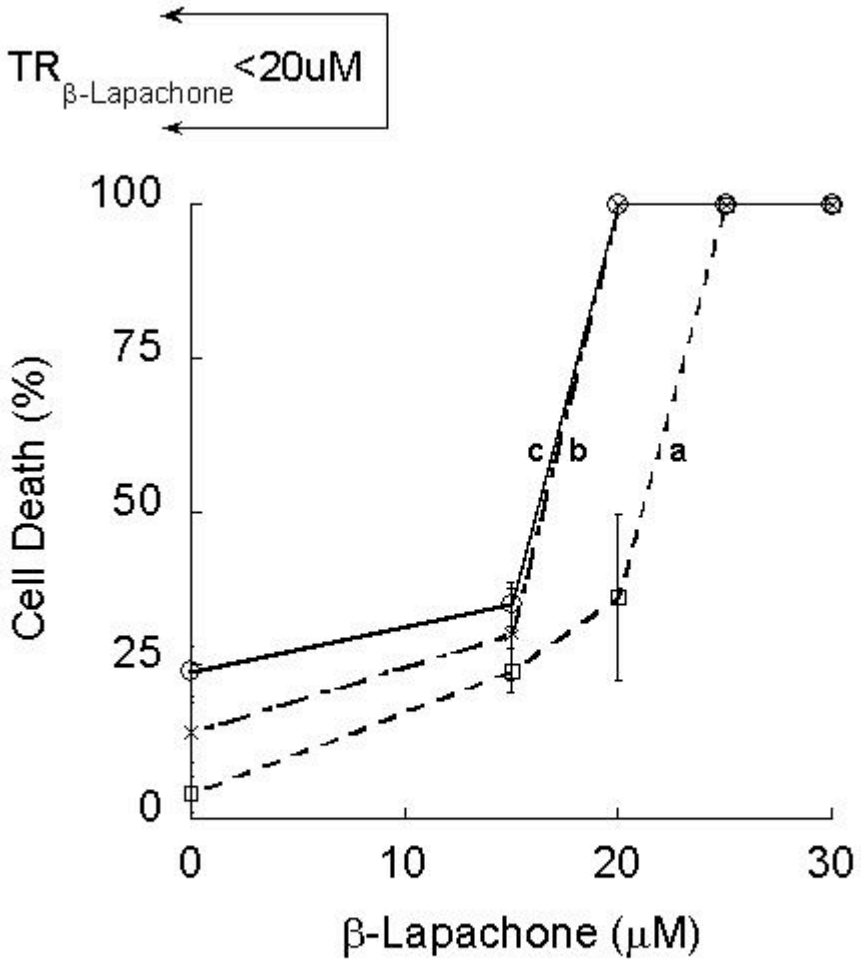

Figure 40 The effect of β-lapachone on the death of MCF-7 cells in the presence of human serum albumin and a combination of selenite and dihydrolipoic acid or flavin adenine dinucleotide.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of β-lapachone in the presence of 40g/L albumin (–□–), 40g/L albumin and 16μM/32μM selenite/dihydrolipoic acid (—o—) or 40g/L albumin and 100μM flavin adenine dinucleotide (—x–). The therapeutic range (TR) in which β-lapachone can be used *in vivo* is illustrated at the top left corner of the figure. Data points are the mean ± SD of three determinations.

Table 38 A statistical analysis of the lethal dose estimates of β-lapachone in the presence of human serum albumin including a combination of selenite and dihydrolipoic acid or flavin adenine dinucleotide in MCF-7 cells.

Description	LD_{100} (μM)	SEM (μM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Lap Alb	33	8	**40a**	
Lap FAD Alb	31	5	**40b**	[**40a**] P > 0.05 (NS) [**39e**] P > 0.05 (NS)
Lap DHLA Senite Alb	29	4	**40c**	[**40a**] P > 0.05 (NS) [**40b**] P > 0.05 (NS) [**39f**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 40 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Lap, β-Lapachone]; [Alb, Albumin]; [FAD, Flavin adenine dinucleotide]; [DHLA, Dihydrolipoic acid]; [Senite, Selenite]; [Ref.Fig , Reference Figure].

In JU77 cells, oxamate or iodoacetate (inhibitors of glycolysis) were combined with a trisulphide combination comprising selenite, selenomethionine and mercaptoethanol in the presence of human serum albumin. Under these conditions both compounds increased cell death with the trisulphide combination reducing the total concentration of trisulphide substantially (Figure 41 and table 39, all $p<0.001$). Increasing glycolysis inhibition enhanced the percentage of cell death (table 39, $p<0.001$).

3.1.9 Combinations with antibiotics

Cytostatic and cytotoxic activity of antibiotics against various tumour cell lines has been reported. Tetracyclines such as doxycycline seem to inhibit the activity of glutathione S-transferase isozymes (Mukanganyama *et.al.* 2002) as well as the cleavage of matrix metalloproteinases (Saikali and Singh 2003). The combination of trimethoprime-sulphamethoxazole is reported to correlate strongly with the reduction of uric acid (Chertow *et.al.* 1996). Both compounds increase cell death of trisulphides.

As illustrated in Figure 42, the three cell lines JU77, MCF-7 and Caco-2 displayed different sensitivities to the combination of cotrim (a two step tetrahydrofolic acid inhibitor) and selenodimethionine in the presence of human serum albumin (table 40). All JU77 cells were killed with this combination in human serum albumin, but the viability of MCF-7 cells and Caco-2 cells was only partially reduced. In contrast, Figure 43 illustrates that combining doxycycline (an inhibitor of the prokaryotic 30S ribosome) with selenodimethionine promoted cell death in all MCF-7 cells in human serum albumin, but JU77 cells and Caco-2 cells were only partially affected (table 41).

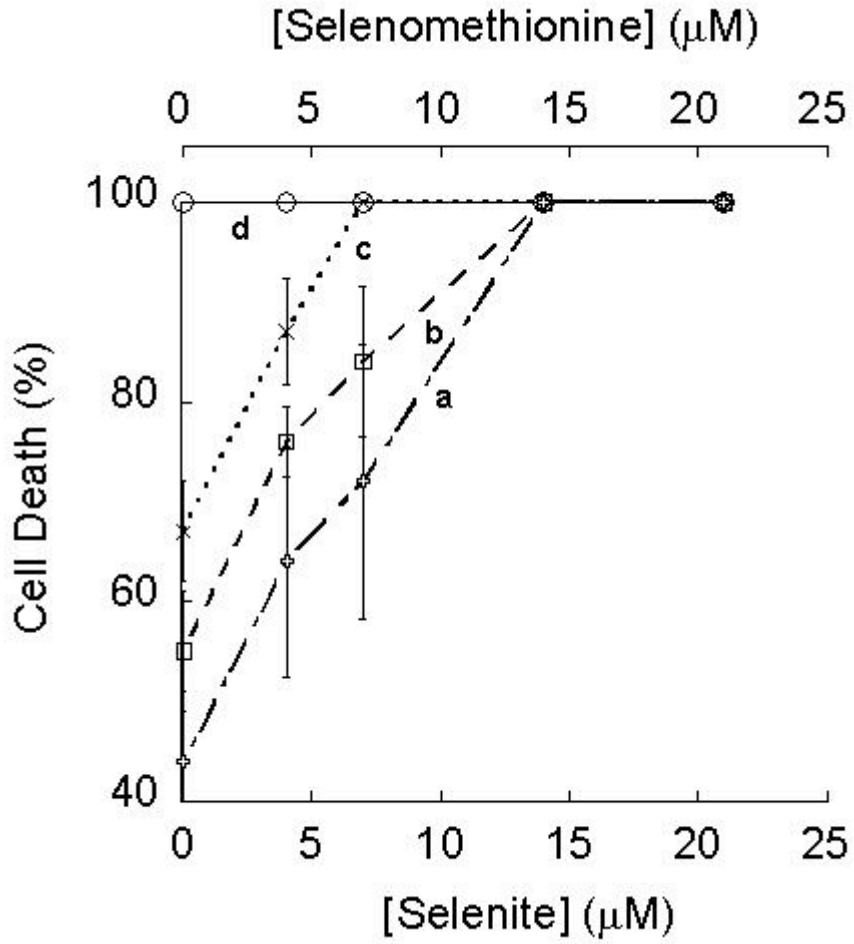

Figure 41 The effect of a combination of selenite and selenomethionine on the death of JU77 cells in the presence of human serum albumin, mercaptoethanol and iodoacetate or oxamate.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenite and selenomethionine in the presence of 40g/L albumin, 40μM mercaptoethanol and 100μM iodoacetate (—**o**—), 40g/L albumin, 40μM mercaptoethanol and 20μM iodoacetate (**-- x --**), 40g/L albumin, 40μM mercaptoethanol and 2mM oxamate (—□—) and 40g/L albumin (40g/L), 40μM mercaptoethanol and 1mM oxamate (—✞—). Data points are the mean ± SD of three determinations.

Table 39 A statistical analysis of the lethal dose estimates of selenite, selenomethionine and mercaptoethanol in the presence of human serum albumin including iodoacetate or oxamate in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
IAA100 Merc Seme Senite Alb	0	0	**41d**	[**41a**] P < 0.001*** [**41b**] P > 0.05 (NS) [**41c**] P < 0.001*** [**36b**] P < 0.001***
IAA20 Merc Seme Senite Alb	16	1	**41c**	[**41a**] P < 0.001*** [**41b**] P < 0.001*** [**36b**] P < 0.001***
Ox2 Merc Seme Senite Alb	8	1	**41b**	[**41a**] P < 0.001*** [**36b**] P < 0.001***
Ox1 Merc Seme Senite Alb	16	2	**41a**	[**36b**] P < 0.001***

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 41 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [IAA, Iodoacetate]; [Ox, Oxamate]; [Merc, Mercaptoethanol]; [Seme, Selenomethionine]; [Senite, Selenite]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

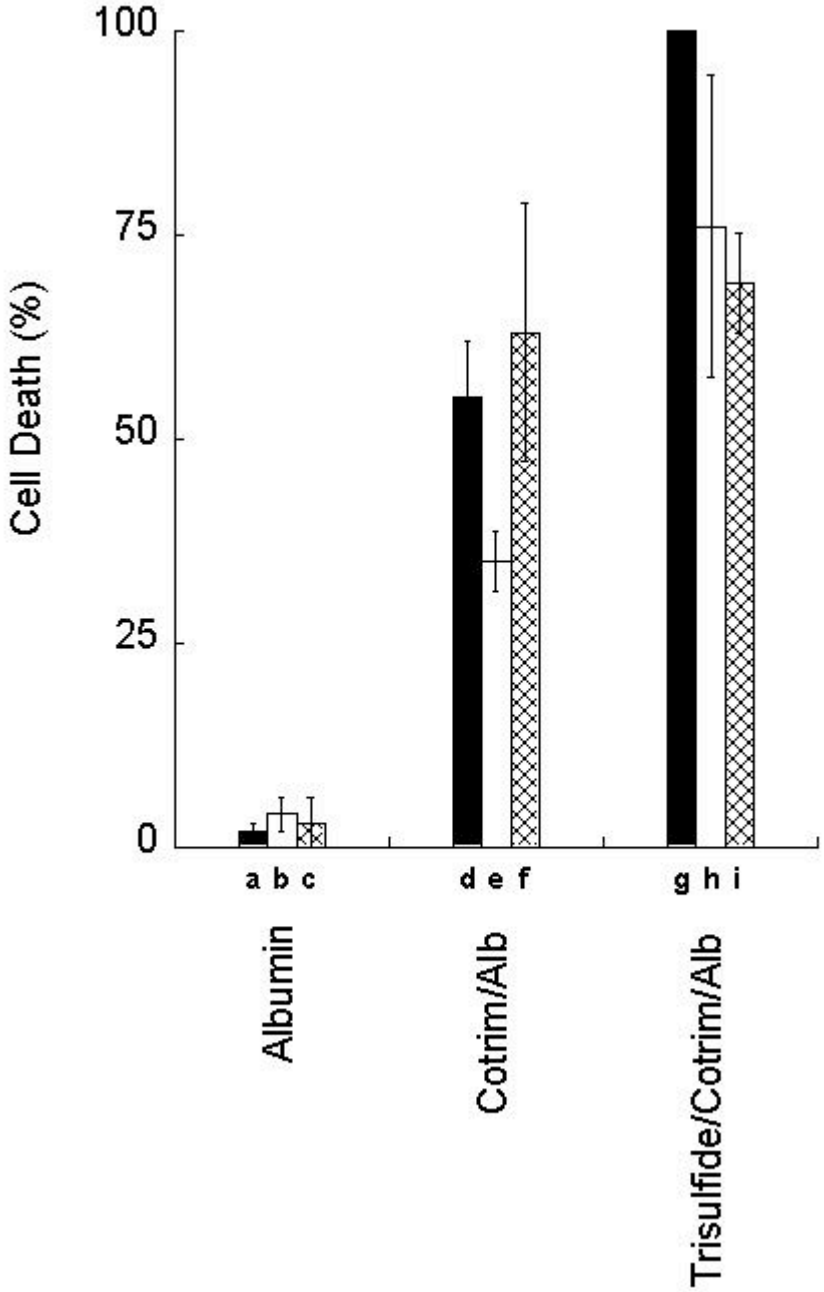

Figure 42 The effect of cotrim (trimethoprime/sulfamethoxazole) and a combination of selenite and selenomethionine on the death of JU77 cells, MCF-7 cells and Caco-2 cells in the presence of human serum albumin. JU77 cells (■), MCF-7 cells (□) and Caco-2 cells (⊠) in their exponential growth phase were treated with 20μM/20μM selenite/selenomethionine and 10μM cotrim in 40g/L albumin or 10μM cotrim in 40g/L albumin. Data points are the mean ± SD of three determinations.

Table 40 A statistical analysis of the lethal effects of selenite and selenomethionine in the presence of human serum albumin including cotrim or a combination of trisulfide and cotrim in JU77, MCF-7 and Caco-2 cells.

Description	LE %CD	SEM	Ref. Fig	Level of Significance Comparison to [Fig x]
Tris Cotr Alb (JU77)	100	0	**40g**	[**40a**] P < 0.001*** [**40d**] P < 0.001*** [**40h**] P > 0.05 (NS) [**40i**] P < 0.05 *
Tris Cotr Alb (MCF-7)	76	10.69	**40h**	[**40b**] P < 0.001*** [**40e**] P < 0.001*** [**40i**] P > 0.05 (NS)
Tris Cotr Alb (Caco-2)	69	3.51	**40i**	[**40c**] P < 0.001*** [**40f**] P > 0.05 (NS)
Cotr Alb (JU77)	55	4.00	**40d**	[**40a**] P < 0.001*** [**40e**] P > 0.05 (NS) [**40f**] P > 0.05 (NS)
Cotr Alb (MCF-7)	35	2.08	**40e**	[**40b**] P < 0.05 * [**40f**] P < 0.05 *
Cotr Alb (Caco-2)	63	9.10	**40f**	[**40c**] P < 0.001***
Alb (JU77)	2	0.58	**40a**	[**40b**] P > 0.05 (NS) [**40c**] P > 0.05 (NS)
Alb (MCF-7)	4	1.15	**40b**	[**40c**] P > 0.05 (NS)
Alb (Caco-2)	3	1.73	**40c**	

Lethal effects of compounds and their combinations were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Tris, Trisulfide]; [Cotr, Cotrim]; [Alb, Albumin]; [LE %CD, Lethal effect in % cell death]; [Ref. Fig , Reference Figure].

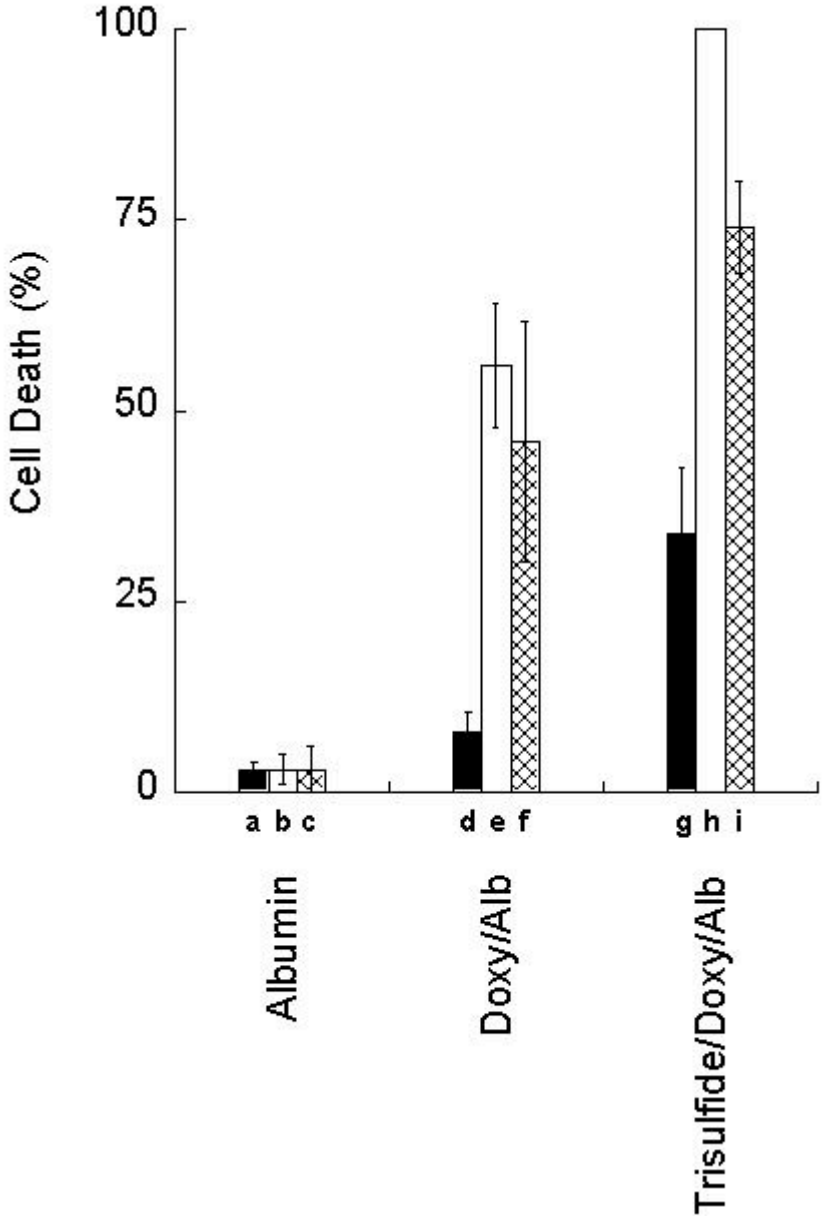

Figure 43 The effect of doxycycline and a combination of selenite and selenomethionine on the death of JU77 cells, MCF-7 cells and Caco-2 cells in the presence of human serum albumin.

JU77 cells (■), MCF-7 cells (□) and Caco-2 cells (⊠) in their exponential growth phase were treated with 20μM/20μM selenite/selenomethionine and 1mM doxycycline in 40g/L albumin or 1mM doxycycline in 40g/L albumin. Data points are the mean ± SD of three determinations.

Table 41 A statistical analysis of the lethal effects of selenite and selenomethionine in the presence of human serum albumin including doxycycline or a combination of trisulfide and doxycycline in JU77, MCF-7 and Caco-2 cells.

Description	LE %CD	SEM	Ref. Fig	Level of Significance Comparison to [Fig x]
Tris Doxy Alb (JU77)	34	4.93	**40g**	[**40a**] P < 0.001*** [**40d**] P < 0.001*** [**40h**] P < 0.001*** [**40i**] P < 0.001***
Tris Doxy Alb (MCF-7)	100	0	**40h**	[**40b**] P < 0.001*** [**40e**] P < 0.001*** [**40i**] P < 0.001***
Tris Doxy Alb (Caco-2)	74	4.04	**40i**	[**40c**] P < 0.001*** [**40f**] P < 0.001***
Doxy Alb (JU77)	8	1.53	**40d**	[**40a**] P > 0.05 (NS) [**40e**] P < 0.001*** [**40f**] P < 0.001***
Doxy Alb (MCF-7)	56	4.73	**40e**	[**40b**] P < 0.001*** [**40f**] P > 0.05 (NS)
Doxy Alb (Caco-2)	46	2.52	**40f**	[**40c**] P < 0.001***
Alb (JU77)	3	0.58	**40a**	[**40b**] P > 0.05 (NS) [**40c**] P > 0.05 (NS)
Alb (MCF-7)	3	1.16	**40b**	[**40c**] P > 0.05 (NS)
Alb (Caco-2)	3	1.16	**40c**	

Lethal effects of compounds and their combinations were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Tris, Trisulfide]; [Doxy, Doxycycline]; [Alb, Albumin]; [LE %CD, Lethal effect in % cell death]; [Ref. Fig , Reference Figure].

3.1.10 Combinations with diethylmaleate

Diethylmaleate depletes glutathione and increases L-cysteine transport across the membrane (Ruiz *et.al.* 2003). The viability of JU77 cells and MCF cells exposed to diethylmaleate decreased in a linear manner in human serum albumin (Figure 44). A combination of 0.4mM diethylmaleate with selenodimethionine reduced the concentration of selenodimethionine in both cell lines (table 42, $p > 0.05$ (NS)). This decreased the minimal concentration needed to kill either cell population to estimated 29μM selenite and selenomethionine, in the presence of human serum albumin (Figure 45, 46 and table 43, 44).

3.1.11 Combinations with hydrogen peroxide

Hydrogen peroxide (H_2O_2) is an important mediator of cellular signalling. H_2O_2 is involved in survival signalling, angiogenesis, cell adhesion and other cell functions. It induces a dose-related apoptosis (Zhou *et.al.* 2007).

Hydrogen peroxide decreased the viability of all four cell lines. However, the sensitivity of the four cell lines differed, decreasing in their estimated LD100 values in the following order: HepG2 cells, Caco-2 cells, JU77 cells and MCF-7 cells (Figure 47and table 45).

The potency of H_2O_2 increased in combination with some β-adrenergic compounds, as illustrated in Figure 48. In JU77 cells, adrenaline effectively reduced the concentration of H_2O_2 by 50% (table 46, $p<0.01$). Salbutamol did not significantly change the concentration of H_2O_2 in the presence of human serum albumin (table 46, $p>0.05$ (NS)).

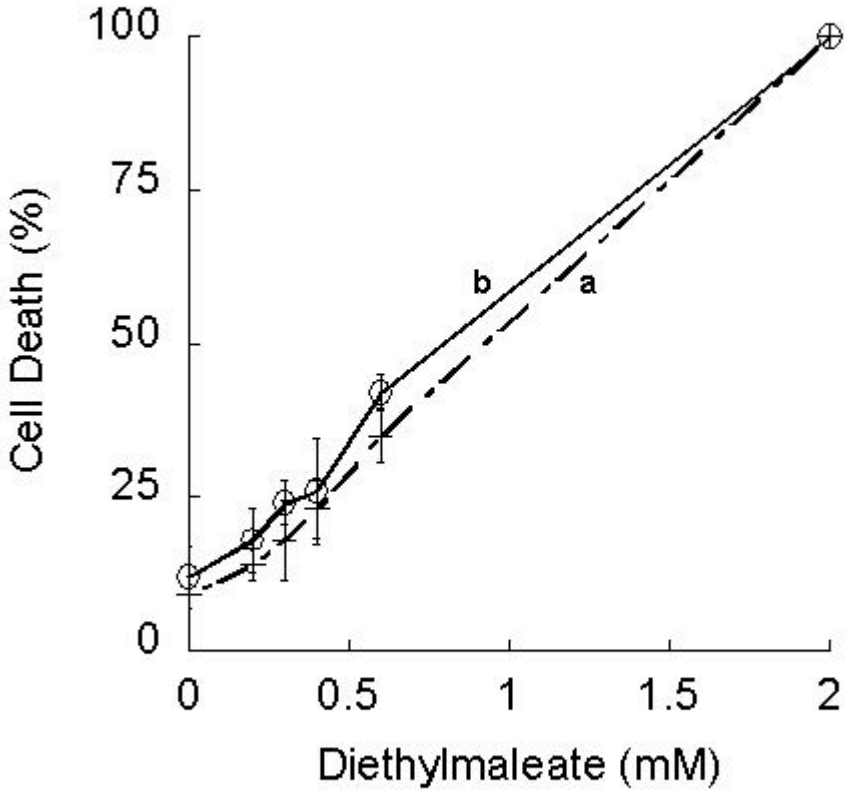

Figure 44 The effect of diethylmaleate on the death of JU77 and MCF-7 cells in the presence of human serum albumin.

JU77 cells (—**o**—) and MCF-7 cells (—**x** -) in their exponential growth phase were treated with varying concentrations of diethylmaleate in the presence of 40g/L albumin. Data points are the mean ± SD of three determinations.

Table 42 A statistical analysis of the lethal dose estimates of diethylmaleate in the presence of human serum albumin in JU77 and MCF-7 cells.

Description	LD_{100} (mM)	SEM (mM)	Ref. Fig	Level of Significance Comparison to [Fig x]
DEM Alb (JU77)	1923	94	[**44b**]	
DEM Alb (MCF-7)	1899	133	[**44a**]	[**44b**] P > 0.05 (NS)

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 44 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [DEM, Diethylmaleate]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

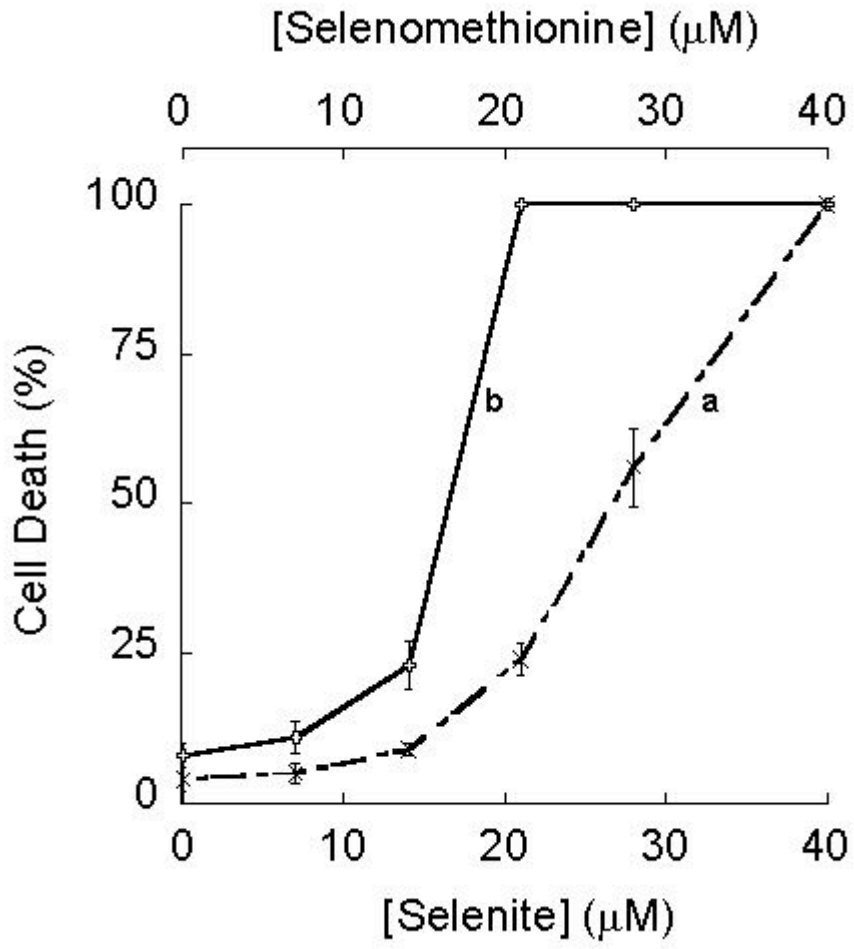

Figure 45 The effect of a combination of selenite and selenomethionine on the death of JU77 cells in the presence of human serum albumin and diethylmaleate.

JU77 cells in their exponential growth phase were treated with varying concentrations of selenodimethionine in 40g/L albumin in the presence (—o–) or absence (—x –) of 400μM diethylmaleate. Data points are the mean ± SD of three determinations.

Table 43 A statistical analysis of the lethal dose estimates of a selenite and selenomethionine in the presence of human serum albumin including diethylmaleate in JU77 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
DEM Tris Alb	29	3	**45b**	[**45a**] $P < 0.001$***
Tris Alb	48	4	**45a**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 45 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Tris, Trisulfide]; [DEM, Diethylmaleate]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

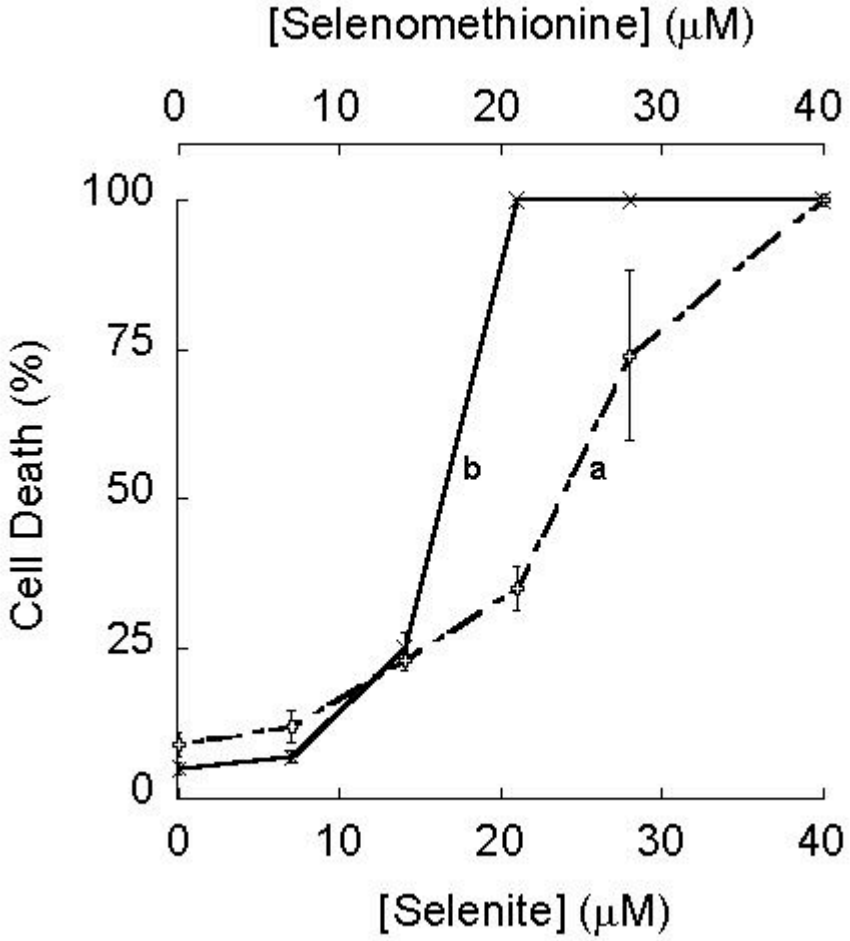

Figure 46 The effect of a combination of selenite and selenomethionine on the death of MCF-7 cells in the presence of human serum albumin and diethylmaleate.

MCF-7 cells in their exponential growth phase were treated with varying concentrations of selenodimethionine in 40g/L albumin in the presence (—x –) or absence (—o—) of 400μM diethylmaleate. Data points are the mean ± SD of three determinations.

Table 44 A statistical analysis of the lethal dose estimates of selenite and selenomethionine in the presence of human serum albumin including diethylmaleate in MCF-7 cells.

Description	LD_{100} (µM)	SEM (µM)	Ref. Fig	Level of Significance Comparison to [Fig x]
DEM Tris Alb	29	3	**46b**	[**46b**] P < 0.001*** [**45b**] P > 0.05 (NS)
Tris Alb	47	3	**46a**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 46 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Tris, Trisulfide]; [DEM, Diethylmaleate]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

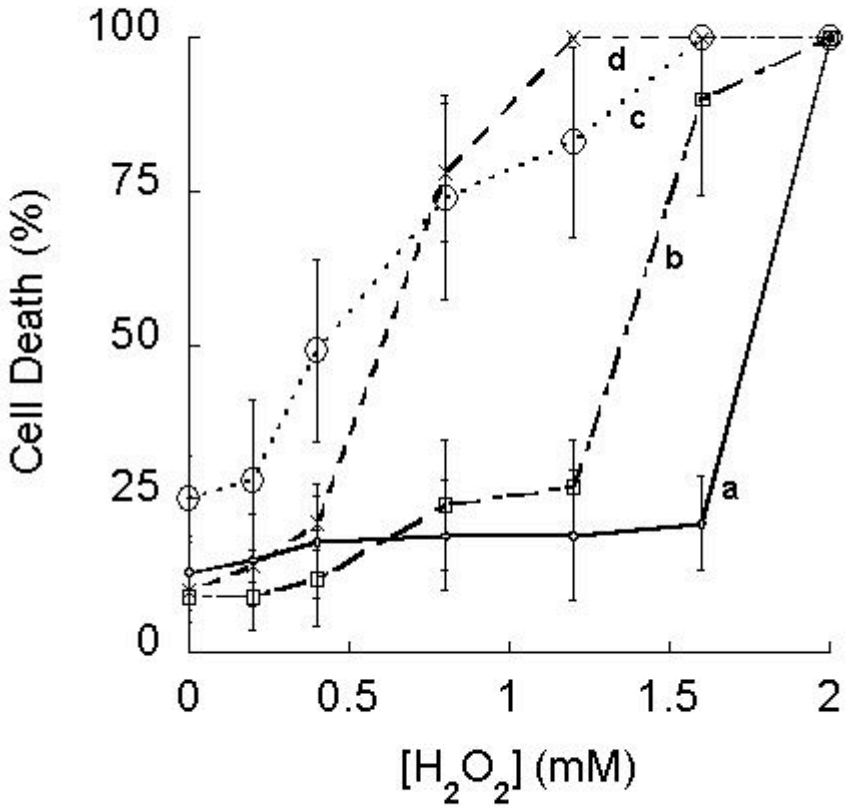

Figure 47 The effect of hydrogen peroxide on the death of JU77, MCF-7, Caco-2 and HepG2 cells.

JU77 cells (···o···), MCF-7 cells (-- x --), Caco-2 cells (—□—) and HepG2 cells (—✞—) in their exponential growth phase were treated with varying concentrations of hydrogen peroxide in the presence of 40g/L albumin. Data points are the mean ± SD of three determinations.

Table 45 A statistical analysis of the lethal dose estimates of hydrogen peroxide in the presence of human serum albumin in JU77, MCF-7, Caco-2 and HepG2 cells.

Description	LD_{100} (mM)	SEM (mM)	Ref. Fig	Level of Significance Comparison to [Fig x]
H_2O_2 Alb (JU77)	2	0	**47c**	[**47a**] P < 0.05 * [**47b**] P < 0.001*** [**47d**] P > 0.05 (NS)
H_2O_2 Alb (MCF-7)	1	0	**47d**	[**47a**] P > 0.05 (NS) [**47b**] P < 0.05 *
H_2O_2 Alb (HepG)	4	1	**47b**	[**47a**] P > 0.05 (NS)
H_2O_2 Alb (Caco-2)	3	0	**47a**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 47 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [H_2O_2, Hydrogenperoxide]; [Alb, Albumin]; [Ref.Fig , Reference Figure].

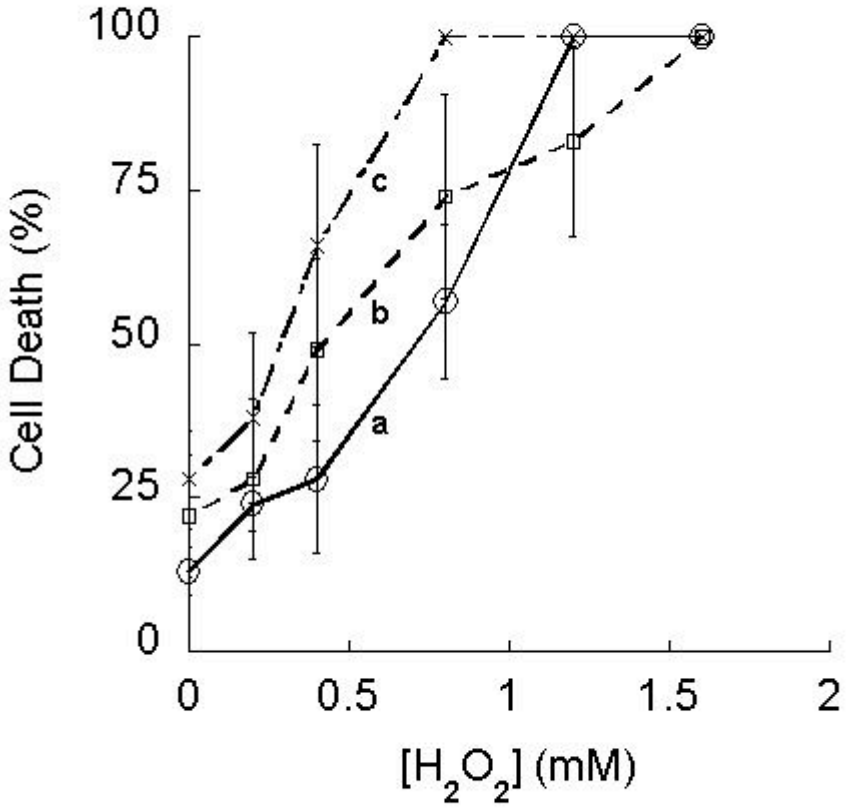

Figure 48 The effect of hydrogen peroxide on the death of JU77 cells in the presence of human serum albumin and β-adrenergic compounds.

JU77 cells in their exponential growth phase were treated with varying concentrations of hydrogen peroxide in the presence of 40g/L albumin (–□–), 40g/L albumin and 1μM salbutamol (—o—) or 40g/L albumin and 50nM adrenaline (—x–). Data points are the mean ± SD of three determinations.

Table 46 A statistical analysis of the lethal dose estimates of hydrogen peroxide in the presence of human serum albumin including β-adrenergic compounds.

Description	LD_{100} (mM)	SEM (mM)	Ref. Fig	Level of Significance Comparison to [Fig x]
Salb/H202/Alb	2	0.2	**48a**	[**48b**] P < 0.01 ** [**48c**] P > 0.05 (NS)
Adren/H202/Alb	1	0.2	**48c**	[**48b**] P < 0.01 **
H202/Alb	2	0.2	**48b**	

Lethal doses were estimated by Probit analysis of the data illustrated in Figure 48 as described in the Methods. Data were compared by ANOVA, Benferroni and Tukey-Kramer Multiple Comparison Tests as detailed in the Methods. By conventional criteria the data is considered to be not statistically significant (NS), statistically significant (*), very statistically significant (**), or extremely statistically significant (***). Abbreviations: [Salb, Salbutamol]; [Adren, Adrenaline]; [H_2O_2, Hydrogenperoxide]; [Ref.Fig , Reference Figure].

3.2 The effect of selenium, selenomethionine and lithium chloride on arachidonic acid metabolism in JU77 cells

Loss of cancer cell viability is reported to be intimately linked to changes in metabolism of arachidonic acid. Inhibiting enzymatic breakdown of arachidonic acid has been reported to decrease viability of cancer cells. Therefore, concentration levels of arachidonic acid metabolites were measured in JU77 cells while treating with lithium chloride, selenite and selenomethionine. As arachidonic acid can be metabolised by cyclooxygenases or lipoxygenases, concentrations of prostaglandin E2, leukotriene C and leukotriene B4 were determined.

Complex and variable changes in the concentration of metabolites of arachidonic acid were noted after six hours of treatment of the cells. Figure 49 illustrates expression of prostaglandin E2 levels by JU77 cells in the presence of the compounds. Lithium chloride or lithium chloride and selenomethionine reduced prostaglandin E2 levels below vehicle levels. All other treatments including the triple compound combination increased the levels of prostaglandin E2.

No significant reduction of leukotriene C concentration was observed when cells were treated with lithium chloride or lithium chloride and selenomethionine relative to the vehicle control (Figure 50). However, selenite or lithium chloride and selenite increased the concentration of leukotriene C. Interestingly, the triple concentration of lithium chloride, selenite and selenomethionine did not alter leukotriene C levels.

Lithium chloride or lithium chloride and selenomethionine reduced the concentration of leukotriene B4 levels below vehicle treatments (Figure

51). All other treatments, including the triple combination increased leukotriene B4 levels.

After treating JU77 cells for 24 hours, prostaglandin E2 levels increased in the presence of lithium chloride and selenite (Figure 49). In all other cases, prostaglandin E2 synthesis plateaued. Expression of leukotriene C was elevated with selenite, lithium chloride and selenite, selenite and selenomethionine or lithium chloride, selenite and selenomethionine (Figure 50). Leukotriene B4 levels increased during treatment with lithium chloride and selenite and also plateaued in all other cases (Figure 51).

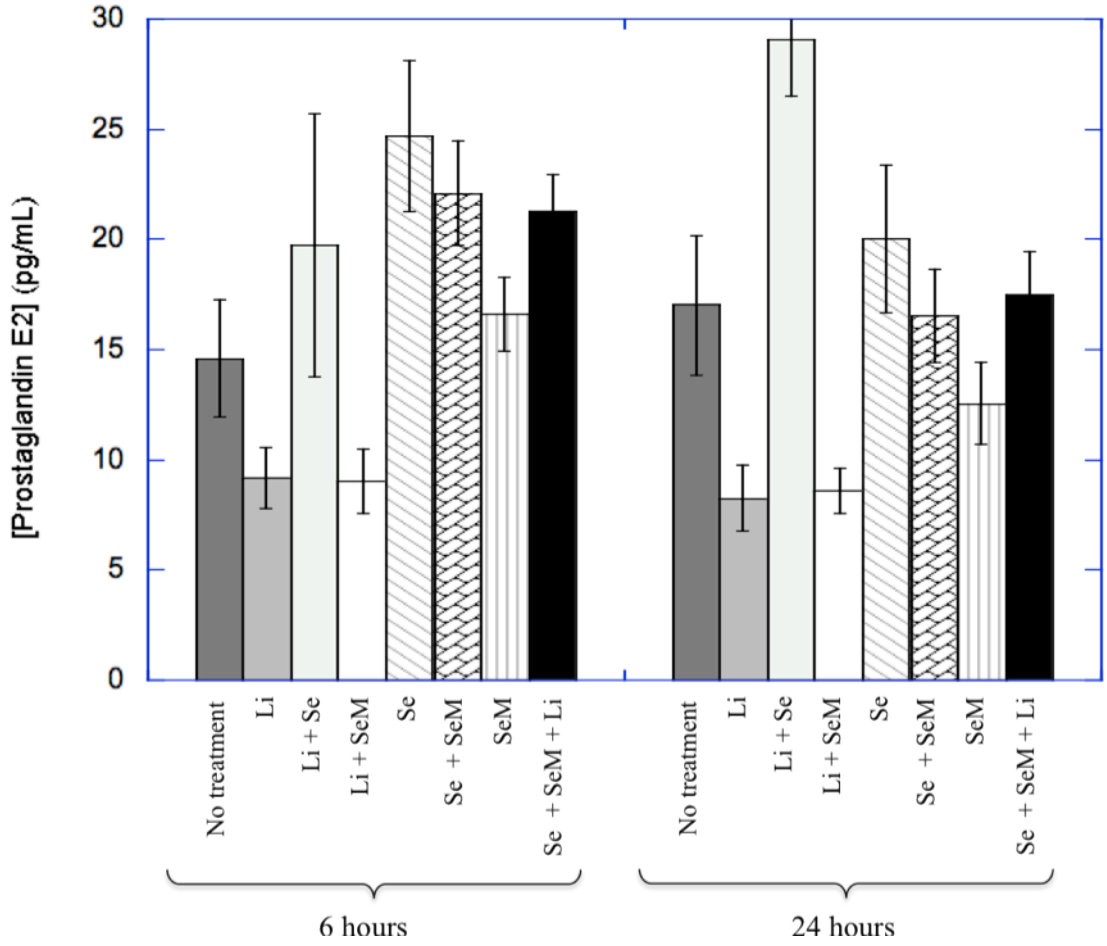

Figure 49 Expression of prostaglandin E2 by JU77 cells in the presence of various compounds.

JU77 cells in their exponential growth phase were treated with 0.5mM lithium chloride (Li), 0.5mM lithium chloride and 30µM selenite (Li+Se), 0.5mM lithium chloride and 30µM selenomethionine (Li+SeM), 30µM selenite (Se), 30µM selenite and 30µM selenomethionine (Se+SeM), 30µM selenomethionine (SeM), 0.5mM lithium chloride and 30µM selenite and 30µM selenomethionine
(Se+SeM+Li), no treatment (No treatment) and prostaglandin E2 levels were measured after 6 and 24 hours.

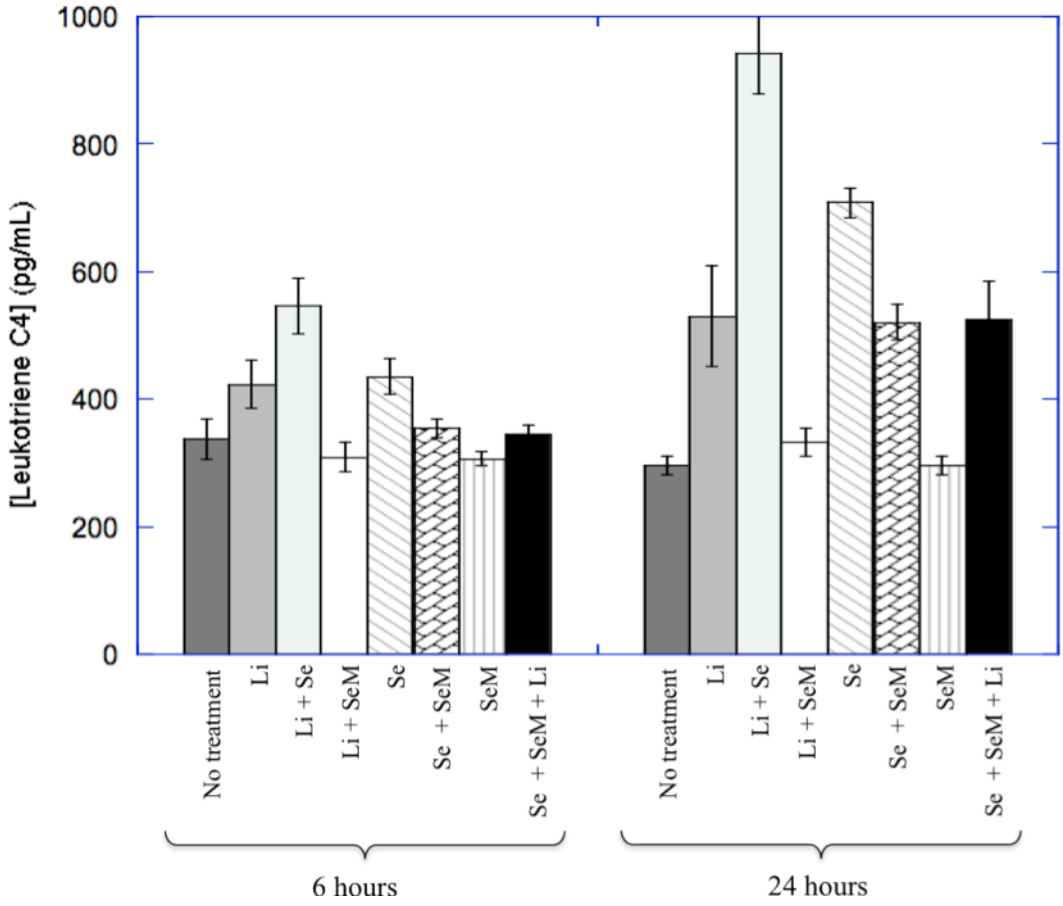

Figure 50 Expression of leukotriene C4 by JU77 cells in the presence of various compounds.

JU77 cells in their exponential growth phase were treated with 0.5mM lithium chloride (Li), 0.5mM lithium chloride and 30µM selenite (Li+Se), 0.5mM lithium chloride and 30µM selenomethionine (Li+SeM), 30µM selenite (Se), 30µM selenite and 30µM selenomethionine (Se+SeM), 30µM selenomethionine (SeM), 0.5mM lithium chloride and 30µM selenite and 30µM selenomethionine (Se+SeM+Li), no treatment (No treatment) and leukotriene C4 levels were measured after 6 and 24 hours.

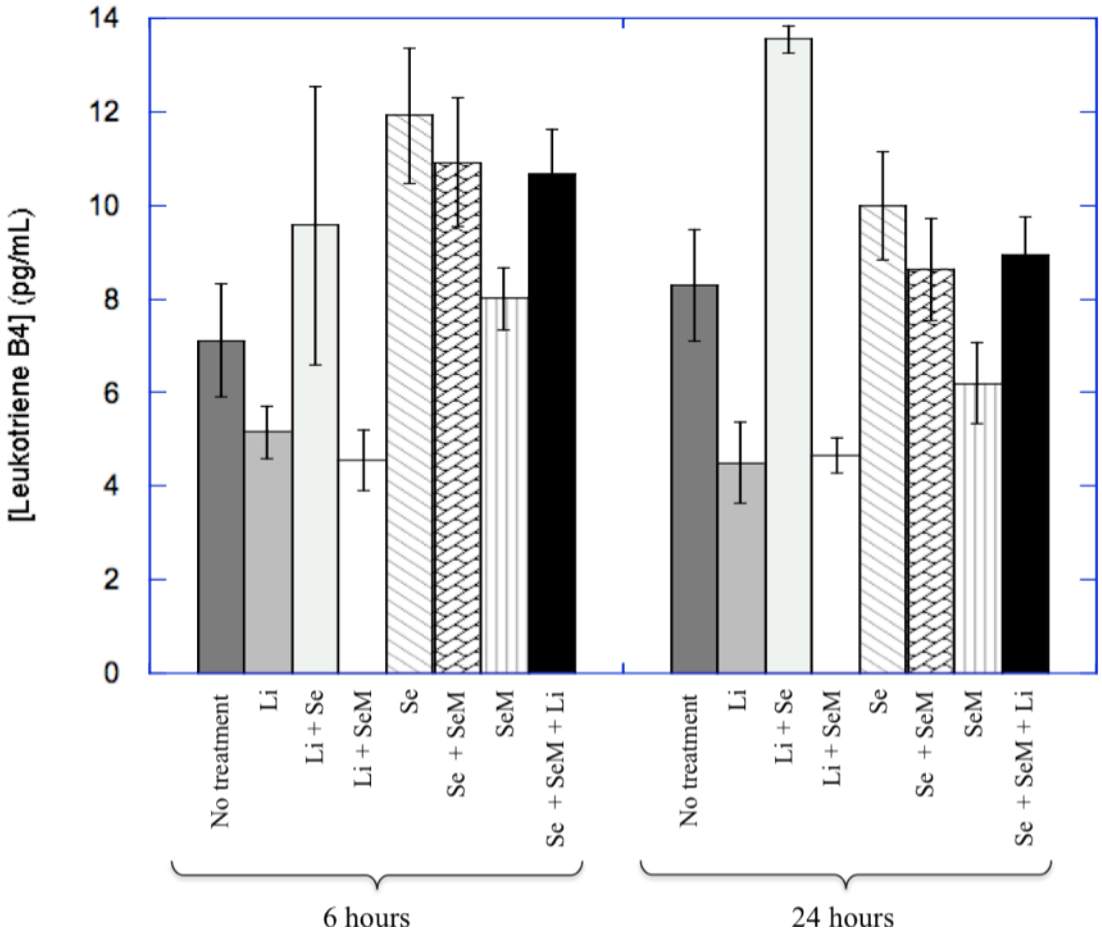

Figure 51 Expression of leukotriene B4 by JU77 cells in the presence of various compounds.

JU77 cells in their exponential growth phase were treated with 0.5mM lithium chloride (Li), 0.5mM lithium chloride and 30µM selenite (Li+Se), 0.5mM lithium chloride and 30µM selenomethionine (Li+SeM), 30µM selenite (Se), 30µM selenite and 30µM selenomethionine (Se+SeM), 30µM selenomethionine (SeM), 0.5mM lithium chloride and 30µM selenite and 30µM selenomethionine(Se+SeM+Li), no treatment (No treatment) and leukotrien B4 levels were measured after 6 and 24 hours.

3.3 The effect of a combinations of lithium chloride, selenite and selenomethionine on JU77 mesothelioma grown in a xenograft model in mice

Tumour-xenografts are useful tools enabling critical comparison between tumourigenic and normal tissues (Double and Bibby 1989). In this study, a mesothelioma tumour model was developed in nude balb/c mice as detailed in the Methods section 3.2.6.2 and 3.2.6.3. A cell number of 10^7 human tumour cells (JU77) were injected into nude balb/c mice in phosphate buffered saline following a standard protocol (Xia *et.al.* 2000). In general, it is presumed that if a tumour cell line is "tumourigenic" and the animals accept the injected tumour cells, self-perpetuation of tumour growth will follow. However, the growth of the mesothelioma tumours developed in this study required that insulin-like growth factor-1 be co-injected daily into the nude balb/c mice. This enhanced tumour onset of JU77 cells and once-daily injections of insulin-like growth factor-1 maintained tumour growth. This observation is consistent with a study of Haluska *et.al.* 2006, who reported that overactivation and/or overexpression of insulin-like growth factor-1 receptor contributes to tumourigenicity.

3.3.1 Animal control studies

JU77 human mesothelioma tumours were grown in nude mice for 4 days. On a daily basis, insulin-like growth factor-1 was injected subcutaneously to enable tumour growth of JU77 cells. Then, tumours were treated with lithium chloride, selenite and selenomethionine and combinations thereof while treatment with insulin-like growth factor-1 was continued (Figure 52-54) Treatments with single compounds did not change the rate of tumour growth relative to the treatment with vehicle alone (Figure 52-53 & Table 47). Similarly, dual treatments using lithium chloride and selenite or lithium chloride and selenomethionine did not change the rate of tumour growth relative to the treatment with vehicle alone (Figure 52-53 & Table 47).

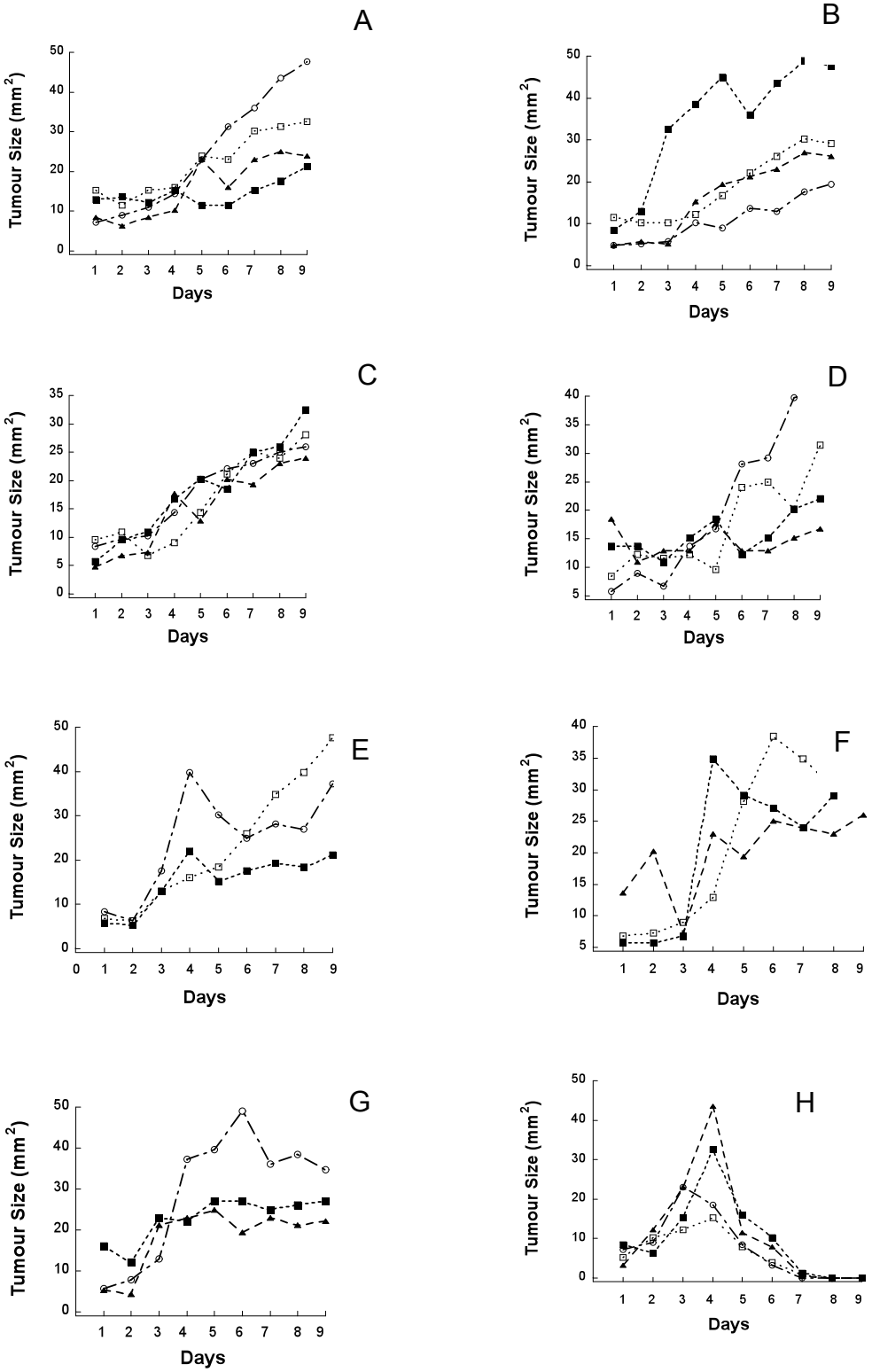

Figure 52 The effect of various treatments on JU77 tumour size in individual nude mice

JU77 human mesothelioma tumours were grown in nude mice as described in the Methods. Treatments began on day 4 of tumour growth: **A** vehicle only (n=4); **B**, selenite (n=4); **C**, selenomethionine (n=4); **D**, tithium (n=4); **E**, selenium and lithium (n=3); **F** selenomethionine and lithium (n=3); (**G**), selenium and selenomethionine (n=3); (**H**) selenium, selenomethionine and lithium (n=4). Data for individual mice are shown.

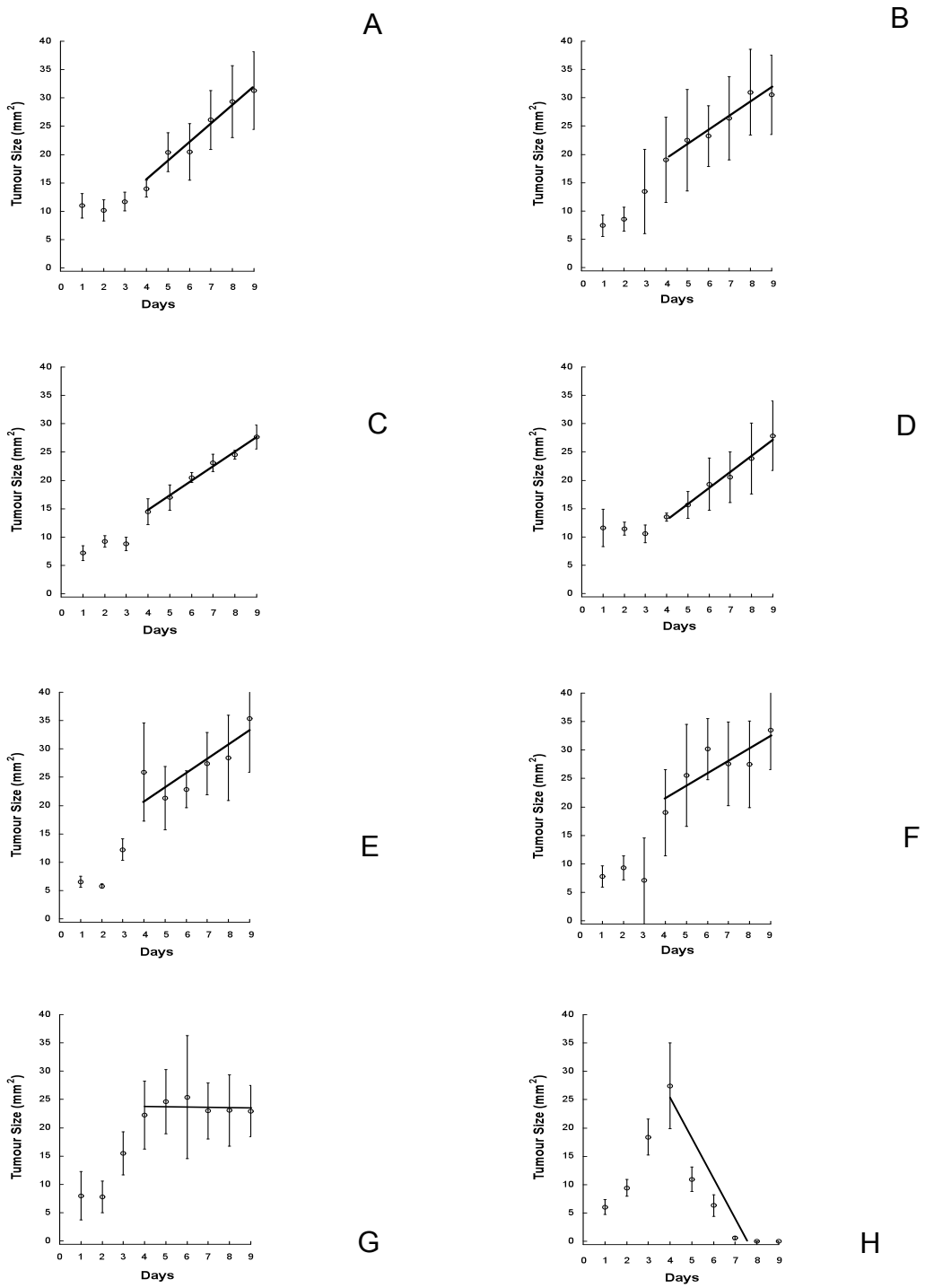

Figure 53 The effect of various treatments on JU77 tumour size in nude mice (mean +/- SE) with trend line following onset of treatment shown

JU77 human mesothelioma tumours were grown in nude mice as described in the Methods. Treatments began on day 4 of tumour growth: **A** vehicle only (n=4); **B**, selenite (n=4); **C**, selenomethionine (n=4); **D**, tithium (n=4); **E**, selenium and lithium (n=3); **F** selenomethionine and lithium (n=3); (**G**), selenium and selenomethionine (n=3); (**H**) selenium, selenomethionine and lithium (n=4). Data is illustrated as the mean plus or minus the standard error. Slopes were fitted to the data from day 4 with statistical comparisons of the trends provided in Table 47.

Table 47 A statistical analysis of the effect of various treatments on JU77 tumour size in nude mice

Treatment	Calculated Slope	Probability that slope fitting treatment is different to vehicle
A) Vehicle alone	3.4 +/- 0.9	
B) Selenite	2.5 +/- 1.4	P = 0.576 (Not Significant)
C) Semethionine	2.6 +/- 0.3	P = 0.431 (Not Significant)
D) Lithium chloride	2.8 +/- 0.8	P = 0.626 (Not Significant)
E) Selenite & Lithium chloride	2.1 +/- 1.2	P = 0.396 (Not Significant)
F) Selenomethionine & Lithium choride	1.5 +/- 0.9	P = 0.168 (Not Significant)
G) Selenite & Selenomethionine	-0.2 +/- 1.1 Slope does not differ significantly from zero (P=0.86)	P = 0.020 (Significant)
H) Selenite, Selenomethionine & Lithium chloride	-5.0 +/- 0.8	P < 0.0001(Extremely Significant)

The slope of each data set in Figure 53 was determined as a function of time following the initiation of a treatment on day 4. The probability that slope fitting the treatment was different to vehicle or different from zero was determined as described in Methods section 2.2.7.4

However, a dual treatment using selenite and selenomethionine reduced the growth of the JU77 mesothelioma tumours (Figure 52G, 53G). This effect was statistically significant (Table 47). Finally, the triple combination of lithium chloride, selenite and selenomethionine dramatically regressed the growth of tumours (Figure 52G, 53G and Table 47). Irrespective of the treatments, there was no significant weight loss with any treatment (Figure 54, Table 48).

To further evaluate the effects of the triple combination of lithium chloride, selenite and selenomethionine, a more detailed study was conducted and both tissue and blood samples were collected at different stages of the treatment. Tumours were grown for four days with daily subcutaneous injections of insulin-like growth factor-1. This ensured tumour growth over the period of treatment (Figure 55B). From day four onwards, a chemotherapeutic combination of lithium chloride (75µg once daily), selenite and selenomethionine (each 70µg twice daily, combined administration) was injected into the peritoneum. This treatment again led to a rapid decrease in tumour size, despite the daily administration of insulin-like growth factor-1. Tumours were not visible after another five days of treatment (Figure 55A). The controls were treated with carrier and insulin-like growth factor-1 twice daily and tumours continued to grow until they finally reached a steady-state (Figure 55B). When chemotherapy began, the weight of tumour-bearing animals dropped about 10% on first day, but this weight loss was not statistically significant and the animals maintained their weight thereafter (Figure 55C). The weight also did not change significantly in carrier-treated animals over the duration of the experiment (Figure 55D).

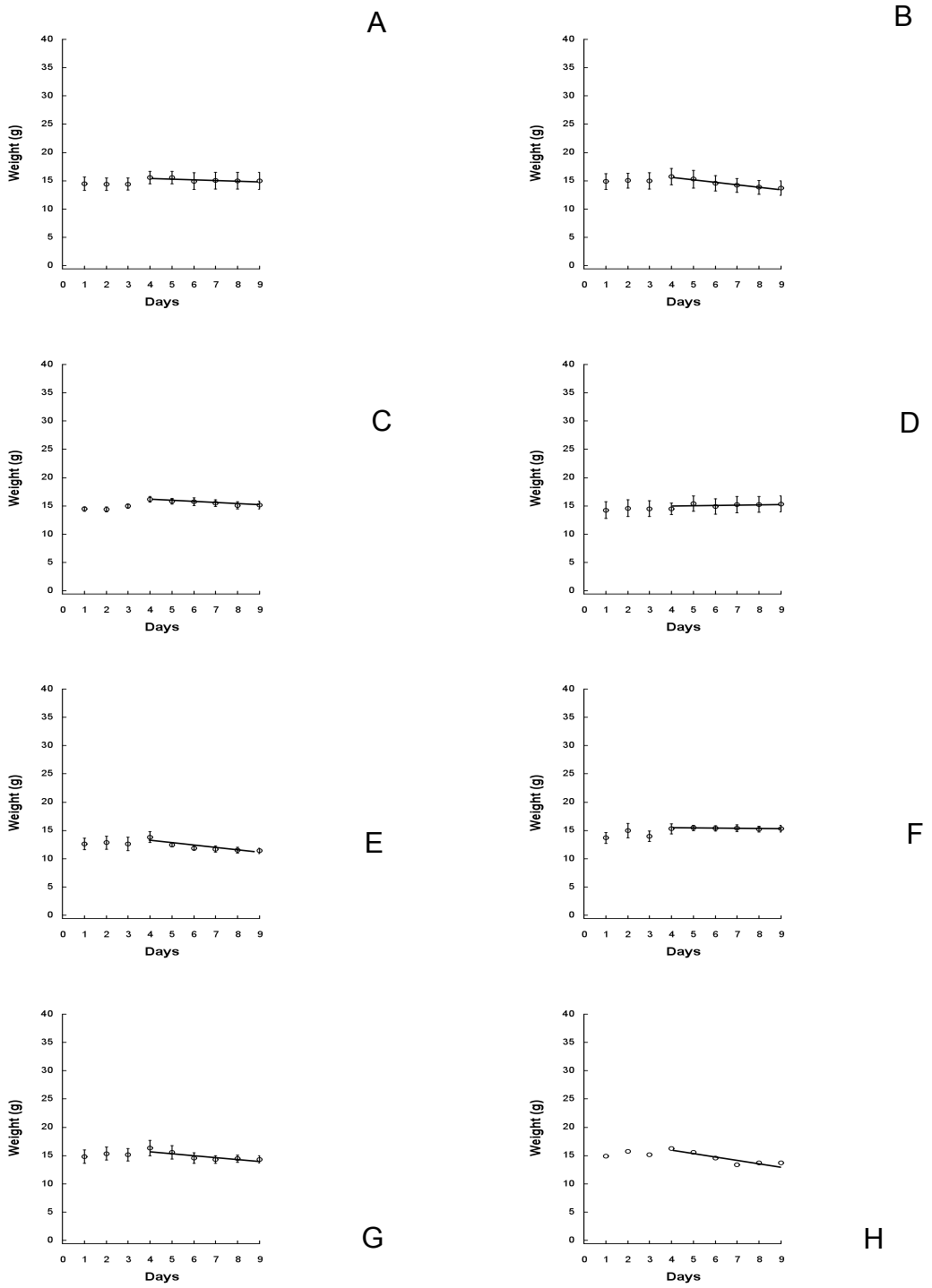

Figure 54 The effect of various treatments on body weight of nude mice (mean +/- SE) with trend line following onset of treatment shown.
JU77 human mesothelioma tumours were grown in nude mice as described in the Methods. Treatments began on day 4 of tumour growth: **A** vehicle only (n=4); **B**, selenite (n=4); **C**, selenomethionine (n=4); **D**, tithium (n=4); **E**, selenium and lithium (n=3); **F** selenomethionine and lithium (n=3); (**G**), selenium and selenomethionine (n=3); (**H**) selenium, selenomethionine and lithium (n=4). Data is illustrated as the mean plus or minus the standard error. Slopes were fitted to the data from day 4 with statistical comparisons of the trends provided in Table 48.

Table 48 A statistical analysis of the effect of various treatments on body weight of nude mice

Treatment	Calculated Slope	Probability that slope fitting treatment is different to vehicle
G) Vehicle alone	-0.1 +/- 0.2	
H) Selenite	-0.4 +/- 0.2	P = 0.412 (Not Significant)
I) Semethionine	-0.2 +/- 0.1	P = 0.769 (Not Significant)
J) Lithium chloride	0.0 +/- 0.2	P = 0.607 (Not Significant)
K) Selenite & Lithium chloride	-0.3 +/- 0.1	P = 0.497 (Not Significant)
L) Selenomethionine & Lithium choride	-0.3 +/- 0.1	P = 0.497 (Not Significant)
G) Selenite & Selenomethionine	-0.3 +/- 0.2	P = 0.449 (Not Significant)
H) Selenite, Selenomethionine & Lithium chloride	-0.5 +/- 0.3	P = 0.279 (Not Significant)

The slope of each data set in Figure 54 was determined as a function of time following the initiation of a treatment on day 4. The probability that slope fitting the treatment was different to vehicle or different from zero was determined as described in Methods section 2.2.7.4

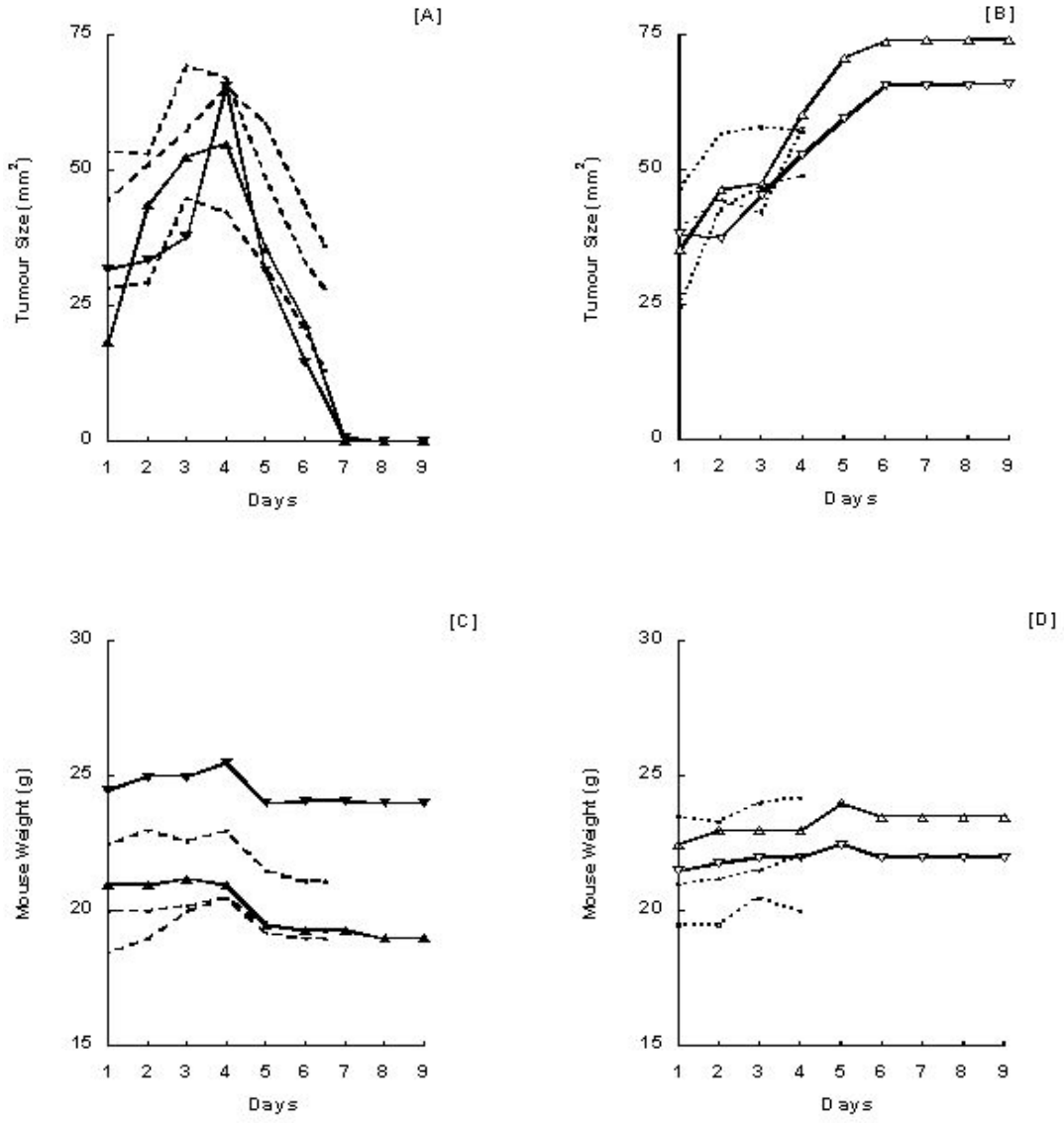

Figure 55 The effect of a combination of lithium chloride, selenite and selenomethionine on the tumour size of growth-factor maintained JU77 tumours in nude balb/c mice.

JU77 cells were injected subcutaneously into the left groin of six week old male, nude balb/c mice. Insulin-like growth factor-1 was injected subcutaneously into the groin on the side opposite to the tumour on a daily basis throughout the entire experiment. After four days, the tumour-bearing nude mice were injected intraperitoneally with either phosphate-buffered saline (captions B & D) or a combination of selenite, selenomethionine and lithium chloride in phosphate-buffered saline (captions A & C). Animals were sacrificed after either 0, 4, 6.5 or 9 days and tissues collected for histopathological analysis. Tumour size (mm^2) was measured daily with an electronic calliper (captions A & B). Animals were weighed daily (captions C & D). Refer to Methods section 3.7.4 and 3.7.5 for additional details of this experiment.

3.2.2 Haematology and biochemistry

Haematological and biochemical parameters were recorded before and after treatment. Data collected for the nude balb/c mice were also compared with control values of normal inbred C57/BL6 mice published in 2007 by Boehm *et.al.*

The haematology parameters are illustrated in Figure 56. A significant change in the red blood cell count of nude balb/c mice was observed when comparing the two treatments (compound/IGF-1 and IGF-1) against no treatment. In both treatments, insulin-like growth factor-1 increased the total red blood cell count. However, no significant difference in red blood cell count was seen between treatment with compound/IGF-1 and treatment with IGF-1 only. In addition, although insulin-like growth factor-1 increased the white blood cell count, the increase in the white cell count was only significant between compound/IGF-1 and the non-treated group and not between IGF-1 and the non-treated group or between the two treated groups. Interestingly, the white cell counts in control nude balb/c mice were significantly elevated relative to reported values of C57BL/6 mice.

Figure 57 compares the biochemical parameters in serum affected by the treatment with insulin-like growth factor-1 or the chemotherapy relative to control animals. The biochemical parameters that were measured in serum were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), lactate dehydrogenase (LDH), bilirubin, albumin, total protein, glucose, calcium, phosphate, alkaline

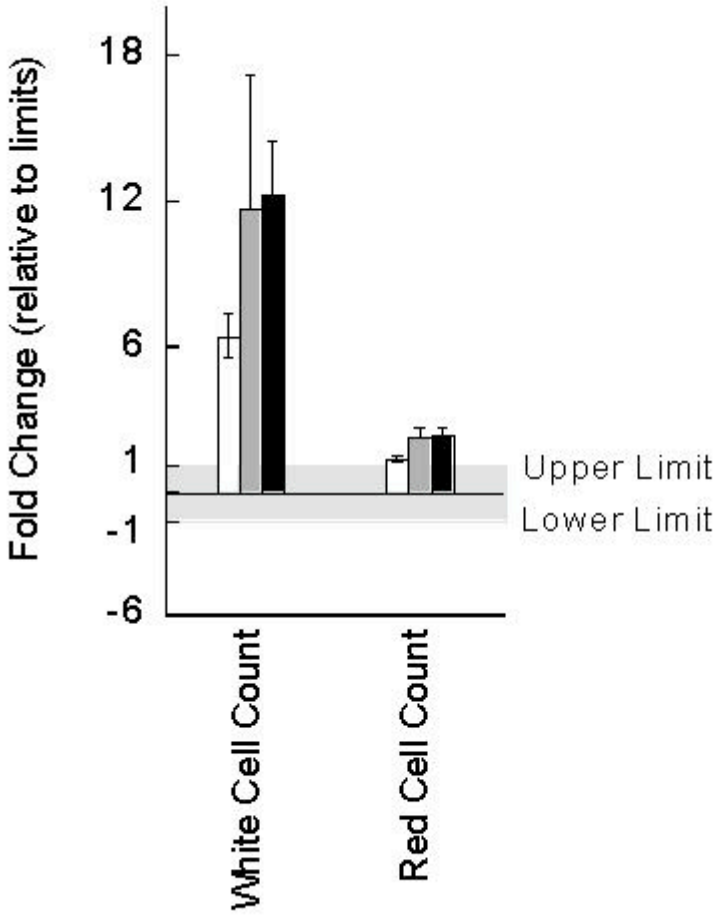

Figure 56 The effect of a combination of lithium chloride, selenite and selenomethionine on the haematological parameters of growth factor-maintained, JU77 tumour-bearing nude mice.

Six week old male nude balb/c mice were treated for nine days either with 75ug lithium chloride once daily, 70ug selenite and 70ug selenomethionine twice daily and insulin-like growth factor-1 (IGF-1) once daily (■) or only with insulin-like growth factor-1 (IGF-1) once daily (■) and haematology parameters were determined. A non-treated control group (□) displayed the normal values in the nude balb/c mouse. The shaded area illustrates the normal range for C57BL/6 mice (adapted from Boehm *et.al.* 2006). Data points are the mean $\pm$ SD of three determinations.

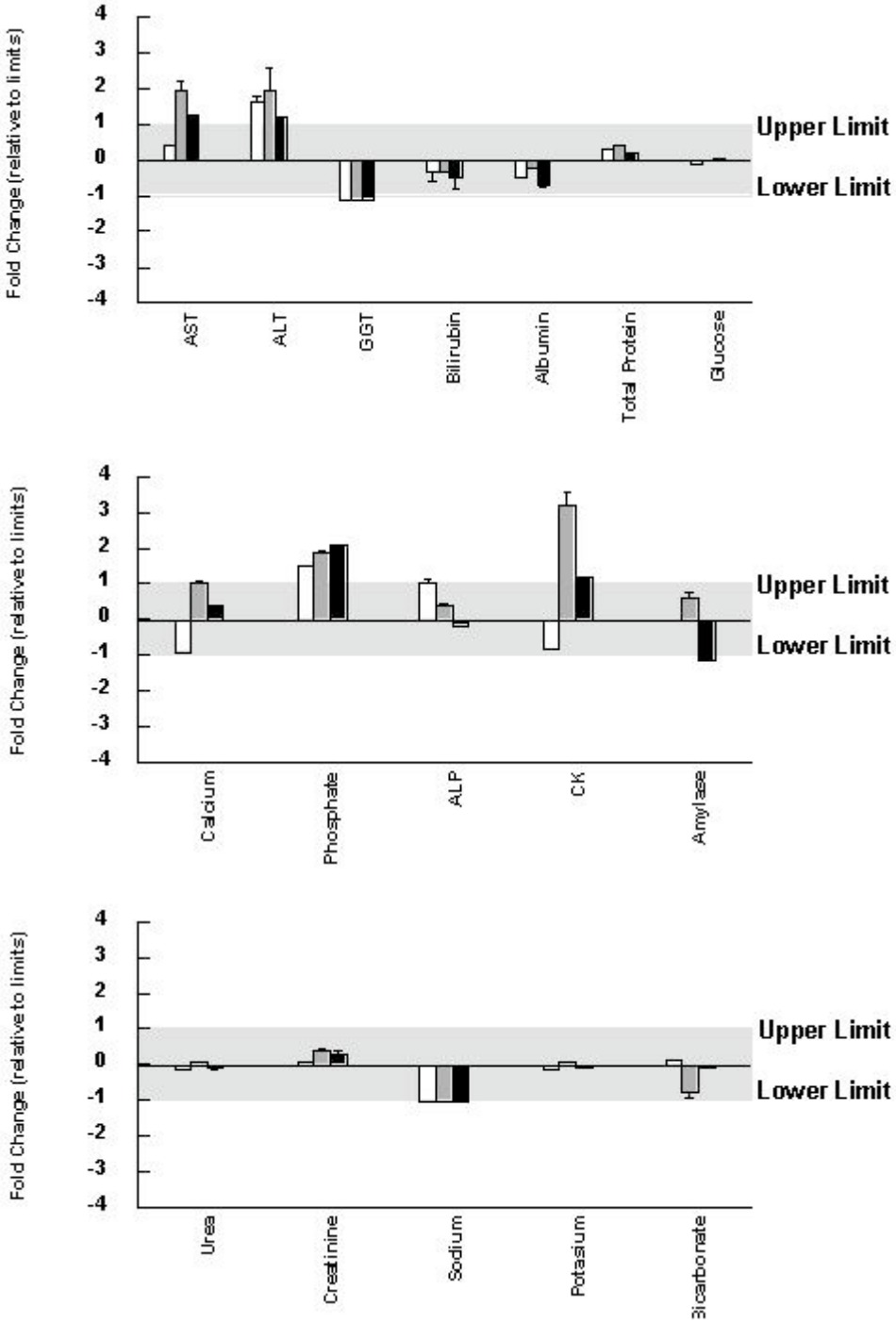

Figure 57 The effect of a combination of lithium chloride, selenite and selenomethionine on the biochemical parameters of growth factor-maintained, JU77 tumour-bearing nude mice.

Six week old male nude balb/c mice were treated for nine days either with 75ug lithium chloride once daily, 70ug selenite and 70ug selenomethionine twice daily and insulin-like growth factor-1 (IGF-1) once daily (■) or only with insulin-like growth factor-1 (IGF-1) once daily (▩) and biochemistry parameters were determined. A non-treated control group (□) displayed the normal values in the nude balb/c mouse. The shaded area illustrates the normal range for C57BL/6 mice (adapted from Boehm *et.al.* 2006). Data points are the mean ± SD of three determinations.

phosphatase (ALP), creatine kinase (CK), amylase, urea, creatinine, sodium, potassium, bicarbonate.

Relevant changes of biochemical parameters were observed with administration of insulin-like growth factor-1 for aspartate aminotransferase, alanine aminotransferase, phosphate and creatine kinase. These parameters did not change more than threefold above the upper limit. Lactate dehydrogenase, a parameter which is very sensitive to hemolysis, was also elevated above the limits. This parameter is not illustrated because it was impossible to distinguish between the effects of (irrelevant) hemolysis and treatment. Chemotherapeutic treatment reversed elevation of parameters due to insulin-like growth factor-1 into the normal range. However, phosphate stayed on a twofold increased level.

3.2.3 Histopathology

Many hundreds of histopathological slides were made from tumours and normal tissues according to standard pathological procedures. The slides were stained with hematoxylin and eosin. Tumour tissues and normal tissues were collected from tumour-bearing animals that had been treated as follows:

- for 4 days with insulin-like growth factor-1
- for 6.5 days with insulin-like growth factor-1 and simultaneously for 2.5 days with lithium chloride and selenodimethionine (days 4-6.5)
- for 9 days with insulin-like growth factor-1 and simultaneously for 5 days with lithium chloride and selenodimethionine (days 4-9)

The chronological sequence of the histological changes in the tumour tissues is illustrated in Figure 58-63.

Figure 58 illustrates a JU77 tumour grown subcutaneously for four days with insulin-like growth factor-1 administration. The neoplasm consists of a

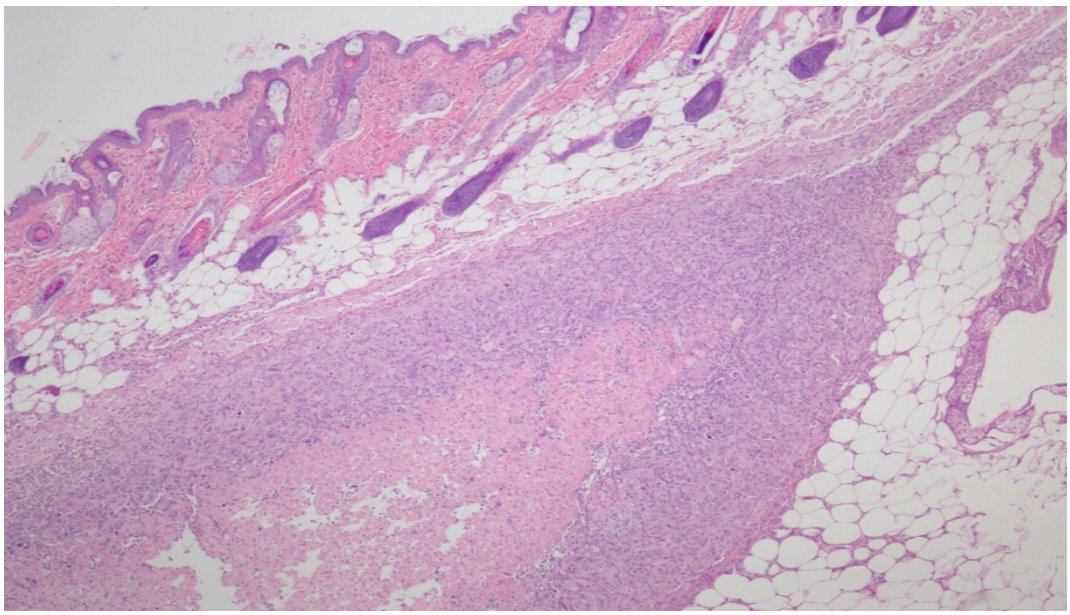

Figure 58 The histopathological structure of a mesothelioma JU77 tumour grown subcutaneously for four days with daily application of IGF-1**:**

The neoplasm consists of a central necrotic area surrounded by a relatively thick peripheral layer of closely packed malignant cells. The thickness of the neoplastic layer is generally regular. H&E, Mag.x100.

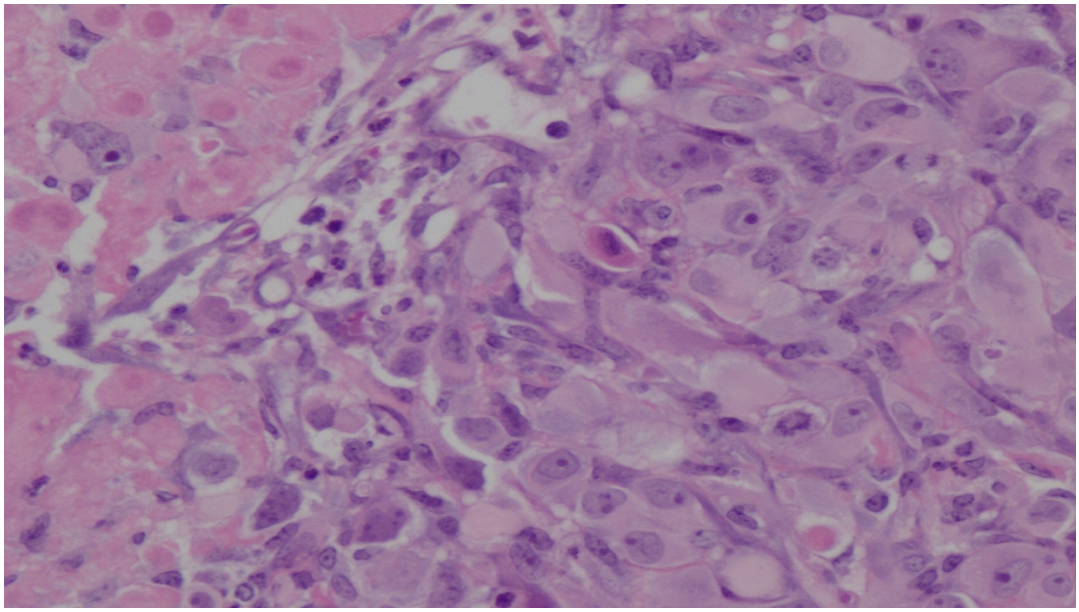

Figure 59 The composition of the peripheral layer of a mesothelioma JU77 tumour grown for four days with daily application of IGF-1:

The peripheral layer of the neoplasm is composed of large polygonal cells which are closely opposed and possess large mildly irregular nuclei with much euchromatin and multiple large nucleoli. A small proportion of these cells are in mitosis while others show evidence of apoptosis. H&E, Mag.x400

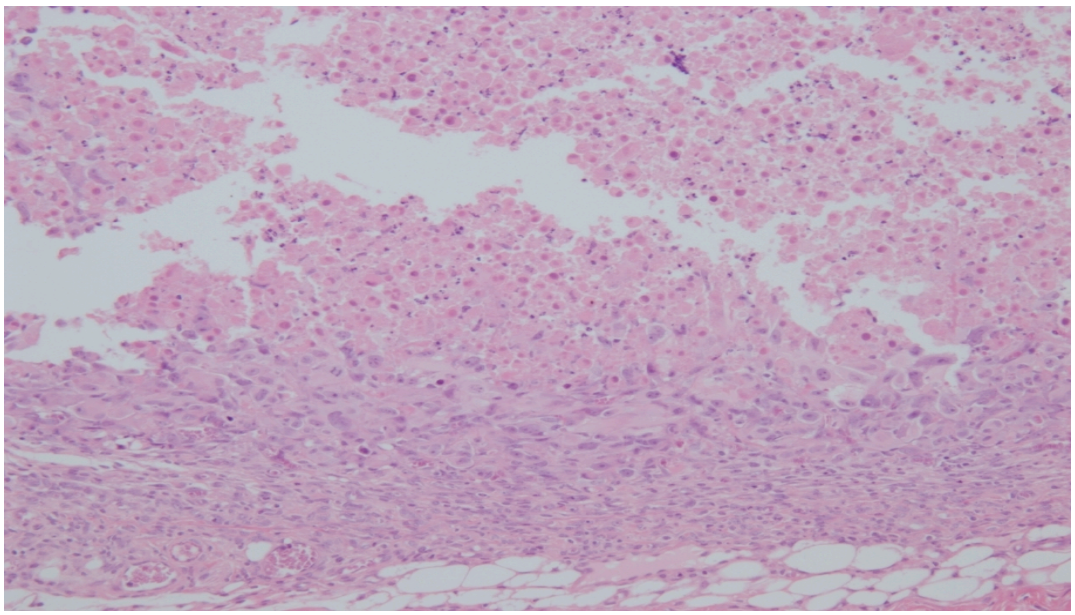

Figure 60 The histopathological structure of a mesothelioma JU77 tumour grown for 6.5 days with daily application of IGF-1 and treated for 2.5 days with application of lithium chloride once daily and selenite and selenomethionine twice daily:

Much of the tumour is necrotic. A thin, irregular, fragmented layer of neoplastic cells can be seen at the periphery. H&E, Mag.x100

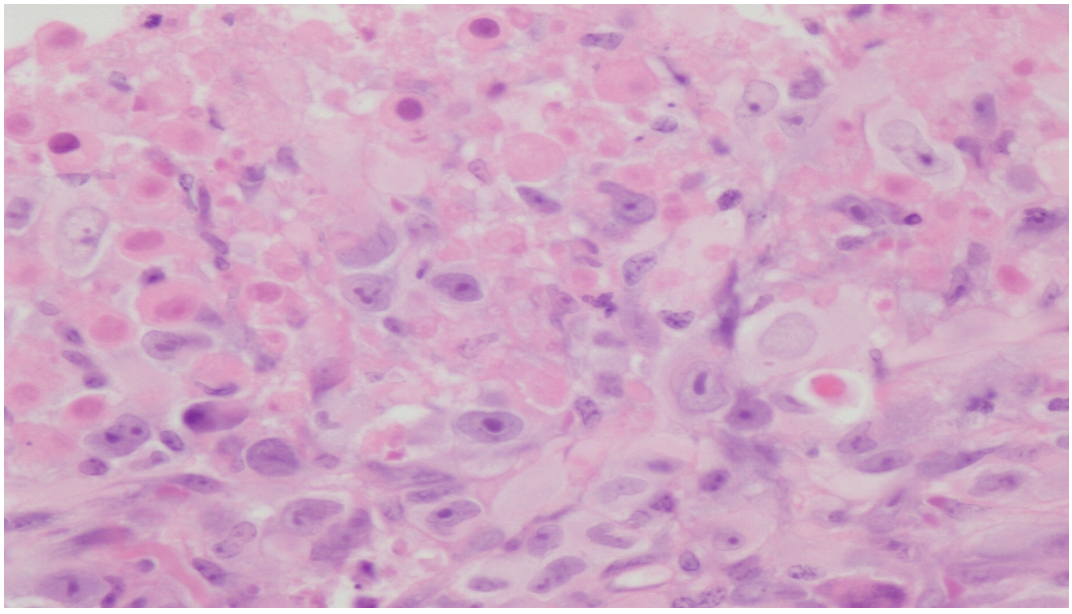

Figure 61 The composition of the peripheral layer of a mesothelioma JU77 tumour grown for 6.5 days with daily application of IGF-1 and treated for 2.5 days with application of lithium chloride once daily and selenite and selenomethionine twice daily:

Most of the tumour is necrotic. However, a few scattered neoplastic cells can still be seen at the periphery. H&E, Mag.x400

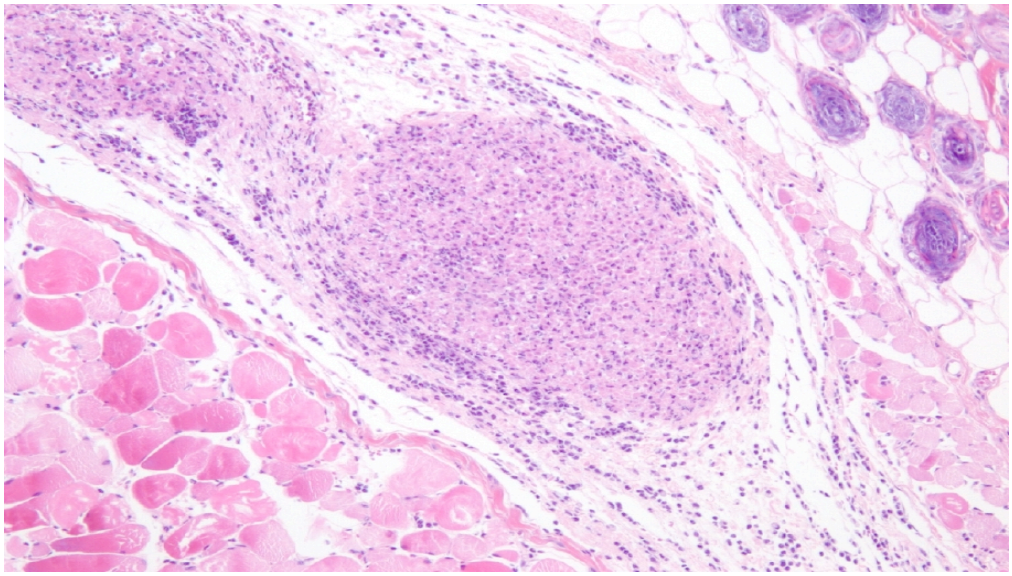

Figure 62 The histopathological structure of a mesothelioma JU77 tumour grown for 9 days with daily application of IGF-1 and treated for 5 days with application of lithium chloride once daily and selenite and seleno-methionine twice daily

No neoplastic cells can be seen, and the tumour consists of scattered small clusters of necrotic tissue. An inflammatory infiltrate surrounds the necrotic tissue. H&E, Mag.x100

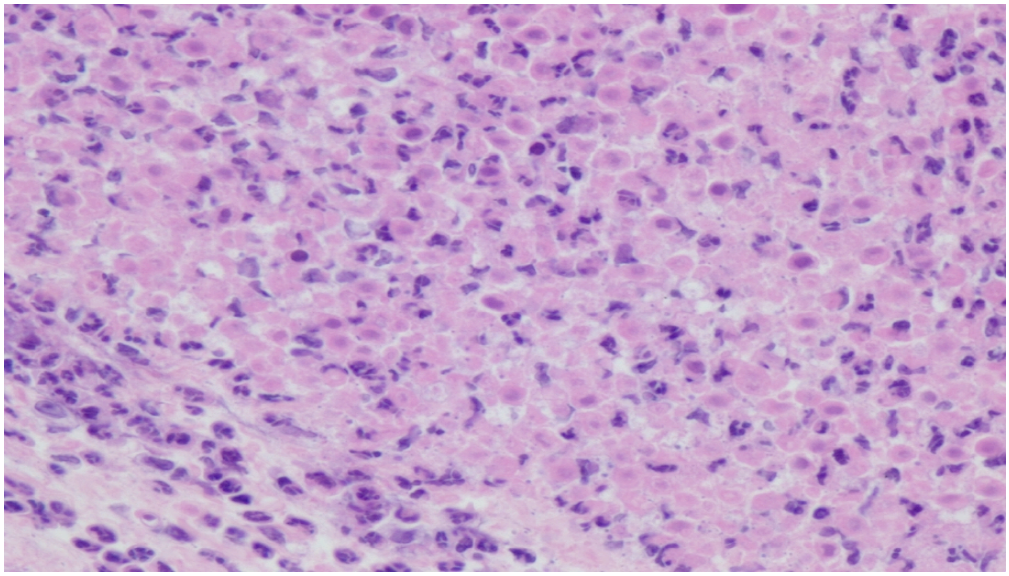

Figure 63 A magnified view of the clusters of necrotic tissue of a mesothelioma JU77 tumour grown for 9 days with daily application of IGF-1 and treated for 5 days with lithium chloride once daily and selenite and selenomethionine twice daily

No intact neoplastic cells can be discerned; only necrotic tissue can be seen. H&E, Mag.x200

central necrotic area surrounded by a thick peripheral layer of closely packed JU77 cells. The thickness of the neoplastic layer is generally regular. As highlighted in Figure 59, large polygonal cells form this layer. They contain large mildly irregular nuclei with much euchromatin and multiple large nucleoli. Some of them are replicating while others show evidence of apoptosis.

Figure 60 illustrates the first structural changes of the mesothelioma JU77 tumour after 2.5 days of treatment with lithium chloride and selenodimethionine and daily administration of insulin-like growth factor-1. Most of the tumour is now necrotic. The peripheral layer is no longer thick. Irregular packing and fragmentation can be observed. Magnification of the peripheral layer reveals a few scattered neoplastic cells at the periphery (Figure 61).

After five days of treatment with lithium chloride, selenite and selenomethionine and administration of insulin-like growth factor-1, the tumor is totally necrotic and surrounded by an inflammatory infiltrate (Figure 62). Even at higher magnification, no intact neoplastic cells can be seen in any of the necrotic clusters (Figure 63). To identify the few remaining, dead tumour cells many hundred histological slides had been performed.

Normal tissues of the tumour-bearing rodents, like liver, lung, spleen and gut were processed at the same time as tumour tissues. Differences between insulin-like growth factor-1 with or without treatment were not observed. However, abnormalities were found in liver and pancreas in both treatments. These abnormalities seemed to be due to the inherited defect of the nude mice and were equally exaggerated by compound treatment with IGF-1 and IGF-1 alone.

4.0 DISCUSSION

Lodish *et.al.* in 2003 described common features of cancers and compared them to their normal counterparts: they characterised "cancer" as having "unlimited replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, tissue invasion during metastasis and sustained angiogenesis". However, despite the observation that all cancers present in a uniform pattern, malignancy must not be interpreted as a uniform default mechanism. Various cancers show great differences in how their malignant transformation has taken place and their signalling has changed. In the first place, their cellular signalling may be different, depending on the tissue they originated from: a mesothelial-derived cancer may behave differently to an epithelial-derived one. However, due to their malignant transformation, even cancers of similar or identical origin display different signalling patterns. Four cancer cell lines have been chosen to illustrate the diversity in cancer cell signalling. The diversity in signalling becomes apparent when monitoring the viability of these cell lines exposed to different chemotherapeutic treatments. Some differences and similarities in signalling of two cell lines are illustrated in Figure 5: the signalling pathways of the mesothelioma cell line JU77 and the breast cancer cell line MCF-7 were targeted with various compound combinations, and it was observed that their sensitivity to various chemotherapeutic compounds was cell line-specific. Presumably, the signaling pathways dictating cancer cell viability is also cell line specific. However, one compound combination affected both cell lines, suggesting that some signalling pathways leading to apoptosis were common between these two different cell lines. These signalling patterns as typified in the above examples may determine the target for treatments of these cancer types.

The process of malignant transformation usually happens spontaneously, but it can also be artificially induced. Cells can be “transformed” by exposing them to chemicals, radiation, viruses and/or other procedures. Tumour cells usually start to proliferate as soon as the transfomation has taken place. Transformed cell lines are valuable to characterise the diversity of cancer-specific biochemical changes for unlimited growth. However, treatment of these transformed cell lines does not necessarily reflect how the corresponding primary tumours will react to the same chemotherapeutic treatments. The transforming process from a primary tumour *in vivo* to an unlimited-growing cell line *in vitro* can change the biochemical structure of the cancer cells significantly and it can be quite challenging to link the results from experiments on transformed cancer cell-lines to treatments of primary tumours *in vivo*. In contrast, patient-derived, non-transformed tumours are usually difficult to cultivate outside their original hosts. However, if they grow, these tumours are unchanged in all the settings, irrespective if they grow in cell culture, in animal tumours or in clinical settings. In the research for this thesis, the non-transformed mesothelioma cell line JU77 and the three transformed cell lines MCF-7 (Breast carcinoma), Caeco-2 (Colon carcinoma) and HepG2 (Hepatocellular carcinoma) were investigated and compared in cell culture. In the beginning it seemed as if it was far less important to find a successful treatment for mesothelioma tumours than for any other tumour; the prevalence of mesothelioma tumours in the general population is known to be rather low compared to the other tumour types. However, this tumour type was finally chosen for ongoing *in vivo* experiments because it offered the opportunity to study the effects of chemotherapeutic treatments in different settings using identical patient-derived cancer cells.

Prior to the work done for this thesis, all these cell lines had been grown in various culture conditions to optimize the doubling times of each cell line.

However in the present study, all four cell lines were grown using the same medium, in which they still replicated exponentially, although not necessarily in their shortest doubling time. Details of the medium composition are specified in Methods section 2.2.1.2. Growing them in similar conditions *in vitro* allowed comparisons between cancer cell lines. No attempt was made to compare normal and malignant cell lines, for several reasons: first, additional growth factors are needed to maintain and grow normal cells in culture; second, the replicating of normal cells is limited; and finally, exponential growth is not a regular feature of normal cells compared to their malignant counterparts. Sometimes, single normal cells like PBMCs (Peripheral blood mononuclear cells) or HUVECs (Human umbilical vein endothelial cells) are treated with the same compounds as single cancer cells to get an idea of the therapeutic index, but these experiments do not reflect the toxicity of compounds for normal tissues and they may also miss significant differences in toxicity between various normal tissues. Therefore, a comparison between normal and malignant cells was made *in vivo* by performing histological examinations of tumours and several normal tissues. This is consistent with the opinion of Double *et.al.* in 1989, who reported that the critical measure between tumourigenic and normal tissues is provided by xenografts models.

Another cornerstone in setting the experimental conditions was to supplement the cell culture medium with high concentrations of insulin-like growth factor-1. Manez and Martinez-A. in 1998, report that tumor cells *in vivo* constitutively express growth factors and growth factor receptors as a result of their malignant transformation, creating autocrine loops by which they perpetuate their own growth. This raises reasonable doubt about the efficacy of chemotherapeutic agents in promoting cancer cell death, because it has been reported that signalling of insulin-like growth factor-1 receptor can substantially inhibit the therapeutic effects of

chemotherapeutic agents under various experimental conditions (Jernberg-Wiklund and Nilsson 2007; Conti *et.al.* 2007). In light of these reports, the *in vitro* experimental conditions in my studies were established to simulate *in vivo* conditions. In an environment with high concentrations of insulin-like growth factor-1, complete cell death of cancer populations was observed at high concentrations of single broad-spectrum compounds. Some compounds were found to be more efficient than others: however, no single compound was found to kill the entire cancer cell population at levels applicable *in vivo* (Figure 5, 10, 18, 21, 25, 33, 39, 44, 47). As an alternative approach different receptor systems were targeted using combinations of inhibitors. This was a reasonable approach considering reports of Navarro and Baserga in 2001, who inhibited an increasing proportion of signalling pathways originating from a single receptor and who discovered that receptor signalling has "limited redundandancy". The approach taken in this study was to inhibit a proportion of different constitutively activated receptor systems in the hope that the survival signals of receptor systems also had "limited redundancy".

However, treatment resistance of tumours against known chemo-therapeutic agents is not only due to high concentrations of insulin-like growth factor-1. Treatment resistance is a much more general problem in oncology. It can develop quite rapidly, it is usually fatal and it has therefore been part of intense research. Usually, a specific treatment is chosen for a particular cancer type due to a different sensitivity of the various cancers to chemotherapeutics but even the most effective anti-cancer agent is usually not eradicating the tumour. While shrinking the size of the tumour, a certain number of cancer cells will still survive inside the tumour mass. An increase in chemotherapeutic concentration is limited by the maximum tolerated dose of the drugs. Tumours that are exposed to such chemical inhibitions for a certain time develop a partial necrosis and some of the

remaining cancer cells inside the tumour mass become resistant. These cells learn how to attenuate the effects of the applied chemicals. They slowly change their inbuilt signalling structure that consists of an enormous redundancy and overlap of signalling pathways and this finally attenuates the so-called “targeted chemical inhibitions”. As mentioned before, the level of redundancy and overlap in signaling structure can differ significantly between different cancer cells and normal cells and this seems to be one reason that different cancer cells develop different mechanisms of resistance. But irrespective that cancer cells might develop different mechanisms of resistance and irrespective of the biochemical mechanism involved therein, the focus should not be on the developing resistance but on the treatment in the first place. If a treatment kills 100% of the cancer cells in a short time after its application, there is no time left for the cancer cells to develop a resistance. Such effective combinations can either be cancer cell-type specific or they may be effective in all different kinds of cancers.

However, the number of inhibitor combinations that could be chosen is virtually unlimited. Some natural compounds which are listed in the literature review were tested as they offered promise as “pre-made inhibition cocktails” targeting various key signalling pathways. Compounds with low toxicity and epidemiologically proven anti-cancer effects were preferred selections. The approach taken was to combine broad-spectrum chemotherapeutic agents with several narrow-spectrum inhibitors. The number of inhibitor combinations that could be chosen from the 99 compounds was virtually unlimited ($9.3x\ 10^{155}$), so combinations were limited to sextuple combinations with a particular focus placed on those compound combinations that include those highlighted in columns A and B of Table 2. In total, approximately 30000 combinations of compounds were tested.

Combinations with narrow-spectrum inhibitors reduced the concentration of the broad-inhibiting compounds needed to effectively kill cancer cell populations. Using the broad-spectrum inhibitors staurosporine, ascorbic acid and selenite, the concentration could be reduced into a therapeutic range (Figure 6, 8, 24, 26, 27, 28, 29, 31). However, as Figure 6 and 7 illustrate, the effective death of cancer cell lines within a therapeutic range required selection of compound combinations that worked in synergy with each other.

As illustrated in Figure 6 and 9, staurosporine concentrations declined into a therapeutic range when combined with narrow-spectrum inhibitors on mesothelioma JU77 cells, but it was impossible to achieve an equivalent result in breast cancer MCF-7 cells despite a wide-spread search for combinations. Staurosporine at a concentration of 60nM seemed to be the minimal critical concentration of staurosporine to promote cell death in MCF-7 cells, but was still too high to be applicable *in vivo*. Interestingly, 60nM staurosporine is known to inhibit the insulin receptor kinase.

The testing of compounds against various cancer cell lines is widely accepted in the literature as a general screening tool (Garrett *et.al.* 2003). Foetal calf serum is usually included as a medium supplement. Usually, the results from these *in vitro* experiments with foetal calf serum are then later transferred into animal studies and clinical settings. However, in animal studies, the compounds are exposed to adult albumin. Results of experiments in this thesis illustrate, that the effects of compounds can change dramatically when exposed to different kinds of albumin: compounds adhere more firmly to adult albumin, leaving only the freely dissociated proportion of the compound active. This is illustrated in Figure 10. In this Figure, genistein attached to adult albumin in the presence of 10% foetal calf serum looses 25% of its potency. This can be reversed by

combining genistein and albumin with warfarin, which displaces genistein from adult albumin and reinstitutes its potency. Thus, the potency of compounds can change by binding to adult albumin. It is surprising that the effect of adult albumin is not considered *in vitro* studies prior to animal testing of compounds. I could not find a publication describing the partial replacement of foetal calf albumin with adult albumin, or of the addition of adult albumin to cancer cell cultures.

Brodersen and Honore reported in 1989 that adult albumin binds chemotherapeutic compounds stronger than neonatal albumin. Research conducted to investigate the change in the binding effects of albumin indicated that an increase in S-nitrosylation of albumin can cause a decrease in ligand-affinity (Kashiba-Iwatsuki, Miyamoto and Inoue 1997). High levels of S-nitrosylated albumin were discovered in fetal and neonatal sera (Christen *et.al.* 2007). In addition, the concentration of alpha 1-acid glycoprotein, a plasma binding protein, is much lower in fetal and neonatal serum than in adult serum (McNamara and Alcorn 2002). Thus, a low binding capacity of fetal serum can result in a high bioavailability of compounds in cell culture which does not reflect the situation in animal studies.

Predicting the bioavailability of compounds is a crucial consideration in any drug study. If compounds have low aqueous solubility, a therapeutic concentration in serum may not be achieved. Lipinski *et.al.* in 1997 stated in his “Rule of Five” that the molecular properties of a compound are important for their absorption, distribution, metabolism and excretion (ADME). Changing the molecular properties of compounds to increase their solubility without destroying or changing their function has attracted wide-spread attention in biomedical and pharmaceutical research. This point is well illustrated in Figure 11 where the solubility of genistein is

considered. Hydrolyzing genistein increases its solubility several thousandfold but decreases its potency by half. Due to its enhanced solubility, its bioavailability increases: genistein chalcone is easily absorbed and distributed *in vivo*, and adherence to albumin does not limit the bioavailability. Other approaches can be used to increase compound solubility. For example, compounds may be transiently dissolved in either protic or aprotic substances (like ethanol, or dimethyl sulphoxide). This increases aqueous solubility *in vitro* slightly. However *in vivo*, these transiently dissolving compounds are not approved for treatment (for example, dimethyl sulphoxide) or have undesired side effects (for example, ethanol). Other pharmaceutical options to increase solubility and bioavailability are the inclusion of compounds into cyclodextrins (Carrier, Miller and Ahmed 2007), the formation of nanoparticles (Uner and Yener 2007), the formation of micelles (Narang, Delmarre and Gao 2007) and the use of predrugs (Steffansen 2004).

Lipinski`s "Rule of Five" characterises the criteria determining the bioavailability of compounds *in vivo*, and predicts adsorption, distribution, metabolism and excretion characteristics of the compounds. This affects their bioavailability at a tumour site. However, therapeutic effects are dependent not only on the bioavailability of compounds but also on the potency and specificity of compounds for their target structure. The problem can be further compounded because there may also be heterogeneity in a cancer cell population. This is illustrated in Figure 18 and 19: a concentration of 30µM mifepristone alone killed only 85% MCF-7 breast cancer cells. The remaining 15% of MCF-7 cells were unaffected by the mifepristone treatment even at very high concentrations. However, the second subpopulation was sensitive to 30µM mifepristone in combination with a low, non-lethal concentration of staurosporine.

In the search for successful chemotherapeutic combinations with satisfactory bioavailability, other broad-spectrum inhibitors were also investigated. Vitamin C is known to prevent lipid peroxidation and protein oxidation (Suh, Zhu and Frei 2003); selenotrisulphides changes methylation patterns (Vanyuschin 2006); β-lapachone activates cell cycle checkpoint regulation (Li *et.al.* 2003); glycolysis inhibitors influence the "aerobic glycolysis" (Pelicano *et.al.* 2006); antibiotics inhibit isozymes of glutathione S-transferase a detoxifying enzyme (Mukanganyama *et.al.* 2002); diethylmaleate changes cellular replication due to a redox mechanism involving glutathione (Fratelli *et.al.* 2005); hydrogen-peroxide reacts as a signalling molecule (Zhung and Schnellmann 2004). Trialling these compounds *in vitro* in the presence of human albumin revealed several combinations that were effective in killing JU77 and MCF-7 cancer cells within a therapeutic range. These are illustrated in Figure 24 for vitamin C, in Figure 34, 35, 36, 37, 38 for selenotrisulphides, Figure 39 and 40 for β-lapachone, Figure 41 for glycolysis inhibitors, Figure 42 and 43 for antibiotics and Figure 45 and 46 for diethylmaleate. However, no combination with hydrogen peroxide was discovered that killed a whole cell population within a therapeutic concentration. A combination of lithium chloride and selenotrisulphides was the most promising of all these successful *in vitro* combinations. This combination proved to be remarkably potent and highly bioavailable, and therefore was chosen as the preferred candidate for an *in vivo* animal trial. Whilst the following discussion focuses on these combinations with lithium chloride and selenotrisulphides, it will be interesting to also explore some of the remaining successful *in vitro* combinations.

Selenium-containing compounds display promising *in vitro* and *in vivo* features for cancer treatment. They appear in either inorganic or organic form. Inorganic selenite dose-dependently killed mesothelioma and breast

cancer cells. This is illustrated in Figure 25 and 30. In human serum albumin, the concentration of selenite needed to kill cells, increased to several times the maximum tolerated dose. However, when combined with sulphydryl group-containing compounds, its potency was restored. Trisulphides were formed by adding sulphydryl group-containing compounds. Combining selenite with different sulphydryl group-containing compounds did not change their efficacy in human serum albumin. As illustrated in Figure 32 and 33, selenite combined with cysteine, selenomethionine, glutathione, α-lipoic acid and dihydrolipoic acid, were equally potent. Controls containing single sulphydryl group-containing compounds had little effect on cancer cells. The trisulphide "selenodimethionine" formed by selenite and selenomethionine, gained potency in the presence of lithium chloride or geldanamycin (Figure 34 and 35), mercaptoethanol (Figure 36) or mercaptoethanol plus gadolinium chloride (Figure 37). All these combinations were effective within the therapeutic range of selenotrisulphide. Remarkably, all four cell lines were similarly sensitive to trisulphide combinations, as illustrated in Figure 38. Other compounds like β-lapachone, glycolysis inhibitors, antibiotics and diethylmaleate also synergised with these trisulphides, as illustrated in Figure 39, 40, 41, 42, 43, 45 and 46. The compound combination "selenotrisulphide" was selected for animal trials *in vivo*.

Holub in 1989 discovered that nude mice grow xenografts after injection of human tumor cells, as a result of the inherited deterioration of their thymus glands and their failure to generate mature T lymphocytes. Double and Bibby in 1989 identified tumor-xenografts as a useful tool that provides a critical measure of tumor versus normal tissue. This process is the generally accepted final step in preclinical drug development (Garrett *et.al.* 2003). The non-transformed human mesothelioma cell line JU77 was chosen for the xenograft model for this study in nude balb/c mice to test the trisulphide

combinations mentioned above.
The nude mice did not grow any mesothelioma tumors in the absence of insulin-like growth factor-1 supplementation in the animals. The mesothelioma cell line JU77 did not seem to be tumorigenic. Either the innate immune system of the nude mice was able to reject the injected tumor cells, or high insulin-like growth factor-1 concentrations co-delivered with the cells into the mice *in vitro* influenced tumorigenicity *in vivo*. It was also possible that both factors were at work.

In general, factors that influence tumorigenicity are considered targets for treatment. Haluska *et.al.* in 2006, stated that overactivation and/or overexpression of insulin-like growth factor-1 receptor and insulin receptor contribute to tumourigenicity, proliferation, metastasis and drug resistance. By enhancing the concentration of insulin-like growth factor-1 in the nude mouse model used in this study, the JU77 cell line switched from a non tumourigenic to a tumourigenic cell line.

While the ratio between insulin-like growth factor-1 and insulin-like growth factor binding protein is usually stable, continuous supplementation of insulin-like growth factor-1 in the nude mouse in this study could have changed the ratio. This may be the reason for the switch in tumourigenicity. A similar imbalance is observed when serum proteases or matrix metalloproteinases (MMPs) cleave the insulin-like growth factor binding proteins. This also creates a change in the ratio between growth factor and binding protein, although at a lower concentration. An important example is provided by Miyamoto *et.al.* in 2004 who characterised matrix metalloproteinase 7 as one of the insulin-like growth factor binding protein 3 proteinases releasing the insulin-like growth factor-1, with a resulting change in the ratio.

In the tumor model developed for this study, the concentrations of insulin-like growth factor-1 and insulin-like growth factor binding proteins were not measured and this limits the conclusions that may be drawn. However, awareness of the axis-dependent promotion of tumour cell replication indicates that there is a need for a review of the "golden standard" of drug development. Future testing of compound combinations in syngeneic and xenograft models *in vivo* should include the simultaneous measurement of the ratio between insulin-like growth factor-1 and the insulin-like growth factor binding proteins to determine whether there is an imbalance in the ratio and if this is shown to be so, to correct the imbalance. It could well be that the potency of chemotherapeutic combinations increases when the freely available concentration of insulin-like growth factor-1 is limited.

As soon as JU77 mesothelioma cells *in vivo* were supplemented with daily injections of insulin-like growth factor-1, tumours started to grow. As illustrated in Figure 52, 53 and 55, tumours grew rapidly and reached a steady state in size after one week. The stagnating size can be explained by the fact that these tumours were mainly agglomerations of single tumour cells depending on diffusion with very little angiogenesis. While a tumour grew in size, the centre of the tumour became necrotic. After a week there was a dynamic steady state between tumour cell replicating in the outer layers and cells dying off towards the centre. The histology of the tumours highlighted thick neoplastic layers surrounding a central necrosis (Figure 58). With the onset of the lithium chloride and selenotrisulphide treatment, a critical lymph concentration was rapidly achieved, and the tumours` size decreased quickly, with the thick cover of neoplastic cells changing into a thin, fragmented and irregular layer (Figure 60). Finally, the tumours disappeared, leaving behind a few clusters of necrotic cells with an inflammatory infiltration (Figure 62).

The combination of lithium chloride and selenotrisulphide was clearly bioavailable and potent, as subcutaneous lymph-concentrations were achieved that are lethal for JU77 mesothelioma cells. Treating the JU77 cells with single compounds, with dual combinations or with the triple combination indicated that using all three compounds was a mandatory requirement and this was confirmed *in vivo* for shrinking the tumours (Figure 52H, 53H). A dual combination of selenite and selenomethionine delayed tumour growth but did not destroy the tumour (Figure 52G, 53G). All other single and dual treatments were statistically indistinguishable from the vehicle (Table 47). The triple combination was highly selective to cancer cells because normal tissues like liver, lung, gut spleen or skin that were exposed to the same lymph-concencentration of lithium chloride and selenotrisulphides were not destroyed. In addition, no significant loss in weight of animals was observed with any of the individual or combinations of treatments (Table 48). The red and white cell count increased substantially, due to daily injections of insulin-like growth factor-1, but did not change further with compound treatment (Figure 56). The white cell count of a nude mouse is extremely high (80000/mm^3) in any case; perhaps this compensates for the missing adaptive immune system. Finally, some biochemical parameters changed about threefold above normal limit. Again this was due to insulin-like growth factor-1, and was not further changed with compound treatment (Figure 57A, B and C).

In an attempt to explore the biochemical mechanism of JU77 cell death, measuring the enzyme activity of arachidonic acid metabolism seemed to be an attractive avenue to gain further insight as it has been reported by (Schroeder et al.2007) that simultaneous inhibition of cyclooxygenases (COX) and lipoxygenases (LOX), both pathways of arachidonic metabolism, can lead to death of cancer cells. Depending on the cell line and the experimental conditions in the literature, COX-activity and -

expression were altered by lithium chloride (Bosetti et al. 2002) and selenomethionine (Cherukuri et al, 2005) while LOX-activity was blocked by selenite (Bjoernstedt et al. 1996). However, whilst various individual treatments with selenium, selenomethionine and lithium chlordie or combinations thereof induced a number of changes and trends in the expressions of prostaglandin E2 and leukotrienes C and B in JU77 cells in this study (Figures 49-51), it was difficult to detect any obvious and logic pattern of changes. Interestingly, and somewhat surprisingly, the patterns of expression of prostaglandin E2 and leukotrience B4 mirrored each other closely. It is apparent that the effect of the compounds on arachidonic acid metabolism, whilst of interest, is highly complex. It is clearly beyond the scope of this study to untangle the specific biochemical pathway(s) leading to the observed changes in arachidonic acid metabolism. Given the overlapping nature of most of the biochemical pathways involved in metabolism, and the highly diverse array of biochemical actions of each of the individual compounds, it will be an extraordinarily difficult task to define the primary pathways involved in the mechanism of action of the compound combinations used in this study.

Nevertheless, it is apparent that the combination of lithium chloride and selenotrisulphides is a new, potent treatment with very few side effects. However, the success of this treatment is conditional: tumourigenicity was achieved by supplementing insulin-like growth factor-1. If tumourigenicity is determined by the balance between growth factor and binding proteins it could also be accomplished by cleaving the binding proteins. Twelve species of these cleaving enzymes exist and each species contains many members. They are involved in cell adhesion, cell fusion, membrane shedding and proteolysis (Mitsui *et.al.* 2006) and called “A disintegrin and metalloproteinases (ADAMs)”. As an example, insulin-like growth factor binding protein 3 (IGFBP-3) is the main binding protein of insulin-like

growth factor-1 and can be cleaved by the membrane-anchored matrix metalloproteinase MMP-1 (Rajah *et.al.* 1996), MMP-2, MMP-3 (Fowlkes *et.al.* 1994), MMP-7 (Miyamoto *et.al.* 2004), the serum protease calpain (Berg, Bang and Carlsson-Skwirut 2007) and growth hormone dependent serum protease (Rutishauser *et.al.* 1993). This list may not be complete.

The regulation of expression and activity of enzymes which cleave insulin-like growth factor binding protein 3 has been further investigated: expression of MMP-7 is dependent on the formation of advanced glycated endproducts (McLennan *et.al.* 2007) and polyamines (Matters, Manni and Bond 2005). Expression of MMP-1 is under the influence of leukotriene D4 (Rajah *et.al.* 1996). Finally, the activity of matrix metalloproteinases and serum proteases is regulated by an orchestra of tissue inhibitors (Fowlkes *et.al.* 1995). The clinical relevance of expression, activity and cleavage of insulin-like growth factor binding proteins is still under investigation.

Further research could investigate compound combinations in a non-growth factor supplemented tumour model. In such a scenario, chemotherapy would have to correct the growth factor/binding protein ratio as well as targeting oncogenic cellular structures. Most of the chemotherapeutic compounds that reduce insulin-like growth factor-1 or enhance insulin-like growth factor binding protein-3 are available and of low toxicity.

Another strategy might be to divert attention to the host response. *In vivo*, an excess of insulin-like growth factor-1 down-regulates apoptosis of granulocytes and maintains ongoing inflammation. This disables responses of the innate immune system (Rossi *et.al.* 2007). Surprisingly, exposing the innate immune system to certain microbial components like mycoplasma or chlamydia or streptococci or heliobacter has a similar effect as an excess of insulin-like growth factor-1. These infections are known to be highly

correlated to different cancers (Huang, *et.al.* 2001). The connection between exposure to microbial components and increased signalling of the insulin-like growth factor-1 receptor was independently investigated by Francois and Into in 2005. They observed that certain microbial components activate Toll receptor pathways which merge with insulin-like growth factor-1 receptor phosphorylation cascades: Francois *et.al.* illustrated in 2005 that Toll receptors activate phosphatidyl inositol 3-kinase/AKT and NF-kappa B. Into and Shibata noted in 2005 that mycoplasmal lipoproteins and staphylococcal peptidoglycans induce apoptosis signal-regulating kinase 1 (ASK1), which mediates sustained activation of the p38 mitogen-activated protein kinase. Constitutively activated Toll receptor signalling and continuous insulin-like growth factor-1 supplementation seem to exert similar immune response changes. Therefore, it might be of interest to investigate the combined inhibition of Toll receptor activation and binding protein cleavage with trisulphides on cancer cells.

Now the circle seems to close. In this thesis combinations of chemotherapeutic compounds were studied that target constitutively active signalling pathways in cancer cells. A mixture of selenite and selenomethionine was discovered to be effective against four different cancer cell lines. While trialling this compound combination *in vivo*, it became apparent that tumour onset and growth switches with supplementation of insulin-like growth factor-1 which may disable the hosts` immune response against cancerous signalling. Future research could investigate if the compound combination containing lithium chloride, selenite and selenomethionine is able to destroy *in vivo* mesothelioma tumours that are grown without injection of growth factors. If not, it will be of great interest to establish whether a combined inhibition of insulin-like growth factor-1 and the aberrant signaling pathways will have the desired

impact on these tumours. The reduction of insulin-like growth factor-1 concentrations could be realised by several means: growth hormone releasing hormone inhibitors reduce growth hormone and insulin-like-growth factor-1 release. They are known to induce apoptosis in many cancers (Rekasi *et.al.* 2005, Szepeshazi *et.al.* 2000). A monoclonal antibody against insulin-like growth factor-1 receptor is available and could inhibit the binding of insulin-like growth factor-1 to the receptor (Scotlandi *et.al.*1998). Applying insulin-like growth factor binding protein 3 could reduce the freely available amount of insulin-like growth factor-1 in serum and is known to induce apoptosis in many cancers (Kim *et.al.* 2004). The options to inhibit simultaneously tumourigenicity and aberrant signalling *in vivo* are manyfold. Further observations from this kind of research may indicate how the default mechanism in cancer may best be targeted in a combined fashion. It seems to me as if this combined approach of controlling endocrine "loops" and/or feedback to the pituitary/hypothalamus together with aberrant cancerous signaling is the roadmap for further research into how it may be possible to achieve the goal of a cancer cure.

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Appendix 1

Compounds	Supplier
17-Aageldanamycin	Sigma Australia
Adrenaline	Point Walter Pharmacy
8Cl-cAMP	Merck Colchester UK
Ascorbate	Dr.W.Barnes
Aspirin	Point Walter Pharmacy
ATP	Sigma Australia
Avandia	Dr.W.Barnes
Bromelin	Sigma Australia
Bromocriptin	Point Walter Pharmacy
tert-Butylbenzoicacid	Sigma Australia
Caesium chloride	Sigma Australia
Chloramphenicol	Biochemistry Curtin
Ciprobay	Point Walter Pharmacy
Citrate	Pharmacy Curtin
Clindamycin	Point Walter Pharmacy
Curcumin	Sigma Australia
Cyproterone acetate	Sigma Australia
2-Deoxyglucose	Biomed.Curtin
Dexamethasone	Biomed Curtin
DHEA	Dr.W.Barnes
DHLA(Trisulphide)	Dr.W.Barnes
Dichloroacetate	Sigma Australia
Dicumarol	Sigma Australia
Diphenyleneiodonium	Sigma Australia
Diethylmaleate	Sigma Australia
Digoxin	Point Walter Pharmacy
Doxycyclin	Point Walter Pharmacy
DMSO	Biomed.Curtin
EGCG	Dr.W.Barnes
Emodin	Dr.W.Barnes
Epichlorohydrin	Sigma Australia
Equol	Sigma Australia
Estradiol	Biomed.Curtin
p-Ethylphenol	Sigma Australia
Forskolin	Merck Colchester UK
Gadolinium chloride	Sigma Australia
Geftinib	Biaffin Kassel Germany
Geldanamycin	Sigma Australia
Genistein	Sigma Australia
Glibenclamid	Point Walter Pharmacy
Glutathione red.	Sigma Australia
Glycerol	Biochemistry Curtin
Glyceryltrinitrate	Point Walter Pharmacy
Haloperidol	Point Walter Pharmacy
Hesperidin	Dr.W.Barnes
Hesperitin	Dr.W.Barnes
1,6-Hexabromocriptin	Sigma Australia
	Sigma Australia

Compounds	Supplier
IBMX	Sigma Australia
Ibuprufen	Point Walter Pharmacy
Iodoacetate	Sigma Australia
β-Lapachone	Sigma Australia
Letrozole	Point Walter Pharmacy
Lithium chloride	Biochemistry Curtin
α-Lipoic acid	Dr.W.Barnes
LY294002	Biaffin Kassel Germany
Magnesium chloride	Biomed.Curtin
Menadione	Sigma Australia
Mercaptoethanol	Pharmacy Curtin
Metformin	Biomed.Curtin
Methylene blue	Pharmacy Curtin
Metoprolol	Point Walter Pharmacy
Mifepristone	Sigma Australia
NADH	Sigma Australia
NADPH	Sigma Australia
Naphtalene	Sigma Australia
Oxamate	Sigma Australia
Oxythiamine	Sigma Australia
Oxytocin	Point Walter Pharmacy
Parthenolide	Dr.W.Barnes
PD153035	Biaffin Kassel Germany
PD98058	Biaffin Kassel Germany
Penicillin/Streptomyc	Invitrogen Australi
Picropodophyllin	Biaffin Kassel Germany
PP2	Biaffin Kassel Germany
Progesteron	Biomed.Curtin
Quercetin	Dr.W.Barnes
Rapamycin	Biaffin Kassel Germany
Red Clover	Dr.W.Barnes
Salbutamol	Point Walter Pharmacy
Salicylic acid	Biochemistry Curtin
Selenate	Biochemistry Curtin
Selenite	Sigma Australia
Selenomethionine	Sigma Australia
Se-piccolinate	Dr.W.Barnes
Sulphamethoxazole	Point Walter Pharmacy
Sulphosalazine	Point Walter Pharmacy
Suramin	Pharmacy Curtin
Staurosporine	Biaffin Kassel Germany
Tamoxifen	Point Walter Pharmacy
Tautomycin	Biaffin Kassel Germany
Tetrathiomolybdate	Dr.W.Barnes
Theophylline	Point Walter Pharmacy
Thiosulphate	Biochemistry Curtin
Trimethoprime	Point Walter Pharmacy
Trypsin	Biomed.Curtin
Verapamil	Point Walter Pharmacy
Warfarin	Point Walter Pharmacy
Wortmannin	Biaffin Kassel Germany

Appendix 2

Table of Compounds

Compounds	MW	Solubility aequous	aqueousConc.range upper/lower limit	
17-Aageldanamycin	585.1	slightly	200nM	20nM
Adrenaline	183.1	100mg/L	1µM	10nM
8Cl-cAMP	363.7	soluble	200µM	2µM
Ascorbate	176.1	620g/L	50mM	500µM
Aspirin	180.2	10g/L	50µM	1µM
ATP	573.1	soluble	1mM	100µM
Avandia	473.5	soluble	10µM	1µM
Bromelin	33000.1	soluble	10µM	10nM
Bromocriptin	654.6	2.4mg/L	1nM	100pM
tert-Butylbenzoicacid	178.1	70mg/L	30µM	3µM
Caesium chloride	168.7	300g/L	1mM	100µM
Chloramphenicol	323.1	slightly	10µM	1µM
Ciprobay	331.4	16.5mg/L	50µM	5µM
Citrate	294.1	6g/L	50mM	1mM
Clindamycin	551.4	30mg/L	50µM	5µm
Curcumin	368.3	30ng/L	200µM	20µM
2-Deoxyglucose	164.2	131g/L	1mM	100µM
Dexamethasone	516.4	insoluble	10µM	1µM
DHEA	288.1	30mg/L	1µM	100nM
DHLA(Trisulphide)	228.3	slightly	1mM	10µM
Dichloroacetate	150.9	slightly	2mM	200µM
Dicumarol	336.3	128mg/L	25µM	5µM
Diphenyleneiodonium	314.6	slightly	10µM	1µM
Diethylmaleate	172.1	slightly	200µM	20µM
Digoxin	780.9	slightly	20nM	2nM
Doxycyclin	444.4	1.8mg/L	4µM	1µM
DMSO	78.1	soluble	250mM	1mM
EGCG	458.2	5g/L	100µM	1µM
Emodin	270.2	slightly	200µM	20µM
Epichlorohydrin	92.1	65g/L	500µM	5µM
Equol	242.2	slightly	400µM	10µm
Estradiol	272.4	slightly	10nM	1pM
p-Ethylphenol	122.2	slightly	100µM	1µM
Forskolin	410.5	10mg/L	500µM	10µM
Gadolinium chloride	263.2	soluble	500µM	50µM
Geftinib	446.9	slightly	2µM	0.5µM
Geldanamycin	560.6	slightly	20nM	2nM
Genistein	270.2	miscible	500µM	50µM
Glibenclamid	494.1	4mg/L	50µM	1µM
Glutathione red.	307.3	soluble	5mM	50µM
Glycerol	92.1	soluble	1mM	10µM
Glyceryltrinitrate	227.1	1g/L	100µM	10µM
Haloperidol	530.1	Soluble	10µM	100nM
Hesperidin	610.1	Slightly	1mM	100µM
Hesperitin	302.3	Slightly	1mM	100µM
1,6-Hexabromocriptin	734.8	Slightly	100µM	10µM
H2O2	38.1	Soluble	10mM	100µM

Compounds	MW	Solubility Aqueous	aqueousConc.range upper/lower limit	
IBMX	222.2	Slightly	1mM	500μM
Ibuprufen	206.3	Slightly	1μM	200nM
Iodoacetate	207.1	103g/L	100μM	10μM
β-Lapachone	242.1	Slightly	100μM	1μM
Letrozole	285.3	41mg/L	10μM	100nM
Lithium chloride	42	630.7g/L	10mM	100μM
α-Lipoic acid	228.3	Slightly	10mM	100μM
LY294002	343.8	Slightly	40μM	10μM
Magnesium chloride	203.3	542g/L	40mM	4mM
Menadione	276.2	Slightly	50μM	0.5μM
Mercaptoethanol	78.1	Miscible	100μM	10μM
Metformin	129.2	Soluble	100μM	10μM
Methylene blue	373.1	35.5g/L	100μM	10μM
Metoprolol	684.1	soluble	100μM	10μM
Mifepristone	429.6	slightly	100μM	1μM
NADH	709.4	soluble	1mM	10μM
NADPH	833.4	soluble	1mM	10μM
Naphtalene	128.1	30mg/L	100μM	10μM
Oxamate	111.2	50g/L	10mM	100μM
Oxythiamine	338.3	soluble	1μM	100nM
Oxytocin	1007.19	soluble	500nM	10nM
Parthenolide	248.3	slightly	2μM	20nM
PD153035	396.7	slightly	10μM	100nM
PD98058	267.3	slightly	40μM	10μM
Picropodophyllin	414.4	slightly	10μM	1μM
PP2	553.6	slightly	10μM	100nM
Progesteron	314.5	6mg/L	10nM	1pM
Quercetin	302.2	slightly	500μM	50μM
Rapamycin	914.2	6mg/L	200nM	20nM
Red Clover	N/A	slightly	N/A	N/A
Salbutamol	576.7	slightly	100μM	1μM
Salicylic acid	138.1	2g/L	100μM	10μM
Selenate	184.1	soluble	100μM	1μM
Selenite	172.9	85g/L	100μM	1μM
Selenomethionine	196.1	soluble	100μM	1μM
Se-piccolinate	N/A	slightly	N/A	N/A
Sulphamethoxazole	253.3	13.4g/L	200μM	5μM
Sulphosalazine	398.4	slightly	50μM	5μM
Suramin	1297.1	soluble	300μM	3μM
Staurosporine	466.5	slightly	10μM	10nM
Tamoxifen	371.5	500mg/L	10μM	1μM
Tautomycin	767.1	slightly	10μM	100nM
Tetrathiomolybdate	260.9	slightly	100μM	10μM
Theophylline	180.2	moderate	100μM	10μM
Thiosulphate	158.3	soluble	10mM	100μM
Trimethoprime	290.3	400mg/L	50μM	5μM
Trypsin	23800.1	soluble	10μM	10nM
Verapamil	496.2	soluble	50μM	2.5μM
Warfarin	308.1	1.7g/L	300μM	100μM
Wortmannin	428.4	miscible	100μM	1μM