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Jivan, Rupal

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CHARACTERISING THE ANTI–PROLIFERATIVE EFFECTS OF METFORMIN AND ASSESSING ITS EFFICACY IN COMBINATION CHEMOTHERAPY STRATEGIES *IN-VITRO* FOR THE TREATMENT OF OESOPHAGEAL SQUAMOUS CELL CARCINOMA

Rupal Jivan

Supervisor: Demetra Mavri-Damelin

Co-Supervisor: Leonard H. Damelin

A Thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

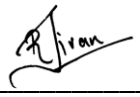
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Declaration

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“Sola dosis facit venenum

(The dose makes the poison)”

- Paracelsus (1493-1541)

Abstract

Oesophageal Squamous cell Carcinoma (OSCC) has a poor survival rate and is highly prevalent in southern Africa. Cisplatin is the standard therapeutic drug for OSCC, but has poor efficacy due to drug resistance and toxicity. Development of therapies that can be used to reduce the dose of cisplatin or offer a more effective tumour response is of great importance. Metformin is an anti-diabetic drug that has demonstrated anti-proliferative effects in various cancer types. Metformin's potential as a chemotherapeutic drug is highlighted by its low toxicity profile, ability to reduce growth factor signalling, and toxic effects against cancer stem cells.

In this study we combined metformin and cisplatin to find that whilst metformin reduced the proliferation of OSCC cell lines, it antagonised the effects of cisplatin. This was attributed to increased levels of reduced thiols as a consequence of enhanced glycolysis, which leads to the formation of reducing equivalents such as NADPH. Since metformin enhances the intracellular reducing potential, we combined metformin with drugs that are activated in reducing environments. Two copper bis(thiosemicarbazones), Cu-ATSM and Cu-GTSM, both retained their toxicity in the presence of metformin. Disulfiram (DSF), an established anti-alcoholism drug, has previously demonstrated chemotherapeutic potential when conjugated to copper (Cu-DSF). DSF and Cu-DSF both exerted potent cytotoxic effects against OSCC cell lines which were enhanced by metformin. Metformin increased intracellular copper accumulation when combined with DSF and we found that DSF perturbed proteasome function, as observed in other studies. Furthermore, we identified a novel target of DSF, the lysosome, and found that DSF reduces lysosomal pH, which led to increased accumulation of lysosomal protein aggregates, thereby inhibiting autophagy in OSCC cell lines.

Therefore, the co-prescription of metformin and cisplatin is not advised for OSCC treatment. However metformin can be effectively combined with DSF, which inhibits multiple protein degradation pathways, to offer a novel treatment option for OSCC.

Research Outputs

Original Publications:

Damelin, L. H.†, Jivan, R.†, Rousseau, A., Veale, R. B., Mavri-Damelin, D. (2014) Metformin induces and intracellular reductive state that protects oesophageal squamous cell carcinoma cells against cisplatin but not copper-bis(thiosemicarbazones). *BMC Cancer*, 14(314), doi:10.1186/1471-2407-14-314

Jivan, R.†, Damelin, L.H.†, Birkhead, M., Rosseau, A.L., Veale, R.B., Mavri-Damelin, D. (2015) Disulfiram/Cu-Disulfiram damages multiple protein degradation and turnover pathways and cytotoxicity is enhanced by metformin in oesophageal squamous cell carcinoma cell lines. *Journal of Cellular Biochemistry*. 30. in press. DOI: 10.1002/jcb.25184.

† Equal contributors

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Poster Presentation: Jivan, R., Damelin, L. H., Veale, R. B., Mavri-Damelin, D. The Effects of Metformin on Oesophageal Squamous Cell Carcinoma Cell Proliferation and Combination Chemotherapy. AORTIC International Cancer Conference, Durban, South Africa, 2013.

Oral Presentation: Jivan, R., Damelin, L. H., Veale, R. B., Mavri-Damelin, D. Metformin Negatively Alters the Response of Oesophageal Squamous Cell Carcinoma Cells to Cisplatin by Reducing DNA Adduct Formation. Young Researchers Forum by the South African Society for Human Genetics, Johannesburg, South Africa, 2013

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Internal Conferences:

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Poster Presentation: Jivan, R., Damelin, L. H., Veale, R. B., Mavri-Damelin, D. Metformin Negatively Alters the Response of Oesophageal Squamous Cell Carcinoma Cells to Cisplatin by Reducing DNA Adduct Formation. 5th Cross Faculty Graduate Symposium, Johannesburg, 2013.

Poster Presentation: Jivan, R., Mavri-Damelin, D. Synergistic Effects of Metformin and Cisplatin on Oesophageal Squamous Cell Carcinoma Cells. 4th Cross Faculty Graduate Symposium, Johannesburg, 2012.

Poster Presentation: Jivan, R., Mavri-Damelin, D. Synergistic Effects of Metformin and Cisplatin on Oesophageal Squamous Cell Carcinoma Cells. Molecular Biosciences Research Thrust Postgraduate Day, Johannesburg, 2012.

Oral Presentation: Jivan, R., Mavri-Damelin, D. The effects of metformin directly and its synergistic effects with cisplatin on oesophageal squamous cell carcinoma (OSCC) cell lines. Molecular Biosciences Research Thrust (MBRT) Postgraduate Day, Johannesburg, 2011

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Guru Brahma Gurur Vishnu
Guru Devo Maheshwaraha
Guru Saaksha Para Brahma
Tasmai Shree Gurave Namaha

Translation: A Guru/teacher is a representative of the Divine. He creates, sustains knowledge and destroys the weeds of ignorance. I salute all Gurus/teachers.

It is with great sincerity and humbleness that I quote the above ancient Vedic Hymn, in order to express my gratitude toward each and every one of my teachers who have all played an important role in bringing me to this stage of personal development that I currently am.

Firstly, I would like to acknowledge my entire family who have raised me and moulded me into the person I am. I am most grateful to my mother, Maya Jivan, who is not only my first teacher but my confidante and support structure. My mother has invested in my future and my development as a human being; she has played the role of two parents and is a pillar of strength for our entire family. I cannot fully express my gratitude in words. I am also very grateful to my sister, Harshna Jivan, for being my best friend and for giving me support when I needed it the most.

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"No country can really develop unless its citizens are educated"

- Nelson Mandela

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List of Abbreviations

4E-BP	-	Eukaryotic translation initiation factor 4E binding protein-1
6PGD	-	6-phosphogluconate dehydrogenase
6PGL	-	6-phosphoglucono-δ-lactone
ADP	-	Adenosine diphosphate
Akt/PKB	-	Protein kinase B
AMP	-	Adenosine monophosphate
AMPK	-	AMP activated protein kinase
AO	-	Acridine orange
APS	-	Ammonium persulfate
ATP	-	Adenosine triphosphate
ATSM	-	Diacetyl-bis(4-methyl-3-thiosemicarbazone)
BCS	-	Bathocuproine disulfonic acid
BSA	-	Bovine serum albumin
BSO	-	Buthionine sulfoximine
CaMKK	-	Calcium/calmodulin dependent protein kinase kinase
cAMP	-	Cyclic AMP
CDDP	-	Cisplatin or cis-diamminedichloroplatinum(II)
CDK	-	Cyclin dependent kinase
CQ	-	Chloroquine
Ctr1	-	Copper transporter 1
Cu(DDC)2	-	Bis(diethyldithiocarbamato) copper
Cu-8HQ	-	Copper-8-hydroxyquinoline
Cu-ATSM	-	Diacetyl-bis(4-methylthiosemicarbazonato) copper(II)
Cu-DSF	-	Copper-Disulfiram complex
Cu-GTSM	-	Glyoxal-bis(4-methylthiosemicarbazonato) copper(II)
DDC	-	Diethyldithiocarbamate
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	Dimethyl sulfoxide
DPTD	-	Dipyrrolidine thiuram disulfide
DSF	-	Disulfiram or tetraethylthiuram disulfide
EDTA	-	Ethylenediaminetetraacetic acid
EGFR	-	Epidermal growth factor receptor
EM	-	Electron Microscopy

FAS	-	Fatty acid synthase
FCS	-	Foetal calf serum
FDA	-	US Food and Drug Administration
G6P	-	Glucose-6-phosphate
G6PD	-	Glucose-6-phosphate dehydrogenase
Glut4	-	Glucose transporter 4
GPx	-	Glutathione peroxidase
GSH	-	Reduced glutathione
GSSG	-	Oxidised glutathione
GS-X	-	Glutathione S-conjugate pump
GTP	-	Guanosine triphosphate
GTPase	-	Guanosine triphosphate hydrolase
GTSM	-	Glyoxal bis(thiosemicarbazone)
GβL	-	G-protein β-subunit-like protein
HER2	-	Human epidermal growth factor receptor 2
ICP-MS	-	Inductively coupled plasma mass spectrometry
IRS	-	Insulin receptor substrate
LC3-B	-	Microtubule-associated protein light chain 3 beta
LC50	-	Lethal concentration required for 50% cell death
LKB1	-	Liver kinase B1
Me-DDC	-	Diethyldithiomethylcarbamate
Met	-	Metformin
Met-DTC	-	Diethylthiomethylcarbamate
mTOR	-	Mammalian target of rapamycin
mTORC1	-	Mammalian target of rapamycin complex 1
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NAC	-	N-acetyl-L-cysteine
NAD+	-	Nicotinamide adenine dinucleotide (Oxidised)
NADH	-	Nicotinamide adenine dinucleotide (Reduced)
NADP+	-	Nicotinamide adenine dinucleotide phosphate (Oxidised)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (Reduced)
NER	-	Nucleotide excision repair
OCT	-	Organic cation transporter
OSCC	-	Oesophageal Squamous Cell Carcinoma
p70S6K	-	70kDa ribosomal protein S6 kinase
PBS	-	Phosphate buffered saline

PDK1	-	Phophoinositide-dependent protein kinase-1
PE	-	Phosphatidylethanolamine
PET	-	Positron emmission tomography
PFKFB3	-	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PI	-	Propidium iodide
PI3K	-	Phosphatidylinositol 3-kinase
PIP2	-	Phosphatidylinositol 4,5-bisphosphate
PIP3	-	Phosphatidylinositol (3,4,5)-trisphosphate
PPP	-	Pentose phosphate pathway
PRAS40	-	Proline-rich Akt substrate 40 kDa
PTEN	-	Phosphatase and tensin homologue
Rag	-	Ras-related GTP binding protein
REDD1	-	Regulated in development and DNA damage responses 1
Rheb	-	Ras homologue enriched in brain
RIPA	-	Radioimmunoprecipitation buffer
ROS	-	Reactive oxygen species
Ru5PI	-	Ribulose-5-phosphate
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electropheresis
Suc-LLVY-AMC	-	Succinyl-Leu-Leu-Val-Tyr-amido methylcoumarin
TAK1	-	TGFβ activated kinase 1
TBS	-	Tris buffered saline
TCA	-	Tricarboxylic acid
TEMED	-	Tetramethylenediamine
TSC	-	Tuberous sclerosis complex
TTD	-	Tetrapropyl thiuram disulfide
Ulk1	-	Unc-51-like kinase
VEGF	-	Vascular endothelial growth factor

Chapter 1: General Introduction and Overview

1.1. A Brief History of Cancer

Many people believe that cancer is as old as the human race. However, paleopathological evidence has shown formation of haemangioma in the bones of dinosaurs and early mammals, which indicates that cancer actually predates the human race (Haddow, 1936). The first medical description of cancer can be traced back to Egypt in 3000 BC, where a case of breast cancer was described on the Edwin Smith Papyrus (Mould, 2008). Hippocrates (460-370 BC) first used the word *carcinos* or *carcinoma* to describe cancer, since the projections of a metastatic tumour which he observed resembled that of a crab. These words were later translated to the Latin word for crab, *cancer* by the Roman physician, Celcus (25-50 BC) (Hajdu, 2011).

By the 18th century, scientists had begun performing autopsies, which allowed them to diagnose and differentiate between cancers that could be removed by surgery and those that had metastasized. In the 19th century, development of microscopy allowed scientists to better examine cancers and ensure that they were completely removed during surgery (Hajdu, 2011). In 1971, US President Richard Nixon signed the National Cancer Act, and declared a war on cancer. The hype about cancer at the time convinced society to invest their money in cancer research, and more government funding meant that cancer research could erupt. However, people had the false notion that cancer could be cured in a matter of 5 years and very soon after the act was passed, people demanded answers.

In the 1970's, technology was not adequate enough to allow for early detection of cancer. Research soon shifted toward identifying the causes of cancer and more emphasis was placed on cancer prevention. The identification of carcinogens such as tobacco and UV radiation raised public awareness campaigns that helped to reduce the onset of cancer. In developed countries, policies were created to increase taxes on cigarettes, which led to an eventual decline in cancer caused by cigarette smoke. Due to a decline in sales, cigarette companies were forced to target foreign markets. As such, less developed countries with fewer restrictions on tobacco policies have now become victim to these tobacco giants (Doku, 2010).

Cancer is one of the leading causes of disease-related death worldwide. Although diseases such as HIV, pneumonia and tuberculosis are more frequently diagnosed than cancer in less developed countries, cancer-related mortality still prevails in more developed regions. This indicates that once healthcare and awareness are improved in less developed countries, and deaths due to non-communicable diseases such HIV and tuberculosis are reduced, cancer will still pose a severe threat. Factors such as genetic predisposition, nutrition and diet further highlight the importance of region-specific cancer research. Improving research and technology will bring humanity closer to finding a solution to this age-old disease.

1.2. Oesophageal Squamous Cell Carcinoma

1.2.1. Oesophageal Squamous Cell Carcinoma in Southern Africa

There are two major histological subtypes of oesophageal cancer, adenocarcinoma and squamous cell carcinoma, which differ in their aetiology and epidemiology (Pickens & Orringer, 2003). Risk factors associated with adenocarcinoma include Barrett's oesophagus and gastroesophageal reflux. Oesophageal squamous cell carcinoma (OSCC) is associated with excessive tobacco and alcohol consumption, low nutrient intake and DNA methylation induced by nitrosamines originating from dietary nitrates and nitrites (Stoner & Gupta, 2001).

Adenocarcinoma is more prevalent in developed regions, whereas OSCC generally occurs more frequently in developing regions, with the exception of China. The frequency of OSCC in sub-Saharan Africa is the second highest in the world, where age standardised incidence rates are 6.4 per 100 000 in men and 4.6 per 100 000 in women (Arnold et al., 2015) (Figure 1.1). According to the South African National Cancer Registry, there is a higher frequency of oesophageal cancer in black people compared to other races, which suggests that there may be a genetic predisposition (National Health Laboratory Service, 2008). Studies in Asian populations suggest that genetic polymorphisms do influence OSCC occurrence (Kuroki et al., 2002; Hao et al., 2004). Genome sequencing in an African cohort would be required to determine whether genetic factors correlate with increased incidence of OSCC in African populations.

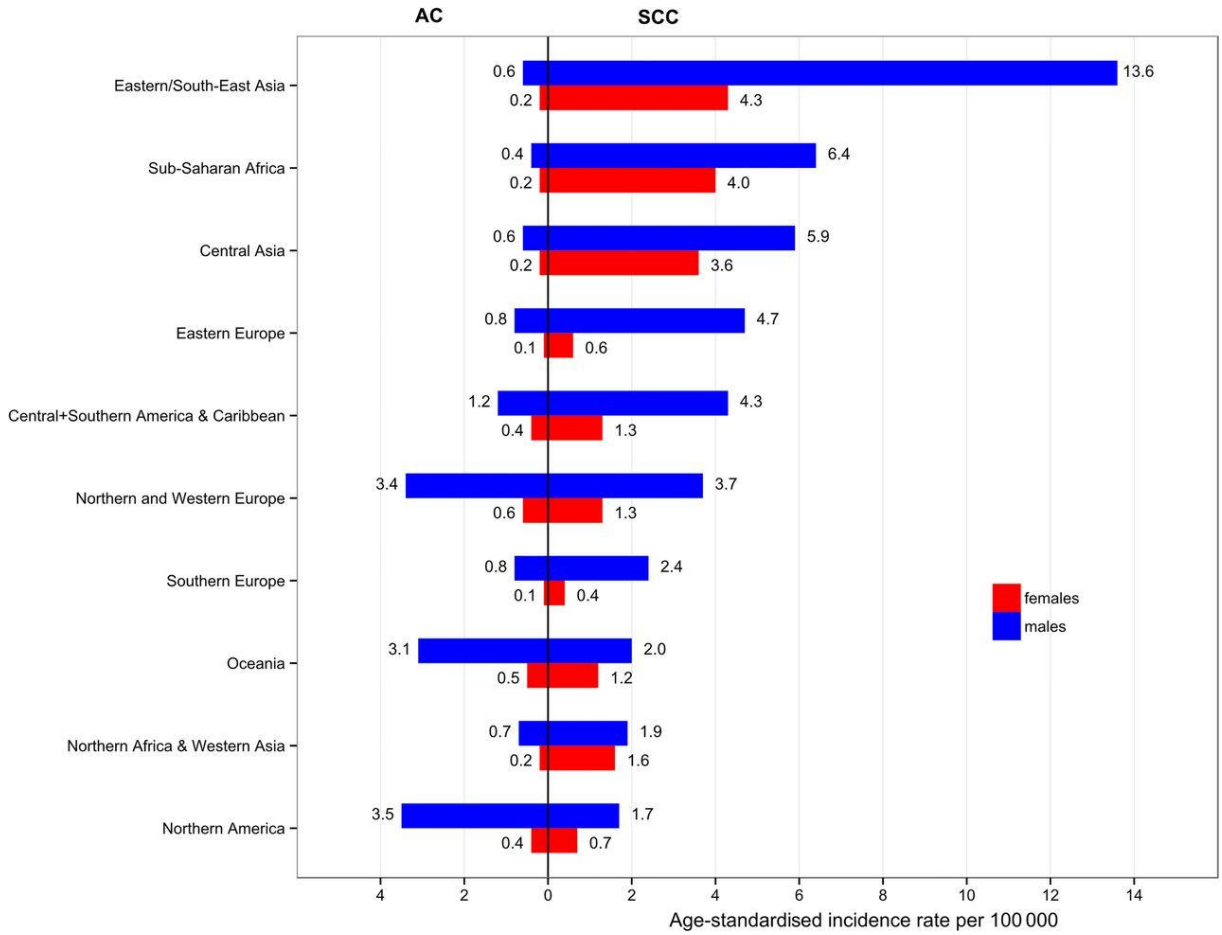


Figure 1.1: Age-standardized incidence rates for oesophageal cancer based on region, subtype and sex. Statistics based on those collected from the International Agency for Research on Cancer (IARC) show that the incidence rate of oesophageal squamous cell carcinoma in sub-Saharan Africa is the second highest in the world. Of the two subtypes squamous cell carcinoma (SCC) is more prevalent than adenocarcinoma (AC) in sub-Saharan Africa. The frequency of oesophageal cancer is also higher in males compared to females. (Arnold et al., 2015)

Another motivating factor for OSCC research in South Africa is the fact that mortality rates are particularly high for this cancer. Based on information obtained from the interactive IARC global cancer statistics website, there are approximately 3868 new cases of oesophageal cancer and 3557 deaths due to oesophageal cancer in South Africa per annum (Ferlay et al., 2013). These staggeringly high mortality rates may be attributed to late detection and a lack of cost effective treatment options. This further emphasises the need for improved treatment options that are affordable in less developed countries.

1.2.3. Stages of Oesophageal Squamous Cell Carcinoma

Treatment of an OSCC patient is decided based on the stage at which the cancer is detected. There are five stages by which oesophageal cancer is graded (Figure 1.2). Differences between these stages are characterised by the depth of invasion, the degree of lymph node metastasis and the degree of differentiation.

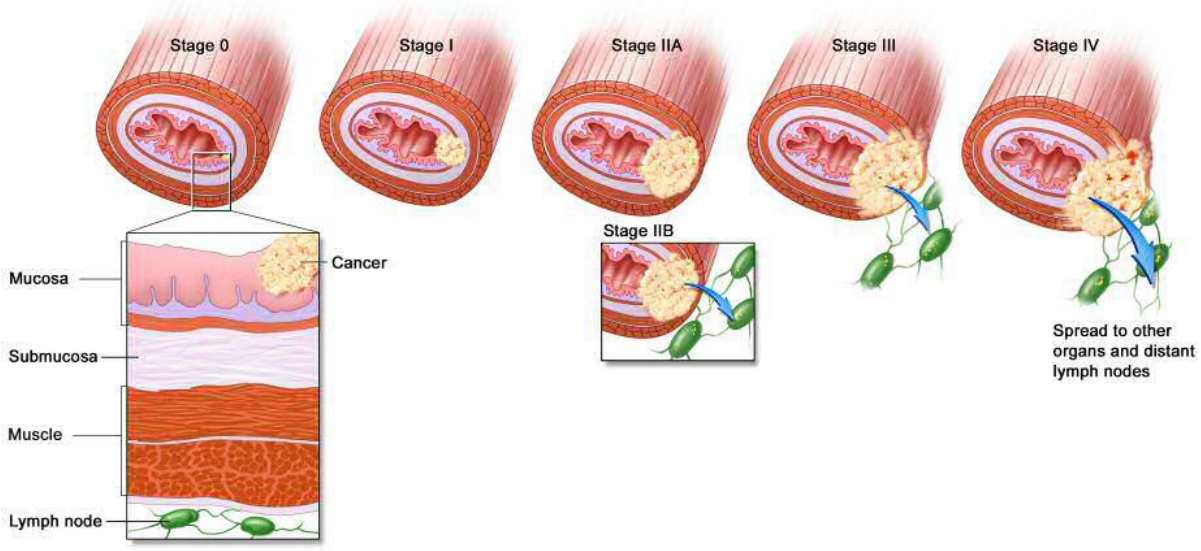


Figure 1.2: Stages of Oesophageal Cancer. Stage 0, a high grade dysplasia is present, but does not spread beyond the mucosa. Stage I – the tumour invades the lamina propia and penetrates the submucosa. Stage IIA – the tumour invades the muscle and adventitia layers but there are no signs of lymph node metastasis. Stage IIB – the tumour invades muscle and adventitia layers and metastasis is detected in 1-2 regional lymph nodes. Stage III – the tumour has invaded neighbouring structures and metastasis can be detected in 3-6 neighbouring lymph nodes. Stage IV – the tumour has invaded adjacent structures and metastasized to lymph nodes and distant organs. (Bhaijee & Akhtar, 2013)

Chemotherapeutic drugs most commonly prescribed for OSCC in South Africa include cisplatin, 5-fluorouracil and mitomycin C. These drugs are often combined with each other and with radiation and surgery to offer more potent effects against the cancer. At present, the primary treatment option for OSCC is surgical resection which is often combined with chemotherapy and radiotherapy to offer a more effective treatment, although the therapeutic approach used depends on the stage of the cancer. For stage 0 and stage I

lesions that do not penetrate into the submucosal layer, there is a slim chance of lymph node metastasis. In these cases the conventional and often curative therapy is endoscopic resection (Higuchi et al., 2009). An improved outcome was shown in a clinical trial where patients with stage 0 oesophageal cancer were treated with cisplatin and 5-fluorouracil combined with radiation compared to radiotherapy alone (Herskovic et al., 1992). However there are still debates about whether this is more successful than surgery. For stage I lesions that penetrate further into the submucosal layer, endoscopic resection is followed by chemoradiotherapy to prevent local relapses (Higuchi et al., 2009).

Due to post-operative complications, in stage II and stage III lesions that have not yet invaded neighbouring structures, pre-operative chemoradiotherapy is first applied in order to reduce tumour size followed by surgical resection (Higuchi et al., 2009). Pre-operative chemotherapy was compared to post-operative chemotherapy with cisplatin and 5-fluorouracil, and the former was shown to be more successful as overall survival rates were higher (Igaki et al., 2008). Post-surgical survival is poor for patients with stage III lymph node metastasis or stage IV distal metastatic cancer. For this reason, chemoradiotherapy without surgery is used to improve 5 year survival rates or as a palliative option. Three year survival rates were improved in stage III and stage IV OSCC patients receiving 5-FU combined with cisplatin and radiation (Ohtsu et al., 1999).

The importance of chemotherapeutic drugs for OSCC treatment is stressed by their frequent prescription in combination chemotherapy as well as in multi-modal therapies involving radiation and surgery. Cisplatin is a first-line drug used for OSCC treatment and was investigated in the present study. The mechanism of action of cisplatin in cancer and modes of resistance will be described further.

1.2.4. Cisplatin – Mode of Action, Resistance and Side Effects

Barnett Rosenburg discovered the anti-proliferative effects of cisplatin while investigating the effects of electrical current on *E. coli* growth in ammonium chloride buffer. *E. coli* were exposed to electrical currents from a platinum electrode and began to appear elongated and growth was stunted. The hydrolysis products of platinum in this buffer led to the formation of cisplatin (cis-diamminedichloroplatinum(II) complex), which was soon discovered to be the cause of altered morphology in *E. coli* (Alderden, Hall & Hambley, 2006). A method for the rapid synthesis of cisplatin was described in 1970 (Dhara, 1970) and cisplatin was approved for the treatment of cancer by the U.S. Food and Drug Administration (FDA) in 1978.

Cisplatin enters the cell either by passive diffusion or active transport (Figure 1.3). A linear correlation between cisplatin dose and accumulation suggested that the drug might enter the cell via passive diffusion. However, combinations of glycolysis and oxidative phosphorylation inhibitors that reduce ATP levels impaired cisplatin entry, which suggested that some of the drug enters the cell via energy dependent transport (Andrews et al., 1988). Transporters that have been implicated in cisplatin uptake include the copper transporter (Ctr1) and the organic cation transporters (OCTs) (Ishida et al., 2002; Ciarimboli et al., 2005). Knockout studies of transporters such as Ctr1 did not completely abolish the effects of cisplatin, suggesting that cisplatin enters the cell via multiple modes (Ishida et al., 2002).

Upon entry into a cancer cell, cisplatin causes DNA damage, which leads to apoptosis via the DNA damage response (Figure 1.3). Cisplatin is administered intravenously and, as the chloride ion concentration in the bloodstream is high, this prevents the displacement of cisplatin chloride ions. Since the intracellular chloride ion concentration is much lower, either one or both of the chloride ions are replaced with water upon cisplatin entry into cells (Alderden, Hall & Hambley, 2006). The hydrolysis products of cisplatin are highly reactive and this forms the basis for its anti-proliferative effects. Purine bases on DNA are particularly susceptible to cisplatin, which causes DNA damage by forming both intrastrand and interstrand DNA cross-links. Cisplatin DNA-adduct formation triggers the binding of DNA damage response proteins to DNA. If DNA damage is too extensive to repair, apoptosis is triggered via DNA damage response pathways (Siddik, 2003).

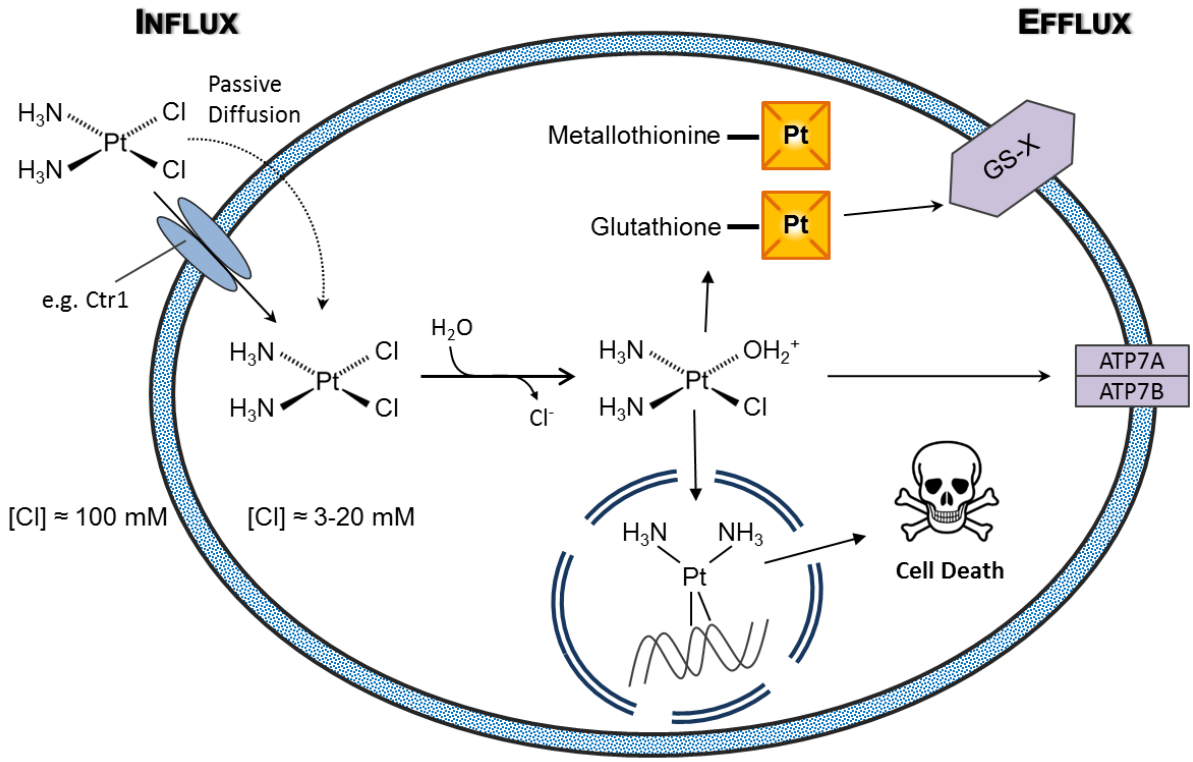


Figure 1.3: Cisplatin Mode of Entry, Action and Resistance. Cisplatin enters the cell by passive diffusion or active transport via transporters such as the copper transporter (Ctr1). Due to the lower intracellular chloride concentration, cisplatin is hydrolysed to its highly reactive form. It then reacts with DNA to form cross-links. This triggers apoptosis due to excessive DNA damage. Cisplatin also reacts with intracellular thiols such as glutathione and metallothionine and can be targeted for export by glutathione S-conjugate (GS-X) pumps. Cisplatin can also be directly removed from the cell by the active transporters, ATP7A and ATP7B.

Two major factors that contribute to cisplatin resistance are reduced formation of cisplatin DNA adducts and increased repair of DNA damage. Reduced cisplatin DNA binding can be caused by reduced drug accumulation and altered drug sequestration. Reduced intracellular accumulation of cisplatin can be caused by reduced influx and increased efflux of the drug. Mutations or altered expression of transporter proteins such as Ctr1, involved in cisplatin uptake, can lead to reduced drug uptake (Song et al., 2004). Extracellular pH and osmolarity may be involved in modulating cisplatin entry via passive diffusion (Andrews et al., 1987). The copper exporters, ATP7A and ATP7B are also involved in the export of cisplatin. Over-expression of ATP7A has been associated with resistance to cisplatin (Samimi et al., 2004).

Intracellular thiols such as glutathione and metallothionine can bind to cisplatin and sequester the drug in the cytoplasm. Glutathione, metallothionine and thiol-containing proteins bind to the central platinum atom of cisplatin, thus preventing the formation of cisplatin DNA-adducts. Elevated glutathione and metallothionine levels have been highly correlated with cisplatin resistance in numerous cancer types, including oesophageal cancer (Andrews, Murphy & Howell, 1987; Godwin et al., 1992; Hishikawa et al., 1997). Similarly, increased activity of the glutathione S-conjugate (GS-X) pump has been correlated with reduced cisplatin accumulation and increased glutathione levels, indicating that reduced drug accumulation is partly due to increased export via GS-X pumps (Kurokawa et al., 1995).

Nucleotide excision repair (NER) can confer resistance to cisplatin whereas DNA mismatch repair can increase cisplatin sensitivity. Cisplatin-DNA adducts can be removed by NER proteins and cells deficient in these proteins were shown to be more sensitive to cisplatin than their non-deficient counterparts (Lee et al., 1993). Additionally, enhanced expression of NER proteins have been detected in cisplatin-resistant tumour cell lines (Dabholkar et al., 1994). Reduced expression of mismatch repair proteins allows for DNA synthesis machinery to overlook cisplatin-DNA adducts and continue synthesising DNA; the down-regulation of mismatch repair genes has been observed in cell lines resistant to cisplatin (Aebi et al., 1996).

The Fanconi anaemia (FA) pathway has also been shown to play a significant role in the repair of DNA cross-links and alterations in this pathway can alter susceptibility to cisplatin (Romick-Rosendale et al., 2013). FA gene products form an FA complex that is involved in the repair of DNA interstrand cross-links (Deans & West, 2011). Numerous proteins that have been shown to increase breast cancer susceptibility, including the BRCA family, are also members of the FA family of proteins. Mutations in these genes leading to non-functional products promote cancer formation, but also improve sensitivity to DNA cross-linking agents such as cisplatin as these cell types are deficient in their ability to repair interstrand cross-links (Romick-Rosendale et al., 2013). However, cells that have fully functional FA gene products are more resistant to cisplatin (Siddik, 1998).

Another major factor contributing to poor cisplatin efficacy is the harsh side-effects of the drug. Common side effects of cisplatin include pain, mild hearing loss, swelling and general

weakness. Cisplatin resistance by tumour cells leads to the prescription of higher doses or prolonged chemotherapy in order to improve the anti-cancer effects. However, patients treated with high doses of cisplatin can suffer from severe nephrotoxicity. Cisplatin is administered intravenously, and apart from having more potent effects on proliferating cells, it is not a targeted treatment. The organic cation transporters have been associated with cisplatin uptake. Studies show that the OCT2 isoform, which is mainly expressed in the kidney, is involved in cisplatin uptake. As such, the expression of OCT2 in kidney cells is associated with cisplatin-mediated nephrotoxicity (Ciarimboli et al., 2005).

Cisplatin accumulation in renal cells results in numerous toxic events. It impairs renal excretion due to increased accumulation in tubules, alters the uptake and metabolism of substrates that are involved in regular kidney function, and causes increased calcium levels and mitochondrial swelling. This results in increased ROS levels which leads to necrotic cell death of kidney cells. Altogether, cisplatin induced nephrotoxicity alters the histology of kidneys and impairs renal function (Yao et al., 2007). This impedes the prescription of high doses of cisplatin for cancer therapy. Therefore, resistance to cisplatin and lack of drug specificity highlights the need for more targeted and less harmful therapies for OSCC.

1.3. Emerging Therapies for Oesophageal Squamous Cell Carcinoma

1.3.1. Potential Drugs for Oesophageal Cancer Treatment

The drugs currently prescribed for oesophageal cancer lack in their ability to effectively reduce the size of late stage tumours. There is a need for more effective chemotherapeutic options that can be combined with surgery for stage II and III tumours and drugs that offer better palliation for non-resectable tumours. Various new drugs are now being tested for the treatment of oesophageal cancer in pre-clinical studies as well as clinical trials. These include monoclonal antibodies, receptor tyrosine kinase inhibitors, angiogenesis inhibitors and drugs that target metabolic pathways.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase which has an extracellular ligand binding domain and an intracellular region containing a tyrosine kinase domain. When a ligand binds to the receptor, this results in dimerization and autophosphorylation of the intracellular kinase domain. Activation of EGFR triggers a signaling cascade that leads to cell survival, proliferation and angiogenesis. EGFR is upregulated in 71-88% of oesophageal cancers, including OSCC (Ozawa et al., 1987; Laskin & Sandler, 2004). Another member of the epidermal growth factor receptor family, HER2, has also been reported to be upregulated in 30% of OSCC cases and expression is associated with poor prognosis (Sato-Kuwabara et al., 2009; Zhan et al., 2012). HER2 is a binding partner for other members of the same family of growth factor receptors.

Monoclonal antibodies targeted at EGFR and HER2, as well as receptor tyrosine kinase inhibitors, are now being developed and tested for cancer therapy. Cetuximab is a monoclonal antibody targeted at EGFR. It is currently prescribed for the treatment of colorectal cancer and ongoing trials are assessing its use in combination with other drugs and radiation for metastatic colorectal cancer (Cutsem et al., 2009). Cetuximab did not have significant effect as a monotherapy for oesophageal cancer treatment, however phase II clinical trials for its use in combination with other drugs are ongoing (De Vita et al., 2011). Trastuzumab (Herceptin) is a monoclonal antibody targeted at HER2. Antibody-mediated apoptotic cell death occurred in OSCC cell lines with various degrees of HER2 expression

treated with Herceptin, and levels of cell death correlated with the degree of HER2 expression (Mimura, Kono, Hanawa, Kanzaki, et al., 2005). Early studies have shown that the combination of Cetuximab and Herceptin is effective against OSCC cell lines that over-express both EGFR and HER2. However, this did not correlate with receptor expression levels, suggesting that other mechanisms may be involved in the anti-proliferative effects of these drugs (Kawaguchi et al., 2007).

Monoclonal antibodies and tyrosine kinase inhibitors both target receptor tyrosine kinases in different ways. Whereas monoclonal antibodies bind to the extracellular domain and inhibit ligand binding, tyrosine kinase inhibitors prevent ATP binding to the intracellular domain to block catalytic activity of the receptor. The receptor tyrosine kinase inhibitors, Gefitinib and Erlotinib, have recently been tested in phase II clinical trials in combination with chemoradiotherapy for the treatment of oesophageal cancer. However, no significant benefit was shown with Gefitinib, and only partial benefit with Erlotinib (Rodriguez et al., 2010; Ilson et al., 2011). Another target for treatment is angiogenesis, the process required by tumours to sustain growth by modifying the surrounding vasculature. Vascular endothelial growth factor (VEGF) is frequently overexpressed in many cancers including oesophageal cancer. Bavacizumab is a monoclonal antibody targeted at VEGF, and acts to block angiogenesis. Some benefit was shown when Bevacizumab was combined with cisplatin and Irinotecan for the treatment of gastroesophageal junction adenocarcinoma (Shah et al., 2006). There is no evidence of the effects of this drug on OSCC.

Although many of the targeted therapies described above have shown some therapeutic benefit, the cost of these drugs make it near to impossible to implement in resource-poor countries where OSCC is prevalent, such as South Africa. HER2 is frequently over-expressed in breast cancer and Herceptin has been approved by the South African Oncology Consortium for the treatment of breast cancer. In 2006, a South African Attorney, Lisa Metzer won a case against Discovery for breach of contract. Discovery was liable to pay for a year's supply of Herceptin for 8 patients who were to be treated once every three weeks. The treatment cost was R450 000 per patient, and Discovery had argued that it was unfeasible to pay these high prices as they would have to increase monthly premiums of other insurers in order to cover the costs. Since then, medical aid schemes have changed their policies so that they require co-payment for Herceptin, which is unaffordable by most

people (Independent Online News - South Africa, 2006). It is therefore crucial to find more effective anti-cancer agents that are affordable in less developed countries such as South Africa.

Over the past few years, there has been growing interest in cancer cell metabolism and its potential as a target for cancer therapy. Fatty acid synthase (FAS) inhibitors have been considered for oesophageal cancer treatment due to the high expression of FAS in this cancer type (Nemoto et al., 2001). However, these drugs are still at the pre-clinical stage and more research is required before they can be developed into a prescribed drug. More recently, there has been heightened interest in the use of metformin for oesophageal cancer treatment. Metformin is an anti-diabetic drug with an established clinical profile. This drug is the topic of investigation for this project and will be described in detail.

1.3.2. Metformin – From Anti-Diabetic to Chemotherapeutic Drug

The use of metformin (dimethyl biguanide hydrochloride) can be traced as far back as medieval Europe, when *Galega officinalis* was used as a herbal medicine for the treatment of diabetes like symptoms. In the late 1800's, it was discovered that *G. officinalis* was rich in guanidine and in 1918 this compound was shown to reduce glucose levels in animals. However, due to the toxicity of guanidine, attention shifted to a second compound found in the plant, galegine (isoamylene guanidine) (Bailey & Day, 2004). Once the structure of galegine was determined, analogues of the compound were soon developed.

Metformin, a galegine analogue synthesised in Dublin in 1922, was shown to have glucose lowering effects in people and was much less toxic than guanidine and galegine (Shenfield, 2013). Due to the development of insulin for diabetes treatment, attention toward biguanide research waned, and it was not until the 1950's that research in this field continued. In 1957, Jean Sterne published research where he proposed using metformin for diabetes treatment under the name "Glucophage". At the same time, two analogues that were slightly more potent than metformin, phenformin and buformin, were also synthesised. But these were retracted by 1970 due to an increased risk of lactic acidosis and cardiac mortality (Bailey & Day, 2004). Metformin did not show severe side-effects like its counterparts, which led to commercialisation of the drug.

Metformin is now the first line drug prescribed for the treatment of type II diabetes and is also prescribed for insulin resistance and metabolic syndrome (American Diabetes Association, 2014). Common side effects of metformin include mild gastrointestinal upset such as diarrhoea, stomach pain and loss of appetite and rare side effects include breathing difficulties, cramps, skin problems and lactic acidosis (Datapharm, 2014). A meta-analysis showed that metformin-associated lactic acidosis mainly occurred in individuals suffering from renal impairment or those who had overdosed (Stades et al., 2004).

The glucose lowering effects of metformin have been attributed to reduced intestinal absorption of glucose, reduced hepatic gluconeogenesis and increased glucose uptake and utilization by muscle cells (Cusi, Consoli & DeFronzo, 1996; Hundal et al., 2000). Additionally, metformin causes reduced insulin secretion from pancreatic β-cells and may therefore reduce pancreatic insulin secretion (Leclerc et al., 2004). Diabetes is associated with a higher risk of cancer, which may be attributed to higher circulating glucose levels and increased expression of insulin and insulin-like growth factor receptors on the surface of cancer cells (Giovannucci et al., 2010).

In 2005, a role for metformin in cancer became evident when a case control study showed that metformin was associated with a lower risk of cancer in diabetic patients (Evans et al., 2005). It was also shown that diabetic patients suffering from breast cancer who were treated with metformin and neoadjuvant chemotherapy had higher pathological complete response rates than diabetics not treated with metformin and non-diabetic breast cancer patients, who also received neoadjuvant chemotherapy (Jiralerspong et al., 2009). Numerous cohort studies and randomized controlled trials have since shown that metformin can reduce the risk of breast, colorectal, liver, lung and pancreatic cancers (Emami Riedmaier et al., 2013). *In vitro* research has shown that metformin reduces the growth of numerous cancer cell lines including breast, ovarian and endometrial cancer and it induced apoptosis in pancreatic cancer cells (Gotlieb et al., 2008; Alimova et al., 2009; Kisfalvi et al., 2009). More interestingly, metformin was shown to target breast cancer stem cells that are frequently resistant to chemotherapy (Hirsch et al., 2009). Therefore metformin may be able to enhance the response of tumours to current chemotherapeutic agents by targeting a different cell population within the tumour microenvironment.

Due to its well-established tolerance profile, there are many clinical trials currently investigating the use of metformin for cancer therapy. Metformin as a monotherapy is being tested for the treatment of non-diabetic patients suffering from early stage cancer, including breast and endometrial cancer (NCT01101438 and NCT01205672). Metformin is being combined with other chemotherapeutic drugs and radiotherapy to treat metastatic cancer of the head and neck or lung (NCT01333852 and NCT02115464). Metformin is also being tested as a pre-surgical therapy for cancers including breast and prostate cancer (NCT00930579 and NCT01433913). Clinical trials for the use of metformin to treat oesophageal cancer are currently focused on adenocarcinoma as this is the more prevalent subtype in western countries (NCT01447927 and NCT01465113).

A meta-analysis of cohort studies testing the effects of metformin on cancer in diabetic patients showed that cancer-related mortality was reduced by at least 20%. However, randomised controlled trial data did not show a significant benefit of metformin (Noto et al., 2012). This indicates that the anti-proliferative effects of metformin may be dependent on cancer type, which highlights the importance of investigating the effects of metformin on each cancer type in order to determine whether it may or may not be useful.

Although metformin is widely prescribed for the treatment of diabetes and is now being tested for cancer therapy, a direct target of the drug has not been identified. Metformin is known to reduce systemic glucose and insulin levels. This alone however, cannot be the reason for its anti-cancer effects as other glucose lowering agents are not as effective as metformin against cancer in diabetic patients (Soranna et al., 2012). Studies have shown that metformin promotes phosphorylation and activation of the AMP-activated protein kinase (AMPK) (Zhou et al., 2001). This kinase plays a central role in metabolic regulation at the whole-body and cellular levels. The roles of metformin and AMPK in metabolic regulation in cancer will be discussed further.

1.4. Targeting Cancer Cell Metabolism

1.4.1. Altered Metabolism is an Emerging Hallmark of Cancer

Growing tumours are able to maintain enhanced cell proliferation and are able to survive nutrient deprivation and hypoxia. In order to maintain their phenotype, cancer cells must reprogram their metabolic activities in order to cope with the requirements of sustained proliferation in the absence of a regular nutrient and oxygen supply. In order to provide for the basic needs of rapidly proliferating cells, cancer cell metabolism is altered in a way that increases ATP generation, increases macromolecular biosynthesis and there is tightened maintenance of an altered redox state. As such, regulation of essential macromolecules such as carbohydrates, lipids, amino acids and nucleic acids is altered in cancer.

Altered metabolism in cancer was first described by Otto Warburg, who showed that cancer cells are more reliant on glycolysis for energy production than oxidative phosphorylation, even in the presence of oxygen (Warburg, 1956). Warburg proposed that cancer cells have mitochondrial defects that impair their ability to break down glucose by oxidative phosphorylation. However, more recent studies indicate that many cancer cell lines have functional mitochondria and still preferentially select glycolysis as opposed to oxidative phosphorylation for their metabolic demands (Moreno-Sánchez et al., 2007; Frezza & Gottlieb, 2009).

Glycolysis is a much less efficient form of energy production compared to oxidative phosphorylation. However, glycolytic degradation may better provide for the biosynthetic requirements of proliferating tumour cells by providing carbon based precursors required for nucleotide, lipid and protein synthesis. The high energy demands of proliferating cells may be compensated for by extremely high rates of glycolysis observed in cancer cells (DeBerardinis et al., 2008). As such, glycolysis is more efficient in catering for the needs of growing tumours than oxidative phosphorylation. This type of metabolic reprogramming is crucial for tumour progression, and is now regarded as an emerging hallmark of cancer (Hanahan & Weinberg, 2011).

Taking this into consideration, these deregulated metabolic pathways may serve as alternative targets for cancer chemotherapy. Therapies that have been used to target a

single rate-limiting step of these metabolic pathways, such as glucose mimetics or hexokinase inhibitors, have shown some benefit but were not cytotoxic. There is now a shift toward designing drugs or drug combinations that have multiple targets in a metabolic pathway to offer a more effective chemotherapeutic approach (Moreno-Sánchez et al., 2010).

1.4.2. Metformin and the AMPK Signaling Pathway

The adenosine monophosphate (AMP) activated protein kinase (AMPK) is an energy sensing molecule involved in metabolic regulation (Hardie, 2005). AMPK is involved in metabolic regulation as it activates pathways that produce energy and inhibits pathways that consume energy. AMPK phosphorylates numerous targets involved in carbohydrate, lipid and protein metabolism and also plays a role in the cell cycle by promoting senescence (Carling, 2004). A role for AMPK in cancer became apparent when studies identified LKB1 (liver kinase B1) as the major AMPK kinase (Hawley et al., 2003). LKB1 is a well-known tumour suppressor gene, inactivation of which leads to Peutz-Jeghers syndrome (Hemminki et al., 1998). Peutz-Jeghers syndrome is a disease that results in the onset of intestinal hamartomous polyps and is highly associated with the onset of colorectal and gastro-intestinal cancers later in life (Volikos et al., 2006). LKB1 may also be involved in familial predisposition to breast cancer (Chen & Lindblom, 2000). Two other less investigated kinases, CaMKK and TAK1, have also been shown to phosphorylate AMPK (Hurley et al., 2005; Momcilovic, Hong & Carlson, 2006).

In 2001, Zhou et al. first showed that metformin promoted the activation of AMPK (Zhou et al., 2001). The exact mechanism responsible for increased AMPK phosphorylation in response to metformin is yet to be determined (Rena, Pearson & Sakamoto, 2013). Some of the interactions between metformin and the AMPK signalling pathway are outlined in Figure 1.4. It is hypothesised that metformin promotes AMPK activation by increasing the AMP:ATP ratio. Metformin has been shown to inhibit complex I of the mitochondrial electron transport chain (El-Mir et al., 2000). This abrogates adequate proton gradient formation that is required to drive ATP synthesis by complex V, thus causing an increase in the AMP:ATP ratio. Complex I inhibition prevents the oxidation of NADH to NAD^+, which is required as a cofactor in the tricarboxylic acid (TCA) cycle. A reduction in TCA flux reduces mitochondrial

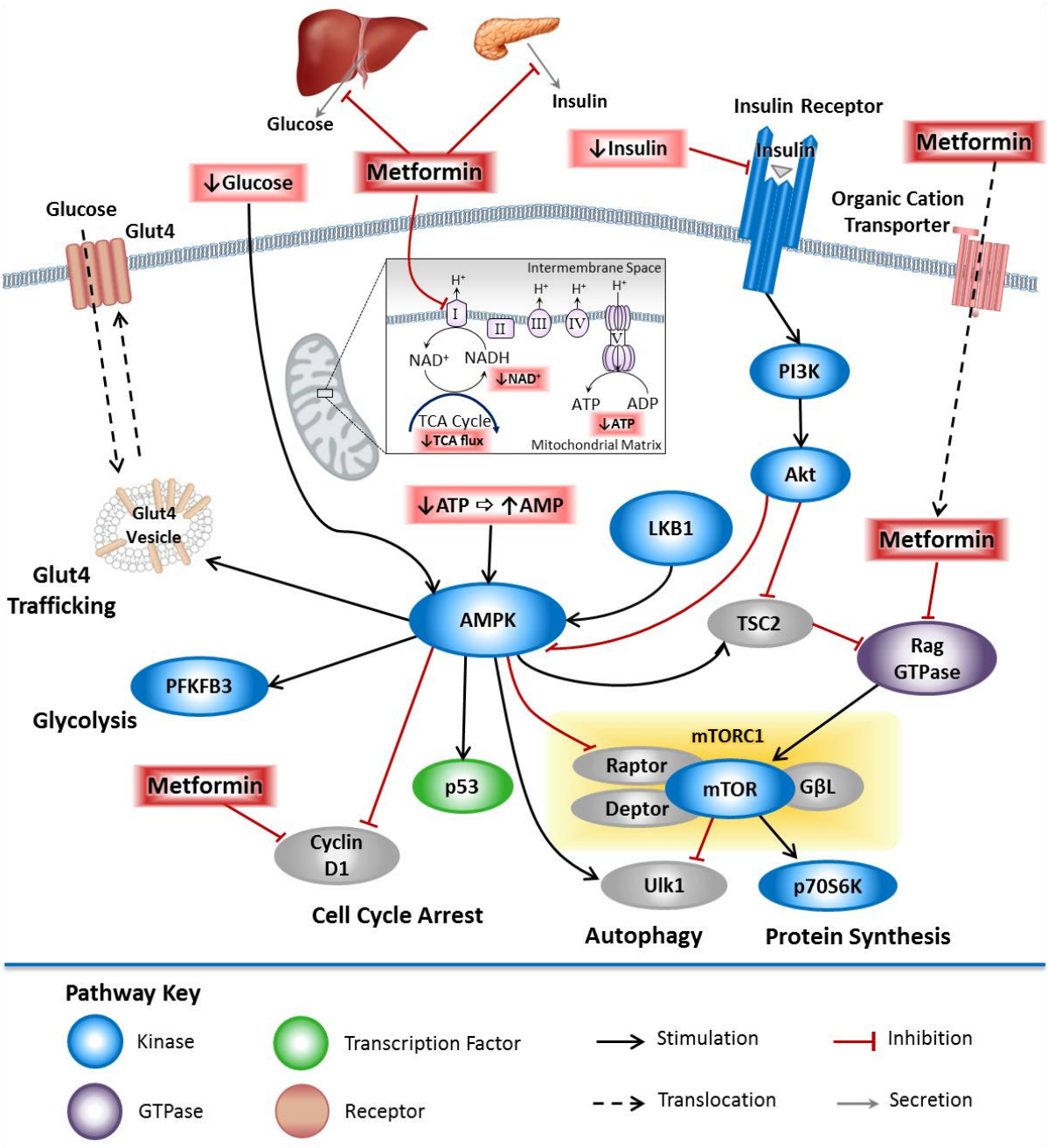


Figure 1.4: Metformin and its Interactions with the AMPK Signaling Pathway. Metformin exerts whole body effects by inhibiting hepatic glucose secretion and insulin secretion by pancreatic β cells. It exerts direct cellular effects upon entry via organic cation transporters. Metformin inhibits complex I of the mitochondrial respiratory chain which results in an increase in the AMP:ATP ratio, this promotes AMPK phosphorylation by an upstream kinase such as LKB1. Upon activation, AMPK promotes GLUT4 translocation to the plasma membrane which facilitates glucose uptake and glycolysis. AMPK promotes cell cycle arrest by inhibiting cyclin D1 and also promotes p53 stabilisation. AMPK and metformin inhibit protein synthesis and promote autophagy via inhibition of the mTOR signaling cascade.

oxidative phosphorylation and further impairs ATP production (Luengo, Sullivan & Heiden, 2014). AMP binding promotes AMPK phosphorylation by upstream kinases, therefore, metformin indirectly activates AMPK due to an increase in the AMP:ATP ratio. AMPK can also be activated in response to low glucose levels (Laderoute et al., 2006).

Upon activation, AMPK phosphorylates multiple downstream targets involved in the degradation of macromolecules such as carbohydrates, lipids and proteins. AMPK plays a direct role in glycolysis by promoting translocation of the GLUT4 transporter to the plasma membrane, thus enabling glucose entry into the cell (Figure 1.5). AMPK activation also promotes transcription of the GLUT4 and GLUT1 genes (Kurth-Kraczek et al., 1999; Zheng et al., 2001). AMPK further promotes glycolysis by activating 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), thus increasing fructose 2,6-bisphosphate levels (Marsin et al., 2000).

AMPK promotes cell senescence by halting cell cycle progression at the G1/G0 stage. Cell division and progression of the cell cycle is an energy consuming processes that must be slowed down to allow a cell to conserve energy. AMPK is involved in regulation of the cell cycle as it was shown to block G1-S transition via phosphorylation of $p53^{Ser15}$ (Jones et al., 2005). Cell cycle progression is tightly regulated by cyclins and cyclin dependent kinases. Cyclin D1 is involved in the transition from G1 to S-phase. Metformin-induced activation of AMPK was shown to block cell cycle progression and reduce cyclin D1 levels in breast cancer cells (Zhuang & Miskimins, 2008).

AMPK also inhibits protein synthesis by suppressing mammalian target of rapamycin (mTOR) complex 1 formation. The mTORC1 complex is a kinase that couples growth factor signaling with amino acid availability in order to regulate cell growth. Growth factor stimuli such as insulin receptor activation trigger the Akt signaling pathway, which promotes activation of mTORC1 via a Rag GTPase. There are various downstream targets of mTORC1, one of them is the ribosomal protein S6 kinase (p70S6K), which plays an important role in ribosomal biogenesis and nascent protein synthesis (Ma & Blenis, 2009). Inhibition of mTOR by AMPK leads to an inhibition of $p70S6K^{Thr389}$ phosphorylation and hence protein synthesis, which is essential for tumour growth, is also reduced.

AMPK and mTORC1 play an important role in regulating autophagy. AMPK activates a mammalian autophagy initiating kinase (Ulk1), whereas phosphorylation of Ulk1 by mTORC1 leads to inhibition of autophagy initiating complex formation (Kim et al., 2011). In addition, mTORC1 exerts a second level of control over autophagy by inhibiting other members of the autophagy initiating complex such as mATG13. Autophagy has mixed roles in cancer progression as turnover of old proteins and organelles can be useful for cell survival by replenishing pools of amino acids required for protein synthesis. On the other hand, over-induction of autophagy can lead to cell death by apoptosis (Morselli et al., 2009).

1.4.3. AMPK Independent Effects of Metformin

In addition to the AMPK-dependent effects of metformin described above, there is growing evidence that metformin also exerts AMPK independent effects. A recent study showed that the effects of metformin in cancer may be attributed to reduced energy charge due to impaired mitochondrial function (Andrzejewski et al., 2014). Metformin has been shown to inhibit complex I of the mitochondrial respiratory chain (El-Mir et al., 2000). Inhibition of complex I hampers electron transfer and disrupts the proton gradient required for ATP production. This leads to an increase in the intracellular AMP:ATP ratio. Although there is still debate about whether complex I is a direct target for metformin, there is a consensus that metformin leads to an increase in the AMP:ATP ratio and as such, alters energy charge in the cell. Apart from AMPK activation, reduced ATP levels may have a range of other effects.

Metformin also inhibits a second mitochondrial enzyme, glycerol-phosphate dehydrogenase (Madiraju et al., 2014), which is involved in shuttling NADH from the cytosol into mitochondria. NADH is utilized as a cofactor by complex I, hence inhibition of mitochondrial glycerol-phosphate dehydrogenase, along with complex I, impairs mitochondrial NADH oxidation and further disrupts electron transfer and ATP production (Luengo, Sullivan & Heiden, 2014). Reduced ATP production by mitochondrial oxidative phosphorylation led to higher levels of aerobic glycolysis in response to metformin treatment in breast cancer cells (Andrzejewski et al., 2014).

Metformin can reduce glucose levels independent of AMPK activation. Glucagon is a peptide hormone produced by pancreatic cells and is involved in promoting hepatic gluconeogenesis.

ATP is required for the synthesis of cyclic AMP (cAMP), a second messenger required for glucagon signaling (Miller et al., 2013). Reduced ATP levels prevent cAMP production via adenylyl cyclase and therefore glucagon signaling is impaired. Reduced glucagon signaling reduces hepatic gluconeogenesis, and as such, metformin can reduce systemic glucose levels in the absence of AMPK. Metformin was shown to reduce gluconeogenesis in hepatocytes that lacked AMPK or LKB1 (Foretz et al., 2010).

Metformin blocks cell cycle progression and inhibits mTOR activity in the absence of AMPK. Cell cycle progression was blocked in prostate cancer cells that lacked AMPK in response to metformin. This was associated with a decrease in cyclin D1 levels (Ben Sahra et al., 2008). Mouse embryonic fibroblasts that expressed mutant AMPK or AMPK double-knockout cells showed reduced p70S6K phosphorylation, indicative of reduced mTORC1 signaling in response to metformin (Kalender et al., 2010). This was dependent on the presence of Rag GTPases. Therefore, mTORC1 inhibition can occur independently of AMPK activation in a Rag GTPase dependent manner.

The hypothesis that metformin exerts its effects through a reduction of energy charge is now gaining more favour. Although metformin has been shown to inhibit complex I, the mechanism by which this occurs is still not known, and a direct target for metformin is yet to be identified. It would be of great benefit to elucidate the exact molecular mechanism by which metformin acts as this would help determine which cancers may be more susceptible to the drug. For now, more rigorous *in vitro* and *in vivo* testing is still required to determine which cancers metformin may be most effective against.

1.4.4. Redox Regulation in Cancer

Reactive oxygen species (ROS) are a group of highly reactive oxygen containing molecules and mainly include the superoxide free radical, $O_2^{\bullet -}$, singlet oxygen, $^1O_2$, the hydroxyl free radical, $OH^{\bullet -}$, and hydrogen peroxide, H_2O_2. ROS play a role in cell signaling due to oxidation of protein kinases and phosphatases. High ROS levels have been shown to promote genomic instability and contribute to tumourigenesis (Fruehauf & Meyskens, 2007). For these reasons, antioxidants are considered to be beneficial for cancer therapy. Although elevated

ROS levels are a feature of many cancers and promote tumourigenesis, excessive ROS levels, above the tumour inducing threshold, can exert chemotherapeutic effects by promoting cancer cell death (Cairns, Harris & Mak, 2011). This forms the basis of using ionizing radiation for cancer therapy which increases ROS levels above this threshold and in turn leads to apoptosis of cancer cells (Wang & Yi, 2008).

Mitochondria are the main producers of intracellular ROS. Damage to mitochondrial DNA has been shown to impair electron transport, as increased intracellular $OH^{\bullet -}$ levels have been observed in osteosarcoma cells. Rotenone is known to inhibit complex I of the mitochondrial respiratory chain and it has been shown to increase intracellular $OH^{\bullet -}$ levels (Indo et al., 2007). However, rotenone has also been shown to reduce ROS production in isolated mitochondria by impairing reverse electron flux through complex I. Similar to rotenone, metformin has been shown to hamper reverse electron flux and ROS production in isolated mitochondria, but not to the same extent as rotenone (Batandier et al., 2006). This suggests that metformin inhibits complex I in a manner slightly different to rotenone, and the exact mechanism by which metformin inhibits complex I is not yet known. Studies have shown that metformin reduced ROS production in bovine aortic endothelial cells but its effects on ROS production in cancer cells are still poorly defined (Ouslimani et al., 2005).

Intracellular ROS levels are tightly regulated by anti-oxidant molecules such as glutathione, thioredoxin, superoxide dismutase and peroxidases (Scherz-Shouval & Elazar, 2011). The glutathione anti-oxidant defence system is a major intracellular pathway for ROS detoxification. Glutathione can exist in either a reduced (GSH) or oxidised (GSSG) state and pools of GSH are replenished due to the activity of glutathione reductase. Glutathione reductase uses NADPH as a cofactor which is oxidised to $NADP^{+}$. Glucose-6-phosphate (G-6-P) produced during the first step of glycolysis can be shunted into the pentose phosphate pathway (PPP). This replenishes NADPH pools as it utilises $NADP^{+}$ as a cofactor for the conversion of G-6-P to 6-phosphogluconolacton via glucose-6-phosphate dehydrogenase (Sattler & Mueller-Klieser, 2009). Therefore, increased shunting of glycolytic intermediates into the PPP provides the NADPH required for glutathione reduction and increased glycolysis contributes to a more reducing intracellular environment.

Considering that cancer cells have increased levels of glycolysis, drugs such as metformin which increase glycolytic flux could enhance the reducing potential of cancer cell lines by increasing NADPH or GSH levels. It would be of value to consider combining metformin with drugs that are activated in reducing environments for cancer treatment. We have shown that metformin increases the reducing potential of OSCC cell lines (Damelin et al., 2014). We also effectively combined metformin with disulfiram and copper bis(thiosemicarbazones) which are cytotoxic agents activated in reducing environments. The combination of metformin and reductively activated drugs with chemotherapeutic potential, may offer a novel approach to OSCC treatment that is both effective and affordable.

1.5. Reductively Activated Drugs for Cancer Therapy

1.5.1. Copper bis(thiosemicarbazones)

The bis(thiosemicarbazone), glyoxal bis(thiosemicarbazone) (GTSM) was first synthesised by Neuberg *et al*. in 1909 (Neuberg, Neimann & Salkowski, 1909). However, commercially available glyoxal used in its synthesis at the time contained impurities such as formaldehyde, glycoaldehyde and glyoxilic acid, which could yield toxic thiosemicarbazone products during synthesis. The preparation of this compound was improved in 1958 and was shown to have anti-cancer effects (French et al., 1958). A marked reduction in DNA synthesis and mild reductions in protein and RNA synthesis were identified as the mechanism responsible for the anticancer effects of bis(thiosemicarbazones) (Sartorelli & Booth, 1967). French and Freedlander showed that GTSM and various other bis(thiosemicarbazone) derivatives improved survival of Sarcoma 480 mice when administered orally, but not when administered via intra-peritoneal injection (French & Freedlander, 1960). It was hypothesised that the anti-cancer effects of these compounds may be dependent on their metal chelating properties (French et al., 1958).

Whilst bis(thiosemicarbazones) showed a potential benefit *in vivo*, initial studies showed that the doses required for anti-cancer effects *in vitro* were particularly high, which may have been due to low copper content in culture medium (Petering, Buskirk & Underwood, 1964). A cupric chelate of kethoxal bis(thiosemicarbazone) synthesised in the late 1960's was shown to be more toxic and also prolonged the life-span of mice bearing sarcoma (Sartorelli & Booth, 1967). However, kethoxal bis(thiosemicarbazone) had also been shown to bind to numerous metabolites, including proteins, in higher animals (Underwood et al., 1959). Therefore, numerous analogues of the bis(thiosemicarbazones) have been synthesised and investigated. Many of these analogues resulted in moderate to severe weight loss in mice. Additionally Cu-GTSM, which was shown to exert beneficial effects against tumour bearing mice, is insoluble in aqueous media, resulting in drug accumulation at the site of injection (French et al., 1958). These effects, along with heightened interest in cisplatin for cancer therapy, led to a decline in interest and research on the bis(thiosemicarbazones).

Copper bis(thiosemicarbazone) research was renewed in the late 1980's, this time to be applied as a radiopharmaceutical for positron emission tomography (PET) (Green, Klippenstein & Tennison, 1988). In 1997, Fujibayashi *et al*. first proposed the use of copper diacetyl-bis(4-methyl-3-thiosemicarbazone) (^{64}Cu-ATSM) as a radiopharmaceutical (Fujibayashi et al., 1997). ^{64}Cu-ATSM showed promise as it was easily distributed and penetrated the brain and heart, it showed selectivity for hypoxic tissue and was easily cleared from the blood pool (Fujibayashi et al., 1997). More recent reports documenting some of the mechanisms responsible for hypoxia selectivity of Cu-ATSM and comparing these effects to other copper bis(thiosemicarbazones) have allowed research on these drugs to progress to clinical trials (Xiao et al., 2008; Paterson & Donnelly, 2011).

Renewed interest in copper bis(thiosemicarbazone) research for radiopharmaceuticals has prompted further studies on the use of these agents for cancer therapy. ^{64}Cu-ATSM is currently undergoing clinical trials for use as an imaging agent for cancer detection (NCT00794339). This highlights the fact that Cu-ATSM is tolerated by humans and that it targets hypoxic cancer tissues. Further studies will be required to confirm whether it can be tolerated for longer periods of time that are effective for cancer therapy. The rationale behind using Cu-ATSM as a chemotherapeutic agent is that it may serve to deliver excess amounts of copper to hypoxic tissue leading to cell death due to heavy metal toxicity. Palanimuthu *et al.* recently synthesised a number of new bis(thiosemicarbazone) complexes. The glyoxal-based copper bis(thiosemicarbazones) were more toxic than Cu-ATSM and resulted in a better tumour response (Palanimuthu et al., 2013). However, these analogues were not targeted or specific to cancer tissue. More studies are required to elucidate their molecular mechanism of action and to determine whether new analogues can be derived to offer better anti-tumour effects with reduced toxicity.

1.5.2. Disulfiram

Disulfiram (tetraethylthiuram disulphide) was first synthesised in 1881 by M. Grodzki, a chemist in Berlin, but received little attention back then (Kragh, 2008). It was introduced in the rubber manufacturing process to accelerate the vulcanising process in the early 1930's. In 1937, a physician at a rubber manufacturing plant, E. E. Williams, first noticed that factory workers exposed to disulfiram experienced unpleasant symptoms upon alcohol consumption and voluntarily abstained from alcohol (Suh et al., 2006). Between 1940 and 1945 in Denmark, Erik Jacobsen and Jens Hald conducted research on disulfiram and noticed that disulfiram caused an unpleasant reaction to alcohol (Kragh, 2008). Tetraethylthiuram monosulfide (Sulfiram) was shown to be effective in the treatment of scabies, which is a skin infection caused by the mite *Sarcoptes scabiei* (Gordon & Seaton, 1942). Disulfiram was also used to treat domestic animals for scabies and intestinal worms in the early 20^{th} century. Jacobsen and Hald discovered that the effects of disulfiram in the treatment of scabies and intestinal worms were due to the formation of disulfiram-copper chelates, as lower life forms rely on copper rather than iron for oxygen transport. In trying to purify disulfiram contaminated with copper, the group figured out a method to improve drug absorption. Jacobsen and Hald patented this method for the preparation of disulfiram under the name "Antabuse" in 1952 (Kragh, 2008).

The prescription of disulfiram (DSF) for alcohol abuse promoted pharmacological research on its biochemical mechanism of action, which showed that there are a number of metabolites of disulfiram. Upon ingestion, disulfiram is partly reduced to diethyldithiocarbamate (DDC), which is unstable in acidic solutions and rapidly converts to carbon disulphide and diethylamine. Alternatively, DDC is a strong metal chelating molecule, which rapidly forms a complex with cupric ions to form a bis(diethyldithiocarbamato) copper complex ($Cu(DDC)_2$). $Cu(DDC)_2$ is acid stable and hydrophobic, hence easily absorbed along the gastrointesintal tract. Upon entry into the bloodstream, DSF and $Cu(DDC)_2$ are reduced to DDC, which can be further metabolised to diethyldithiomethylcarbamate (Me-DDC) and the glucuronic acid of DDC. Me-DDC is converted to diethylthiomethylcarbamate (Me-DTC) by oxidative desulfuration mediated by microsomal p450 mono-oxygenases. Me-DTC is involved in the disulfiram-ethanol reaction as it inhibits mitochondrial aldehyde

dehydrogenase leading to accumulation of acetaldehyde and the harsh side effects of DSF in the presence of alcohol (Johansson, 1992).

During early stages of investigation, high doses of disulfiram were prescribed (2000-3000 mg per day) which led to psychosis in some patients (Martensen-Larson, 1951). The dose was then reduced to a more tolerable 500-2000 mg per day, however this led to the deaths of 4 patients who had consumed large amounts of alcohol (Jacobsen, 1952). Today, the recommended daily dose is 250 mg, and should not exceed 500 mg. In the presence of alcohol, a severe disulfiram ethanol reaction results, causing facial flushing, throbbing in the head and neck, headache, nausea, vomiting, sweating, thirst, chest pain, palpitations, dyspnea, hyperventilation, tachycardia, confusion, arrhythmias, and convulsions (Elenbaas, 1977). However, in the absence of alcohol, the side effects of disulfiram are mild, and include drowsiness, headache, skin rash and impotence. Hepatotoxicity occurs in approximately 1 in 30 000 patients treated with disulfiram, but can be reversed if treatment with disulfiram is stopped upon first signs of liver damage (Chick, 1999). As such, liver function is strictly monitored and patients receiving disulfiram are educated on the potential signs and symptoms of liver disease.

The first evidence for the use of disulfiram in cancer was in 1974, when Lee Wattenberg showed that disulfiram suppressed the formation of benzopyrene or dimethylbenzanthracene induced gastric tumours in Sprague-Dawley rats (Wattenberg, 1974). Wattenburg later showed that disulfiram also prevented the formation of dimethylhydrazine induced neoplasias of the large intestine in mice (Wattenberg, 1975). Considering that mice were treated with tumour supressing agents either prior to or during treatment with the tumour-inducing agent, it was difficult to tell from these findings alone whether disulfiram could reduce the size of a pre-existing tumour. In the 1980's, it was shown that disulfiram potentiated the effects of various anti-cancer agents in mice (Hacker et al., 1982; Valeriote & Grates, 1989). However, little was known about the mechanism of action of disulfiram against cancer at the time.

Many studies have now been conducted toward elucidating a mechanism for the anti-cancer effects of disulfiram. Collectively, these studies demonstrate that disulfiram exerts a variety of effects in cancer cells, which impacts on at least three cancer hallmarks. Disulfiram has

been shown to induce apoptosis in a variety of cancer cell lines, reduce angiogenesis and also reduce tumour invasiveness and metastasis (Chen et al., 2001, 2006; Shiah et al., 2003; Cho et al., 2007). Apoptosis induction in response to disulfiram treatment is attributed to DNA damage as a result of increased ROS levels and inhibition of NFκB, which has been associated with increased inflammation and tumour survival (Yip et al., 2011). Furthermore, disulfiram altered DNA transcriptional regulation by inhibiting supercoiling activity of DNA topoisomerases and also inhibits DNA methyltransferase 1 (Yakisich et al., 2001; Lin et al., 2011). In the majority of the above-mentioned cases, the effects of disulfiram were copper dependent.

The role of copper in disulfiram induced toxicity is two-fold. Firstly, disulfiram may act as a copper delivery agent. Increased intracellular copper levels can lead to cell death due to heavy metal toxicity. Copper is also a redox active metal, delivery of this metal into cells promotes oxidative stress and leads to cell death (Stefan et al., 1995). Secondly, disulfiram may alter cell function by chelating intracellular copper. Chelation of intracellular copper can inhibit the activity of Cu/Zn superoxide dismutase, this promotes oxidative stress, inhibits angiogenesis and may lead to cell death (Marikovsky et al., 2002). In addition, disulfiram and other copper-binding agents have been shown to inhibit proteasome activity. Protein degradation is essential to replenish amino acid pools required for *de novo* protein synthesis and cell division. As such, proteasome inhibition is associated with reduced tumour growth and cell proliferation, further highlighting the chemotherapeutic potential of disulfiram (Daniel et al., 2005; Rajkumar et al., 2005; Chen et al., 2006; Wickström et al., 2007).

There are a number of phase I clinical trials that are ongoing or have been recently completed for the use of disulfiram alone or in combination with standard chemotherapy or chemoradiotherapy to treat individuals with various cancer types. These include solid tumours of the liver, non-small cell lung cancer, prostate cancer, metastatic melanoma and glioblastoma (NCT00742911, NCT00312819, NCT01118741, NCT00256230, NCT01907165). While results from these clinical trials are keenly awaited, *in vitro* and mouse studies on the effects of disulfiram in cancer show promising evidence for its repurposing as a chemotherapeutic agent. Further studies are still required in order to elucidate a direct cellular target for disulfiram.

1.6. Aim and Objectives

Aim

To investigate the anti-proliferative effects of metformin on oesophageal squamous cell carcinoma cell lines and determine whether its combination with other anti-cancer agents can offer novel combination chemotherapeutic strategies.

Objective 1

To determine the effects of metformin on OSCC cell proliferation and the AMPK signalling pathway

1.1. Determine the effects of metformin on OSCC cell proliferation by direct cell counts.
1.2. Assess the stage of the cell cycle after metformin treatment by flow cytometry.
1.3. Assess changes in phospho-AMPKα^{Thr172} levels in response to metformin treatment by western blotting.
1.4. Assess changes in phospho-p70S6K^{Thr389} levels in response to metformin treatment by western blotting.

Objective 2

To investigate the effects of metformin on cisplatin mediated cytotoxicity in OSCC cell lines.

2.1. Determine the effects of metformin in combination with cisplatin on OSCC cytotoxicity by MTT assay.
2.2. Determine whether metformin alters cisplatin DNA adduct formation by quantifying platinum in DNA by inductively coupled plasma mass spectrometry (ICP-MS).
2.3. Assess the effects of metformin on glycolysis levels by quantifying lactate secreted into cell culture medium after metformin treatment.
2.4. Determine whether metformin alters intracellular reduced thiol levels based on monobromobimane fluorescence.
2.5. Determine whether modulation of intracellular thiols alters the effects of metformin on cisplatin-mediated cytotoxicity.
2.6. Assess whether metformin alters the reducing potential of OSCC cells by MTT assay.

Objective 3

To investigate the effects of metformin in combination with drugs activated in reducing environments as novel combination chemotherapeutic strategies for OSCC.

3.1. Evaluate the effects of metformin combined with DSF, Cu-ATSM or Cu-GTSM on OSCC cytotoxicity by MTT assay.
3.2. Determine whether DSF, Cu-ATSM and Cu-GTSM exert copper mediated DNA damage based on their ability to alter plasmid DNA supercoiling.
3.3. Determine whether metformin combined with DSF, Cu-ATSM or Cu-GTSM alters intracellular copper levels by ICP-MS.
3.4. Determine whether DSF, Cu-ATSM or Cu-GTSM, with or without metformin alters lysosomal acidity based on acridine orange fluorescence.
3.5. Determine whether copper modulation alters the effects of DSF.
3.6. Determine the effects of DSF with or without metformin on protein degradation by quantifying total ubiquitin and LC3-B levels by western blotting.
3.7. Evaluate the effects of DSF and metformin on autophagosome formation by transmission electron microscopy.
3.8. Determine the effects of DSF with or without metformin on proteasome activity based on cleavage and fluorescence of succinyl-Leu-Leu-Val-Tyr-amido methylcoumarin (Suc-LLVY-AMC).
3.9. Compare the effects of disulfiram with analogues that have lower hydrolysis rates on OSCC cytotoxicity by MTT assay.
3.10. Assess the effects of DSF analogues on lysosomal acidity by acridine orange fluorescence.

1.7. Synopsis

Chapter 2: Characterising the Anti-Proliferative Effect of Metformin in OSCC Cell Lines

Oesophageal squamous cell carcinoma (OSCC) is a highly aggressive cancer with a poor survival rate. The poor survival rate may be attributed to a lack of cost-effective chemotherapeutic strategies for this cancer. We hope to find effective chemotherapeutic drugs that are also affordable for the treatment of OSCC. Metformin is a widely prescribed anti-diabetic drug that shows chemotherapeutic potential, and is also affordable.

In this chapter, we show that metformin inhibits the proliferation of OSCC cell lines as fewer cells were present after 10 mM metformin treatment for 24 hours compared to untreated controls. Flow cytometry assessment with propidium iodide staining showed that cells accumulated at the G1/G0 stage of the cell cycle in response to metformin, suggesting that the drug halts cell cycle progression. Metformin treatment was associated with increased phosphorylation and activation of AMPK, evaluated by western blotting. There was also a reduction in the phosphorylation of p70S6 kinase which is inhibited by AMPK, thus proving that AMPK signalling is increased in the presence of metformin.

These results show that metformin halts OSCC cell proliferation, and as such may potentially be used to halt tumour progression. However, the treatment was not cytotoxic and would have to be combined with more potent drugs in order to offer an effective chemotherapeutic strategy.

Chapter 3: Metformin Negatively Alters the Effects of Cisplatin in OSCC Cell Lines

Cisplatin is currently the first-line drug prescribed for OSCC treatment. However, cisplatin resistance is a pitfall associated with frequent therapy and the prescription of high doses. The combination of metformin and cisplatin has shown both positive and negative effects, depending on cancer type (Gotlieb et al., 2008; Janjetovic et al., 2011; Lesan et al., 2014). We sought to determine the effects of metformin on cisplatin mediated cytotoxicity in OSCC

cell lines. Furthermore, we investigated the mechanisms responsible for the effects of metformin and cisplatin when combined.

Cytotoxicity was evaluated using the MTT assay to calculate the drug concentration required for 50% cell death (LC50). Metformin-cisplatin treated cells displayed higher LC50 values versus cisplatin treatment alone, suggesting that metformin negatively alters the effects of cisplatin in these cell lines. Platinum levels in DNA quantified by inductively coupled plasma mass spectrometry indicated that protective effects of metformin are attributed to reduced cisplatin-DNA adduct formation. Metformin also increased glycolytic rates in these cell lines, which suggested that it could modulate the glutathione redox defence system. We quantified levels of reduced low-molecular weight thiols based on monobromobimane fluorescence, which was higher in cells treated with metformin. This indicates that metformin increases the levels of reduced thiols in these cell lines. Increased thiols are a plausible explanation for the negative effects of metformin on cisplatin mediated cytotoxicity as treatment with N-acetyl-L-cysteine, a glutathione pre-cursor, also negatively altered the effects of cisplatin. Glutathione depletion by buthionine sulfoximine blocked the protective effects of metformin on cisplatin mediated toxicity. Increased levels of intracellular thiols suggested that metformin could alter the redox state of these cells. Reducing potential was higher in the presence of metformin, as indicated by its ability to reduce MTT.

Metformin reduced cisplatin cytotoxicity by reducing DNA adduct formation. This occurred due to altered drug sequestration or increased drug export by intracellular thiols such as glutathione. For these reasons, metformin should not be combined with cisplatin for OSCC treatment. Extra caution must be heeded in the case of cancer patients that also suffer diabetes. Interestingly, metformin increased the reducing potential of OSCC cell lines, which suggests that it may work well when combined with cytotoxic agents that are activated in reducing environments.

Chapter 4: Metformin has a positive effect on anti-cancer agents which are activated in reducing environments

The effects of metformin in combination with Cu-ATSM, Cu-GTSM and disulfiram on OSCC cytotoxicity were investigated. These drugs were chosen as they are activated in reducing environments. Disulfiram showed the most promising response, and therefore the mechanisms of toxicity were further investigated. Disulfiram has been shown to inhibit proteasomal function. Therefore, the effects of this drug in combination with metformin on protein degradative pathways were further investigated.

Cu-ATSM or Cu-GTSM both exerted toxicity toward OSCC cell lines at particularly low doses and this was not altered in the presence of metformin. Metformin enhanced the cytotoxic effects of DSF. This indicated that metformin can be combined with these drugs for OSCC treatment. DSF is the most promising agent for potential application as an anti-cancer drug as it is FDA approved and has a well-established low toxicity profile. Our findings corroborate other studies which show that the effects of DSF are significantly enhanced as copper levels are increased, which indicate that copper plays a significant role in the mechanism of action of this drug. The DSF-copper complex, Cu-DSF, has been shown to inhibit proteasome activity. We found that DSF and Cu-DSF treatment led to a significant increase in the accumulation of ubiquitinated proteins, but only partially inhibited proteasome function. This suggested that DSF could be involved in inhibiting other pathways of protein degradation. We showed that DSF still exerted cytotoxic effects without additional copper and after copper depletion. We therefore hypothesised that the hydrolysis products of DSF may be involved in its toxic effects, and that diethylamine may accumulate in lysosomal vesicles, reduce their acidity and inhibit autophagy. We proved this by showing that DSF treatment reduced lysosomal acidity as indicated by acridine orange staining. We also showed that cells treated with DSF or Cu-DSF had increased levels of LC3B-II and electron microscopy showed an accumulation of protein aggregates in autolysosomes. This indicated that DSF inhibited autophagy in OSCC cell lines.

Therefore, metformin enhanced the effects of DSF, which indicates that these drugs can be used in combination for OSCC therapy. DSF owes its anticancer effects to the inhibition of multiple protein degradation pathways.

Chapter 2: Characterising the Anti-Proliferative Effect of Metformin on OSCC Cell Lines

2.1. Introduction

Metformin is a widely prescribed anti-diabetic drug that has recently demonstrated chemotherapeutic potential (Evans et al., 2005). It has been shown to inhibit the proliferation of numerous cancer cell lines including breast, prostate, endometrial, ovarian, melanoma, glioma, pancreatic and colon cancers (Zakikhani et al., 2006; Buzzai et al., 2007; Isakovic et al., 2007; Ben Sahra et al., 2008; Wang et al., 2008; Cantrell et al., 2010; Rattan et al., 2011; Tomic et al., 2011). In addition, metformin was cytotoxic towards glioma cells and melanoma cell lines (Isakovic et al., 2007; Wang et al., 2008; Tomic et al., 2011). Since metformin is already FDA approved for the treatment of diabetes and has a well-established tolerance profile, numerous clinical trials are being conducted to elucidate whether metformin can be used for cancer therapy. There is extensive evidence supporting the anti-proliferative effects of metformin; however, an exact molecular mechanism of action for metformin in cancer is still not known. Although metformin was shown to reduce the proliferation of many cancer cell types, the effects of metformin on OSCC were undefined at the beginning of this study. Kobayashi *et al.* have recently shown that metformin inhibits the proliferation of Japanese derived OSCC cell lines (Kobayashi et al., 2013).

Reduced cell proliferation in response to metformin is associated with cell cycle arrest. Metformin induces cell cycle arrest at the G1/G0 stage with a concomitant reduction in cyclin D1 levels in breast cancer cells (Ben Sahra et al., 2008; Alimova et al., 2009). In order to acquire replicative immortality, cancer cells must bypass the G1/S checkpoint to allow cell cycle progression. Cyclin D1 regulates G1 to S phase transition partly by repressing the E2F transcription factor (Figure 2.1). E2F promotes the expression of genes required for the transition from G1 to S phase (Tashiro, Tsuchiya & Imoto, 2007). Cyclin D1 is also a member of a complex that sequesters and blocks the action of cyclin dependent kinase (CDK) inhibitors, $p27^{kip}$ and $p21^{cip}$. The CDK inhibitors bind to and inhibit cyclin E which promotes G1-S cell cycle progression (Zhuang & Miskimins, 2008). Therefore, inhibition of cyclin D1

leads to release of these CDK inhibitors, which then sequester cyclin E and halt cell cycle progression.

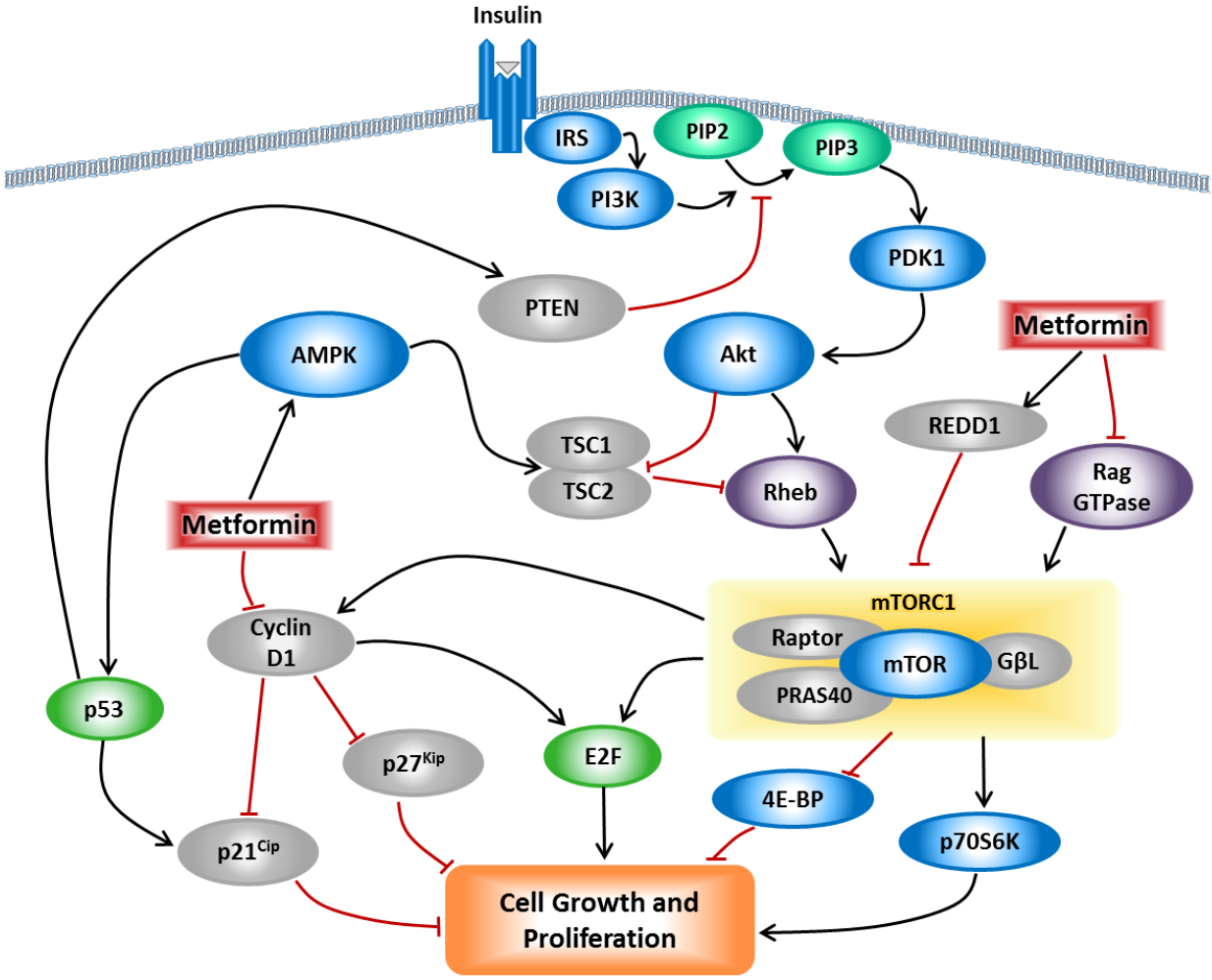


Figure 2.1: Metformin interacts with the AMPK and mTOR signalling pathways leading to reduced cell proliferation. Metformin inhibits the cell cycle via the AMPK or mTOR signalling pathways and independent of these pathways via cyclin D1. Metformin activates AMPK. AMPK promotes p53 activation which is associated with increased levels of the cyclin-dependent kinase inhibitor, $p21^{cip}$, and therefore inhibits cell cycle progression. Metformin has been shown to reduce cyclin D1 expression independent of AMPK. This leads to elevated levels of $p27^{kip}$ and $p21^{cip}$, which inhibit cell cycle progression. Cyclin D1 also promotes the action of eukaryotic initiation factor, E2F, which is inhibited in the presence of metformin. Metformin can inhibit mTORC1 via Rag GTPases, REDD1 or via AMPK. Inhibition of mTORC1 leads to reduced levels of p70S6K and increased levels of 4E-BP, which results in reduced cell growth proliferation. Metformin can therefore inhibit cell cycle progression via numerous routes.

AMPK is linked to reduced cell proliferation due to its role in inhibiting mTOR at various levels and its involvement in promoting $p53^{Ser15}$ phosphorylation which leads to activation of $p21^{cip}$ (Imamura et al., 2001). Metformin induced G1/G0 stage cell cycle arrest in prostate cancer cells and was associated with reduced cyclin D1 expression and elevated $p27^{kip}$ levels. However, AMPK knockdown in those cell lines did not alter the effects of metformin on cell proliferation, which indicates that AMPK is not fully required for the growth inhibitory effects of metformin (Ben Sahra et al., 2008).

In addition to p53-dependent effects, the anti-proliferative effects of metformin, in breast cancer cells, have also been found to be AMPK dependent but not dependent on p53. AMPK activation has been shown to be associated with down-regulation of cyclin D1, and increased binding of $p27^{kip}$ and $p21^{cip}$ to cyclin E, which is required for G1-S phase transition. These effects were shown to be AMPK-dependent, as treatment with the AMPK inhibitor, compound C, reduced the anti-proliferative effects of metformin (Zhuang & Miskimins, 2008). Although compound C has been widely used as an AMPK inhibitor, it has also been shown to exert AMPK-independent effects, which raises questions about the use of this agent as a specific AMPK inhibitor (Vucicevic et al., 2014). Metformin has also been shown to inhibit cell cycle progression after siRNA mediated knockdown of AMPK, which indicates that metformin can halt cancer cell proliferation irrespective of its AMPK or p53 status (Ben Sahra et al., 2011).

Metformin and AMPK have been shown to inhibit mammalian target of rapamycin (mTOR) signalling. There are two major complexes containing mTOR, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which play a role in promoting cell growth and proliferation in response to growth factors, nutrients, energy and stress. Due to the fact that mTOR signalling is upregulated in numerous cancers, inhibition of mTOR has been considered for cancer therapy (Chan, 2004). Two main proteins activated by mTORC1 and have growth promoting effects due to their roles in protein translation are the 70kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding protein-1 (4E-BP1) (Hara et al., 1998). Downstream mTORC1 signalling is dependent on recruitment of three complex members to mTOR, the rapamycin-sensitive adaptor protein of mTOR (raptor), the G protein β-subunit-like protein (GβL) and the proline-rich Akt substrate 40 kDa (PRAS40). Upon activation, p70S6K regulates ribosomal protein translation and ribosome

biogenesis. Phosphorylation of 4E-BP1 by mTORC1 leads to the release of eIF4E which is required for translation of mRNA encoding proteins required for G1 to S phase transition such as cyclin D1 (Bjornsti & Houghton, 2004). Inhibition of mTORC1 results in G1 phase cell cycle arrest.

Phosphorylation of $p53^{Ser15}$ by AMPK was also shown to activate the phosphatase and tensin homologue (PTEN) which consequently inhibits PI3K-AKT signalling by inhibiting the conversion of PIP2 to PIP3 (Levine et al., 2006). PTEN is mutated in numerous cancers including brain, breast, prostate cancer, bladder, lung and renal carcinomas, and is one of the most frequently mutated genes in cancer (Li, 1997; Guertin & Sabatini, 2005). This further emphasises requirement of enhanced mTOR signalling by cancer cells. Interestingly, metformin was shown to inhibit mTORC1 independent of AMPK or TSC2, in a Rag GTPase dependent manner (Kalender et al., 2010). Metformin also promoted mTORC1 inhibition by activating REDD1 (regulated in development and DNA damage responses 1), and these effects are dependent on p53 (Ben Sahra et al., 2011). However, the mechanism by which REDD1 inhibits mTORC1 is still largely unknown.

In this chapter, we aimed to determine the effects of metformin on OSCC cell proliferation. Metformin treatment reduced proliferation and halted cell cycle progression at the G1/G0 stage in these cell lines. Preliminary findings indicated that mTOR levels were particularly high in these cell lines and did not seem to be altered in the presence of metformin. Metformin treatment was associated with increased phosphorylation of AMPK and inhibition of p70S6K in the three cell lines tested.

2.2. Methods

The three human oesophageal squamous cell carcinoma (OSCC) cell lines used in this study, WHCO1, WHCO5 (Veale & Thornley, 1989) and SNO (Bey et al., 1976) were originally derived from moderately differentiated tumour. Since OSCC has been shown to have a higher prevalence amongst the black male population, these cell lines are an ideal model for the target population as they are derived from males of African descent. The effects of metformin on cell proliferation were investigated by determining cell viability and stage of the cell cycle in the absence and presence of metformin. We also aimed to determine whether the effects of metformin on cell proliferation were attributed to alterations in metabolic regulation by assessing levels of P-AMPKα^{Thr172} and P-p70S6K^{Thr389}. All reagents used in this study were purchased from Sigma-Aldrich, unless otherwise specified.

2.2.1. Cell Culture

All cell lines used in this study were a kind gift from Professor Robin Veale. Cells were cultured in medium made of three parts Dulbecco's Modified Eagle's Medium (DMEM) to one part Hams F12 supplemented with 10% foetal calf serum (FCS) (Highveld Biological) and maintained in a humid incubator at 37 °C with 5% carbon dioxide. Cells were routinely sub-cultured when 70-90% confluence was reached by removing culture medium, rinsing cells three times in 1 x PBS (*137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4,* pH 7.2, autoclaved), followed by the addition of 1.2 ml Trypsin:EDTA (0.25% Trypsin: 0.02% EDTA in Hanks' Balanced Salt Solution) for 2-5 min at 37 °C. Approximately 300 µl of the cell suspension was re-suspended in 8 ml fresh culture medium for maintenance of the cell line. Culture medium was replaced 24 hour after seeding and every second day thereafter.

2.2.2. Evaluation of Cell Proliferation with Trypan Blue Staining

To determine the effects of metformin on OSCC cell proliferation, untreated and metformin treated cells were counted and compared. Cells were equally seeded in 24-well plates (4 x 10^4 cells/well) and allowed to settle for 24 hours. Cells were then treated with 10 mM metformin for a further 24 hours, or culture medium was replaced for all untreated controls. After treatment, cells were trypsinized for 5-10 min at 37 °C, and trypsin was inactivated by re-suspending cells in 2 volumes culture medium. The cell suspension was then added to an

equal volume of Trypan blue (0.2% in 1 x PBS, filter sterilized), mixed for 2 min and counted using a haemocytometer slide. Counts were used to calculate cell concentration (cells/ml) and total number of cells per well, values were represented as an average (n = 3 ± SD).

2.2.3. Cell Imaging by Microscopy

An equal number of cells were seeded per well (200 000 cells/well) on autoclaved glass cover-slips in 6-well plates. After 48 hours, cells were treated with 10 mM metformin for 24 hours or cells were incubated in fresh culture medium for 24 hours in the case of untreated cells. Following treatment, glass coverslips with adhered cells were rinsed in 1 x PBS, placed cell side down on glass slides and immediately viewed with an Olympus BX63 microscope. Images were captured at 10 x magnification in bright-field.

2.2.4. Flow Cytometry for Cell Cycle Evaluation

Propidium iodide (PI) stained DNA in OSCC cells were assessed by flow cytometry in order to determine the stage of the cell cycle in OSCC cell lines (Liu et al., 2001). Cells were evenly seeded in 10 cm cell culture dishes and after 24 hours they were treated with 10 mM metformin for a further 24 hours, or medium was replaced for untreated controls. Additionally, cells deprived of serum for 8 hours were used as positive controls for G1/G0 cell cycle arrest. Treated cells were rinsed in 1 x PBS three times, removed by trypsinisation for 5 min, two volumes fresh culture medium was added to block the effects of trypsin. Cells were counted on a haemocytometer slide, and approximately 3 x 10^6 cells were centrifuged at 3000 rpm for 10 min, culture medium was removed and cells were re-suspended in 500 µl 1 x PBS prior to analysis. The DNA Prep Reagent Kit (Beckman Coulter) was used for cell cycle analysis. An equal volume of DNA prep LPR lysis solution was added to cells and vortexed for 10 sec to permeabilize cell membranes. DNA prep stain solution composed of PI and RNAse was then added to each sample and vortexed for a further 5 sec to facilitate DNA binding of PI. Cell suspensions were then passed through the BD LSRFortessa™ cell analyser (BD Biosciences), excited with a 488nm laser and detected with a 584/42nm band pass emission filter. The data was used to create DNA histograms using FlowJo v10 software and the percentage of cells in the G0/G1, S, and G2/M phase of the cell cycle were calculated (n = 3 ± SD).

2.2.5. Western Blot for AMPK and p70S6 Kinase

Western blotting was used to detect AMPKα, phospho-AMPKα^{Thr172}, p70S6K and phospho-p70S6K^{Thr389} levels in the absence and presence of metformin in OSCC cell lines. Following treatment, cytoplasmic proteins were extracted and quantified using the Bradford assay. Proteins were resolved by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE), with a 5% stacking gel and 10% resolving gel. Proteins were then transferred onto nitrocellulose membranes, blocked and rinsed. Membranes were then incubated in primary antibodies (1 in 1000 in 5% BSA made up in 1 x TBS/T) of interest followed by incubation in the goat anti-rabbit IgG horse-radish peroxidase-linked secondary antibody (SantaCruz, sc-2004) (1/2000 in 5% fat-free milk powder made up in 1 x TBS/T). Bands were detected after exposure to chemiluminescent substrates with X-ray film. Full descriptions of these steps are detailed below.

2.2.5.1. Protein Sample Preparation

2.2.5.1.1. Treatment and Collection of Cells

Cells were evenly seeded in 100 mm cell culture dishes, after 24 hours they were treated with 10 mM metformin for 24 hours or medium was replaced for untreated controls. After treatment, cells were harvested and kept on ice throughout the procedure. Cells were rinsed in 5 ml ice cold 1 x PBS three times, scraped in 1 ml 1 x PBS and collected in 1.5 ml microcentrifuge tubes. Tubes were centrifuged at 4300 x g for 10 min at 4 °C to pellet cells, excess 1 x PBS was removed and cells were stored at -70 °C until required.

2.2.5.1.2. Preparation of lysates containing cytoplasmic proteins

Frozen cell pellets were lysed in ½ x radio-immunoprecipitation (RIPA) buffer (*25 mM Tris-HCl, pH 8.0; 75 mM NaCl; 0.05% SDS; 0.5% Triton X-100; 0.25% sodium deoxycholate*) containing inhibitors. Each of the following inhibitors were added per 1 ml RIPA buffer immediately before use: 10 µl phenylmethylsulfonylfluoride (1 mM stock solution dissolved in ethanol); 1 µl leupeptin (10 µM stock); 10 µl sodium orthovanadate (1 mM stock); 20 µl sodium fluoride (10 mM stock). Approximately 80-100 µl RIPA buffer was added to each cell pellet, samples were incubated on ice and vortexed every 5 min at high speed for 20 min to facilitate lysis of the cell membrane. Cytoplasmic fractions were used as all proteins

investigated exist in the cytoplasmic compartment. Samples were centrifuged at 9600 x g for 5 min at 4 °C to remove cell debris and nuclei. Aliquots of the supernatants containing cytoplasmic protein fractions were stored at -70 °C for further analysis.

2.2.5.1.3. Protein concentration estimation

Proteins were quantified using the method by Bradford (Bradford, 1976). For the microplate assay, standard solutions of bovine serum albumin (BSA) in the linear range of 9 – 75 µg/ml were prepared. Cell lysates from test samples with unknown concentrations were diluted by a factor of 1/100 or 1/200, blank solutions were made up of the corresponding dilutions of ½ x RIPA buffer. 160 µl of each solution was added per well in triplicate on a 96-well plate and 40 µl of Bio-Rad protein dye reagent concentrate was added to each sample (Bio-Rad). Samples were mixed by pipetting and absorbance readings at 595 nm were recorded using the MULTISKAN GO microplate reader (ThermoScientific). Absorbance values for BSA standard solutions were plotted against their respective concentrations and a linear trendline was fitted to data using MS Excel. Concentrations of unknown protein solutions were determined from equation of the standard curve.

2.2.5.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Mini-PROTEAN® Tetra Cell system (Bio-Rad 165-800) was used for SDS-PAGE. Two glass plates, one with a 1 mm spacer were sandwiched against each other in a gel cassette. A 10% resolving gel and a 5% stacking gel were prepared as detailed in Table 2.1. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added immediately before the solution was poured between the glass plates and a layer of distilled water was poured over the resolving gel to prevent evaporation. The resolving gel was allowed to set for 30 min, excess water overlay was then removed and a layer of stacking gel with freshly added APS and TEMED was poured above the resolving gel with a comb and allowed to set for a further 30 min. The SDS-PAGE gel and glass plates were then removed from the cassette and assembled with the gasket in the running tank. Running buffer (*192 mM Glycine, 25 mM Tris pH 8.8; 0.1 % SDS*) was poured between the electrode assembly and into the mini tank. Protein samples were then loaded onto the gel.

Table 2.1: Resolving gel and stacking gel recipes for SDS-PAGE.

	Resolving Gel (10 ml)			Stacking Gel (5 ml)	
	10%	**12%**	**14%**	**5%**	**7%**
ddH_20	4 ml	3.3 ml	2.63 ml	3.4	3.1 ml
29:1 Acryl/Bisacryl	3.3 ml	4.0 ml	4.7 ml	830 µl	1.2 ml
Tris (1.5M pH 8.8)	2.5 ml	2.5 ml	2.5 ml	630 µl	630 µl
10% SDS	100 µl	100 µl	100 µl	50 µl	50 µl
10% APS	100 µl	100 µl	100 µl	50 µl	50 µl
TEMED	5 µl	4 µl	4 µl	4 µl	3 µl

Proteins samples were prepared by first adding 3 parts protein to 1 part 4x loading buffer (*40% glycerol; 240 mM Tris-HCl pH 6.8; 8% SDS; 0.04% bromophenol blue, 5% β-mercaptoethanol*) in microcentrifuge tubes. Samples were placed in boiling water for 5 min before loading equal amounts of protein (µg) per well. The PageRuler^{TM} prestained protein ladder (Fermentas) was used as a molecular weight marker. Proteins were separated by electrophoresis at a constant current of 25 mA per gel till the dye front had reached the bottom of the gel (±60 min). The regions of gel containing antigens of interest were cut out and prepared for electrophoretic transfer. Excess pieces of gel were stained in Coomassie blue gel staining solution (3 *mM Coomassie blue (Merck); 50 % methanol; 10 % acetic acid*) for 1 hour and de-stained by incubating in destain solution (*10 % acetic acid; 10 % methanol*) overnight with shaking at 60 rpm at room temperature.

2.2.5.3. Electrophoretic Transfer

Proteins were transferred from the gel onto nitrocellulose with the Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad 170-3930) system. SDS-PAGE gels and nitrocellulose membranes were sandwiched between four sheets of filter paper and two fibre pads. The electrode module was set up such that the gel was closer to the negative electrode and nitrocellulose was closer to the positive electrode. The electrode module was placed in a tank with an ice pack and immersed in transfer buffer (*192 mM glycine; 25 mM Tris; 20% methanol; pH 8.3*). A current of 300 mA was passed through the system for 2.5 hours in a 4°C cold room, the current was then stopped and nitrocellulose membranes were removed and rinsed in distilled water prior to blocking.

2.2.5.4. Blocking, Antibody Binding and Detection

Nitrocellulose membranes were blocked in 5% fat-free milk powder made up in 1 x TBS/T (*20 mM Tris-HCl pH 7.6; 137 mM NaCl; 0.01 % Tween-20*) for 1 hour at room temperature with gentle agitation at 60 rpm. After blocking, membranes were washed in three changes of 1 x TBS/T with shaking at 120 rpm for 5 min each time and then incubated in primary antibody. All primary antibodies used were purchased from Cell Signaling Technology unless otherwise specified. In this chapter rabbit anti-AMPKα (#2532), rabbit anti-phospho AMPKα (Thr172) (#2531), rabbit anti-p70S6K (#2708), rabbit anti-phospho p70S6K (Thr389) (#9234) and β-actin (Santa Cruz, sc-130656) were used. Nitrocellulose membranes were incubated in primary antibodies diluted in 5% bovine serum albumin (BSA) in 1 x TBS/T (1/1000) overnight at 4 °C. Excess antibody was removed by washing membranes in cold 1 x TBS/T five times for 5 min per wash at 120 rpm, and membranes were incubated in secondary antibody (Santa Cruz, sc2004) for 1 hour at room temperature. Membranes were washed in cold 1 x TBS/T five times for 5 min per wash to remove excess secondary antibody. Excess secondary antibody was removed by washing membranes in cold 1 x TBS/T five times for 5 min per wash at 120 rpm prior to antibody detection.

Bands were detected using the SuperSignal® West Pico Chemiluminescent Substrate Kit (Thermo Scientific). Equal volumes of stable peroxide solution and luminol/enhancer solution were mixed and layered on the surface of nitrocellulose containing proteins for 5 min. Membranes were dabbed on paper towel to remove excess liquid, then covered in cling film and blue X-ray film was used to detect bands. X-ray film was then placed in developing solution (*6.4 M metol; 0.6 M sodium sulphite; 80 mM hydroquinone; 45 mM sodium carbonate; 34 mM potassium bromide*), rinsed in water and then placed in fixer solution (*0.8 M sodium thiosulphate; 0.2 M sodium metasulphite*) and rinsed in water once again. Bands were scanned with a GS-800 calibrated densitometer (Bio-Rad, 170-7980) and quantified using LabWorks 3.0 software.

2.2.8. Statistical Analysis

Results are expressed as means ± SD and statistical significance was calculated using a paired Student's t-test on MS Excel 2007. In all cases, p-value stars represent the following:

$p \leq 0.001$	***
$p \leq 0.01$	**
$p \leq 0.05$	*

2.3. Results

2.3.1. Metformin Reduces OSCC Cell Proliferation and Promotes Cell Accumulation at the G1/G0 stage

Metformin has been shown to reduce proliferation of various cancers. We aimed to determine whether metformin could also reduce proliferation of South African derived OSCC cell lines. Studies in mice show that metformin accumulates in tissues at concentrations several fold higher than blood circulating conditions, tissue accumulating concentrations are reported to be between 1 – 10 mM metformin (Martin-Castillo et al., 2010). Early studies in our laboratory showed that 10 mM metformin had the most promising effects on inhibition of cell proliferation with minimal toxicity. Therefore, the clinically attainable concentration of 10 mM metformin was used throughout this study. Direct cell counts indicated that OSCC cell proliferation was significantly reduced after 24 hour treatment with 10 mM metformin compared to untreated controls with a reduction of 18% in WHCO1, 32% in WHCO5 and 33% in SNO cell lines (Figure 2.2). Images of cells treated with metformin clearly depict the reduction in cell growth compared to untreated cells (Figure 2.3).

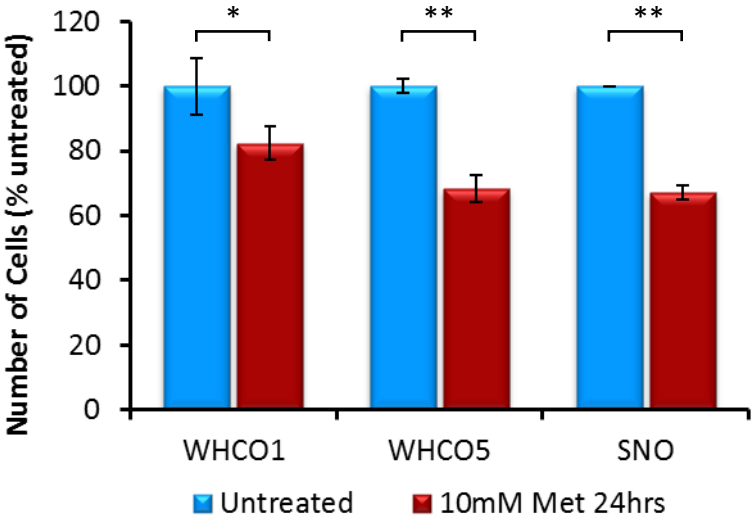


Figure 2.2: Metformin reduces OSCC cell proliferation. Cells were counted after treatment with 10 mM metformin for 24 hrs (red bars) or no treatment (blue bars). Cell numbers were represented as a percentage of untreated controls. The graph indicates that there were significantly fewer cells present after metformin treatment compared to cells that received no treatment for WHCO1, WHCO5 and SNO cell lines (n=3±SD).

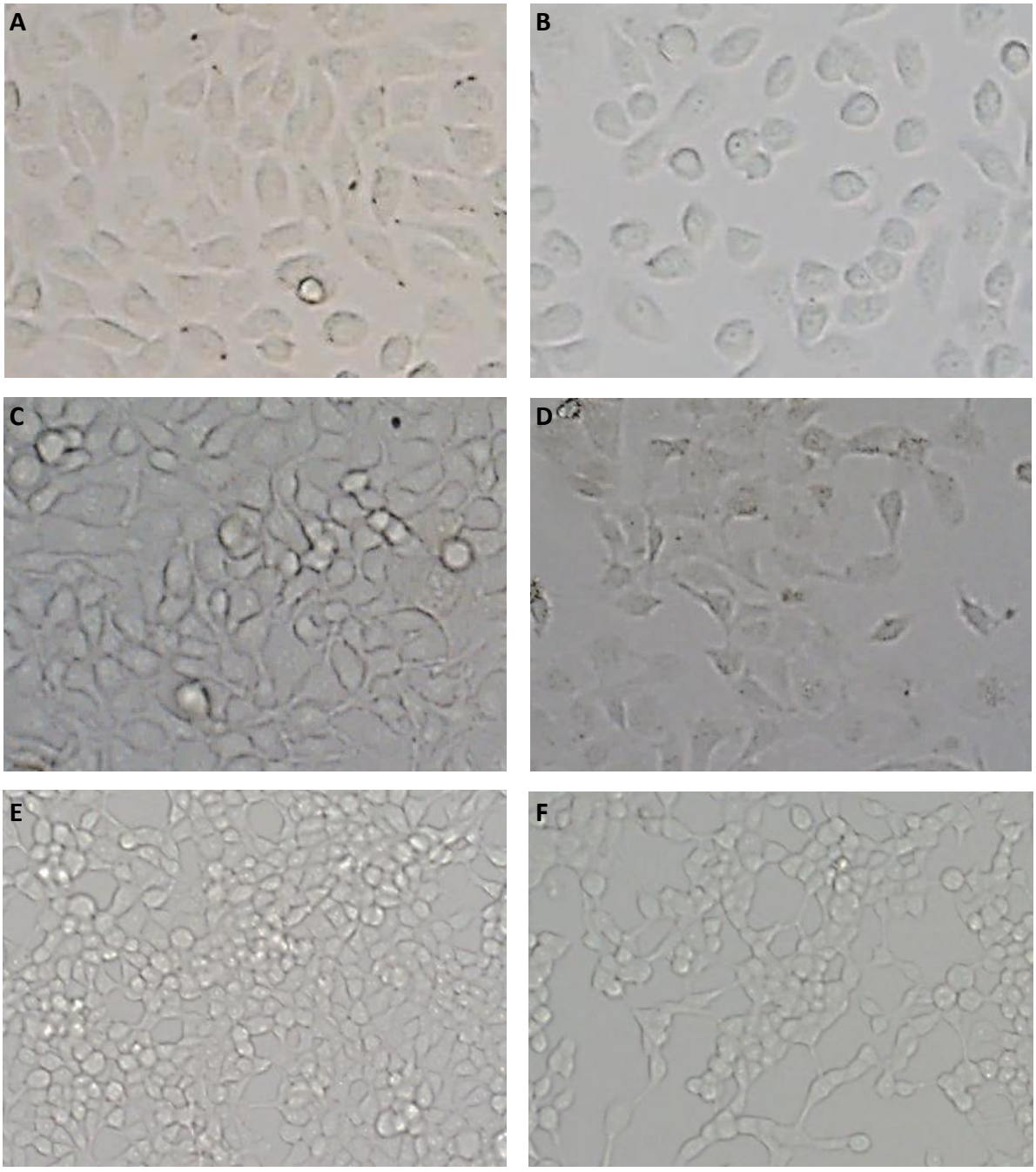


Figure 2.3: Cells are less confluent after treatment with metformin. Images of untreated cells or 10 mM metformin treated cells were captured at 10X magnification under normal light using the Olympus BX63 microscope. (A) WHCO1 untreated, (B) WHCO1 metformin, (C) WHCO5 untreated, (D) WHCO5 metformin, (E) SNO untreated, (F) SNO metformin.

The inhibitory effects of metformin on cell proliferation have been partly attributed to accumulation of cells at the G1/G0 stage of the cell cycle (Zhuang & Miskimins, 2008; Tomic et al., 2011). Flow cytometric evaluation of PI stained cells show OSCC cell accumulation at the G1/G0 stage of the cell cycle was higher after treatment with 10 mM metformin for 24 hours in WHCO1, WHCO5 and SNO cell lines (Figure 2.4). A concomitant reduction in S-phase and G2/M phase cells was also observed. Pooled data for replicates are shown in Table 2.2.

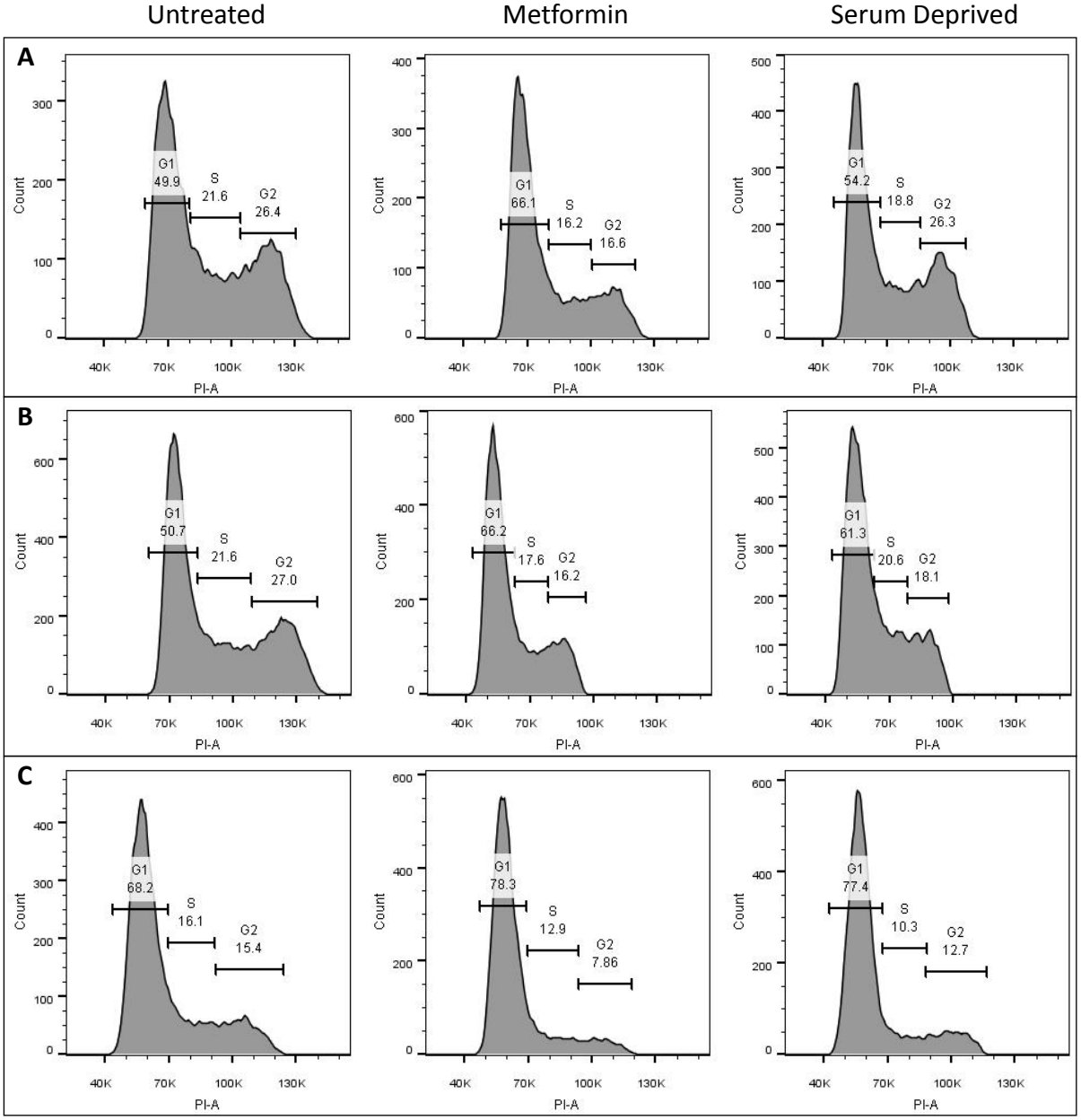


Figure 2.4: Metformin promotes cell accumulation at the G1/G0 stage. PI fluorescence per cell was measured in **(A)** WHCO1, **(B)** WHCO5 and, **(C)** SNO cell lines that were untreated **(left)**, 10 mM metformin for 24 hr treated **(centre)**, or deprived of foetal calf serum for 8 hrs **(right)**. Metformin halted cell cycle progression at the G1/G0 stage. Results for a single set of data are depicted.

Table 2.2: Metformin increases the proportion of cells in the G1/G0 stage and reduces proportion in G2/M and S stages of the cell cycle (n=3±SD).

Cell line	Stage	Cell Proportion (%)		
		Untreated	Metformin †	Serum Deprived †
WHCO1	G1/G0	50.5 ± 0.7%	61.6 ± 1.2% ***	55.2 ± 0.8% ***
	S	24.6 ± 1.1%	17.4 ± 0.9% ***	20.0 ± 2.0% *
	G2/M	24.4 ± 0.5%	21.1 ± 1.9% *	23.4 ± 1.6% ns
WHCO5	G1/G0	52.5 ± 2.3%	64.0 ± 1.9% **	62.0 ± 2.0% **
	S	21.2 ± 0.7%	17.8 ± 0.3% **	18.5 ± 2.9% ns
	G2/M	26.1 ± 2.7%	18.2 ± 1.7% **	18.4 ± 0.4% *
SNO	G1/G0	67.5 ± 2.5%	80.4 ± 1.7% **	76.5 ± 2.6% **
	S	16.1 ± 0.7%	9.9 ± 2.4% *	11.4 ± 1.4% **
	G2/M	16.3 ± 2.5%	11.2 ± 1.8% *	12.1 ± 1.7% *

† Stars represent P-value significance for metformin treatment compared to untreated cells or serum deprived cells compared to untreated cells.

There was a significant increase in the proportion of cells in the G1/G0 stage and a reduction in G2/M and S stage cells with metformin treatment. This result therefore supports other studies that have also shown increased accumulation of cells in the G1/G0 stage of the cell cycle in response to metformin treatment (Buzzai et al., 2007; Tomic et al., 2011). AMPK activation and inhibition of p70S6K as a result of hampered mTOR signalling may contribute to the effects of metformin on OSCC cell lines. We next evaluated the effects of metformin on these two members of these signalling pathways as their regulation plays a key role in cell growth or growth inhibition.

2.3.2. Metformin increases phospho-AMPK^{Thr172} levels

Metformin mediated cell cycle arrest at the G1/G0 stage was shown to be dependent on AMPK in breast cancer cells (Zhuang & Miskimins, 2008). AMPK has also been shown to inhibit the mTOR signalling pathway, which may contribute reduced cell proliferation (Bolster et al., 2002). The effects of metformin on AMPK in OSCC cell lines was evaluated by determining levels of P-AMPKα^{Thr172} and comparing these to total AMPKα levels in untreated and metformin treated cells. There was a slight increase in total AMPK levels with metformin treatment in OSCC cell lines. A significant increase in P-AMPK^{Thr172} levels were observed presence of metformin in all three OSCC cell lines tested (Figure 2.5).

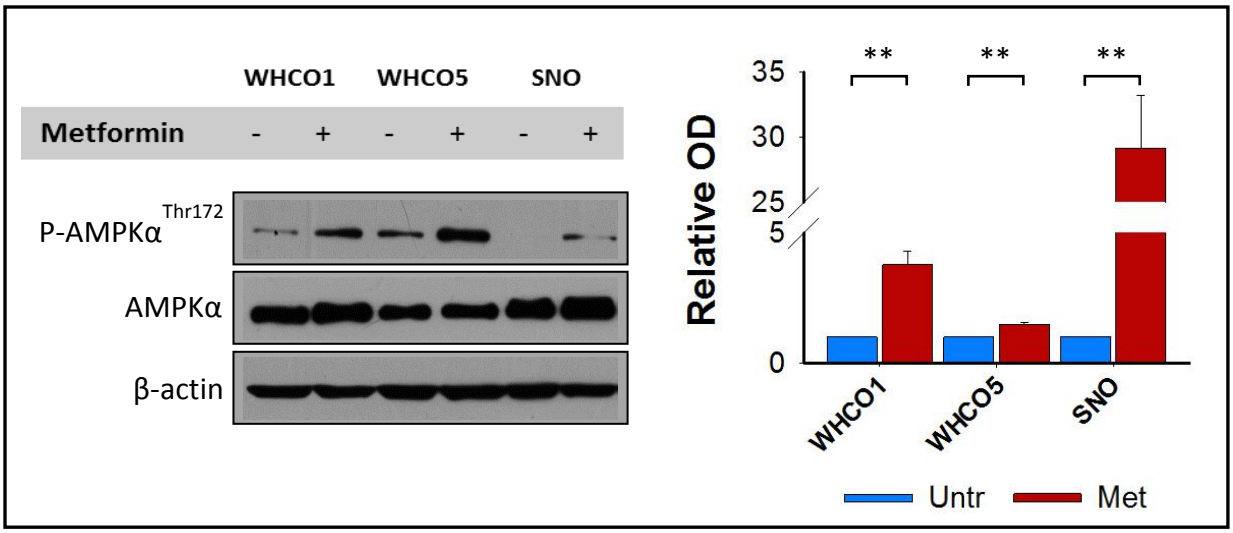


Figure 2.5: Metformin increases P-AMPKα^{Thr172} levels in OSCC cell lines. Cytoplasmic protein extracts from OSCC cells that were untreated or 10 mM metformin treated for 24 hrs were used in the detection of P-AMPKα^{Thr172}, total AMPKα and β-actin levels by western blotting. Relative optical densities indicate levels of P-AMPKα^{Thr172} compared to total AMPKα relative to their respective untreated controls. P-AMPKα^{Thr172} levels were higher in metformin treated cells (red bars) compared to untreated controls (blue bars) in WHCO1, WHCO5 and SNO cell lines.

2.3.3. Metformin reduces phospho-p70S6K^{Thr389} levels

Signalling via the mTORC1 pathway is upregulated in many cancers and activation of its downstream targets promotes cell growth and proliferation (Zoncu, Efeyan & Sabatini, 2011). Activation of mTOR promotes protein synthesis and cell growth by activating p70S6K, which promotes translation of proteins required for ribosomal biogenesis (Wullschleger, Loewith & Hall, 2006). Therefore, the mTOR-p70S6K signalling axis promotes cell growth and proliferation. Metformin was shown to directly inhibit p70S6K activity in breast cancer cells (Vazquez-Martin, Oliveras-Ferraros & Menendez, 2009). Additionally, activation of AMPK suggested that the mTOR-p70S6K signaling axis could be inhibited in the presence of metformin. We investigated the effects of metformin on p70S6K activity in OSCC cell lines and as expected, we found that metformin reduced P-p70S6K^{Thr389} in the OSCC cell lines (Figure 2.6). Total p70S6K were not significantly altered in the presence of metformin.

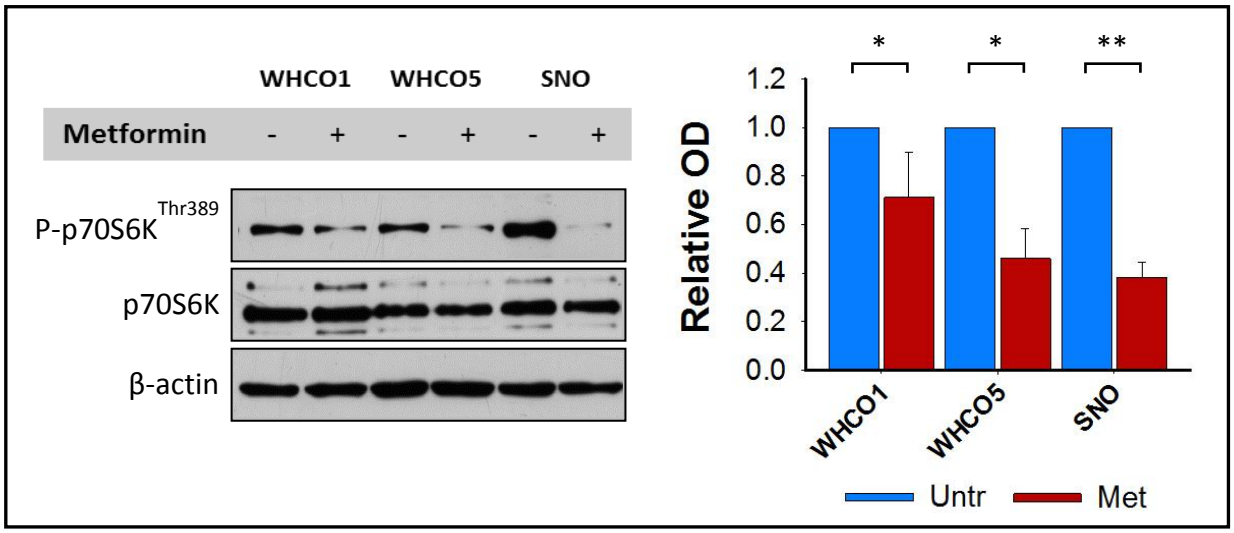


Figure 2.6: Metformin reduces P-p70S6K^{Thr389} levels in OSCC cell lines. Cytoplasmic protein extracts from OSCC cells that were untreated or 10 mM metformin treated for 24 hrs were used in the detection of P-p70S6K^{Thr389}, total p70S6K and β-actin levels by western blotting. Relative optical densities were expressed as P-p70S6K^{Thr389} compared to p70S6K with respect to corresponding untreated controls. P-p70S6K^{Thr389} levels were significantly lower in metformin treated cells (red bars) compared to untreated controls (blue bars) in WHCO1, WHCO5 and SNO cell lines.

2.4. Discussion

We have shown that metformin reduces the proliferation of OSCC cell lines. This was attributed to cell accumulation at the G1/G0 stage of the cell cycle. Reduced proliferation is a promising result as it suggests that metformin could be used to halt OSCC progression prior to surgical resection for early stage cancer. Metformin treatment also resulted in increased activation of AMPK and inhibition of mTOR signalling as indicated by reduced p70S6K phosphorylation. The AMPK and mTOR signalling pathways play critical roles in the cells response to nutrient deprivation and stress. Both AMPK and mTOR signalling pathways are frequently deregulated in cancer as cancer cells must bypass the ability to sense nutrient deprivation yet retain the ability to promote cell proliferation. Increased AMPK activation and inhibition of mTOR by metformin may serve as means to normalise signalling via these pathways and promote cell senescence.

We show in this study that metformin increased AMPK activation in OSCC cell lines. However, we cannot confirm whether AMPK is required for the anti-proliferative effects of metformin from these findings alone. The anti-proliferative effects of AMPK are partly attributed to p53-mediated activation of $p21^{cip}$ (Motoshima et al., 2006). However the proliferation of SNO cells, which harbour a mutated p53, was still significantly reduced and AMPK was also activated in response to metformin (Fanucchi & Veale, 2009). This indicates that p53 activation via AMPK signalling may not play a significant role in the anti-proliferative effects of metformin in OSCC cell lines. Western blot evaluation of P-$p53^{Ser15}$ levels could be used to confirm the involvement of p53 in metformin-induced cell cycle arrest. AMPK activation is also associated with inhibition of the mTOR signalling pathway and inhibition of mTOR-p70S6K signalling may play a prominent role in the anti-proliferative effects of metformin in these cell lines. Further evaluation of the expression levels of other components of this pathway such as $p27^{kip}$ and $p21^{cip}$, cyclin D1, E2F, REDD1 and 4E-BP will allow for a better understanding of the way metformin alters these pathways in OSCC.

We found that metformin inhibited p70S6K in OSCC cell lines. Protein translation is critical for cell survival and proliferation, p70S6K is of critical importance as it plays a role in promoting the translation of proteins required for ribosomal biogenesis (Bjornsti & Houghton, 2004). Inhibition of p70S6K in response to metformin may therefore be central to

its anti-proliferative effects. Previous studies indicate that metformin can inhibit mTOR independently of AMPK (Kalender et al., 2010; Ben Sahra et al., 2011). Further investigations would be required to determine whether p70S6K inhibition is dependent on or occurs independently of AMPK in OSCC cell lines. Inhibition of p70S6K with metformin in breast cancer cells was associated with reduced levels of the Her2 receptor (Vazquez-Martin, Oliveras-Ferraros & Menendez, 2009). Her2 is up-regulated in many cancers including OSCC and over-expression of this growth factor receptor is associated with poor prognosis (Mimura, Kono, Hanawa, Mitsui, et al., 2005). Therefore, inhibition of p70S6K is a promising effect that can be harnessed for chemotherapy.

Metformin reduced OSCC cell proliferation, but was not toxic at 10 mM, which has been shown to be the highest clinically achievable concentration (Martin-Castillo et al., 2010). It is possible that transient exposure to higher doses of metformin may exert cytotoxic effects that could be exploited for cancer therapy. However, these doses are not clinically achievable via oral administration. Additionally, the side-effects of this type of administration are not yet known and further studies are required to determine whether this type of application will be effective with *in vivo* studies. Drug administration via non-conventional routes such as intravenous injection, topical ointments for skin cancer or suppositories for rectal cancer may potentially be used to obtain higher drug concentrations at the site of the cancer (Menendez et al., 2014). We have proven that metformin reduces OSCC cell proliferation at a dose that is clinically achievable and this corresponds with a recent study investigating the effects of metformin on OSCC cell lines (Kobayashi et al., 2013). To exploit the anti-proliferative effects of metformin for cancer therapy, it can be combined with cytotoxic agents in order to offer a more effective therapeutic response.

Chapter 3: Metformin Negatively Alters the Effects of Cisplatin in OSCC Cell Lines

3.1. Introduction

Cisplatin is one of the most widely prescribed chemotherapeutic drugs and is used for the treatment of many different cancers including oesophageal, non-small cell lung, breast, cervical, stomach and bladder cancers (Griffiths et al., 2011; Kitagawa et al., 2012; Koo et al., 2012; Lee et al., 2012; Paz-Ares et al., 2012; Byrski et al., 2014). Cisplatin has been used since the 1980's either as a monotherapy or combination therapy agent for the treatment of OSCC (Leichman, 1989). Despite its widespread use, cisplatin can lead to severe renal toxicity which is a clinical limitation and major setback to continued treatment or the prescription of high doses. For this reason, there is an ongoing search for drugs that can enhance the anti-cancer effects of cisplatin or reduce cisplatin-induced toxicity (Pinzani et al., 1994). Over 3000 analogues of cisplatin have been synthesised. Of these, only carboplatin showed benefit over cisplatin with regards to its toxicity profile, but was less effective in terms of tumour response (Weiss & Christian, 1993; Stewart, 2007). In addition to renal toxicity, many patients treated with cisplatin acquire resistance to the drug (Perez, 1998).

Cisplatin resistance occurs when cancer cells continue to survive in the presence of the drug. Cisplatin exerts cytotoxic effects by forming covalent bonds with nitrogen atoms on DNA bases. These platinum-DNA adducts hamper DNA replication and promote activation of DNA damage response pathways (Jamieson & Lippard, 1999). Alterations in DNA damage response signalling can therefore contribute to cisplatin resistance. Mutations in DNA damage recognition machinery allow cancer cells to by-pass DNA damage check-points and continue to proliferate, whereas over-induction of DNA damage response pathways leads to increased repair of platinum-DNA adducts. Both of these effects contribute to cisplatin resistance. Conversely, mutations in DNA repair pathways can enhance the sensitivity to cisplatin due to reduced repair of platinum-DNA adducts (Basu & Krishnamurthy, 2010). Aside from alterations in DNA damage signalling, cisplatin resistance can also be caused

when there is reduced cisplatin entry into the nucleus due to altered drug sequestration or increased drug export.

Although DNA binding is thought to be the primary mechanism by which cisplatin exerts cytotoxic effects, only a small percentage of cisplatin that enters the cell binds to DNA. This may be explained by cross reactivity of cisplatin with other cell components such as RNA, proteins, membrane phospholipids and cytoskeletal microfilaments (Gonzalez et al., 2001). Platinum-RNA adducts can also disrupt cellular processes that involve the RNA to which it binds (Hostetter, Osborn & DeRose, 2012). In addition to its high affinity for nitrogen atoms, cisplatin can also form covalent bonds with sulfhydryl groups, such as those in cysteine containing proteins. DNA-protein cross-links mediated by cisplatin have been implicated in DNA synthesis inhibition (Chválová, Brabec & Kaspárková, 2007). Cisplatin treatment leads to the collapse of the cytoskeleton due to it binding thiols in tubulin and to proteins that interact with cytoskeletal components (Köpf-Maier & Mühlhausen, 1992). Cisplatin, in its aquated form, can also bind to phosphatidylserine moieties of the plasma membrane, which may disrupt downstream signalling by membrane proteins (Speelmans et al., 1996). Although many of these alternative targets of cisplatin could contribute to its anti-cancer effects, they may also act to sequester cisplatin in the cytoplasm and limit its interaction with DNA, thereby limiting its cytotoxicity.

Binding of cisplatin to intracellular thiols such as glutathione (GSH), metallothionein or thioredoxin is a major factor that contributes to cisplatin resistance. Increased glutathione levels correlated with cisplatin resistance in ovarian cancer cell lines, along with elevated levels of γ-glutamylcysteine synthetase, which is required for GSH synthesis (Godwin et al., 1992). Conversely, over-expression of glutamate cysteine ligase, also required for GSH synthesis, increased cisplatin sensitivity in small-cell lung cancer cell lines and this was attributed to up-regulation of the copper transporter which is involved in cisplatin uptake (Chen et al., 2008). A plausible mechanism for cisplatin resistance was discovered when it was shown that cytoplasmic GSH can bind to cisplatin and target the drug for export via glutathione-S conjugate (GS-X) pumps in leukaemia cells (Ishikawa & Ali-Osman, 1993). This leads to reduced cisplatin-DNA binding and therefore promotes drug resistance. Cisplatin is also able to bind with the cysteine-rich protein, metallothionein, which is involved in heavy metal detoxification. Higher levels of metallothionein were correlated with cisplatin

resistance in ovarian cancer cell lines (Andrews, Murphy & Howell, 1987). Another redox active small protein, thioredoxin, also reduced cisplatin sensitivity when it was over-expressed in bladder and prostate cancer cell lines (Yokomizo et al., 1995). The level of intracellular reduced thiols or thiol containing anti-oxidant molecules may therefore play an important role in resistance to cisplatin.

The primary function for intracellular GSH is the detoxification of reactive oxygen species (ROS) and GSH levels are maintained via the reducing potential of NADPH (Figure 3.1). The ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG), respectively, is reported to be between 100:1 and 300:1 (Dooley et al., 2004). The pentose phosphate pathway (PPP) links glycolysis to the GSH redox system. $NADP^+$ is converted to NADPH during reactions catalysed by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), thus providing NADPH which is required for the reduction of GSSG (Mehta, Mason & Vulliamy, 2000).

Metformin promotes glycolysis as increased lactate levels have been observed in diabetic patients treated with metformin and higher amounts of glycolytic intermediates were found in livers of metformin treated rats (Owen, Doran & Halestrap, 2000). Increased levels of glycolysis can also promote PPP flux as glucose-6-phosphate (G6P), produced during the first step of glycolysis, can be shunted into the PPP for the production of ribose-5-phosphate which is essential for DNA replication. This leads to increased NADPH production which promotes the reduction of GSSG to GSH, contributing to a further increase the intracellular reducing potential. These effects of metformin on GSH metabolism may therefore impact on cisplatin mediated cytotoxicity.

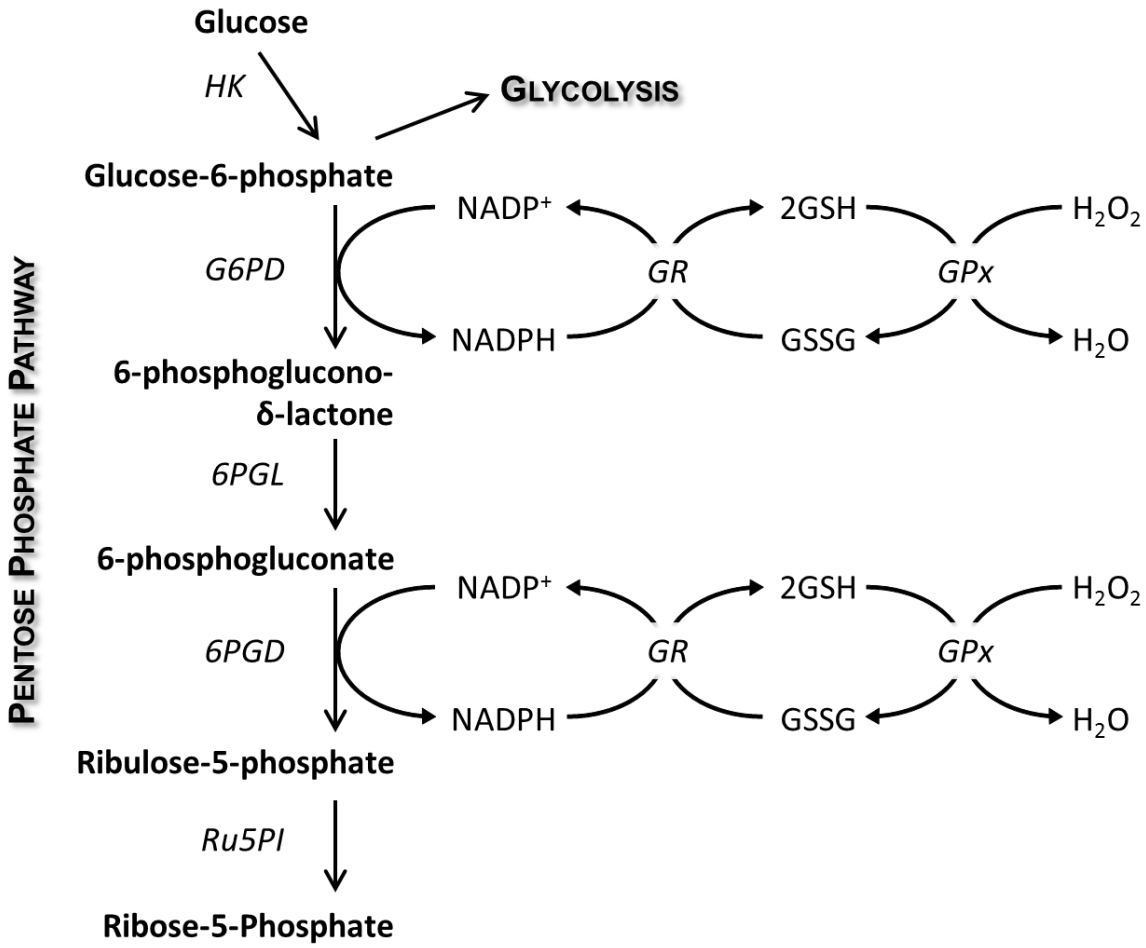


Figure 3.1: Relationship between glucose metabolism and the glutathione regulation. G6P produced during the first step of glycolysis can be shunted into the PPP. $NADP^+$ is utilised as a co-factor during the oxidative reactions of the PPP, thus providing NADPH which is subsequently utilised for the reduction of GSSG to GSH. GSH plays an important role in ROS detoxification, and increased GSH also contributes to cisplatin resistance. Hexokinase, HK; glucose-6-phosphate dehydrogenase, G6PDH, 6-phosphogluconolactonase, 6PGL; 6-phosphogluconate dehydrogenase, 6PGD; ribulose-5-phoshate isomerase, Ru5PI; nicotinamide adenine dinucleotide phosphate, NADP; nicotinamide adenosine dinucleotide phosphate, NADPH; reduced glutathione, GSH; oxidised glutathione, GSSG; glutathione reductase, GR; glutathione peroxidase, GPx.

There are numerous studies that have evaluated the effects of metformin combined with cisplatin for cancer therapy and these have shown both positive and negative effects. Metformin enhanced the effects of cisplatin in ovarian cancer cell lines and ovarian cancer xenografts (Gotlieb et al., 2008; Rattan et al., 2011). Metformin also augmented cisplatin cytotoxicity in melanoma cells, but it reduced cisplatin toxicity in glioma, neuroblastoma and leukaemia cell lines (Janjetovic et al., 2011). Metformin was also shown to enhance the effects of cisplatin in lung cancer cell lines, independent of the AMPK signalling pathway and via a pathway that interfered with ROS generation (Lin et al., 2013). A recent study showed that metformin antagonised the effects of cisplatin in gastric cancer cell lines, and this was shown to involve Akt upregulation (Lesan et al., 2014). Therefore, it is evident that the effects of metformin combined with cisplatin may differ depending on the cancer type.

In this chapter, we investigated the effects of metformin in combination with cisplatin on OSCC cytotoxicity. We found that metformin decreased the cytotoxic effects of cisplatin and was associated with reduced platinum-DNA adducts. Metformin also increased glycolysis and the reducing potential of OSCC cell lines. We show that metformin increased intracellular thiols and that excessive thiols contribute to cisplatin resistance in OSCC cell lines. Therefore, metformin should not be combined with cisplatin for OSCC therapy.

3.2. Methods

3.2.1. Cytotoxicity Assessment by MTT assay

The effects of metformin on cisplatin mediated toxicity in OSCC cell lines were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cells were evenly seeded (7500 cells per well) in 96-well plates and allowed to settle. On the following day they were either treated with 10 mM metformin for 24 hours or medium was replaced for cells that did not receive metformin. This was followed by treatment with cisplatin (linear range 0 – 400 µM) for a further 48 hours, either in the presence or absence of 10 mM metformin.

Following treatment, cell culture medium was removed and replaced with 100 µl of 0.5 mg/ml MTT solution diluted in culture medium and incubated at 37 °C for 1.25 hrs. This allowed for the reduction of MTT to formazan by viable cells. After incubation, 80 µl of the MTT solution was removed, 20 µl was left in the well to ensure formazan crystals were not disturbed, 100 µl dimethyl sulfoxide (DMSO) was then added per well to dissolve formazan crystals. Plates were incubated at 37 °C for 5 minutes, followed by 10 min of shaking in the dark at room temperature.

Absorbance readings at 570 nm were recorded using the MULTISKAN GO microplate reader (n = 3 ± SD). Raw absorbance values expressed as a percent of the control, indicative of cell viability, were plotted against drug concentration. SigmaPlot 11.0 was used fit sigmoidal dose response curves to the data and to calculate the lethal drug concentration required for 50% cell death (LC50) for each drug treatment.

3.2.2. Platinum Content in DNA Quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The cytotoxic action of cisplatin is mainly attributed to its ability to form cisplatin-DNA adducts. In order to determine whether metformin alters cisplatin-DNA adduct formation, total genomic DNA was extracted from OSCC cells and platinum content in DNA was quantified by inductively coupled plasma mass spectrometry (ICP-MS). OSCC cells were treated with LC30 concentrations of cisplatin for 48 hours either with or without prior exposure to 10 mM metformin for 24 hours.

Cells were harvested and collected as previously described (section 2.2.5.1.1.) and genomic DNA was immediately isolated using a phenol: chloroform extraction method. Cells in 50 µl 1x PBS were resuspended in 450 µl genomic lysis buffer (*10mM Tris pH 8; 100mM EDTA pH8; autoclaved; 0.5% SDS in nuclease-free water*) and incubated at 37 °C for 1 hour. Proteinase K (Fermentas, EO0491) was added to a final concentration of 100 µg/ml and samples were incubated at 50 °C for 2 hours with swirling every 20 min in order to degrade proteins. An equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol was added to each sample, vortexed on medium speed for 5 sec and then centrifuged at 12 500x g for 20 min at room temperature to allow for phase separation. The upper aqueous phase was removed into a clean tube and the previous step was repeated. An equal volume of 24:1 chloroform: isoamly alcohol was then added to remove excess phenol and samples were centrifuged at 12 500x g for 20 min at room temperature once again.

Two volumes of 100% ethanol and a tenth of the volume of 3M sodium acetate (pH 5.2) was added to each sample in order to precipitate DNA. Samples were gently mixed and incubated at -20 °C for 24 hours. Samples were centrifuged at 10 000x g for 5 min at 4 °C to pellet DNA precipitate, and pellets were resuspended in 70% ethanol in order to wash DNA. After centrifugation to collect DNA, excess ethanol was removed, and pellets were dried and resuspended in nuclease-free water. DNA solutions were quantified using the NanoDrop spectrophotometer (Thermo Scientific, ND1000).

For ICP-MS, equal amounts of DNA (40 µg) were hydrolysed in 1% HNO_3 at 70°C for 24 hours and analysed for platinum content on an Agilent 7700 ICP-MS (n = 3 ± SD). ICP-MS was performed as a charged service by Riana Rossouw at Stellenbosch University. The instrument

was optimised for sensitivity and low oxides. Analysis was done in no-gas mode, and the instrument was calibrated for platinum analysis using National Institute of Standards and Technology traceable standards.

3.2.3. Lactate Quantification for Evaluation of Glycolysis

Glycolysis levels were determined by quantifying lactate secreted in culture medium by the lactate dehydrogenase assay (Cowley et al., 2004). During the conversion to lactate to pyruvate via lactate dehydrogenase, NAD^+ is utilized as a cofactor which results in NADH release at a rate that is directly proportional to the concentration of lactate. NADH strongly absorbs light at 340 nm whereas NAD^+ does not. Cells were either not treated or treated with 10 mM metformin for 24 hours after which culture medium and cells were collected. Cells were counted as described in section 2.2.2. For lactate quantification, 50 µl of culture medium was added to 950 µl glycine-hydrazine buffer (*640 mM glycine, 640 mM hydrazine, 4.8 mM NAD^+, 16 U/ml lactate dehydrogenase, pH 9.2*) and incubated at 37°C for 2 minutes. NADH was then quantified spectrophotometrically at 340 nm and values corrected for cell number (n = 3 ± SD).

3.2.4. MTT Assay to Determine Reducing Potential in the Presence of Metformin

The effect of metformin on the reducing potential of OSCC cell lines was measured using the MTT assay. Cells were evenly seeded in 96-well plates (8500 cells/well) and allowed 24 hours to settle. Cells were then treated with 10 mM metformin for a further 24 hours or medium was replaced for untreated controls. For the MTT assay, cells were treated with 100 µl of a 0.5 mg/ml MTT solution made up in cell culture medium for 1 hour. Once the precipitate had formed, 80 µl of the supernatant was removed from each well to ensure that the formazan precipitate was not disturbed and 100 µl dimethyl sulfoxide (DMSO) was added per well in order to dissolve formazan. Plates were incubated at 37 °C in the dark for 5 min, followed by 15 min shaking at room temperature. Absorbance values at 570 nm were recorded using the Multiskan GO microplate spectrophotometer (Thermo Scientific, 51119200). Absorbance values were corrected against live cells counted from replicate wells using Trypan blue exclusion as described in section 2.2.2.

3.2.5. Low Molecular Weight Thiol levels Determined by Monobromobimane Fluorescence

Cisplatin can bind to intracellular thiols which reduces its efficacy due to altered drug sequestration and reduced DNA binding. Metformin was shown to increase reducing potential in OSCC cell lines, and the intracellular redox state is tightly controlled by the thiol redox defence system. We hypothesised that metformin contributes to cisplatin resistance by modulating intracellular thiols. Monobromobimane was used to quantify levels of reduced low molecular weight thiols determined by the fluorescence of thiol-bimane adducts.

Cells were evenly seeded in 10 cm culture dishes and allowed 24 hours to settle. They were then treated with 10 mM metformin for 24 hours or medium was replaced for untreated controls. Cells were then treated with 0.3mM monobromobimane made up in DMSO for 10 min and harvested as previously described. Cells were lysed in triple detergent lysis buffer (*50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate*), then centrifuged at 10 000x g for 5 min to remove cell debris and 100 µl of the resulting supernatants were used in the assay.

Samples were analysed using the Ascent multi-well plate fluorimeter (Thermo Scientific) at excitation/emission wavelengths of 360/460 nm. Fluorescence readings were corrected against cell number (n = 3 ± SD) for cells seeded in parallel dishes that were counted using trypan blue exclusion as described in section 2.2.2.

3.2.6. The Effect of Glutathione Depletion by Buthionine Sulfoximine on Cisplatin Toxicity

We showed that metformin increased intracellular thiols in OSCC cell lines. Glutathione is the major intracellular thiol and may play a role in the negative effects of metformin on cisplatin cytotoxicity. In order to determine whether the observed increase in thiols in response to metformin treatment negatively alters the effects of cisplatin, cytotoxicity of cisplatin or metformin and cisplatin was assessed during glutathione (GSH) depletion by buthionine sulfoximine (BSO). BSO acts to deplete GSH by inhibiting γ-glutamyl cysteine ligase, which is required for GSH synthesis (Griffith, 1982).

An equal number of cells were seeded (7500 cells per well) in a 96-well plate and allowed 18 hours to settle. They were then treated with 10 mM metformin for 18 hours or 10 mM metformin combined with 0.4 mM BSO for 18 hours; followed by the addition of 12 fold dilutions of cisplatin (400 – 0 μM) for 36 hours. Control cells were untreated, treated with 10 mM metformin or treated with metformin and BSO for the entire duration. Cytotoxicity was then evaluated using the MTT assay as described in section 3.2.1 (n = 3 ± SD).

3.2.7. The Effect of Thiol Repletion with N-acetyl-L-cysteine on Cisplatin Toxicity

In order to confirm that increased thiols contribute to cisplatin resistance in OSCC cell lines, the effects of increased intracellular thiols on the cytotoxicity of cisplatin was determined using the MTT assay. N-acetyl-L-cycteine (NAC) is a cell permeable cysteine derivative that was used to increase intracellular thiol pools. Cytotoxicity assays were performed as previously described (section 3.2.1.). Cells were evenly seeded at 7500 cells/well, after 24 hours they were treated with 10 mM NAC for 24 hours or replaced with fresh culture medium. They were then treated with a cisplatin or 10 mM NAC and cisplatin for a further 48 hours and the MTT assay was performed.

3.3. Results

3.3.1. Metformin increases resistance to cisplatin in OSCC cell lines by reducing platinum-DNA adduct formation.

In chapter 1 we showed that metformin exerts anti-proliferative effects on OSCC cell lines, but it was not cytotoxic at the concentration used. We combined metformin with cisplatin and evaluated cytotoxicity in OSCC cell lines. MTT assay data indicated that metformin reduced the toxic effects of cisplatin on OSCC cell lines (Figure 3.2) as LC50 values were higher when 10 mM metformin was combined with cisplatin (Table 3.1). This indicates that metformin negatively alters the response to cisplatin in OSCC cell lines. The percentage increase in LC50 for metformin cisplatin treatment compared to cisplatin alone was 77 ± 6% for WHCO1, 128 ± 65% for WHCO5 and 173 ± 38% for SNO.

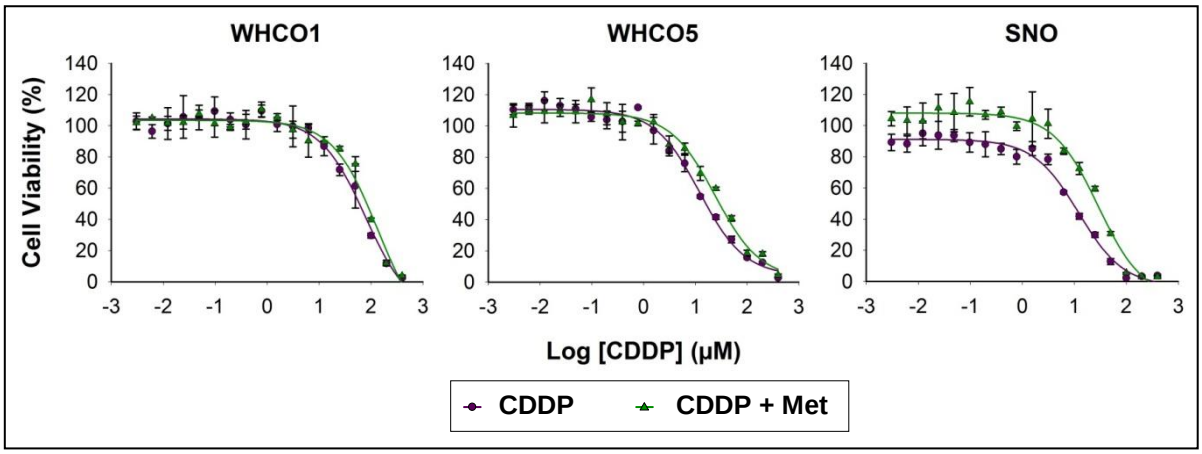


Figure 3.2. Cytotoxicity curves indicate that metformin causes resistance to cisplatin. Cells were treated with 18 fold dilutions of cisplatin (CDDP) for 48 hrs or pre-treated with 10 mM metformin (Met) for 24 hrs followed by 48 hr co-treatment with metformin and cisplatin. A) MTT assay data expressed as a percent of respective controls are depicted on the graphs and sigmoidal dose response curves were fitted to the data (n=3 ± SD).

Table 3.1: Metformin antagonises the cytotoxicity of cisplatin. LC50 values were higher when metformin was combined with cisplatin compared to cisplatin alone (n=3 ± SD), which confirms that metformin promotes cisplatin resistance.

	LC50 (µM CDDP)		
	WHCO1	**WHCO5**	**SNO**
CDDP	70.88 ± 13.80	11.68 ± 3.62	11.01 ± 1.62
CDDP + Met	126.02 ± 26.57	28.03 ± 15.81	28.16 ± 3.37
P-value	0.009	0.075	0.001

Although resistance to cisplatin is not desirable from a chemotherapeutic perspective, it was important to determine the mechanisms responsible for the altered susceptibility to cisplatin in the presence of metformin as both these drugs are already being prescribed for the treatment of cancer and diabetes. Diabetes is associated with an increased risk of cancer and as such, it is possible that these drugs may be co-prescribed to individuals suffering from both cancer and diabetes. By determining the mechanisms of metformin-induced cisplatin-resistance, it may also be possible to identify other cancer types that could become cisplatin-resistant.

Since cisplatin primarily exerts cytotoxic effects by forming platinum-DNA adducts, we quantified platinum levels in DNA extracted from cells treated with cisplatin alone or metformin combined with cisplatin. Platinum content in DNA was lower when metformin was combined with cisplatin compared to cisplatin alone in all three OSCC cell lines tested (Figure 3.3). There was a 19 ± 0.9% decrease in the WHCO1 cell line, a 14 ± 0.4% decrease in the WHCO5 cell line and a 27 ± 4.9% decrease in the SNO cell line.

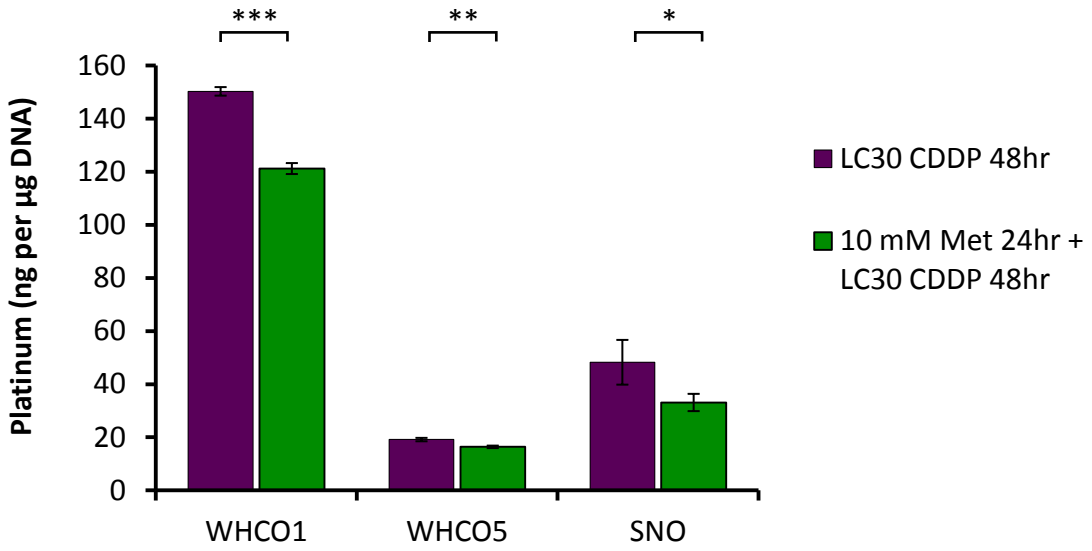


Figure 3.3: Metformin reduces platinum-DNA adduct formation. Cells were treated with cisplatin alone for 48 hours or pre-treated with 10 mM metformin for 24 hours followed by treatment with 10 mM metformin combined with cisplatin for 48 hours. Platinum content in genomic DNA extracted from these samples were quantified by ICP-MS. Results showed that platinum levels were significantly lower when metformin was combined with cisplatin compared to cisplatin alone (n=3 ± SD). Therefore, metformin reduces cisplatin toxicity by inhibiting platinum-DNA adduct formation.

ICP-MS data indicates that the addition of metformin led to a reduction in the formation of platinum-DNA adducts in OSCC cell lines. Therefore, metformin antagonized the effects of cisplatin by inhibiting its primary mechanism of cytotoxicity. We hypothesised that reduced formation of platinum-DNA adducts in the presence of metformin may be attributed to reduced drug entry into the nucleus due to altered drug sequestration. Due to the thiol binding properties of cisplatin, we investigated whether metformin may increase the pool of reduced thiols which contribute to cisplatin resistance.

3.3.2. Metformin promotes glycolytic flux and increases the reducing potential of OSCC cell lines.

Enhanced glycolysis contributes to the glucose lowering effects of metformin in diabetes and may also be responsible for the increased serum lactate levels observed in diabetic patients. Increased glycolysis has been observed in prostate cancer cells treated with metformin (Ben Sahra, Laurent, et al., 2010). Enhanced glycolysis contributes to increased PPP flux, and this may alter the intracellular redox state due to alterations in the $NADP^+$/NADPH ratio. Lactate levels serve as a marker of glycolysis. Hence lactate secreted into cell culture medium after OSCC cell lines were treated with metformin were quantified. Metformin treatment resulted in higher lactate secretion, indicative of increased glycolysis in OSCC cell lines (Figure 3.4).

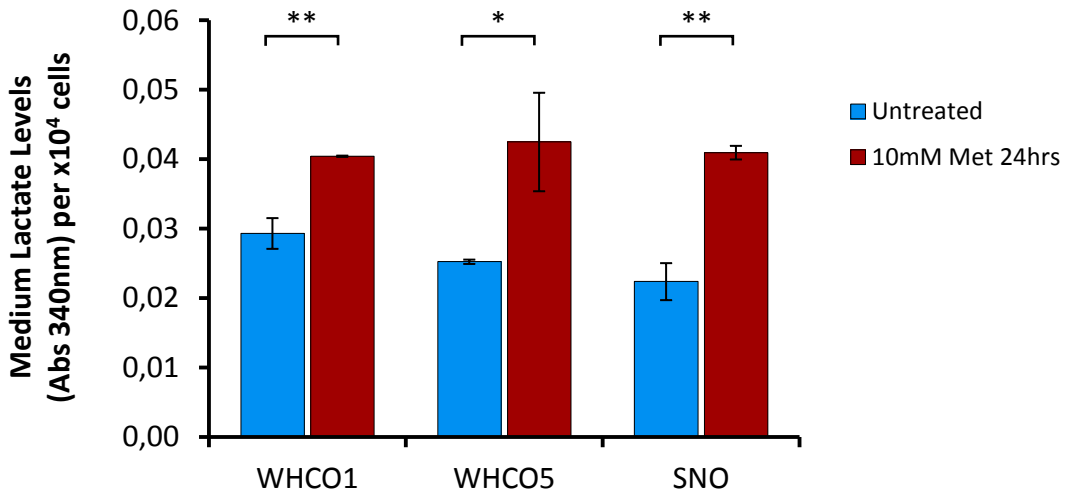


Figure 3.4: Metformin increases glycolysis levels as determined by medium lactate levels. Lactate and NAD^+ are converted to pyruvate and NADH in the presence of LDH. Lactate levels in culture medium of cells that were not treated or treated with 10 mM metformin for 24 hrs are indicated by NADH absorbance values at 340 nm. Absorbance values were corrected against cell number and represented above, untreated (blue bars); metformin (red bars) (n=3±SD). Increased lactate secreted by OSCC cell lines after metformin treatment show that metformin increases glycolysis.

Next, we determined whether metformin could alter the reducing potential of OSCC cell lines as G6P shunted into the PPP can contribute to an increase in the NADPH/$NADP^+$ ratio, which would consequently increase the reducing potential of OSCC cell lines. The

intracellular reducing environment was determined based on the ability of OSCC cell lines to reduce MTT to formazan in the absence or presence of metformin. We found that metformin increased the intracellular reducing environment in all three OSCC cell lines (Figure 3.5)

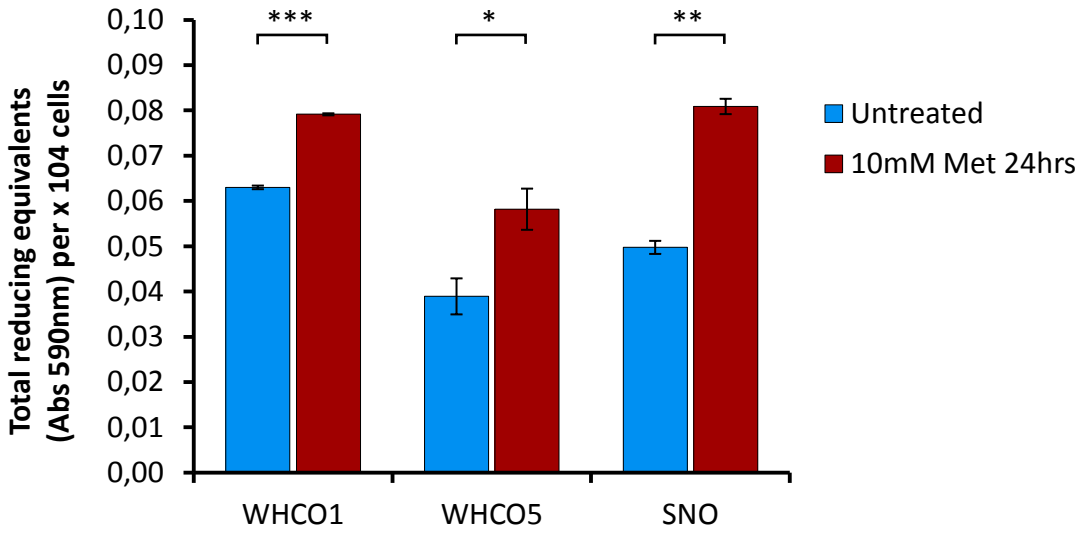


Figure 3.5: Metformin increases reducing equivalents in OSCC cell lines. Reducing potential was measured in OSCC cell lines in the absence and presence of 10 mM metformin for 24 hrs by quantifying the reduction of MTT to formazan. Absorbance values (590 nm) of formazan produced by cells were corrected for cell number to represent total reducing equivalents per x 10^4 cells. Data indicated that metformin treated cells had a higher reducing potential than untreated cells (n=3±SD).

3.3.3. Modulation of intracellular thiols by metformin contributes to cisplatin resistance.

Metformin increased glycolytic flux and also increased the reducing potential of OSCC cell lines. We hypothesised that increased glycolysis may increase PPP flux and NADPH levels which fuels the reduction of GSSG to GSH. This contributes to cisplatin resistance by reducing cisplatin-DNA binding due to altered drug sequestration or increased export (Ishikawa & Ali-Osman, 1993).

Levels of reduced intracellular thiols after 24 hour treatment with 10 mM metformin were quantified and compared to that of untreated controls. Metformin increased intracellular reduced thiol levels as monobromobimane fluorescence was higher in metformin treated samples (Figure 3.6). For each cell line, fluorescence per 1 x 10^4 cells in untreated and metformin treated cells, respectively, were WHCO1 - 4.3 ± 0.13 and 8.8 ± 0.28; WHCO5 – 5.4 ± 0.36 and 9.3 ± 0.27; SNO – 6.5 ± 0.18 and 11.8 ± 0.62. We concluded that increased levels of intracellular reduced thiols may alter the sequestration of cisplatin which reduces the formation of platinum-DNA adducts and contributes to cisplatin resistance in OSCC cell lines.

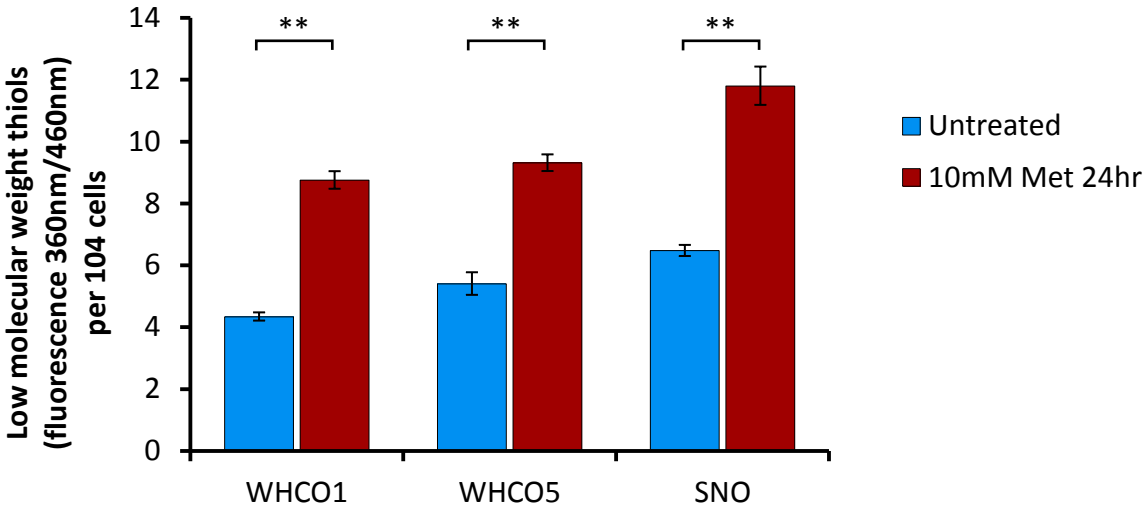


Figure 3.6: Metformin increases the level of reduced low molecular weight thiols in OSCC cell lines. Untreated (blue bars) and 10 mM metformin treated cells (red bars) were treated with 0.3 mM monobromobimane for 10 min and fluorescence was recorded (excitation 360 nm/emission 460 nm). Cells exhibited higher fluorescence in the presence of metformin indicating higher levels of reduced molecular weight thiols.

In order to confirm that thiols were involved in the mechanism of resistance to cisplatin in the presence of metformin, we sought to determine the effects of buthionine sulfoximine (BSO) on cisplatin and metformin. We considered using BSO as it inhibits synthesis of the major intracellular thiol, glutathione (GSH). Cytotoxicity was evaluated in cells treated with cisplatin only, cisplatin plus BSO, cisplatin and metformin or cisplatin and metformin plus BSO. We found that thiol depletion blocked the protective effects of metformin on cisplatin chemotoxicity (Figure 3.7). Predictably, thiol depletion enhanced toxicity of cisplatin as lower LC50 values were observed.

LC50 values for cisplatin and metformin combined with BSO were much lower compared to cisplatin and metformin without BSO. In fact, LC50 values for metformin and cisplatin therapy combined with BSO were very similar to that of cisplatin alone, which shows that GSH is involved in the metformin-induced cisplatin resistance. Glutathione depletion completely abolished the chemoprotective effects of metformin when combined with cisplatin, which indicates that glutathione plays a significant role in metformin induced cisplatin resistance.

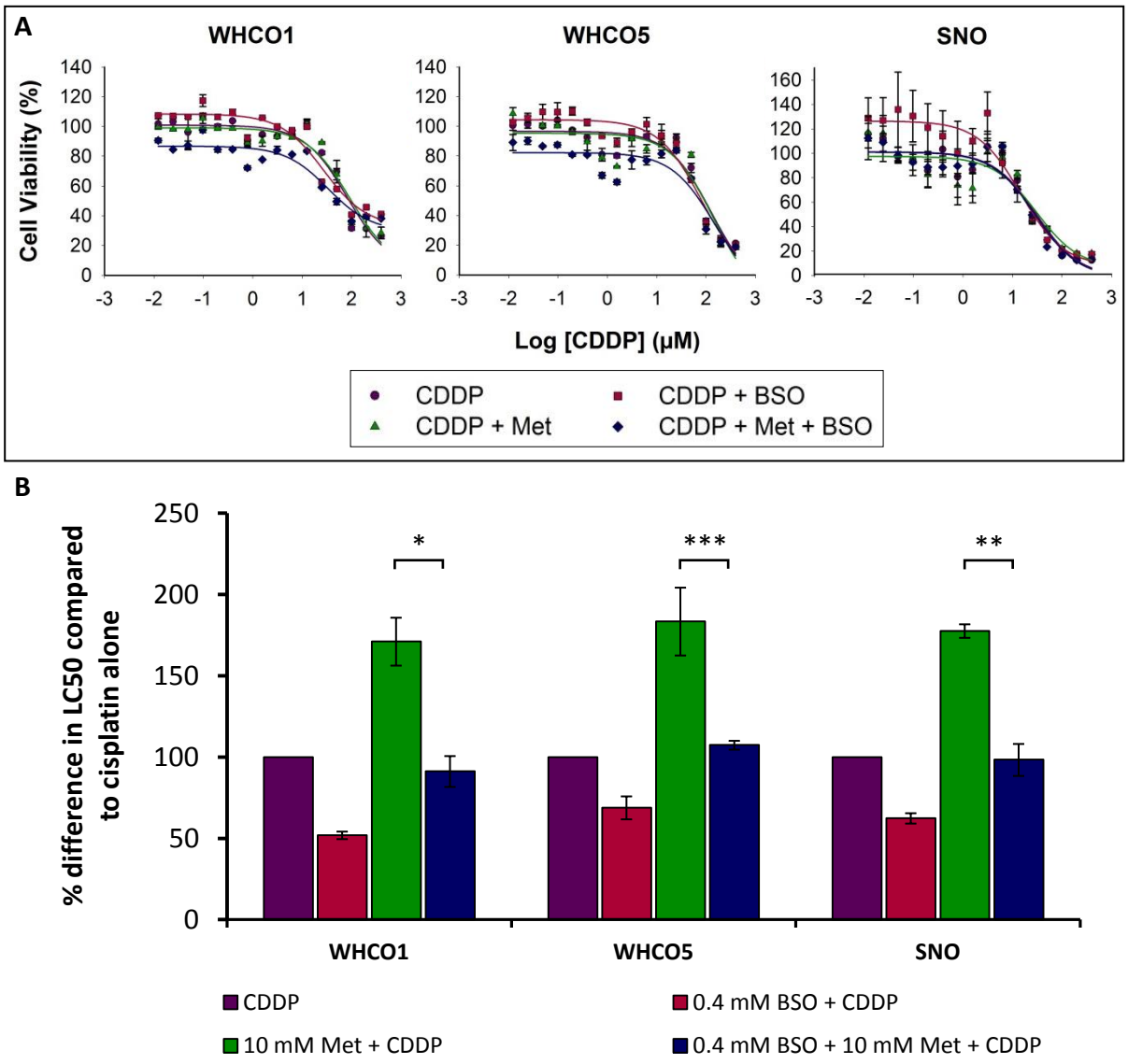


Figure 3.7: Cisplatin resistance in the presence of metformin is reversed by glutathione inhibition. Cells were treated with 0-400μM CDDP for 36 hrs; 0.4 mM BSO plus CDDP; 10 mM metformin and CDDP; or 0.4 mM BSO, 10 mM metformin and CDDP. Cytotoxicity was then evaluated using the MTT assay. **A)** MTT assay curves display the effects of the various drug combinations on OSCC cell lines. **B)** LC50 values were expressed as a percentage of cisplatin alone and depicted on the graph. As expected, BSO enhanced the toxicity of CDDP alone. LC50 values for BSO, metformin and CDDP treatment were reduced to similar levels as CDDP alone which indicates that BSO abolished metformin induced cisplatin resistance in OSCC cell lines.

In order to confirm that increased levels of intracellular thiols is a plausible mechanism of resistance to cisplatin in OSCC cell lines, higher levels of intracellular thiols were induced with N-acetly-L-cysteine (NAC) and the effects of this treatment on cisplatin-mediated cytotoxicity was measured with the MTT assay. Cells treated with NAC combined with cisplatin exhibited higher LC50 values compared to cells treated with cisplatin only, as seen by the considerable rightward shift in toxicity curves for NAC treated cells (Figure 3.8). The percentage change in LC50 compared to cisplatin alone is represented in Table 3.2. As expected, NAC increased LC50 values in OSCC cell lines.

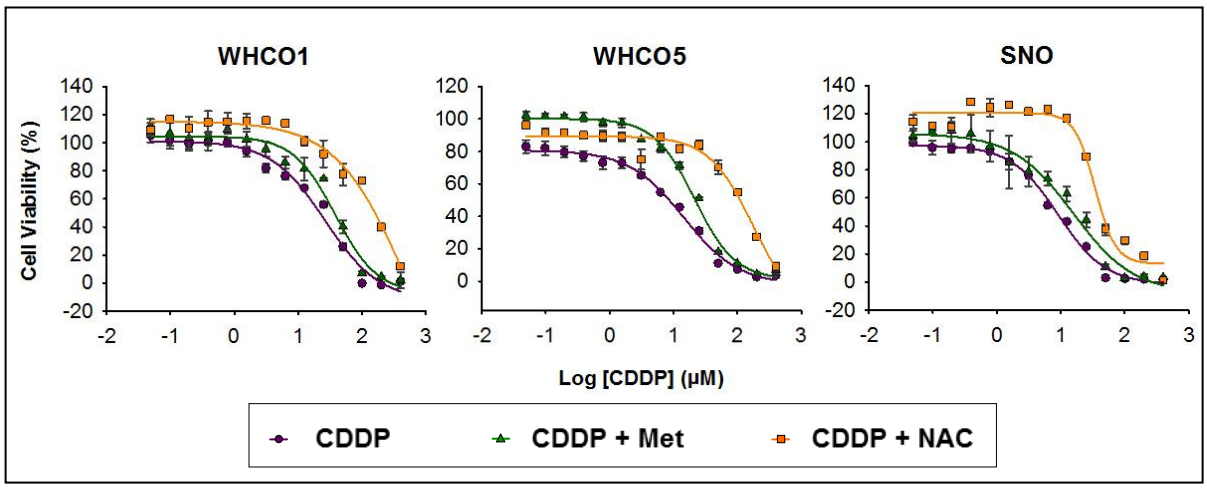


Figure 3.8: Increased thiols promote cisplatin resistance in OSCC cell lines. Cytotoxicity was evaluated by MTT assay in cells treated with 0-400 μM CDDP, CDDP plus 10 mM metformin or CDDP plus 10 mM NAC. LC50 values were higher when CDDP was combined with 10 mM NAC. MTT assay graphs depict a rightward shift in orange curves compared to purple curves. Metformin and NAC both increase LC50 values with regard to CDDP, indicating that increased intracellular thiols contribute to cisplatin resistance and metformin induced CDDP resistance may be due to increased thiols.

Table 3.2: Percentage change in LC50 values indicate that cisplatin-induced toxicity is reduced in the presence of NAC. NAC reduced cisplatin induced toxicity by 3- to 12-fold in OSCC cell lines, which indicates that higher levels of intracellular thiols are a major factor contributing to cisplatin resistance. Metformin also caused resistance to cisplatin, but to a lower extent than NAC alone.

		WHCO1	WHCO5	SNO
% Increase in LC50 with respect to CDDP	**CDDP**	100 ± 1.2	100 ± 12.8	100 ± 4.5
	CDDP + 10 mM Met	145 ± 13.2	147 ± 2.5	174 ± 5.0
	P-Value	0.011	0.015	5×10^{-5}
	CDDP + 10 mM NAC	591 ± 71.6	1253 ± 317.8	380 ± 23.7
	P-Value	0.003	0.013	0.002

The significant reduction in cisplatin-mediated toxicity proves that intracellular thiols play a major role in cisplatin resistance in these OSCC cell lines. Metformin did not reduce the toxic effects of cisplatin to the same extent as NAC, which is expected as the cysteine based molecule contains a thiol group. Metformin did, however increase the levels of low molecular weight reduced thiols in OSCC cell lines. Therefore, increased levels of reduced thiols provides a valid explanation for the reduced toxicity of cisplatin in the presence of metformin.

3.4. Discussion

We show in this chapter that metformin diminished the cytotoxic effects of cisplatin in OSCC cell lines. Studies by Janjetovic et al. support this finding as they showed that metformin antagonized the toxicity of cisplatin in numerous other cancer cell lines (Janjetovic et al., 2011). They suggested that reduced toxicity of cisplatin occurred because metformin treatment results in hyper-activation of Akt independent of AMPK. Hyperactivation of Akt is known to promote tumour survival and stimulate glycolysis (Elstrom et al., 2004). Additionally, complex I inhibition by metformin could lead to reduced oxidative phosphorylation, which increases the AMP:ATP ratio and up-regulates glycolysis via AMPK (Ben Sahra, Marchand-Brustel, et al., 2010). We showed that metformin also increased glycolysis in OSCC cell lines. Increased glycolysis can provide G6P which fuels the PPP and promotes the reduction of $NADP^+$ to NADPH. NADPH also acts as a co-factor for the reduction of glutathione, and this promotes resistance to cisplatin.

Studies have shown that ovarian cancer cell lines that have increased GSH levels are highly resistant to cisplatin (Godwin et al., 1992). Cell lines isolated from cisplatin-resistant patients also displayed elevated GSH, however, increased GSH did not correlate with cisplatin-resistance in all cancer types as some cell lines that display cisplatin resistance do not have increased GSH levels (Kelland, 1993). Other thiol containing molecules such as metallothionein and thioredoxin are also associated with cisplatin resistance (Andrews, Murphy & Howell, 1987; Yokomizo et al., 1995). We showed that metformin increased levels of low molecular weight reduced thiols in OSCC cell lines and this contributes to cisplatin resistance.

We concluded that GSH plays an important role in metformin induced cisplatin resistance as co-treatment with BSO abolished metformin induced cisplatin resistance. GSH depletion enhanced the toxicity of cisplatin and increased thiol levels achieved by treatment with NAC negated the effects of cisplatin in OSCC cell lines. This result provides conclusive evidence in support of the hypothesis that metformin negatively alters the cytotoxic effects of cisplatin by increasing the levels of intracellular reduced thiols. Although this finding is not beneficial from a cancer treatment perspective, it is important to report that caution should be used if these drugs are co-prescribed for diabetes and cancer.

Increased levels of reduced thiols and glycolysis in response to metformin also support our finding that metformin increases the reducing potential of OSCC cell lines. Although metformin did not work well with cisplatin, its anti-proliferative effects make it an attractive drug for use with other chemotherapeutic drugs. We have shown that metformin does not significantly alter the effects of mitomycin C, which is also commonly prescribed for OSCC in South Africa (Damelin et al., 2014). Preliminary data from our laboratory also indicate that metformin does not enhance the effects of 5-fluorouracil, which is also prescribed for OSCC. Given the fact that metformin inhibits the proliferation of OSCC cell lines, and that it is less toxic than most currently prescribed chemotherapeutic drugs, it is a promising agent for the treatment of OSCC. Since we showed that metformin increased the reducing potential of OSCC cell lines, we aimed to determine whether it would work well in combination with drugs that are activated in reducing environments.

Chapter 4: Metformin promotes the effects of anti-cancer agents which are activated in reducing environments

4.1. Introduction

We have proven that metformin exerts chemostatic effects in OSCC cell lines and other studies have shown that it has cytotoxic effects against cancer stem cells (Hirsch et al., 2009; Song et al., 2012). Due to its low toxicity profile, as evidenced by its frequent prescription for diabetes and the fact that it has demonstrated chemotherapeutic potential, metformin is a promising agent for the treatment of OSCC. Whilst we have shown that metformin induces cisplatin resistance (Damelin et al., 2014), we determined that metformin increased the reducing potential in OSCC cell lines. For this reason, the effects of metformin in combination with reductively activated drugs on OSCC cell lines will be evaluated in this chapter. The drugs tested include two copper bis(thiosemicarbazones), diacetyl bis(thiosemicarbazonato) copper(II) (Cu-ATSM) and glyoxal bis(thiosemicarbazonato) copper(II) (Cu-GTSM), and disulfiram (DSF).

4.1.1. Copper Bis(thiosemicarbazones)

Bis(thiosemicarbazones) are biologically active tetradentate ligands that chelate transition metals such as Cu(II). Chelates of these coordination complexes have a neutral charge and are therefore lipophyllic and membrane permeable (Paterson & Donnelly, 2011). Copper bis(thiosemicarbazones) exert toxic effects primarily by promoting intracellular copper accumulation. When copper bis(thiosemicarbazones) enter a cancer cell, cupric ions (Cu^{2+}) are reduced to cuprous ions (Cu^{1+}), which become trapped and accumulate within the cell resulting in cytotoxic effects due to general copper toxicity and DNA damage. The cuprous ions are easily re-oxidised in normal cells due to the higher oxygen content, unlike tumour cells which are often hypoxic and this adds to the tumour specificity of copper bis(thiosemicarbazones) (Palanimuthu et al., 2013). Copper accumulation results in cell poisoning due to inhibition of DNA synthesis and oxidative phosphorylation as well as the

oxidation of thiols to form disulfides (Paterson & Donnelly, 2011). Cupric ions exert DNA damage by binding to DNA bases and cycling between cupric and cuprous states in the presence of reducing agents such as ascorbic acid and glutathione, this promotes the formation of hydroxyl free radicals, an additional factor contributing to DNA damage (Samuni et al., 1983).

Cu-ATSM is a promising anti-cancer agent as it is tolerated *in vivo* and shows hypoxia selectivity. Due to it hypoxia selectivity, ^{64}Cu-ATSM is currently undergoing clinical trials (NCT00794339) to be used as an imaging agent in positron emission tomography to detect hypoxic regions in tumours (Vāvere & Lewis, 2007). Tumour hypoxia is a characteristic feature of highly aggressive solid tumours and increased hypoxia is associated with drug resistance and resistance to radiotherapy. Over-expression of the hypoxia-inducible transcription factor, which is activated in response to hypoxia, is a prognostic feature of OSCC and correlates with the stage and severity of the cancer (Ping et al., 2014). Therefore, Cu-ATSM may show beneficial anti-cancer effects toward OSCC. Cu-GTSM has been shown to induce cell cycle arrest, followed by apoptosis in a neuroblastoma cell line (Bica et al., 2011). Both Cu-ATSM and Cu-GTSM and other bis(thiosemicarbazones) have been shown to exert anti-cancer effects by inhibiting DNA and RNA synthesis (Palanimuthu et al., 2013). These effects are likely due to bis(thiosemicarbazone) mediated inhibition of uridine and thymidine incorporation into RNA and DNA, respectively (Bhuyan & Betz, 1968). The effects of copper bis(thiosemicarbazones) in OSCC have not been previously characterised and therefore, they were investigated in this study.

4.1.2. Disulfiram

DSF is a member of the dithiocarbamate family of drugs and is a heavy metal chelating agent with particularly high affinity for copper (Johansson, 1992). DSF can be metabolised to various hydrolysis products (Figure 4.1). Upon reduction, DSF is broken down to its monomer, diethyldithiocarbamate (DDC). DDC is unstable and readily breaks down to form diethylamine and carbon disulfide. Two molecules of DDC can combine with copper to form the more stable bis(diethyldithiocarbamato)copper(II) ($Cu(DDC)_2$) complex. DSF can also directly chelate copper to corm the copper disulfiram (Cu-DSF) complex.

DSF is approved by the FDA for treatment of alcoholism, it does not have any notable side effects in the absence of alcohol and has demonstrated anti-cancer effects at doses much lower than those currently prescribed for the treatment of alcohol abuse (Chen et al., 2006). *In vitro* studies suggest that the anti-cancer effects of DSF are dependent on copper as Cu-DSF exerted high levels of cytotoxicity toward glioblastoma cell lines, whereas DSF alone did not show any effective cytotoxic response (Liu et al., 2012). On the other hand, an *in vivo* study indicated that while copper enhanced the effects of DSF, an effective chemotherapeutic response toward neuroblastoma and glioblastoma xenografts in nude mice was observed without additional copper (Rae et al., 2013). DSF was shown to reduce the growth of breast tumours *in vivo*, but did not exert harmful effects toward normal breast tissue (Daniel et al., 2005). Cancer tissues have been shown to have elevated copper levels and this may partly explain why DSF exerts potent cytotoxicity toward malignant cells, but not toward normal cells (Rizk & Sky-Peck, 1984). High copper levels are associated with increased angiogenesis which is a hallmark of cancer and is contributes to tumour progression (Brewer, 2001). DSF has been shown to inhibit angiogenesis, which is another factor contributing to its anti-cancer effects (Shian et al., 2003).

Numerous copper-binding compounds such as 8-hydroxyquinoline and chloroquine have been shown to act as proteasome inhibitors and inducers of apoptosis (Daniel et al., 2007). Protein degradation is required for the recycling of old, damaged or unused proteins and in the regulation of short-lived proteins such as those which play a role in cell cycle regulation. As such, protein degradation is essential for cell survival and proliferation. Cancer cells have been shown to have increased proteasomal activity and an increased reliance on autophagy (Chen & Madura, 2005; O'Donovan, O'Sullivan & McKenna, 2014). As such, proteasome inhibition is known to be an important target for cancer therapy and this has led to the advent of proteasome targeting drugs, such as bortezomib which target the proteasome (Nencioni et al., 2006). DSF and its analogue, pyrrolidine dithiocarbamate, have both been shown to inhibit proteasome activity in breast cancer cells, but only when complexed with copper (Daniel et al., 2005; Chen et al., 2006). Interestingly, these compounds did not inhibit proteasome function in normal breast cancer cells (Daniel et al., 2005). We have shown in this study that another analogue of DSF, di-N-propyl dithiocarbamate also inhibits proteasome activity. The proteasome is a promising target for cancer therapy and inhibition

of proteasome activity by DSF is considered to be one of the major mechanisms contributing to its anti-cancer effects.

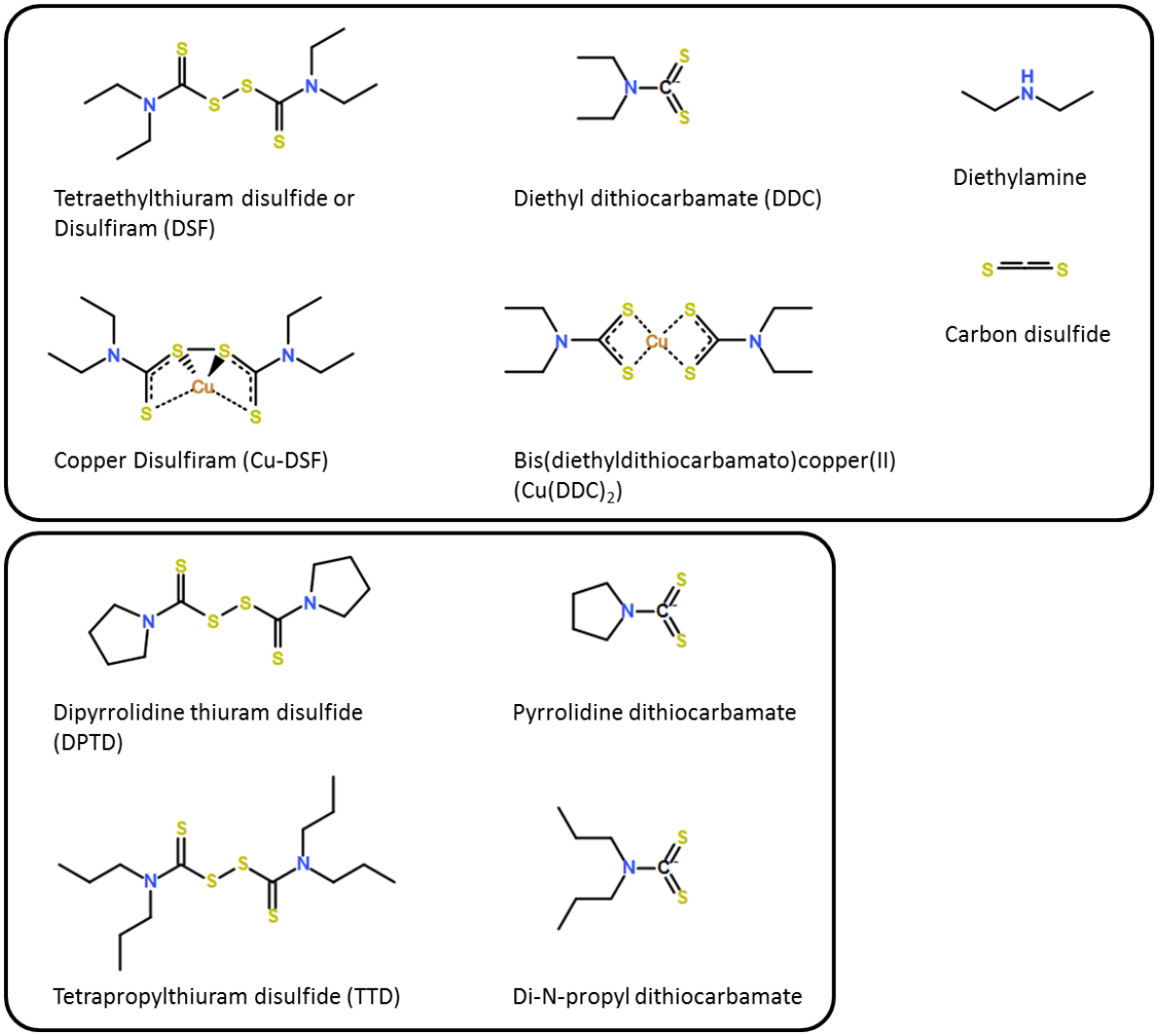


Figure 4.1: Disulfiram, its metabolites and analogues. DSF is reduced to form DDC, which can be further reduced to form diethylamine and carbon disulfide. DSF can bind copper directly to form the Cu-DSF complex, alternatively, two molecules of DDC can combine to chelate copper to form the $Cu(DDC)_2$ complex. The effects of DSF were compared to two thiuram disulfide analogues, DPTD and TTD. The monomeric structures of these molecules are also represented.

4.1.3. Protein Degradation Pathways

There are two major pathways known to mediate protein degradation in eukaryotic cells, the ubiquitin-proteasome system (UPS) and lysosomal proteolysis (summarised in Figure 4.2). The UPS is responsible for the degradation of 80-90% of proteins in eukaryotic cells such as regulated, short-lived, and damaged proteins. It involves the attachment and ATP dependent activation of ubiquitin, a 76 amino acid polypeptide, to an activating enzyme, E1. Activated ubiquitin is then transferred to a conjugating enzyme, E2, and then to the target protein via an E3 ubiquitin ligase. This leads to the formation of a polyubiquitin chain on the substrate, which is then recognised and targeted for degradation by the 26S proteasome (Lilienbaum, 2013). The target substrate then unfolds and is cleaved by the protesomal enzymes which include two trypsin-like, two chymotrypsin-like and two caspase-like proteolytic sites that each recognise and cleave proteins at specific amino acid sequences (Heinemeyer et al., 1997). Substrates are cleaved into short peptides of 3 - 25 amino acids in length. Studies have shown that copper binding agents such as disulfiram, pyrrolidine dithiocarbamate, clioquinol and 8-hydroxyquinoline act as proteasome inhibitors when they are in complex with copper and that these agents are able to induce apoptosis in tumour cells known to contain elevated copper levels (Daniel et al., 2005).

Lysosomes are organelles that are involved in the degradation of proteins and other macromolecules (Figure 4.2). These membrane enclosed structures contain hydrolytic enzymes which are active in an acidic pH. The intralysosomal pH is maintained at 4.6 - 5.0 by a proton pumping ATPase in the lysosomal membrane. Lysosomes receive and degrade macromolecules from the autophagic, endocytic, phaogocytic and secretory pathways. (Luzio, Pryor & Bright, 2007). Although lysosomes are responsible for the degradation of fewer proteins than the UPS, they are active in most cells and are involved in multiple proteolytic pathways which include macroautophagy, chaperone mediated autophagy, microautophagy and endocytosis (Dice, 2007).

Macroautophagy is initiated in response to environmental stresses such as nutrient deprivation, hypoxia, radiation or anti-cancer drug treatment. This triggers activation of AMPK and inhibition of mTOR, which promotes the formation of an autophagy (ATG) protein complex. The complex then binds to a portion of membrane believed to originate from the

endoplasmic reticulum and promotes the formation of an isolation membrane. The isolation membrane then elongates to form a phagophore, this process involves the ATG5-ATG12 complex and microtubule light chain protein (LC3). During macroautophagy, pro-LC3 is first cleaved at its C-terminus to form LC3-I followed by conjugation with the lipid phosphotidylethanolamine (PE) moiety to form LC3-II (Mizushima, Yoshimori & Levine, 2010). The phagophore then engulfs target substrates such as old or unused proteins and organelles. Once the vesicle closes to form the autophagosome and the ATG complex is released, the autolysosome fuses with a lysosome and the contents of this acidic vesicular organelle (AVO) including LC3 are degraded (Yang & Klionsky, 2010).

Chaperone mediated autophagy is activated during long term starvation. During this process, substrate proteins are directed to the lysosome by a molecular chaperone complex. The chaperone complex then recognises and binds to a receptor on the lysosomal membrane followed by import and degradation of the substrate in the intralysosomal lumen (Dice, 2007). Microautophagy can be selective or non-selective and is involved in the degradation of cytosolic components containing proteins as well as membrane enclosed organelles such as peroxisomes, mitochondria and nuclei. Microautophagy is distinct from other autophagic pathways as it involves invagination of the lysosomal membrane followed by engulfment and scission of the vesicle (Mijaljica, Prescott & Devenish, 2014). In addition to the autophagic pathways, substrates are also targeted to the lysosome via the endosomal pathway. Extracellular proteins such as hormones and receptor proteins are absorbed into the cytoplasm and form an endosome. Endosomes may enfold and engulf other vesicles to form a multivesicular body which then fuses with a lysosome resulting in the degradation of its components (Dice, 2007). These pathways highlight the importance of lysosomes in the recycling and effective turnover of both proteins and organelles.

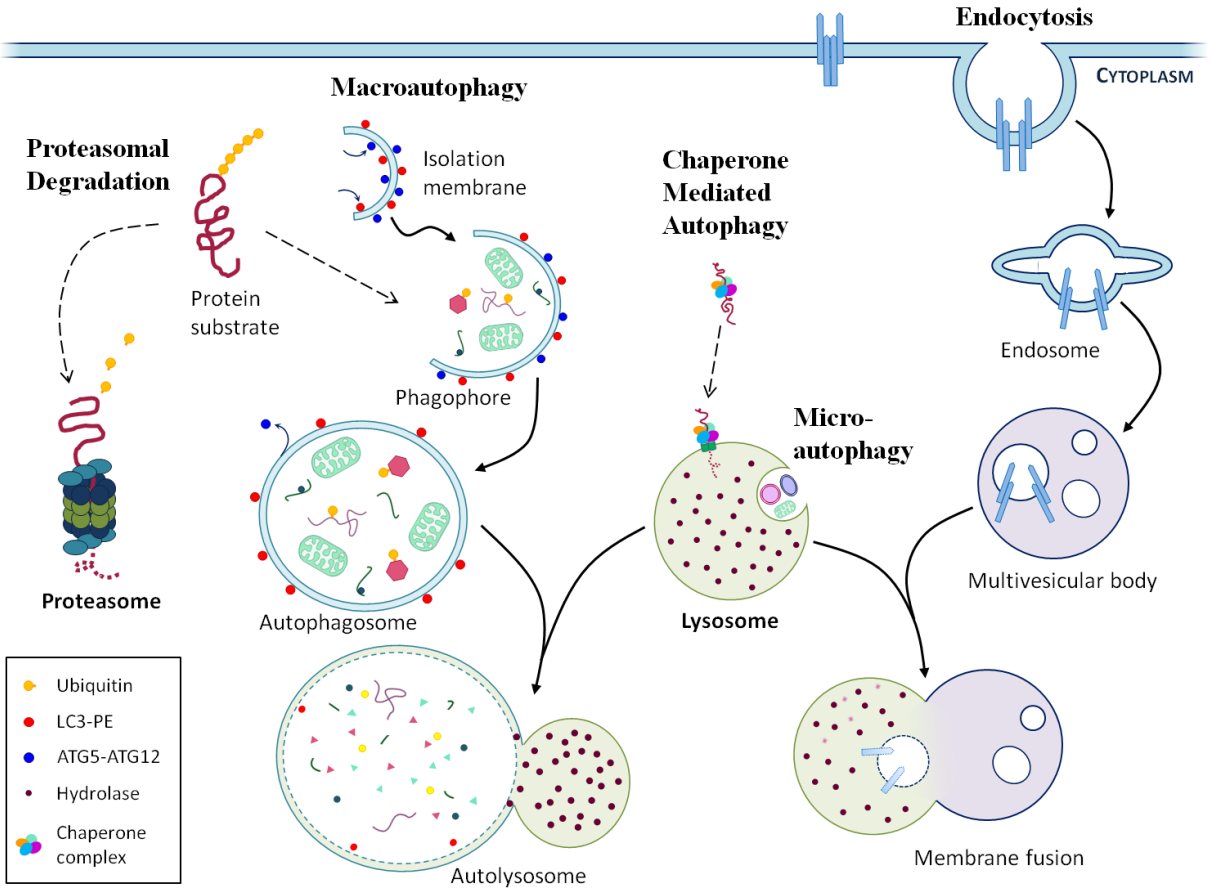


Figure 4.2: Protein degradation pathways. The ubiquitin proteasome system is involved in the degradation of approximately 90% of intracellular proteins. Other proteins and macromolecules are degraded by lysosomal hydrolytic enzymes. Numerous pathways are involved in targeting proteins and organelles for degradation by lysosomal hydrolases. These include the autophagic pathways, macroautophagy, microautophagy and chaperone mediated autophagy as well as the endosomal pathway.

In this chapter, we aimed to determine whether metformin can be combined with drugs activated in reducing environments to offer a novel therapeutic strategy for OSCC. We evaluated the effects of these drug combinations on OSCC cytotoxicity. DSF is a well-established drug that has potential to enter into phase II clinical trials for cancer therapy as it is already approved for the treatment of alcoholism and as such, we investigated the mechanisms responsible for the anti-proliferative effects of DSF. Identifying these mechanisms will be useful for the identification of diagnostic markers that can be used to differentiate between individuals who are likely to respond to this treatment and those who are not. We hypothesised that DSF exerted cytotoxic effects toward OSCC cell lines by impairing the function of both proteasomal and lysosomal protein degradation pathways.

4.2. Methods

4.2.1. Synthesis of Compounds

The bis(thiosemicarbazones, diacetyl-bis(4-methylthiosemicarbazone) (ATSM) and glyoxal-bis(4-methylthiosemicarbazone) (GTSM) were synthesised by Dr. Leonard H. Damelin according to the method of French et al. (French et al., 1958; Damelin et al., 2014). Two analogues of disulfiram, dipyrrolidine thiuram disulfide (DPTD) and tetrapropyl thiuram disulfide (TTD) were also synthesised by Dr. Leonard H. Damelin as previously described (Cramer, 1935). Copper conjugates of each compound were synthesised by reacting 100 µl of a 50 µM solution of each drug, prepared in DMSO, with 2.5 µl of 2M copper chloride ($CuCl_2$). Structure of these compounds were assessed by NMR which was conducted by Dr. Amanda L. Rosseau.

ATSM: ^{1}H NMR (500 MHz, DMSO) 11.74 (2H, s, 2 × CH=N), 8.48 (2H, d, J = 4.2, 2 × NH), 7.72 (2H, s, 2 × NH), 2.96 (6H, d, J = 4.4, 2 × CH3); ^{13}C NMR (126 MHz, DMSO) 177.55 (2 × C= S), 140.02 (2 × C=N), 30.89 (2 × CH3).

GTSM: ^{1}H NMR (500 MHz, DMSO) 10.20 (2H, s, 2 × NH), 8.36 (2H, d, J = 4.1, 2 × NH), 3.02 (6H, d, J = 4.5, 2×CH3), 2.20 (6H, s, 2 × CH3); ^{13}C NMR (126 MHz, DMSO) 178.47 (2 × C= S), 147.95 (2 × C=N), 31.18 (2 × CH3), 11.64 (2 × CH3).

DPTD: ^{1}H NMR (500 MHz, DMSO-d6) δ 3.86 (dt, J = 61.6, 7.0 Hz, 1H), 2.03 (dt, J = 73.0, 6.9 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d6) δ 187.71, 57.43, 51.40, 26.63, 24.21.

TTD: ^{1}H NMR (500 MHz, DMSO-d6) δ 3.89 (dt, J = 10.1, 4.9 Hz, 1H), 1.78 (dq, J = 87.4, 7.6 Hz, 1H), 0.92 (dt, J = 57.1, 7.4 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d6) δ 192.10, 58.88, 54.89, 21.73, 19.49, 11.42.

4.2.2. Cytotoxicity Assessment of Metformin Combined with DSF, Cu-ATSM or Cu-GTSM

In order to determine the effects of metformin in combination with drugs that are activated in reducing environments, cells were first pre-treated with 10 mM metformin for 24 hours followed by combination therapy with metformin and ATSM, Cu-ATSM, GTSM, Cu-GTSM, DSF or Cu-DSF for a further 48 hours. MTT assays were then performed as described in section 3.2.1.

The dependence on copper for the cytotoxic effects of DSF was evaluated by reacting copper with DSF in 0.25:1, 0.5:1 and 1:1 molar ratios, respectively. Cells were seeded as described in section 3.2.1, and after 48 hours they were treated with DSF or each of the above-mentioned compounds for 48 hours. Cytotoxicity was assessed using the MTT assay as described.

To confirm the role of copper in the mechanism of action of DSF, cytotoxicity was assessed after intracellular copper was depleted with the cell impermeable Cu^{2+} chelator, bathocuproine disulfonic acid (BCS) (Furuta et al., 2002). Once settled, cells were pre-treated with 10 mM metformin for 24 hours followed by treatment with DSF or DSF and 200 µM BCS for a further 48 hours. MTT assays were then conducted as described in section 3.2.1 in order to determine the concentration of DSF required for 50% cell death.

Cytotoxicity of DSF was compared to two DSF analogues, DPTD and TTD. These analogues have lower hydrolysis rates and therefore, are not metabolised to the same extent as DSF. Briefly, cells were equally seeded in 96-well plates and allowed 24 hours to settle. Cells were then pre-treated with metformin for 24 hours, followed by treatment with DSF, DPTD, TTD or their respective copper conjugates, in the absence or presence of metformin for 48 hours. MTT assays were then conducted as described in section 3.2.1.

4.2.3. Microscopic Imaging of Disulfiram Treated Cells

The effects of DSF on cell morphology were captured using bright-field microscopy. Cells were equally seeded on glass coverslips in 6-well plates. After 48 hours, cells were treated with 10 mM metformin or treated with LC50 concentrations of DSF or Cu-DSF with or without metformin for 24 hours. Coverslips were rinsed in 1 x PBS, placed with the cell

surface down on glass slides, and images were immediately captured with the Olympus BX63 microscope in bright-field at 10X magnification.

4.2.4. Assessing Plasmid DNA Damage Due to Redox Cycling of Copper

The pBR322 plasmid in JM109 *E. coli* cells stored in glycerol at -70 °C, were placed into LB broth containing 100 µg/ml ampicillin and incubated at 37 °C overnight with shaking at 220 rpm. Plasmid DNA was isolated using the Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research) for use in the experiment. For the assay, 100 ng pBR322 was incubated with 50 µM of the drug of interest in the absence of presence of 0.6 mM ascorbic acid. Stock solutions (0.5 mM) of each of the following drugs were prepared for use in the assay, metformin, metformin-copper chloride, DSF, Cu-DSF, DSF-metformin, Cu-DSF-metformin. Reaction components were added to 10 µl 2 x assay buffer (*100 mM Tris-HCl (pH 7.2); 36 mM NaCl*) and made up to a final volume of 20 µl with nuclease-free water. Samples were incubated at 37 °C for 1 hour and reactions were stopped by the addition of quench buffer (*0.25% bromophenol blue, 50% glycerol, 0.5 mM EDTA*). Samples were immediately loaded onto a 1% agarose gel containing ethidium bromide, and resolved at constant 100V for 1 hour. Images were captured under UV light using the Bio-Rad Gel Doc Imaging System and QuantityOne Software.

4.2.5. Determining Intracellular Copper Accumulation by ICP-MS

ICP-MS was used to quantify total intracellular copper levels as previously described (Price et al., 2011). Cells were seeded in 10 cm dishes and treated with metformin, DSF or metformin and DSF, as previously indicated. Additionally, the effects of the copper depleting agent, bathocuproine disulfonic acid (BCS) on intracellular copper levels were also assessed. Cells were pre-treated with 200 µM BCS or BCS and 10 mM metformin, followed by treatment with LC30 concentrations of DSF or DSF and 10 mM metformin combined with BCS for 24 hrs. Cells were washed with cold 1x PBS three times, scraped and collected by centrifugation for 10 minutes at 8000 rpm at 4 °C. A relative portion of cells was removed for protein quantification by the Bradford assay (Bradford 1976). Cell pellets were then incubated with 65% nitric acid (Suprapure, Merck) at 65 °C for 2 hours. Samples were then diluted to a final

concentration of 5% nitric acid and analysed for copper on an Agilent 7700 ICP-MS with He collision gas. Copper levels were corrected for protein (µg) and compared to untreated cells (n=3 ± SD).

4.2.6. Determining Proteasome Activity based on Amidomethylcoumarin Fluorescence

Proteasome function was assessed in treated cells using the fluorogenic proteasome substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC), which indicates levels of chymotrypsin-like activity, as previously described (Daniel et al., 2004). Cells were equally seeded in 100 mm dishes, allowed 24 hours to settle and treated accordingly. To assess the effects of metformin on DSF mediated proteasome inhibition, cells were treated with 10 mM metformin only for 24 hours, 24 hours with 10 mM metformin followed by 24 hours with the LC30 concentration of DSF and metformin for 24 hours, 24 hours with 10 mM metformin followed by 24 hours with the LC30 concentration of Cu-DSF and metformin for 24 hours, 5 µM copper-8-hydroxyquinoline (Cu-8HQ), an established proteasome inhibitor, for 24 hours. In order to assess the involvement of DSF metabolites on proteasome function, cells were treated with LC30 concentrations of DSF, DPTD or TTD for 24 hours. Following treatment, cells were washed in 1x PBS, collected by scraping and centrifugation, lysed in lysis buffer (*25 mM Tris-HCl, pH 8.0; 75 mM NaCl; 0.05 % SDS; 0.5 % Triton X-100; 0.25 % Sodium deoxycholate)*, and lysates cleared by centrifugation. Protein was quantified by Bradford assay. Equal amounts of protein (40 µg) were added to assay buffer (50 mM Tris-HCl pH 7.5) to a final volume of 400 µl and Suc-LLVY-AMC (6 mM in DMSO) was added to a final concentration of 40 µM, the reaction mixture was incubated at 37 °C for 1 hour, followed by the addition of trichloroacetic acid to a final concentration of 4% to stop the reaction. Fluorescence was measured 360nmexcitation/460nmemission using an Ascent multi-well plate fluorimeter (Thermo Scientific) (n=3 ± SD).

4.2.7. Acridine Orange Staining for Lysosomal Acidity Assessment

Lysosomal acidity was visualised by staining cells with the pH sensitive dye, acridine orange (Paglin et al., 2001). OSCC cells were evenly seeded on glass coverslips in 6-well plates and after 48 hours, were treated with LC30 concentrations of DSF, Cu-DSF, DPTD or TTD, for 24

hours, with or without 10 mM metformin or incubated with 1 mM chloroquine (CQ) for 1 hour as a positive control. LC30 concentrations were chosen in order to observe the effects of these drugs prior to cell death. Cells were incubated with 3 µM acridine orange added to culture medium at 37 °C for 1 hour and immediately visualised using the Olympus BX41 fluorescence microscope (100 W equipped with a camera and lamp using a 490 nm band-pass blue excitation filter and a 515 nm long-pass barrier filter). Images captured and processed with CellSens Dimension software.

4.2.8. Autophagosomes Visualised by Transmission Electron Microscopy

Electron Microscopy (EM) was used to look for autophagosome formation (Yla-Antilla et al. 2009). Cells were equally seeded in 10 cm culture dishes and after 24 hours, they were treated with 10 mM metformin for 24 hours, the LC30 concentration of DSF or DSF and 10 mM metformin for 24 hours, the LC30 concentration of Cu-DSF or Cu-DSF and 10 mM metformin for 24 hours. Cells were then rinsed with cold with 1x PBS three times and fixed in EM fixative (*2.5% gluteraldehyde in 0.1 M HEPES buffer pH 7.2*) for 1 hour. Cells were then scraped into a microcentrifuge tube and collected by centrifugation at 1000 x g for 10 minutes. Fixative was removed and cells were processed for EM as previously described (Yla-Antilla et al. 2009). The following parts of the EM experiments were conducted by Dr. Monica Birkhead at the National Institute for Communicable Diseases (NICD). Pelleted cells were fixed in EM fixative overnight, then rinsed several times in HEPES buffer followed by post-fixation in 1% buffered osmium tetroxide for 1 hour. Cells were then rinsed in HEPES buffer, then dehydrated in graded ethanol solutions for 30 minute intervals with three repeated changes of absolute ethanol. Cells were then infiltrated with low viscosity epoxy resin (Agar Scientific®) and polymerised in BEEM® capsules at 70 °C overnight. Sections (70 nm) were cut with the Leica EM UC6 ultramicrotome, picked up on 0.25% formvar-coated copper slot grids, double stained with uranyl acetate and lead citrate and viewed at 80 kV on an FEI BioTwin Spirit transmission electron microscope fitted with an Olympus Quemesa CCD Camera.

4.2.9. Western Blotting to Determine Autophagy Levels and Protein Degradation

Autophagy and protein ubiquitination were assessed by western blotting to determine LC3-B and total ubiquitinated protein levels, respectively. Cells were treated with 10 mM metformin for 24 hours, 10 mM metformin for 24 hours followed by 24 hours with the LC30 concentration of DSF and metformin for 24 hours, 10 mM metformin for 24 hours followed by 24 hours with the LC30 concentration of Cu-DSF and metformin for 24 hours or 50 μM CQ for 24 hours. Cells were harvested and quantified, and western blots were conducted as described in section 2.2.5, with the following exceptions. Proteins were separated on a 12% SDS-PAGE gel for LC3-B blots. Membranes were incubated in 5% bovine serum albumin in 1xTBS-T with the primary antibodies (1 in 1000 dilution was used for all primary antibodies), rabbit anti-LC3B (#2775), rabbit anti-ubiquitin (#3933) or β-actin (Santa Cruz, sc-130656) overnight, following which, membranes were washed in 1xTBS-T and then incubated with a goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnologies). Densitometry was performed using QuantityOne software.

4.3. Results

4.3.1. Metformin works in combination with drugs activated in reducing environments to exert cytotoxic effects against OSCC cell lines

In chapter 3, we found that metformin increases the intracellular reducing environment in OSCC cell lines. Therefore, it is likely that metformin could work well in combination with drugs that are activated in reducing environments to offer a potentially new treatment option for OSCC. Copper bis(thiosemicarbazones) are activated in reducing environments and exert cytotoxic activity by promoting intracellular copper accumulation. Cu-ATSM and Cu-GTSM release their copper upon intracellular reduction and unlike normal cells which can readily re-oxidise the drug, copper accumulates in hypoxic tissues such as cancer. DSF is also a copper chelating drug that can act as copper delivery agents. DSF toxicity is mainly attributed to formation of the Cu-DSF complex. However, DSF also forms numerous metabolites that may also contribute to its anti-cancer effects. The effects of Cu-ATSM, Cu-GTSM and DSF, with and without metformin, on OSCC cytotoxicity were evaluated.

4.3.1.1. Copper bis(thiosemicarbazones) retain their toxicity in the presence of metformin

Cu-ATSM and Cu-GTSM both displayed cytotoxic effects toward OSCC cell lines, whereas ATSM and GTSM not reacted with copper did not induce an effective cytotoxic response. The cytotoxic effects of Cu-ATSM and Cu-GTSM were retained in the presence of metformin, which indicates that metformin can be combined with Cu-ATSM or Cu-GTSM should they be used for cancer therapy. Figure 4.3 displays cytotoxicity curves for ATSM, Cu-ATSM, GTSM and Cu-GTSM in the absence and presence of metformin for each of the three OSCC cell lines used in this study. LC50 values determined from these curves (Table 4.1) for metformin in combination with Cu-ATSM or Cu-GTSM were not significantly different compared to Cu-ATSM or Cu-GTSM alone. Although a slight increase in LC50 was observed in the case of the SNO cell line treated with Cu-ATSM and metformin, these agents exert cytotoxic effects at doses much smaller than conventional drugs such as cisplatin, which highlights that their potential as anti-cancer agents.

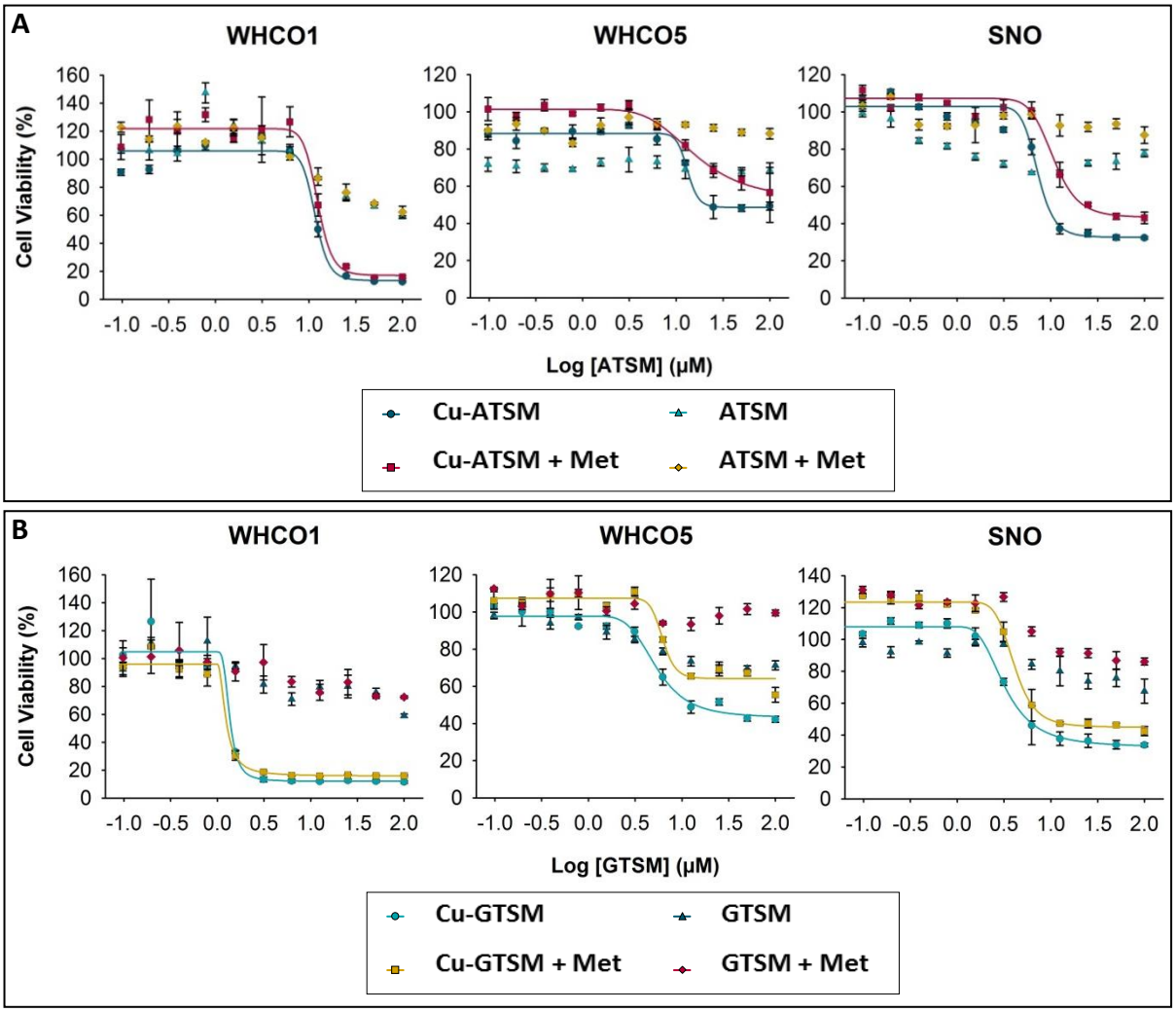


Figure 4.3: Cu-ATSM and Cu-GTSM are toxic toward OSCC cell lines alone and in the presence of metformin. WHCO1, WHCO5 and SNO cell lines were pre-treated with 10 mM metformin for 24 hours, followed by co-treatment with A) ATSM or Cu-ATSM, or B) GTSM or Cu-GTSM, for 48 hours and cytotoxicity was evaluated by MTT assay. Absorbance values were taken as a percentage of the control and plotted against the Log transform of drug concentration. In the absence of reacted copper, ATSM and GTSM did not exert significant cytotoxic effects. Cu-ATSM and Cu-GTSM exerted cytotoxic effects toward all three OSCC cell lines alone and in the presence of metformin.

Table 4.1: Cu-ATSM and Cu-GTSM retain their toxicity in the presence of metformin. LC50 values obtained from cytotoxicity curves are tabulated below. Metformin did not significantly alter the cytotoxicity of Cu-ATSM and Cu-GTSM in all three OSCC cell lines. This indicates that Cu-ATSM and Cu-GTSM retain their toxicity in the presence of metformin.

Compounds	LD50 (μM)		
	WHCO1	WHCO5	SNO
Cu-ATSM	11.93 ± 0.47	13.51 ± 0.81	7.10 ± 0.57
Cu-ATSM + Met	12.54 ± 0.19 ^{ns}	12.05 ± 0.83 ^{ns}	10.90 ± 0.77 *
ATSM	>200	>200	>200
ATSM + Met	>200	>200	>200
Cu-GTSM	1.14 ± 0.16	5.39 ± 0.9	3.37 ± 0.23
Cu-GTSM + Met	1.16 ± 0.12 ^{ns}	6.24 ± 0.01 ^{ns}	4.23 ± 0.70 ^{ns}
GTSM	>200	>200	>200
GTSM + Met	>200	>200	>200

Although the copper bis(thiosemicarbazones) are promising chemotherapeutic agents that can be combined with metformin, more studies are still required before these drugs can be approved for cancer therapy. Cu-ATSM is still undergoing phase I trials for use as a radiopharmaceutical. The copper bis(thiosemicarbazones) are not as well established as DSF. For these reasons, we chose to proceed with investigating the mechanisms of action of DSF rather than the copper bis(thiosemicarbazones). DSF has been used in the treatment of alcoholism for more than 50 years and is known to be tolerated at high doses in the absence of alcohol (Suh et al., 2006). Additionally, DSF is currently undergoing clinical trials for the treatment of various different cancer types. The effects of metformin and DSF on OSCC cytotoxicity is the area of focus for the remaining part of this chapter.

4.3.1.2. Metformin enhances the cytotoxic effects of DSF toward OSCC cell lines and Cu-DSF toxicity is retained

Cytotoxicity was assessed in OSCC cell lines treated with DSF or Cu-DSF in the absence or presence of metformin. MTT assay data (Figure 4.4 A) indicated that DSF and Cu-DSF both exert cytotoxic effects toward the three OSCC cell lines at doses as low as between 6 μM – 12 μM. Metformin enhanced the effects of DSF by 28.5% in WHCO1, 25.9% in WHCO5 and 43.1% in SNO. The effects of copper alone at the doses used during Cu-DSF therapy were

also assessed by MTT assay (Figure 4.4 B). The graphs do not form an S-shape as is typical for cytotoxicity curves, and cells are still viable at the highest concentrations of copper used (up to 200 µM). This indicates that the effects of Cu-DSF on OSCC cytotoxicity are not due to the effects of copper alone.

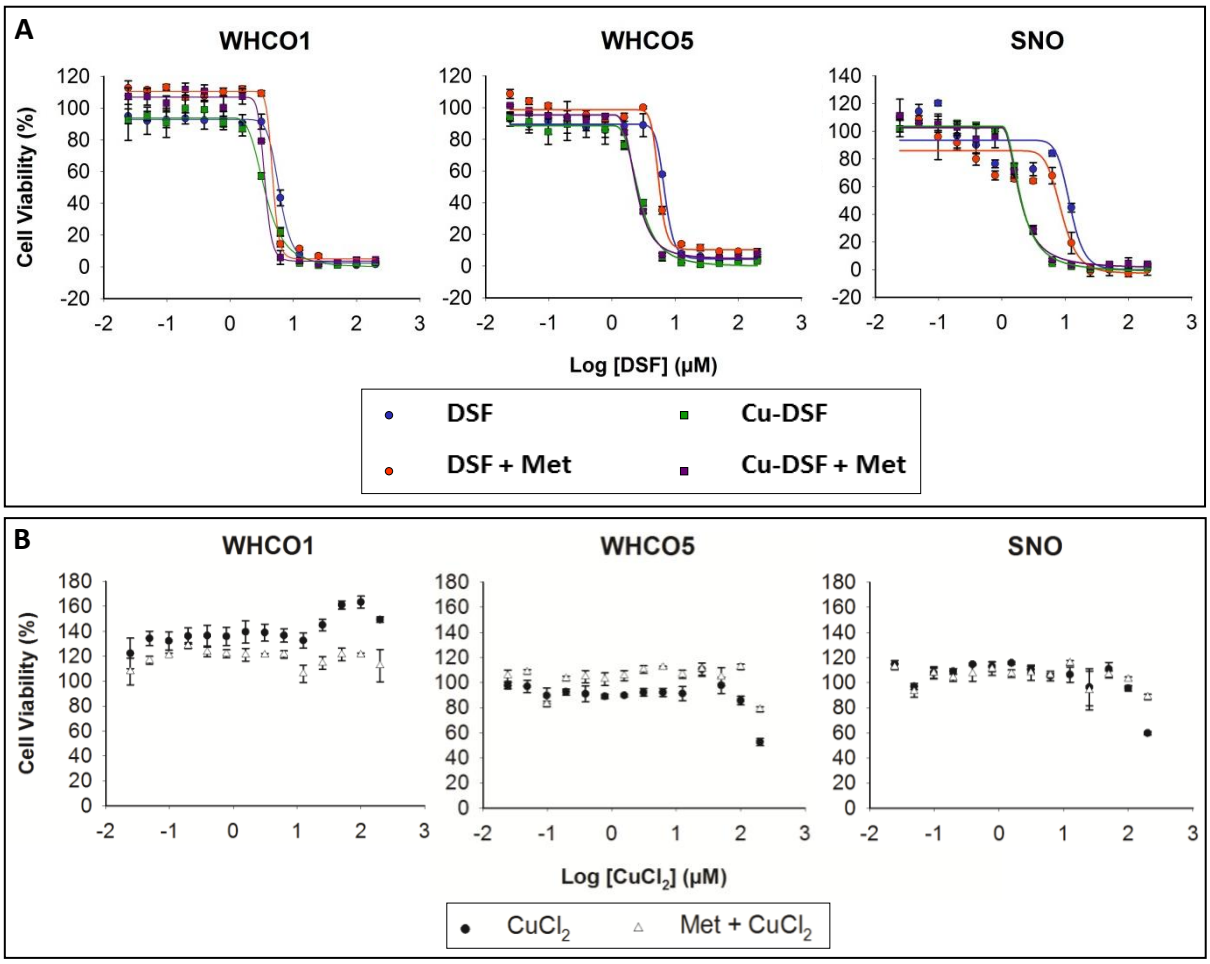


Figure 4.4: Cytotoxic effects of DSF are significantly enhanced in the presence of metformin and copper in OSCC cell lines but minimal cytotoxicity occurs in response to copper alone. **A)** Cells were pre-treated with 10 mM metformin for 24 hrs, followed by co-treatment with DSF or Cu-DSF for 48 hours. Cytotoxicity curves show that DSF and Cu-DSF exert toxic effects toward OSCC cell lines and metformin treatment increased the effects of DSF. **B)** Cells were pre-treated with 10 mM metformin for 24 hours followed by combination therapy with metformin and copper for 48 hours. MTT assays were conducted and results for each cell line are depicted on the graph above. Low concentrations of copper (≤30 µM) did not induce any cytotoxic effects toward OSCC cell lines and mild cytotoxicity was observed at higher concentrations of copper (≥80 µM).

Table 4.2: Metformin and copper enhance the toxicity of DSF in OSCC cell lines. LC50 values were calculated from cytotoxicity curves for DSF and Cu-DSF with or without metformin. There was a significant reduction in LC50 values when metformin was added to DSF therapy, which indicates that metformin enhances the effect of DSF. There was a mild, yet insignificant reduction in LC50 values when metformin was added to Cu-DSF. This indicates that Cu-DSF toxicity is retained in the presence of metformin.

Compounds	LD50 (μM DSF)		
	WHCO1	WHCO5	SNO
DSF	6.04 ± 0.39	6.85 ± 0.32	11.89 ± 0.56
DSF + Met †	4.70 ± 0.14 *	5.44 ± 0.20 **	8.31 ± 1.30 **
Cu-DSF †	3.63 ± 0.31 **	2.80 ± 0.23 ***	2.01 ± 0.08 ***
Cu-DSF + Met	3.44 ± 0.22	2.42 ± 0.10	1.95 ± 0.07

† Significance stars represent P-values for DSF + Met or Cu-DSF compared to DSF alone in the corresponding cell line.

To complement MTT assay data, images of cells with treatments corresponding to MTT assays were captured, in all cases, LC30 concentrations of DSF or Cu-DSF were used (Figure 4.5). When comparing the effects of metformin to untreated cells, it is apparent that cells are less confluent and cell morphology is unaltered, as found previously. The cytotoxic effects of DSF are clearly depicted as there is a visible reduction in cell viability. Cytotoxicity of DSF is enhanced by metformin as cells appeared more rounded with severe alterations to membrane integrity for DSF-metformin treated samples. Cell morphology and viability was significantly altered by Cu-DSF and there was more cell debris in the regions surrounding cells. Whilst not seen with the MTT assays, it was observed that metformin also enhanced the effects of Cu-DSF on OSCC cell viability as very few cells were still attached after Cu-DSF-metformin therapy. Surviving cells had significantly altered morphology with more rounded cells and higher amounts of cell debris.

In vitro studies suggest that the effects of DSF in cancer are copper dependent, but DSF has also been shown to exert anti-cancer effects *in vivo* without additional copper (Chen et al., 2001; Conticello et al., 2012; Rae et al., 2013). Although DSF has been prescribed for years in the treatment of alcoholism, its anti-cancer effects were only recently described. As such, a direct mechanism of action for this drug in cancer is still not known. There are various

actions of DSF that have been shown to contribute to its anti-cancer effects and most of these effects involve additional copper. The contribution of copper to DSF mediated toxicity was therefore investigated further.

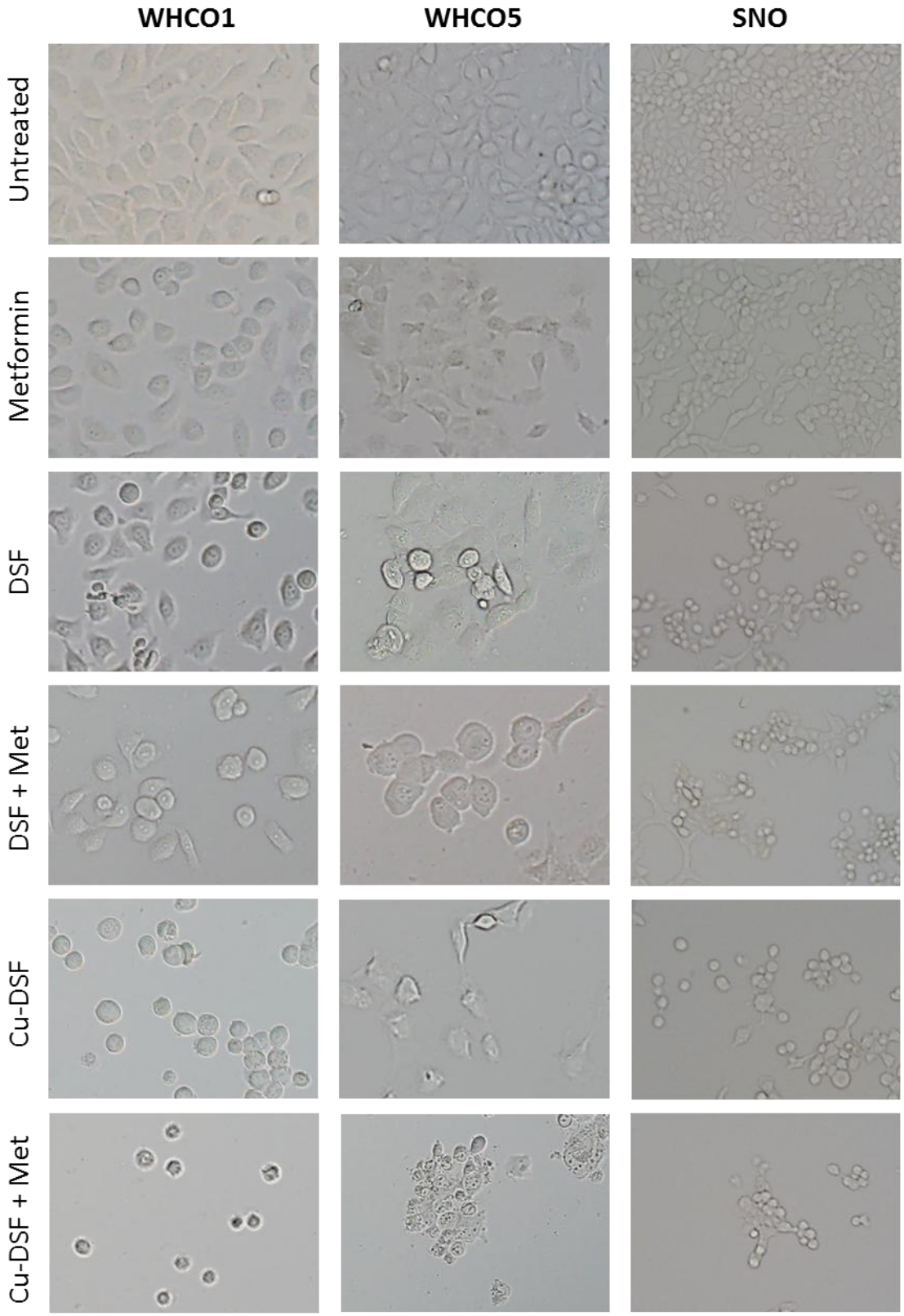


Figure 4.5: Effects of DSF and Cu-DSF in the absence and presence of metformin on cell morphology observed by light microscopy. Images captured in brightfield (100x) demonstrate the cytotoxic effects of DSF and Cu-DSF exert cytotoxic effects toward WHCO1, WHCO5 and SNO cell lines which are enhanced by metformin.

4.3.2. Copper enhances, but is not exclusively required for the cytotoxic effects of DSF in OSCC cell lines

4.3.2.1. Increasing levels of Cu-DSF complex enhances DSF toxicity

Copper and DSF were reacted in 0.25:1, 0.5:1 and 1:1 ratios respectively, and the effects of these complexes on OSCC cytotoxicity were evaluated. As the copper:DSF ratio was increased, cytotoxicity toward OSCC cell lines also increased (Figure 4.6). This indicates that copper plays a significant role in DSF mediated cytotoxicity in OSCC cell lines.

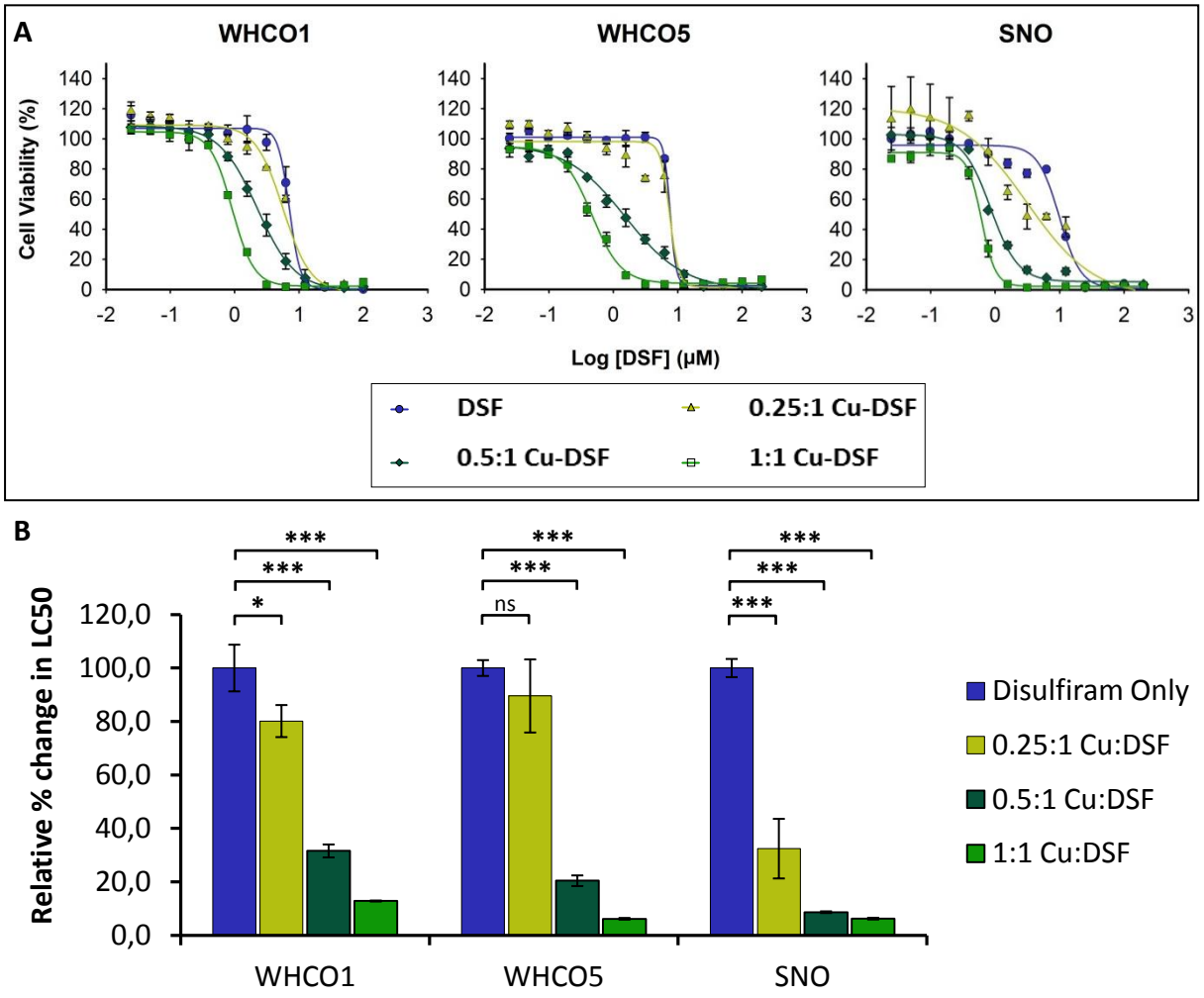


Figure 4.6: DSF toxicity is enhanced by increasing concentrations of copper. OSCC cell lines were exposed to DSF alone or copper reacted with DSF in three molar ratios, 0.25:1, 0.5:1 and 1:1 for 48 hours. **A)** There is a leftward shift in dose response curves as copper levels are increased. **B)** LC50 values expressed as a percent of DSF decreased as copper levels increased, which shows that DSF toxicity is enhanced by copper in OSCC cell lines.

4.3.2.2. DSF treatment leads to increased intracellular copper levels

Intracellular copper levels were quantified after OSCC cells were treated with DSF or DSF and metformin in order to assess the contribution of copper to DSF-metformin mediated toxicity. DSF alone significantly increased intracellular copper levels in all three OSCC cell lines (Figure 4.7). Interestingly, co-therapy with DSF and metformin resulted in a further increase in intracellular copper levels, which was significant in the WHCO1 and WHCO5 cell lines. This finding is not unusual as a recent study revealed that two molecules of metformin can act as a chelating agent with particularly high affinity for copper (Logie et al., 2012). Although metformin did not significantly alter copper uptake in these cell lines, studies have shown that two molecules of metformin can bind to copper, and thus alter copper homeostasis by transporting it into the cell or by altering its sub-cellular localisation.

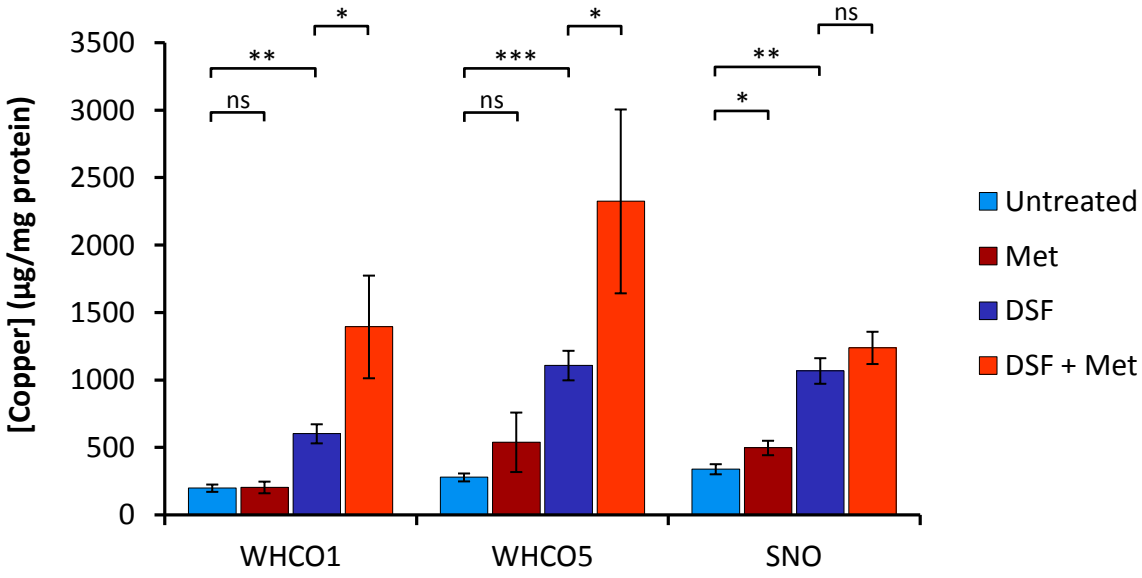


Figure 4.7: Intracellular copper levels in OSCC cell lines are significantly higher in the presence of DSF, and further increased by metformin. Cells were treated with 10 mM metformin for 24 hrs, LC30 concentrations of DSF for 24 hrs, or pre-treated with metformin for 24 hrs followed by combination therapy with DSF and metformin for 24 hours. Intracellular copper levels assessed by ICP-MS, are expectedly higher in the presence of DSF. Metformin combined with DSF led to a further increase in intracellular copper levels compared to DSF alone in the WHCO1 and WHCO5 cell lines.

4.3.2.3. Copper depletion reduces but does not abolish the cytotoxic effects of DSF

Based on the previous two experiments, it is evident that copper plays a significant role in DSF mediated toxicity toward OSCC cell lines. In order to fully assess the contribution of copper, the effects of copper depletion on DSF cytotoxicity were determined. Bathocuproine disulfonate (BCS), a cell impermeable copper chelator, was used to deplete intracellular copper levels by reducing copper uptake. DSF or DSF and metformin were combined with BCS and cytotoxicity was assessed by MTT assay. Additionally, we confirmed that this agent reduces intracellular copper levels by ICP-MS. Treatment with 200 µM BCS effectively reduced intracellular copper levels to those similar to untreated cells in all three OSCC cell lines (Figure 4.8). The percent reduction in intracellular copper levels for DSF-BCS compared to DSF treatment or DSF-BCS-Met compared to DSF-Met treatment respectively, was 62 ± 2.9% and 59 ± 9.6% in WHCO1; 41 ± 1.6% and 43 ± 17.0% in WHCO5; and 32 ± 7.4% and 63 ± 12.6% in SNO.

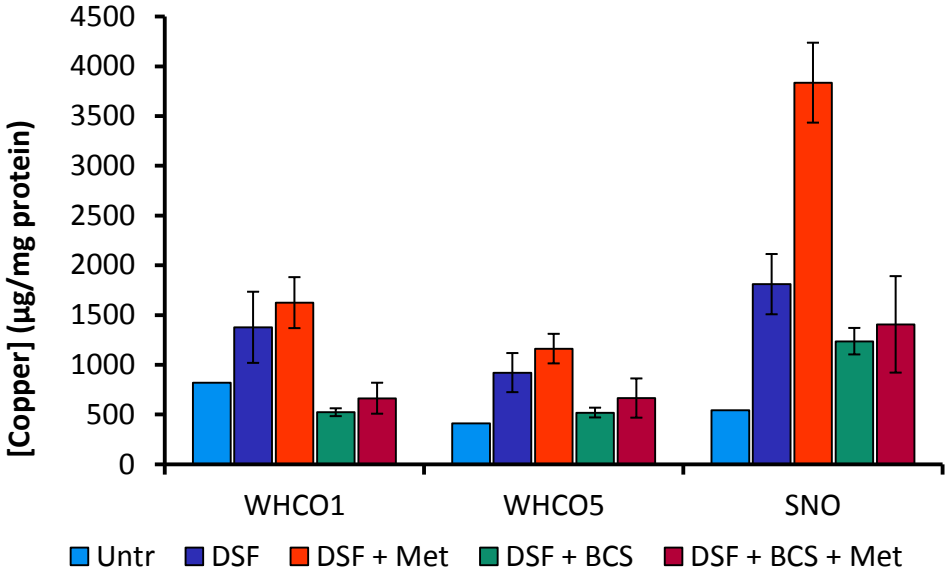


Figure 4.8: Bathocuproine disulfonate (BCS) effectively reduces the DSF or DSF-metformin induced increase in intracellular copper levels. Cells were pre-treated with 200 µM BCS, 10 mM metformin, or BCS and metformin for 24 hrs, followed by combination therapy with DSF for 48 hrs and compared to untreated cells. ICP-MS data indicated that BCS significantly reduced copper levels in OSCC cell lines as depicted in the graph.

As expected, copper depletion inhibited the cytotoxic effects of DSF in OSCC cell lines as higher LC50 values were observed when BCS was combined with DSF or DSF and metformin (Figure 4.9). The percent increase in LC50 for DSF and BCS compared to DSF alone was 42% for WHCO1; 58% for WHCO5 and 182% for SNO. This confirms that copper plays a significant role in DSF mediated cytotoxicity toward OSCC cell lines. However, DSF still induced cell death toward OSCC cell lines even in the presence of BCS, as can be seen by the characteristic S-shape of the dose response curves. This suggests that formation of DSF-copper complexes may not be the only factor contributing to the cytotoxic effects of DSF. DSF toxicity has been attributed to general copper induced toxicity as well as proteasome inhibition caused by Cu-DSF. Accumulation of copper promotes DNA damage and oxidative stress which eventually leads to cell death. We therefore assessed the effects of DSF on copper-induced DNA damage and proteasome activity.

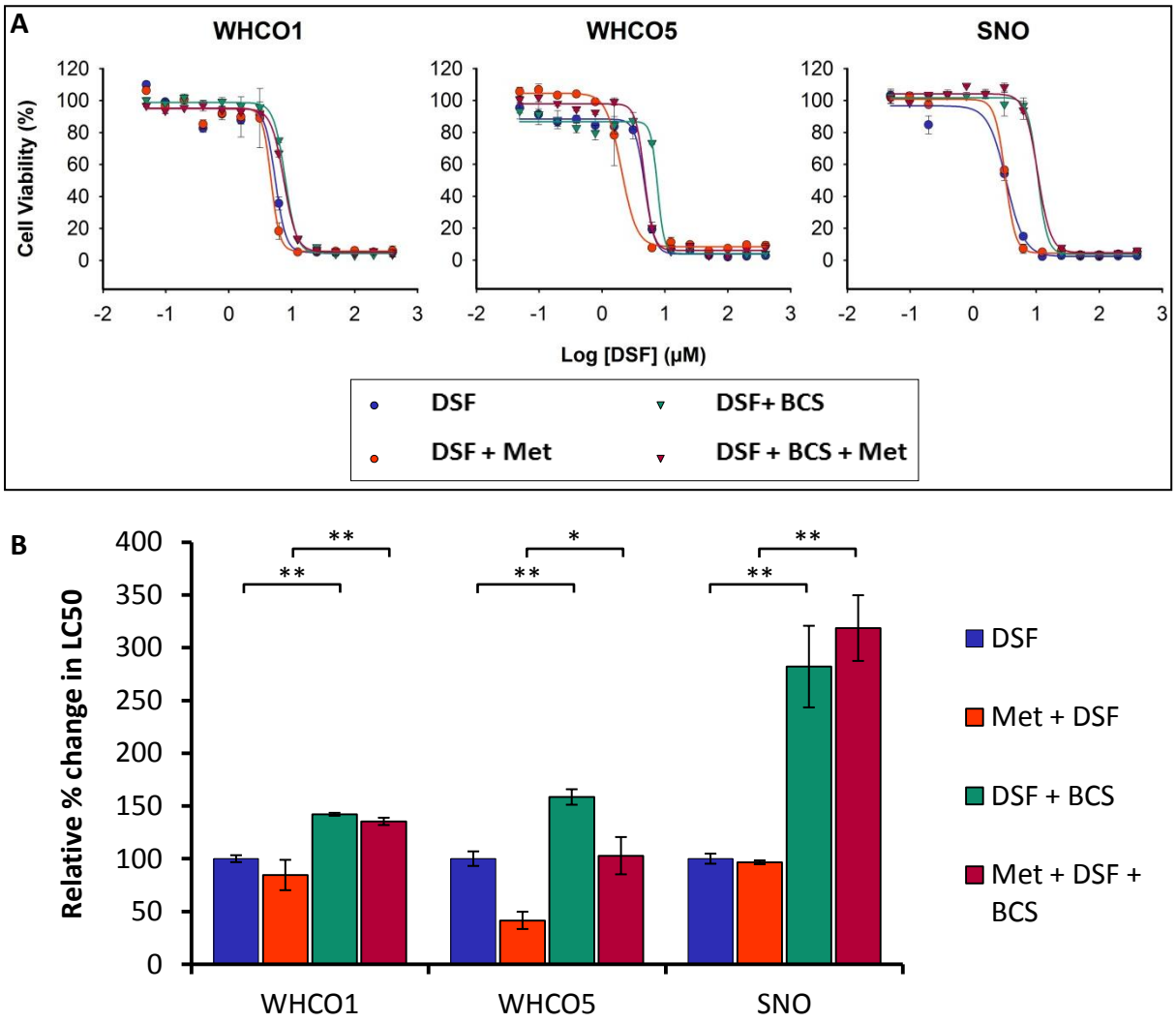


Figure 4.9: Copper depletion partially reduces DSF cytotoxicity in OSCC cell lines. Cells were pre-treated with 200 μM BCS, 10 mM metformin, or BCS and metformin for 24 hrs, followed by combination therapy with DSF for 48 hrs. **A)** MTT assay curves indicate that BCS reduces the toxicity of DSF. However, DSF still exerted potent cytotoxic effects during copper reduction, as can be deduced from the characteristic S-shaped dose response curves. **B)** LC50 values, represented as a percent of DSF alone, are higher in the presence of BCS.

4.3.2.4. Metformin interferes with copper and Cu-DSF induced DNA damage

Copper toxicity can occur due to various reasons and one of these is its ability to cleave DNA in the presence of reducing agents such as ascorbic acid, GSH or H_2O_2. Formation of the Cu(I)-DNA complex results in DNA base modifications, whereas free Cu^{1+} causes random DNA strand breaks (Drouin et al., 1996). We determined the effects of DSF and Cu-DSF with or without metformin on plasmid DNA cleavage in the absence and presence of ascorbic acid. Results indicated that metformin completely blocked DNA damage induced by copper and inhibited Cu-DSF mediated DNA damage (Figure 4.10).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Ascorbic Acid	-	-	+	-	+	-	+	-	+	-	+	-	-	+	-	+	-	+
Metformin	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+
$CuCl_2$	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-
DSF	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-
Cu-DSF	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+

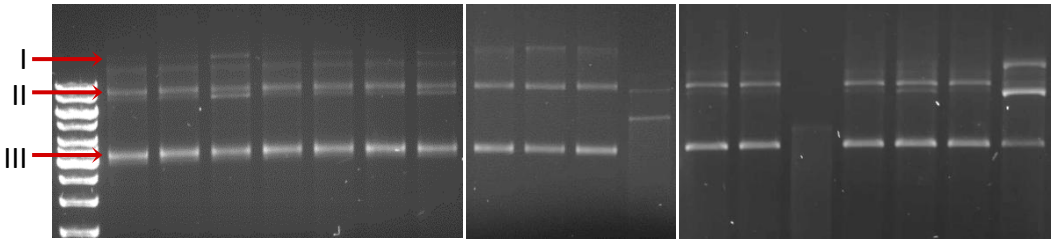


Figure 4.10: Metformin inhibits DNA damage caused by the reduction of copper and Cu-DSF. The pBR322 plasmid was exposed to DSF or Cu-DSF in the absence or presence of metformin or ascorbic acid at 37°C for 1 hr. Bands are labelled based on various degrees of plasmid DNA supercoiling; I- Supercoiled, II- Circular, III- Linear. DNA damage was not induced in the absence of ascorbic acid. Reduction of Cu^{2+} ions to Cu^{1+} resulted in shearing of plasmid DNA, as can be seen in lane 14. Metformin acted to block Cu^{1+} induced DNA damage, lane 7. DSF alone did not alter plasmid DNA, whereas reduced Cu-DSF promoted DNA damage indicated by the absence of supercoiled and circular plasmid DNA in lane 11. Metformin also inhibited Cu-DSF induced DNA damage, as supercoiled plasmid DNA is still present in lane 18.

Plasmid DNA was cleaved upon the reduction of copper and Cu-DSF but not DSF. As this experiment was conducted in a cell-free system, the absence of copper explains why DSF does not induce any DNA damage. The reduction of Cu^{2+} ions from $CuCl_2$ to form Cu^{1+} resulted in extensive DNA damage, and interestingly, this effect was completely blocked with the addition of metformin. Metformin also inhibited Cu-DSF induced DNA damage, as more supercoiled and circular plasmid DNA were observed when metformin was combined with Cu-DSF and ascorbic acid compared to Cu-DSF and ascorbic acid alone which resulted in the formation of linear and cleaved DNA fragments.

Although metformin inhibits copper and Cu-DSF induced DNA damage, it enhanced DSF cytotoxicity and also seemed to add to cytotoxic effects of Cu-DSF based on cell morphological features. This indicates that metformin induced inhibition of DNA damage by copper did not reduce the effects of DSF or Cu-DSF toward OSCC cell lines. This implies that there are other mechanisms, apart from copper induced DNA damage that may play a significant role in DSF mediated toxicity toward OSCC cell lines. Studies have shown that the Cu-DSF complex inhibits proteasomal chymotrypsin-like activity and that this is a major factor contributing to its cytotoxic effects (Chen et al., 2006). We therefore investigated the effects of DSF on proteasome activity in OSCC cell lines.

4.3.3. DSF alters multiple pathways involved in protein degradation and this contributes to DSF induced toxicity

Protein degradation pathways are crucial for regular homeostasis. This process is tightly controlled as it is involved in regulating short-lived proteins, recognising and degrading damaged proteins and also provides amino acids required for nascent polypeptide synthesis. There are three major mechanisms by which proteins are degraded, these include proteasomal, endosomal and autophagic protein degradation (Clague & Urbé, 2010). The Ubiquitin-Proteasome system is responsible for the degradation of approximately 90% of intracellular proteins (Cvek & Dvorak, 2008). Ubiquitin ligases catalyse the binding of ubiquitin to proteins in an ATP dependent manner. Additional ubiquitin molecules then bind to this residue to form a polyubiquitin chain that can adopt various 3D conformations and may be recognised by the 26S proteasome and target the protein for degradation (Lecker, Goldberg & Mitch, 2006). Another mechanism of protein degradation is via lysosomal pathways. Lysosomes are membrane enclosed organelles containing hydrolytic enzymes that can degrade proteins, other major macromolecules and organelles. Lysosomes play an important role in both endosomal and autophagic pathways.

The endosomal pathway is involved in the degradation of membrane proteins. Plasma membrane proteins are engulfed by endocytosis to form early endosomes, which fuse with lysosomes and lead to the degradation of membrane proteins (Cooper, 2000). Ubiquitin was also shown to play a role in the endosomal pathway as some receptors use monoubiquitination as a signal for endocytosis (Clague & Urbé, 2010). Autophagy involves sequestration of cytosolic proteins or organelles in a double membrane vesicle called the autophagosome. The autophagosome then fuses with lysosomes to form an autolysosome that is involved in the degradation of its contents. Numerous autophagy related proteins are involved in regulating the process. One of these proteins, LC3-I (microtubule-associated protein light chain 3) becomes conjugated to a phosphatidylethanolamine (PE) moiety to form LC3-II which is conjugated to the autophagosome. LC3-II acts as an adaptor protein that is involved in recognising protein aggregates and organelles that are to be engulfed by the autophagosome (Glick, Barth & Macleod, 2010). Monoubiquitination has also been linked to autophagic degradation of damaged mitochondria (Clague & Urbé, 2010).

4.3.3.1. Disulfiram inhibits proteasomal function

Studies have suggested that the Cu-DSF complex can inhibit chymotrypsin-like activity of the proteasome (Chen et al., 2006). The 26S proteasome complex contains two trypsin-like, two chymotrypsin-like and two caspase-like proteolytic sites. These sites each recognise and cleave proteins at specific amino acid sequences. The effects of DSF on overall protein degradation were assessed by western blotting for ubiquitin or ubiquitin-conjugated proteins. There was an accumulation of ubiquitinated proteins in samples treated with DSF or Cu-DSF compared to untreated samples which indicates that DSF inhibits protein degradation in OSCC cell lines (Figure 4.11 A). Metformin increased protein degradation as total ubiquitin levels were lower when cells were treated with metformin alone or metformin combined with DSF or Cu-DSF.

The effects of DSF or Cu-DSF with or without metformin, or chloroquine, which is a known proteasome inhibitor, on chymotrypsin-like activity of the proteasome were assessed based on cleavage of Suc-LLVY-AMC. Suc-LLVY-AMC is a proteasome specific substrate and that is cleaved at the tyrosine residue by chymotrypsin-like activity, it then releases the AMC group which emits fluorescence. Fluorescence was lower in DSF and Cu-DSF treated samples indicating that DSF and Cu-DSF inhibit chymotrypsin-like activity (Figure 4.11 B). However, proteasomal chymotrypsin-like activity was only reduced by 15% to 20% for the three cell lines. Interestingly, metformin enhanced proteasomal inhibition as fluorescence was further reduced in cells treated with DSF and metformin or Cu-DSF and metformin. Although metformin enhanced DSF and Cu-DSF mediated inhibition of chymotrypsin-like proteasome activity, ubiquitin western blot data show that it increased overall protein degradation in the presence of DSF. This indicates that other protein degradative pathways are still active in the presence of metformin and proteasome inhibition. Since DSF and Cu-DSF were mild inhibitors of proteasomal activity in OSCC cell lines, this suggests that proteasome inhibition may not be sufficient for the cytotoxic effects of these drugs in OSCC cell lines. For this reason we compared the effects of DSF to two thiuram disulfide analogues, which are more potent proteasome inhibitors, on OSCC cytotoxicity.

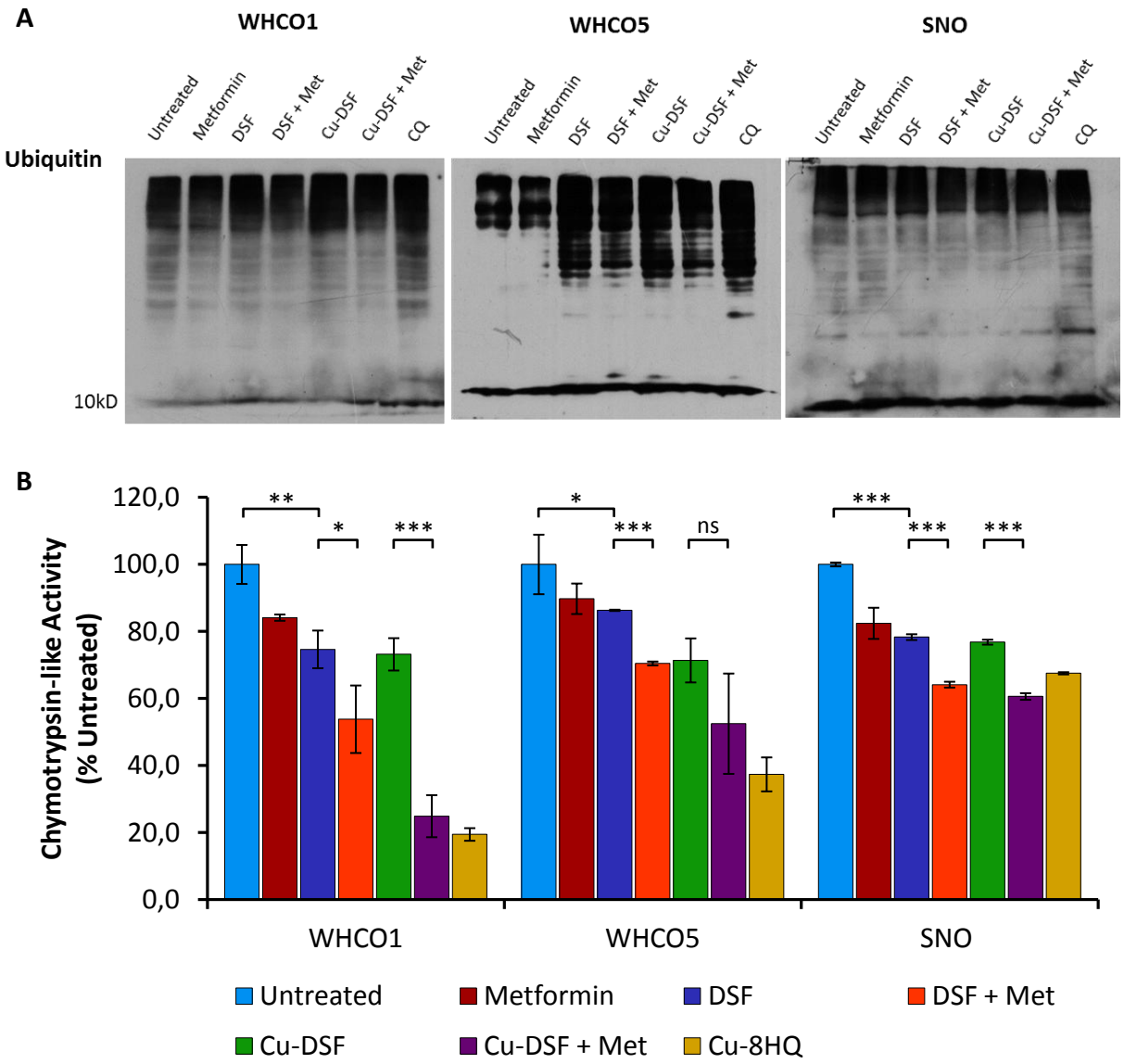


Figure 4.11: Disulfiram inhibits proteasomal activity in OSCC cell lines. Cells were treated with 10 mM metformin for 24 hrs, LC30 concentrations of DSF or Cu-DSF for 24 hrs, or pre-treated with metformin for 24 hrs followed by combination therapy with DSF or Cu-DSF for 24 hrs. Positive control treatments were 50 µM chloroquine (CQ) for 24 hrs to assess ubiquitin accumulation and 5 µM copper 8-hydroxyquinoline (Cu-8HQ) for 24 hrs to assess inhibition of chymotrypsin-like activity. **A)** DSF and Cu-DSF treatment led to an accumulation of ubiquitinated proteins, and therefore inhibited proteasomal function. Metformin was associated with reduced levels of ubiquitinated proteins. **B)** DSF and Cu-DSF both inhibit chymotrypsin-like activity of the proteasome, and interestingly, this is enhanced by metformin as fluorescent signals are further reduced.

4.3.3.2. DSF analogues are more effective inhibitors of proteasomal chymotrypsin-like activity but are less toxic than DSF

Various drugs capable of binding copper have been shown to inhibit proteasomal activity and the dithiocarbamate family of drugs fall in this category. As a members of this drug family, DSF and DDC are known to inhibit chymotrypsin-like proteasome activity when they are in complex with copper (Daniel et al., 2007). Since the Cu-DSF or $Cu(DDC)_2$ complexes are implicated in proteasome inhibition, rather than their unconjugated counterparts, we investigated the effects of two DSF analogues that retain their copper to a better extent than DSF, on proteasome activity.

The two DSF analogues used in this study, dipyrrolidine thiuram disulfide (DPTD) and tetrapropyl thiuram disulfide (TTD) were derived from their monomers, pyrrolidine dithiocarbamate and di-n-propyl dithiocarbamate, respectively. These analogues were chosen as they have lower decomposition rates and are therefore more stable than DSF. Second order decomposition rates for DSF, DPTD and TTD are 2.5×10^4, 0.1 and 500 $L.mol^{-1}.min^{-1}$, respectively (Topping & Jones, 1988). This means DPTD is the most stable and would retain copper better than DSF, which has a high decomposition rate and would easily release its copper. Pyrrolidine dithiocarbamate has been shown to inhibit 26S proteasome activity, and this was due to formation of a copper complex (Daniel et al., 2005). However, DSF analogues do not form DSF hydrolysis products such as diethylamine or modified diethyldithiocarbamates. DPTD and TTD were used to differentiate between the roles of Cu-DSF or $Cu(DDC)_2$ and the effect of DSF metabolites.

We first determined the effects of DSF analogues on OSCC cytotoxicity with the MTT assay. DPTD, TTD and their copper conjugates were less toxic than DSF and Cu-DSF, respectively (Figure 4.12). DPTD reduced proteasome activity by 98%, 73% and 103% and TTD reduced proteasome activity by 49%, 52% and 115% in WHCO1, WHCO5 and SNO cell lines respectively. LC30 values for these treatments are recorded in Table 4.3. DPTD and TTD were also less toxic than DSF when all drugs were combined with metformin; however, metformin did not significantly alter the toxicity of DPTD and TTD in all cell lines.

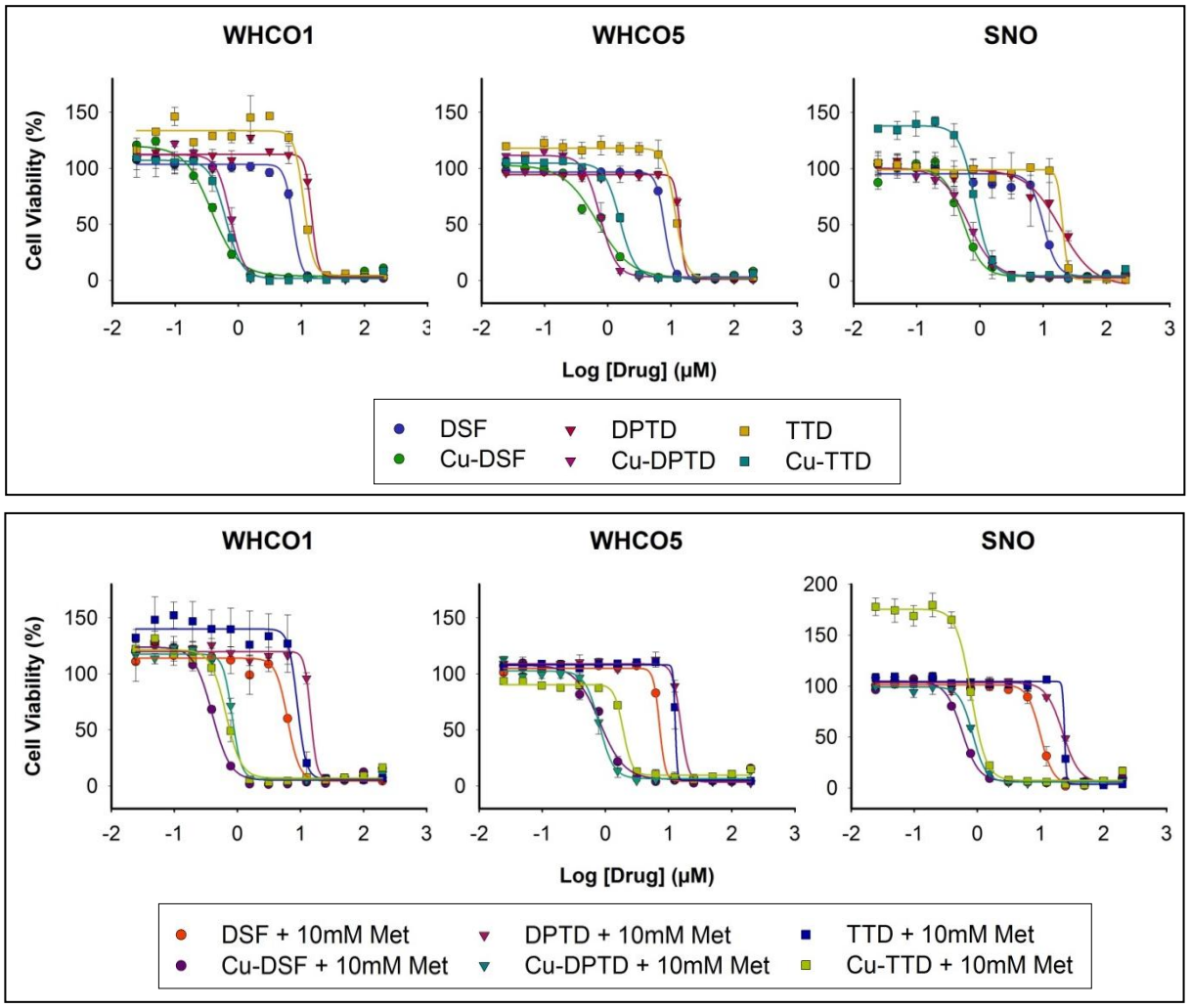


Figure 4.12: DSF analogues with lower hydrolysis rates are less toxic than DSF. Cells were treated with DSF, DPTD, TTD, Cu-DSF, Cu-DPTD or Cu-TTD either with or without 10 mM metformin pre-treatment and combination therapy. Absorbance values were expressed as a percent of their respective control and represented on the graphs. DPTD and TTD were less toxic than DSF and Cu-DPTD and Cu-TTD were less toxic than Cu-DSF. This indicated that DSF hydrolysis products may play a significant role in its cytotoxic effects.

Table 4.3: LC50 values for DSF and DSF analogues. DSF analogues displayed higher LC50 values compared to DSF and this trend was maintained in the presence of metformin and when drugs were reacted with copper.

	LC50 (μM)		
	WHCO1	**WHCO5**	**SNO**
DSF	7.35 ± 0.270	7.87 ± 0.139	10.20 ± 0.724
DSF + Met †	6.26 ± 0.176 *	7.04 ± 0.442 *	10.01 ± 1.118 ns
Cu-DSF †	0.37 ± 0.024 ***	0.67 ± 0.023 ***	0.56 ± 0.113 ***
Cu-DSF + Met ‡	0.40 ± 0.026 ns	0.81 ± 0.026 *	0.57 ± 0.006 ns
DPTD ⁋	14.52 ± 0.264 ***	13.64 ± 0.652 ***	20.68 ± 1.335 ***
DPTD + Met †	14.38 ± 0.702 ns	15.45 ± 0.888 ns	22.89 ± 0.484 ns
Cu-DPTD †	0.75 ± 0.055 ***	0.76 ± 0.099 ***	0.66 ± 0.131 *
Cu-DPTD + Met ‡	0.82 ± 0.025 ns	0.80 ± 0.096 ns	0.85 ± 0.033 *
TTD ⁋	10.98 ± 0.605 **	11.99 ± 0.284 ***	21.92 ± 1.637 ***
TTD + Met †	8.97 ± 1.275 *	13.65 ± 1.643 ns	23.48 ± 1.214 *
Cu-TTD †	0.64 ± 0.064 ***	1.56 ± 0.042 ***	0.83 ± 0.036 ***
Cu-TTD + Met ‡	0.66 ± 0.04 ns	1.90 ± 0.057 **	0.80 ± 0.023 ns

⁋ Significance stars for DPTD and TTD were calculated by comparing these treatments to DSF.

† Significance stars were determined from P-values comparing DSF + Met or Cu-DSF with DSF alone, DPTD + Met and Cu-DPTD with DPTD alone and, TTD + Met or Cu-TTD with TTD alone.

‡ Significance stars were determined from P-values comparing Cu-DSF + Met with Cu-DSF, Cu-DPTD + Met with Cu-DPTD and, Cu-TTD + Met with Cu-TTD.

Next, we investigated the effects of DPTD and TTD on proteasomal chymotrypsin-like activity. DPTD and TTD inhibited proteasome activity to a greater extent than DSF alone (Figure 4.13). DPTD reduced proteasomal activity by 43%, 15% and 29% in WHCO1, WHCO5 and SNO cell lines, respectively compared to DSF. TTD reduced proteasomal activity by 18% in WHCO1 and WHCO5 cell lines and 19% in the SNO cell line compared to DSF. DPTD was a more efficient proteasome inhibitor than TTD and DSF in WHCO1 and SNO cell lines. This is in line with decomposition rates of the compounds, as DPTD is the most stable, followed by TTD and DSF.

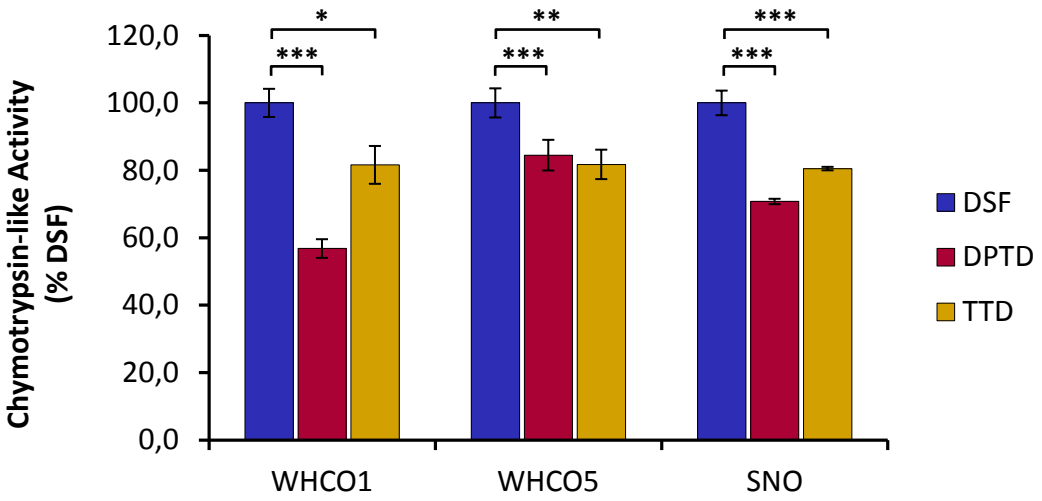


Figure 4.13: DPTD and TTD inhibit proteasomal chymotrypsin-like activity to a greater extent than DSF. Cells were treated with LC30 concentrations of each drug for 24 hours and chymotrypsin-like activity was assessed. Fluorescence of AMC groups was expressed as a percent of DSF and represented on the graph. DSF analogues had lower fluorescence readings which indicate that DPTD and TTD are more effective proteasome inhibitors compared to DSF.

The cytotoxic effects of DSF have been attributed to Cu-DSF or $Cu(DDC)_2$ complex mediated proteasome inhibition (Chen et al., 2006). However, we show here that thiuram disulfide analogues, which inhibit proteasome activity to a greater extent than DSF, have reduced cytotoxicity toward OSCC cell lines. Cytotoxicity of DSF analogues also correlated with their hydrolysis rates, as thiuram disulfides with higher rates of hydrolysis were more toxic toward OSCC cell lines. This finding suggests that copper complexes of DSF are not solely responsible for its cytotoxic effects. Therefore, we hypothesised that the hydrolysis products of DSF also contribute to its cytotoxic effects and that diethylamine may accumulate in lysosomes and reduce lysosomal acidity, which could add to the cytotoxic effects of DSF.

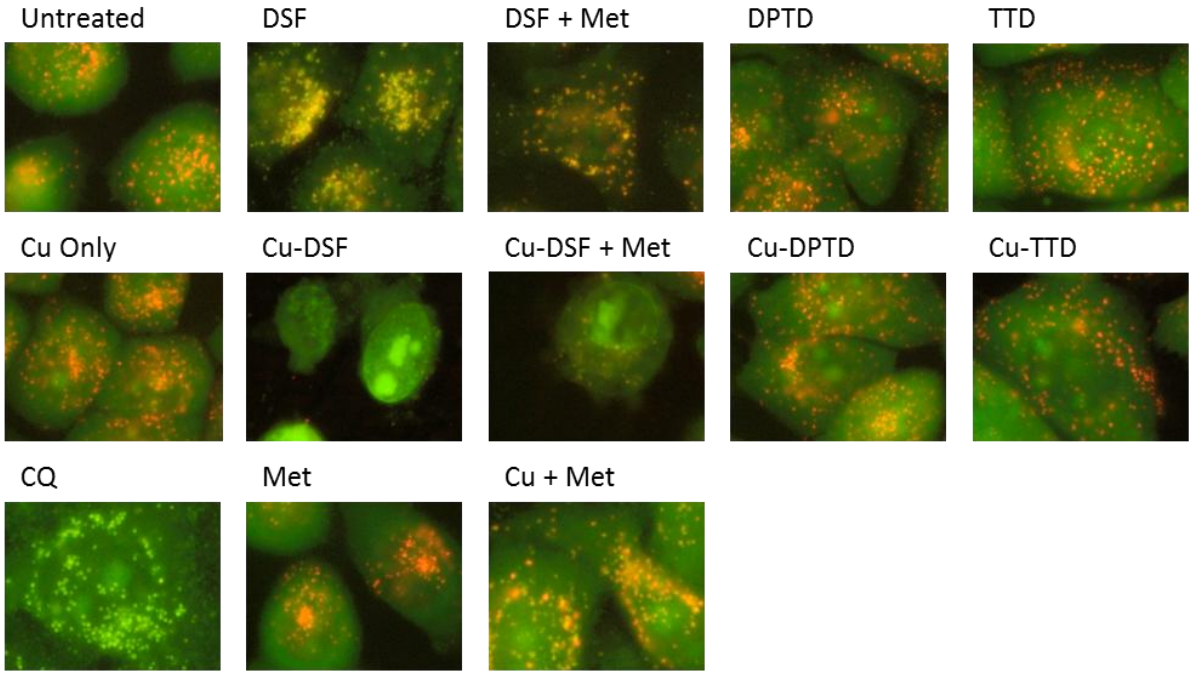
4.3.3.3. DSF reduces lysosomal acidity

We hypothesised that DSF could enter the lysosome, where it is reduced to its hydrolysis products, diethylamine and carbon disulfide, and reduce lysosomal acidity due to accumulation of diethylamine. DSF has been shown to bind to proteins and form mixed disulfides (Strömme, 1965) and may therefore enter lysosomes when bound to proteins. Alternatively, DSF could also enter lysosomes via diffusion. DSF is unstable in low pH environments and the hydrolytic enzymes of the lysosome will reduce DSF to its monomer, DDC, which will be further degraded to form diethylamine and carbon disulfide (Martin, 1953; Johansson, 1992). Increased levels of diethylamine can promote lysosomal alkalinisation and block autophagosome maturation, thus inhibiting another pathway for protein degradation and this would add to DSF toxicity. In order to test this hypothesis, cells that were treated with DSF or DSF analogues were stained with acridine orange (AO) in order to assess the pH of acidic vesicular organelles (AVOs) which include lysosomes and autolysosomes.

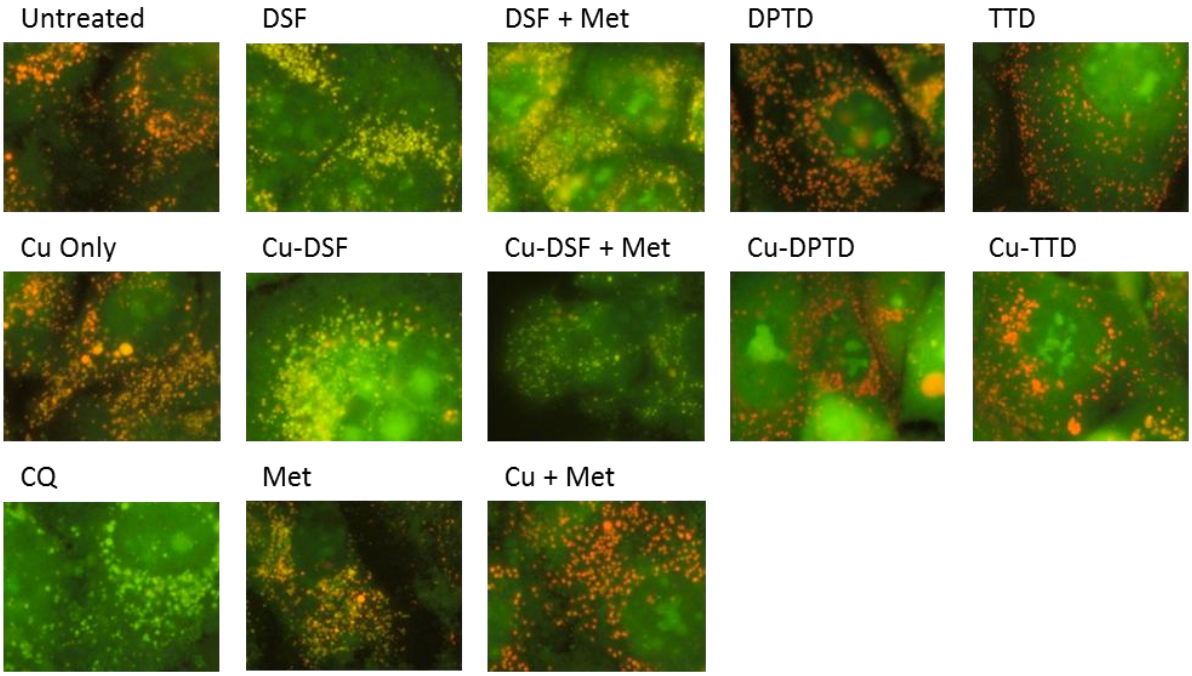
In untreated cells, lysosomes were orange in colour, which indicates that these compartments were still highly acidic (Figure 4.14). Chloroquine (CQ) is a weak base that can be trapped in lysosomes and therefore increase lysosomal pH. As expected, CQ treated cells had completely green lysosomes. DSF treatment reduced lysosomal acidity as can be deduced from the yellow to green appearance of lysosomes. Cu-DSF treated cells also exhibited yellow to green organelles, which indicates that lysosomal function is hampered in the presence of DSF and Cu-DSF. Although lysosomes appeared slightly more orange when DSF or Cu-DSF were combined with metformin compared to DSF or Cu-DSF, lysosomal acidity was significantly reduced compared to untreated cells. Metformin or metformin combined with copper did not significantly alter lysosomal acidity.

Treatment with DPTD and TTD did not alter lysosomal acidity as organelles appeared orange and not much different compared to untreated cells. This confirms that it is a DSF metabolite and not the Cu-DSF complex that is involved in reducing lysosomal acidity. In order to confirm that DSF does alter lysosome function, autophagy was assessed by western blotting for LC3-B and also by transmission electron microscopy.

WHCO1


Untreated
DSF
DSF + Met
DPTD
TTD
Cu Only
Cu-DSF
Cu-DSF + Met
Cu-DPTD
Cu-TTD
CQ
Met
Cu + Met

WHCO5


Untreated
DSF
DSF + Met
DPTD
TTD
Cu Only
Cu-DSF
Cu-DSF + Met
Cu-DPTD
Cu-TTD
CQ
Met
Cu + Met

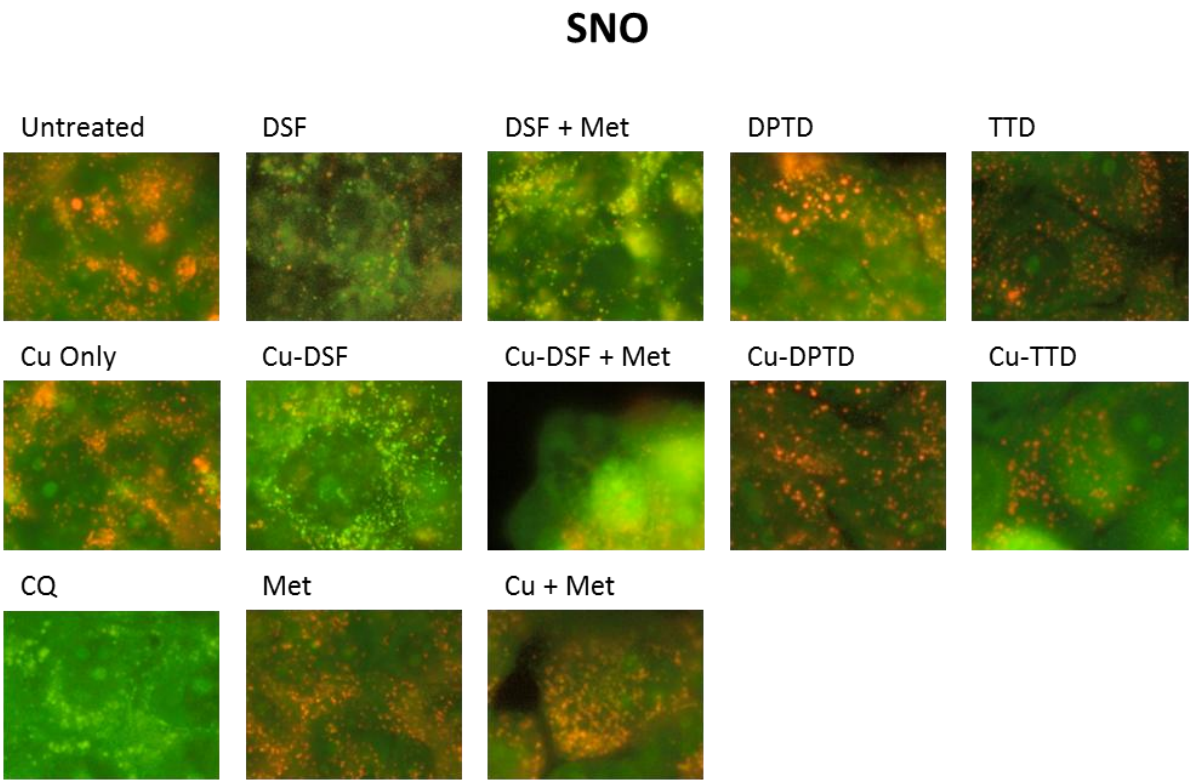


Figure 4.14: DSF reduces lysosomal acidity. OSCC cells were treated with LC30 concentrations of the drugs of interest, in the absence or presence of 10 mM metformin. Cells were treated with 1 mM CQ for 1 hr as a positive control. Lysosomal acidity was visualised by staining cells with 2.7 µM AO for 1 hr and observing cells with the Olympus BX41 microscope (400x). AO is a dye that appears orange in acidic environments and green in alkali environments. Lysosomes appeared yellow to green in cells treated with DSF or DSF + Met, compared to untreated cells where lysosomes were orange. This indicates that lysosomal acidity was significantly reduced in the presence of DSF. A similar trend was observed in Cu-DSF and Cu-DSF + Met treated cells. As expected, lysosomal vesicles were completely green in the presence of CQ. The DSF analogues, DPTD and TTD did not significantly alter lysosomal acidity as indicated by the orange colour of lysosomes.

4.3.3.4. DSF inhibits autophagy

We found that there was a significant increase in ubiquitinated proteins in the presence of DSF, but DSF was only a partial inhibitor of proteasome activity. DSF and Cu-DSF treatment led to reduced lysosomal acidity and this effect was maintained in the presence of metformin. We therefore hypothesised that DSF may inhibit the autophagic pathway, which is also involved in protein degradation. In order to test this hypothesis, autophagosomes were viewed using EM and LC3B levels were evaluated by western blotting. WHCO1 was chosen as a representative cell line for EM experiments.

There was an increase in the number of enlarged autolysosomes in metformin treated cells (Figure 4.15), compared to untreated cells which displayed smaller autophagosomes. There was an accumulation of protein aggregates in AVOs after DSF treatment, as indicated by dark spots within the autolysosomes, which suggests that DSF treatment blocks lysosome mediated protein degradation. Metformin exacerbated the effects of DSF, as there were numerous autolysosomes containing non-degraded protein aggregates in cells treated with DSF and metformin. This suggests that DSF blocked the degradation of components within autolysosomes, which is likely due to reduced lysosomal acidity.

The effects of Cu-DSF were slightly different, protein aggregates were not as common as seen in DSF or DSF + Met treated cells. AVO's were much larger and characteristic of autophagic cell death. Treatment with Cu-DSF combined with metformin resulted in a more toxic response as there was a severe loss in cell membrane integrity and hardly any organelles, apart from the nucleus, could be seen in the cytoplasm. These results confirm our findings which indicate that Cu-DSF is more toxic than DSF alone. However, DSF alone was still able to exert an effective cytotoxic response in OSCC cell lines. Due to the observed accumulation of lysosomes containing non-degraded protein aggregates, we hypothesised that DSF also interferes with autophagy. To test whether DSF inhibits autophagy in OSCC cell lines, we determined the levels of LC3B-II by western blotting.

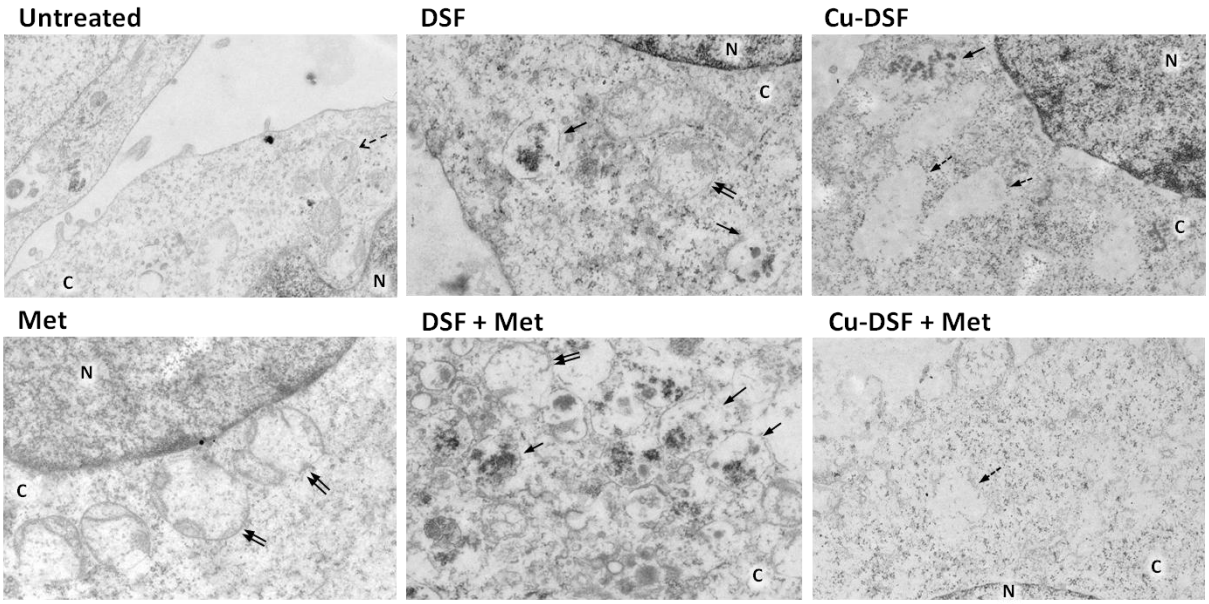


Figure 4.15: DSF inhibits autolysosome maturation. Cells were treated with LC30 concentrations of DSF for 24 hrs, or pre-treated with 10 mM metformin for 24 hrs followed by combination with DSF for a further 24 hrs. Fixed cells were osmicated and evaluated by EM ($1x10^6$ x). Untreated cells had few lysosomes (↑), but autolysosmes were not observed. Autolysosomes (⇑) were observed in the presence of metformin. Many uncleared autolysosmes containing protein aggregates (↑) were observed in cells treated with DSF or DSF + Met. Few autolysomes containing protein aggregates were observed in cells treated with Cu-DSF. Cells treated with Cu-DSF or Cu-DSF and metformin showed evidence of vacuolisation (↑). N-nucleus; C-cytoplasm.

Autophagy initiation leads to cleavage of proLC3 at the C terminus to the soluble form, LC3-I. Autophagy related proteins are involved in conjugating LC3-I to the lipid PE moiety so it can associate with the autophagic membrane both internally and externally (Mizushima, 2004). Internal LC3-II is degraded by hydrolytic enzymes of the lysosome upon maturation, whereas external LC3-II dissociates and is re-converted to LC3-I. Rapid degradation of LC3B-II is indicative of high levels of autophagy. On the other hand, inhibition of autophagy is associated with reduced degradation and increased accumulation of LC3B-II (He & Klionsky, 2009).

DSF and Cu-DSF both inhibited autophagy in OSCC cell lines as indicated by higher LC3B-II levels compared to untreated cells (Figure 4.16). Chloroquine treatment, which inhibits autophagy due to its effects on increasing lysosomal pH, resulted in a large increase in LC3B-II levels as expected. DSF treatment lead to a slight increase in LC3B-II which was further

improved by metformin in WHCO1 and WHCO5 cell lines. This effect was more pronounced in the SNO cell line. Cu-DSF and Cu-DSF with metformin lead to even larger increases in LC3B-II levels in all three cell lines. This indicated that DSF and Cu-DSF both inhibit autophagy in OSCC cell lines.

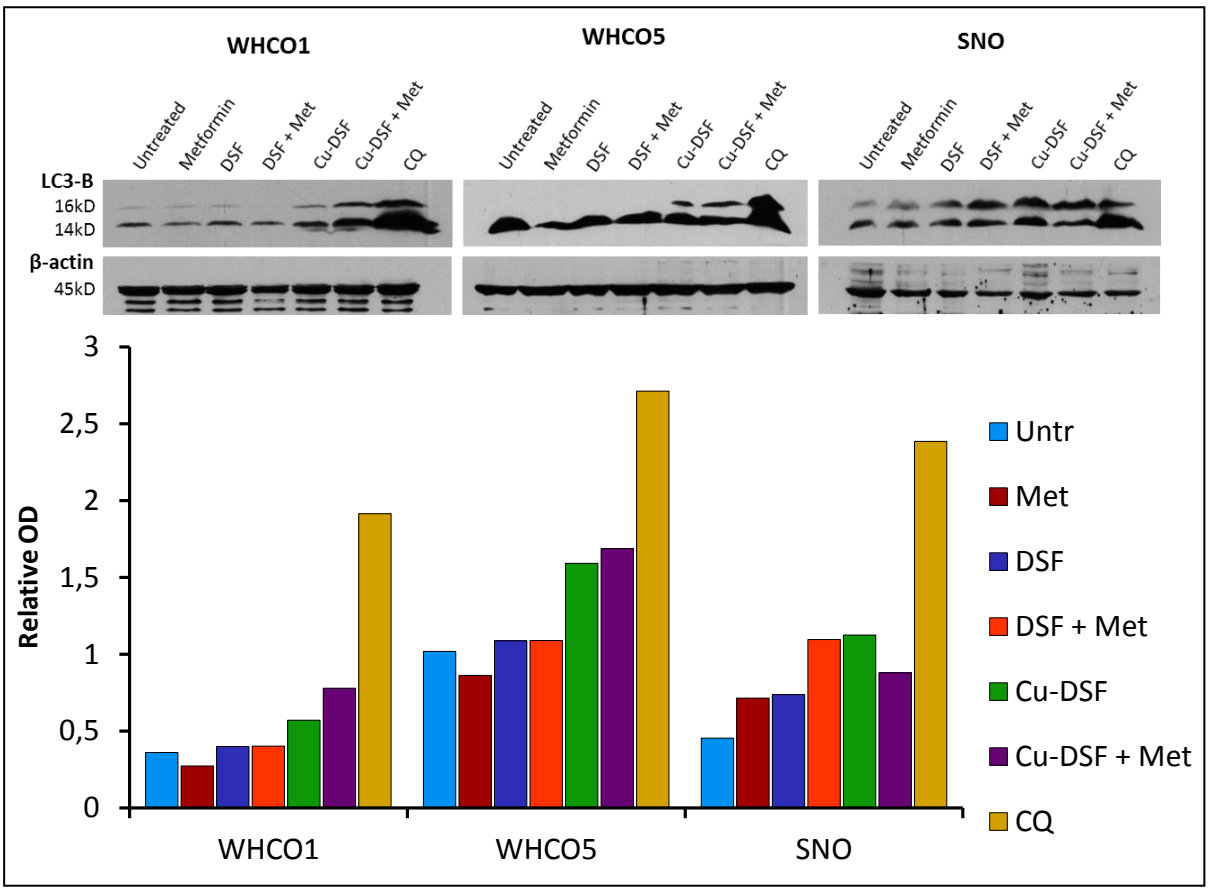


Figure 4.16: DSF increases LC3B-II accumulation and therefore inhibits autophagy. Cells were treated with LC30 concentrations of DSF or Cu-DSF for 24 hrs, or pre-treated with 10 mM metformin for 24 hrs followed by combination with DSF or Cu-DSF for a further 24 hrs. Cells were treated with 50 µM CQ for 24 hrs as a positive control for autophagy inhibition. The bar graph represents the relative optical densities of LC3-B II with respect to β-actin (n = 1). As expected, CQ treatment resulted in an accumulation of LC3B-II (16 kDa), indicative of autophagy inhibition. DSF and Cu-DSF treatment resulted in increased levels of LC3B-II in all three cell lines, which indicates that these drugs and inhibit autophagy. LC3B-II levels were slightly reduced in WHCO1 and WHCO5 cells treated with metformin only, but elevated in SNO cells treated with metformin, suggesting that metformin does not alter autophagy in these cell lines. Metformin did not hamper autophagy inhibition by DSF or Cu-DSF.

4.4. Discussion

The effects of Cu-ATSM and Cu-GTSM toward OSCC cell lines were retained in the presence of metformin. Metformin enhanced the effects of DSF and did not significantly alter the cytotoxic effects of Cu-DSF. The addition of metformin did seem to reduce cell number and morphology when observed under the microscope when combined with Cu-DSF, which suggests that the difference in LC50 value for Cu-DSF compared to Cu-DSF and metformin could be insignificant due to approaching saturating levels of toxicity induced by Cu-DSF. Based on these findings, we concluded that metformin worked well in combination with drugs that are activated in reducing environments and therefore, we suggest that it can be combined with either of these agents for cancer therapy. However, of the three drugs, DSF is the most promising agent for a cancer treatment option that can be implemented relatively sooner than the copper bis(thiosemicarbazones). This is because DSF is already prescribed for the treatment of alcoholism and has passed phase I clinical trials for the treatment of alcohol abuse, if DSF is prescribed at similar doses for cancer therapy, it may easily move to phase II trials for cancer therapy. For this reason, we investigated the mechanistic effects of DSF toward OSCC cell lines.

Our findings clearly indicate that copper plays a major role in DSF mediated toxicity, as an increase in the amount of copper bound DSF enhanced the toxicity toward OSCC cell lines. Treatment with DSF also led to increased intracellular copper levels, which suggests that DSF can bind to copper in cell culture medium and transport it into the cell. Intracellular copper levels were even higher when cells were treated with metformin combined with DSF, which explains the effects of metformin in enhancing DSF toxicity. Although the evidence presented here largely points at copper being the distinguishing factor in DSF mediated toxicity, we also showed that DSF can exert cytotoxic effects during copper depletion. Therefore, although copper plays a major role in enhancing the effects of DSF, DSF may also exert significant cytotoxic effects toward OSCC cell lines in a copper independent manner.

Studies have indicated that DSF promotes apoptosis by inhibiting chymotrypsin-like activity of the proteasome in the presence of copper (Chen et al., 2006). Therefore, we assessed chymotrypsin-like proteasome activity in response to DSF in OSCC cell lines and found that DSF is a mild inhibitor of proteasome function in OSCC cell lines. Metformin enhanced the

effects of DSF and Cu-DSF on proteasome inhibition. Since it is the Cu-DSF complex or $Cu(DDC)_2$ that has been credited with the effects of DSF on proteasome inhibition and the 1:1 Cu-DSF complex had the highest toxicity, we expected that Cu-DSF would be a more effective inhibitor of proteasomal activity than DSF alone. However, inhibition of proteasome activity was not much lower with Cu-DSF compared to DSF treatment, with a difference of 1.5% in WHCO1, 14.9% in WHCO5 and 1.5% in SNO between the two treatments. Therefore, these results indicate that inhibition of proteasomal chymotrypsin-like activity is only a partial contributor to DSF mediated toxicity in OSCC cell lines.

An accumulation in ubiquitinated proteins suggests that DSF and Cu-DSF inhibit protein degradation in OSCC cell lines. However, we showed that there was reduced accumulation or increased clearance of ubiquitinated proteins when metformin was combined with DSF or Cu-DSF, most apparent in the WHCO1 and WHCO5 cell lines. DSF and Cu-DSF were mild inhibitors of proteasomal chymotrypsin-like activity, which explains the observed increase in ubiquitinated proteins. Although there was a reduction in the accumulation of ubiquitinated proteins in the presence of metformin, we found that it improved DSF and Cu-DSF mediated inhibition of chymotrypsin-like activity. This result may seem counter-intuitive at first, however, mono-ubiquitination can be utilized as a signal in other protein degradative pathways such as autophagy (Clague & Urbé, 2010). Which indicates that metformin may promote the degradation of ubiquitin by other protein degradative pathways which are still to be explored. Metformin did improve proteasome inhibition by DSF and Cu-DSF and this corresponds with its ability to enhance the cytotoxicity of these drugs. However, DSF analogues with lower hydrolysis rates were more effective proteasome inhibitors, but were less toxic toward OSCC cell lines. For these reasons, we deduced that DSF may be involved in inhibiting other protein degradative pathways and that this process may involve one or more of its metabolites.

In addition to binding copper, DSF has a high affinity for protein thiols and is involved in the formation of mixed disulfides (Strömme, 1965). DSF-protein binding would inhibit the regular function of these proteins and lead to an accumulation of damaged or unused proteins. We hypothesised that DSF can enter lysosomal compartments, perhaps when when bound to these proteins or by passive diffusion, and alter lysosomal function. DSF is highly unstable in a low pH environment, and would readily degrade to its hydrolysis

products, diethylamine and carbon disulfide (Martin, 1953). The accumulation of diethylamine in lysosomal compartments would reduce lysosomal acidity and thus inhibit the regular function of these acidic organelles. As expected, we saw that both DSF and Cu-DSF significantly reduced lysosomal acidity as acridine orange stained lysosomes were green in the presence of DSF and Cu-DSF in all OSCC cell lines tested. DSF hampered lysosomal function with a concomitant reduction in autophagy based on reduced clearance of LC3B-II. We therefore conclude that DSF exerts its cytotoxic effects by inhibiting multiple protein degradative pathways. The combination of metformin and DSF is a promising new strategy for the treatment of OSCC as it is both effective and affordable.

Chapter 5: Conclusion and Future Prospects

OSCC is among the top 10 most prevalent cancers in South Africa and has a high mortality rate (National Health Laboratory Service, 2008). High mortality rates arise from late detection and lack of cost-effective treatment options. Drugs that are currently prescribed for OSCC lack in their ability to target late stage cancers and harmful side effects of these drugs often lead to discontinuation of the therapy. New drugs that are currently being developed for oesophageal cancer offer a more targeted approach to cancer therapy, however, most of these drugs are unlikely to be implemented in developing or moderately developed countries like South Africa as they are unaffordable. Furthermore, introduction of these drugs in the clinical setting may still be many years away. This highlights the need for more cost effective chemotherapeutic options for OSCC that can be rapidly implemented.

Metformin is a cost effective and frequently prescribed drug, which is known to have a low toxicity profile. Studies have shown that metformin has anti-proliferative effects toward various cancer types *in vitro* and in mouse models (Zakikhani et al., 2006; Gotlieb et al., 2008). In this study we demonstrate metformin also inhibits the growth of OSCC cell lines. The chemostatic effects of metformin are promising as metformin will be useful as a pre-surgical therapy for OSCC. Studies have shown that metformin can target and selectively kill cancer stem cells (Hirsch et al., 2009). The tumour microenvironment consists of multiple cell types, and different parts of the tumour have different levels of access to nutrients, depending on their proximity to blood vessels. Therefore, tumours may contain cancer cells of various levels of differentiation in their microenvironment. Metformin slowed down the proliferation of the moderately differentiated OSCC cell lines and was able to kill cancer initiating stem cells. If combined with other drugs that are cytotoxic toward cancer cell lines, metformin may offer a novel chemotherapeutic option that is both cost-effective and has fewer side effects.

The most commonly prescribed drugs for OSCC are cisplatin, mitomycin C and 5-fluorouracil. We have shown that metformin inhibits the effects of cisplatin and also mildly inhibited the effects of mitomycin C (Damelin et al., 2014). Preliminary data in our lab indicates that

metformin also inhibits the effects of 5-fluorouracil toward OSCC cell lines. Therefore, metformin should not be used in combination with these commonly prescribed chemotherapeutic drugs. Should metformin reach the stage where it can be prescribed for OSCC, caution must be used when treating diabetic patients with these chemotherapeutic drugs.

Metformin increased the intracellular reducing environment and also enhanced the effects of anti-cancer drugs that are reductively activated. DSF is approved for the treatment of alcoholism, and also has a low toxicity profile. DSF exerted cytotoxic effects toward OSCC cell lines which were enhanced in the presence of metformin. This drug combination is a very promising chemotherapeutic approach for OSCC as these drugs are both affordable and individually, they both have a low toxicity profile. DSF was effective against cancer at particularly low doses and may be more effective toward tumours that have elevated copper levels.

We have therefore identified a novel treatment option for OSCC that may be superior to currently prescribed chemotherapeutic drugs as this novel combination is cost effective and less harmful. For this reason, this study has been extended to include an *in vivo* component. Future work that will be conducted in this laboratory will involve studies on nude mice. The effects of DSF, Cu-DSF, Cu-ATSM and Cu-GTSM in combination with metformin will be assessed in nude mice. This will allow us to determine drug dosage that is tolerated and acceptable for cancer therapy. Copper content and redox state of the xenografts will be determined in order to identify whether the effects of these drugs *in vitro* match its *in vivo* effects. We will also investigate whether OSCC tumours will be susceptible to reductively activated drugs such as DSF, Cu-ATSM or Cu-GTSM using immunohistochemistry to detect biomarkers of redox state in human tumour biopsies.

This study holds promise for a novel treatment option for OSCC that is likely to move to clinical trials should mouse trials be successful. Rather than investigating new drugs, drug repurposing may be a more effective approach in the identification of novel chemotherapeutic drugs. This study may be the first step toward the implementation of a new treatment option for OSCC patients in this country and other developing countries.

References:

Aebi, S., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R.D., Boland, C.R., et al. 1996. Loss of DNA Mismatch Repair in Acquired Resistance to Cisplatin. *Cancer Research*. 56(13):3087–3090. Available: http://cancerres.aacrjournals.org/content/56/13/3087.short [2014, December 19].

Alderden, R.A., Hall, M.D. & Hambley, T.W. 2006. The Discovery and Development of Cisplatin. *Journal of Chemical Education*. 83(5):728–734.

Alimova, I.N., Liu, B., Fan, Z., Edgerton, S.M., Dillon, T., Lind, S.E. & Thor, A.D. 2009. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle*. 8(6):909–915. Available: http://www.ncbi.nlm.nih.gov/pubmed/19221498.

American Diabetes Association. 2014. Standards of medical care in diabetes-2014. *Diabetes Care*. 37(Suppl_1):S14–S80. DOI: 10.2337/dc14-S014.

Andrews, P.A., Mann, S.C., Velury, S. & Howell, S.B. 1987. Cisplatin Uptake Mediated Cisplatin-Resistance in Human Ovarian Carcinoma Cells. In *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*. M. Nicolini, Ed. Padua, Italy: Martinus Nijhoff Publishing. 248–254. DOI: 10.1007/978-1-4613-1717-3.

Andrews, P.A., Murphy, M.P. & Howell, S.B. 1987. Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. *Cancer Chemotherapy and Pharmacology*. 19(2):149–154. DOI: 10.1007/BF00254568.

Andrews, P.A., Velury, S., Mann, S.C. & Howell, S.B. 1988. cis-Diamminedichloroplatinum(II) Accumulation in Sensitive and Resistant Human Ovarian Carcinoma Cells. *Cancer Research*. 48(1):68–73. Available: http://cancerres.aacrjournals.org/content/48/1/68.short [2014, December 18].

Andrzejewski, S., Gravel, S.-P., Pollak, M. & St-Pierre, J. 2014. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer & Metabolism*. 2(1):12. DOI: 10.1186/2049-3002-2-12.

Arnold, M., Soerjomataram, I., Ferlay, J. & Forman, D. 2015. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut*. 64(3):381–387. DOI: 10.1136/gutjnl-2014-308124.

Bailey, C.J. & Day, C. 2004. Metformin: its botanical background. *Practical Diabetes International*. 21(3):115–117. DOI: 10.1002/pdi.606.

Basu, A. & Krishnamurthy, S. 2010. Cellular Responses to Cisplatin-Induced DNA Damage. *Journal of Nucleic Acids*. 2010:16. DOI: 10.4061/2010/201367.

Batandier, C., Guigas, B., Detaille, D., El-Mir, M.-Y., Fontaine, E., Rigoulet, M. & Leverve, X.M. 2006. The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. *Journal of Bioenergetics and Biomembranes*. 38:33–42. DOI: 10.1007/s10863-006-9003-8.

Bey, E., Alexander, J., Whitcutt, J.M., Hunt, J.A. & Gear, J.H.S. 1976. Carcinoma of the Esophagus in Africans: Establishment of a Continuously Growing Cell Line from a Tumor Specimen. *Society for In vitro Biology*. 12(2):107–114.

Bhaijee, F. & Akhtar, I. 2013. *Esophagus - Staging of esophageal carcinoma*. Available: http://www.pathologyoutlines.com/topic/esophagusstaging.html [2014, December 17].

Bhuyan, B.K. & Betz, T. 1968. Studies on the Mode of Action of the Copper(II)Chelate of 2-Keto-3-ethoxybutyraldehyde-bis(thiosemicarbazone). *Cancer Research*. 28(4):758–763. Available: http://cancerres.aacrjournals.org/content/28/4/758.short [2015, March 19].

Bica, L., Meyerowitz, J., Parker, S.J., Caragounis, A., Du, T., Paterson, B.M., Barnham, K.J., Crouch, P.J., et al. 2011. Cell cycle arrest in cultured neuroblastoma cells exposed to a bis(thiosemicarbazonato) metal complex. *Biometals*. 24(1):117–133. DOI: 10.1007/s10534-010-9380-7.

Bjornsti, M.-A. & Houghton, P.J. 2004. The TOR pathway: a target for cancer therapy. *Nature reviews. Cancer*. 4(5):335–48. DOI: 10.1038/nrc1362.

Bolster, D.R., Crozier, S.J., Kimball, S.R. & Jefferson, L.S. 2002. AMP-activated Protein Kinase Suppresses Protein Synthesis in Rat Skeletal Muscle through Down-regulated Mammalian Target of Rapamycin (mTOR) Signaling. *The Journal of Biological Chemistry*. 277(27):23977–80. DOI: 10.1074/jbc.C200171200.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*. 72:248–254.

Brewer, G.J. 2001. Copper control as an antiangiogenic anticancer therapy: lessons from treating Wilson's disease. *Experimental Biology and Medicine*. 226(7):665–673. Available: http://ebm.sagepub.com/content/226/7/665.full [2015, March 20].

Buzzai, M., Jones, R.G., Amaravadi, R.K., Lum, J.J., Deberardinis, R.J., Zhao, F., Viollet, B. & Thompson, C.B. 2007. Systemic Treatment with the Antidiabetic Drug Metformin Selectively Impairs p53-Deficient Tumor Cell Growth. *Cancer Research*. 67(14):6745–6752. DOI: 10.1158/0008-5472.CAN-06-4447.

Byrski, T., Huzarski, T., Dent, R., Marczyk, E., Jasiowka, M., Gronwald, J., Jakubowicz, J., Cybulski, C., et al. 2014. Pathologic complete response to neoadjuvant cisplatin in BRCA1-positive breast cancer patients. *Breast cancer research and treatment*. 147(2):401–5. DOI: 10.1007/s10549-014-3100-x.

Cairns, R. a, Harris, I.S. & Mak, T.W. 2011. Regulation of cancer cell metabolism. *Nature reviews. Cancer*. 11(2):85–95. DOI: 10.1038/nrc2981.

Cantrell, L.A., Zhou, C., Mendivil, A., Malloy, K.M., Gehrig, P.A. & Bae-jump, V.L. 2010. Metformin is a potent inhibitor of endometrial cancer cell proliferation — implications for a novel treatment strategy. *Gynecologic Oncology*. 116:92–98. DOI: 10.1016/j.ygyno.2009.09.024.

Carling, D. 2004. The AMP-activated protein kinase cascade – a unifying system for energy control. *Trends in Biochemical Sciences*. 29(1):18–24. DOI: 10.1016/j.tibs.2003.11.005.

Chan, S. 2004. Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer. *British journal of cancer*. 91(8):1420–4. DOI: 10.1038/sj.bjc.6602162.

Chen, J. & Lindblom, A. 2000. Germline mutation screening of the STK11/LKB1 gene in familial breast cancer with LOH on 19p. *Clinical Genetics*. 57(5):394–397. DOI: 10.1034/j.1399-0004.2000.570511.x.

Chen, L. & Madura, K. 2005. Increased proteasome activity, ubiquitin-conjugating enzymes, and eEF1A translation factor detected in breast cancer tissue. *Cancer research*. 65(13):5599–606. DOI: 10.1158/0008-5472.CAN-05-0201.

Chen, D., Cui, Q.C., Yang, H. & Dou, Q.P. 2006. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts via inhibition of the proteasome activity. *Cancer Research*. 66(21):10425–10433. DOI: 10.1158/0008-5472.CAN-06-2126.

Chen, H.H.W., Song, I., Hossain, A., Choi, M., Yamane, Y., Liang, Z.D., Lu, J., Wu, L.Y.-H., et al. 2008. Elevated Glutathione Levels Confer Cellular Sensitization to Cisplatin Toxicity by Up-Regulation of Copper Transporter hCtr1. *Molecular Pharmacology*. 74(3):697–704. DOI: 10.1124/mol.108.047969.head.

Chen, S.H., Liu, S.H., Liang, Y.C., Lin, J.K. & Lin-Shiau, S.Y. 2001. Oxidative stress and c-Jun-amino-terminal kinase activation involved in apoptosis of primary astrocytes induced by disulfiram-Cu(2+) complex. *European Journal of Pharmacology*. 414(2-3):177–88. Available: http://www.ncbi.nlm.nih.gov/pubmed/11239917.

Chick, J. 1999. Safety Issues Concerning the Use of Disulfiram in Treating Alcohol Dependence. *Drug Safety*. 20(5):427–435. DOI: 10.2165/00002018-199920050-00003.

Cho, H., Lee, T., Park, J., Park, K., Choe, J., Sin, D., Park, Y., Moon, Y., et al. 2007. Disulfiram Suppresses Invasive Ability of Osteosarcoma Cells Via the Inhibition of MMP-2 and MMP-9 Expression. *Journal of Biochemistry and Molecular Biology*. 40(6):1069–1076.

Chválová, K., Brabec, V. & Kaspárková, J. 2007. Mechanism of the formation of DNA-protein cross-links by antitumor cisplatin. *Nucleic Acids Research*. 35(6):1812–1821. DOI: 10.1093/nar/gkm032.

Ciarimboli, G., Ludwig, T., Lang, D., Pavenstädt, H., Koepsell, H., Piechota, H.-J., Haier, J., Jaehde, U., et al. 2005. Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2. *The American Journal of Pathology*. 167(6):1477–84. DOI: 10.1016/S0002-9440(10)61234-5.

Clague, M.J. & Urbé, S. 2010. Ubiquitin: same molecule, different degradation pathways. *Cell*. 143(5):682–685. DOI: 10.1016/j.cell.2010.11.012.

Conticello, C., Martinetti, D., Adamo, L., Buccheri, S., Giuffrida, R., Parrinello, N., Lombardo, L., Anastasi, G., et al. 2012. Disulfiram, an old drug with new potential therapeutic uses for human hematological malignancies. *International Journal of Cancer*. 131(9):2197–203. DOI: 10.1002/ijc.27482.

Cooper, G.M. 2000. *The Cell - A Molecular Approach*. 2nd ed. G.M. Cooper, Ed. Sunderland (MA): Sinauer Associates. Available: http://www.ncbi.nlm.nih.gov/books/NBK9957/ [2015, March 02].

Cowley, A.R., Dilworth, J.R., Donnelly, P.S., Gee, A.D. & Heslop, J.M. 2004. Acetylacetonate bis(thiosemicarbazone) complexes of copper and nickel: towards new copper radiopharmaceuticals. *Dalton transactions (Cambridge, England : 2003)*. (16):2404–12. DOI: 10.1039/B406429A.

Cramer, H.I. 1935. *Patent No. 2014353*. United States. Available: http://www.google.com/patents/US2014353 [2015, February 05].

Cusi, K., Consoli, A. & DeFronzo, R.A. 1996. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *The Journal of clinical endocrinology and metabolism*. 81(11):4059–67. DOI: 10.1210/jcem.81.11.8923861.

Cutsem, E. Van, Köhne, C.-H., Hitre, E., Zaluski, J., Chien, C.-R.C., Makhson, A., D'Haens, G., Pintér, T., et al. 2009. Cetuximab and Chemotherapy as Initial Treatment for Metastatic Colorectal Cancer. *The New England Journal of Medicine*. 360(14):1408–1417. Available: http://www.nejm.org/doi/full/10.1056/NEJMoa0805019 [2014, December 19].

Cvek, B. & Dvorak, Z. 2008. The value of proteasome inhibition in cancer. Can the old drug, disulfiram, have a bright new future as a novel proteasome inhibitor? *Drug Discovery Today*. 13(15-16):716–722. DOI: 10.1016/j.drudis.2008.05.003.

Dabholkar, M., Vionnet, J., Bostick-Bruton, F., Yu, J.J. & Reed, E. 1994. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *The Journal of clinical investigation*. 94(2):703–8. DOI: 10.1172/JCI117388.

Damelin, L.H., Jivan, R., Veale, R.B., Rousseau, A.L. & Mavri-Damelin, D. 2014. Metformin induces an intracellular reductive state that protects oesophageal squamous cell carcinoma cells against cisplatin but not copper-bis(thiosemicarbazones). *BMC Cancer*. 14(1):314. DOI: 10.1186/1471-2407-14-314.

Daniel, K.G., Gupta, P., Harbach, R.H., Guida, W.C. & Dou, Q.P. 2004. Organic copper complexes as a new class of proteasome inhibitors and apoptosis inducers in human cancer cells. *Biochemical pharmacology*. 67(6):1139–51. DOI: 10.1016/j.bcp.2003.10.031.

Daniel, K.G., Chen, D., Orlu, S., Cui, Q.C., Miller, F.R. & Dou, Q.P. 2005. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. *Breast Cancer Research*. 7(6):R897–R908. DOI: 10.1186/bcr1322.

Daniel, K.G., Chen, D., Yan, B. & Dou, Q.P. 2007. Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer. *Frontiers in Bioscience*. 12:135–144. Available: http://www.ncbi.nlm.nih.gov/pubmed/17127289 [2015, February 25].

Datapharm. 2014. *Metformin hydrochloride*. Department of Health. Available: http://services.medicines.org.uk/assethosting/assets/printable/m/e/metformin hydrochloride/printable.1255_3_1803.pdf [2014, December 22].

Deans, A.J. & West, S.C. 2011. DNA interstrand crosslink repair and cancer. *Nature reviews. Cancer*. 11(7):467–80. DOI: 10.1038/nrc3088.

DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G. & Thompson, C.B. 2008. The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metabolism*. 7:11–20. DOI: 10.1016/j.cmet.2007.10.002.

Dhara, S. 1970. A rapid method for the synthesis of cis-[Pt(NH3)2Cl2]. *Indian Journal of Chemistry*. 8:193–194.

Dice, J.F. 2007. Lysosomal Degradation of Proteins. In *Encyclopedia of Life Sciences*. Chichester: John Wiley & Sons, Ltd. 1–7. DOI: 10.1002/9780470015902.a0000646.pub2.

Doku, D. 2010. The tobacco industry tactics-a challenge for tobacco control in low and middle income countries. *African Health Sciences*. 10(2):201–203. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2956281&tool=pmcentrez&rendertype=abstract [2015, March 22].

Dooley, C.T., Dore, T.M., Hanson, G.T., Jackson, W.C., Remington, S.J. & Tsien, R.Y. 2004. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *The Journal of Biological Chemistry*. 279(21):22284–22293. DOI: 10.1074/jbc.M312847200.

Drouin, R., Rodriguez, H., Gao, S.-W., Gebreyes, Z., O'Connor, T.R., Holmquist, G.P. & Akman, S.A. 1996. Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. *Free Radical Biology and Medicine*. 21(3):261–273. DOI: 10.1016/0891-5849(96)00037-8.

Elenbaas, R.M. 1977. Drug therapy reviews: management of the disulfiram-alcohol reaction. *American Journal of Hospital Pharmacy*. 34(8):827–831. Available: http://www.ncbi.nlm.nih.gov/pubmed/331944 [2015, January 05].

El-Mir, M.Y., Nogueira, V., Fontaine, E., Avéret, N., Rigoulet, M. & Leverve, X. 2000. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *The Journal of biological chemistry*. 275(1):223–228. Available: http://www.ncbi.nlm.nih.gov/pubmed/10617608.

Elstrom, R.L., Bauer, D.E., Buzzai, M., Karnauskas, R., Harris, M.H., Plas, D.R., Zhuang, H., Cinalli, R.M., et al. 2004. Akt stimulates aerobic glycolysis in cancer cells. *Cancer research*. 64(11):3892–9. DOI: 10.1158/0008-5472.CAN-03-2904.

Emami Riedmaier, A., Fisel, P., Nies, A.T., Schaeffeler, E. & Schwab, M. 2013. Metformin and cancer: from the old medicine cabinet to pharmacological pitfalls and prospects. *Trends in Pharmacological Sciences*. 34(2):126–135. DOI: 10.1016/j.tips.2012.11.005.

Evans, J.M.M., Donnelly, L.A., Emslie-Smith, A.M., Alessi, D.R. & Morris, A.D. 2005. Metformin and reduced risk of cancer in diabetic patients. *Biomedical Journal*. 330:1304–1305. DOI: 10.1136/bmj.38393.572188.EB.

Fanucchi, S. & Veale, R.B. 2009. Role of p53/FAK association and p53 Ser46 phosphorylation in staurosporine-mediated apoptosis: Wild type versus mutant p53-R175H. *FEBS Letters*. 583(22):3557–3562. DOI: 10.1016/j.febslet.2009.10.059.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., et al. 2013. *GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*. Available: http://globocan.iarc.fr [2015, January 09].

Foretz, M., Hébrard, S., Leclerc, J., Zarrinpashneh, E., Soty, M., Mithieux, G., Sakamoto, K., Andreelli, F., et al. 2010. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *The Journal of Clinical Investigation*. 120(7):2355–2369. DOI: 10.1172/JCI40671.

French, F.A. & Freedlander, B.L. 1960. Chemotherapy Studies on Transplanted Mouse Tumors. *Cancer Research*. 20:505–538.

French, F.A., Freedlander, B.L., Hoskino, A. & French, J. 1958. Carcinostatic Action of Polycarbonyl Compounds and Their Derivatives: IV. Glyoxal Bis(thiosemicarbazone) and Derivatives. *Cancer Research*. 18(11):1290–1300. Available: http://cancerres.aacrjournals.org/content/18/11/1290.short [2014, December 30].

Frezza, C. & Gottlieb, E. 2009. Mitochondria in cancer: not just innocent bystanders. *Seminars in Cancer Biology*. 19(1):4–11. DOI: 10.1016/j.semcancer.2008.11.008.

Fruehauf, J.P. & Meyskens, F.L. 2007. Reactive oxygen species: a breath of life or death? *Clinical Cancer Research*. 13(3):789–794. DOI: 10.1158/1078-0432.CCR-06-2082.

Fujibayashi, Y., Taniuchi, H., Yonekura, Y., Ohtani, H., Konishi, J. & Yokoyama, A. 1997. Copper-62-ATSM: A New Hypoxia Imaging Agent with High Membrane Permeability and Low Redox Potential. *Journal of Nuclear Medicine*. 38(7):1155–1160.

Furuta, S., Ortiz, F., Zhu Sun, X., Wu, H.-H., Mason, A. & Momand, J. 2002. Copper uptake is required for pyrrolidine dithiocarbamate-mediated oxidation and protein level increase of p53 in cells. *The Biochemical Journal*. 365(Pt 3):639–648. DOI: 10.1042/BJ20011251.

Giovannucci, E., Harlan, D.M., Archer, M.C., Bergenstal, R.M., Gapstur, S.M., Habel, L. a, Pollak, M., Regensteiner, J.G., et al. 2010. Diabetes and Cancer: A consensus report. *Diabetes Care*. 33(7):1674–1685. DOI: 10.2337/dc10-0666.

Glick, D., Barth, S. & Macleod, K.F. 2010. Autophagy: cellular and molecular mechanisms. *The Journal of Pathology*. 221(1):3–12. DOI: 10.1002/path.2697.

Godwin, A.K., Meistert, A., Dwyer, P.J.O., Huangt, C.S., Hamilton, T.C. & Andersont, M.E. 1992. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proceedings of the National Academy of Sciences*. 89(7):3070–3074.

Gonzalez, V.M., Fuertes, M. a, Alonso, C. & Perez, J.M. 2001. Is cisplatin-induced cell death always produced by apoptosis? *Molecular pharmacology*. 59(4):657–63. Available: http://www.ncbi.nlm.nih.gov/pubmed/11259608.

Gordon, R.M. & Seaton, D.R. 1942. Treatment of Scabies. *British Medical Journal*. 1(4248):685–687. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2161678&tool=pmcentrez&rendertype=abstract [2015, January 05].

Gotlieb, W.H., Saumet, J., Beauchamp, M., Gu, J., Lau, S., Pollack, M.N. & Bruchim, I. 2008. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. *Gynecologic Oncology*. 110:246 – 250. DOI: 10.1016/j.ygyno.2008.04.008.

Green, M.A., Klippenstein, D.L. & Tennison, J.R. 1988. Copper(II) Bis(thiosemicarbazone) Complexes as Potential Tracers for Evaluation of Cerebral and Myocardial Blood Flow with PET. *Journal of Nuclear Medicine*. 29(9):1549–1557.

Griffith, O.W. 1982. Mechanism of Action, Metabolism, and Toxicity of Buthionine Sulfoximine and Its Higher Homologs, Potent Inhibitors of Glutathione Synthesis. *The Journal of Biological Chemistry*. 257(22):13704–13712.

Griffiths, G., Hall, R., Sylvester, R., Raghavan, D. & Parmar, M.K.B. 2011. International phase III trial assessing neoadjuvant cisplatin, methotrexate, and vinblastine chemotherapy for muscle-invasive bladder cancer: long-term results of the BA06 30894 trial. *Journal of Clinical Oncology*. 29(16):2171–2177. DOI: 10.1200/JCO.2010.32.3139.

Guertin, D.A. & Sabatini, D.M. 2005. An expanding role for mTOR in cancer. *Trends in molecular medicine*. 11(8):353–61. DOI: 10.1016/j.molmed.2005.06.007.

Hacker, M.P., Ershler, B., Newman, A. & Camelli, L. 1982. Effect of Disulfiram (Tetraethylthiuram Disulfide) and Diethyldithiocarbamate on the Bladder Toxicity and Antitumor Activity of Cyclophosphamide in Mice1. *Cancer Research*. 42:4490–4494.

Haddow, A. 1936. Historical Notes on Cancer from the MSS. of Louis Westenra Sambon. *Proceedings of the Royal Society of Medicine*. 29(9):1015–1028.

Hajdu, S.I. 2011. A note from history: landmarks in history of cancer, part 1. *Cancer*. 117(5):1097–102. DOI: 10.1002/cncr.25553.

Hanahan, D. & Weinberg, R.A. 2011. Hallmarks of cancer: the next generation. *Cell*. 144(5):646–674. Available: http://www.ncbi.nlm.nih.gov/pubmed/21376230.

Hao, B., Wang, H., Zhou, K., Li, Y., Chen, X., Zhou, G., Zhu, Y., Miao, X., et al. 2004. Identification of genetic variants in base excision repair pathway and their associations with risk of esophageal squamous cell carcinoma. *Cancer research*. 64(12):4378–84. DOI: 10.1158/0008-5472.CAN-04-0372.

Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M.T., Belham, C. & Avruch, J. 1998. Amino Acid Sufficiency and mTOR Regulate p70 S6 Kinase and eIF-4E BP1 through a Common Effector Mechanism. *Journal of Biological Chemistry*. 273(23):14484–14494. DOI: 10.1074/jbc.273.23.14484.

Hardie, D.G. 2005. New roles for the LKB1 -> AMPK pathway. *Current Opinion in Cell Biology*. 17:167–173. DOI: 10.1016/j.ceb.2005.01.006.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Mäkelä, T.P., Alessi, D.R. & Hardie, D.G. 2003. Complexes between the LKB1 tumor suppressor , STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade. *Journal of Biology*. 2(4):28.1–28.16.

He, C. & Klionsky, D.J. 2009. Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics*. 43:67–93. DOI: 10.1146/annurev-genet-102808-114910.

Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U. & Wolf, D.H. 1997. The Active Sites of the Eukaryotic 20 S Proteasome and Their Involvement in Subunit Precursor Processing. *Journal of Biological Chemistry*. 272(40):25200–25209. DOI: 10.1074/jbc.272.40.25200.

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., et al. 1998. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature*. 391(6663):184–187. DOI: 10.1038/34432.

Herskovic, A., Martz, K., Al-Sarraf, M., Leichman, L., Brindle, J., Vaitevicius, V., Cooper, J., Byhardt, R., et al. 1992. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *New England Journal of Medicine*. 326(24):1593–1598. Available: http://www.nejm.org/doi/pdf/10.1056/NEJM199206113262403 [2014, December 17].

Higuchi, K., Koizumi, W., Tanabe, S., Sasaki, T., Katada, C., Azuma, M., Nakatani, K., Ishido, K., et al. 2009. Current Management of Esophageal Squamous-Cell Carcinoma in Japan and Other Countries. *Gastrointestinal Cancer Research*. 3:153–161.

Hirsch, H.A., Iliopoulos, D., Tsichlis, P.N. & Struhl, K. 2009. Metformin Selectively Targets Cancer Stem Cells, and Acts Together with Chemotherapy to Block Tumor Growth and Prolong Remission. *Cancer Research*. DOI: 10.1158/0008-5472.CAN-09-2994.

Hishikawa, Y., Abe, S., Kinugasa, S., Yoshimura, H., Monden, N., Igarashi, M., Tachibana, M. & Nagasue, N. 1997. Overexpression of Metallothionein Correlates with Chemoresistance to Cisplatin and Prognosis in Esophageal Cancer. *Oncology*. 54(4):342–347. DOI: 10.1159/000227714.

Hostetter, A.A., Osborn, M.F. & DeRose, V.J. 2012. RNA-Pt adducts following cisplatin treatment of Saccharomyces cerevisiae. *ACS Chemical Biology*. 7(1):218–225. DOI: 10.1021/cb200279p.

Hundal, R.S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S.E., Schumann, W.C., et al. 2000. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes*. 49(12):2063–9. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2995498&tool=pmcentrez&rendertype=abstract [2014, October 22].

Hurley, R.L., Anderson, K.A., Franzone, J.M., Kemp, B.E., Means, A.R. & Witters, L.A. 2005. The Ca 2+/Calmodulin-dependent Protein Kinase Kinases Are AMP-activated Protein Kinase Kinases. *The Journal of iological Chemistry*. 280(32):29060 –29066. DOI: 10.1074/jbc.M503824200.

Igaki, H., Kato, H., Ando, N., Shinoda, M., Shimizu, H., Nakamura, T., Ozawa, S., Yabusaki, H., et al. 2008. A randomized trial of postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil versus neoadjuvant chemotherapy for clinical stage II/III squamous cell carcinoma of the thoracic esophagus (JCOG 9907). *Journal of Clinical Oncology (Meeting Abstracts)*. 26(15S):4510. Available: http://hwmaint.meeting.ascopubs.org/cgi/content/abstract/26/15_suppl/4510 [2014, December 17].

Ilson, B.D.H., Saltz, L., Enzinger, P., Huang, Y., Kornblith, A., Gollub, M., Reilly, E.O., Schwartz, G., et al. 2011. Phase II Trial of Weekly Irinotecan Plus Cisplatin in Advanced Esophageal Cancer. *October*. 17(10):3270–3275.

Imamura, K., Ogura, T., Kishimoto, a, Kaminishi, M. & Esumi, H. 2001. Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator, 5-aminoimidazole- 4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochemical and biophysical research communications*. 287(2):562–7. DOI: 10.1006/bbrc.2001.5627.

Independent Online News - South Africa. 2006. *Discovery agrees to pay for Herceptin*. Available: http://www.iol.co.za/news/south-africa/discovery-agrees-to-pay-for-herceptin-1.269423#.VJRRV14CZA [2014, December 19].

Indo, H.P., Davidson, M., Yen, H.-C., Suenaga, S., Tomita, K., Nishii, T., Higuchi, M., Koga, Y., et al. 2007. Evidence of ROS generation by mitochondria in cells with impaired electron

transport chain and mitochondrial DNA damage. *Mitochondrion*. 7(1-2):106–18. DOI: 10.1016/j.mito.2006.11.026.

Isakovic, A., Harhaji, L., Stevanovic, D., Markovic, Z., Sumarac-Dumanovic, M., Starcevic, V., Micic, D. & Trajkovic, V. 2007. Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cellular and molecular life sciences : CMLS*. 64(10):1290–1302. DOI: 10.1007/s00018-007-7080-4.

Ishida, S., Lee, J., Thiele, D.J. & Herskowitz, I. 2002. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proceedings of the National Academy of Sciences of the United States of America*. 99(22):14298–302. DOI: 10.1073/pnas.162491399.

Ishikawa, T. & Ali-Osman, F. 1993. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *Journal of Biological Chemistry*. 268(27):20116–20125. Available: http://www.jbc.org/content/268/27/20116.short [2015, January 23].

Jacobsen, E. 1952. Deaths of alcoholic patients treated with disulfiram (tetraethylthiuram disulfide) in Denmark. *Quarterly Journal of Studies on Alcohol*. 13(1):16–26. Available: http://www.ncbi.nlm.nih.gov/pubmed/14912292 [2015, January 05].

Jamieson, E.R. & Lippard, S.J. 1999. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. *Chemical Reviews*. 99(9):2467–98. Available: http://www.ncbi.nlm.nih.gov/pubmed/11749487 [2015, January 21].

Janjetovic, K., Vucicevic, L., Misirkic, M., Vilimanovich, U., Tovilovic, G., Zogovic, N., Nikolic, Z., Jovanovic, S., et al. 2011. Metformin reduces cisplatin-mediated apoptotic death of cancer cells through AMPK-independent activation of Akt. *European Journal of Pharmacology*. 651:41–50. DOI: 10.1016/j.ejphar.2010.11.005.

Jiralerspong, S., Palla, S.L., Giordano, S.H., Meric-Bernstam, F., Liedtke, C., Barnett, C.M., Hsu, L., Hung, M.-C., et al. 2009. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 27(20):3297–302. DOI: 10.1200/JCO.2009.19.6410.

Johansson, B. 1992. A review of the pharmacokinetics and pharmacodynamics of disulfiram and its metabolites. *Acta Psychatrica Scandinavica*. 86:15–26.

Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J. & Thompson, C.B. 2005. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Molecular cell*. 18(3):283–93. DOI: 10.1016/j.molcel.2005.03.027.

Kalender, A., Selvaraj, A., Kim, S.Y., Gulati, P., Brûlé, S., Viollet, B., Kemp, B.E., Bardeesy, N., et al. 2010. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metabolism*. 11(5):390–401. DOI: 10.1016/j.cmet.2010.03.014.

Kawaguchi, Y., Kono, K., Mimura, K., Mitsui, F., Sugai, H., Akaike, H. & Fujii, H. 2007. Targeting EGFR and HER-2 with cetuximab- and trastuzumab-mediated immunotherapy in oesophageal squamous cell carcinoma. *British Journal of Cancer*. 97(4):494–501. DOI: 10.1038/sj.bjc.6603885.

Kelland, L.R. 1993. New platinum antitumor complexes. *Critical Reviews in Oncology/Hematology*. 15(3):191–219. DOI: 10.1016/1040-8428(93)90042-3.

Kim, J., Kundu, M., Viollet, B. & Guan, K.-L. 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology*. 13(2):132–41. DOI: 10.1038/ncb2152.

Kisfalvi, K., Eibl, G., Sinnett-Smith, J. & Rozengurt, E. 2009. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. *Cancer Research*. 69(16):6539–6545. DOI: 10.1158/0008-5472.CAN-09-0418.

Kitagawa, R., Katsumata, N., Shibata, T., Nakanishi, T., Nishimura, S., Ushijima, K., Takano, M., Satoh, T., et al. 2012. A randomized, phase III trial of paclitaxel plus carboplatin (TC) versus paclitaxel plus cisplatin (TP) in stage IVb, persistent or recurrent cervical cancer: Japan Clinical Oncology Group study (JCOG0505). *ASCO Meeting Abstracts*. 30(15_suppl):5006. Available: http://hwmaint.meeting.ascopubs.org/cgi/content/abstract/30/15_suppl/5006 [2015, January 20].

Kobayashi, M., Kato, K., Iwama, H., Fujihara, S., Nishiyama, N., Mimura, S., Toyota, Y., Nomura, T., et al. 2013. Antitumor effect of metformin in esophageal cancer: In vitro study. *International Journal of Oncology*. 42(2):517–524. Available: http://www.spandidos-publications.com/ijo/42/2/517/abstract [2014, November 17].

Koo, D.H., Park, S.-I., Kim, Y.-H., Kim, J.H., Jung, H.-Y., Lee, G.-H., Choi, K.D., Song, H.J., et al. 2012. Phase II study of use of a single cycle of induction chemotherapy and concurrent chemoradiotherapy containing capecitabine/cisplatin followed by surgery for patients with resectable esophageal squamous cell carcinoma: long-term follow-up data. *Cancer chemotherapy and pharmacology*. 69(3):655–63. DOI: 10.1007/s00280-011-1750-5.

Köpf-Maier, P. & Mühlhausen, S.K. 1992. Changes in the cytoskeleton pattern of tumor cells by cisplatin in vitro. *Chemico-Biological Interactions*. 82(3):295–316. DOI: 10.1016/0009-2797(92)90002-3.

Kragh, H. 2008. From Disulfiram to Antabuse: The Invention of a Drug. *Bulletin for the History of Chemistry*. 33(2):82–88.

Kurokawa, H., Ishida, T., Nishio, K., Arioka, H., Sata, M., Fukumoto, H., Miura, M. & Saijo, N. 1995. Gamma-glutamylcysteine synthetase gene overexpression results in increased activity of the ATP-dependent glutathione S-conjugate export pump and cisplatin resistance. *Biochemical and Biophysical Research Communications*. 216(1):258–264. DOI: 10.1006/bbrc.1995.2618.

Kuroki, T., Trapasso, F., Shiraishi, T., Alder, H., Mimori, K., Mori, M. & Croce, C.M. 2002. Genetic Alterations of the Tumor Suppressor Gene WWOX in Esophageal Squamous Cell Carcinoma. *Cancer Res.* 62(8):2258–2260. Available: http://cancerres.aacrjournals.org/content/62/8/2258.short [2014, December 17].

Kurth-Kraczek, E.J., Hirshman, M.F., Goodyear, L.J. & Winder, W.W. 1999. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes*. 48(8):1667–1671. DOI: 10.2337/diabetes.48.8.1667.

Laderoute, K.R., Amin, K., Calaoagan, J.M., Knapp, M., Le, T., Orduna, J., Foretz, M. & Viollet, B. 2006. 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. *Molecular and Cellular Biology*. 26(14):5336–47. DOI: 10.1128/MCB.00166-06.

Laskin, J.J. & Sandler, A.B. 2004. Epidermal growth factor receptor: a promising target in solid tumours. *Cancer Treatment Reviews*. 30(1):1–17. DOI: 10.1016/j.ctrv.2003.10.002.

Lecker, S.H., Goldberg, A.L. & Mitch, W.E. 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology : JASN*. 17(7):1807–1819. DOI: 10.1681/ASN.2006010083.

Leclerc, I., Woltersdorf, W.W., da Silva Xavier, G., Rowe, R.L., Cross, S.E., Korbutt, G.S., Rajotte, R. V, Smith, R., et al. 2004. Metformin, but not leptin, regulates AMP-activated protein kinase in pancreatic islets: impact on glucose-stimulated insulin secretion. *American Journal of Physiology. Endocrinology and Metabolism*. 286(6):E1023–E1031. DOI: 10.1152/ajpendo.00532.2003.

Lee, J., Lim, D.H., Kim, S., Park, S.H., Park, J.O., Park, Y.S., Lim, H.Y., Choi, M.G., et al. 2012. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 30(3):268–73. DOI: 10.1200/JCO.2011.39.1953.

Lee, K.B., Parker, R.J., Bohr, V., Cornelison, T. & Reed, E. 1993. Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. *Carcinogenesis*. 14(10):2177–2180. DOI: 10.1093/carcin/14.10.2177.

Leichman, L. 1989. The role of chemotherapy in the treatment of squamous cell tumours of the esophagus. In *Cancer Chemotherapy: Concepts, Clinical Investigations and Therapeutic Advances*. 1st ed. V. 42. F.M. Muggia, Ed. (Cancer Treatment and Research). Boston, MA: Springer US. 127–149. DOI: 10.1007/978-1-4613-1747-0.

Lesan, V., Ghaffari, S.H., Salaramoli, J., Heidari, M. & Rostami, M. 2014. Evaluation of Antagonistic Effects of Metformin with Cisplatin in Gastric Cancer Cells. *International Journal of Hematology-Oncology and Stem Cell Research*. 8(3):12–19.

Levine, A.J., Feng, Z., Mak, T.W., You, H. & Jin, S. 2006. Coordination and communication between the p53 and IGF-1–AKT–TOR signal transduction pathways. *Autophagy*. 20:267–275. DOI: 10.1101/gad.1363206.GENES.

Li, J. 1997. PTEN, a Putative Protein Tyrosine Phosphatase Gene Mutated in Human Brain, Breast, and Prostate Cancer. *Science*. 275(5308):1943–1947. DOI: 10.1126/science.275.5308.1943.

Lilienbaum, A. 2013. Relationship between the proteasomal system and autophagy. *International Journal of Biochemistry and Molecular Biology*. 4(1):1–26.

Lin, C.-C., Yeh, H.-H., Huang, W.-L., Yan, J.-J., Lai, W.-W., Su, W.-P., Chen, H.H.W. & Su, W.-C. 2013. Metformin enhances cisplatin cytotoxicity by suppressing signal transducer and activator of transcription-3 activity independently of the liver kinase B1-AMP-activated protein kinase pathway. *American journal of respiratory cell and molecular biology*. 49(2):241–50. DOI: 10.1165/rcmb.2012-0244OC.

Lin, J., Haffner, M.C., Zhang, Y., Lee, B.H., Brennen, W.N., Britton, J., Kachhap, S.K., Shim, J.S., et al. 2011. Disulfiram is a DNA demethylating agent and inhibits prostate cancer cell growth. *The Prostate*. 71(4):333–343. DOI: 10.1002/pros.21247.

Liu, B., Fang, M., Lu, Y., Mendelsohn, J. & Fan, Z. 2001. Fibroblast growth factor and insulin-like growth factor differentially modulate the apoptosis and G1 arrest induced by anti-epidermal growth factor receptor monoclonal antibody. *Oncogene*. 20(15):1913–22. DOI: 10.1038/sj.onc.1204277.

Liu, P., Brown, S., Goktug, T., Channathodiyil, P., Kannappan, V., Hugnot, J.-P., Guichet, P.-O., Bian, X., et al. 2012. Cytotoxic effect of disulfiram/copper on human glioblastoma cell lines and ALDH-positive cancer-stem-like cells. *British Journal of Cancer*. 107(9):1488–1497. DOI: 10.1038/bjc.2012.442.

Logie, L., Harthill, J., Patel, K., Bacon, S., Hamilton, D.L., Macrae, K., Mcdougall, G., Wang, H., et al. 2012. Cellular Responses to the Metal-Binding Properties of Metformin. *Diabetes*. 61:1423–1433. DOI: 10.2337/db11-0961.

Luengo, A., Sullivan, L.B. & Heiden, M.G. Vander. 2014. Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy. *BMC Biology*. 12:82. DOI: 10.1186/s12915-014-0082-4.

Luzio, J.P., Pryor, P.R. & Bright, N.A. 2007. Lysosomes: fusion and function. *Nature Reviews. Molecular Cell Biology*. 8:622–632. DOI: 10.1038/nrm2217.

Ma, X.M. & Blenis, J. 2009. Molecular mechanisms of mTOR-mediated translational control. *Nature reviews. Molecular cell biology*. 10(5):307–18. DOI: 10.1038/nrm2672.

Madiraju, A.K., Erion, D.M., Rahimi, Y., Zhang, X.-M., Braddock, D.T., Albright, R.A., Prigaro, B.J., Wood, J.L., et al. 2014. Metformin suppresses gluconeogenesis by inhibiting

mitochondrial glycerophosphate dehydrogenase. *Nature*. 510(7506):542–6. DOI: 10.1038/nature13270.

Marikovsky, M., Nevo, N., Vadai, E. & Harris-Cerruti, C. 2002. Cu/Zn superoxide dismutase plays a role in angiogenesis. *International journal of cancer. Journal international du cancer*. 97(1):34–41. Available: http://www.ncbi.nlm.nih.gov/pubmed/11774241 [2015, January 07].

Marsin, A.-S., Bertrand†, L., Rider, M.H., Deprez, J., Beauloye, C., Vincent‡, M.F., Van den Berghe‡, G., Carling, D., et al. 2000. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Current Biology*. 10(20):1247–1255. DOI: 10.1016/S0960-9822(00)00742-9.

Martensen-Larson, O. 1951. Psychotic phenomena provoked by tetraethylthiuram disulfide. *Quarterly Journal of Studies on Alcohol*. 12(2):206–216. Available: http://www.ncbi.nlm.nih.gov/pubmed/14844646 [2015, January 05].

Martin, A.E. 1953. Instability of Diethyldithiocarbamic Acid at Low pH. *Analytical Chemistry*. 25(8):1260–1261. DOI: 10.1021/ac60080a035.

Martin-Castillo, B., Vazquez-Martin, A., Oliveras-Ferraros, C. & Menendez, J.A. 2010. Metformin and cancer - Doses, mechanisms and the dandelion and hormetic phenomena. *Cell Cycle*. 9(6):1057–1064.

Mehta, A., Mason, P.J. & Vulliamy, T.J. 2000. Glucose-6-phosphate dehydrogenase deficiency. *Best Practice & Research Clinical Haematology*. 13(1):21–38. DOI: 10.1053/beha.1999.0055.

Menendez, J.A., Quirantes-Piné, R., Rodríguez-Gallego, E., Corominas-Faja, B., Cuyàs, E., Bosch-Barrera, J., Martin-castillo, B., Segura-Carretero, A., et al. 2014. Oncobiguanides: Paracelsus' law and nonconventional routes for administering diabetobiguanides for cancer treatment. *Oncotarget*. 5(9):2344 – 2348.

Mijaljica, D., Prescott, M. & Devenish, R.J. 2014. Microautophagy in mammalian cells: Revisiting a 40-year-old conundrum. *Autophagy*. 7(7):673–682. DOI: 10.4161/auto.7.7.14733.

Miller, R.A., Chu, Q., Xie, J., Foretz, M., Viollet, B. & Birnbaum, M.J. 2013. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature*. 1–6. DOI: 10.1038/nature11808.

Mimura, K., Kono, K., Hanawa, M., Kanzaki, M., Nakao, A., Ooi, A. & Fujii, H. 2005. Trastuzumab-mediated antibody-dependent cellular cytotoxicity against esophageal squamous cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 11(13):4898–904. DOI: 10.1158/1078-0432.CCR-04-2476.

Mimura, K., Kono, K., Hanawa, M., Mitsui, F., Sugai, H., Miyagawa, N., Ooi, A. & Fujii, H. 2005. Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal

squamous cell carcinoma. *British journal of cancer*. 92(7):1253–60. DOI: 10.1038/sj.bjc.6602499.

Mizushima, N. 2004. Methods for monitoring autophagy. *The International Journal of Biochemistry & Cell Biology*. 36(12):2491–2502. DOI: 10.1016/j.biocel.2004.02.005.

Mizushima, N., Yoshimori, T. & Levine, B. 2010. Methods in Mammalian Autophagy Research. *Cell*. 140:313–326. DOI: 10.1016/j.cell.2010.01.028.

Momcilovic, M., Hong, S.-P. & Carlson, M. 2006. Mammalian TAK1 Activates Snf1 Protein Kinase in Yeast and Phosphorylates AMP-activated Protein Kinase in Vitro. *Journal of Biological Chemistry*. 281(35):25336 –25343. DOI: 10.1074/jbc.M604399200.

Moreno-Sánchez, R., Rodríguez-Enríquez, S., Marín-Hernández, A. & Saavedra, E. 2007. Energy metabolism in tumor cells. *The FEBS Journal*. 274(6):1393–418. DOI: 10.1111/j.1742-4658.2007.05686.x.

Moreno-Sánchez, R., Saavedra, E., Rodríguez-Enríquez, S., Gallardo-Pérez, J.C., Quezada, H. & Westerhoff, H. V. 2010. Metabolic control analysis indicates a change of strategy in the treatment of cancer. *Mitochondrion*. 10(6):626–39. DOI: 10.1016/j.mito.2010.06.002.

Morselli, E., Galluzzi, L., Kepp, O., Vicencio, J.-M., Criollo, A., Maiuri, M.C. & Kroemer, G. 2009. Anti- and pro-tumor functions of autophagy. *Biochimica et biophysica acta*. 1793(9):1524–32. DOI: 10.1016/j.bbamcr.2009.01.006.

Motoshima, H., Goldstein, B.J., Igata, M. & Araki, E. 2006. AMPK and cell proliferation - AMPK as a therapeutic target for atherosclerosis and cancer. *The Journal of Physiology*. 574(1):63–71. DOI: 10.1113/jphysiol.2006.108324.

Mould, R.F. 2008. Evolution of the knowledge of cancer from earliest times to the end of the 18 th century. *Journal of Oncology*. 58(2):103–115.

National Health Laboratory Service. 2008. *Summary Statistics of Cancer Diagnosed Histologically in 2008*. Available: http://www.nioh.ac.za/assets/files/FINAL_2014_NCR_2008 tables(2).pdf [2014, December 17].

Nemoto, T., Terashima, S., Kogure, M., Hoshino, Y., Kusakabe, T., Suzuki, T. & Gotoh, M. 2001. Overexpression of fatty acid synthase in oesophageal squamous cell dysplasia and carcinoma. *Pathobiology*. 69(6):297–303. DOI: 64636.

Nencioni, A., Grünebach, F., Patrone, F., Ballestrero, A. & Brossart, P. 2006. Proteasome inhibitors: antitumor effects and beyond. *Leukemia*. 21(1):30–36. DOI: 10.1038/sj.leu.2404444.

Neuberg, C., Neimann, W. & Salkowski, H. 1909. Eine Methode zur Isolierung von Aldehyden und Ketonen. *Zeitschrift für Analytische Chemie*. 48(1):58–59. DOI: 10.1007/BF01349841.

Noto, H., Goto, A., Tsujimoto, T. & Noda, M. 2012. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. *PloS one*. 7(3):e33411. DOI: 10.1371/journal.pone.0033411.

O'Donovan, T.R., O'Sullivan, G.C. & McKenna, S.L. 2014. Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. *Autophagy*. 7(5):509–524. DOI: 10.4161/auto.7.5.15066.

Ohtsu, A., Boku, N., Muro, K., Chin, K., Muto, M., Yoshida, S., Satake, M., Ishikura, S., et al. 1999. Definitive Chemoradiotherapy for T4 and/or M1 Lymph Node Squamous Cell Carcinoma of the Esophagus. *Journal of Clinical Oncology*. 17(9):2915. Available: http://jco.ascopubs.org/content/17/9/2915.short [2014, December 17].

Ouslimani, N., Peynet, J., Bonnefont-Rousselot, D., Thérond, P., Legrand, A. & Beaudeux, J.-L. 2005. Metformin decreases intracellular production of reactive oxygen species in aortic endothelial cells. *Metabolism: clinical and experimental*. 54(6):829–34. DOI: 10.1016/j.metabol.2005.01.029.

Owen, M.R., Doran, E. & Halestrap, A.P. 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal*. 348:607–614.

Ozawa, S., Ueda, M., Ando, N., Abe, O. & Shimizu, N. 1987. High incidence of egf receptor hyperproduction in esophageal squamous-cell carcinomas. *International Journal of Cancer*. 39(3):333–337. DOI: 10.1002/ijc.2910390311.

Palanimuthu, D., Shinde, S.V., Somasundaram, K. & Samuelson, A.G. 2013. In Vitro and in Vivo Anticancer Activity of Copper Bis(thiosemicarbazone) Complexes. *Journal of Medicinal Chemistry*. 56:722–734.

Paterson, B.M. & Donnelly, P.S. 2011. Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals. *Chemical Society Reviews*. 40(5):3005–3018. DOI: 10.1039/c0cs00215a.

Paz-Ares, L.G., Biesma, B., Heigener, D., von Pawel, J., Eisen, T., Bennouna, J., Zhang, L., Liao, M., et al. 2012. Phase III, randomized, double-blind, placebo-controlled trial of gemcitabine/cisplatin alone or with sorafenib for the first-line treatment of advanced, nonsquamous non-small-cell lung cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 30(25):3084–92. DOI: 10.1200/JCO.2011.39.7646.

Perez, R.P. 1998. Cellular and Molecular Determinants of Cisplatin Resistance. *European Journal of Cancer*. 34(10):1535–1542.

Petering, H.G., Buskirk, H.H. & Underwood, G.E. 1964. The Anti-Tumor Activity of 2-Keto-3-ethoxybutyraldehyde Bis (thiosemicarbazone) and Related Compounds. *Cancer Research*. 24:367–372.

Pickens, A. & Orringer, M.B. 2003. Geographical Distribution and Racial Disparity in Esophageal Cancer. *Annals of Thoracic Surgery, The*. 76:1367–1369.

Ping, W., Sun, W., Zu, Y., Chen, W. & Fu, X. 2014. Clinicopathological and prognostic significance of hypoxia-inducible factor-1α in esophageal squamous cell carcinoma: a meta-analysis. *Tumour Biology*. 35(5):4401–4409. DOI: 10.1007/s13277-013-1579-0.

Pinzani, V., Bressolle, F., Haug, I.J., Galtier, M., Blayac, J.P. & Balmès, P. 1994. Cisplatin-induced renal toxicity and toxicity-modulating strategies: a review. *Cancer Chemotherapy and Pharmacology*. 35(1):1–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/7987972.

Price, K.A., Crouch, P.J., Volitakis, I., Paterson, B.M., Lim, S., Donnelly, P.S. & White, A.R. 2011. Mechanisms controlling the cellular accumulation of copper bis(thiosemicarbazonato) complexes. *Inorganic Chemistry*. 50(19):9594–9605. DOI: 10.1021/ic201334q.

Rae, C., Tesson, M., Babich, J.W., Boyd, M., Sorensen, A. & Mairs, R.J. 2013. The Role of Copper in Disulfiram-Induced Toxicity and Radiosensitization of Cancer Cells. *Journal of Nuclear Medicine*. 54(6):953–960. DOI: 10.2967/jnumed.112.113324.

Rajkumar, S.V., Richardson, P.G., Hideshima, T. & Anderson, K.C. 2005. Proteasome inhibition as a novel therapeutic target in human cancer. *Journal of Clinical Oncology: Biology of Neoplasia*. 23(3):630–639. DOI: 10.1200/JCO.2005.11.030.

Rattan, R., Graham, R.P., Maguire, J.L., Giri, S. & Shridhar, V. 2011. Metformin Suppresses Ovarian Cancer Growth and Metastasis with Enhancement of Cisplatin Cytotoxixity In Vivo. *Neoplasia*. 13(5):483–491. DOI: 10.1593/neo.11148.

Rena, G., Pearson, E.R. & Sakamoto, K. 2013. Molecular mechanism of action of metformin: old or new insights? *Diabetologia*. 56:1898–1906. DOI: 10.1007/s00125-013-2991-0.

Rizk, S.L. & Sky-Peck, H.H. 1984. Comparison between concentrations of trace elements in normal and neoplastic human breast tissue. *Cancer Research*. 44(11):5390–5394. Available: http://www.ncbi.nlm.nih.gov/pubmed/6488192 [2015, March 05].

Rodriguez, C.P., Adelstein, D.J., Rice, T.W., Rybicki, L.A., Videtic, G.M.M., Saxton, J.P., Murthy, S.C., Mason, D.P., et al. 2010. A Phase II Study of Perioperative Concurrent Chemotherapy, Gefitinib, and Hyperfractionated Radiation Followed by Maintenance Gefitinib in Locoregionally Advanced Esophagus and Gastroesophageal Junction Cancer. *Journal of Thoracic Oncology*. 5(2):229–235. DOI: 10.1097/JTO.0b013e3181c5e334.

Romick-Rosendale, L.E., Lui, V.W.Y., Grandis, J.R. & Wells, S.I. 2013. The Fanconi anemia pathway: repairing the link between DNA damage and squamous cell carcinoma. *Mutation research*. 743-744:78–88. DOI: 10.1016/j.mrfmmm.2013.01.001.

Ben Sahra, I., Laurent, K., Loubat, A., Giorgetti-Peraldi, S., Colosetti, P., Auberger, P., Tanti, J.F., Le Marchand-Brustel, Y., et al. 2008. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*. 27(25):3576–3586. DOI: 10.1038/sj.onc.1211024.

Ben Sahra, I., Laurent, K., Giulliano, S., Larbret, F., Ponzio, G., Gounon, P., Marchand-Brustel, Y. Le, Giorgetti-Peraldi, S., et al. 2010. Targeting cancer Cell Metabolism: The combination of metformin and 2-deoxyglucose inhibits autophagy and induces AMPK-dependent apoptosis in prostate cancer cells. *Autophagy*. 6(5):2465–2475. DOI: 10.1158/0008-5472.CAN-09-2782.

Ben Sahra, I., Marchand-Brustel, Y. Le, Tanti, J.-F. & Bost, F. 2010. Metformin in Cancer Therapy: A New Perspective for an Old Antidiabetic Drug? *Molecular Cancer Therapeutics*. 9:1092–1099. DOI: 10.1158/1535-7163.MCT-09-1186.

Ben Sahra, I., Regazzetti, C., Robert, G., Laurent, K., Le Marchand-Brustel, Y., Auberger, P., Tanti, J.-F., Giorgetti-Peraldi, S., et al. 2011. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Research*. 71(13):4366–4372. DOI: 10.1158/0008-5472.CAN-10-1769.

Samimi, G., Safaei, R., Katano, K., Holzer, A.K., Rochdi, M., Tomioka, M., Goodman, M. & Howell, S.B. 2004. Increased Expression of the Copper Efflux Transporter ATP7A Mediates Resistance to Cisplatin , Carboplatin , and Oxaliplatin in Ovarian Cancer Cells. 10(858):4661–4669.

Samuni, A., Aronovitch, J., Godinger, D., Chevion, M. & Czapski, G. 1983. On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism. *European Journal of Biochemistry/FEBS*. 137(1-2):119–124. Available: http://www.ncbi.nlm.nih.gov/pubmed/6317379 [2015, February 24].

Sartorelli, A.C. & Booth, B.A. 1967. Inhibition of the Growth of Sarcoma 180 Ascites Cells by Combinations of Inhibitors of Nucleic Acid Biosynthesis and the Cupric Chelate of Kethoxal Bis-(thiosemicarbazone). *Cancer Research*. 27:1614–1619.

Sato-Kuwabara, Y., Neves, J.I., Fregnani, J.H.T.G., Sallum, R.A. & Soares, F.A. 2009. Evaluation of gene amplification and protein expression of HER-2/neu in esophageal squamous cell carcinoma using Fluorescence in situ Hybridization (FISH) and immunohistochemistry. *BMC cancer*. 9(1):6. DOI: 10.1186/1471-2407-9-6.

Sattler, U.G. a & Mueller-Klieser, W. 2009. The anti-oxidant capacity of tumour glycolysis. *International journal of radiation biology*. 85(11):963–71. DOI: 10.3109/09553000903258889.

Scherz-Shouval, R. & Elazar, Z. 2011. Regulation of autophagy by ROS: physiology and pathology. *Trends in Biochemical Sciences*. 36(1):30–8. DOI: 10.1016/j.tibs.2010.07.007.

Shah, M.A., Ramanathan, R.K., Ilson, D.H., Levnor, A., D'Adamo, D., O'Reilly, E., Tse, A., Trocola, R., et al. 2006. Multicenter phase II study of irinotecan, cisplatin, and bevacizumab in patients with metastatic gastric or gastroesophageal junction adenocarcinoma. *Journal of Clinical Oncology*. 24(33):5201–5206. DOI: 10.1200/JCO.2006.08.0887.

Shenfield, G. 2013. Metformin: myths, misunderstandings and lessons from history. *Australian Prescriber*. 36(2):38–39.

Shiah, S., Kao, Y., Wu, F.Y. & Wu, C. 2003. Inhibition of Invasion and Angiogenesis by Zinc-Chelating Agent Disulfiram. *Molecular Pharmacology*. 64(5):1076–1084.

Shian, S.-G., Kao, Y.-R., Wu, F.Y.-H. & Wu, C.-W. 2003. Inhibition of invasion and angiogenesis by zinc-chelating agent disulfiram. *Molecular Pharmacology*. 64(5):1076–1084. DOI: 10.1124/mol.64.5.1076.

Siddik, Z.H. 1998. Cisplatin resistance - Molecular basis of a Multifaceted Impediment. In *Cancer Drug Discovery and Development: Cancer Drug Resistance*. V. 1. B. Teicher, Ed. New Jersey: Humana Press Inc. 283–307. DOI: 10.1016/S0169-5002(98)90048-4.

Siddik, Z.H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*. 22:7265–7279. DOI: 10.1038/sj.onc.1206933.

Song, C.W., Lee, H., Dings, R.P.M., Williams, B., Powers, J., Santos, T. Dos, Choi, B.-H. & Park, H.J. 2012. Metformin kills and radiosensitizes cancer cells and preferentially kills cancer stem cells. *Scientific reports*. 2:362. DOI: 10.1038/srep00362.

Song, I.-S., Savaraj, N., Siddik, Z.H., Liu, P., Wei, Y., Wu, C.J. & Kuo, M.T. 2004. Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells. *Mol. Cancer Ther.* 3(12):1543–1549. Available: http://mct.aacrjournals.org/content/3/12/1543.short [2014, December 18].

Soranna, D., Scotti, L., Zambon, A., Bosetti, C., Grassi, G., Catapano, A., La Vecchia, C., Mancia, G., et al. 2012. Cancer Risk Associated with Use of Metformin and Sulfonylurea in Type 2 Diabetes: a Meta-Analysis. *The Oncologist*. 17(6):813–822. DOI: 10.1634/theoncologist.2011-0462.

Speelmans, G., Sips, W.H.H.M., Grisel, R.J.H., Staffhorst, R.W.H.M., Fichtinger-Schepman, A.M.J., Reedijk, J. & De Kruijff, B. 1996. The interaction of the anti-cancer drug cisplatin with phospholipids is specific for negatively charged phospholipids and takes place at low chloride ion concentration. *Biochimica et Biophysica Acta - Biomembranes*. 1283:60–66. DOI: 10.1016/0005-2736(96)00080-6.

Stades, A.M.E., Heikens, J.T., Erkelens, D.W., Holleman, F. & Hoekstra, J.B.L. 2004. Metformin and lactic acidosis: cause or coincidence? A review of case reports. *Journal of Internal Medicine*. 255(2):179–187. DOI: 10.1046/j.1365-2796.2003.01271.x.

Stefan, C., Nobel, I., Kimland, M., Lind, B., Orrenius, S. & Slater, A.F.G. 1995. Dithiocarbamates Induce Apoptosis in Thymocytes by Raising the Intracellular Level of Redox-active Copper. *Journal of Biological Chemistry*. 270(44):26202–26208. DOI: 10.1074/jbc.270.44.26202.

Stewart, D.J. 2007. Mechanisms of resistance to cisplatin and carboplatin. *Critical Reviews in Oncology/Hematology*. 63(1):12–31. DOI: 10.1016/j.critrevonc.2007.02.001.

Stoner, G.D. & Gupta, A. 2001. Etiology and chemoprevention of esophageal squamous cell carcinoma. *Commentary*. 22(11):1737–1746.

Strömme, J.H. 1965. Interactions of disulfiram and diethyldithiocarbamate with serum proteins studied by means of a gel-filtration technique. *Biochemical Pharmacology*. 14(4):381–391. DOI: 10.1016/0006-2952(65)90212-1.

Suh, J.J., Pettinati, H.M., Kampman, K.M. & O'Brien, C.P. 2006. The Status of Disulfiram A Half of a Century Later. *Journal of Clinical Psychopharmacolgy*. 26(3):290–302. DOI: 10.1097/01.jcp.0000222512.25649.08.

Tashiro, E., Tsuchiya, A. & Imoto, M. 2007. Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression. *Cancer science*. 98(5):629–35. DOI: 10.1111/j.1349-7006.2007.00449.x.

Tomic, T., Botton, T., Cerezo, M., Robert, G., Luciano, F., Puissant, A., Gounon, P., Allegra, M., et al. 2011. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death & Disease*. 2(e199):10. DOI: 10.1038/cddis.2011.86.

Topping, R.J. & Jones, M.M. 1988. Optimal dithiocarbamate structure for immunomodulator action. *Medical Hypotheses*. 27(1):55–57. DOI: 10.1016/0306-9877(88)90084-9.

Underwood, G.E., Siem, R.A., Gerpheide, S.A. & Hunter, J.H. 1959. Binding of an Antiviral Agent (Kethoxal) by Various Metabolites. *Experimental Biology and Medicine*. 100(2):312–315. DOI: 10.3181/00379727-100-24611.

Valeriote, F. & Grates, H.E. 1989. Potentiation of Nitrogen Mustard Cytotoxicity by Disulfiram, Diethyldithiocarbamic Acid, and Diethylamine in Mice. *Cancer Research*. 49:6658–6662.

Vāvere, A.L. & Lewis, J.S. 2007. Cu-ATSM: a radiopharmaceutical for the PET imaging of hypoxia. *Dalton transactions (Cambridge, England : 2003)*. 43:4893–4902. DOI: 10.1039/b705989b.

Vazquez-Martin, A., Oliveras-Ferraros, C. & Menendez, J.A. 2009. The antidiabetic drug metformin suppresses HER2 (erbB-2) oncoprotein overexpression via inhibition of the mTOR effector p70S6K1 in human breast carcinoma cells. *Cell Cycle*. 8(1):88–96.

Veale, R.B. & Thornley, A.L. 1989. Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. *South African Journal of Science*. 85:375–379.

De Vita, F., Orditura, M., Martinelli, E., Vecchione, L., Innocenti, R., Sileni, V.C., Pinto, C., Di Maio, M., et al. 2011. A multicenter phase II study of induction chemotherapy with FOLFOX-4 and cetuximab followed by radiation and cetuximab in locally advanced oesophageal cancer. *British journal of cancer*. 104(3):427–32. DOI: 10.1038/sj.bjc.6606093.

Volikos, E., Robinson, J., Aittomaki, K., Mecklin, J.-P., Jarvinen, H., Westerman, A.M., de Rooij, F.W.M., Vogel, T., et al. 2006. LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome. *Journal of Medical Genetics*. 43:e18. DOI: 10.1136/jmg.2005.039875.

Vucicevic, L., Misirkic, M., Kristina, J., Vilimanovich, U., Sudar, E., Isenovic, E., Prica, M., Harhaji-Trajkovic, L., et al. 2014. Compound C induces protective autophagy in cancer cells through AMPK inhibition-independent blockade of Akt/mTOR pathway. *Autophagy*. 7(1):40–50. DOI: 10.4161/auto.7.1.13883.

Wang, J. & Yi, J. 2008. Cancer cell killing via ROS. *Cancer Biology and Therapy*. 7(12):1875–1884.

Wang, L.-W., Li, Z.-S., Zou, D.-W., Jin, Z.-D., Gao, J. & Xu, G.-M. 2008. *Metformin induces apoptosis of pancreatic cancer cells*. Available: http://www.wjgnet.com/1007-9327/E-Journal/WJGv14i47.pdf#page=55 [2015, January 13].

Warburg, O. 1956. On the Origin of Cancer Cells. *Science*. 123(3191):309–314.

Wattenberg, L.W. 1974. Inhibition of Carcinogenic and Toxic Effects of Polycyclic Hydrocarbons by Several Sulfur-Containing Compounds. *Journal of the National Cancer Institute*. 52(5):1583–1587.

Wattenberg, L.W. 1975. Inhibition of Dimethylhydrazine-Induced Neoplasia of the Large Intestine by Disulfiram. *Journal of the National Cancer Institute*. 54(4):1005–1006.

Weiss, R.B. & Christian, M.C. 1993. New Cisplatin Analogues in Development. *Drugs*. 46(3):360–377. DOI: 10.2165/00003495-199346030-00003.

Wickström, M., Danielsson, K., Rickardson, L., Gullbo, J., Nygren, P., Isaksson, A., Larsson, R. & Lövborg, H. 2007. Pharmacological profiling of disulfiram using human tumor cell lines and human tumor cells from patients. *Biochemical pharmacology*. 73(1):25–33. DOI: 10.1016/j.bcp.2006.08.016.

Wullschleger, S., Loewith, R. & Hall, M.N. 2006. TOR signaling in growth and metabolism. *Cell*. 124(3):471–84. DOI: 10.1016/j.cell.2006.01.016.

Xiao, Z., Donnelly, P.S., Zimmermann, M. & Wedd, A.G. 2008. Transfer of copper between bis(thiosemicarbazone) ligands and intracellular copper-binding proteins. insights into mechanisms of copper uptake and hypoxia selectivity. *Inorganic Chemistry*. 47(10):4338–47. DOI: 10.1021/ic702440e.

Yakisich, J.S., Sidén, A., Eneroth, P. & Cruz, M. 2001. Disulfiram is a potent in vitro inhibitor of DNA topoisomerases. *Biochemical and Biophysical Research Communications*. 289(2):586–590. DOI: 10.1006/bbrc.2001.6027.

Yang, Z. & Klionsky, D.J. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. *Current Opinion in Cell Biology*. 22(2):124–131. DOI: 10.1016/j.ceb.2009.11.014.

Yao, X., Panichpisal, K., Kurtzman, N. & Nugent, K. 2007. Cisplatin Nephrotoxicity: A Review. *American Journal of the Medical Sciences*. 334(2):115–124.

Yip, N.C., Fombon, I.S., Liu, P., Brown, S., Kannappan, V., Armesilla, A.L., Xu, B., Cassidy, J., et al. 2011. Disulfiram modulated ROS-MAPK and NFκB pathways and targeted breast cancer cells with cancer stem cell-like properties. *British Journal of Cancer*. 104(10):1564–1574. DOI: 10.1038/bjc.2011.126.

Yokomizo, A., Ono, M., Nanri, H., Makino, Y., Ohga, T., Wada, M., Okamoto, T., Yodoi, J., et al. 1995. Cellular Levels of Thioredoxin Associated with Drug Sensitivity to Cisplatin, Mitomycin C, Doxorubicin, and Etoposide. *Cancer Res.* 55(19):4293–4296. Available: http://cancerres.aacrjournals.org/content/55/19/4293.short [2015, January 26].

Zakikhani, M., Dowling, R., Fantus, I.G., Sonenberg, N. & Pollak, M. 2006. Metformin Is an AMP Kinase – Dependent Growth Inhibitor for Breast Cancer Cells. *Cancer Research*. 66(21):10269–10273. DOI: 10.1158/0008-5472.CAN-06-1500.

Zhan, N., Dong, W.-G., Tang, Y.-F., Wang, Z.-S. & Xiong, C.-L. 2012. Analysis of HER2 gene amplification and protein expression in esophageal squamous cell carcinoma. *Medical oncology (Northwood, London, England)*. 29(2):933–40. DOI: 10.1007/s12032-011-9850-y.

Zheng, D., MacLean, P.S., Pohnert, S.C., Knight, J.B., Olson, A.L., Winder, W.W. & Dohm, G.L. 2001. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. *J Appl Physiol*. 91(3):1073–1083. Available: http://jap.physiology.org/content/91/3/1073 [2014, November 23].

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., et al. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation*. 108(8):1167–1174. DOI: 10.1172/JCI200113505.Introduction.

Zhuang, Y. & Miskimins, W.K. 2008. Cell cycle arrest in Metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27Kip1 or p21Cip1. *Journal of Molecular Signaling*. 3(18):11. DOI: 10.1186/1750-2187-3-18.

Zoncu, R., Efeyan, A. & Sabatini, D.M. 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology*. 12(1):21–35. DOI: 10.1038/nrm3025.

Appendix A

First publication from this Thesis:

Metformin induces an intracellular reductive state that protects oesophageal squamous cell carcinoma cells against cisplatin but not copper-bis(thiosemicarbazones)

Leonard Howard Damelin[1,2†], Rupal Jivan[3†], Robin Bruce Veale[3], Amanda Louise Rousseau[4] and Demetra Mavri-Damelin[3*]

[†]Equal contributors

Author details

[1] School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg 2193, South Africa.

[2] Cell Biology Group, Centre for HIV and STI's, National Institute for Communicable Diseases, Private Bag X4, Sandringham, Johannesburg 2131, South Africa.

[3] School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

[4] Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

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Metformin induces an intracellular reductive state that protects oesophageal squamous cell carcinoma cells against cisplatin but not copper-bis(thiosemicarbazones)

Leonard Howard Damelin[1,2†], Rupal Jivan[3†], Robin Bruce Veale[3], Amanda Louise Rousseau[4] and Demetra Mavri-Damelin[3*]

Abstract

Background: Oesophageal squamous cell carcinoma (OSCC) is a highly aggressive carcinoma with a poor survival rate. One of the most commonly used chemotherapeutic drugs, cisplatin, displays varied and often poor efficacy in vivo. Therefore, alternative, cost-effective and more efficacious treatments are required. Metformin has been previously shown to reduce proliferative rates in various carcinoma cell lines. We report for the first time, the effect of metformin on OSCC cell proliferation and show that it antagonises cisplatin-induced but not copper-bis (thiosemicarbazone)-induced cytotoxicity in OSCC cells.

Methods: Cell proliferation and stage of the cell cycle were quantified by trypan blue counts and flow cytometry, respectively. All cytotoxicity measurements were made using the tetrazolium based MTT assay. Metabolic alterations to cells were determined as follows: glycolysis via a lactate dehydrogenase assay, reducing equivalents by MTT reduction and reduced intracellular thiols by monobromobimane-thiol fluorescence, and glutathione depletion using buthionine sulfoximine. Inductively coupled plasma mass spectrometry was used to quantify cisplatin-DNA adduct formation.

Results: Metformin was found to reduce cell proliferation significantly in all OSCC cell lines, with an accumulation of cells in G0/G1 phase of the cell cycle. However, metformin significantly protected OSCC cells against cisplatin toxicity. Our results indicate that a major mechanism of metformin-induced cisplatin resistance results from a significant increase in glycolysis, intracellular NAD(P)H levels with a concomitant increase in reduced intracellular thiols, leading to decreased cisplatin-DNA adduct formation. The glutathione synthesis inhibitor buthionine sulfoximine significantly ablated the protective effect of metformin. We subsequently show that the copper-bis (thiosemicarbazones), Cu-ATSM and Cu-GTSM, which are trapped in cells under reducing conditions, cause significant OSCC cytotoxicity, both alone and in combination with metformin.

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* Correspondence: Demetra.Mavri-Damelin@wits.ac.za
†Equal contributors
[3]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa
Full list of author information is available at the end of the article

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Conclusions: This is the first study showing that metformin can be used to decrease cell proliferation in OSCC cells. However, metformin protects against cisplatin cytotoxicity by inducing a reducing intracellular environment leading to lower cisplatin-DNA adduct formation. As such, we advise that caution be used when administering cisplatin to diabetic patients treated with metformin. Furthermore, we propose a novel combination therapy approach for OSCC that utilises metformin with metformin-compatible cytotoxic agents, such as the copper-bis (thiosemicarbazones), Cu-ATSM and Cu-GTSM.

Keywords: Oesophageal squamous cell carcinoma, Metformin, Copper bis(thiosemicarbazones), Metabolism, Cisplatin, Thiol, Glutathione

Background

Oesophageal carcinoma, of which there are two subtypes, adenocarcinoma and squamous cell carcinoma, is the sixth most common cause of cancer-related death [1]. Oesophageal squamous cell carcinoma (OSCC) is a highly aggressive carcinoma with a very poor survival rate that occurs with particularly high frequency in developing countries including Iran, China, South Africa, and Brazil, where mortality rates can exceed 100 per 100,000 population. In developed countries, incidence rarely exceeds 10 per 100,000 population [2,3], with the exceptions of certain regions in North-West France and Northern Italy where incidence may reach 30 and 2 per 100,000 in males and females, respectively [4]. The causes of OSCC are multiple and varied, probably reflecting repeated exposure to dietary components, such as N-nitroso compounds, excessive smoking and alcohol consumption, chronic inflammation and possibly, genetic predisposition [5]. Current commonly used therapies for OSCC include 5-fluorouracil and cisplatin, which show poor efficacy and often display both chemotoxicity and chemoresistance [6]. Cisplatin has multiple mechanisms of cytotoxicity including the formation of DNA and protein adducts, as well as via oxidative stress. Many resistance mechanisms for cisplatin have been identified, including, pertinent to this study, sequestering of cisplatin by glutathione (a major species of intracellular thiols). This results in export of cisplatin-glutathione adducts leading to a reduction in cisplatin-mediated DNA damage [7-9]. Investigations into more effective targeted treatment options for OSCC using monoclonal antibody therapies are very promising [10] however, access to such therapies in developing countries is extremely limited, primarily due to cost. Therefore, there is a continued and urgent requirement for alternative, effective and economical treatment options.

Recently, the well characterized and tolerated anti-diabetic drug, metformin has been the subject of intense investigations in cancer research. Population studies have shown that this biguanide, conventionally used to decrease peripheral glucose levels and increase insulin sensitivity in diabetic and pre-diabetic patients [11,12], reduced breast cancer occurrence in female patients with type 2 diabetes [13]. Since then, metformin has been observed to reduce the proliferation of many types of carcinoma cell lines and diabetic patients taking metformin have been found to have better recovery rates from breast cancer [14-17]. Furthermore, metformin has been shown to target cancer stem cells [18]. However, whilst metformin reduces cell proliferation in most cancer types, it rarely causes apoptosis, and is therefore being combined with conventional chemotherapeutic drugs, including cisplatin. This treatment combination has mixed results, with some studies showing that metformin can enhance the effectiveness of chemotherapeutic drugs whilst others have shown increased chemoresistance in the presence of metformin [19,20]. With regards to cisplatin, metformin has been shown to reduce cisplatin sensitivity through the AMPK-independent upregulation of the Akt survival pathway [20]. A search on *clinicaltrials.gov* found over 40 clinical trials investigating metformin and a variety of chemotherapeutic drugs, for breast, ovarian and prostate cancer amongst a number of others.

In this study, we investigated the effect of metformin on OSCC cell proliferation and on the cytotoxicity of cisplatin for OSCC cells. We show that whilst metformin markedly reduces OSCC cell proliferation and causes cells to accumulate in the G0/G1 phase of the cell cycle, it also significantly protects against cisplatin cytotoxicity. The protective effect is not solely due to reduced cell-proliferation, as the biguanide minimally to partially protects against the DNA-crosslinker, mitomycin C, but is dependent on a metformin-induced increase in glycolysis and intracellular NAD(P)H levels with a concomitant increase in reduced intracellular thiols, which coincides with decreased cisplatin-DNA adduct formation. The glutathione synthesis inhibitor buthionine sulfoximine (BSO) significantly reverses this protective effect, confirming the role of reduced glutathione in cisplatin detoxification by metformin-treated cells. In light of these findings, we investigated the copper-bis(thiosemicarbazones), copper diacetyl-bis(4-methylthiosemicarbazonato)copper(II) (Cu-ATSM) and copper glyoxal-bis (4-methylthiosemicarbazonato)copper(II) (Cu-GTSM).

Copper-bis(thiosemicarbazones) induce cytotoxicity through a number of mechanisms, including inhibition of DNA synthesis [21]. Importantly, as these compounds are known to be trapped in cells under reducing conditions, they are therefore compatible with a reducing intracellular state [22]. We show that both Cu-ATSM and Cu-GTSM display significant levels of cytotoxicity at LD_{50} values comparable to or lower than cisplatin, both alone or in combination with metformin, highlighting the use of metformin and reduction-compatible cytotoxic drugs as a novel combination therapy strategy for the treatment of OSCC.

Methods

Reagents

Reagents for flow cytometry were purchased from Beckman Coulter. All other reagents were purchased from Sigma Aldrich unless otherwise specified.

Synthesis of bis(thiosemicarbazones)

The bis(thiosemicarbazones), ATSM and GTSM, were synthesised from 4-methyl thiosemicarbazide and butanedione or glyoxal, respectively, according to the method of French *et al.* [23].

ATSM: ^{1}H NMR (500 MHz, DMSO) 11.74 (2H, s, 2 × C*H* = N), 8.48 (2H, d, J = 4.2, 2 × NH), 7.72 (2H, s, 2 × NH), 2.96 (6H, d, J = 4.4, 2 × CH_3); ^{13}C NMR (126 MHz, DMSO) 177.55 (2 × C = S), 140.02 (2 × C = N), 30.89 (2 × CH_3).

GTSM: ^{1}H NMR (500 MHz, DMSO) 10.20 (2H, s, 2 × NH), 8.36 (2H, d, J = 4.1, 2 × NH), 3.02 (6H, d, J = 4.5, 2 × CH_3), 2.20 (6H, s, 2 × CH_3); ^{13}C NMR (126 MHz, DMSO) 178.47 (2 × C = S), 147.95 (2 × C = N), 31.18 (2 × CH_3), 11.64 (2 × CH_3).

Cu-ATSM and Cu-GTSM were synthesized from ATSM and GTSM and cupric chloride as previously described [24].

Cell culture

The human OSCC cell lines were a kind gift from Professor Robin Veale. These cells, WHCO1, WHCO5 [25] and SNO [26] were maintained in Dulbecco's Modified Eagles Medium/Hams F12 (DMEM/Hams F12, 3:1) supplemented with 10% FCS at 37°C and 5% CO_2.

Cell proliferation

Cell proliferation was assessed by cell counts using trypan blue exclusion. Cells were seeded in 48-well plates at 1×10^4 cells per well. After 24 hours, cells were incubated with or without 10 mM metformin for an additional 24 hours. Cells were then trypsinized, resuspended in 1×PBS and incubated in 2% trypan blue for 2 minutes and counted using a haemocytometer (n = 5 ± SD).

Cell cycle analysis

Cell cycle analysis was by flow cytometry as previously described [27]. Cells were seeded equally in 10 cm dishes and cultured for 48 hours (~60% confluent). At this time, the medium was replaced and cells incubated with or without 10 mM metformin for 24 hours. Control cells were serum-deprived for 8 hours. Cells were then harvested and prepared for analysis using the DNA Prep Reagent kit according to manufacturers' instructions (Beckman Coulter). Briefly, cells were treated with DNA prep LPR (lysis) solution in order to facilitate propidium iodide (PI) entry and samples vortexed for 10 seconds followed by the addition of DNA prep stain (PI and RNAse) and additional vortexing. Samples were then immediately analysed on an LSRFortessa™ cell analyser, BD Biosciences. DNA histograms were analysed using FlowJo v10 software and the percentage of cells in the G0/G1, S, and G2/M phase of the cell cycle calculated (n = 3 ± SD).

Cytotoxicity assays

Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cells (8500 cells per well) were seeded into 96-well plates and after 24 hours exposed to cytotoxic agents for varying times. After treatment, the medium was replaced with 100 μl of MTT solution (0.5 mg/ml in cell culture medium) and incubated at 37°C for 2 hours. MTT solution was then removed, and MTT formazan dissolved in 100 μl dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using the Bio-Rad iMark microplate reader (n = 3 ± SD).

ICP-MS analysis of platinum-DNA adducts

Inductively-coupled plasma mass spectrometry (ICP-MS) was performed as previously described [28]. Briefly, cells were treated with cisplatin (LD_{30} concentrations) for 48 hours with or without 24 hour prior exposure to 10 mM metformin. Total genomic DNA was extracted, resuspended in water and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA samples were hydrolysed in a final concentration of 1% HNO_3 at 70°C for 24 hours and analysed for platinum (n = 3 ± SD) on an Agilent 7700 ICP-MS. The instrument was optimised for sensitivity and low oxides. Analysis was done in no-gas mode, and the instrument was calibrated for platinum analysis using National Institute of Standards and Technology traceable standards.

Determination of glycolysis via lactate production

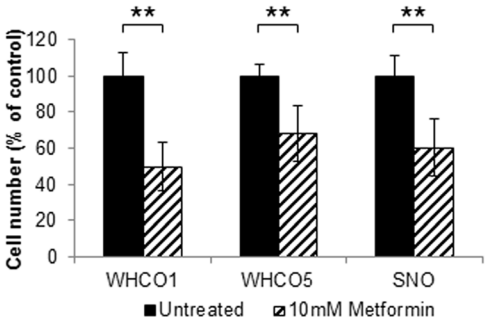
As an indicator of levels of glycolysis, lactate levels in culture medium were quantified using a lactate dehydrogenase assay [29] where the production of NADH from NAD via the conversion of lactate to pyruvate is directly proportional to lactate concentration. Cells were seeded and treated as for cell cycle analysis and both conditioned

culture medium and cells collected after 24 hours. Cells were counted using trypan blue exclusion as described above. For lactate quantification, 50 μl of medium was added to 950 μl glycine-hydrazine buffer (0.64 M glycine, 0.64 M hydrazine, 4.8 mM NAD^+, 16 U/ml lactate dehydrogenase, pH 9.2) and incubated at 37°C for 2 minutes. NADH was then quantified spectrophotometrically at 340 nm and values corrected for cell number (n = 3 ± SD).

Quantification of reducing equivalents

Total cellular reducing equivalents were quantified by tetrazolium (MTT) assay as previously described [30]. An equal number of cells were seeded into 96-well plates (8500 cells per well) and after 24 hours cells were incubated for 24 hours with or without 10 mM metformin and MTT assay performed. Values were corrected for cell number using trypan blue exclusion as described above (n = 3 ± SD).

A



B

		% of cells at cell cycle phase	
		Untreated	Metformin
WHCO1	G0/G1	50.8±1.4%	62.8±3.3%
	S	33.1±3.4%	23.5±1.9%
	G2/M	15.1±2.7%	12.7±1.2%
WHCO5	G0/G1	52.8±3.0%	60.9±5.3 %
	S	30.5±0.6%	21.6±4.7%
	G2/M	15.4±2.9%	16.5±1.8%
SNO	G0/G1	62.6±3.0%	73.9±4.0%
	S	24.1±4.5%	16.4±5.2%
	G2/M	13.2±4.9%	8.8±2.9%

C

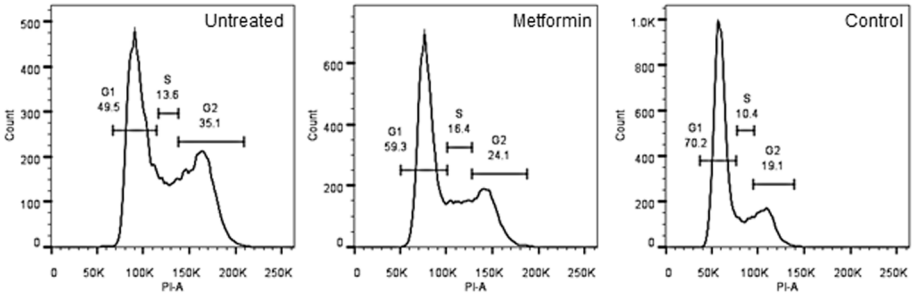


Figure 1 Anti-proliferative effects of metformin on OSCC cells. A, Cells exposed to 10 mM metformin for 24 hours showed a decrease in cell number in comparison to untreated controls across all cell lines, n = 4, mean ± SD. **B**, Quantification and **C**,representative figures of flow cytometry analysis (from SNO cells) for untreated cells (Untreated), FCS deprived control (FCS Control) and cells exposed to 10 mM metformin for 24 hours (Metformin). Metformin treated cells exhibited an accumulation at the G0/G1 stage of the cell cycle across all cell lines, expressed as % of cells in phase of cell cycle (n = 3, mean ± SD), where for all cells there was a statistically significant increase in cells in G0/G1 in metformin treated cells relative to untreated controls with WHCO1 p = 0.05, WHCO5 p = 0.04 and SNO p = 0.01.

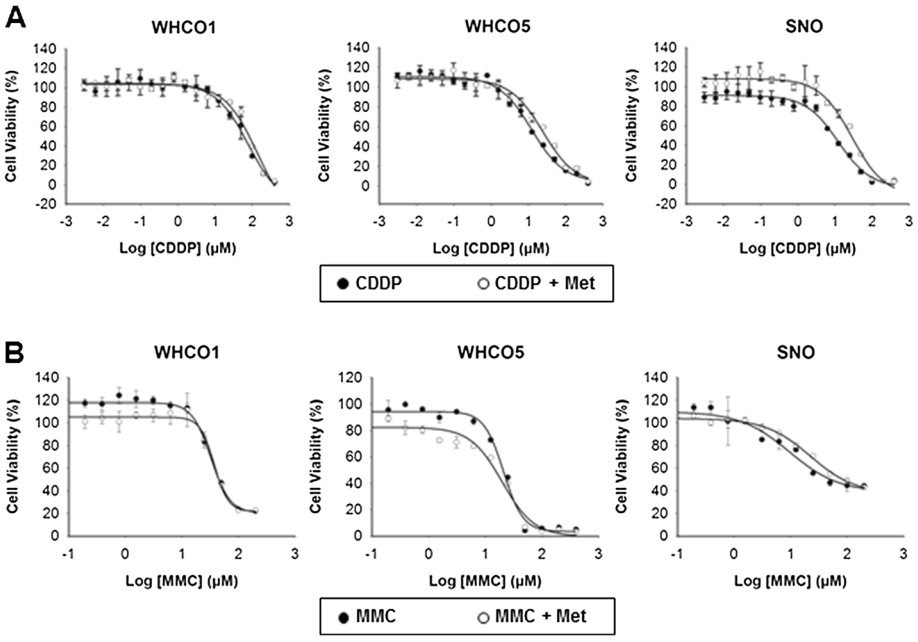


Figure 2 Effect of metformin on cisplatin and mitomycin C cytotoxicity for OSCC cells. OSCC cells, untreated or treated with 10 mM metformin for 24 hours and then treated with **(A)** cisplatin or **(B)** mitomycin C for a further 48 hours, were assessed by MTT assay. All metformin-cisplatin treated cells displayed a trend for higher LD_{50} values, with WHCO1 and SNO cells statistically higher (n = 3, mean ± SD).

Low-molecular weight thiol quantification

Total low-molecular weight thiols were quantified using monobromobimane, which forms fluorescent thiol conjugates [31]. Cells were seeded and treated as for cell cycle analysis with or without 10 mM metformin for 24 hours. Cells were subsequently washed three times with 1×PBS and incubated in 1 ml of 1×PBS with 18 μl monobromobimane solution (stock 5 mg/ml in DMSO) for 10 minutes. Cells were then lysed in 1 ml of triple detergent lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Triton ×-100, 0.5% sodium deoxycholate), the lysate centrifuged at 10000 g and 100 μl of the resultant supernatant fluorescently analysed at $360nm_{excitation}/460nm_{emission}$ using an Ascent multi-well plate fluorimeter (Thermo Scientific). Cells seeded in parallel dishes were counted using trypan blue exclusion as above and fluorescence values were corrected for cell number (n = 3 ± SD).

Glutathione depletion assay

The glutathione synthesis inhibitor BSO was used in order to deplete intracellular glutathione levels and thereby assess the involvement of thiols (glutathione) in the cytoprotective effects of metformin on cisplatin toxicity [32]. Cytotoxicity assays were performed as above with the following modifications, cells were seeded at 7500 cells per well in 96-well plates and allowed to settle for 18 hours. Cells were then treated with 0.4 mM of BSO for 18 hours, followed by the addition of 10 mM metformin (or metformin diluent for control cells) and subsequently cisplatin, and cytotoxicity determined by MTT assay as above (n = 3 ± SD).

Table 1 Cytotoxicity in OSCC cells treated with or without metformin and cisplatin or mitomycin C

Compounds	LD_{50} (μM)					
	WHCO1		WHCO5		SNO	
Cisplatin	70.88 ± 13.8	p = 0.009	11.68 ± 3.62	p = 0.075	11.01 ± 1.62	p = 0.0001
Met + Cisplatin	126.02 ± 26.57		28.03 ± 15.81		28.16 ± 3.37	
Mitomycin C	32.73 ± 2.49	p = 0.003	32.87 ± 3.03	p = 0.25	9.92 ± 1.80	p = 0.011
Met + Mitomycin C	37.15 ± 0.79		30.19 ± 4.36		16.64 ± 2.77	

OSCC cells were treated with 10 mM metformin (Met) and either cisplatin or mitomycin C and MTT assays performed. LD_{50} (μM) was calculated on replicates (n = 3, mean ± SD).

Statistical analysis

Comparisons were by two-tailed Student's t-tests and $p < 0.05$ was considered statistically significant. LD_{50} and LD_{30} values were calculated using GraphPad Prism version 6.

Results

OSCC cells exhibit decreased cell proliferation and cell cycle arrest in response to metformin

We investigated the effect of metformin on three OSCC cell lines (WHCO1, WHCO5 and SNO), previously derived from South African OSCC patients [25,26]. All cell lines exhibited a significant reduction in cell proliferation in response to 10 mM metformin after 24 hour treatment, in comparison to untreated controls. There was 50%, 32% and 39% reduction in cell proliferation in WHCO1, WHCO5 and SNO cells, respectively (Figure 1A). In addition, we assessed cell cycle progression using flow cytometry with propidium iodide staining of cellular DNA content. Cells deprived of foetal calf serum (FCS) for 8 hours were used as the control, which as expected, showed an increase in the number of cells in G0/G1 phase of the cell cycle. Metformin treatment (10 mM for 24 hours), as anticipated, caused an increase in the number of cells in G0/G1 phase relative to untreated controls (Figure 1B and C).

Metformin protects cells from cisplatin cytotoxicity

Next, we assessed the effect of metformin on cisplatin cytotoxicity by MTT assay. Cells pre-treated with 10 mM metformin for 24 hours and then treated with 10 mM metformin and cisplatin for 48 hours (Figure 2A), exhibited significantly lower cytotoxicity than cells

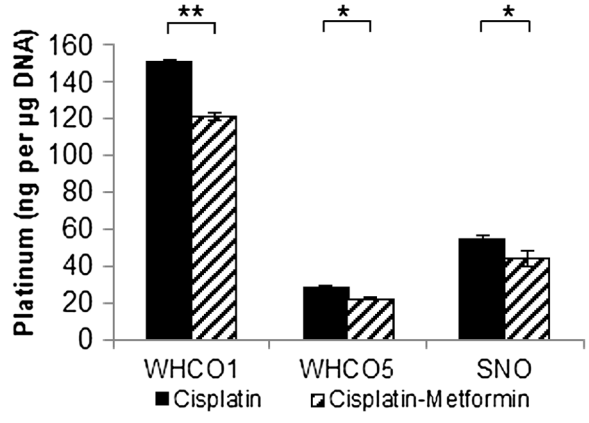


Figure 3 Decreased platinum-DNA adduct formation in cisplatin-metformin treated OSCC cells. OSCC cells were treated with LD_{30} concentrations of cisplatin, either alone or in combination with 10 mM metformin. Genomic DNA was extracted and platinum quantified by ICP-MS which showed a decrease in platinum in cisplatin-metformin treated cells in comparison to cells treated with cisplatin alone (values expressed per µg of DNA) (n = 3, mean ± SD).

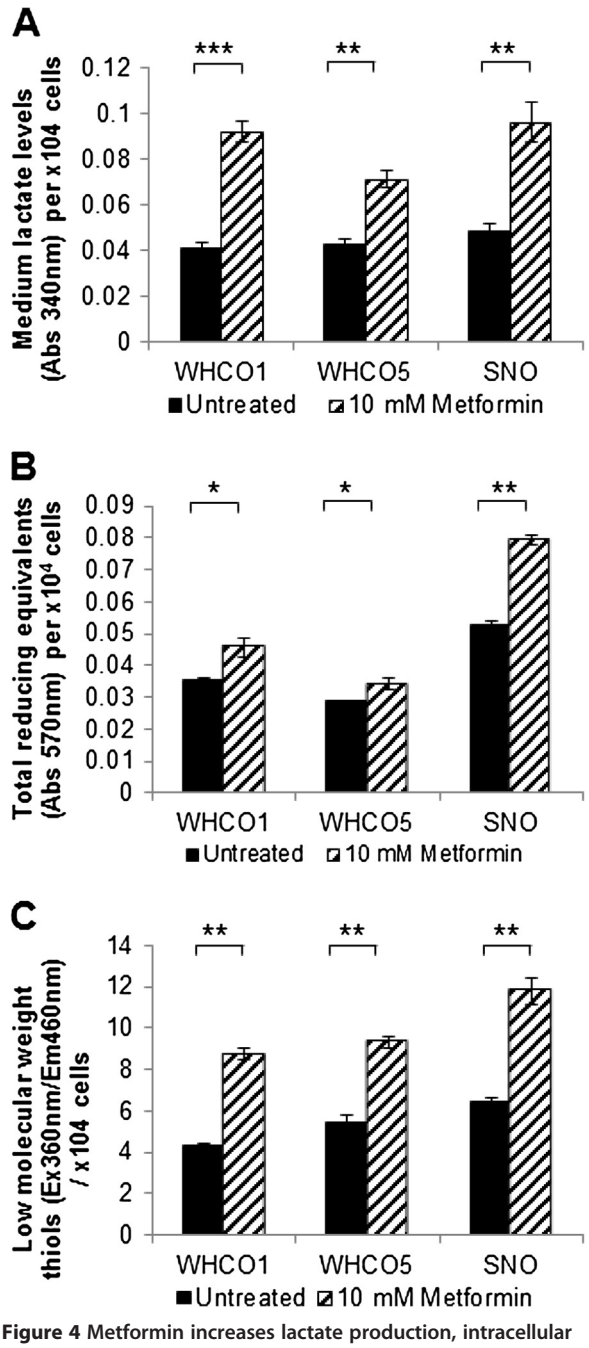


Figure 4 Metformin increases lactate production, intracellular NAD(P)H and low molecular weight reduced thiols in OSCC cells. A, Increased secretion of lactate (per 10^4 cells) indicated increased glycolysis levels in OSCC cells treated with 10 mM metformin for 24 hours in comparison to untreated cells (n = 3, mean ± SD). **B**, Elevated total reducing equivalents (per 10^4cells) in OSCC cells treated with 10 mM metformin for 24 hours in comparison to untreated cells (n = 3, mean ± SD). **C**, Low molecular weight thiols levels (per 10^4 cells) is higher in OSCC cells treated with 10 mM metformin for 24 hours, in comparison to untreated cells (n = 3, mean ± SD).

treated with cisplatin alone; thus displaying higher LD_{50} values for cisplatin in the presence of metformin, with a 78% increase for WHCO1, 140% increase for WHCO5 and 156% increase for SNO cells (Table 1). We assessed the effects of metformin on the formation of cisplatin-DNA adducts, by treating cells as above but using the calculated LD_{30} of cisplatin. DNA-bound platinum, as quantified by ICP-MS, showed a significant reduction in metformin treated cells by 19.3% in WHCO1, 14.1% in WHCO5 and 18.4% in SNO cells (Figure 3). To determine whether reduced cytotoxicity and cisplatin-DNA adduct levels was principally due to the observed metformin-induced reduction in cell proliferation, cells were treated with an alternative DNA crosslinker, mitomycin C with or without metformin as above (Figure 2B). Partial to no protection from mitomycin C was observed after metformin pre-treatment across the cell lines (Table 1), indicating that factors other than decreased proliferation were the major contributors to metformin-dependent cisplatin resistance.

Metformin treatment increases lactate production, intracellular NAD(P)H and low molecular weight reduced thiols in OSCC cells

Metformin has been shown to increase cellular glucose transport and glycolytic rates [33]. We hypothesized that such an occurrence in OSCC cells could result in an enhanced intracellular reducing environment (increased NAD(P)H levels) and the potential for increased intracellular reduced thiol levels, thus contributing to the observed metformin-induced protection against cisplatin. Cisplatin cytotoxicity has been previously shown to be antagonized by low-molecular-weight reduced thiols via cisplatin-thiol adduct formation, specifically with glutathione. Glutathione is the major contributor to intracellular thiols, existing in millimolar amounts in the cytosol [7,8,34]. We found that glycolysis (as measured by lactate output), and indirectly, glucose utilization, was indeed significantly increased for all OSCC cell lines after treatment with 10 mM metformin for 24 hours relative to untreated controls (Figure 4A). As predicted, total intracellular NAD(P)H levels (quantified by tetrazolium (MTT) reduction) (Figure 4B) and low-molecular weight thiol levels (monobromobimane-thiol adduct fluorescence) (Figure 4C) were significantly elevated for all OSCC cell lines following metformin treatment relative to untreated controls.

Intracellular thiols mediate metformin induced cisplatin protection in OSCC cells

To confirm that increased thiol levels can protect OSCC cells against cisplatin, cells were treated with the cell permeable thiol derivative, N-acetyl cysteine (NAC) (10 mM) prior to cisplatin exposure [35]. Predictably, all OSCC cell lines were significantly protected against cisplatin cytotoxicity by NAC pre-treatment (Figure 5). Therefore, our hypothesis, that a metformin-dependent increase in intracellular thiols is primarily responsible for the observed protection against cisplatin, seemed highly plausible. Since glutathione is the major thiol species within the cells, we confirmed its role in metformin-induced cisplatin resistance using the glutathione synthase inhibitor, BSO [32], to deplete intracellular glutathione pools. Cells were treated with metformin in the presence of BSO, prior to cisplatin exposure. Glutathione depletion by BSO almost completely reversed the protective effect of metformin for all OSCC cell lines, confirming the role of reduced-glutathione in metformin-induced cisplatin resistance (Figure 6). We also observed that BSO increased cisplatin cytotoxicity, with lower LD_{50} values, and this was anticipated as decreased intracellular glutathione levels would result in less cisplatin-thiol sequestration and an increase in cisplatin-DNA adduct formation.

OSCC cells are highly susceptible to copper-bis (thiosemicarbazones)

Given the above observations, we considered the role of cytotoxic molecules that are compatible with increased

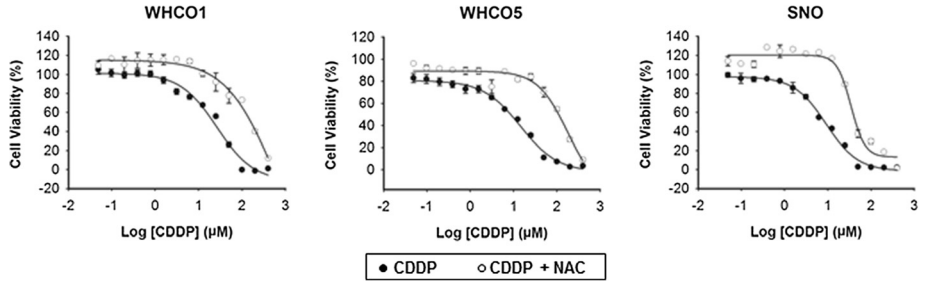


Figure 5 Increased intracellular thiols causes cisplatin resistance in OSCC cells. The cell permeable thiol derivative NAC was used to confirm the role of thiols in cisplatin resistance in OSCC cells. Cells were either untreated or treated with 10 mM NAC for 24 hours and then treated with a concentration range of cisplatin for a further 48. Cytotoxicity was assessed by MTT assay. All NAC-cisplatin treated cells displayed higher LD_{50} values than cisplatin treated cells alone (n = 3, mean ± SD).

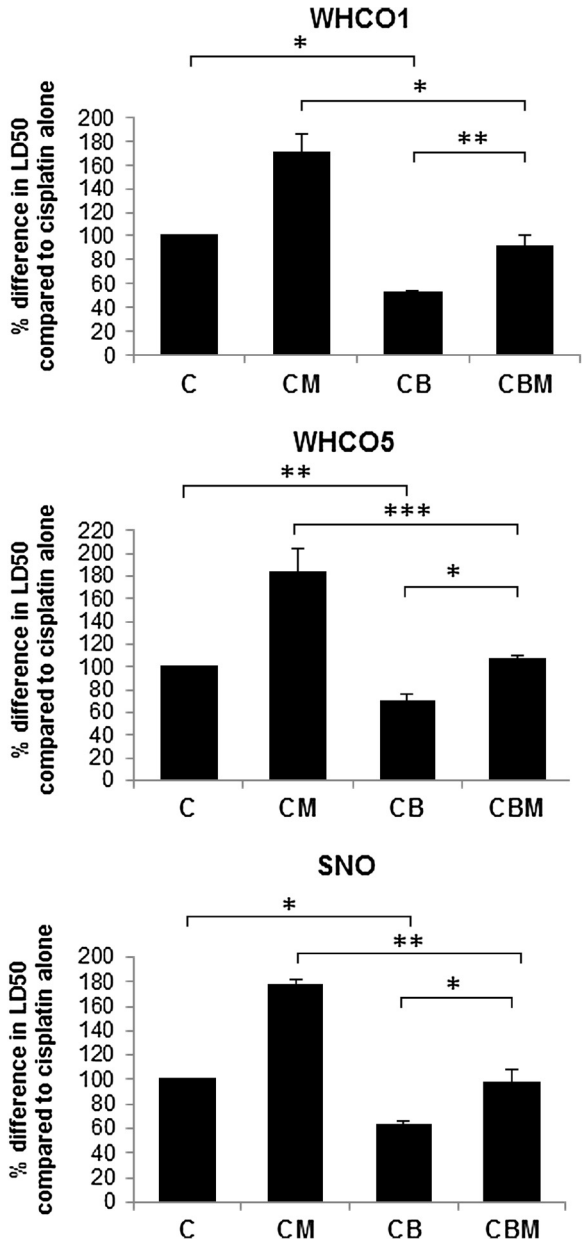


Figure 6 Metformin-induced cisplatin resistance is reversed by glutathione depletion in OSCC cells. The glutathione synthesis inhibitor, BSO was used to confirm the involvement of elevated glutathione levels in metformin induced cisplatin resistance in OSCC cells. MTT assays for cytotoxicity were performed as described, with cells treated with cisplatin alone (C), or in the presence 0.4 mM BSO (CB), or metformin and cisplatin (CM), or metformin and cisplatin in the presence of 0.4 mM BSO (CMB). Data is expressed as the percentage difference of LD_{50} values for each treatment relative to cisplatin alone (n = 3, mean ± SD). Predictably, the inhibition of glutathione synthesis increased cisplatin toxicity as LD_{50} values for cisplatin-BSO treated cells were significantly lower than cisplatin alone. Importantly, the presence of the inhibitor ablates the protective effect of metformin, with LD_{50} values for cisplatin-metfomin-BSO treated cells approaching those of cisplatin alone.

intracellular reducing conditions and could therefore be used in conjunction with metformin. In this way, the cytostatic effects of metformin could be utilised when combined as an adjuvant in chemotherapy regimens; since there is also evidence that metformin targets cancer stem cells, this would offer a considerable added advantage [18]. The copper bis(thiosemicarbazone) derivatives ATSM and GTSM have been previously shown to be trapped intracellularly under reducing conditions [22]. We therefore tested their efficacy as cytotoxic agents against OSCC cell lines with or without metformin. OSCC cells were pre-treated with or without 10 mM metformin for 24 hours and then treated with copper-bis(thiosemicarbazones) and 10 mM metformin for 48 hours (Figure 7). Interestingly, we found that both Cu-ATSM and Cu-GTSM displayed significant cytotoxicity for all cell lines, both in the presence and absence of metformin treatment, with LD_{50} values lower than or comparable to cisplatin alone. Cu-GTSM displayed lower LD_{50} levels than Cu-ATSM (Table 2). Statistically there was no difference between untreated and metformin treated samples ($p > 0.05$). Non-cop per-conjugated bis(thiosemicarbazone) compounds displayed far lower levels of cytotoxicity than their copper-conjugated counterparts, with LD_{50} concentrations over 200 μM; copper alone had minimal effect on cells at the concentrations used in this study.

Discussion

We have established that metformin significantly reduces the proliferation of OSCC cells. However, we observed that metabolic alterations caused by metformin rendered cells less sensitive to the commonly used chemotherapeutic agent, cisplatin. Previous studies have shown that metformin can reduce sensitivity to cisplatin through the activation of pro-survival signals via Akt [20] and hyperactivation of Akt has been linked to increased glycolysis [36]. Those studies therefore support our findings, which show that metformin increases glycolysis with a subsequent increase in intracellular reducing equivalents and a concomitant increase in intracellular reduced thiols.

Since cisplatin is ineffective in a reducing intracellular environment, our findings also support observations regarding cisplatin chemoresistance in tumours; cancer cells within the tumour are known to display a highly reducing phenotype and resist cisplatin chemotherapy [37]. However, in recent years, the observation that tumours consist of cells in differing metabolic states to surrounding normal tissue [38] has encouraged the concept of cancer-cell specific metabolic targeting as an increasingly popular strategy in cancer therapy [39]. Our study highlights the use of metformin with cytotoxic agents that are compatible with or remain active under

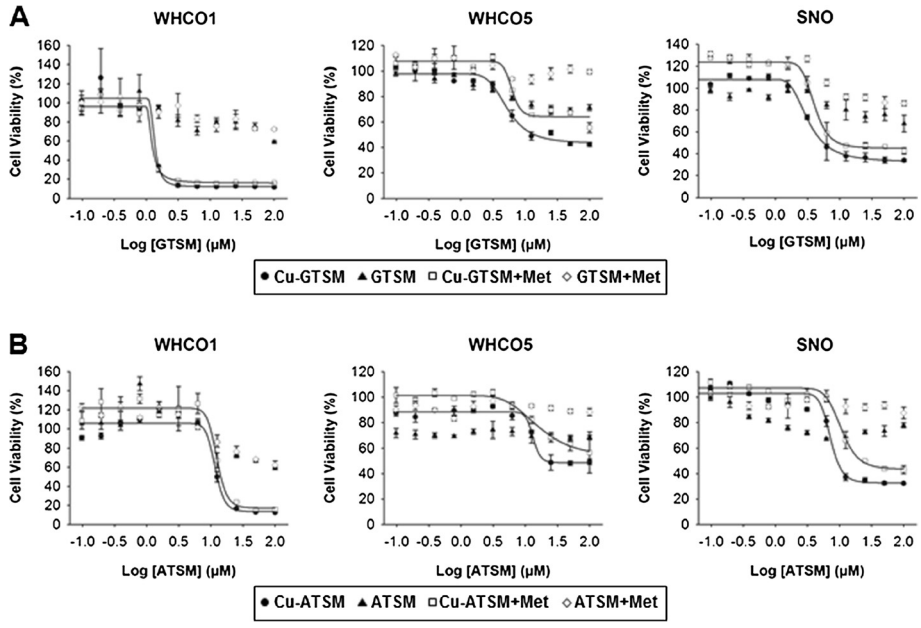


Figure 7 Copper bis(thiosemicarbazones) are highly toxic to OSCC cells in the presence of metformin. OSSC cells, untreated or treated with 10 mM metformin for 24 hours and then treated with **(A)** GTSM or Cu-GTSM, or **(B)** ATSM or Cu-ATSM for a further 48 hours were assessed by MTT assay. The non-copper-conjugated bis(thiosemicarbazones) showed relatively little toxicity with LD_{50} values greater than 200 μM in both the presence and absence of metformin. The copper-conjugated compounds however displayed considerable toxicity to OSCC cells with similar LD_{50} values for metformin treated-and untreated compounds for WHCO1, WHCO5, and SNO cells.

reducing conditions, thus paving the way for novel drug therapy combinations for the treatment of this highly aggressive malignancy.

Mitomycin C, which must be reductively activated to exert its biological effects [40], is one potential candidate for this strategy as partial to no protection from this drug was observed after metformin pre-treatment. However, an obvious concern with the use of mitomycin C and related DNA crosslinkers in combination with metformin would be the potential for decreased drug effectiveness or the emergence of drug resistance *in vivo,* due to the anti-proliferative effects of the biguanide. Therefore, agents that are either reductively activated or tolerant, and that target proliferating and non-proliferating tumour cells, would be a more logical choice for use in combination with metformin in OSCC. We have established that a potential highly efficacious combination strategy of this kind, could be metformin and the copper-bis (thiosemicarbazones), Cu-GTSM or Cu-ATSM. Bis (thiosemicarbazones) have been considered for cancer treatment since the 1950's [23], whilst the copper-bis (thiosemicarbazones) have been shown to possess potent anti-cancer activities and are attractive candidates for use as chemotherapeutics as they often preferentially accumulate in tumour tissue and are retained in cells under reducing conditions [22]. We have shown that Cu-ATSM and Cu-GTSM, in contrast to non-copper conjugated bis(thiosemicarbazones), are highly cytotoxic to OSCC cells, both in the presence and absence of metformin, and are thus metformin-compatible. The fact that an increase in toxicity was not observed for Cu-ATSM or Cu-GTSM in the presence of metformin suggests that: (1) there already exists a sufficiently high intracellular reducing environment in the OSCC cell lines used (a common observation in cancer cells [37]) to allow for the intracellular accumulation of these compounds to toxic levels, and (2) that the mechanisms of toxicity of these compounds, are compatible with, but not necessarily dependent on a intracellular reducing environment.

Table 2 Cytotoxicity in OSCC cells treated with or without metformin and with Cu-GTSM or Cu-ATSM

Compounds	LD_{50} (μM)		
	WHCO1	WHCO5	SNO
Cu-GTSM	1.14 ± 0.16	5.39 ± 0.9	3.37 ± 0.23
Metformin + Cu-GTSM	1.16 ± 0.12	6.24 ± 0.01	4.23 ± 0.7
GTSM	>200	>200	>200
Metformin + GTSM	>200	>200	>200
Cu-ATSM	11.93 ± 0.47	13.51 ± 0.81	7.099 ± 0.57
Metformin + Cu-ATSM	12.54 ± 0.19	12.05 ± 0.83	10.90 ± 0.77
ATSM	>200	>200	>200
Metformin + ATSM	>200	>200	>200

OSCC cells were treated with 10 mM metformin and either GTSM, Cu-GTSM, ATSM or Cu-ATSM and the MTT assay performed. LD_{50} (μM) was calculated on replicates (n = 3, mean ± SD).

Predictably we found that Cu-GTSM exhibited lower LD_{50} values than Cu-ATSM as Cu-GTSM is known to be rapidly reduced by intracellular thiols resulting in cell retention, copper release and ultimately apoptosis via oxidative stress, and/or the inhibition of DNA synthesis and oxidative phosphorylation [41,42]; Cu-ATSM on the other hand has been shown to be poorly reduced by intracellular thiols and thought to be maintained in a reduced state (and thus retained intracellularly) only under hypoxic conditions [41]. Recently, however, Donnelly *et al.* have shown that Cu-ATSM can be retained in cells under normoxic conditions when the intracellular reducing environment is increased due to factors such as impaired mitochondrial electron transport chain function [22]. These findings appear to agree with the findings of our study as SNO cells, which exhibit the greatest intracellular reducing environment (in the absence of metformin) of all the OSCC cell lines tested (Figure 4B), exhibit the greatest sensitivity to Cu-ATSM (Table 2). Nonetheless, the fact that all OSCC cell lines were highly sensitive to Cu-ATSM alone or in combination with metformin, at LD_{50} values comparable to or lower than those for cisplatin for all OSCC cell lines used, is extremely promising given its increased stability over Cu-GTSM and investigatory Food and Drug Administration approval of ^{64}Cu-ATSM for use as a hypoxia contrast agent [43].

Conclusions

Metformin, which has an extensive track record and is well tolerated by individuals, has been shown to suppress cancer cell proliferation. We have established that metformin significantly reduces cell proliferation in OSCC cell lines. However, we found metformin causes resistance to cisplatin in OSCC cell lines and as such we advise that caution be used when administering cisplatin to diabetic patients treated with metformin and in the use of metformin as an adjuvant to cisplatin chemotherapy. Furthermore, we have shown that two copper-conjugated bis(thiosemicarbazones), Cu-ATSM and Cu-GTSM, exhibit marked cytotoxicity in OSCC cells in the presence of metformin. The preliminary data presented in this study justifies further investigations into the therapeutic effects of copper-bis(thiosemicarbazones) in both the presence and absence of metformin, for OSCC. In addition metformin lends itself to combination therapy with reduction compatible or activated compounds (unlike cisplatin) for both OSCC and potentially other cancers where similar metabolic changes are observed.

Abbreviations

BSO: Buthionine sulfoximine; Cu-ATSM: Copper diacetyl-bis(4-methylthiosemicarbazonato)copper(II); Cu-GTSM: Copper glyoxal-bis(4-methylthiosemicarbazonato)copper(II); DMSO: Dimethyl Sulfoxide; FCS: Foetal calf serum; ICP-MS: Inductively coupled plasma mass spectrometry; LDH: Lactate dehydrogenase; NAC: N-acetyl-L-cysteine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide.

Competing interests

Authors LHD and DMD are co-applicants on a patent for the treatment of cancer and OSCC using metformin and copper-conjugated compounds. The authors declare that they have no competing interests.

Authors' contributions

LHD conceived the study, participated in its design, performed low-molecular weight thiol quantification, synthesized bis(thiosemicarbazone) compounds, conducted data analysis and interpretation, and assisted in drafting the manuscript; RJ carried out the cell proliferation studies, cell cycle analysis, prepared samples for ICP-MS analysis, and contributed to the MTT and LDH assays and statistical analysis; AR assessed bis(thiosemicarbazone) structures by NMR spectroscopy; RV participated in manuscript preparation; DMD conceived the study, participated in its design, acquired data for the MTT, LDH, thiol, reducing equivalents, and glutathione depletion assays, conducted data analysis and interpretation, and drafted the manuscript. All authors read and approved the final manuscript.

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Author details

[1]School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg 2193, South Africa. [2]Cell Biology Group, Centre for HIV and STI's, National Institute for Communicable Diseases, Private Bag X4, Sandringham, Johannesburg 2131, South Africa. [3]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa. [4]Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa.

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References

1. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM: **Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.** *Intl J Cancer* 2008, **2010**(127):2893–917.
2. Enzinger PC, Mayer RJ: **Esophageal cancer.** *N Engl J Med* 2003, **349:**2241–52.
3. Holmes RS, Vaughan TL: **Epidemiology and pathogenesis of esophageal cancer.** *Semin Radiat Oncol* 2007, **17:**2–9.
4. La Vecchia C, Bosetti C, Lucchini F, Bertuccio P, Negri E, Boyle P, Levi F: **Cancer mortality in Europe, 2000–2004, and an overview of trends since 1975.** *Ann Oncol* 2010, **21:**1323–60.
5. Stoner GD, Gupta A: **Etiology and chemoprevention of esophageal squamous cell carcinoma.** *Carcinogenesis* 2001, **22:**1737–1746.
6. Ilson DH: **Esophageal cancer chemotherapy: recent advances.** *Gastrointest Cancer Res* 2008, **2:**85–92.
7. Siddik ZH: **Cisplatin: mode of cytotoxic action and molecular basis of resistance.** *Oncogene* 2003, **22:**7265–7279.
8. Arn'er ESJ, Nakamura H, Sasada T, Yodoi J, Holmgren A, Spyrou G: **Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by cis-diamminedichloroplatinum (II) and its major**

metabolite the glutathione-platinum complex. *Free Rad Biol Med* 2001, **31**(10):1170–1178.
9. Ishikawa T: **The ATP-dependent glutathione S-conjugate export pump.** *Trends Biochem Sci* 1992, **17:**463–468.
10. Tew WP, Kelsen DP, Ilson DH: **Targeted therapies for esophageal cancer.** *Oncologist* 2005, **10:**590–601.
11. Kitabchi AE, Temprosa M, Knowler WC, Kahn SE, Fowler SE, Haffner SM, Andres R, Saudek C, Edelstein SL, Arakaki R, Murphy MB, Shamoon H: **Role of insulin secretion and sensitivity in the evolution of type 2 diabetes in the diabetes prevention program: effects of lifestyle intervention and metformin.** *Diabetes* 2005, **54:**2404–2414.
12. Prager R, Schernthaner G, Graf H: **Effect of metformin on peripheral insulin sensitivity in non-insulin dependent diabetes mellitus.** *Diabetes Metab* 1986, **12:**346–350.
13. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD: **Metformin and reduced risk of cancer in diabetic patients.** *BMJ* 2005, **330:**1304–1305.
14. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M: **Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells.** *Cancer Res* 2006, **66:**10269–10273.
15. Sahra IB: **The combination of metformin and 2-deoxyglucose inhibits autophagy and induces AMPK-dependent apoptosis in prostate cancer cells.** *Autophagy* 2010, **6:**670–671.
16. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, Kobara H, Mori H, Himoto T, Okano K, Suzuki Y, Murao K, Masaki T: **The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo.** *Mol Cancer Ther* 2012, **11:**549–60.
17. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, Hsu L, Hung MC, Hortobagyi GN, Gonzalez-Angulo AM: **Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer.** *J Clin Oncol* 2009, **27:**3297–3302.
18. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K: **Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission.** *Cancer Res* 2009, **69:**7507–7511.
19. Rattan R, Graham RP, Maguire JL, Giri S, Shridhar V: **Metformin suppresses ovarian cancer growth and metastasis with enhancement of cisplatin in vivo.** *Neoplasia* 2011, **13:**483–491.
20. Janjetovic K, Vucicevic L, Misirkic M, Vilimanovich U, Tovilovic G, Zogovic N, Nikolic Z, Jovanovic S, Bumbasirevic V, Trajkovic V, Harhaji-Trajkovic L: **Metformin reduces cisplatin-mediated apoptotic death of cancer cells through AMPK-independent activation of Akt.** *Eur J Pharmacol* 2011, **651:**41–50.
21. Palanimuthu D, Shinde SV, Somasundaram K, Samuelson AG: **In vitro and in vivo anticancer activity of copper bis(thiosemicarbazone) complexes.** *J Med Chem* 2013, **56:**722–734.
22. Donnelly PS, Liddell JR, Lima S, Paterson BM, Cater MA, Savva MS, Mot AI, James JL, Trounce IA, White AR, Crouch PJ: **An impaired mitochondrial electron transport chain increases retention of the hypoxia imaging agent diacetylbis(4-methylthiosemicarnazonato)copper^{II}.** *Proc Natl Acad Sci U S A* 2012, **109:**47–52.
23. French FA, Freedlander BL: **Carcinostatic action of polycarbonyl compounds and their derivatives: IV. Glyoxal bis(thiosemicarbazone) and derivatives.** *Cancer Res* 1958, **18:**1290–1300.
24. Crowley AR, Dilworth JR, Donnelly PS, Gee AD, Heslop JM: **Acetylacetone bis(thiosemicarbazone) complexes of copper and nickel: towards new copper radiopharmaceuticals.** *Dalton Trans* 2004, 2404–2412.
25. Veale RB, Thornley AL: **Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines.** *S Afr J Sci* 1989, **85:**375–379.
26. Bey E, Alexander J, Whitcutt JM, Hunt JA, Gear JH: **Carcinoma of the esophagus in Africans: establishment of a continuously growing cell line from a tumor specimen.** *In Vitro* 1976, **12:**107–114.
27. Liu B, Fang M, Lu Y, Fan Z: **Fibroblast growth factor and insulin-like growth factor differentially modulate the apoptosis and G1 arrest induced by anti-epidermal growth factor receptor monoclonal antibody.** *Oncogene* 2001, **20:**1913–1922.
28. Brouwers EEM, Tibben MM, Pluim D, Rosing H, Boot H, Cats A, Schellens JHM, Beijnen, JH: **Inductively coupled plasma mass spectrometric analysis of the total amount of platinum in DNA extracts from peripheral blood mononuclear cells and tissue from patients treated with cisplatin.** *Anal Bioanal Chem* 2008, **391:**577–585.
29. Smith DJ, Cossins LR, Hatzinisiriou I, Haber M, Nagley P: **Lack of correlation between MYCN expression and the Warburg effect in neuroblastoma cell lines.** *BMC Cancer* 2008, **8:**259.
30. Damelin LH, Coward S, Choudhury SF, Chalmers S, Cox IJ, Robertson NJ, Revial G, Miles M, Tootle R, Hodgson HJF, Selden C: **Altered mitochondrial function and cholesterol synthesis influences protein synthesis in extended HepG2 spheroid cultures.** *Arch Biochem Biophys* 2004, **434:**167–77.
31. Kosower NS, Kosower EM, Newton GL, Ranney HM: **Bimane fluorescent labels: labelling of normal human red cells under physiological conditions.** *Proc Natl Acad Sci U S A* 1979, **79:**3382–3386.
32. Griffith OW: **Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs potent inhibitors of glutathione synthesis.** *J Biol Chem* 1982, **257:**13704–13712.
33. Shaw RJ, Lamia K, Vasquez D, Koo S-H, Bardeesy N, Depinho R, Montminy M, Cantley LC: **The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin.** *Science* 2005, **310:**1642–6.
34. Lu SC: **Regulation of glutathione synthesis.** *Mol Aspects of Med* 2009, **30:**42–59.
35. Zafarullaha M, Lia WQ, Sylvestera J, Ahmad M: **Molecular mechanisms of N-acetylcysteine actions.** *Cell Mol Life Sci* 2003, **60:**6–20.
36. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB: **Akt stimulates aerobic glycolysis in cancer cells.** *Cancer Res* 2004, **64:**3892–3899.
37. Jorgenson TC, Zhong W, Oberley TD: **Redox imbalance and biochemical changes in cancer.** *Cancer Res* 2013, **73:**6118–6123.
38. Cairns R, Harris IS, Mak TW: **Regulation of cancer cell metabolism.** *Nat Rev Cancer* 2011, **11:**85–95.
39. Vander Heiden MG: **Targeting cancer metabolism: a therapeutic window opens.** *Nat Rev Drug Discovery* 2011, **10:**671–84.
40. Paz MM: **Reductive activation of mitomycin C by thiols: kinetics, mechanism, and biological implications.** *Chem Res Toxicol* 2009, **22:**1663–1668.
41. Paterson BM, Donnelly PS: **Copper complexes of bis(thiosemicarbazones): from chemotherapeutics to diagnostic and therapeutic radiopharmaceuticals.** *Chem Soc Rev* 2011, **40:**3005–18.
42. Tardito S, Marchiò L: **Copper compounds in anticancer strategies.** *Curr Med Chem* 2009, **16:**1325–48.
43. Shankar LK: **The clinical evaluation of novel imaging methods for cancer management.** *Nat Rev Clinical Oncology* 2012, **9:**738–744.

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Appendix B

Second Publication from thesis:

Disulfiram/Copper-Disulfiram damages multiple protein degradation and turnover pathways and cytotoxicity is enhanced by metformin in oesophageal squamous cell carcinoma cell lines†

Rupal Jivan[1,#], Leonard Howard Damelin[2,3,#], Monica Birkhead[4], Amanda Louise Rousseau[5], Robin Bruce Veale[1], Demetra Mavri-Damelin[1]

Author details:

[1] The School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg, 2050, South Africa.

[2] The School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg, 2193, South Africa.

[3] Cell Biology Group, Centre for HIV and STI's, National Institute for Communicable Diseases (NHLS), Private Bag X4, Sandringham, Johannesburg 2131, South Africa.

[4] Electron Microscope Laboratory, Centre for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases (NHLS), Private Bag X4, Sandringham, Johannesburg 2131, South Africa. 5Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg, 2050, South Africa.

[#]Joint first authors

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Article

Disulfiram/Copper-Disulfiram damages multiple protein degradation and turnover pathways and cytotoxicity is enhanced by metformin in oesophageal squamous cell carcinoma cell lines†

Rupal Jivan[1,#], Leonard Howard Damelin[2,3,#], Monica Birkhead[4], Amanda Louise Rousseau[5], Robin Bruce Veale[1], Demetra Mavri-Damelin[1,*]

[1] The School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg, 2050, South Africa.
[2] The School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg, 2193, South Africa.
[3] Cell Biology Group, Centre for HIV and STI's, National Institute for Communicable Diseases (NHLS), Private Bag X4, Sandringham, Johannesburg 2131, South Africa.
[4] Electron Microscope Laboratory, Centre for Emerging and Zoonotic Diseases, National Institute for Communicable Diseases (NHLS), Private Bag X4, Sandringham, Johannesburg 2131, South Africa.
[5]Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, Private Bag X3, Johannesburg, 2050, South Africa.

[#]Joint first authors

***Corresponding Author**
Dr. Demetra Mavri-Damelin
The School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag X3, Johannesburg, 2050, South Africa
Tel: +27 11 717 6339
Fax: +27 11 717 6351
Email: Demetra.Mavri-Damelin@wits.ac.za

Running head: Disulfiram is cytotoxic to OSCC cells

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Key words:

- **Metformin**
- **Disulfiram**
- **Autophagy**
- **Proteasome**
- **Lysosome**
- **Copper**
- **Cancer**

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Disclosure of Potential Conflicts of Interest
Authors LHD and DMD are co-applicants on a patent for the treatment of OSCC using disulfiram and copper-disulfiram with and without metformin.

List of Abbreviations
AO Acridine orange
AVO Acidic vesicular organelles
BCS Bathocuproinedisulfonic acid
Cu-8HQ Copper-8-hydroxyquinoline
Cu-DSF Copper-disulfiram
DMSO Dimethyl sulfoxide
DPTD Dipyrrolidine thiuram disulphide
DSF Disulfiram
Suc-LLVY-AMC N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MeDTC-SO Methyl diethylthiocarbamoyl sulfoxide
OSCC Oesophageal squamous cell carcinoma
TTD Tetrapropyl thiuram disulphide

Authors' Contributions
RJ carried out toxicity assays, western blots, fluorescence microscopy, proteasome assays, statistical analysis and assisted in drafting the manuscript; LHD conceived the study, participated in its design, synthesised the disulfiram analogues, performed fluorescence microscopy, conducted data analysis and interpretation, and assisted in drafting the manuscript; MB prepared electron microscopy cells and images; ALR performed NMR spectroscopic analysis; RBV assisted in manuscript preparation; DMD conceived the study, participated in its design, conducted data analysis and interpretation, drafted and approved the final manuscript.

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ABSTRACT

Disulfiram (DSF), used since the 1950s in the treatment of alcoholism, is reductively activated to diethyldithiocarbamate and both compounds are thiol-reactive and readily complex copper. More recently DSF and copper- DSF (Cu-DSF) have been found to exhibit potent anticancer activity. We have previously shown that the anti-diabetic drug metformin is anti-proliferative and induces an intracellular reducing environment in oesophageal squamous cell carcinoma (OSCC) cell lines. Based on these observations, we investigated the effects of Cu-DSF and DSF, with and without metformin, in this present study. We found that Cu-DSF and DSF caused considerable cytotoxicity across a panel of OSCC cells, and metformin significantly enhanced the effects of DSF. Elevated copper transport contributes to DSF and metformin-DSF-induced cytotoxicity since the cell-impermeable copper chelator, bathocuproinedisulfonic acid, partially reversed the cytotoxic effects of these drugs, and interestingly, metformin-treated OSCC cells contained higher intracellular copper levels. Furthermore, DSF may target cancer cells preferentially due to their high dependence on protein degradation/turnover pathways, and we found that metformin further enhances the role of DSF as a proteasome inhibitor. We hypothesized that the lysosome could be an additional, novel, target of DSF. Indeed, this acid-labile compound decreased lysosomal acidification, and DSF-metformin co-treatment interfered with the progression of autophagy in these cells. In summary, this is the first such report identifying the lysosome as a target of DSF and based on the considerable cytotoxic effects of DSF either alone or in the presence of metformin, *in vitro,* we propose these as novel potential chemotherapeutic approaches for OSCC. This article is protected by copyright. All rights reserved

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Disulfiram (DSF) (tetraethylthiuram disulfide) has been used since the 1950's to treat alcohol abuse (Johansson 1992). *In vivo,* DSF is rapidly converted to its monomer, diethyldithiocarbamate (DDC), which is then methylated and subsequently oxidised to methyl diethylthiocarbamoyl sulfoxide (MeDTC-SO) (Ververka et al., 1997). Both DSF and MeDTC-SO can irreversibly inhibit aldehyde dehydrogenase, which leads to an accumulation of acetaldehyde in the blood upon alcohol intake, causing, amongst other effects, severe nausea, vomiting and tachycardia (Johansson 1992; Ververka et al., 1997).

DSF was shown to have anticancer activity in the 1970's (Wattenberg 1974) and in recent years, there has been renewed interest in DSF for cancer therapy primarily because DSF is an attractive candidate for drug 'repurposing' given its well characterised toxicity profile and good safety track record (Cvek 2011; Johansson 1992). DSF and DDC are thiol-reactive and can react with the free thiol groups on proteins and glutathione; they also have high affinity for, and form stable complexes with, heavy metals such as copper and zinc (Johansson 1992). Much of the anticancer activity of DSF has been attributed to its copper complex, which has been found to exhibit highly efficacious and specific toxicity both *in vitro* and *in vivo* through a variety of mechanisms, including acting as an inhibitor of activating transcription factor/cyclic AMP-responsive element binding protein (ATF/CREB), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), P-glycoprotein, DNA topoisomerases, DNA methyltransferases, as a potent inhibitor of the proteasome and of invasion and angiogenesis (Cen et al., 2004; Brar et al., 2004; Wang et al., 2003; Loo et al., 2004; Yakisich et al., 2001; Lin et al., 2011; Lovberg et al. 2006; Chen et al., 2006).

In a previous study, in which we investigated the effects of metformin on cisplatin cytotoxicity in oesophageal squamous cell carcinoma (OSCC) cell lines, we established that metformin induces a reducing intracellular environment, which whilst protective against cisplatin toxicity, allows cells to be targeted by compounds that are activated under reducing conditions. As a case in point, we showed that two copper-bis(thiosemicarbazone) compounds, copper diacetyl-bis(4-methylthiosemicarbazonato)copper(II) (Cu-ATSM) and copper glyoxal-bis(4-

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methylthiosemicarbazonato)copper(II) (Cu-GTSM), which are understood to be reductively activated, are highly cytotoxic to OSCC cells in the presence of metformin (Damelin et al. 2014). Given these promising results, we decided to investigate the effect of the reductively activated DSF and Cu-DSF on OSCC cells in combination with metformin; we and others have shown that metformin exhibits excellent antiproliferative effects on carcinoma cell lines (Damelin et al. 2014; Quinn et al. 2013).

In this study, we show that Cu-DSF and interestingly, DSF alone, display considerable cytotoxic activity towards OSCC cell lines, and that this cytotoxicity is enhanced by metformin co-treatment. In addition, we show that DSF toxicity is only partially copper-dependent and, for the first time, we identify that a major contributing mechanism of DSF toxicity (in OSCC cells) is perturbed lysosomal acidification and autophagic activity, which in combination with proteasomal inhibition, confer damage to multiple protein degradation/ turnover pathways in these cells. This study therefore identifies a novel mechanism of action for DSF and highlights the potential use of DSF, with or without metformin as a potential chemotherapy strategy for the treatment of OSCC.

MATERIALS AND METHODS

REAGENTS

All reagents were purchased from Sigma Aldrich unless otherwise specified.

SYNTHESIS OF CU-DSF AND DSF ANALOGUES

Cu-DSF was synthesized by adding equimolar amounts of DSF and cupric chloride in DMSO. Analogues of DSF were synthesized, as previously described (Cramer 1935).

Dipyrrolidine thiuram disulphide (DPTD)

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^{1}H NMR (500 MHz, DMSO-d_6) δ 3.86 (dt, J = 61.6, 7.0 Hz, 1H), 2.03 (dt, J = 73.0, 6.9 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 187.71, 57.43, 51.40, 26.63, 24.21.

Tetrapropyl thiuram disulphide (TTD)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.89 (dt, J = 10.1, 4.9 Hz, 1H), 1.78 (dq, J = 87.4, 7.6 Hz, 1H), 0.92 (dt, J = 57.1, 7.4 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 192.10, 58.88, 54.89, 21.73, 19.49, 11.42.

CELL CULTURE

The human oesophageal squamous carcinoma cell (OSCC) lines, WHCO1, WHCO5, (Veale and Thornley, 1989) and SNO (Bey et al., 1976) were cultured in DMEM/F12 (3:1) with 10% foetal bovine serum (Lonza) and 100 U/ml penicillin/0.1 mg/ml of streptomycin, in a humidified atmosphere at 37°C with 5% CO_2. OSCC cell lines were isolated from moderately differentiated tumours from South African OSCC patients. The SNO cell line harbours an p53-R175H mutation (Fanucchi and Veale, 2009).

CYTOTOXICITY ASSAYS

Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, 7500 cells per well were seeded into 96-well plates and after 24 hours, exposed to cytotoxic agents DSF, Cu-DSF or the DSF analogues DPTD or TTD for a further 48 hours. To determine the effect of metformin on cytotoxicity, cells were pre-treated with 10 mM metformin for 24 hours then co-treated with metformin and DSF, Cu-DSF or the DSF analogues DPTD or TTD for a further 48 hours. To investigate the role of copper in DSF mediated cytotoxicity, cells were treated as described but with the addition of the cell impermeable copper chelator, bathocuproinedisulfonic acid (BCS) (200 µM) (Furuta et al., 2002). After treatments, the medium was replaced with 100 µl of MTT solution (0.5 mg/ml MTT in cell culture medium) and incubated at 37°C for 1.5 hours. MTT solution was then removed, and MTT formazan dissolved in 100 µl dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a Thermo Scientific Multiskan GO microplate reader (n=3±SD). Cells

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were also visualized microscopically and images captured on an Olympus BX63 microscope at 40x magnification and images processed using CellSense Dimension software.

INDUCTIVELY COUPLED PLASMA MASS SPECTOMETRY FOR COPPER QUANTIFICATION

Inductively Coupled Plasma - Mass Spectometry (ICP-MS) was used to quantify total intracellular copper levels as previously described (Price et al., 2011). Cells were seeded in 100 mm dishes and treated with metformin, DSF or metformin and DSF, as previously indicated. Cells were washed with cold 1x Phosphate Buffered Saline (PBS) three times, scraped and collected by centrifugation for 10 minutes at 8000 rpm at 4 °C. A relative portion of cells was removed for protein quantification by the Bradford assay (Bradford 1976). Cell pellets were then incubated with 65% nitric acid (Suprapure, Merck) at 65 °C for 2 hours. Samples were then diluted to a final concentration of 5% nitric acid and analysed for copper on an Agilent 7700 ICP-MS with He collision gas (n=3±SD).

WESTERN BLOTTING FOR AUTOPHAGY AND PROTEIN UBIQUITINATION

Autophagy and protein ubiquitination were assessed by western blotting by LC3B and total ubiquitinated protein levels, respectively. Cells were treated as described above (the positive control was 50 µM chloroquine (CQ) for 24 hrs) and harvested in triple detergent cell lysis buffer. Protein was quantified (Bradford assay), 40 µg electrophoresed on 5-10% polyacrylamide gels at 25 mA for 45 minutes and protein was subsequently electroblotted for 2 hours at 200 mA onto nitrocellulose (Bradford 1976). Membranes were blocked in 5% fat-free milk powder in 1xTris Buffered Saline with 0.1% Tween (TBS-T) then incubated with the primary antibodies, rabbit anti-LC3B or rabbit anti-ubiquitin (Cell Signalling Technology) in 5% bovine serum albumin in 1xTBS-T overnight, following which, membranes were washed in 1xTBS-T and then incubated with a goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnologies). Chemiluminescent signal was obtained with SuperSignal®

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West Pico Chemiluminescent substrate (Thermo Scientific) and detected using x-ray film. Densitometry was performed using QuantityOne software.

PROTEASOME FUNCTION

Proteasome function was assessed in treated cells using the fluorogenic proteasome substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC), which indicates levels of chymotrypsin-like activity, as previously described (Daniel et al. 2004). Cells seeded at equal density in 100mm dishes were left to settle for 24 hours then treated with LD_{30} concentrations of DSF, Cu-DSF or the DSF analogues DPTD and TTD, as for the MTT assays, or with the positive control copper-8-hydroxyquinoline (5 µM for 24 hours) (Cu-8HQ), which is a previously established inhibitor of proteasomal function (Daniel et al., 2004). Cells were then washed in 1xPBS, collected by scraping and centrifugation, lysed in 25 mM Tris-HCl, pH 8.0; 75 mM NaCl; 0.05 % SDS; 0.5 % Triton X-100; 0.25 % Sodium deoxycholate, and lysates cleared by centrifugation. Protein was quantified by Bradford assay and 40 µg added to assay buffer (50 mM Tris-HCl pH 7.5) to a final volume of 400 µl. Suc-LLVY-AMC in DMSO was added to a final concentration of 40 µM and the reaction mixture incubated at 37 ^{o}C for 1 hour, followed by the addition of trichloroacetic acid to a final concentration of 4% to stop the reaction. Fluorescence was measured $360nm_{excitation}/460nm_{emission}$ using an Ascent multi-well plate fluorimeter (Thermo Scientific) (n=3±SD).

FLUORESCENCE MICROSCOPY FOR ACIDIC VESICULAR ORGANELLE ACIDIFICATION

Acidic vesicular organelles (AVO), including lysosomes, were visualised using the pH sensitive dye acridine orange (AO), which displays a clear shift from red to yellow to green fluorescence, with increasing pH, unlike other commercially available dyes (Paglin et al., 2001; Pierzynska-Mach et al., 2014). OSCC cells were grown on glass coverslips until 50% confluent then treated with LD_{30} concentrations of DSF, Cu-DSF, DPTD or TTD, for 24 hours, with or without 10 mM metformin. Chloroquine was used as the positive control (1 mM for 1 hour). Cells

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were then incubated with 3 µM AO added to culture medium for 1 hour at 37 °C. Cells were visualised using the Olympus BX41 fluorescence microscope (100 W equipped with a camera and lamp using a 490 nm band-pass blue excitation filter and a 515 nm long-pass barrier filter) and images captured and processed with CellSense Dimensions software.

ELECTRON MICROSCOPY FOR AUTOPHAGY

Electron microscopy (EM) was used to look for autophagosome formation (Yla-Antilla et al. 2009). Cells were cultured as previously described in 100 mm dishes and when 60% confluent were incubated with 10 mM metformin for 24 hours, or LD_{30} dose of DSF, metformin plus DSF, Cu-DSF or CuDSF plus metformin. Cells were then rinsed with cold 1x PBS three times and then fixed with 2.5% glutaraldehyde in 0.1 M HEPES buffer pH 7.2 for 1 hour. Cells were then scraped into a microfuge tube and collected by centrifugation at 1000 xg for 10 minutes. Pelleted cells were routinely prepared for transmission electron microscopy as previously described (Hayat 2000). Briefly, pelleted cells were fixed overnight in 2.5% glutaraldehyde in 0.1M HEPES buffer (pH 7.2), rinsed several times in HEPES buffer followed by post-fixation in 1% buffered osmium tetroxide for 1 hour, repeatedly rinsed, dehydrated in a graded ethanol series (30 minute intervals, absolute ethanol thrice), infiltrated with a low viscosity epoxy resin (Agar Scientific®) and polymerised in BEEM® capsules at 70°C overnight. 70nm sections were cut on a Leica EM UC6, picked up on 0.25% formvar-coated copper slot grids, double stained with uranyl acetate and lead citrate, and viewed at 80kV on an FEI BioTwin Spirit transmission electron microscope fitted with an Olympus Quemesa CCD camera.

STATISTICAL ANALYSIS

All experiments were performed a minimum of three times as indicated. Comparisons were made by Student's t-tests and $p < 0.05$ was considered statistically significant. LD_{50} and LD_{30} values were calculated using GraphPad Prism version 6.

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RESULTS

OSCC CELLS ARE HIGHLY SUSCEPTIBLE TO CU-DSF AND DSF ALONE AND METFORMIN ENHANCES DSF CYTOTOXICTY

MTT cytotoxicity assays indicated that Cu-DSF was highly toxic to all OSCC cell lines (IC_{50} values: WHCO1 3.6±0.3 µM, WHCO5 2.8±0.2 µM and SNO 2±0.08 µM) (Fig. 1A and 1B). Interestingly, DSF alone was also highly cytotoxic to OSCC cells with IC_{50} values of 6±0.4 µM for WHCO1, 6.8±0.3 µM for WHCO5 and 11.9±0.6 µM for SNO cells (Fig. 1A and 1B). The addition of metformin significantly enhanced the cytotoxicity of DSF alone (by 25-40% across the three cell lines), but not of Cu-DSF (Fig. 1A and 1B). Cell morphology following treatments reflected the findings of the MTT assays (Fig. 1C).

PERTURBED COPPER HOMEOSTASIS IS PARTIALLY RESPOSNSIBLE FOR DSF AND METFORMIN-ENHANCED DSF CYTOTOXICTY

The treatment of OSCC cell lines with DSF and doubling cupric chloride concentrations, with or without metformin, indicated that DSF-induced cytotoxicity increased with increasing cupric chloride concentrations (Fig. 2A), but interestingly, metformin-enhanced cytotoxicity was only observed at low and not at high levels of cupric chloride concentrations. This suggested that metformin-enhanced DSF-cytotoxicity was due to increased copper transport (leading to increased intracellular Cu-DSF and/or Cu-dithiocarbamate formation), and that this effect is saturating at high copper concentrations. To confirm this, intracellular copper levels for DSF treated cells, with or without metformin co-treatment, were quantified using ICP-MS analysis. Predictably, DSF-treated cells exhibited a significant increase in intracellular copper levels vs. untreated controls; DSF facilitates increased copper import via the formation of stable, neutral lipophilic copper complexes (Fig. 2B). Metformin treated cells

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exhibited higher intracellular copper levels overall vs. untreated controls while DSF-metformin co-treated cells exhibited an additive increase in intracellular copper levels (Fig. 2B), confirming that metformin treatment did indeed facilitate increased copper transport and this was most likely responsible for the observed increase in cytotoxicity for DSF/metformin co-treatment vs DSF alone. These findings agree with previous investigations, which have established that metformin can indeed bind copper and alter intracellular copper levels (Zhu et al, 2002; Logie et al., 2012).

Whilst copper-enhanced DSF toxicity in all OSCC cell lines was significant, we also determined the contribution of copper–independent mechanisms of DSF cytotoxicity using the cell impermeable copper chelator, BCS (200 μM), which was in considerable excess to medium copper levels (range 1.4-4 μM). ICP-MS on cells treated with BCS confirmed the reduction in intracellular copper levels (Figure 2B). Interestingly, a significant but only partial (approximately 50% for WHCO1 and WHCO5 cells) increase in the LD_{50} value was observed for DSF-treated cells in the presence of BCS vs. DSF alone (Fig. 2C), indicating that while copper significantly contributed to DSF-cytotoxicity, copper-independent mechanisms were also involved in DSF-mediated cytotoxicity in OSCC cell lines.

DSF PERTURBS MULTIPLE PROTEIN DEGRADATION AND TURNOVER PATHWAYS:

1. DSF INHIBITS PROTEASOME FUNCTION

The above observation that DSF alone was cytotoxic to OSCC cells was interesting as previous investigations have indicated that cytotoxicity occurs largely through proteasome inhibition by Cu-DSF, and is the primary contributor to DSF-cytotoxicity (Cvek and Dvorak 2008). To confirm that DSF treatment inhibited proteasome function in OSCC cell lines, we quantified levels of ubiquitinated proteins as a gross indicator of protein turnover and observed an increase in total ubiquitinated proteins for DSF and Cu-DSF treated cells, (WHCO1 cells shown in Fig. 3A, WHCO5 and SNO cells in supplementary data). Next, proteasome chymotrypsin-like activity was fluorescently quantified for DSF-treated cells using the fluorogenic substrate Suc-LLVY-AMC. This assay

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indicated that DSF and Cu-DSF markedly reduced proteasome function, with metformin co-treatment exacerbating this effect. Cu-8HQ served as an effective positive control for proteasome inhibition (WHCO1 cells shown in Fig. 3B, WHCO5 and SNO cells in supplementary data). In order to gain further insight into the contribution of proteasome inhibition to DSF-cytotoxicity in OSCC cell lines, two DSF analogues, dipyrrolidine thiuram disulphide (DPTD) and tetrapropyl thiuram disulphide (TTD) were synthesized and their effect on proteasome activity in OSCC cells tested. The monomers of DPTD and TTD, namely pyrrolidine dithiocarbamate and dipropyl dithiocarbamate, respectively, have significantly smaller decomposition rates than the diethyl dithiocarbamate monomer of DSF: second order decomposition rate constants for pyrrolidine dithiocarbamate, dipropyl dithiocarbamate and diethyl dithiocarbamate are 0.1, 500 and 2.5x104 L mol-1 min-1, respectively (Topping and Jones, 1998), but their copper complexes are still expected to exhibit proteasome inhibition, as previously established for Cu-(DPTD). We found that, DPTD and TTD were significantly better at inhibiting proteasome function in OSCC cells in comparison to DSF (40% and 20% more effective, respectively) (WHCO1 cells shown in Fig. 3C, WHCO5 and SNO cells in supplementary data), but importantly, MTT assays revealed DPTD and TTD to be far less toxic than DSF for all OSCC cell lines (approximately a log fold and half a log fold less toxic, respectively) (WHCO1 cells shown in Fig. 3D, WHCO5 and SNO cells in supplementary data).

Collectively, these results suggested that: (1) proteasome inhibition by Cu-DSF was only a partial contributor to DSF-induced cytotoxicity in OSCC cell lines and (2), that the intracellular decomposition of the DSF monomer, DDC, may be important for DSF-induced cytotoxicity.

2. DSF ALTERS LYSOSOMAL ACIDIFICATION

Dithiocarbamates are acid labile and rapidly break down at low pH to their parent amine and carbon disulfide. We therefore hypothesized that since the decomposition rates of the monomers (dithiocarbamates) for DSF, TTD and DPTD were found to be proportional to their cytotoxicities, a potential novel mechanism of toxicity for DSF could be via its diffusion into lysosomes, its conversion to acid labile DSF and subsequent decomposition of

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DSF within the low pH (~4) lysosomal environment to diethylamine and carbon disulfide, resulting in amine accumulation within lysosomes, amine-dependent lysosomal alkalinisation, and ultimately, reduced lysosomal function. Therefore to investigate the effect of DSF and DSF analogues on lysosomal pH, cells were treated with LD_{30} levels of DSF, DPTD and TTD with or without cupric chloride and then stained with AO and examined by fluorescent microscopy. AO is a weekly basic, lysomsomotropic fluorescent dye, which upon accumulation in AVOs, including lysosomes, exhibits a red shift in fluorescence. Therefore acidic lysosomes stained with AO display orange/red fluorescence while lysosomes with reduced acidity display a progressive shift to yellow then green fluorescence with increasing alkalinisation. DSF treated cells displayed a marked increase in AVO (lysosomal) pH and this effect was exacerbated by the presence of copper, where overall fluorescence was both shifted towards green (DSF alone) and also reduced in intensity (Cu-DSF) in comparison to untreated controls, as seen across all OSCC cell lines (Fig. 4). DPTD had a minimal effect on lysosomal pH, with and without copper, while TTD treatment alone had a minimal effect on lysosomal pH while some reduction in overall fluorescence was observed in the presence of copper (Fig. 4). Chloroquine, a standard lysosomal alkaliniser, which served as a positive control, completely dissipated lysosomal pH as expected, as evidenced by bright green lysosomes (Fig. 4). These results strongly indicated that DSF did indeed perturb lysosomal pH/function and supported the above hypothesis that diethyl dithiocarbamate decomposition within lysosomes and subsequent diethylamine-dependent lysosomal alkalinisation may be responsible for this effect. DPTD and TTD, that have monomers with significantly smaller decomposition rates than the monomer of DSF (diethyl dithiocarbamate), exhibited a minimal effect on lysosomal pH, as would be expected for this model to be valid.

3. DSF INHIBITS AUTOPHAGY

Damaged lysosomal function leads to perturbed autophagy (Yang et al. 2011). We observed, by electron microscopy, that a representative OSCC cell line (WHCO1) exhibited numerous autophagic vesicles, which increased in number and size after metformin treatment (Fig. 5A), indicating a highly autophagic phenotype for

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OSCC cells and potentially, as with many other cancer cell lines, a high susceptibility to autophagic perturbation. We therefore investigated the effect of DSF on autophagic activity by assessing LC3B levels in OSCC cell lines. Cells treated with DSF exhibited a significant increase in LC3B-II relative to β-actin in response to DSF treatment and this effect was highly exacerbated by metformin, copper and copper/metformin co-treatment (Fig. 5B), suggesting that both perturbed lysosomal function and copper-DSF mediated oxidative effects acted in concert to severely damage autophagic function in Cu-DSF treated cells, and this is not surprising given the sensitivity of the autophagic cascade to altered redox state (Filomeni et al., 2010). Electron microscopy further supported the above findings in that DSF-treated cells contained large irregular autophagosomes while DSF-metformin treated cells exhibited a considerable accumulation of autophagosomes with poorly cleared autolysosomes. This suggested an inhibition in the maturation of autophagosomes to autolysosomes, which occurs upon fusion of the autophagosome with the lysosome, and is a potential consequence of perturbed lysosomal function (Fig. 5A). Cu-DSF and Cu-DSF-metformin treated cells exhibited vacuolization (Fig. 5A) along with a significant increase in LC3B-II (Fig. 5B), which is likely to be indicative of cell death with autophagy (Kroemer et al 2009).

DISCUSSION

Cancer cells have a higher dependence on protein degradation and turnover pathways than normal cells, in order to accommodate for their elevated metabolic rates (Mancias and Kimmelman, 2011; Molineaux 2012). These pathways comprise the ubiquitin-proteasome system, where proteins are targeted by polyubiquitination for degradation by the proteasome; and lysosomal proteolysis, which involves the degradation of proteins by proteases in this acidic organelle. This latter pathway also includes autophagy since autophagosomes, containing cellular material for degradation, fuse with lysosomes to form autolysosomes, in which organelles and proteins are degraded (Yang et al. 2011). Cancer cells are therefore very sensitive to inhibition of these

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pathways and this has led to the identification and development of new classes of inhibitors that have a greater effect on cancer cells over normal cells; proteasome inhibitors and modifiers of autophagy such as bortezomib and hydroxychloroquine, respectively, are under extensive clinical trial evaluation (Molineaux 2012).

In recent years, DSF (and Cu-DSF), has been shown to exhibit anticancer properties both *in vitro* and *in vivo* (Cvek, 2011). We have found in this study that both Cu-DSF and DSF are highly cytotoxic to OSCC cells lines. OSCC is a particularly aggressive carcinoma and there are very few treatment options, especially in the resource poor countries in which OSCC is prevalent (Ilson 2008; Ferlay et al., 2010), and as such, drug 'repurposing' is an attractive prospect. DSF displays a high specificity in its toxicity towards cancer cells. We show that in addition to proteasome inhibition, which is evidenced in this study and has been previously documented (Cvek and Dvorak 2008), DSF also perturbs AVO (lysosomal) acidification and subsequently autophagy (an effect greatly enhanced by copper) in OSCC cells. This simultaneous inhibition of multiple protein degradation/turnover pathways in cells that are so highly dependent on these processes, may additionally explain the cancer-cell specific toxicity of DSF over normal cells.

In this study we also show that metformin, which is highly anti-proliferative in OSCC cells (Damelin et al., 2014), significantly increases DSF cytotoxicity. We have shown that a major reason for this is a metformin-dependent increase in copper transport but as metformin also induces a reductive phenotype in OSCC cells (Damelin et al., 2014), metformin treatment may increase the rate of reduction of DSF to its monomer, diethyl dithiocarbamate, the decomposition of which is likely responsible for lysosomal alkalinisation. Electron microscopy in this study, and in previous studies, also indicate that metformin may increase autophagy (Tomic et al., 2011), therefore also potentially increasing cell susceptibility to DSF-dependent autophagic perturbation.

Our findings therefore highlight the potential use of DSF in OSCC therapy and the use of metformin/DSF as a highly efficacious and economical drug therapy combination in the treatment of this highly aggressive malignancy. Such 'repurposing' of existing drugs that are already very well characterised could be particularly beneficial in resource poor settings where OSCC is prevalent.

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REFERENCES

Bey E, Alexander J, Whitcutt JM, Hunt JA, Gear JH. 1976. Carcinoma of the esophagus in Africans: establishment of a continuously growing cell line from a tumor specimen. In Vitro 12:107-114.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72; 248-254.

Brar SS, Grigg C, Wilson KS, Holder, Jr. WD, Dreau D, Austin C, Foster M, Ghio AJ, Whorton AR, Stowell GW, Whittall LB, Whittle RR, White DP, Kennedy TP. 2004. Disulfiram inhibits activating transcription factor/cyclic AMP-responsive element binding protein and human melanoma growth in a metal-dependent manner in vitro, in mice and in a patient with metastatic disease Mol Cancer Ther 3; 1049-1060.

Cen D, Brayton D, Shahandeh B, Meyskens FL, Jr., Farmer PJ. 2004. Disulfiram facilitates intracellular copper uptake and induces apoptosis in human melanoma cells. J Med Chem 47:6914–20.

Chen D, Cui QC, Yang H, Dou QP. 2006. Disulfiram, a clinically used antialcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts via inhibition of the proteasome activity. Cancer Res 66: 10425–10433.

Cramer HI. Method of preparing thiuram disulphides. US Patent: 2014353. September 1935.

Cvek B. 2011. Targeting malignancies with disulfiram (Antabuse): multidrug resistance, angiogenesis, and proteasome. Curr Cancer Drug Targets. 11(3):332-7.

This article is protected by copyright. All rights reserved

Accepted Article

Cvek B, Dvorak Z. 2008. Can the old drug, disulfiram, have a bright new future as a novel proteasome inhibitor? Drug Disc Today 13 (15/16):716-722.

Damelin LH, Jivan R, Veale RB, Rousseau AL, Mavri-Damelin D. Metformin induces an intracellular reductive state that protects oesophageal squamous cell carcinoma cells against cisplatin but not copper-bis(thiosemicarbazones). BMC Cancer 2014: 14:314.

Daniel KG, Gupta P, Harbach RH, Guida WC, Dou QP. 2004. Organic copper complexes as a new class of proteasome inhibitors and apoptosis inducers in human cancer cells. Biochem Pharmacol 67(6):1139-51.

Fanucchi S and Veale RB. 2009. Role of p53/FAK association and p53Ser46 phosphorylation in staurosporine-mediated apoptosis: Wild type versus mutant p53-R175H. FEBS Letters 583(22):3557–3562.

Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Intl J Cancer 127:2893–917.

Filomeni G, Desideri E, Cardaci S, Rotilio G, Ciriolo MR. 2010. Under the ROS…Thiol network is the principal suspect for autophagy commitment. Autophagy 6:7, 999-1005.

Furuta S, Ortiz F, Zhu Sun X, Wu HH, Mason A, Momand J. 2002. Copper uptake is required for pyrrolidine dithiocarbamate-mediated oxidation and protein level increase of p53 in cells. Biochem J 1;365(Pt 3):639-48.

This article is protected by copyright. All rights reserved

Hayat MA. 2000. Principles and techniques of electron microscopy biological applications. Cambridge University Press.

Ilson DH. 2008. Esophageal cancer chemotherapy: recent advances. Gastrointest Cancer Res 2:85–92.

Johansson, B. 1992. A review of the pharmacokinetics and pharmaco- dynamics of disulfiram and its metabolites. Acts Psychiactr Scanda 86:15–26.

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES,H Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nun G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G. 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16, 3–11.

Lin J, Haffner MC, Zhang Y, Lee BH, Brennen WN, Britton J, Kachhap S K, et al. 2011. Disulfiram is a DNA demethylating agent and inhibits prostate cancer cell growth. The Prostate 71(4):333–43.

Logie L, Harthill J, Patel K, Bacon S, Hamilton DL, Macrae K, McDougall G, Wang HH, Xue L, Jiang H, Sakamoto K, Prescott AR, Rena G. 2012. Cellular responses to the metal-binding properties of metformin. Diabetes 61(6):1423-33.

Loo TW, Bartlett MC, Clarke DM. 2004. Disulfiram metabolites permanently inactivate the human multidrug resistance P-glycoprotein. Mol Pharm 1(6):426-33.

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Lovborg H, Oberg F, Rickardson L, Gullbo J, Nygren P, Larsson R. 2006. Inhibition of proteasome activity, nuclear factor-KB translocation and cell survival by the antialcoholism drug disulfiram
Int J Cancer 118, 1577–1580.

Mancias JD, Kimmelman AC. 2011. Targeting autophagy addiction in cancer. Oncotarget 2(12):1302-6.

Molineaux SM. 2012. Molecular pathways: targeting proteasomal protein degradation in cancer. Clin Cancer Res 18(1):15-20.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. 2001. A Novel Response of Cancer Cells to Radiation Involves Autophagy and Formation of Acidic Vesicles Advances in Brief A Novel Response of Cancer Cells to Radiation Involves Autophagy and Formation of Acidic Vesicles. Cancer Res 61, 439–444.

Pan J, Zhang Q, Wang Y, You M. 2010. 26S Proteasome Activity is Down-Regulated in Lung Cancer Stem-Like Cells Propagated In Vitro. PLoS One 5(10), 9 pages.

Pierzyńska-Mach A, Janowski PA, Dobrucki JW. 2014. Evaluation of Acridine Orange, LysoTracker Red, and Quinacrine as Fluorescent Probes for Long-Term Tracking of Acidic Vesicles. Cytometry Part A 85(8):729-37.

Price KA, Crouch PJ, Volitakis I, Paterson BM, Lim SC, Donnelly PS, White AR. 2011. Mechanisms controlling cellular accumulation of copper-bis(thiosemicarbazone) complexes. Inorg Chem 50,9594-9605.

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Quinn BJ, Kitagawa H, Memmott RM, Gills JJ, Dennis PA. 2013. Repositioning metformin for cancer prevention and treatment. Trends Endocrinol Metab 24 (9): 469-480.

Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, Gounon P, Allegra M, Bertolotto C, Bereder J-M, Tartare-Deckert S, Bahadoran P, Auberger P, Ballotti R, Rocchi S. 2011. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. Cell Death and Disease 2, e199.

Topping RJ, Jones MM. 1998. Optimal dithiocarbamate structure for immunomodular action. Med Hypotheses 27:55-57.

Veale RB, Thornley AL. 1989. Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. S Afr J Sci 85:375-379.

Veverka KA, Johnson KL, Mays DC, Lipsky JJ, Naylor S. 1997. Inhibition of aldehyde dehydrogenase by disulfiram and its metabolite methyl diethylthiocarbamoyl-sulfoxide. Biochem Pharmacol 53(4):511–518.

Wang W, McLeod HL, Cassidy J. 2003. Disulfiram-mediated inhibition of NF-kB activity enhances cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines. Int J Cancer 104, 504–511.

Wattenberg LW. 1974. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by several sulfur-containing compounds. J Natl Cancer Inst. 52(5):1583-7.

Yakisich JS, Sidén A, Eneroth P, Cruz M. 2001. Disulfiram is a potent in vitro inhibitor of DNA topoisomerases. Biochem Biophys Res Commun 289(2):586-90.

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Yang ZJ, Chee CE, Huang S, Sinicrope FA. 2011. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 10(9):1533-41.

Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL. 2009. Monitoring autophagy by electron microscopy in mammalian cells. Methods Enzymol 452:143-64.

Zhu M, Lu L, Yang P, Jin X. 2002. Bis (1,1-dimethylbiguanido)copper(II)octahydrate. Acta Cryst E58:m217-m219.

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FIGURE LEGENDS

Fig. 1. Cu-DSF and DSF are highly cytotoxic to OSCC cells and DSF cytotoxicity is enhanced by metformin. 1A. Cells exposed to Cu-DSF and DSF for 48 hours show considerable cytotoxicity across all three OSCC cell lines with a significant leftward shift in toxicity curves for Cu-DSF (n=3, mean±SD). 1B. The addition of 10mM metformin further enhanced DSF cytotoxicity and whilst Cu-DSF was considerably more toxic than DSF alone, the addition of metformin to Cu-DSF did not significantly change cytotoxicity (n=3, mean±SD). 1C. Cell morphology mirrored the observations of the MTT assays.

Fig. 2. Copper is partially responsible for DSF toxicity and metformin causes increased copper uptake by OSCC cells

2A. The role of copper in DSF toxicity was observed by increasing copper concentrations to equimolar molar levels of copper and DSF, which had an increasing effect on cytotoxicity as seen by the significant leftward shift in toxicity curves with increasing copper concentration (n=3, mean±SD). 2B. Measurement of intracellular copper by ICP-MS showed that as expected DSF treated cells had significantly higher copper levels than untreated cells. Interestingly OSCC cells treated with 10 mM metformin had, overall, higher intracellular levels of copper in comparison to untreated controls. The effect of metformin compounded that of DSF, with DSF-metformin co-treated cells displaying higher copper levels than DSF alone (values expressed per µg of protein), (n=3, mean±SD. 1D). Copper content of cells was also assessed in the presence of a cell impermeable copper chelator bathocuproinedisulfonic acid (BCS) to confirm the reduction in intracellular copper, as anticipated. 2C. The contribution of copper to cell cytotoxicity was further assessed by the addition of BCS (100 µm), which partially reversed the cytotoxic effects of DSF. All values are expressed as % change in LD_{50} relative to DSF alone (n=3, mean±SD).

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Fig. 3. DSF inhibits proteasomal function.

3A. OSCC cells treated with LD_{30} concentrations of DSF for 48 hours had higher amounts of total ubiquitinated proteins in comparison to untreated controls. Metformin (Met) treated cells (10 mM for 24 hours) had slightly lower levels, as did cells treated with both DSF and metformin; as expected, chloroquine treated cells showed high levels of total ubiquitinated proteins. 3B. Proteasomal function for chymotrypsin-like activity using the fluorogenic peptide LLVY-AMC showed that, as expected, DSF decreased proteasome function. Interestingly, metformin also had an inhibitory effect with slightly decreased fluorescence exhibited, and when combined with DSF or copper-DSF, proteasome activity was even further diminished. The positive control, Cu-8HQ, caused a considerable reduction in fluorescence as expected, (n=3, mean±SD). 3C. DPTD and TTD, two analogues of DSF that have lower rates of decomposition and thiol exchange, inhibited proteasome function more effectively in comparison to disulfiram. 3D. DPTD and TTD displayed lower levels of OSCC cell toxicity in comparison to their respective controls (n=3, mean±SD) (all data shown for WHCO1 cells).

Fig. 4. DSF decreases AVO (lysosomal) acidification.

Lysosomal acidification as visualised by AO staining shows a shift from red (low pH) to yellow to green (higher pH). DSF treated and metformin-DSF (DSF + Met) co-treated cells clearly had yellow lysosomes, in comparison the red lysosomes in untreated cells, clearly indicating an increase in pH of these organelles upon DSF treatment. Cu-DSF and metformin-Cu-DSF (Cu-DSF + Met) treatment was highly cytotoxic to cells. Cells treated with the known lysosomal inhibitor chloroquine (CQ) (1 mM for 1 hour) as the positive control, had only green lysosomes, as expected. The DSF analogues DPTD and TTD did not show the same extent of perturbation of lysosomal acidification as DSF.

Fig. 5. DSF inhibits autophagy.

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5A. Electron microscopy showed a small increase in the numbers of autophagosomes and autolysosomes in metformin (Met) only treated cells. DSF only treated cells also exhibited increased numbers of autophagosomes, in comparison to untreated control. Interestingly, metformin-DSF (DSF+Met) co-treated cells, showed a considerable accumulation of autophagosomes (single arrow) with protein aggregates and far fewer cleared autolysosomes (double arrow). Cu-DSF and metformin-CU-DSF (Cu-DSF+Met) treated cells show considerable vacuolization (dashed arrow). 5B. Autophagy by LC3B western blot showed that metformin (Met) alone did not significantly alter autophagy but that DSF alone treated cells had slightly higher LC3B-II levels relative to β-actin in comparison to untreated controls (Un), but that for metformin-DSF (DM), Cu-DSF (CuD) and metformin-CuDSF (CuDM) treated cells this increase in LC3B-II was significant. The positive control chloroquine (CQ) displayed a large proportion of the LC3B-II form as expected.

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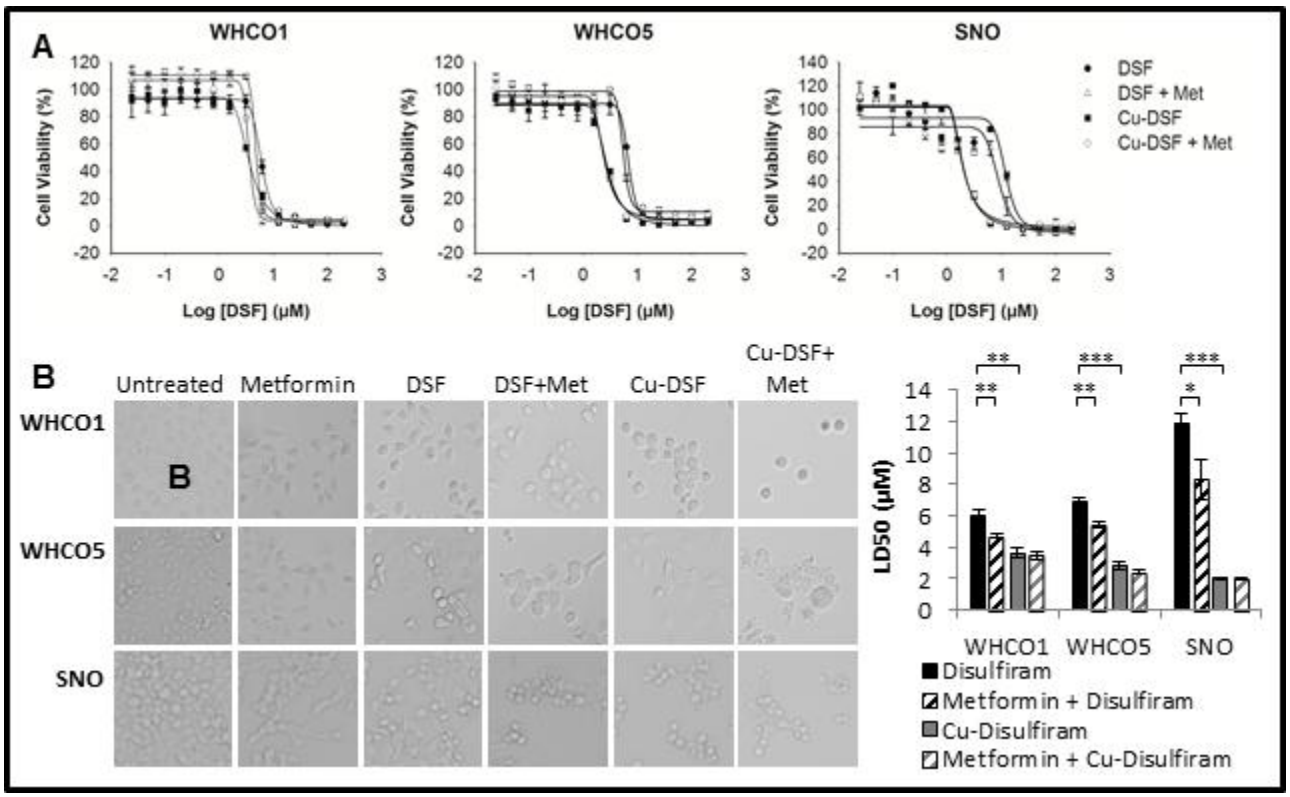


Figure 1

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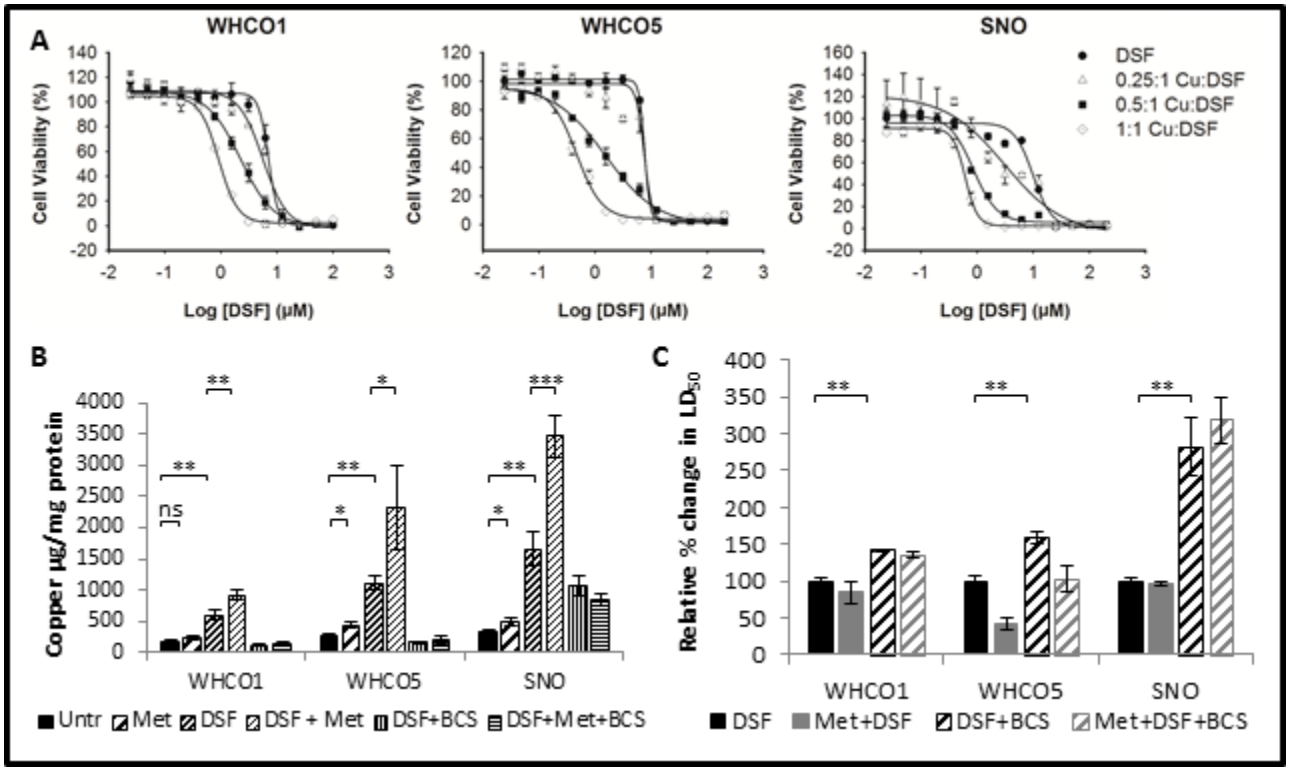


Figure 2

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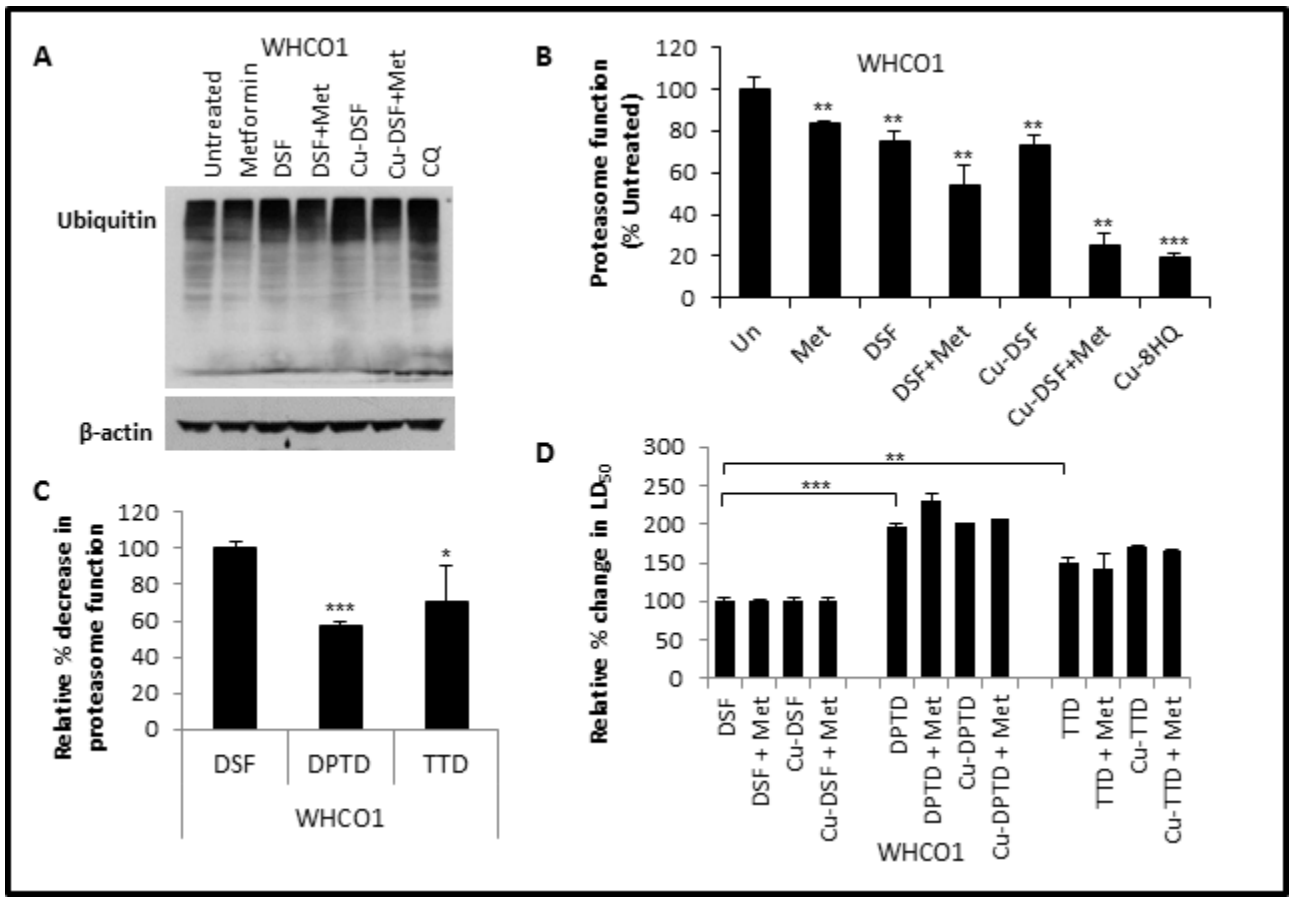


Figure 3

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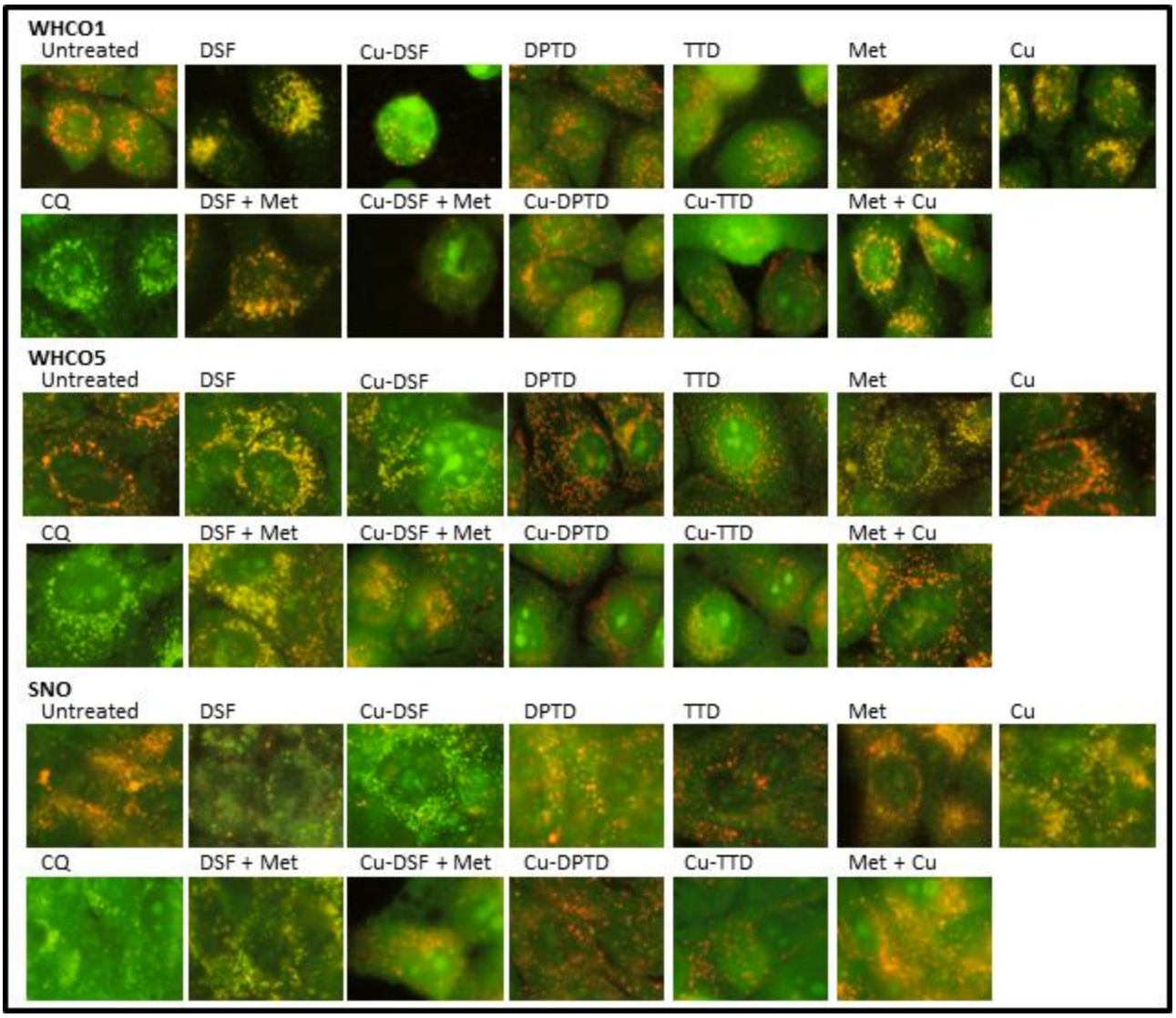


Figure 4

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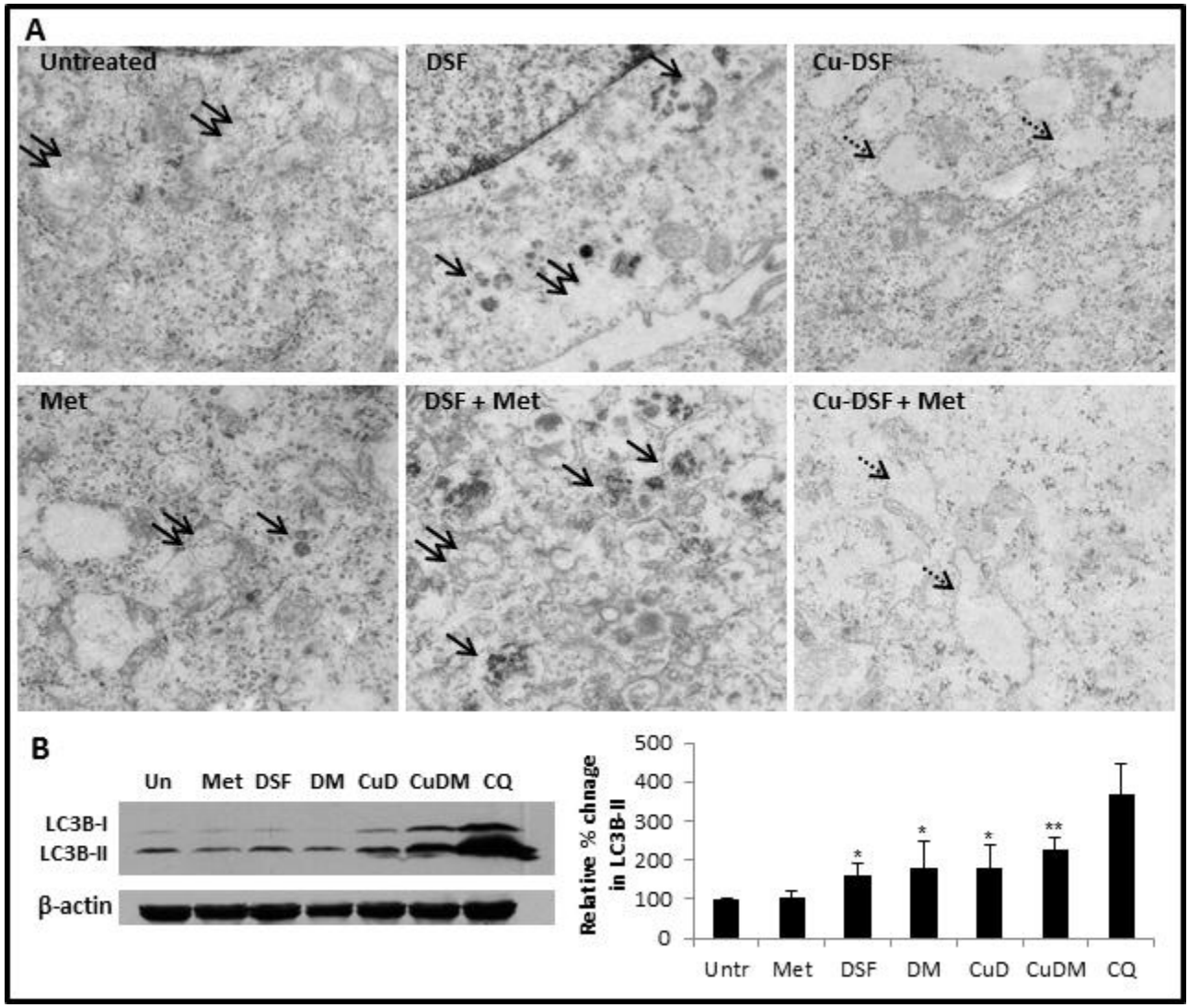


Figure 5

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