Targeting Antioxidant Systems to Treat Pancreatic Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine & Pathobiology
University of Toronto

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Abstract

Pancreatic cancer has the worst prognosis of all cancers. Pancreatic tumours are characterized by excessive oxidative stress that may provide a unique vulnerability to target in the development of new therapeutic strategies. I investigated a novel combination therapy targeting the two main intracellular antioxidant systems, glutathione and thioredoxin. Buthionine sulfoximine (BSO) targets glutathione synthesis and auranofin targets thioredoxin recycling. These drugs have been tested in human clinical trials and are strong candidates for repurposing in cancer therapy. I found that BSO potentiated auranofin’s cytotoxic effects \textit{in vitro}. Furthermore, the combination augmented oxidative stress in cells. Auranofin engaged its target TrxR \textit{in vitro} at concentrations causing cell death. I also assessed the biodistribution of auranofin \textit{in vivo} and observed a preferential accumulation in the stroma 24 hours after treatment. Overall, I conclude that this treatment strategy can be effective against pancreatic cancer and deserves attention for future development.
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<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signaling kinase 1</td>
</tr>
<tr>
<td>Bax</td>
<td>bcl-2-like protein 4</td>
</tr>
<tr>
<td>BCNU</td>
<td>carmustine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>cellular FLICE-inhibitory protein</td>
</tr>
<tr>
<td>CETSA</td>
<td>cellular thermal shift assay</td>
</tr>
<tr>
<td>DCF</td>
<td>2’,7’-dichlorofluorescein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed, paraffin-embedded</td>
</tr>
<tr>
<td>G6PD</td>
<td>glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCL</td>
<td>glutamate-cysteine ligase</td>
</tr>
<tr>
<td>GCLC</td>
<td>glutamate-cysteine ligase catalytic subunit</td>
</tr>
<tr>
<td>GCLM</td>
<td>glutamate-cysteine ligase modifier subunit</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyltranspeptidase</td>
</tr>
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</table>
GLUD1  glutamate dehydrogenase
GOT1  aspartate transaminase
GPx  glutathione peroxidase
GR  glutathione reductase
Grx  glutaredoxin
GS  glutathione synthetase
GSH  glutathione
GSSG  oxidized glutathione
GST  glutathione S-transferase
HNE  4-hydroxynonenal
IKK-β  inhibitor of nuclear factor kappa-B kinase subunit beta
IMC  imaging mass cytometry
Keap1  kelch-like ECH-associated protein 1
mBBr  monobromobimane
MDH1  malate dehydrogenase
ME1  malic enzyme 1
MMP  mitochondrial membrane potential
Msr  methionine sulfoxide reductase
NADPH  nicotinamide adenine dinucleotide phosphate
Nrf2  nuclear factor, erythroid derived 2, like 2
P-gp  multidrug resistance protein 1
PanIN  pancreatic intraepithelial neoplasia
PBS  phosphate-buffered saline
PD-L1  programmed death ligand 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>PDX</td>
<td>patient-derived xenograft</td>
</tr>
<tr>
<td>PEGPH20</td>
<td>pegvorhyaluronidase alfa</td>
</tr>
<tr>
<td>PHGDH</td>
<td>phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPP</td>
<td>pentose phosphate pathway</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>System X&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cystine/glutamate antiporter system</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>vascular endothelial growth factor A</td>
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1.1 Pancreatic Cancer

Pancreatic cancer has the worst prognosis amongst all cancers; 5 year survival rates for the disease range from less than 5 to 8%\textsuperscript{1-3}. Although it is only the twelfth most commonly diagnosed, it is the fourth most common cause of cancer death in Canada\textsuperscript{2}. Unfortunately, mortality from pancreatic cancer has not improved during the era of modern medicine\textsuperscript{4,5}. Estimates predict that pancreatic cancer will become the second most common cause of cancer death behind non-small-cell lung cancer by 2030\textsuperscript{6}. The dismal outlook for patients with pancreatic cancer can be partially attributed to the lack of progress made in the development of diagnostics and therapeutics, which have remained stagnant over the last 50 years\textsuperscript{7}. During this time vast improvements have been made for the diagnosis and treatment of other tumour types. For example, the development of the Pap smear can reduce cervical cancer death by up to 80\%\textsuperscript{8}, and the development of trastuzumab has increased overall survival by 33\% in HER2+ breast cancers\textsuperscript{9,10}. The identification of new biomarkers and treatment strategies is required to overcome pancreatic cancer.

Early stage pancreatic cancer is generally asymptomatic, leading to difficulties in detection. The disease may be identified secondary to non-specific symptoms, or as an incidental finding. In addition to this, screening tests have not been developed. Currently the only treatment that offers the potential for long-term survival is surgery in early-stage disease, however the 5 year survival rate in patients with resectable tumours remains only 15-20\%\textsuperscript{11}. For patients with advanced metastatic disease there are few systemic options that offer little benefit in survival.
1.1.1 Pancreatic Cancer Therapy Development

The first systemic therapy used in the treatment of pancreatic cancer was 5-fluorouracil (5-FU). 5-FU is a non-specific agent that inhibits the synthesis of the nucleoside thymidine. 5-FU provided a 4 month survival benefit when used in combination with radiotherapy, compared to radiotherapy alone\textsuperscript{12}. Since the introduction of 5-FU in pancreatic cancer clinics some small advancements have been made. Gemcitabine became the new standard in the late 1990s when a clinical trial showed it offered a significant improvement in median overall survival when compared to 5-FU (5.6 months vs. 4.4 months)\textsuperscript{13}. However, gemcitabine is another non-specific agent. It is a nucleoside analog that replaces cytidine during DNA replication. Since the introduction of gemcitabine in the treatment of pancreatic cancer, two therapies have been developed for clinical use. The combination therapy FOLFIRINOX (folinic acid, 5-FU, irinotecan, and oxaliplatin) improved median overall survival compared to gemcitabine in a clinical trial (11.1 months vs. 6.8 months)\textsuperscript{14}, and the combination of nab-paclitaxel with gemcitabine improved median overall survival compared to gemcitabine alone (8.5 months vs. 6.7 months)\textsuperscript{15}. Although there appears to be a survival difference between FOLFIRINOX and gemcitabine/nab-paclitaxel, the FOLFIRINOX regimen is more toxic and the patient population was skewed slightly in favour of the FOLFIRINOX cohort, leading many investigators to consider the two regimens to be similar\textsuperscript{16–18}. Although these therapies are an improvement on past therapies, median survival falls short of one year with either regimen.

Great interest has centred on the development of targeted therapies to provide effective non-toxic treatment for pancreatic cancer. There have been many proposed targets and clinical trials\textsuperscript{19}, however no such therapy has entered clinical practice in Canada to date. Trials have examined agents such as bevacizumab, inhibiting angiogenesis by targeting vascular endothelial growth
factor A (VEGF-A), or cetuximab, inhibiting epidermal growth factor receptor (EGFR), in combination with gemcitabine to no benefit\textsuperscript{20,21}, among many other attempts. From the trials, only erlotinib in combination with gemcitabine has shown a significant effect on overall survival (6.2 months vs. 5.9 months), although this is not clinically relevant\textsuperscript{22}. In summary, current attempts at targeted therapy have proven ineffective in the management of pancreatic cancer. This may change in the future, however current prospects seem bleak and new biomarkers may be required to develop these therapies further\textsuperscript{23}.

Beyond targeted therapy there have been attempts to develop immunotherapies for pancreatic cancer. Immunotherapy is a treatment approach that relies on augmenting the host’s immune response against cancer\textsuperscript{24}. This method derives from the observation that the immune system can eliminate malignant cells during the early stages of transformation\textsuperscript{25}. While there have been many unequivocal successes in other tumour types, particularly in advanced melanoma\textsuperscript{26–28}, pancreatic cancer has proven resistant to these approaches. Clinical trials have observed a lack of response to immune checkpoint blockade with ipilimumab\textsuperscript{29} and programmed death ligand 1 (PD-L1) directed monoclonal antibodies\textsuperscript{30}. These failures have resulted from the poorly immunogenic and immunosuppressive nature of the pancreatic cancer microenvironment\textsuperscript{7,31}. A small number of patients enrolled in these trials have received some therapeutic benefit, leading to continued interest in developing new immunotherapy strategies. One such strategy includes attempting to achieve a synergistic response through the use of multiple agents. This may include combining immune checkpoint blockade, inhibition of immunosuppressive signalling, stimulation of T cells, and/or conventional cytotoxic therapies\textsuperscript{32}.

In relation to immunotherapy, other strategies attempt to target additional features of the tumour microenvironment. Attention has focused on the role of the stroma in determining therapy
response. These tumours are characterized by a dense desmoplastic reaction comprised of fibrotic tissue, extracellular matrix proteins, pancreatic stellate cells, immune cells, and blood vessels. The extensiveness of desmoplasia correlates with worse disease outcome. Furthermore, desmoplasia limits tumour vascularization, creating a barrier to drug uptake by tumours. A clinical trial using the modified hyaluronidase molecule pegvorhyaluronidase alfa (PEGPH20) to degrade stromal hyaluronan in combination with nab-paclitaxel and gemcitabine has shown improvement in progression free survival by the perceived mechanism of increasing tumour vasculature to aid drug delivery. However, there is evidence to suggest that the stroma may have a role in protection against tumour progression and an increased tumour vasculature may contribute to accelerated growth if not targeted properly. Therefore, there is a difficult balance to achieve via this strategy. Clinical trials have also given attention to hypoxia, which is a feature of solid tumours that can result from the lack of vasculature in the stroma.

Hypoxia is a tumour microenvironment state where oxygen utilization exceeds its supply. Traditionally, pancreatic tumours have been considered to be characterized by extensive hypoxia, which may be caused by the lack of blood supply resulting from the desmoplastic reaction. In patient-derived xenograft (PDX) mouse models, hypoxia correlates with aggressive growth and metastasis, making it a logical choice to target. The prodrug evofosfamide is activated under hypoxic conditions to release a DNA alkylating agent that inhibits cancer cell replication. However, a phase III clinical trial observed no significant improvement in median overall survival using evofosfamide/gemcitabine compared to gemcitabine alone (8.7 months vs. 7.6 months). It is possible that hypoxia in patient tumours is more variable than previously believed, which would limit the effectiveness of therapy across the entire patient population. This can be addressed by non-invasive imaging using positron-emitting hypoxia tracers in order to stratify patients towards hypoxia-targeting clinical trials.
In summary, the most effective systemic therapies available for metastatic pancreatic cancer provide only a small benefit to patients. Although there are promising avenues to explore further, attempts to develop new strategies against pancreatic cancer have fallen short of expectations. Further studies are required to identify and examine novel, exploitable targets in pancreatic cancer.

1.1.2 Pancreatic Carcinogenesis

Four driver genes are frequently mutated in pancreatic cancer. These are the oncogene \textit{KRAS} and the tumour suppressors \textit{CDKN2A}, \textit{SMAD4}, and \textit{TP53}\textsuperscript{41–44}. These mutations are shared by pancreatic intraepithelial neoplasia (PanIN), which is a precursor lesion to pancreatic cancer. Sequencing of these genes in PanINs suggests a stepwise progression of pancreatic cancer. Activation of \textit{KRAS} is present in the earliest PanIN-1 lesions, making it the initiating event in pancreatic cancer tumorigenesis. Inactivation of \textit{CDKN2A} is found in PanIN-2, followed by inactivation of \textit{SMAD4} and \textit{TP53} in PanIN-3 lesions\textsuperscript{45}. However, more recent whole-genome sequencing of tumour cell-enriched primaries showed cases that displayed either simultaneous inactivation of multiple drivers or tumours that did not follow the predicted mutation sequence. These data are consistent with punctuated rather than gradual evolution\textsuperscript{46}. Regardless, \textit{KRAS} activation occurs in more than 90\% of human pancreatic cancer and its effects likely have the greatest impact on pancreatic cancer\textsuperscript{41}. Interestingly, \textit{KRAS} has been implicated in mediating pancreatic carcinogenesis in part through its impact on oxidative stress. Its most important effect on oxidative stress is the promotion of low intracellular reactive oxygen species (ROS) levels by inducing nuclear factor, erythroid derived 2, like 2 (Nrf2) activation\textsuperscript{47}. This aspect will be discussed further in the next section.
1.2 Oxidative Stress

Pancreatic cancer, among other cancers, is characterized by excessive oxidative stress. Oxidative stress is caused by the overproduction of ROS, leading to damage of lipids, proteins, and DNA. It occurs when there is an imbalance between intracellular ROS and antioxidants in favour of the oxidants. ROS are produced at an extreme rate due to the high metabolism inherent in transformed cells, and may promote aggressive tumour features. Oxidative stress and ROS represent an interesting target in pancreatic cancer because ROS exhibit dual roles; lower concentrations of ROS can stimulate cancer cell proliferation, whereas higher concentrations of ROS are damaging to cells. Cancer cells adapt during oxidative stress conditions to maintain low ROS levels to avoid cell death and promote proliferation by enhancing antioxidant defences.

ROS exist as multiple species including superoxide (O$_2^•$), hydroxyl radical (HO$^•$), and hydrogen peroxide (H$_2$O$_2$). They are produced in various subcellular compartments: the cytoplasm, the mitochondria, peroxisomes, and the endoplasmic reticulum. The major source of cytoplasmic ROS in pancreatic cancer is NADPH oxidase, which produces superoxide anion by facilitating the reduction of molecular oxygen by NADPH. Mitochondrial ROS are produced in dysfunctional mitochondria that result from inefficient hypermetabolism in rapidly proliferating cancer cells. This can cause leakage of electrons at complex I and complex III of the mitochondrial electron transport chain, leading to the production of superoxide and its dismutation to hydrogen peroxide by superoxide dismutases. Peroxisomal ROS are produced during the $\beta$-oxidation of fatty acids. Lastly, endoplasmic reticulum can contribute to ROS production through its role in providing an oxidizing environment to promote disulfide bonding and protein folding. Although ROS themselves are short-lived molecules, they can generate longer lasting lipid peroxides such as 4-hydroxynonenal (HNE). HNE is capable of reacting with cellular macromolecules such as
proteins, lipids, and DNA. In addition, HNE can stimulate signaling cascades to affect cell proliferation and differentiation\textsuperscript{61}. As such, it has been implicated in different cancers.

1.2.1 Hypoxia

Hypoxic tumours experience both chronic and acute hypoxia. Chronic hypoxia exists in regions located away from blood vessels and is limited by the diffusion of oxygen through the dense tumour stroma. Acute or cycling hypoxia exists in regions proximal to blood vessels and is perfusion-limited in that there are temporal instabilities in blood flow through a tumour. Cycling hypoxia generates greater oxidative stress because the acute influx of oxygen can contribute to an increase in ROS\textsuperscript{62}. In pancreatic xenografts hypoxia is associated with aggressive growth and metastasis\textsuperscript{37}. However, human pancreatic tumours may not possess extensive hypoxia as previously suggested\textsuperscript{40}. Although hypoxia stems from a lack of oxygen, hypoxic tumours have been stated to overproduce ROS\textsuperscript{54}. However, direct evidence suggesting this phenomenon is lacking. Hypoxia can contribute to resistance to chemo and radiotherapy, which rely on ROS to mediate their effects\textsuperscript{63}. This evidence contradicts the idea hypoxia of greater oxidative stress during hypoxia.

1.2.2 The Impact of High ROS Levels

At high levels ROS disrupt cellular processes by non-specifically damaging proteins, lipids, and DNA. Furthermore, ROS are implicated in both the extrinsic (death receptor-dependent) and intrinsic (mitochondrial) pathways of apoptosis. The extrinsic pathway is mediated by death-inducing ligands, such as TNF-related apoptosis-inducing ligand (TRAIL)\textsuperscript{64}. Following ligand-receptor interaction the caspase cascade is activated, leading to apoptosis\textsuperscript{65}. ROS negatively regulate cellular FLICE-inhibitory protein (c-FLIP) by a post-transcriptional method to target it for degradation and activate the extrinsic apoptosis pathway. The intrinsic pathway is mediated by
a permeability transition in the mitochondria. Mitochondrial stress, including oxidative stress, stimulates pore formation in the inner mitochondrial membrane which causes cytochrome-c mobilization and mitochondrial swelling\textsuperscript{66}. This can lead to rupture of the outer membrane, and with the help of pro or anti-apoptotic Bcl-2 family member proteins can stimulate or prevent apoptosis. Bcl-2-like protein 4 (Bax) in particular mediates the formation of the mitochondrial apoptosis-induced channel (MAC) to release mitochondrial pro-apoptotic factors, such as cytochrome-c, which leads to activation of the executioner caspase proteins\textsuperscript{67–69}. As mitochondria are a significant source of ROS production, the intrinsic pathway is the largest contributor to ROS-mediated apoptosis. In this pathway, electron transport chain-produced superoxide triggers eventual cytochrome-c release, leading to the induction of apoptosis\textsuperscript{70}. Apoptosis following oxidative stress displays several distinct steps that can be measured by flow cytometry methods. Following glutathione (GSH) depletion in cells, ROS production causes the mitochondrial permeability transition and the subsequent loss of mitochondrial membrane potential (MMP), due to cellular damage. Finally, cells lose viability after perforation of the cell membrane\textsuperscript{71}.

In addition to apoptosis, ROS contribute to ferroptosis, a form of cell death that depends on intracellular iron\textsuperscript{72}. NADPH oxidase-induced accumulation of ROS was identified to contribute to ferroptosis. Furthermore, the compounds erastin and sulfasalazine, which inhibit the cystine/glutamate antiporter system (system X\textsubscript{C}^-), lead to toxic accumulation of ROS and ferroptosis\textsuperscript{72,73}. The lethal effect can be reversed by addition of the lipophilic antioxidants ferrostatin-1 and vitamin E, suggesting an important role for lipid radicals in mediating ferroptosis\textsuperscript{72,74}. \textit{KRAS} driven cancer cells have been shown to be susceptible to ROS-mediated ferroptosis following treatment with lanperisone, an FDA-approved muscle relaxant\textsuperscript{75}, suggesting that ROS can be utilized to induce ferroptosis in a \textit{KRAS} driven tumour such as pancreatic cancer.
High ROS also have indirect effects on cell death by sensitizing cancer cells to chemo and radiotherapy. The multidrug resistance protein 1 (P-gp) facilitates the removal of several anticancer agents from cells. High ROS concentrations have been associated with decreased P-gp expression, which allows for cytotoxic drugs to accumulate within a cell\textsuperscript{76}. Furthermore, ROS have been identified to mediate the effects of some cytotoxic chemotherapies. For example, gemcitabine acts through a ROS-dependent mechanism that may promote metastasis due to the action of antioxidants\textsuperscript{77}. This feature may contribute to gemcitabine’s limited effectiveness in pancreatic cancer. Moreover, both 5-FU and oxaliplatin have been observed to augment oxidative stress in patients with colon cancer\textsuperscript{78}. These drugs are two of the components of the combination FOLFIRINOX, which is one of the most effective systemic therapies against metastatic pancreatic cancer. Paclitaxel, which is the main component of the nab-paclitaxel/gemcitabine therapy for pancreatic cancer, and doxorubicin also enhance oxidative stress\textsuperscript{79,80}. While augmented oxidative stress is not the primary mechanism of action of these therapies, it is interesting to note that the most effective therapies in clinical use for pancreatic cancer elicit an effect on the redox environment of the tumour.

In addition to chemosensitization, high ROS levels are involved in mediating the cytotoxic effects of radiotherapy. Radiotherapy utilizes ionizing radiation to generate ROS, particularly hydroxyl radical. Hydroxyl radicals enact the therapeutic and side effects of radiotherapy. It propagates by reacting with other molecules including DNA, leading to abundant DNA lesions and subsequent cell death\textsuperscript{81}. It has been observed that radical scavengers are effective at attenuating the cytotoxic effects of radiation, indicating that ROS are required\textsuperscript{82–84}. The evidence presenting the role of ROS in chemo and radiosensitization suggests that exploiting an increase in ROS can be effective in the development of new therapies.
Cancer cells require multiple adaptations to protect from inherently high ROS levels allowing them to survive and proliferate. Signaling through the oxidative stress response mediates this adaptation. The master regulator of this response is the transcription factor Nrf2. Nrf2’s activity is modulated by its repressor Kelch-like ECH-associated protein 1 (Keap1). In a normal redox state Nrf2 is constitutively degraded. Keap1 promotes its ubiquitination by Cullin E3 ubiquitin ligase to label Nrf2 for proteasome degradation. During oxidative stress conditions, redox-sensitive cysteine residues on Keap1 are oxidized by ROS. In this state Keap1 is unable to bind Nrf2 to recruit ubiquitin ligases to target it for degradation. Nrf2 subsequently translocates to the nucleus where it heterodimerizes with small Maf proteins to stimulate transcription of genes associated with antioxidant response element (ARE) promoters. The lipid peroxide HNE is a potent inducer of Nrf2 by covalent binding to Keap1 redox-sensitive cysteines and by activating upstream kinases such as protein kinase C. It has been observed that nuclear Nrf2 expression associates with poor survival in pancreatic cancer, whereas cytoplasmic Keap1 expression associates with reduced metastasis and membrane-associated Keap1 displays a protective effect on survival.

1.2.3 Adaptations to High ROS Levels

Nrf2 and ARE positively regulate the transcription of several cytoprotective genes including drug-metabolizing enzymes, drug transporters, and antioxidants. Nrf2 regulates the expression of many phase I and II drug-metabolizing enzymes, as well as members of the multi-drug resistance-associated gene family of multidrug transporters. It can indirectly modulate ROS levels by regulating free Fe (II) homeostasis. This prevents the Fenton reaction, where Fe(II) catalyses the oxidation of hydrogen peroxide to the more reactive hydroxyl radical. This function occurs by promoting the release of Fe (II) from heme, and its subsequent sequestration. However, Nrf2’s most important role is functioning as the master regulator of antioxidants. The production
and regeneration of the most abundant antioxidant cofactor, GSH, is controlled exclusively by Nrf2\textsuperscript{97}. In addition, Nrf2 supports the expression of enzymes involved in GSH utilization\textsuperscript{98}, as well as enzymes involved in the production, regeneration, and utilization of thioredoxin (Trx)\textsuperscript{99}. These actions of Nrf2 are responsible for direct modulation of oxidative stress. These antioxidants will be discussed in further detail in section 1.3. In parallel to antioxidant production, cancer cells require the redirection of cellular metabolism to support antioxidant function.

Cancer cell metabolism differs from that of normal cells and requires abundant ATP production to support rapid proliferation. Cancer cells experience a metabolic switch to rely on aerobic glycolysis, rather than oxidative phosphorylation, to supplement their needs. This phenomenon is termed the Warburg effect\textsuperscript{100,101}. Constitutively active KRAS in pancreatic cancer plays a key role in activating the metabolic switch towards supporting anabolic pathways\textsuperscript{102}. In addition to this switch, Nrf2 reprograms metabolism in oxidative stress conditions to support the antioxidants it promotes. It reprograms metabolism by increasing transcription of NADPH-generating enzymes to modulate glucose, glutamine, and serine metabolism\textsuperscript{103,104}. NADPH is an important cofactor that is required by antioxidant enzymes to supply reducing potential. Nrf2 promotes a proliferative phenotype through the PI3K-Akt axis, which redirects glucose into anabolic pathways to increase metabolism\textsuperscript{103}. Three major pathways that produce NADPH are regulated by Nrf2: the pentose phosphate pathway (PPP), the serine synthesis and folate pathway, and the malic enzyme pathway of glutamine metabolism.

The PPP is a metabolic pathway that runs in parallel to glycolysis. It consists of oxidative and non-oxidative phases. The oxidative arm is responsible for NADPH production, while the non-oxidative arm is responsible for the generation of ribonucleotides\textsuperscript{105}. Both phases are critical to cancer cell survival. Glucose 6-phosphate dehydrogenase (G6PD) is the first enzyme in the
oxidative arm of the PPP and is positively regulated by Nrf2\textsuperscript{93}. G6PD commits glucose 6-phosphate to the PPP, rather than glycolysis\textsuperscript{105}. The PPP has been observed to promote cell survival and proliferation during neoplastic transformation\textsuperscript{106}. Although the oxidative phase has generally been implicated to supply cancers with NADPH, constitutively active \textit{KRAS} has been observed to uncouple ribose biogenesis from NADPH production in pancreatic cancer\textsuperscript{107}. Glycolytic intermediates are utilized specifically in the non-oxidative arm of the PPP, but not the oxidative arm. Inhibition of the PPP by knockdown of G6PD, as well as glucose deprivation, in pancreatic cancer cells led to minimal effect on ROS, indicating that the oxidative arm is not required to maintain ROS levels in pancreatic cancer\textsuperscript{108}. ROS could be affected, however, by modulating the other two NADPH production pathways.

The serine synthesis pathway is another glycolytic shunt responsible for NADPH production. In this pathway the glycolytic intermediate 3-phosphoglycerate is converted to serine via three reactions. The first committed step is catalyzed by phosphoglycerate dehydrogenase (PHGDH). After its production serine can be used in the folate cycle to generate glycine and NADPH\textsuperscript{109}. The transcription of the enzymes involved in this process is regulated by Nrf2\textsuperscript{104}. Overexpression of PHGDH has been reported in non-small cell lung, colorectal, breast, and pancreatic cancers\textsuperscript{104,110–112}. In pancreatic cancer, PHGDH has been described as an independent prognostic indicator for patients; increased expression is associated with poor prognosis. Furthermore, PHGDH promotes proliferation, migration, and invasion of pancreatic cancer cells \textit{in vitro}\textsuperscript{112}. Reduced levels of serine hydroxymethyltransferase, the enzyme responsible for preparing serine for the folate cycle, were responsible for decreasing NADPH while increasing ROS\textsuperscript{113}. Therefore, Nrf2 promotes the serine synthesis and folate pathway to produce NADPH, which allows the cancer cells to adapt to oxidative stress and may contribute to worse outcomes in pancreatic cancer.
Finally, glutamine metabolism through the malic enzyme (ME1) pathway is another source of NADPH production that may be the most important in pancreatic cancer\(^{108}\). ME1 is positively regulated by Nrf2\(^{99}\). Cancer cells exhibit glutamine addiction because of its essential canonical roles in supplying intermediates for the Kreb’s cycle through anaplerosis, and its roles in nucleotide, amino acid, and lipid biosynthesis\(^{114-116}\). Most cells utilize the enzyme glutamate dehydrogenase (GLUD1) to convert glutamine-derived glutamate into \(\alpha\)-ketoglutarate to fuel the Krebs cycle, however constitutively active KRAS in pancreatic cancer downregulates GLUD1 and upregulates aspartate transaminase (GOT1) to shunt glutamine into the non-canonical ME1 pathway for NADPH production\(^{108}\). In this pathway glutamine-derived aspartate is converted to oxaloacetate via GOT1 in the cytoplasm, and then oxaloacetate is metabolized by malate dehydrogenase (MDH1) into malate. The final step involves the oxidation of malate to pyruvate by ME1. This reaction is responsible for NADPH regeneration and assists in protecting from damage during oxidative stress\(^{117}\). Confirming this, glutamine deprivation and ME1 knockdown led to elevated ROS and decreased clonogenicity in pancreatic cancer cells. The cells could be rescued with supplemented oxaloacetate, malate, or GSH, indicating that glutamine is required for redox homeostasis in pancreatic cancer\(^{108}\). Further demonstrating the importance of glutamine metabolism in pancreatic cancer, ME1 pathway inhibition diminished tumour xenograft growth\(^{108}\).

### 1.2.4 The Impact of Low ROS Levels

The adaptations controlled by Nrf2 maintain ROS at low to moderate levels (ie. above background levels but below cytotoxic levels) to prevent cell death. These low ROS levels are implicated in stem cell phenotype and in tumour cell proliferation and act as important signaling molecules capable of regulating various functions including proliferation, apoptosis, and tumour cell invasion\(^{48}\). Low ROS levels are associated with maintaining cancer stem cell-like properties in
cells that confers therapy resistance\textsuperscript{118}. For example, human breast cancer stem cells possess robust ROS scavenging systems that maintain low ROS levels\textsuperscript{84}. This feature prevents ROS-induced DNA damage following ionizing radiation, protecting the cells from death. Furthermore, pharmacologic depletion of antioxidants results in loss of stem cell properties\textsuperscript{84}.

Low ROS are involved in signal transduction pathways to regulate various cellular responses. This function is made possible because of ROS-mediated inactivation of protein-tyrosine phosphatases, which allows for increased signaling through receptor tyrosine kinases\textsuperscript{119}. As such, ROS activate signaling of the MAPK pathway by activating p38 MAPK to promote tumour cell differentiation and proliferation\textsuperscript{120}. In addition, ROS can inactivate PI3K/Akt phosphatases like PTEN. This stimulates signaling of the PI3K pathway to promote proliferation and apoptosis evasion\textsuperscript{55,121}. Proliferation promotion by ROS has been observed in various cancer cell types\textsuperscript{122,123}. These functions implicate ROS in carcinogenesis. Moreover, ROS may contribute to the promotion of invasion and metastasis. Tumour cell invasion and metastasis requires cell mobility through the extracellular matrix (ECM). Cells degrade glycosaminoglycan in the ECM to allow for motility. ROS-dependent degradation of the ECM occurs due to the stimulation of matrix metalloproteinase activity, expression, and secretion\textsuperscript{124}. ROS-activated MAPK pathways contribute to the expression of matrix metalloproteinases. Specifically, extracellular signal-regulated kinase (ERK) activation by ROS promotes invasion and metastasis\textsuperscript{125}. The metastatic phenotype is enabled and sustained by concomitant ROS production and the ability to protect from the cytotoxicity of oxidative stress with antioxidants to maintain low ROS levels\textsuperscript{126}. These functions of low ROS levels allow for tumour growth and spread that may contribute to worse prognosis in pancreatic cancer.

Taken together this information indicates that low ROS levels contribute to many of the tumorigenic properties of cancer cells. Cancer cells utilize antioxidants to protect themselves from
ROS and maintain these low ROS levels. These antioxidants are regulated by the master regulator Nrf2 during the oxidative stress response. ROS scavenging prevents ROS-induced toxicity and assists in ROS-mediated proliferation. Strategies designed to exacerbate ROS through targeted inhibition of antioxidants may provide a relatively non-toxic therapy, as cancer cells are reliant on antioxidants whereas normal cells can survive change. This strategy has been employed using various drugs against a number of targets to great success\textsuperscript{75,127,128}.

### 1.3 The Role of Antioxidants

The most important factor in the cell that moderates ROS levels are the antioxidants regulated by Nrf2\textsuperscript{129}. The role of antioxidants and Nrf2 in cancer is controversial as studies suggest them to be either tumour suppressive or oncogenic. The answer to this controversy is likely to be that the role of antioxidants is context specific\textsuperscript{91,130}. Nrf2-knockout mice are more susceptible to carcinogenesis and show diminished efficacy of chemopreventive enzymes\textsuperscript{131}. Furthermore, the natural compound sulforaphane activates Nrf2 and was found in a phase II clinical trial to protect against hepatocellular carcinoma by preventing DNA adduct formation\textsuperscript{132}. Although these studies suggest that the antioxidant response mediated by Nrf2 is required for chemoprevention, most clinical trials suggest that dietary antioxidants do not provide this effect\textsuperscript{133,134}, and may even increase cancer risk\textsuperscript{135,136}.

Nrf2 may promote tumorigenesis by protecting cells from oxidative stress and maintaining low ROS. Oncogenes such as KRAS in pancreatic cancer increase Nrf2 to provide cytoprotection and decrease ROS levels\textsuperscript{47}. Loss-of-function mutations in KEAP1, resulting in constitutive Nrf2 activation, have been identified in various solid tumours\textsuperscript{137}. Lastly, high Nrf2 expression has been correlated with resistance to platinum-based chemotherapy in ovarian cancer\textsuperscript{138}. 

These contrasting sets of data suggest that the role of Nrf2 may depend on the stage of tumorigenesis. Before the cancer development, Nrf2 and antioxidants may help to suppress cancer by the prevention of mutations and oxidative stress. Once the cancer develops, Nrf2 and the antioxidants it regulates may promote cancer progression\textsuperscript{91,130}. Therefore, once a tumour has been developed, antioxidants may prove to be an important target for cancer therapy. As previously discussed, the GSH and Trx systems are both coordinated by Nrf2. These are likely two of the main factors contributing to tumour cytoprotection. Not only is the production of GSH and Trx controlled by Nrf2, but also the production of the enzymes that recycle and utilize them to evoke their effects.

1.3.1 The Glutathione Antioxidant System

GSH is the most abundant non-protein thiol in cells and is critical to providing protection from oxidative stress\textsuperscript{139}. GSH exists in two forms: reduced GSH, or oxidized glutathione (GSSG) which is a dimer. A number of enzymes responsible for GSH homeostasis are regulated by Nrf2 during periods of oxidative stress. GSH synthesis is performed in a two-step process from the amino acids cysteine, glutamate, and glycine. The first reaction in GSH biosynthesis is the rate-limiting step catalyzed by glutamate-cysteine ligase (GCL), which has both a catalytic (GCLC) and modifier (GCLM) subunit\textsuperscript{140}. GCLC performs the catalytic activity of the enzyme\textsuperscript{141}, while GCLM is an important regulatory subunit that lowers the \(K_m\) for glutamate\textsuperscript{142}. In this reaction glutamate and cysteine are ligated to form \(\gamma\)-glutamylcysteine. This dimer contains a non-conventional peptide bond between the \(\gamma\)-carboxyl group of glutamate and the amine of cysteine, rather than the \(\alpha\)-carboxyl group. Hydrolysis of this bond can only be performed by \(\gamma\)-glutamyltranspeptidase (GGT) which is found on the external surface of certain cell types and supplies cells with GSH precursors\textsuperscript{143}. As such GSH resists intracellular degradation. The second reaction to add glycine
to γ-glutamylcysteine is catalyzed by GSH synthetase (GS). Overexpression of GS fails to increase GSH levels, whereas GCL overexpression leads to an increase in GSH\textsuperscript{144}, supporting the notion that GCL is more important in the regulation of GSH synthesis. Since γ-glutamylcysteine is found at very low intracellular concentrations, GCL is considered to be rate-limiting\textsuperscript{140}. Cystine uptake through the system X\textsubscript{C}– transporter can also be a limiting step in GSH synthesis, as intracellular cysteine concentrations are low\textsuperscript{145}. Cysteine auto-oxidizes extracellularly to cystine, which can then be taken up by the system X\textsubscript{C}– transporter before rapid intracellular reduction\textsuperscript{145}. The expression of both GCL subunits and the critical xCT subunit of the system X\textsubscript{C}– are regulated by Nrf2\textsuperscript{146,147}.

### 1.3.1.1 Functions of the Glutathione System

As a redox sensitive thiol the primary function of GSH is to scavenge ROS\textsuperscript{148}. Supporting the antioxidant role of GSH, depletion of GSH leads to increases in ROS and subsequent ROS-induced toxicity\textsuperscript{149}. GSH is particularly required in the mitochondria to protect from pathologically generated oxidative stress\textsuperscript{150}. This antioxidant function is performed in reduction reactions catalyzed by glutathione peroxidase (GPx). GPx catalyzes the reduction of hydrogen peroxide and lipid peroxides, which in turn oxidizes GSH to GSSG\textsuperscript{151}. Members of the GPx enzyme family, including GPx2 and GPx4, are regulated by Nrf2\textsuperscript{94,152}. GSH recycling is performed when cells reduce GSSG using the enzyme glutathione reductase (GR) and NADPH to maintain a reducing environment. This key enzyme for maintaining GSH homeostasis is regulated by Nrf2\textsuperscript{153}. Since the GSH to GSSG ratio is a significant contributor to the redox equilibrium, cells require substantially more GSH than GSSG to maintain a reducing environment. During periods of oxidative stress when GSSG accumulates, cells can export GSSG when the capacity of GR to recycle GSH is outmatched\textsuperscript{154}. Another mechanism to prevent toxic GSSG accumulation during
oxidative stress is the reaction of GSSG with a protein thiol catalyzed by protein disulfide isomerase to form a protein mixed disulfide\textsuperscript{155}.

GSH can contribute to redox signaling in addition to its antioxidant function. This role is executed by modifying the oxidation state of critical modifying cysteine residues in proteins\textsuperscript{156}. Mechanistically this is performed by the reversible binding of GSH to protein thiols to alter the protein structure and function. This process is called glutathionylation and can activate or inactivate proteins\textsuperscript{157}. Glutathionylation can happen enzymatically with the assistance of GSH S-transferase (GST) or non-enzymatically\textsuperscript{158,159}. This mechanism exists to protect sensitive proteins from irreversible oxidation. Deglutathionylation is catalyzed by the oxidoreductase glutaredoxin (Grx) which is itself reduced by GSH\textsuperscript{160}. A significant number of proteins critical in cell signaling have been identified to be regulated by glutathionylation. These include GAPDH, inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-\(\beta\)), and p53\textsuperscript{161–164}. This process may affect ROS generation, as glutathionylation of electron transport chain proteins can alter their function\textsuperscript{165}. GST has an additional role in phase II detoxification of xenobiotics, including chemotherapeutic drugs. In this process GSH is conjugated to xenobiotics during metabolism to prepare for excretion\textsuperscript{166}. GST-mediated detoxification can contribute to chemoresistance\textsuperscript{167}.

GSH contributes to the regulation of growth in both normal and transformed cells. Increased GSH levels are essential for cell cycle progression and are associated with proliferation\textsuperscript{168–170}. Hepatocytes increase GSH in response to injury, as has been observed during liver regeneration following partial hepatectomy\textsuperscript{171}. Blocking this GSH generation causes a reduction in the total amount of DNA synthesis associated with cell division\textsuperscript{170}. GSH levels in various cancer types including hepatocellular carcinoma, melanoma, and pancreatic cancer have been correlated with cell and tumour growth\textsuperscript{170,172,173}. Interestingly, the redox environment of the nucleus has been
observed to be critical during the cell cycle. During proliferation GSH localizes to the nucleus and can affect gene expression of multiple proteins through influence on histones\textsuperscript{174}. Cell cycle progression requires a reducing environment moderated by GSH to occur. In addition to cell growth regulation, GSH is also involved in cell death regulation. This regulation is carried out through effects on the mechanism of apoptosis. GSH levels impact the expression and activity of caspases that are involved in effecting apoptosis\textsuperscript{175}. As previously mentioned, GSH levels fall as ROS propagate during the apoptotic cascade\textsuperscript{149}. This can occur because of decreased GCL activity following GCLC cleavage by caspase 3 during apoptosis\textsuperscript{176}.

1.3.1.2 Targeting the Glutathione System in Cancer

GSH is critical for malignant behaviour of cancer cells. GSH is required during tumour initiation, as its depletion impairs tumorigenesis\textsuperscript{127}. This is because GSH prevents oxidative stress from enacting its cytotoxic effects on transformed cells. Following tumour formation GSH becomes important to maintain high growth rates, as GSH levels are correlated to growth\textsuperscript{177}. These levels are elevated in cancer due to increased expression of GCL and the system $X_C$\textsuperscript{178,179}. In addition, GGT is an early marker of neoplastic transformation and assists with the GSH cycle to supply cancer cells with GSH metabolic precursors\textsuperscript{180}. GGT overexpression has been observed in melanoma and hepatocellular carcinoma\textsuperscript{181,182}, among other cancers. Lastly, GSH is required for establishing distant metastases because metastatic cells experience higher oxidative stress. In response they elevate GSH levels to combat the microenvironment and maintain redox homeostasis\textsuperscript{126}. To highlight the importance of GSH in cancer, many studies have attempted pharmacologic GSH depletion and observed sensitization to various therapies. Cancer cells are sensitized to radiation upon GSH depletion\textsuperscript{84}. In addition, chemoresistance has been associated with high GSH levels in multiple myeloma and ovarian cancer\textsuperscript{165,166}. This chemoresistance can be eliminated following depletion of GSH\textsuperscript{184}. 
As GSH is critical to cancer progression and survival there is reason to believe that it can be exploited in cancer therapy. Various key nodes in GSH metabolism can be targeted as an anticancer strategy. First, substrate supply for GSH biosynthesis can be targeted. Glutamine analogs targeting GGT have been evaluated in humans but have been found too toxic for clinical use. Newer uncompetitive inhibitors are in development but have yet to prove effective and will require time before clinical application. The system $XC^{-}$ transporter may be a critical target of an anti-GSH cancer therapy as it is required to regulate intracellular cysteine and has been observed to be necessary for cancer cell survival. Multiple inhibitors against this system have been described. Sulfasalazine is a drug that has been used in clinics for rheumatoid arthritis, ulcerative colitis, and Crohn’s disease, and inhibits the system $XC^{-}$. However, the mechanism of action is poorly understood, it is metabolically unstable, and lacks potency. Erastin is a newer molecule that is more potent than sulfasalazine and more selective for system $XC^{-}$. However, it has yet to be tested in humans.

A second potential target critical to GSH metabolism is GSH recycling. The nitrogen mustard compound carmustine (BCNU) is primarily an alkylating agent used in chemotherapy for brain cancer and lymphomas, however its off-target effect is GR inhibition. Unfortunately, BCNU is in short supply, costly, and displays prolonged myelosuppression and potentially lethal pulmonary toxicity. A newer molecule 2-AAPA has been described as a GR inhibitor that increases GSSG and stimulates protein glutathionylation. However, 2-AAPA has yet to be tested in humans and possesses off-target effects including Grx and thioredoxin reductase (TrxR) inhibition. These off-target effects and potentially others that have yet to be uncovered make 2-AAPA too unpredictable for use in cancer therapy.
Lastly, a third strategy involves direct targeting of GSH synthesis. GCL, the rate-limiting enzyme, is more important for GSH levels than GS, the enzyme catalyzing the second step of GSH synthesis\textsuperscript{144}. Therefore, GCL would be the ideal target. Fortunately, an irreversible inhibitor of GCL, buthionine sulfoximine (BSO), has been studied extensively \textit{in vitro}, \textit{in vivo}, and in human clinical trials\textsuperscript{127,197,198}. BSO has proven to be minimally toxic in humans when used on its own\textsuperscript{199}. Although it is rapidly metabolized following intravenous bolus, concentrations of \textasciitilde500\textmu M following continuous infusion are achievable in humans while maintaining safe toxicity profiles\textsuperscript{200}.

BSO has garnered great interest for use in drug combinations against cancers that have been observed to require GSH for chemoresistance\textsuperscript{197}. Mechanistically, it contributes to oxidative stress in the cells by acute GSH depletion and long-term ROS exacerbation\textsuperscript{149}. This can lead to both apoptosis and ferroptosis\textsuperscript{149,201}. BSO’s safety profile, specificity, low cost, previous clinical trials, and mechanistic understanding make it an excellent drug to develop further for cancer therapy that modulates oxidative stress.

BSO on its own is not very cytotoxic with short-term treatments and many cell lines are resistant to acute GSH depletion\textsuperscript{197}. It is likely that therapy involving BSO would require a second agent to enhance toxicity. Adaptation to GSH depletion involves Nrf2 activation to upregulate other antioxidants, including Trx\textsuperscript{202}. Since antioxidants have similar, sometimes overlapping functions, compensation from alternative antioxidant systems is a plausible mechanism for resistance to GSH depletion. Compensation between antioxidants has been observed in other contexts including between GSH and Trx, and between Trx and heme oxygenase\textsuperscript{127,203,204}. Therefore, therapy tailored to inhibit more than one antioxidant system may be able to overcome compensation and produce strong anti-cancer effects.
1.3.2 The Thioredoxin Antioxidant System

The Trx system is a reductase system that is required for DNA synthesis and protection from oxidative stress\textsuperscript{203}. The three major components of the system are Trx, TrxR, and NADPH. As with other antioxidant genes, Trx and TrxR are transcriptionally regulated during oxidative stress by Nrf2\textsuperscript{99,205}. Trx is a small (~12 kDa) reductase that is the effector of the Trx system. It catalyzes the reduction of protein disulfides. The active site dithiol in reduced Trx interacts with various protein substrates via a thiol-disulfide exchange reaction that forms a disulfide in Trx (oxidized Trx). This oxidized form is reduced by electron transfer from NADPH via the reaction catalyzed by TrxR. There is a mitochondrial Trx2 with two active site cysteines, and cytosolic and nuclear Trx1 with 3 additional cysteines that contribute to enzymatic regulation\textsuperscript{206}. TrxR is a homodimeric oxidoreductase that recycles Trx, and belongs to the same protein family as GR\textsuperscript{207}. Mammalian cells contain three TrxRs: cytosolic TrxR1, mitochondrial TrxR2, and testis-specific TrxR3\textsuperscript{208}. Of these, TrxR1 has been identified as a fitness gene\textsuperscript{209}. Although structurally and functionally similar, the TrxR enzymes have different amino acid sequences\textsuperscript{210}. These enzymes contain a unique C-terminal selenocysteine residue that contributes to its reaction mechanism.

In the mechanism of TrxR reduction electrons from NADPH are first transferred to enzyme-bound FAD, then FAD transfers reducing equivalents to the N-terminal disulfide in the redox centre of the same subunit. This reduces the disulfide to a dithiol, which then transfers the electrons to the C-terminal selenenylsulfide of the other subunit. Addition of another NADPH molecule reduces these residues to a selenol-thiol pair\textsuperscript{211}. The reduced enzyme can transfer these electrons to its substrates, including proteins such as oxidized Trx and Grx2, and small molecules such as hydrogen peroxide and cytochrome c\textsuperscript{212}. When transferring electrons to Trx the selenol of TrxR is deprotonated to a selenolate anion. This anion attacks the disulfide of oxidized Trx, resulting in a
TrxR-Trx mixed selenenylsulfide. The free thiol on the C-terminal TrxR reduces this bond, causing the reformation of the selenenylsulfide in TrxR. This allows the now reduced Trx to leave with reduced dithiols in its active site. This mechanism is made possible because of the selenocysteine residue. Selenocysteine is a cysteine analog that replaces sulfur with selenium. This maintains many chemical properties of the amino acid, however the pKₐ of the selenol group in selenocysteine is significantly lower than the thiol group in cysteine (cysteine pKₐ = ~5.8 vs. selenocysteine pKₐ = ~8.3). This property allows the selenol to exist primarily as the ionized selenolate at physiological conditions, whereas the thiol exists in the protonated form. The ionized molecule is more reactive than the protonated version. In fact, replacement of selenocysteine with cysteine dramatically decreases activity of TrxR.

1.3.2.1 Functions of the Thioredoxin System

As one of the major thiol-dependent electron donor systems in the cell, the Trx system carries out multiple important functions. The Trx system can perform mechanisms of both direct and indirect ROS scavenging. First, Trx can directly neutralize ROS. This action involves quenching hydroxyl radical by providing electrons, and requires Trx’s catalytic cysteines, as shown by loss of ROS scavenging following mutation of these cysteines to alanine. Second, TrxR can indirectly scavenge ROS by providing electrons to other endogenous antioxidants, including ubiquinone. The reduced form of ubiquinone, ubiquinol, can act as a ROS scavenger by reducing radicals to protect from lipid peroxidation. The reduction of ubiquinone is catalyzed by TrxR under physiologic conditions, and may contribute to protection from oxidative stress.

A critical function of the Trx system involves the regulation of protein disulfide formation. During oxidative stress ROS enact damage to cellular macromolecules. As proteins are the most highly concentrated macromolecule in cells, they are likely the most significant target of ROS.
proteins are equally susceptible to oxidative damage, as subcellular localization and content of oxidation-sensitive amino acids may contribute\(^\text{219}\). The most oxidation-sensitive amino acid residue is cysteine, that readily forms disulfide bridges with other cysteine residues upon oxidation\(^\text{220}\). These aberrant disulfides can cause structural changes that impact protein function. Trx maintains protein redox status via its reductase activity which can reduce disulfides in an extensive number of target proteins to dithiols\(^\text{221}\). Trx targets regulate a vast array of cellular processes including proliferation, metabolism, and apoptosis. In general, the role of Trx is to provide a reducing environment to protect from oxidative stress to prevent cell death and promote cell growth. The most important targets of Trx are reductive enzymes including Prx, ribonucleotide reductase, and methionine sulfoxide reductase. This highlights the importance of Trx in the maintenance of other reductive pathways\(^\text{222}\).

Prxs are a peroxidase family that act primarily as cellular antioxidants. Three subgroups of Prx exist: 2-cysteine Prx isoforms (Prx1-4), atypical 2-cysteine Prx isoform (Prx5), and 1-cysteine Prx isoform (Prx6)\(^\text{223}\). Reduced Trx is the electron donor for Prx1-5 which can then remove ROS such as hydrogen peroxide, lipid hydroperoxides, and peroxynitrite. Hyperoxidation of Prx occurs from ROS overexposure. The catalytic cysteine residues oxidize to form disulfide bonds, which must be reversed by Trx\(^\text{224}\). This establishes a redox cascade where the flow of electrons travels from NADPH through the Trx system to Prx and finally to neutralize toxic ROS\(^\text{225}\). The speed of hydrogen peroxide scavenging by Prx is comparable to other significant ROS scavenging enzymes like glutathione peroxidase, on the magnitude of \(10^7 – 10^8 \text{ M}^{-1}\text{s}^{-1}\), indicating that it is a major contributor to peroxide scavenging in cells\(^\text{226,227}\). Prxs can inhibit cancer development or promote tumorigenesis depending on the specific Prx member and cancer context\(^\text{228}\). However, high levels of Prxs are often associated with radioresistance and chemoresistance. For example, high Prx2 levels correlates to radioresistance in breast cancer cells, as well as with cisplatin resistance in
gastric cancer cells\textsuperscript{229,230}. Silencing of Prx2 sensitizes colorectal cancer cells to both radiation and oxaliplatin treatment\textsuperscript{231}. These data indicate that Trx may have an indirect role in promoting therapy resistance through Prx.

Another significant reductase target of Trx is ribonucleotide reductase (RNR). RNR reduces all four ribonucleotides to deoxyribonucleotides for use in DNA synthesis. During ribonucleotide reduction the two active site cysteines of RNR form a double bond which must be reduced by a C-terminal dithiol through a thiol-disulfide exchange reaction\textsuperscript{232}. The original conformation of the C-terminal cysteines is restored by an external reductase system, either Trx or Grx which both have similar catalytic efficiency with mammalian RNR\textsuperscript{233–235}. This redundancy allows for cells to withstand the loss of one reductase system. This is supported by studies showing that TrxR downregulation causes no change in deoxyribonucleotide triphosphate pools in mouse cancer cells\textsuperscript{236}. Some differences may exist as Trx is believed to support S phase electron donation, whereas Grx is believed to support DNA repair and mitochondrial DNA synthesis\textsuperscript{235}. Interestingly, Grx is an important effector of the GSH system, supporting compensatory mechanisms between Trx and GSH.

Methionine is another oxidation-sensitive amino acid. However, protein reduction by Trx is limited to disulfides. Methionine sulfoxide reductase (Msr) is capable of reducing oxidized methionine in proteins and acquires its reducing potential from Trx\textsuperscript{237}. Although there are multiple Msr isoforms with different numbers of catalytic cysteines and mechanisms, they all require Trx to maintain their reductive capacity\textsuperscript{222}. This is yet another pathway by which Trx is required to protect cells from damage caused by oxidative stress.

Beyond reductase systems, Trx is responsible for post-translational modifications to regulate numerous signaling cascades and transcription factors. Apoptosis signaling kinase 1 (ASK1) is
part of the MAPKKK family that activates the JNK and p38 MAP kinase pathway. Reduced Trx can bind ASK1 to inhibit its activation, directing it to ubiquitin-mediated degradation, which prevents ASK1 from activating proapoptotic gene expression. Inactivation of PTEN, a negative regulator of the PI3K-Akt pathway, results in the stimulation of proliferation. Trx1 binds PTEN and inhibits its activity, which may stimulate cancer cell proliferation. These data indicate a potential role for Trx in cancer proliferation and highlight the importance of Trx inhibition in cancer therapy.

1.3.2.2 Targeting the Thioredoxin System in Cancer

A novel strategy for pancreatic cancer therapy may involve Trx system inhibition, as the Trx system is involved in protection from oxidative stress that may promote cancer. Excluding downstream targets of Trx, three major targets can be identified within the Trx system: NADPH, Trx, and TrxR. Since NADPH is ubiquitous and required in a vast array of major biosynthetic pathways, complete inhibition of NADPH production would not be feasible. The individual pathways of NADPH production could potentially be targeted. The molecule dichloroacetate inhibits the oxidative phase of the PPP and has been clinically tested. However, this likely may not be effective in a KRAS driven tumour like pancreatic cancer since the oxidative phase of the PPP is less involved in NADPH production than other pathways. When targeting the serine biosynthesis pathway PHGDH knockdown in overexpressing cancers inhibits proliferation. However, inhibitors of PHGDH, including PKUMDL-WQ-2101 and PKUMDL-WQ-2201, have only recently been identified and require further development before use in humans. Targeting glutamine metabolism through inhibition of ME1 has shown to be promising by inducing senescence and apoptosis in vitro. However, an inhibitor of ME1 has yet to be characterized. An inhibitor of the mitochondrial isoform ME2, NPD389, has been described but not developed.
As the effector of most functions of the Trx system, Trx would seem to be an ideal target. The synthetic molecule PX-12 is the most developed Trx inhibitor that covalently binds to Trx cysteine residues, forming mixed disulfides. PX-12 inhibits breast cancer cell proliferation \textit{in vitro} and reduced tumour volume \textit{in vivo}. Interestingly, PX-12 can be a substrate for TrxR causing competitive inhibition of TrxR for its normal substrate, oxidized Trx\textsuperscript{246}. PX-12 has been tested in clinical trials, and was shown to decrease plasma Trx in a phase I trial\textsuperscript{247}. However, a phase II trial of PX-12 in patients with advanced pancreatic cancer refractory to gemcitabine therapy showed no antitumour activity\textsuperscript{248}. These results may stem from the inability to achieve adequate plasma concentrations of PX-12 without significant toxicities because of rapid binding to plasma components. Combined results of clinical trials suggest that development of PX-12 for intravenous infusion is not feasible\textsuperscript{249}.

The third and last potential target, TrxR, may contain the most potential for anticancer therapy. High TrxR1 expression correlates to poor prognosis in hepatocellular carcinoma, breast, and pancreatic cancer, among others\textsuperscript{250,251}. TrxR1 inhibition following siRNA knockdown dramatically reduced tumour progression in xenograft models of lung cancer\textsuperscript{252}. In contrast, normal adult tissues can survive TrxR1 loss\textsuperscript{253}. Recent development has centered around attempts to develop specific inhibitors of TrxR1 with some success\textsuperscript{254}. However, these molecules require extensive development and testing before potential clinical use. The pan-TrxR inhibitor auranofin is an FDA approved drug for the treatment of rheumatoid arthritis that has recently garnered interest for repurposing in cancer therapy\textsuperscript{255}. Auranofin is an orally available gold complex that irreversibly inhibits all TrxR isoforms, and is inexpensive, well-characterized, and has relatively minor toxicities\textsuperscript{256}. Although it has been reported to inhibit proteasome deubiquitinases in addition to TrxR\textsuperscript{257}, evidence to support this is sparse and seems to be secondary to cell death. Auranofin is capable of inducing apoptotic cell death following the proliferation of ROS\textsuperscript{258,259}. However, it
appears to work best in combination with other drugs after being testing as a combination therapy against various cancers\textsuperscript{260-262}. In addition, auranofin has been used in phase I and II clinical trials for chronic lymphocytic leukemia therapy (see www.clinicaltrials.gov NCT01419691). Therefore, auranofin is likely the most ideal candidate to develop for pancreatic cancer therapy that targets the Trx system to augment oxidative stress.

1.4 A New Therapeutic Strategy for Pancreatic Cancer

The hallmarks of cancer describe essential alterations involved in tumour development. They include the sustainment of proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, enabling replicative immortality, induction of angiogenesis, resisting cell death, deregulation of cellular energetics, and avoidance of immune destruction\textsuperscript{263}. Redox signaling and the responses to oxidative stress are involved in every hallmark of cancer\textsuperscript{264}. Therefore, consideration for cancer promotion by antioxidants should be made when developing novel anti-cancer therapies. Normal tissues are resistant to antioxidant loss, which may prevent harmful toxicities of antioxidant inhibition.

As previously discussed, BSO and auranofin appear to be useful drug candidates for combined targeting of the GSH and Trx systems. Evidence suggests that these systems overlap to compensate and protect against inefficiencies or inhibition of either system. The Trx system can be an alternative route for cells to reduce oxidized GSH \textit{in vivo}\textsuperscript{265}. In addition, physiologic concentrations of GSH and Grx are capable of reducing oxidized Trx \textit{in vitro}, suggesting GSH as a backup to TrxR function\textsuperscript{266}. However, this observation was made in a cell-free system which may limit its applicability in the complex cell environment.
Combination therapy using both BSO and auranofin can help to overcome compensation. Ideally the combination of the two drugs will produce improved effectiveness compared to monotherapy with either compound. This result can either be stated as synergy or potentiation. Synergy is observed when the effect of two or more drugs is greater than the sum of effects when the drugs are administered as monotherapies. The effect is mutual in that each drug enhances the effect of the other\textsuperscript{267}. In contrast, a scenario by which the enhancement of effect is one-sided can be deemed a potentiation or augmentation. Evaluation of a potentiation can be stated as a fold change in the effect of one drug, caused by the effect of the other drug\textsuperscript{267}.

Previous studies have shown that dual-inhibition of the GSH and Trx systems can lead to greater anti-cancer responses\textsuperscript{127}. Use of BSO and auranofin in combination sensitized mouse tumours to radiotherapy\textsuperscript{83}, and mesothelioma cells show extensive oxidative stress and cell death following combination of the drugs\textsuperscript{268}. These studies do not evaluate potential synergism of the combination of BSO and auranofin. Nor do any studies evaluate this combination in pancreatic cancer, which seems to be an exceptional target of pro-oxidant therapy.

Considering that the Trx and GSH systems comprise the main antioxidant systems in the cell, I hypothesize that combination therapy using auranofin and BSO will cause primary pancreatic cancer cells to undergo apoptosis by exacerbating oxidative stress. Auranofin will be identifiable in pancreatic PDX tumours using IMC. I have three aims to test this hypothesis:

Aim 1: Evaluate the effect of combination therapy on cell viability and death.

Aim 2: Evaluate the effect of combination therapy on the redox environment of the cell.

Aim 3: Evaluate the drug-target interaction of auranofin-TrxR \textit{in vitro} and biodistribution of auranofin \textit{in vivo}. 
Targeting the Thioredoxin and Glutathione Antioxidant Systems to Treat Pancreatic Cancer

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The work presented in this chapter is presented as a manuscript for future submission.
2.1 Introduction

Pancreatic cancer is the 4\textsuperscript{th} leading cause of cancer death in Canada, however it is only the 12\textsuperscript{th} most commonly diagnosed\textsuperscript{2}. There is poor prognosis with an overall 5-year survival rate of 5\%\textsuperscript{4}. Significant advancements in the development of new treatment options for the disease have not been made. Many patients with advanced disease are given standard chemotherapy drugs that provide little benefit, leading to an urgent need for new strategies to combat this disease.

Pancreatic cancer, among other cancers, is characterized by excessive oxidative stress\textsuperscript{48}, which occurs when production of intracellular reactive oxygen species (ROS) outpaces the capacity of antioxidants to protect from ROS. These ROS are produced in mitochondria at an extreme rate in transformed cells due to inherently high metabolism, and may promote aggressive tumour features\textsuperscript{50}. Adaptations to protect from ROS are required for cancer cells to survive. These adaptations primarily involve oxidative stress signaling through Nrf2, which mediates the oxidative stress response to promote the production of the antioxidants glutathione (GSH) and thioredoxin (Trx)\textsuperscript{54}. The main oncogenic driver of pancreatic cancer, KRAS, induces Nrf2 activation to maintain antioxidant defenses\textsuperscript{47}. In conjunction to stimulating antioxidant production, pancreatic cancer relies on glutamine metabolism for the production of NADPH\textsuperscript{108}. This adaptation occurs because of the need to protect against high ROS during oxidative stress, as NADPH provides reducing power to antioxidant systems in the cell to prevent cytotoxic damage caused by high ROS levels.

ROS exhibit dual roles; lower concentrations of ROS that are promoted by antioxidants can stimulate cancer cell proliferation, whereas higher concentrations of ROS are damaging to cells\textsuperscript{49,52}. Therefore, antioxidants may contribute to aggressive growth and metastasis in cancer because of their functions in ROS scavenging, preventing ROS-mediated cellular damage, and
promoting ROS-mediated proliferative signalling. The reliance of pancreatic cancer on antioxidants to protect from oxidative stress may provide a unique vulnerability in the tumour. Targeting antioxidants may promote cytotoxic damage specifically in tumours by augmenting oxidative stress.

Both the GSH and Trx systems have been extensively studied and tested inhibitors exist. GSH is the most abundant antioxidant cofactor in cells and is responsible for ROS scavenging. The rate-limiting step of GSH synthesis is catalyzed by glutamate-cysteine ligase (GCL). An inhibitor of GCL, buthionine sulfoximine (BSO), has been well characterized and previously tested in human subjects with minimal toxicity. Trx reduces aberrant protein disulfides caused by oxidative stress. Oxidized Trx is recycled by thioredoxin reductase (TrxR) using NADPH to replenish its reducing power. Three TrxR isozymes exist: cytosolic TrxR1 that has been identified as a fitness gene, mitochondrial TrxR2, and testes-specific TrxR3. The gold complex auranofin is an inhibitor of all three TrxR proteins and has previously been used in the treatment of rheumatoid arthritis. It has garnered interest for repurposing as a therapy in various cancers.

Many recent studies have identified oxidative stress as an effective target in cancer therapy, including in KRAS driven tumours. Targeting one antioxidant pathway may allow for compensation from another to continue providing protection to the cell. This may possibly be overcome by a double hit. To our knowledge no studies have attempted a double hit in pancreatic cancer. Considering that Trx and GSH comprise the main antioxidant systems in the cell, combination therapy using auranofin and BSO may cause cell death by depleting antioxidant capacity and increasing ROS in primary pancreatic cancer cells.
2.2 Materials & Methods

Cell Culture and Reagents

Primary cell lines (9A and OCIP23) were derived from human pancreatic cancer tumours. 9A was maintained in Roswell Park Memorial Institute (RPMI) 1640 (Wisent) medium supplemented with 10% fetal bovine serum (FBS) (VWR Seradigm Life Science) and 1x penicillin/streptomycin (Wisent). OCIP23 was maintained in Dulbecco’s Modified Eagle Medium (DMEM)/F-12 (Wisent) supplemented with 2.5% FBS and 1x penicillin/streptomycin. Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Auranofin (Sigma-Aldrich) was diluted in DMSO (Sigma-Aldrich) at 100mM, aliquoted and stored at -10°C to be used once without refreezing. BSO (Sigma-Aldrich) was prepared fresh in sterilized MilliQ water at 100mM.

MTS Assay

Cell viability was assayed by MTS assay and performed according to the manufacturer’s protocol (Promega). In summary, 96 well plates were seeded with 5000 cells/100µL/well and incubated for 24 hours. Medium was replaced with medium containing DMSO (Sigma-Aldrich) [0.003% for OCIP23, 0.004% for 9A], auranofin, BSO, or combination of auranofin and BSO at the indicated concentrations. Each treatment was performed in triplicate. Cells were incubated for 24 hours, then cell viability was determined by adding 20µL of MTS reagent followed by incubation for 3 hours at 37°C and absorbance reading at 492nm using the Multiskan EX microplate spectrophotometer (Thermo Fisher).

Clonogenic Assay
Clonogenic assays evaluated drug treatment effects on colony formation and was performed as previously described\textsuperscript{274}. Briefly, cells were grown to 70-80\% confluence in 10cm plates then treated with vehicle (1\(\mu\)L/mL DMSO), auranofin, BSO, or a combination at the indicated concentrations for 24 hours. Cells were harvested and reseeded in 6-well plates at 10\(^5\), 10\(^4\), 10\(^3\), and 10\(^2\) cells/well in triplicate, then allowed to incubate for 12 days before fixing and staining with 0.2\% methylene blue in 50\% ethanol/water. Colonies were counted using the Gel Count (Oxford Optronix) counter. Plating efficiency was calculated as the average number of colonies divided by the number of cells plated. Surviving fraction was expressed as the plating efficiency of the sample divided by the plating efficiency on vehicle control plates.

**Multiparametric Flow Cytometry**

Apoptosis, ROS levels, and GSH levels were assayed by flow cytometry. 7x10\(^5\) cells/plate were seeded in 6cm plate. After 24 (OCIP23) or 48 hours (9A), media was replaced with either normal medium or BSO-supplemented medium. Auranofin was diluted in DMSO to 1000x the indicated concentrations and added at 1\(\mu\)L/mL. After 24 hour treatment the cells were trypsinized and resuspended in medium. Cells were centrifuged at 1100rpm for 5 minutes, then the pellets were resuspended in 0.5mL serum free media. Cell suspensions were then incubated in a water bath at 37\(^\circ\)C with 40nM DiIC1(5) (ThermoFisher Scientific) and 5\(\mu\)M 2',7'-dichlorofluorescein (DCF) (ThermoFisher Scientific) for 30 minutes. 5 minutes before the end of the incubation, 40\(\mu\)M monobromobimane (mBBr) (ThermoFisher Scientific) and 1\(\mu\)g/mL propidium iodide (PI) (ThermoFisher Scientific) were added. Following incubation, samples were cooled on ice for 10 minutes then analyzed with the CytoFLEX flow cytometer (Beckman Coulter). During the time course experiment samples were prepared in the same manner, however the drug treatments were
for 2 and 5 hours, and mBBr was excluded from the panel of fluorescent probes. Data analysis was performed using FlowJo Software (BD).

**Tumour sample preparation**

Animal experiments were carried out according to protocols approved by the University Health Network Animal Care Committee. Patient-derived xenograft (PDX) mice were established subcutaneously in 5 week old severe combined immunodeficiency (SCID) mice. Two models, OCIP83 and 9A were used. Mice were given an intraperitoneal injection of 10mg/kg auranofin for a 24 hour treatment. Mice were later given 120mg/kg pimonidazole (Hypoxyprobe, Inc.), followed by 30mg/kg EF5 (obtained from Dr. Cameron J. Koch, University of Pennsylvania) 5 and 3 hours prior to sacrifice, respectively. Tumours were excised, fixed, and processed for paraffin embedding.

**Immunohistochemistry (IHC)**

Formalin-fixed, paraffin-embedded (FFPE) sections of subcutaneous PDX tumours were incubated with mouse pimonidazole antibody (Hypoxyprobe, Inc.) at 1:400, overnight at 4°C. Biotinylated horse anti-mouse IgG was added at 1:200 concentration and incubated for 1 hour. Detection was performed using the HRP labeling (Vector Labs) and DAB chromogen (Dako).

**Imaging Mass Cytometry (IMC) Staining, Data Acquisition, and Visualization**

Imaging mass cytometry was used to directly image the distribution of atomic gold in tissue sections following treatment with auranofin. Tissue staining for IMC was performed as previously described\(^{275}\). In summary, FFPE sections were dewaxed and rehydrated as routine. Antigen retrieval and blocking with BSA was performed, followed by incubation with primary antibody
against pimonidazole for 1 hour at room temperature (RT). Samples were then incubated overnight at 4°C with a metal-conjugated antibody cocktail diluted in PBS/0.5% BSA (Table 1). Following this, samples were washed and exposed to 25μM Ir-Intercalator (Fluidigm) for 30 minutes at RT. Lastly, the samples were rinsed then dried before IMC ablation.

Regions of interest were determined by assessing IHC samples stained for pimonidazole. During data acquisition, the IMC immunostained samples are inserted into a laser ablation chamber where the tissue is ablated in a 1μm diameter spot. The ablation spot is vaporized, and the plume is carried with high time-fidelity into the inductively coupled plasma ion source for simultaneous analysis by the mass cytometer (Fluidigm). The metal isotopes located on each spot are simultaneously measured. This process continues for the entire area, eventually yielding an intensity map of the targets throughout the tissue region of interest.

Data-processing and image visualization was performed using the in-house-developed MCD Viewer (Fluidigm). The signal data from the mass cytometer was exported in text format, then reconstructed into images. The epithelial cells were defined by E-cadherin and pan-keratin staining, and the stromal cells were defined by collagen and αSMA. Areas within these regions were selected in the MCD viewer to semi-quantitatively determine the average number of gold ions within them.

**Cell Lysis and Protein Determination**

Lysis was performed using a lab-made lysis buffer containing 50mM Hepes (pH 8.00), 10% glycerol, 1% Triton-X, 150mM NaCl, 1mM EDTA, 1.5mM MgCl₂, 100mM NaF, 10mM Na₄P₂O₇·10H₂O, 1mM Na₃VO₄, and 1 tablet/7mL buffer cOmplete protease inhibitor cocktail (Sigma-Aldrich). Cell cultures were incubated with 700μL lysis buffer for 1 hour at 4°C, then
collected and centrifuged at 14,000rpm for 15 minutes at 4°C. Supernatants were collected and stored at -80°C until use. Protein determination was performed using the BCA Assay kit (ThermoFisher Scientific) as per the manufacturer’s protocol.

**Gel Electrophoresis and Immunoblot**

Samples were resolved using 10% SDS-PAGE. Following transfer to polyvinylidene difluoride membranes, proteins were detected with antibodies to TrxR1 1:1000 (Cell Signaling Technology), TrxR2 1:1000 (Cell Signaling Technology), β-actin 1:7000 (Abcam), GAPDH 1:4000 (Cell Signaling Technology). Horseradish peroxidase (HRP) conjugated secondary sheep anti-mouse (Abcam) and donkey anti-rabbit (Abcam) were used for detection at 1:5000. Quantification was performed by densitometry using ImageJ (NIH).

**Cellular Thermal Shift Assay**

Cellular Thermal Shift Assay (CETSA) was performed as previously described to assess auranofin-TrxR engagement. Briefly, to determine the optimal temperature at which maximal thermal shifts were observed, cells were treated with vehicle or 10μM auranofin for 1 h, then divided into equal portions, centrifuged, and resuspended in PBS with protease inhibitors. Cells were then heated at temperatures ranging from 46-70°C for 3 min in a thermal cycler (SimpliAmp, Applied Biosystems), cooled at RT for 3 min, snap-frozen in liquid nitrogen, thawed at RT, and then lysed by 3 freeze/thaw cycles with vortexing in between. Samples were then centrifuged at 20,000 x g for 20 min at 4°C and supernatants were collected. Lysates were analyzed by immunoblotting. When evaluating changes with different auranofin concentrations the same procedure was followed, however cells were treated with increasing auranofin concentrations at the determined optimal temperature of 56°C.
Statistical Analysis

Data are presented as the mean value ± S.E.M. GraphPad Prism version 7.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. A value of P<0.05 was considered to indicate statistically significant differences.

2.3 Results

Auranofin, but not BSO, inhibits cell growth and viability of primary pancreatic cancer cell lines.

To evaluate the effect of Auranofin and BSO on cell viability, human primary pancreatic cancer cells were treated with different concentrations of either drug for 24 hours. MTS assay measured cell metabolic activity. BSO alone displayed no significant effect on the viability of the primary cells (Fig. 2.1A). In contrast, auranofin reduced viability in a concentration dependent manner with IC50 values 1.40μM and 2.35μM in OCIP23 and 9A, respectively (Table 2.1; Fig. 2.1B). These data suggest that after 24 hours, these two primary pancreatic cancer cell lines are susceptible to auranofin, but not BSO. Since BSO does not reduce viability at this time point, any interaction between the two drugs cannot be deemed synergy.

BSO potentiates auranofin’s inhibitory effect on cell viability of primary pancreatic cancer cells.

Using the MTS assay, we evaluated cell viability following treatment of cells with a combination of auranofin and BSO. Co-treatment with 10μM BSO caused a 61.1-fold decrease in the IC50 of auranofin in OCIP23 (IC50 = 22.9nM), and a 4.07-fold decrease in 9A (IC50 = 578nM) (Fig. 2.1B). The addition of 50μM BSO caused a 238-fold decrease in the IC50 of auranofin in OCIP23 (IC50 = 5.88nM), and a 11.0-fold decrease in the IC50 in 9A (IC50 = 213nM) (Fig. 2.1B). These
data support a strong BSO-mediated potentiation of auranofin cytotoxicity. The higher IC50 and lower potentiation observed with 9A cells suggest they possess a higher reserve capacity of TrxR1/2 activity that makes them more resistant to drug-induced oxidative stress. To test this hypothesis, we assessed expression levels of Trx1/2 in 9A and OCIP23 cells. 9A cells showed a significantly higher expression level of TrxR1, but not TrxR2, compared to OCIP23 cells (p=0.0007) (Fig. 2.1C and D). This may explain, at least in part, the differential response of OCIP23 and 9A to auranofin and combination with BSO.

**Combination of BSO and auranofin inhibits clonogenicity of primary pancreatic cancer cells.**

We then conducted clonogenic assay to determine whether the BSO-auranofin combination inhibited long-term viability and if low auranofin concentrations on their own improved viability as observed by MTS assay. There was a significant decrease in clonogenicity following combination treatment in 9A (p=0.0155) with 43.1% surviving fraction (Fig. 2.1E) and OCIP23 (p=0.0088) with 43.8% surviving fraction (Fig. 2.1F) when compared to vehicle controls. Single treatment with auranofin (100nM or 400nM) showed no increase in clonogenicity, which contrasts the improved viability of low auranofin concentrations observed via MTS assay (Fig. 2.1B). The results of these two assays may differ because of the different endpoints measured, metabolism versus colony growth. These data support the potentiation of auranofin by BSO at low auranofin concentrations that otherwise have no effect on cell death and viability when used as monotherapy.

**Table 2.1.** IC50 values of auranofin monotherapy and in combination with BSO in primary pancreatic cancer cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>BSO Concentration (μM)</th>
<th>Auranofin IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCIP23</td>
<td>-</td>
<td>1400</td>
</tr>
<tr>
<td>OCIP23</td>
<td>10</td>
<td>22.9</td>
</tr>
<tr>
<td>OCIP23</td>
<td>50</td>
<td>5.88</td>
</tr>
<tr>
<td>9A</td>
<td>-</td>
<td>2350</td>
</tr>
<tr>
<td>9A</td>
<td>10</td>
<td>578</td>
</tr>
<tr>
<td>9A</td>
<td>50</td>
<td>213</td>
</tr>
</tbody>
</table>
Figure 2.1. Dose-response of antioxidant pathway inhibitors, TrxR isozyme levels, and colony formation potential. (A and B) Viability of primary pancreatic cancer cells was determined after 24 hour treatment using an MTS assay. Shown are the toxicity profiles of A) BSO monotherapy (n=3), and B) auranofin monotherapy or combination (n=3). (C and D) Relative amounts of C) TrxR1 and TrxR2 in untreated cell lines (n=3) and their corresponding D) immunoblots. Levels were normalized to the first OCIP23 sample. (E and F) Colony formation of primary cell lines was determined after 24 hour treatment. Cells were reseeded and incubated for 12 days before colonies were assessed for E) 9A (n=3) (Combination of Au 400nM and BSO 10μM) and F) OCIP23 (n=3) (Combination of Au 100nM and BSO 10μM). Combinations were compared to vehicle controls. Surviving fraction is represented as a percentage of vehicle controls. Shown are the S.E.M. Significant decrease from vehicle control, * = p<0.05, ** = p<0.01, *** = p<0.001.
**BSO-auranofin combination induces apoptosis in primary pancreatic cancer cells.**

We conducted live cell flow cytometry to determine whether apoptosis contributed to cell death after BSO-auranofin combination. The loss of mitochondrial membrane potential (MMP) has been observed to be one of the first steps of apoptosis, and has previously been observed following auranofin treatment. In addition, the loss of membrane integrity, and subsequent uptake of propidium iodide (PI), is a marker of cell death. Here we used DiIC1(5), a marker of MMP, and PI to assess cell death after combination treatment. Auranofin or BSO alone did not increase the MMP negative or PI positive populations. In contrast, combinations of these low concentrations increased the proportion of cells observed to be starting apoptosis and those that have already died in both OCIP23 (Fig. 2.2) and in 9A. These results indicate that combinations of low concentrations of auranofin and BSO induces apoptosis in primary pancreatic cancer cells.

**BSO-auranofin combination reduces thiol levels and induces an acute increase in ROS levels.**

We also used live cell flow cytometry to assess the effect of combinatorial treatment on oxidative stress. First, GSH was examined after 24 h treatment by using mBBr, which fluoresces after binding to free thiols. GSH is the most prominent free thiol in cells, and contributes to approximately 50% of the mBBr signal. The treated cells were gated for PI negative populations to restrict analysis to viable cells. Auranofin did not affect GSH levels when applied as a monotherapy in OCIP23 but had a significant impact in 9A (p=0.0345). However, BSO had a significant impact (OCIP23 p<0.0001; 9A p=0.0003) on GSH levels (Fig. 2.4A). The GSH status of MMP negative populations, both PI negative and positive, was assessed during the same live cell flow cytometry assay. Following 24 hour treatment both populations displayed depleted GSH compared to MMP positive populations (Fig. 2.3). These data suggest that the combination therapy may lead to cytotoxicity by depletion of GSH levels.
Figure 2.2. Representative two parameter plots measuring cell death and apoptosis in OCIP23. Cells were treated with either monotherapy or combination therapy for 24 hours followed by flow cytometry. Apoptotic cell populations are MMP-, PI- (bottom left quadrant); Dead cell populations are MMP-, PI+ (Upper left quadrant); Live cells are MMP+, PI- (bottom right quadrant). Shown are the percentages of the total population for each subpopulation.
Figure 2.3. Representative two parameter plots assessing the GSH content of apoptotic cells in OCIP23. Cells were treated with either monotherapy or combination therapy for 24 hours followed by flow cytometry. MMP is a marker for apoptosis, mBBr is a marker for GSH, and PI is a marker for cell viability. PI positive (non-viable) cells are shown in red, and PI negative (viable) cells are shown in grey.

Second, ROS were examined following 2 and 5 hour treatments by 2’,7’-dichlorofluorescin (DCF), which fluoresces following oxidation by ROS. Again, treated cells were gated for PI negative populations to restrict analysis to viable cells. At these time points, ROS were not affected by auranofin alone. However, BSO alone and in combination with auranofin displayed a significant dose-dependent increase (OCIP23 p<0.0001; 9A p<0.0001) in ROS levels (Fig. 2.4B). Combined, these results indicate that the combination of auranofin and BSO leads to oxidative stress in the
treated cells. This suggests that depletion of GSH and increase in ROS may contribute to cell death after combination therapy.

Auranofin binds to TrxR1 in primary pancreatic cancer cells at concentrations associated with oxidative stress and cell death.

We performed a cellular thermal shift assay (CETSA) to observe whether auranofin engages its targets TrxR1 and TrxR2 in intact primary pancreatic cancer cells at different concentrations (Fig.
This assay is based on thermostabilization of protein targets upon binding chemical ligands. It is more clinically relevant as it allows to test drug-target engagement in the complex cellular environment, as opposed to cell-free assays. As an initial step in CETSA, we treated 9A cells with a saturating concentration (10 µM) of auranofin and determined the optimal temperature at which we observed maximal thermostabilization of TrxR1/2 in treated cells compared to untreated controls. By analysis of thermal shift blots, we found 56°C to be the optimal temperature for TrxR1 and TrxR2 (Fig. 2.5A).

We then tested auranofin engagement after treating 9A and OCIP23 cells with increasing concentrations to determine whether it binds TrxR1/2 at concentrations associated with cell death. In both 9A and OCIP23 we observed TrxR1-auranofin binding at concentrations as low as 78nM (Fig. 2.5B and C). Binding was observed at concentrations much below the IC50’s of auranofin monotherapy in both cell lines. In addition, our CETSA data suggest that auranofin-mediated cell death is attributable to targeting TrxR1, but not TrxR2, at IC50’s observed after BSO combination (Table 2.1). Since OCIP23 and 9A cells exhibit different TrxR1 but not TrxR2 expression levels, our CETSA data provide further explanation of greater potentiation observed with OCIP23 compared to 9A cells. Taken together, our data show that auranofin bound TrxR1 in intact primary pancreatic cancer cells at concentrations capable of inducing cell death, and auranofin targets TrxR1 not TrxR2 at concentrations associated with BSO-mediated potentiation.
Figure 2.5. Auranofin-TrxR interaction in vitro. A) Engagement of auranofin and TrxR was assessed by CETSA. 9A was treated with 10µM auranofin for 1 hour. Following drug treatment, cells were exposed to the indicated temperatures for 3 minutes prior to lysis and immunoblot (n=1). B) 9A and C) OCIP23 were treated with different auranofin concentrations for 2 hours before heat treatment at 56°C and immunoblot (n=1).
Table 2.2. Panel of markers measured by IMC using subcutaneous PDX tumours.

<table>
<thead>
<tr>
<th>Antibody/Reagent</th>
<th>Antibody Clone</th>
<th>Metal</th>
<th>Final Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avanti Lipid</td>
<td>N/A</td>
<td>^115^In</td>
<td>10µM</td>
</tr>
<tr>
<td>α-SMA</td>
<td>1A4</td>
<td>^141^Pr</td>
<td>1.25</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>2G11/TRX</td>
<td>^146^Nd</td>
<td>10</td>
</tr>
<tr>
<td>EF5</td>
<td>ELK3-51</td>
<td>^149^Sm</td>
<td>10</td>
</tr>
<tr>
<td>Pimonidazole</td>
<td>N/A</td>
<td>^155^Gd</td>
<td>10</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>24E10</td>
<td>^155^Gd</td>
<td>2.5</td>
</tr>
<tr>
<td>Pan-keratin</td>
<td>C11</td>
<td>^164^Dy</td>
<td>0.6</td>
</tr>
<tr>
<td>Goat Anti-Mouse (Egr1 detection)</td>
<td>N/A</td>
<td>^165^Ho</td>
<td>1:100</td>
</tr>
<tr>
<td>Collagen</td>
<td>Goat polyclonal</td>
<td>^169^Tm</td>
<td>1.25</td>
</tr>
<tr>
<td>Intercalator</td>
<td>N/A</td>
<td>^191/^193^Ir</td>
<td>0.25µM</td>
</tr>
<tr>
<td>Auranofin</td>
<td>N/A</td>
<td>^197^Au</td>
<td>10mg/kg</td>
</tr>
</tbody>
</table>

Auranofin distributes within PDX tumours 24 hours after treatment, and associates with the stroma.

Auranofin contains gold in its molecular structure, which suggested the possibility to detect it in tissues using imaging mass cytometry (IMC), as we have recently described for platinum-based drugs. This novel high throughput technology detects heavy metal isotopes on a histological section. These heavy metals can either be linked to antibodies or found within the tissue. Data gained from this method can give information on the distribution of auranofin, its interactions with the tumour tissue, and an approximation of its concentration within different compartments of a tumour as long as auranofin is detectable with IMC. The antibody panel used can be seen in Table 2.2. In this pilot study we observed auranofin distributed throughout the two PDX tumours (Fig. 2.6). Auranofin appeared to be more closely associated with collagen in the stromal compartment of the tumour (Fig. 2.6A and C), rather than E-cadherin in the epithelial compartment (Fig. 2.6B and D). We then approximated the average number of gold ions per pixel (each pixel has a defined volume of 5µm^3) in each compartment (Table 2.3). This semi-quantitative method showed a
preferential accumulation of auranofin in the stroma. This method can be expanded in the future to gain a greater understanding of auranofin’s effects \textit{in vivo}.

\textbf{Figure 2.6. Distribution of auranofin in subcutaneous PDX tumours.} Mice were treated with 10mg/kg auranofin for 24 hours, followed by pimonidazole for 5hr and EF5 for 3 hours prior to sacrifice. Tissue section ablations were performed using IMC and markers were false-coloured. The relation of auranofin (green) to (A and C) stromal collagen (blue), (B and D) epithelial E-cadherin (blue), and pimonidazole (red) in OCIP83 (A and B) and 9A (C and D) tumours is shown. Scale bars are provided for reference. Arrows highlight regions of interest.
Table 2.3. Estimation of the mean ions/pixel in the stroma and epithelium of subcutaneous tumours from auranofin treated PDX mice.

<table>
<thead>
<tr>
<th>Model</th>
<th>Ions/pixel (Stroma)</th>
<th>Ions/pixel (Epithelium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCIP83</td>
<td>4.75</td>
<td>2.83</td>
</tr>
<tr>
<td>9A</td>
<td>8.67</td>
<td>8.28</td>
</tr>
</tbody>
</table>

2.4 Discussion

New strategies are required in the treatment of pancreatic cancer. Oxidative stress is an inherent feature in pancreatic cancer that can be potentially exploited by therapy. The described results provide evidence that a combination of BSO and auranofin at low clinically achievable drug concentrations\textsuperscript{278,279} yields anticancer efficacy \textit{in vitro} by augmenting oxidative stress.

In this study we assessed the effect of combination therapy on cell death and the redox environment of the cell. Through an MTS assay and flow cytometry-based assays we identified a strong potentiation of the cytotoxic effects of auranofin by BSO. Although BSO did not contribute to cell death on its own after 24 hour treatment, it was responsible for causing increased ROS and decreased GSH during the combination therapy. Low auranofin concentrations did not independently affect ROS and had a minimal effect on GSH in one cell line. However, it produced cytotoxic effects when combined with BSO. Overall, these findings are consistent with the predicted mechanism of action of a decreased antioxidant capacity and increased oxidative stress following combination of two antioxidant inhibitors\textsuperscript{64,83,204}. However, it was surprising to observe that low auranofin concentrations did not independently affect ROS. Other studies have assessed ROS following treatment with higher auranofin concentrations, and observed substantial increases\textsuperscript{280}. Our results suggest redundancy between the antioxidant pathways in that both GSH
and Trx moderate ROS levels, however the low auranofin concentrations are not required to affect ROS because of the effect of BSO in this context. It is possible that only a modest increase in ROS is required to kill the cells because of the concomitant depletion of antioxidants, although to our knowledge literature does not exist that elucidates the amount of ROS required for cytotoxicity in the context of antioxidant depletion. Moreover, some cytotoxicity can likely be attributed to downstream action of TrxR. As protein disulfides accumulate under oxidative stress in the cell, Trx is unable to remedy the damage because of impaired recycling through TrxR. Cells would lose their ability to maintain critical functions. These data intimate that these two antioxidant systems are critical to cancer cell survival. It is possible that other inhibitors towards these systems can be used to achieve similar results.

When assessing the engagement of auranofin and TrxR we utilized CETSA. This assay provided the capability to determine whether auranofin bound its target in the context of a complex biological system. Our observations show that auranofin engages TrxR1 at low concentrations compatible with cell death. Engagement with TrxR2 was not observed in this assay. This data is significant as it shows that auranofin interacts with its target in intact cells, whereas results obtained in cell-free systems eliminate the presence of other proteins that may interfere with this binding. This result provides greater support for auranofin’s proposed target, TrxR1, mediating the observed cytotoxicity. Future studies to show auranofin-TrxR1 engagement in vivo can further support this finding.

To assess the biodistribution of auranofin in the tumour we used IMC. This provided the unique insight into auranofin’s distribution 24 hours following treatment. In this pilot study we observed auranofin associated with collagen in the stromal compartment of the tumour. Although this has not been previously reported, IMC has previously observed cisplatin association with collagen in
PDX tumours. This finding is corroborated by the long half-life that has been observed in patients taking oral auranofin. Collagen may serve as a reservoir for the drug and contribute to its slow elimination. Our semi-quantitative approach to estimate the number of ions in each compartment trended towards an increase in the stroma compared to the epithelium, although the data was not significant. In the future this method can be applied to gaining an in depth understanding of the interaction of auranofin and molecular markers of oxidative stress to observe how auranofin affects the molecular biology of the tumour.

This study is limited in a few different aspects. First, the measure of GSH by mBBr fluorescence using flow cytometry is not specific for GSH. mBBr binds to all free thiols in cells. However, the background we captured is consistent with previously reported results. Second, although our data shows that there is an interaction of auranofin and TrxR1 in the cells, there is no functional output to measure the effect on TrxR1 activity. Therefore, it is difficult to determine how effective the low auranofin concentrations are at inhibiting TrxR1 activity. Third, the semi-quantitative method of determining the number of ions in the tumour can only provide an estimate, as only small regions of stroma and epithelium in each section could be analyzed, and the number of ions lost during acquisition cannot be accounted for.

In conclusion, we propose that targeting antioxidants can prove to be an effective new strategy in treating pancreatic cancer. In the context of the tested combination of BSO and auranofin, we observed that BSO potentiates auranofin by augmenting oxidative stress, allowing auranofin to elicit its cytotoxic effects at lower concentrations. Future in vivo studies are required to assess whether this method is feasible in the context of a living organism, as it is possible that the double hit may cause undesired toxicities in normal tissues. However, if basal oxidative stress occurs in the tumour then the reliance on antioxidants should make them more vulnerable to antioxidant...
inhibition. Furthermore, normal tissues, including hepatocytes, can withstand oxidative stress following Trx and GSH loss with dietary methionine\textsuperscript{253}. This work gives further evidence to support the growing body of evidence that suggests that cancers rely on antioxidants for their survival. Hopefully, this can be a step towards the future development of effective treatments for pancreatic cancer.
Chapter 3

Overall Discussion, Future Directions, and Conclusions
3.1 Overall Discussion

Oxidative stress is an interesting target for future cancer therapy development. Pancreatic cancer relies on antioxidants to protect from oxidative stress and promote proliferation. As such, it is sensitive to antioxidant depletion which may alter the redox environment. The reliance on antioxidants is specific to tumour tissue, whereas normal tissues may resist antioxidant loss\textsuperscript{253}. Targeting antioxidant systems may require a double hit as they can compensate for each other’s functions.

A common barrier to the development of novel therapies is the length of time from drug discovery to implementation in clinics\textsuperscript{282}. However, the strategy of augmenting oxidative stress avoids this barrier with the use of auranofin, which has been used in clinics, and BSO, which has been used in human clinical trials. Before a combination of the two can be used in human trials, preliminary tests are required to examine their effects \textit{in vitro} and \textit{in vivo}. Therefore, the goal of this thesis is to test the combination against pancreatic cancer and identify how the drugs interact.

3.1.1 BSO potentiates the acute cytotoxicity of auranofin

I first sought to examine the dose-response of primary human pancreatic cancer cells to both BSO and auranofin. I showed that BSO potentiates auranofin’s cytotoxicity at 24 hours and that response may depend partially on TrxR1 levels. Previous studies have examined the effect of these drugs on cell death, in particular apoptosis. At 24 hours BSO does not induce apoptotic cell death\textsuperscript{149}. In contrast, treatment of less than 24 hours with auranofin is sufficient to induce the mitochondrial permeability transition indicative of apoptosis\textsuperscript{258}. Of the TrxR isoforms present in the cell, TrxR1 inhibition is likely most responsible for mediating the response to auranofin, as it has been identified as a fitness gene\textsuperscript{209}. 
Following 24 hour treatment BSO did not affect viability of either cell line, indicating that the cells were resistant to acute GSH loss. Over the same timespan auranofin reduced viability in a dose-dependent manner. OCIP23 was more sensitive than the 9A cell line. Since BSO was not lethal on its own after 24 hours, synergy was excluded because by definition synergy requires an independent effect of each drug. I then treated cells with both drugs simultaneously for 24 hours and observed a strong potentiation of auranofin’s cytotoxic effects by BSO. Again, OCIP23 was more sensitive. The required concentrations of both drugs was within the safe plasma concentrations that have been achieved during human clinical trials of either drug\(^{278,279}\). This supports the idea that these cytotoxic concentrations can be attained if administered to a human patient. Although the discrepancy in sensitivity between the two cell lines can result from numerous factors, I observed a significant decrease in TrxR1, but not TrxR2, levels in OCIP23. As TrxR1 has been identified as a fitness gene this difference may contribute to sensitivity to this combination therapy and may potentially be validated in the future as a biomarker to predict response to auranofin therapy.

The MTS data showed an odd result in that low auranofin concentrations seemed to improve viability. However, this may be an artifact of the MTS method itself, in that it measures NADH production from mitochondrial metabolism which may accumulate in dysfunctional mitochondria during oxidative stress\(^{283}\). The clonogenic assay showed that these concentrations did not improve long-term cell growth, and when used in the combination therapy effectively inhibited clonogenicity.

### 3.1.2 Apoptosis contributes to cell death

I also identified that apoptosis contributes to the mechanism of cell death of the combination therapy. Previous work has shown apoptosis caused by BSO following treatment of 48 hours and
longer\textsuperscript{149}, and apoptosis following short-term auranofin treatment\textsuperscript{258}. The combination also displayed apoptosis as shown by the mitochondrial permeability transition. However, this cannot exclude other mechanisms of cell death as contributors, including senescence and ferroptosis. Ferroptosis in particular may likely contribute as a result of augmented oxidative stress but was not assessed using the current assays\textsuperscript{284}.

3.1.3 The drugs engage their expected targets

An important observation when assessing a therapy is to determine whether the drugs enact their expected primary effect. In the case of BSO, GCL inhibition is expected to decrease GSH levels as previously observed\textsuperscript{83}. I identified that in the primary pancreatic cancer cells there was a decrease in GSH following both BSO monotherapy and in combination with auranofin. Thus, BSO enacted its primary role of preventing GSH synthesis. Auranofin is expected to decrease TrxR activity through direct interactions with TrxR. Although this thesis does not include an assay for TrxR activity, it is well-established that auranofin can reduce or eliminate TrxR activity\textsuperscript{83,254}. Instead I used CETSA to examine drug-target interaction in the context of the complex cellular environment. I observed that auranofin engaged TrxR1 at low concentrations compatible with cell death during combination therapy, indicating that auranofin does in fact bind its proposed target in the cell when used at low concentrations.

3.1.4 Oxidative stress follows combination therapy

The downstream effects following target inhibition are important to understand when assessing a potential new therapy. I observed that simultaneous inhibition of GSH and Trx augments oxidative stress by increasing ROS. Previous studies have observed this phenomena following both BSO and auranofin monotherapy\textsuperscript{83,149,254}. As such, the studied combination therapy caused the expected downstream effect. Of note, the increase in ROS was modest and of lower magnitude than
previously reported with higher auranofin concentrations\textsuperscript{83,149}. However, the observed increase in ROS was still capable of inducing cell death. Unfortunately, it is difficult to make comparisons to other work. This is because other studies mostly focus on the amount of exogenous ROS required to kill cells, rather than ROS from endogenous sources\textsuperscript{285}. Furthermore, the amount of ROS required to kill in the context of a cell with compromised antioxidant defences has not been previously determined. It is likely that antioxidant depletion allows for this modest acute spike in ROS to elicit cytotoxic effects. Lastly, it should be noted that higher auranofin concentrations (ie. monotherapy IC50 concentrations) have been shown to induce ROS\textsuperscript{83}, which would resolve their cytotoxic effects as a monotherapy in light of my results showing that low auranofin concentrations do not independently augment ROS.

3.1.5 IMC is an effective tool to examine auranofin in vivo

Powerful analytical tools are required to understand the full effect of a therapy in vivo. Auranofin’s unique chemistry provides the potential of IMC identification in tissues, as has been reported with cisplatin\textsuperscript{275}. My pilot study determined that gold from auranofin can be identified by IMC 24 hours after drug administration. As with cisplatin, it displays a preferential association with collagen in the PDX tissue stroma versus E-cadherin in the epithelium\textsuperscript{275}. However, this data does not provide insight to the biodistribution at shorter time points and may be suggestive of the auranofin reservoir following elimination of most auranofin molecules. This would be consistent with auranofin’s exceptionally long total body elimination (55-80 days) observed during clinical trials\textsuperscript{286}. A larger number of samples are required to prove these results, as this was only a pilot study. Lastly, it should be noted that IMC cannot distinguish between auranofin and atomic gold. Therefore, the observations of auranofin distribution in the stroma may be indicative of gold following auranofin
breakdown. However, this is consistent with previous methods that restrict detection to atomic gold when evaluating auranofin\textsuperscript{287}.

The significance of this finding is that IMC can be a tool for the assessment of a larger cohort to develop an in-depth study of auranofin’s effects in mouse and/or human clinical trials. An antibody panel can be developed to examine auranofin’s interactions with different tissue structural features, cell proliferation or growth arrest, hypoxia, cell signaling, and many other potential features of interest.

3.2 Future Directions

A number of different avenues can be explored following the work described in this thesis. The first possibility would be to study the mechanism of action greater detail. As previously mentioned, apoptosis contributes to cell death following the combination therapy. However, the presented data cannot exclude other mechanisms of death such as ferroptosis, which can result from excessive lipid peroxidation that occurs during oxidative stress\textsuperscript{284}. The fluorescent probe C11-BODIPY can be used to assess the extent of lipid peroxidation in a convenient flow cytometry assay that includes probes for ROS, GSH, viability, and MMP\textsuperscript{288}. Another avenue could be to perform experiments to further support the current data. The current method for GSH measurement uses mBBr fluorescence, which also detects protein thiols. A modified version of the Tietze enzymatic recycling assay can provide a direct biochemical measurement of GSH following BSO treatments\textsuperscript{289}. The current method to assess auranofin efficacy detects whether auranofin binds its target. However, this does not specify the extent of TrxR activity loss. An insulin reduction assay can be performed to assess TrxR activity at the same concentrations\textsuperscript{290,291}. 
To optimize therapeutic results alterations to the dosing regimen can be tested. For example, BSO concentrations can be decreased while increasing auranofin. This can be changed to ensure that doses of each drug that are easiest to achieve in humans are used. Furthermore, alternative treatment lengths can be tested. This may maximize cytotoxic effects of BSO that have been observed when used for 48 hours and longer\(^{149}\). Lastly, the strategy for therapy can be altered. The current study uses simultaneous administration of BSO and auranofin. However, since GSH depletion by BSO requires time to commence it is possible that an alternative strategy may take advantage of this. BSO pre-treatment for 24 hours may possibly maximize the effect of auranofin as the second hit would be administered in a GSH knockdown system. Other alterations to the strategy may include additions to the current combination. For example, lower doses of BSO and auranofin may sensitize tumours to chemo or radiotherapy\(^{83,202}\). Chemotherapy drugs like gemcitabine or ionizing radiation can be used in combination with reduced BSO and auranofin doses to optimize responses.

Lastly, the most significant option for future studies would be to examine the efficacy of this combination therapy in orthotopic PDX mice. This approach would allow for testing in a model that is the most representative of tumours seen in clinics. First, a few mice can be used to determine the safety of the combination, as adverse events can be unpredictable. If the therapy is deemed safe a larger experiment can be performed. The design of such an experiment would place mice in 4 treatment arms: control, auranofin, BSO, and combination therapy. Numerous endpoints can be assessed including survival, changes in tumour volume, and number of metastases. In addition, a comparison can be made to the current standard of care if another group is used. It is possible that sensitivity to therapy may depend on TrxR1 levels. As such, tumours bearing different amounts of TrxR1 can be tested.
This experiment also provides the opportunity to assess the effects of auranofin using IMC. Various questions can be postulated and answered if the correct antibody panel is used on the tumour tissue. This can also compare differences between monotherapy and combination therapy. In conjunction, a shorter time-course experiment can examine the timing of different effects following therapy. These experiments would provide unique insights into how auranofin affects tumour tissue \textit{in vivo}, and whether this combination therapy is safe and effective enough to receive attention in human clinical trials. An additional benefit of running a trial like this would be that CETSA can be performed on tumours to assess auranofin engagement with TrxR \textit{in vivo}.

3.3 Conclusions

Oxidative stress is a hallmark of pancreatic cancer that deserves consideration in the development of new therapies against pancreatic cancer. I conclude in chapter 2 that the current study supports the efficacy of a combination therapy using BSO and auranofin to treat pancreatic cancer. In this combination BSO potentiates auranofin’s cytotoxic effects. This therapy results in both antioxidant depletion and augmentation of oxidative stress. Furthermore, auranofin is detectable by IMC analysis. Future work can further characterize the effects of this combination and identify whether it is feasible for clinical use in human pancreatic cancer patients.
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