INHIBITION OF THE THIOREDOXIN SYSTEM: REGULATION BY THE CANCER CELL ENVIRONMENT

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ABSTRACT

The oxygen environment in tumors is not static and involves constant cycling between hypoxic and re-oxygenation phases, a phenomenon known as intermittent hypoxia. Hypoxic and redox pathways are upregulated in response to intermittent hypoxia. The thioredoxin system, comprised of thioredoxin and thioredoxin reductase, is one of the main antioxidant systems, while hypoxia inducible factor 1 (HIF1) is the major hypoxia responsive system. High levels of both the thioredoxin system proteins and HIF1α have been correlated with extremely aggressive and highly metastatic tumors. Both these systems have also been linked to development of resistance against anti-cancer therapies. Moreover, HIF1α is indirectly redox regulated by thioredoxin, suggesting a potential cross-talk between the two systems, which becomes more apparent under intermittent hypoxia. Therefore, an understanding of these two systems under different oxygen conditions occurring in cancers may aid in the designing more effective therapeutics.

Initial studies were performed using auranofin, a gold-based thioredoxin reductase inhibitor as a tool to inhibit the thioredoxin system. The response of MDA-MB-231 breast and A549 lung cancer cells to this inhibition, with respect to the thioredoxin system, was studied under different oxygen parameters involving normoxia (20% oxygen), hypoxia (0.1% oxygen), and preconditioning with intermittent hypoxia (short cycles of hypoxia/re-oxygenation prior to a prolonged hypoxic insult). Cell proliferation assays revealed that the different oxygen conditions did not affect auranofin associated cytotoxicity in cancer cells. However, the oxygen conditions influenced auranofin’s mode of action by affecting its ability to inhibit the thioredoxin reductase activity. Promoter reporter assays demonstrated that cells respond to auranofin induced inhibition by upregulating the promoter activity of both thioredoxin and thioredoxin reductase, which was also influenced by the oxygen conditions. Western blotting showed that preconditioning cells with intermittent hypoxia prior to auranofin treatment increased the levels of thioredoxin but not thioredoxin reductase protein in cancer cells. Heme oxygenase-1, another antioxidant protein, was found to provide cytoprotection
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against auranofin induced inhibition of the thioredoxin system under all the tested oxygen conditions.

The effect of auranofin induced inhibition of the thioredoxin system on the HIF1α system was also studied under the different oxygen parameters. Preconditioning with intermittent hypoxia enhanced the stabilisation of HIF1α protein but did not affect its transcriptional activity, which was examined directly by HIF1α-CAD activity assays and indirectly through the expression of vascular endothelial growth factor (VEGF). The hypoxic response, with respect to HIF1α stabilisation and activation, was found to be cell-line specific and was not statistically significantly influenced by auranofin treatment.

The role of thioredoxin’s redox activity was also studied in the migration and clonogenic activity of cancer cells. The MDA-MB-231 cells over-expressing thioredoxin and its redox inactive mutated form (1SS) were used along with auranofin in monolayer scratch assays. Inhibition of the thioredoxin system either by 1SS over-expression or by auranofin resulted in a decrease in cancer cell migration. Thioredoxin over-expression did not affect this migration. Moreover, auranofin treatment inhibited the clonogenic ability of cancer cells, thereby implying that the thioredoxin system may be a potential anti-metastatic target.

Auranofin’s cytotoxicity was evaluated in a 3D culture model compared to a 2D monolayer model. The results suggested that auranofin was less potent towards MDA-MB-231 breast cancer cells grown as 3D spheroids in comparison to those grown as 2D monolayers, indicating that the tissue architecture may play a role in influencing the sensitivity of cancer cells towards chemotherapeutics.
STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Maneet Bhatia
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  (Includes in part the work presented in Chapter 5)

• Maneet Bhatia, Carrie J. Lovitt, Prahlad V. Raninga, Vicky M. Avery, Giovanna DiTrapani, and Kathryn F. Tonissen (2016). Expression of the Thioredoxin System is Modulated by the *In Vivo*-like Cancer Cell Environment upon Auranofin Treatment. *Manuscript submitted*

  (Includes work presented in Chapters 3 and 6)
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<td>Two-dimensional</td>
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<tr>
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<td>ANOVA</td>
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<td>Activator Protein-1</td>
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<td>Ammonium Persulphate</td>
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<td>ARE</td>
<td>Antioxidant Responsive Element</td>
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<td>ARNT</td>
<td>Aryl hydrocarbon Receptor Nuclear Translocator</td>
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<td>Apoptosis Signalling Kinase-1</td>
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<td>Arsenic trioxide</td>
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<td>Area Under Curve</td>
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<td>Basic helix-loop-helix</td>
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<td>Bovine Serum Albumin</td>
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<td>Carbonic Anhydrase-IX</td>
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<tr>
<td>CBP</td>
<td>CREB-Binding Protein</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>Carbon dioxide</td>
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<td>3-(Dimethylamino)propionitrile</td>
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<td>Dimethyl Sulfoxide</td>
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<td>Dithiothreitol</td>
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<td>Full Form</td>
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<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>Factor Inhibiting HIF-1</td>
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<td>Gal Responsive Element</td>
</tr>
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<td>Green Fluorescent Protein</td>
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<td>Growth Factor Reduced</td>
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<td>Hydrogen peroxide</td>
</tr>
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<td>Heme Oxygenase-1</td>
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<tr>
<td>HRE</td>
<td>Hypoxia Responsive Element</td>
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<tr>
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<td>Hypoxia Re-oxygenation</td>
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<td>Horseradish Peroxide</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half-maximum Inhibitory Concentration</td>
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<td>IF</td>
<td>Immunofluorescence</td>
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<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
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<td>IPAS</td>
<td>Inhibitory PAS</td>
</tr>
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<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like Erythroid cell-derived Protein1</td>
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<td>MDR</td>
<td>Multidrug Resistance</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MSR</td>
<td>Methionine Sulfoxide Reductase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
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<td>NAD</td>
<td>Amino-terminal transactivation domain</td>
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<td>NAPDH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NF-κβ</td>
<td>Nuclear Factor-κβ</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear Factor (erythroid-derived 2)-like 2</td>
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<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>ODD</td>
<td>Oxygen-dependent Degradation Domain</td>
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<td>OSA</td>
<td>Obstructive Sleep Apnea</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>PAS</td>
<td>Per-ARNT-Sim</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<td>PC</td>
<td>Preconditioning</td>
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<td>Polymerase Chain Reaction</td>
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<td>PEG</td>
<td>Polyethylene Glycol</td>
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<td>Pgp</td>
<td>P-glycoprotein</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PHD</td>
<td>Prolyl Hydroxylase Domain-containing proteins</td>
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<td>Prx</td>
<td>Peroxiredoxin</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<td>Ref-1</td>
<td>Redox Factor-1</td>
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<tr>
<td>RFU</td>
<td>Relative Fluorescence Unit</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Luminescence Unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleotide Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-PCR</td>
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<tr>
<td>SCM</td>
<td>Serum Containing Media</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>Sec</td>
<td>Selenocysteine</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum Free Media</td>
</tr>
<tr>
<td>siRNA</td>
<td>small inhibitory RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>tBHQ</td>
<td>tert-Butylhydroquinone</td>
</tr>
<tr>
<td>TGR</td>
<td>Thioredoxin Glutathione Reductase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Matrix Metalloproteinase</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin Reductase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
</tr>
<tr>
<td>ZnPP</td>
<td>Zinc Protoporphyrin</td>
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CHAPTER 1

INTRODUCTION
1.1 General Introduction

The tumor microenvironment has several components, including hypoxia, the extracellular matrix (ECM), and different cell types. The interaction between these elements is an important determinant of carcinogenesis. With respect to the oxygen availability, the tumor environment is quite dynamic and cycles between low and high oxygen conditions, a phenomenon termed as intermittent hypoxia. Under these unstable oxygen conditions two important regulatory systems come into play- the redox and the hypoxic systems.

The redox systems involve antioxidants that maintain the oxygen homeostasis of cells by scavenging reactive oxygen species (ROS). The thioredoxin system, comprised of thioredoxin and thioredoxin reductase, is one of the most important antioxidant systems that is present in all species. Additional functions of thioredoxin include regulation of transcription factors, control of apoptosis, and role as a growth factor (as reviewed by Holmgren, 1985). The hypoxic systems are activated under low oxygen availability and upregulate genes involved in the protection of cells under hypoxia. Hypoxia inducible factor 1 (HIF1) is an important transcription factor that regulates the expression of several proteins required during low oxygen conditions (Greer et al., 2012). Interestingly, HIF1 is redox regulated by thioredoxin (Welsh et al., 2002).

Both thioredoxin and HIF1 are expressed at higher levels under intermittent hypoxia and have also been linked to not only the invasive phenotype of cancer cells but also to the development of resistance against anticancer drugs (Semenza and Prabhakar, 2007, Liu et al., 2010, Malec et al., 2010). Thus, the oxygen environment is extremely important in cancer biology because cancer cells constantly switch between high and low oxygen conditions. These dynamic changes may influence how anti-cancer drugs that are designed and tested under environmental oxygen conditions perform in vivo.

Tumor architecture plays a critical role in regulating cancer cell signalling and metastasis (Gilkes et al., 2014). In recent years, the in vivo cell-cell and cell-ECM interactions have been established as important determinants of drug response (Ivascu and Kubbies, 2007, Lovitt et al., 2014). This has led to the development of 3D culturing
models, involving the ECM components, and is expected to bridge the gap between 2D in vitro culturing and the in vivo animal models.

Therefore, the overall aim of this project was to study how inhibition of the thioredoxin system is influenced by the cancer cell microenvironment.

1.2 Oxygen Homeostasis

Oxygen is essential for the survival of all living beings. A balanced oxygen environment is required since both lower and higher than the required oxygen levels can be detrimental to the cells (Figure 1.1). Different organs in the body exist under different oxygen environments, depending on the location and function of the cells in an organ. Most healthy organs reside in approximately 3-6% oxygen, which can be termed as ‘tissue normoxia’ or ‘physioxia’ (Carreau et al., 2011) while conditions lower than 3% oxygen are described as hypoxia. Cells also survive in hypoxic environments during normal development (Muniyappa et al., 2009). However, hypoxia is mostly detrimental to the cells by disrupting the oxygen homeostasis.

![Figure 1.1: Oxygen homeostasis. Low cellular oxygen results in hypoxic stress causing cells to upregulate pathways involved in increasing the oxygen supply. On the other hand, higher oxygen levels result in oxidative stress and many antioxidants are induced in response to reduce the available oxygen and prevent the subsequent cellular damage (Bhatia, 2013).](image)
Cancer cells are capable of surviving under hypoxic conditions by inducing the expression of metabolic enzymes required for anaerobic metabolism. To fulfill their oxygen and nutritional requirements, cancer cells can also stimulate the formation of blood vessels by a process called angiogenesis. The transcription factor HIF1 is responsible for induction of specific gene expression by binding to hypoxic response elements (HRE) present in the promoters of these target genes, which are essential for cells to survive under a low oxygen environment, as reviewed recently by Greer and co-workers (Greer et al., 2012). When hypoxic tumor cells are re-oxygenated due to angiogenesis, oxidative stress may occur. However, angiogenesis in tumors is aberrant due to sparse arteriolar supply (Dewhirst et al., 1999), low vascular density (Dewhirst et al., 1996), and inefficient orientation of microvessels (Secomb et al., 1993). This creates a scenario where cancer cells are in flux, where they cycle between hypoxia and the re-oxygenated state. There are two dominant timescales that contribute to the cycling kinetics. One is of a faster frequency with a few cycles per hour and primarily arises from fluctuations in red blood cell flux (Lanzen et al., 2006). The slower timescale varies from hours to days and is due to vascular remodelling (Nehmeh et al., 2008). This makes angiogenesis irregular with respect to both space and time, thereby leading to an unstable cancer environment that oscillates between low and high oxygen conditions. This cycling phenomenon is termed as intermittent hypoxia or cycling hypoxia (Dewhirst, 2007). The involvement of re-oxygenation phases in intermittent hypoxia suggests that cancer cells experience oxidative stress in addition to hypoxic stress and may utilise discreet mechanisms to counteract these stresses.

1.3 Oxidative Stress

Oxidative stress occurs when there is more than the required amount of oxygen in the cellular environment. Due to incomplete reduction of excess oxygen, highly reactive and unstable molecules known as ROS are produced. As a by-product of respiration, the majority of ROS are generated in the mitochondria; however ROS are also produced in other organelles, such as the cytosol, peroxisomes, nuclear membrane and endoplasmic reticulum. ROS can also be produced due to external factors, such as UV exposure (as reviewed by Nordberg and Arner, 2001).
ROS have several beneficial roles, such as intracellular signalling and redox regulation; serving as a mediator for the activity of important transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1); and acting as a secondary messenger for several cytokines, growth factors and hormones (Thannickal and Fanburg, 2000). Phagocytes also produce ROS to kill pathogens (Vignais, 2002). However, being highly reactive and unstable, ROS are generally detrimental to the cells by causing toxicity and mutagenesis. To protect themselves from the harmful effects of ROS, cells utilise redox systems, such as antioxidant proteins.

### 1.3.1 Redox System under Oxidative Stress

An important antioxidant system that is present in all species and is conserved through evolution is the thioredoxin system. It comprises thioredoxin and thioredoxin reductase and catalyses oxidoreductase reactions through a dithiol-disulfide exchange mechanism (Figure 1.2) (as reviewed by Holmgren, 1985). Thioredoxin is a small 12kDa protein containing an active site motif of Cys-Gly-Pro-Cys. Reduced thioredoxin catalyses the reduction of disulphide bonds in other oxidised proteins and in the process itself becomes oxidised such that a disulphide bond forms between the two cysteine residues in its active site. Thioredoxin is then restored to a reduced state by thioredoxin reductase with the use of NADPH as an electron source (as reviewed by Holmgren, 1985).

![Figure 1.2: Mechanism of action of the thioredoxin system.](image)

Thioredoxin in its active form [TRX-(SH)₂] catalyses the reduction of several cellular proteins and in this process, it itself gets oxidised [TRX-S₂]. The oxidised thioredoxin can be restored to its active reduced form by thioredoxin reductase in presence of NADPH (adapted from Nordberg and Arner, 2001 with permission from Elsevier).
Thioredoxin is a globular protein and has a hydrophobic core formed by five β-pleated sheet strands. The core is surrounded by four helices towards the periphery. Two highly conserved cysteine residues (Cys32 and Cys35) form the redox active site (Jeng et al., 1994). A protrusion is formed between one of the helices and the middle of a pleated sheet by the two cysteine residues, making the redox-active site accessible from the side of the protein (Figure 1.3). There are three isoforms of mammalian thioredoxin: the cytosolic thioredoxin1, the mitochondrial thioredoxin2, and Sp thioredoxin, which is expressed in the sperm (as reviewed by Holmgren, 1985). This thesis will focus on thioredoxin1 unless otherwise specified.

**Figure 1.3: Structure of thioredoxin protein.** The thioredoxin fold comprises five β-pleated sheet strands (blue) surrounded by four α-helices (purple). The redox active site contains two important cysteine residues (yellow) (adapted from Jeng et al., 1994 with permission from Elsevier).

Thioredoxin reductase is a 55-65kDa selenoenzyme. It has an active site motif of Cys-Val-Asn-Val-Gly-Cys (as reviewed by Holmgren, 1985). Thioredoxin reductase also contains a selenocysteine (Sec), which is crucial for the enzymatic activity of the protein (Figure 1.4) (Zhong et al., 2000). Thioredoxin reductases have a wide range of substrates, such as 5,5´-dithio-bis(2-15 nitrobenzoic acid) (DTNB) (Holmgren, 1977), hydrogen peroxide (H₂O₂) (Sun et al., 1999), and selenium-containing compounds (Kumar et al., 1992). Until recently, thioredoxin reductase was the only known protein capable of reducing thioredoxin, however, the glutathione system has now been identified to act as a backup for reducing thioredoxin in absence of thioredoxin reductase (Du et al., 2012). Mammalian thioredoxin reductase also has three isoforms:
cytosolic thioredoxin reductase1, mitochondrial thioredoxin reductase2, and testis specific thioredoxin glutathione reductase (TGR) (Sun et al., 2005). This project will focus on thioredoxin reductase1.

Figure 1.4: A schematic of the thioredoxin reductase protein. Thioredoxin reductase has an active site motif of CVNVGC and contains a selenocysteine residue that is critical for its activity (reprinted with permission from Zhong et al., 2000).

1.3.1.1 Functions of Thioredoxin

Thioredoxin is found in the cytoplasm, in the nucleus and also in the extracellular environment and it has distinct functions in each location (Figure 1.5). The key function of the thioredoxin system is to maintain the redox balance of cells by either directly scavenging highly unstable and reactive molecules known as ROS (Das and Das, 2000) or by regulating the activity of several other important enzymes, such as peroxiredoxins (Prx) (Rhee et al., 2005) and methionine sulfoxide reductase (MSR) (Kim and Kim, 2008), which also maintain the cellular oxygen balance. Peroxiredoxins are a family of small (22-27 kDa) peroxidases comprised of 6 isoforms. They use their -SH groups as reducing equivalents and act to reduce peroxides such as H$_2$O$_2$, organic hydroperoxides and peroxynitrite (Rhee et al., 2005). The oxidised form of peroxiredoxins can then be recycled back to their active reduced form through the action of an electron donor, which for peroxiredoxins 1-5 is thioredoxin.

The MSR family consists of MSRA and MSRB antioxidant proteins and provides an indirect defense against ROS. Methionine residues in several proteins become oxidised by ROS to Met-S-O and Met-R-O, epimers of methionine sulfoxide (Met-O). This can render the proteins non-functional. MSRA and MSRB can restore the functionality of proteins by reducing the Met-S-O and Met-R-O bound proteins respectively (Zhang et
al., 2010). During this process the MSR proteins become oxidised, but are reduced to their active form by thioredoxin.

Thioredoxin also directly interacts with the apoptotic pathway by binding to apoptosis signal-regulating kinase-1 (ASK-1), a member of the MAPKKK family. The reduced form of thioredoxin binds to ASK-1 but in the presence of ROS, thioredoxin becomes oxidised and dissociates. This allows the free ASK-1 to promote apoptosis (Saitoh et al., 1998).

In the nucleus, thioredoxin is responsible for regulating the activity of several transcription factors. NF-κβ is a transcription factor involved in the regulation of apoptosis and is activated in response to ROS (Hirota et al., 1999). Under normal conditions, NF-κβ is inhibited by I-κβ, which keeps NF-κβ sequestered in the cytosol. In response to oxidative stress, I-κβ is degraded and releases NF-κβ, which is translocated to the nucleus. In the nucleus, thioredoxin directly reduces Cys62 in the p50 subunit of NF-κβ, which allows NF-κβ to bind to the specific recognition sequence in the promoter.

Figure 1.5: Localisation of thioredoxin with some of its functions and the pathways it regulates (Bhatia, 2013).
of its target genes, such as those involved in cell survival, to induce their expression (Hirota et al., 1999). Thus, thioredoxin contributes to the upregulation of anti-apoptotic proteins.

Thioredoxin can also regulate transcription factors via indirect mechanisms through redox factor-1 (Ref-1), which is an intermediate protein that reduces several other transcription factors to enhance their binding to the promoters of their target genes (Xanthoudakis and Curran, 1992). AP-1 is a heterodimeric complex of Fos and Jun proteins that binds to the DNA regulatory element known as the AP-1 binding site (Abate et al., 1990). AP-1 mediates growth of cells in response to external stimuli. Thioredoxin acts on Ref-1, which in turn activates AP-1 by reducing the highly conserved cysteine residues in the DNA-binding domains of Fos (Cys154) and Jun (Cys272) (Xanthoudakis and Curran, 1992). Therefore, thioredoxin is also involved in cell growth. Furthermore, under hypoxic conditions, thioredoxin activates HIF1 through Ref-1 (Ema et al., 1999).

Thioredoxin is also secreted by a variety of normal and neoplastic cells through an as yet unknown pathway (Rubartelli et al., 1992). Secreted thioredoxin has been implicated in immune responses (Angelini et al., 2002, Schwertassek et al., 2007) and in cell survival mechanisms (Backman et al., 2007, Mougiakakos et al., 2011). Extracellular thioredoxin has been suggested to have chemotactic activity and to act as chemo-attractant for neutrophils, monocytes and T-cells (Bertini et al., 1999). Extracellular thioredoxin has also been associated with cancer cell metastasis (Farina et al., 2001) and the promotion of a matrix metalloproteinase-9 (MMP-9) dependent invasive phenotype in malignant breast cancer cells (Farina et al., 2011).

### 1.3.1.2 Induction of the Thioredoxin System by Oxidative Stress

In order to maintain the cellular oxygen homeostasis, antioxidant systems are upregulated under oxidative stress. The induction of thioredoxin expression during oxidative stress occurs primarily through an antioxidant response element (ARE) in the thioredoxin gene promoter. ARE elements are short cis-acting elements found in the
promoter regions of many genes encoding antioxidant enzymes and they regulate gene expression during oxidative stress (Rushmore et al., 1991). A redox-sensitive transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) plays a critical role in mediating the antioxidant gene expression via the ARE element (Ishii et al., 2000). Nrf2 is ubiquitously expressed in most tissues and is continuously degraded in the cytosol under normal oxygen conditions via its inhibitor “kelch-like erythroid cell-derived protein1” (Keap1) (Itoh et al., 1999). Keap1 contains several cysteine residues that act as redox sensors. Upon changes in the cellular oxygen environment, these cysteine residues are oxidised (Zhang and Hannink, 2003). As a result, Keap1 undergoes a conformational change and releases Nrf2, which is translocated into the nucleus (Rushmore et al., 1991). In the nucleus, Nrf2 forms a heterodimer with small maf proteins and binds to the ARE of the target antioxidant genes (Itoh et al., 1997), including thioredoxin (Kim et al., 2001) and thioredoxin reductase (Hintze et al., 2003) (Figure 1.6).

Figure 1.6: Antioxidant gene expression via the ARE/Nrf2 pathway (Bhatia, 2013).
1.4 Hypoxic Stress

The other type of stress encountered by the cells is known as hypoxic stress and occurs under lower than the normal oxygen conditions or physioxia. As stated earlier, cells survive in a hypoxic environment during development but hypoxia is mostly harmful to the cells. Several vital genes are expressed under hypoxia, including genes encoding metabolic enzymes to allow growth under hypoxia and proteins that assist hypoxic tissues to re-establish oxygen supply. The hypoxia mediated gene response is different from that mediated under oxidative stress and occurs through the HRE present in the promoter of the target genes. This pathway involves HIF1 that regulates the expression of several vital genes in response to oxygen deficient conditions (Greer et al., 2012). HIF1 is a complex of two subunits: aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF1β, which is constitutively expressed in all cells, and HIFα, which is stabilised under hypoxia. Normally, HIFα is synthesised and continuously degraded in the cytosol, but in response to a low oxygen environment it starts accumulating rapidly (Salceda and Caro, 1997). Under these low oxygen conditions, HIFα is translocated into the nucleus, where it dimerises with HIF1β to form the HIF1 complex, which then binds to the HRE in the promoters of target genes to activate their expression (Greer et al., 2012) (Figure 1.7).

![Figure 1.7: Regulation of the HIF1 signalling pathway and the expression of its target genes](Bhatia, 2013)
1.4.1 HIF Proteins and Hypoxic Regulation

Both HIF1 subunits belong to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription factors. The bHLH domain aids in DNA-binding while the PAS domain mediates protein-protein interaction. Both domains also act as an interface for dimerisation of the α and β subunits (Huang et al., 1993). There are three identified HIFα subunits (Greer et al., 2012) and one β subunit, which is alternatively spliced (Qin et al., 2001). HIF1α is the most characterised form and will be discussed in this thesis. HIF1α and HIF2α have structurally similar DNA binding and dimerisation domains, but they differ in their transactivation domains. This may explain why a genome wide screen detected both HIF1α and HIF2α bound to the same HRE consensus sites, but without initiating the same transcriptional response (Mole et al., 2009). Moreover, HIF2α is only expressed in certain tissues (Wiesener et al., 2003), while HIF1α is ubiquitously expressed.

Overall the biological actions of HIF1α and HIF2α in response to hypoxia are distinct, as reviewed by Loboda and colleagues (Loboda et al., 2010). For example, HIF1α, but not HIF2α regulates the transcription of genes encoding enzymes involved in glycolysis (Hu et al., 2003), while HIF2α has been associated with adaptation to high altitude exposure (van Patot and Gassmann, 2011). Furthermore, Bracken and co-workers showed in PC12 rat cells that HIF1α required a shorter duration (4 hours) under hypoxia to be stabilised, whereas a longer hypoxic exposure (16 hours) was required for HIF2α stabilisation. However, this difference was cell-line specific (Bracken et al., 2006). In human colon cancer, advanced tumors displayed strong HIF1α staining and weak HIF2α staining, while in early stage tumors, strong HIF2α and weak HIF1α staining was observed. This implies that HIF1α and HIF2α have different roles in colon cancer (Imamura et al., 2009). The third HIFα subunit, HIF3α has an inhibitory function since it lacks the transactivation domain, but binds to HIF1α and prevents it from activating transcription. Therefore, HIF3α is also called ‘inhibitory PAS domain’ (IPAS) and arises as an alternatively spliced product of the HIF1α gene (Makino et al., 2001).
There are two transactivation domains in HIF1α: the amino-terminal transactivation domain (NAD) and the carboxy-terminal transactivation domain (CAD) (Jiang et al., 1997, Pugh et al., 1997). These domains (Figure 1.8) are involved in the transcriptional activation of HIF1α under hypoxia. The NAD overlaps the oxygen-dependent degradation domain (ODD), linking the transcriptional activity of HIF1 with the stabilisation of the protein (Pugh et al., 1997). On the other hand, the transcriptional activity of the CAD is associated with the binding of transcriptional co-activators, including CREB-binding protein (CBP)/p300 (Kallio et al., 1998).

**Figure 1.8: Schematic of HIF1α domains.** A representation of HIF1α domains, including the two transactivation domains (NAD and CAD), DNA-binding domain, oxygen-dependent degradation domain, and binding sites for prolyl hydroxylase domain-containing proteins (PHDs), factor inhibiting HIF-1 (FIH-1) and thioredoxin/Ref-1 (Bhatia, 2013).

The recruitment of the co-activators is redox-regulated and requires Ref-1, which reduces the cysteine residue at position 800 of HIF1α within the CAD region (Ema et al., 1999). The co-activators are then able to bind HIF1 and subsequently initiate transcription. It should be noted that Ref-1 is an intermediate protein that is regulated by thioredoxin.
INTRODUCTION

1.4.2 HIF1 Regulation under Normoxia

Although the HIF1α proteins are activated in response to hypoxia, they do not sense the changes in the oxygen environment themselves. Sensors to such changes have been identified as oxygen-dependent hydroxylases. The hydroxylases responsible for modifying HIF1α are the ‘prolyl hydroxylase domain-containing proteins’ (PHDs) and an asparaginyl hydroxylase called ‘Factor Inhibiting HIF-1’ (FIH-1) (Lando et al., 2002b). These hydroxylases continuously modify HIF1α in presence of oxygen. When there is a negative change in oxygen availability, PHDs and FIH-1 can no longer hydroxylate HIF1α, which is stabilised and translocated to the nucleus (Pouyssegur et al., 2006) (Figure 1.9).

Under higher oxygen conditions, PHDs modify distinct proline residues (Pro 402 and Pro 564) in the ODD domain of HIF1α (Bruick and McKnight, 2001), leading to the recruitment of von Hippel-Lindau (VHL) proteins (Maxwell et al., 1999) and subsequent degradation of HIF1α (Jaakkola et al., 2001). The PHD family has three members: PHD1, PHD2 and PHD3, with PHD2 being the most abundant and highly active towards HIF1α (Huang et al., 2002). PHDs require only a short stretch of HIF1α amino acids (as short as 20 residues) for the selective recognition of proline hydroxylation sites and subsequent VHL-binding. These sites reside within an LXXLAP motif, which is highly conserved between the HIFα isoforms as well as across species (Jaakkola et al., 2001). The hydroxylation enables the VHL protein to bind HIF1α, which initiates degradation via the ubiquitination pathway (Cockman et al., 2000, Jaakkola et al., 2001). VHL-deficient cells have the HIF1α subunit constitutively stabilised and thus, HIF1 is constantly activated in these cells (Maxwell et al., 1999).
An additional hydroxylation event in the CAD domain ensures that any HIF1α that escapes degradation is rendered inactive. This process involves the hydroxylation of an asparagine residue instead of a proline and suppresses the recruitment of CBP/p300 co-activators (Lando et al., 2002b). This asparaginyl hydroxylase is the FIH-1, and uses both HIF1α and HIF2α as substrates. In HIF1α, FIH-1 hydroxylates an asparagine residue at position 803. FIH-1 is an Fe(II)-dependent enzyme and plays the role of a second oxygen sensor within the hypoxic response pathway (Lando et al., 2002a).

Thus, under normoxia, prolyl and asparaginyl hydroxylases prevent the activation of HIF1α by acting on the NAD and CAD domains respectively. However, when oxygen levels decrease, these hydroxylases become inactive; HIF1α proteins are stabilised and translocated to the nucleus where they dimerise with HIF1β. The reduction of Cys 800 in the CAD by Ref-1, through the action of thioredoxin, results in the recruitment of transcriptional co-activators and subsequent expression of the target genes.
1.4.3 Regulation of the HIF1 System by ROS

While HIF1 is stabilised and active under conditions of low oxygen, paradoxically ROS can also stabilise HIF1. Under normoxia, the addition of \( \text{H}_2\text{O}_2 \) caused HIF1\( \alpha \) stabilisation and enhanced expression from HRE-reporter constructs (Chandel \textit{et al.}, 2000). In addition, Hep3B \( p^0 \) cells, which do not have mitochondrial electron transport function, can exhibit HRE-luciferase reporter activity under normoxia upon addition of \( \text{H}_2\text{O}_2 \) (Chandel \textit{et al.}, 2000). The molecular basis for ROS stabilising HIF1 was shown by exposing murine breast tumor cells to nitric oxide (NO). Addition of NO caused nitrosylation of a specific cysteine residue in the ODD domain of HIF1\( \alpha \) under normoxia. The VHL protein was therefore unable to bind to HIF1\( \alpha \), thereby preventing its degradation (Li \textit{et al.}, 2007). This represents a control mechanism that bypasses the function of the PHD enzymes under normoxia, since the nitrosylation did not prevent or change the level of proline hydroxylation detected in the NAD domain.

ROS is also believed to play a role in the HIF1 signalling pathway during hypoxia. Cells with non-functional mitochondria, and therefore, reduced ROS levels, were unable to stabilise HIF1\( \alpha \) in response to hypoxia (Chandel \textit{et al.}, 2000, Mansfield \textit{et al.}, 2005). When \( \text{H}_2\text{O}_2 \) was inhibited by catalase over-expression in human 293 cells under hypoxia, there was reduced HRE-luciferase reporter activity, suggesting lower HIF1\( \alpha \) activity, which was restored by the addition of \( \text{H}_2\text{O}_2 \) (Chandel \textit{et al.}, 2000). These observations suggest that the presence of \( \text{H}_2\text{O}_2 \) in the cytosol is necessary for HIF1\( \alpha \) stabilisation under hypoxia. One possible role of ROS may be to inhibit the PHD enzymes. Addition of 10\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) showed more than 50\% inhibition of PHD enzyme function \textit{in vitro} but did not increase HIF1\( \alpha \) transcriptional activity in Hep3B cells (Pan \textit{et al.}, 2007). This implies that HIF1\( \alpha \) activation by ROS can occur through multiple pathways including both stabilisation and recruitment of co-activators.

While ROS appears to exert some regulatory function on HIF1, there is still debate as to whether ROS levels are increased (Hohler \textit{et al.}, 1999, Chandel \textit{et al.}, 2000, Kolamunne \textit{et al.}, 2011) or decreased (Paky \textit{et al.}, 1993, Michelakis \textit{et al.}, 2002, Karlenius \textit{et al.}, 2012) during hypoxia. Contradictory results may occur due to differences in cell type, mode of generating hypoxia, oxygen levels and assays used to measure ROS. Previous
work from our laboratory demonstrated that MDA-MB-231 breast cancer cells grown under hypoxia have reduced ROS levels (Karlenius et al., 2012). However, it was found that how the cells were processed was extremely important. If cells were processed under normoxic conditions following the hypoxic growth then increased ROS levels were observed. When cells were maintained under hypoxia throughout the processing steps, then a decrease was evident (Karlenius et al., 2012). This indicates that cells grown in hypoxia must be maintained in hypoxia during processing to avoid introduction of an inadvertent re-oxygenation step (however brief), thus, mimicking the intermittent hypoxia observed in tumors.

1.4.4 Redox Regulation of the HIF1 System

The activity of the HIF1 system is regulated by the thioredoxin redox system, via Ref-1. Thioredoxin provides the reducing potential for Ref-1 to reduce a cysteine residue in the CAD domain of HIF1α that enhances the ability of HIF1 to recruit co-activators (Ema et al., 1999). Consequently, cell lines engineered to over-express thioredoxin also displayed increased HIF1α levels, enhanced HIF1 DNA binding and upregulated activation of HIF1 regulated gene promoters. This results in increased levels of hypoxia regulated proteins such as vascular endothelial growth factor (VEGF) (Welsh et al., 2002, Jones et al., 2006) and cyclooxygenase-2 (COX-2) (Csiki et al., 2006). In contrast, when cells were transfected with the dominant negative redox inactive thioredoxin protein, VEGF and COX-2 levels were decreased. Other small molecule inhibitors of the thioredoxin system, such as quinols, also led to down regulation of HIF1 activity (Jones et al., 2006) and subsequently to a decrease in VEGF and inducible nitric oxide synthase (iNOS) expression in MCF-7 breast cancer cells (Welsh et al., 2003).

A recent study showed that thioredoxin reductase levels were decreased during hypoxia and as a consequence higher ROS levels were observed (Naranjo-Suarez et al., 2012). It was concluded that hypoxia does not increase mitochondrial ROS production, but that lower thioredoxin reductase levels are responsible for higher ROS levels. Since HIF1 is also regulated by ROS, this study demonstrated that the thioredoxin redox system could
modulate HIF1 signalling by indirectly affecting ROS levels, in addition to the direct interaction described above.

1.5 Redox and Hypoxic Systems: the Intermittent Hypoxia Link

Hypoxia induces the formation of new blood vessels, which are often poorly formed, causing an inconsistent oxygen supply (Figure 1.10) (Lanzen et al., 2006). Therefore, cells can experience a cycling between hypoxia and re-oxygenation. Hypoxic pathways are induced during periods of low oxygen while the re-oxygenation results in induction of antioxidant proteins, including the redox enzymes. Thus, the interplay between the two systems is important to study in tumors to not only increase the understanding of the molecular mechanisms involved in carcinogenesis but also to aid in designing of effective therapeutics.

![Figure 1.10: Abnormal tumor vasculature compared to the normal tissue.](image)

Upon experiencing hypoxia, tumor cells induce angiogenesis. However, the blood vessels are irregular, leaky, heterogeneous and tortuous. These aberrant vessels can also influence drug delivery and make tumors less sensitive to chemoradiation (reprinted from Brown and Wilson, 2004 with permission from Nature Publishing Group).
1.5.1 HIF1 System under Intermittent Hypoxia

Several studies have implicated an upregulation of HIF1α under intermittent hypoxia. This increase supersedes the HIF1α levels found in acute hypoxia (Yuan et al., 2005, Semenza and Prabhakar, 2007). Yuan and co-workers found this to be Ca^{2+} dependent (Yuan et al., 2005). They demonstrated the involvement of calcium-calmodulin dependent kinase II (CaMK II) under intermittent hypoxia. CaMK II phosphorylates p300, a co-activator required for the transcriptional activity of HIF1α, thereby increasing the HIF1α transactivation (Yuan et al., 2005). In contrast, under acute hypoxia, HIF1α transcriptional activity is increased as a result of a decrease in the O_{2} dependent asparaginyl hydroxylation in the CAD region of HIF1α, assisting in the recruitment of co-activators (Lando et al., 2002b).

Intermittent hypoxia has been linked to increased tumor invasion and resistance against radiotherapy (Yao et al., 2005, Martinive et al., 2006) and to enhanced metastasis in rodent lungs (Cairns et al., 2001). Liu and colleagues demonstrated that intermittent hypoxia treated H446 lung cancer cells displayed a greater metastatic ability and radio-resistance. They found HIF1α was found to be involved in both processes (Liu et al., 2010). Endothelial cells exposed to intermittent hypoxia also showed enhanced migration and exhibited an increased resistance against irradiation as compared to their counterparts grown in normoxia or acute hypoxia. This effect was also mediated by HIF1α since siRNA targeting HIF1α abolished the radiation resistance (Martinive et al., 2006). Therefore, HIF1α may be expected to have a role in tumor invasion observed under intermittent hypoxia.

Differences in HIF1α and HIF2α expression under acute and intermittent hypoxia have been shown in obstructive sleep apnea. While intermittent hypoxia caused an upregulation in HIF1α levels, the HIF2α levels were downregulated in intermittent hypoxia treated rat PC12 cells and also in in vivo rat models. In contrast, both HIF1α and HIF2α were upregulated by acute hypoxia (Nanduri et al., 2009). It was proposed that downregulation of HIF2α contributes to oxidative stress, at least in part via transcriptional downregulation of a HIF2 target gene, an antioxidant called superoxide dismutase (SOD). Intermittent hypoxia also increased ROS by decreasing the
mitochondrial complex I activity. The increase in ROS levels was linked to the upregulated HIF1α levels under intermittent hypoxia (Peng et al., 2003). Therefore, the differential regulation of HIF1α and HIF2α is believed to cause oxidative stress resulting from an imbalance between ROS and antioxidants (Nanduri et al., 2009). Similar mechanisms may contribute to higher levels of ROS in cancer cells. However, as antioxidants are proteins that scavenge ROS, antioxidant levels may be expected to be augmented in such a scenario. Interestingly, a number of studies implicate an upregulation of antioxidants in cancer cells cultured under intermittent hypoxia.

1.5.2 Redox System under Hypoxia and Intermittent Hypoxia

Since intermittent hypoxia involves phases of re-oxygenation, it is reasonable to expect that redox enzymes would be induced during these re-oxygenation phases. The expression of thioredoxin during the hypoxic phase has been less clear. In hypoxic regions of tumors, thioredoxin expression has been reported as high (Hedley et al., 2004), but intermittent hypoxia may contribute to this high expression. In cells cultured in vitro, there have been conflicting reports regarding thioredoxin expression levels under hypoxia.

Thioredoxin protein levels were increased in A549 human lung cancer cells during growth under 0.05% oxygen (Kim et al., 2003a) and in both human endothelial progenitor cells and human umbilical vein endothelial cells cultured under 1% oxygen (Park et al., 2010), as assessed by western blotting. Work done previously in our laboratory (Karlenius et al., 2012), showed a visible increase (by western blotting) in thioredoxin levels in MDA-MB-231 cells cultured under 0.1% hypoxia, however, this increase was not statistically significant. In addition, neither thioredoxin nor thioredoxin reductase promoter activity was increased under hypoxia (Karlenius et al., 2012). Ref-1 protein levels were also not increased (Karlenius et al., 2012) and other studies reported that peroxiredoxin protein levels were not increased in A549 cells cultured under hypoxia (Kim et al., 2007b). A recent study demonstrated a decrease in thioredoxin reductase protein levels under hypoxia (Naranjo-Suarez et al., 2012). Previously, it was
reported that thioredoxin reductase was increased in human endothelial progenitor cells but not in human umbilical vein endothelial cells under hypoxia (Park et al., 2010).

This conflicting data suggests that as with the variable ROS levels reported under hypoxia, expression of the thioredoxin system under hypoxia may depend on the specific cell line, oxygen levels or how samples are processed. For example, Jewell and co-workers observed that thioredoxin levels in the nucleus were increased after as little as 30 seconds of oxygen exposure following hypoxic growth (Jewell et al., 2001). Thus, in some reported cases, cells may have received an inadvertent re-oxygenation stimulus during processing of cells after hypoxic growth, which may be sufficient to induce the antioxidant gene expression.

Since re-oxygenation stimulates the production of ROS, one might expect that high levels of thioredoxin would be detected in cells re-oxygenated after hypoxia (Figure 1.11). As stated previously, in the study from our laboratory (Karlenius et al., 2012), MDA-MB-231 cells cultured under 0.1% oxygen followed by re-oxygenation had increased thioredoxin levels as assessed visually on western blots, but this was statistically non-significant when quantitated by densitometry. In addition, after 6 hours of re-oxygenation, the levels were visually decreasing. This correlates with other studies that reported a visible decrease in thioredoxin protein levels in A549 cells grown under 0.2% oxygen followed by 6 hours or more of re-oxygenation (Kim et al., 2010b). Their work showed that thioredoxin was oxidised during the re-oxygenation phase (Kim et al., 2010b), probably by the increased ROS levels (Karlenius et al., 2012). After 6 hours of re-oxygenation, ROS levels start to decrease and it is possible that the cells no longer require thioredoxin.
When conditions mimicking intermittent hypoxia are utilised, the involvement of thioredoxin is quite apparent. Malec and co-workers utilised several different schemes to grow A549 cells alternating between hypoxia and re-oxygenation (Malec et al., 2010). While a maximum of three 2-hour cycles were used for hypoxia or re-oxygenation, the schemes with the greatest number of cycles of hypoxia and re-oxygenation resulted in the highest thioredoxin levels. Nrf2 was also upregulated under these conditions and may be responsible for the increased thioredoxin expression (Malec et al., 2010).

A technique called ischemic preconditioning is commonly used for myocardial preservation (Murry et al., 1986, Li et al., 1990, Das, 1993). It has been shown that when hearts are exposed to short periods of ischemia (restricted blood flow to a tissue) followed by reperfusion (re-establishment of blood flow), they become resistant to the future prolonged ischemic injury, required during myocardial surgeries. The ischemic preconditioning has been shown to be redox-regulated (Kihlstrom, 1990, Yagi et al., 1994, Nakamura et al., 1998, Isowa et al., 2000). A similar phenomenon called hypoxic preconditioning involves short periods of hypoxia followed by re-oxygenation. As described in 1.2, the cycling phenomenon in cancer cells involves two different timescales. The one with the shorter durations, caused by fluctuations in red blood influx, is comparable to the hypoxic preconditioning. Previous work from our laboratory (Karlenius et al., 2012) used a scheme (Figure 1.12) that mimicked an ischemia/reperfusion study performed in the heart (Turoczi et al., 2003). In the heart
study, 4 short cycles of ischemia and reperfusion (of 10 and 20 minutes respectively) prior to longer-term growth in ischemia and subsequent reperfusion led to very high levels of thioredoxin. These preconditioning conditions also provided the heart protection from damage otherwise caused by the longer-term ischemia and reperfusion. In the study from our laboratory these oxygen growth conditions were applied to cancer cells and high levels of thioredoxin were detected in cells preconditioned with short cycles of hypoxia and re-oxygenation followed by a longer exposure to hypoxia and re-oxygenation (Karlenius et al., 2012).

Maximum thioredoxin protein levels were obtained after 4 hours of re-oxygenation, which was confirmed to be statistically significant. These short cycles may also represent what happens in tumors due to red blood cell flux (Lanzen et al., 2006) and may provide the tumor with protection against subsequent oxidative insult. Without the preconditioning cycling, thioredoxin levels were not increased by as much during re-oxygenation, indicating that the cycling may provide an advantage to the cells. Of interest is that Ref-1 levels were also higher in MDA-MB-231 cells subjected to the

![Figure 1.12: Oxygen growth conditions used in the study with MDA-MB-231 cell line. Schematic representation outlining the different combinations of hypoxia and re-oxygenation and their respective length of exposure used to grow MDA-MB-231 cells. Red indicates growth under 20% oxygen. Blue indicates growth under 0.1% oxygen. N: normoxia (20% oxygen); R: re-oxygenation; H: hypoxia; PC: preconditioning (reused from Karlenius et al., 2012 with permission from the authors).](image-url)
preconditioning, but not in cells grown without this step (Karlenius et al., 2012). Since Ref-1 and thioredoxin regulate HIF1 activity, the short pulses of hypoxia may be responsible for their induction. The promoter activity of both thioredoxin and thioredoxin reductase were found to be dependent on Nrf2 in the re-oxygenation phase, and that cells cultured with the preconditioning cycles did not exhibit higher promoter activity (Karlenius et al., 2012). Therefore, the mechanism for inducing higher thioredoxin protein levels in cells subjected to cycling may not be at the transcriptional level.

1.5.3 The Interaction between Redox and Hypoxic Pathways under Intermittent Hypoxia

Cancer cells can reside in conditions of hypoxic as well as oxidative stress. HIF1 and Nrf2 are two important transcription factors that play a crucial role in each of these conditions. While HIF1 is important for cell survival under low oxygen conditions, Nrf2 provides cytoprotection against oxidative stress by upregulating antioxidants such as thioredoxin. Both HIF1 and Nrf2 are induced in cells under intermittent hypoxia. This presents a possible link between the oxygen- and redox-dependent regulatory pathways. As described in 1.5.1, the increase in HIF1 levels under intermittent hypoxia supersedes the HIF1 levels observed under acute hypoxia. Similarly, higher thioredoxin levels were observed under intermittent hypoxia in comparison to acute hypoxia (Malec et al., 2010, Karlenius et al., 2012). Higher levels of other antioxidants have also been observed under intermittent hypoxia. For example, Prx1 was upregulated in response to hypoxia/re-oxygenation, through the action of Nrf2, which binds to the ARE in the Prx1 promoter. In cells lacking Nrf2, Prx1 expression was compromised (Kim et al., 2007b). ROS levels are also higher under intermittent hypoxia (Karlenius et al., 2012) and are involved in both HIF1 regulation and induction of antioxidant expression through the action of Nrf2. This may induce higher thioredoxin levels, which in turn results in the higher HIF1 levels observed under intermittent hypoxia.
The high levels of both thioredoxin and HIF1 in cancer cells cultured under intermittent hypoxia also have implications for tumor metastasis (Semenza and Prabhakar, 2007, Malec et al., 2010). Thioredoxin enhances the invasive behavior of tumor cells by regulating MMP activity, which is required for extracellular matrix (ECM) degradation (Farina et al., 2001, Farina et al., 2011). HIF1 over-expression during hypoxia has also been associated with ECM degradation by upregulating MMP-2 (Krishnamachary et al., 2003) and MMP-9 gene expression (Choi et al., 2011). In a separate study, intermittent hypoxia treated A549 and H446 lung cancer cells exhibited increased invasion in comparison to normoxic cells (Liu et al., 2010). Downregulation of the HIF1α gene decreased the cellular migration in these cells, thereby linking HIF1 to cancer cell invasion under intermittent hypoxia (Liu et al., 2010). Moreover, both thioredoxin and HIF1 have been linked to the development of resistance against anticancer therapeutics (Sasada et al., 1996, Kim et al., 2005, Martinive et al., 2006). These common outcomes suggest an interplay of redox and hypoxic systems under intermittent hypoxia (Figure 1.13), with possible consequences for the design and testing of therapeutics.

**Figure 1.13:** Interaction between thioredoxin and HIF1 under intermittent hypoxia and consequences for cancer progression. Intermittent hypoxia involves cycling between hypoxia and re-oxygenation and as a result upregulates both hypoxic (HIF1) and redox (thioredoxin) systems. HIF1 is redox regulated by thioredoxin. Both HIF1 and thioredoxin regulate proteins involved in tumor invasion and development of resistance against therapeutics (Bhatia, 2013).
1.6 Intermittent Hypoxia and Anti-Cancer Therapies

Development of resistance in cancer cells against chemotherapies presents a major setback in the prevention and cure of the disease that kills millions of people every year. Many chemotherapeutics are based on heavy metals, such as gold and platinum that generate ROS in cells, causing damage to DNA, proteins and lipids, and ultimately leading to apoptosis (Desoize, 2002). However, cells upregulate their antioxidant defenses in order to scavenge ROS, and as a result cancer cells become resistant to these drugs. High levels of thioredoxin and other antioxidant proteins in tumors are correlated with resistance to various chemotherapeutic agents, including cisplatin (Sasada et al., 1996), docetaxel (Kim et al., 2005) and tamoxifen (Schiff et al., 2000). Furthermore, breast tumors with high levels of thioredoxin and other antioxidants prior to treatment with docetaxel were correlated with a high likelihood of developing resistance during therapy (Iwao-Koizumi et al., 2005). Therefore, anti-cancer therapies could be designed to inhibit the thioredoxin system in combination with radiation or chemotherapy.

Radiation treatment has been shown to cause re-oxygenation and perfusion of hypoxic tumors (Bussink et al., 2000). This has been linked with accumulation of ROS. Moeller and colleagues observed an elevation in levels of HIF1 regulated proteins after 72 hours of radiation exposure (Moeller et al., 2004). Inhibition of ROS by a SOD mimetic prevented the stabilisation of HIF1α and sensitised the tumor to the damage caused by radiation (Moeller et al., 2005a). In a separate study, a delay in tumor growth following radiation was observed when HIF1 was inhibited using an antisense knockdown technique (Moeller et al., 2005b). HIF1β null tumor lines were also found to be sensitive to radiotherapy as they prevent the HIF1 response (Williams et al., 2005). All these studies suggest that radiation treatment causes an increase in ROS levels that stabilise HIF1α and leads to the subsequent increase in levels of HIF1 mediated proteins.

Given its immense importance in cancer biology, the thioredoxin system is being considered as a potent anti-cancer target (Powis and Kirkpatrick, 2007). Thioredoxin system inhibition can stimulate apoptosis through the activation of ASK-1 (Saitoh et al., 1998) and the inhibition of NF-κβ (Matthews et al., 1992). Cellular ROS levels are
expected to be increased upon impediment of the thioredoxin system, also leading to apoptosis. Figure 1.14 summarises the various effects of inhibition of the thioredoxin system. Some widely used thioredoxin system inhibitors are listed in Table 1.1.

Figure 1.14: Possible physiological consequences of inhibition of the thioredoxin system. Inhibition of the thioredoxin system leads to an accumulation of ROS, activation of ASK-1, and inhibition of NF-κB, ultimately leading to apoptosis (adapted from Tonissen and Di Trapani, 2009 with permission from the authors).

Table 1.1: Thioredoxin system inhibitors (adapted from Tonissen and Di Trapani, 2009 with permission from the authors).

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Mode of action</th>
<th>Targets</th>
<th>Example</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum compounds</td>
<td>Irreversible DNA damage</td>
<td>Testicular, ovarian, cervical, non-small lung cancer</td>
<td>Cisplatin, carboplatin, oxaliplatin</td>
<td>(Wang and Lippard, 2005)</td>
</tr>
<tr>
<td>Arsenic compounds</td>
<td>Mitochondrial apoptosis</td>
<td>Acute promyelocytic leukemia</td>
<td>Arsenic trioxide (ATO)</td>
<td>(Lu et al., 2007)</td>
</tr>
<tr>
<td>Gold compounds</td>
<td>Interaction with DNA</td>
<td>Rheumatoid arthritis</td>
<td>Auranofin</td>
<td>(Kean et al., 1997, Urg and Becker, 2006)</td>
</tr>
</tbody>
</table>
1.6.1 Auranofin

Chrysotherapy, using gold compounds in medicine, dates back to 2500 BC. In ancient times, gold was presumed to have mystical properties that enhanced the quality of medicines. Although these notions are obsolete in today’s time, gold compounds are still being tested as candidates for chemotherapeutics (Omata et al., 2006, Marzano et al., 2007, Rigobello et al., 2009). Gold has a high affinity for thiols and an even higher affinity for selenolates (Rigobello et al., 2002), making the thioredoxin system a potential target for gold-based drugs. Due to the extremely expensive and highly time-consuming process of drug discovery, development and registration, a lot of interest is being generated to ‘repurpose’ already approved drugs (Roder and Thomson, 2015). Not only is the process of drug ‘repurposing’ quicker and more affordable than novel drug discovery, since these drugs are already FDA approved, they have a well-known toxicity profile and are safe for human use (Roder and Thomson, 2015). In fact, auranofin, a monomeric phosphine gold compound (Figure 1.15) has been used as an anti-rheumatic drug that targets thioredoxin reductase in the synovial fluids of arthritic patients since late 1970s (Yoshida et al., 1999, Tiekink, 2002) and is gaining a lot of attention to be repurposed for the treatment of several other diseases, such as HIV/AIDS, parasitic and bacterial infections, and cancer (Roder and Thomson, 2015).

![Figure 1.15: Chemical structure of auranofin](CSID:21242895 obtained from www.chemspider.com)
Auranofin is considered as a pro-drug that delivers gold(I) into the cells. Before the transportation of the compound into the cells, the acetyl-thioglucose ligand is displaced. The triethylphosphine then enters the cells and confers membrane permeability (Shaw, 1999). Auranofin is an orally administered drug, which can be detected in the plasma, mainly bound to albumin. Most of it is absorbed within the first 20 minutes and the plasma half-life is approximately 25 days (Roder and Thomson, 2015).

Thioredoxin reductase has a solvent exposed flexible C-terminal extension that contains a Cys-Sec redox centre (Zhong et al., 2000). This makes thioredoxin reductase’s Sec residue easily accessible to electrophiles such as auranofin, which irreversibly bind to the thioredoxin reductase active site and inhibit its activity (Gromer et al., 1998). As a consequence of inhibiting the thioredoxin reductase activity the cellular pool of reduced thioredoxin decreases. As a result, the redox state of the cells is expected to shift towards a more oxidised state, leading to the accumulation of ROS (Rigobello et al., 2009). This in turn causes changes to the mitochondrial functions and loss of membrane potential (Rigobello et al., 2002, Rigobello et al., 2004, Rigobello et al., 2005). As stated previously, the inhibition of the thioredoxin system also leads to the activation of ASK-1 and inhibition of NF-κβ, causing the cells to undergo apoptosis. Therefore, auranofin is a promising candidate for anti-cancer therapies.

Surprisingly, the fluctuations between low and high oxygen concentrations in the in vivo tumor environment are not considered while designing and testing anti-cancer drugs, which are done under normoxia (20% oxygen), which is not physiologically relevant. Thus, the drugs tested under normoxia may respond differently in the patient body where most healthy organs contain an average of 3-6% oxygen and tumor cells constantly experience hypoxia. Although the effects of some anti-cancer drugs, such as cisplatin have been studied under hypoxia (Wohlkoenig et al., 2011), in vitro studies involving auranofin have only been performed under normoxia. Hence, it is important to assess auranofin under intermittent hypoxia conditions, which closely resemble the in vivo cancer environment.
1.7 Cancer Metastasis

The leading cause of cancer-related mortality is metastasis. It is the process by which the tumor spreads from its site of origin to new secondary locations (Fidler, 1990). As reviewed by Martin and colleagues, at least half of the patients present clinical signs of metastasis at the time of diagnosis and an even higher number of patients have micrometastasis, which remains undetected by the conventional diagnostic techniques (Martin, 2000). Thus, metastasis is the most life-threatening aspect of cancer.

Cancer metastasis is a multi-step cascade involving a group of coordinated processes, which requires blood and/or lymphatic vessels to serve as a route for metastatic cancer cells to reach distant locations. Prevascular tumors persist as thin lesions due to restricted oxygen and nutrient supply while vascularised tumors not only grow efficiently but are also capable of metastasising (Liotta et al., 1974). The process of angiogenesis (Figure 1.16), itself is a multi-step process consisting of activation, proliferation and migration of endothelial cells resulting in sprouting of new blood vessels (Martin, 2000, Pandya et al., 2006).

Figure 1.16: Key steps involved in the process of angiogenesis. The process of angiogenesis involves release of angiogenic signals by tumor cells; endothelial cell (EC) activation, proliferation and migration through existing blood vessels; remodelling of the extracellular matrix; tube and loop formation; and vascular stabilization (reprinted from Pandya et al., 2006 with permission from Elsevier).
As stated earlier, metastasis involves several sequential steps (Figure 1.17). The first stage of metastasis is the detachment of cells from the primary tumor mass and degradation of the surrounding extracellular matrix (ECM), followed by the subsequent invasion of these primary tumor cells into the newly formed blood/lymphatic vessels (Saiki, 1997). It has been shown that at this stage, less than 0.1% of tumor cells that enter the blood stream survive to ensue metastasis (Fidler, 1970). Moreover, a number of factors, including the tissue of origin, intrinsic tumor properties, and circulation patterns influence the course and severity of metastasis as well as the site of tumor spread (Martin, 2000). The cells that survive to metastasise form an embolism by getting arrested in a distant tissue/organ capillary bed. The next step involves the extravasation of the emboli from circulation by degradation of the ECM at the secondary tumor site. The last step is colonisation of the primary tumor cells at the secondary location, completing the metastatic cascade (Fidler, 1990).

Figure 1.17: Metastatic cascade. Upon formation of new blood vessels in primary tumor mass, some cells detach and invade the blood stream, get arrested at a distant organ and proliferate to give rise to a secondary tumor (adapted from Fidler, 2003 with permission from Nature Publishing Group).
An important aspect of metastasis is ECM degradation and involves the MMP family of proteins (Sato et al., 1994), which are regulated by tissue inhibitor of matrix metalloproteinases (TIMPs) (Nagase, 1997). In healthy cells, TIMPs maintain the MMP levels and ECM degradation is inhibited. However, the MMP/TIMP balance is disturbed in tumor cells, which causes ECM degradation and subsequent tumor invasion (Farina et al., 2001). Thioredoxin has been linked to the invasion process in studies performed by Farina and co-workers. Increased thioredoxin levels inhibited TIMPs in neuroblastoma cells, causing an upregulation in the MMP activity, thereby stimulating neuroblastoma invasion (Farina et al., 2001). In a separate study, these researchers showed that MMP transcription was stimulated by thioredoxin over-expression in MDA-MB-231 cells (Farina et al., 2011).

Cancer cell invasion has also been correlated with the cellular redox state in prostate cancer cell lines (Chaiswing et al., 2007). Over-expression of thioredoxin in human lung carcinoma cell lines resulted in more aggressive tumors being formed when injected into immune-compromised mice (Ceccarelli et al., 2008). Previous studies performed in our laboratory also showed thioredoxin to increase the invasion of MDA-MB-231 cells in vitro when over-expressed in the cells as well as when added extracellularly (Bloomfield, 2003). These studies suggest that thioredoxin plays a critical role in cancer invasion and progression.

1.8 3D Culturing: Recreating the Tumor Microenvironment in vitro

The preclinical anti-cancer drug development involves testing of drugs in a series of biochemical and cell-based assays, which is followed by evaluation in animal models. Although these cell-based models are straightforward and convenient, their main limitation is that they lack the complexity of in vivo tumor tissues and therefore, offer little value in predicting clinical efficacy of drugs. To overcome this limitation of the simple two-dimensional (2D) cell culturing involving growing cells as flat monolayers on a plastic substrate, a lot of interest is being generated by three-dimensional (3D) culture systems.
As discussed in the previous sections, the cellular microenvironment plays a crucial role in influencing tumor cell responses. The roles of tissue architecture and the ECM in promoting tumor growth and responses to anti-cancer therapies have also been studied. 3D culture systems better reflect the in vivo conditions with respect to nutrient and oxygen gradients, cellular heterogeneity, and cell-cell communications (Fischbach et al., 2007, Friedrich et al., 2007). As reviewed by Friedrich and co-workers, different types of 3D model systems are used by researchers and are summarised in Table 1.2.

Table 1.2: Different types of 3D culture systems (Friedrich et al., 2007).

<table>
<thead>
<tr>
<th>Model description</th>
<th>To study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multilayer cells cultured on top of porous membranes</td>
<td>Drug transport, drug resistance, tumor cell invasion</td>
<td>(Hicks et al., 1997, Padron et al., 2000)</td>
</tr>
<tr>
<td>Matrix-embedded cultures using ECM components</td>
<td>Cell-matrix interactions, cell differentiation, migration and invasion</td>
<td>(Debnath and Brugge, 2005, Lee et al., 2007)</td>
</tr>
<tr>
<td>Hollow-fiber bioreactors using cells cultured within artificial capillaries</td>
<td>Metabolism, drug resistance</td>
<td>(Gillies et al., 1993, Dulong et al., 2002)</td>
</tr>
<tr>
<td>Ex vivo cultures of excised tumor tissues grown in vitro</td>
<td>Drug resistance</td>
<td>(Berglund et al., 2002, Ochs et al., 2003)</td>
</tr>
</tbody>
</table>

The 3D spheroid model system was introduced in the early 1970s by Sutherland and colleagues (Sutherland et al., 1970). To mimic the avascular microregions of solid tumors, the 3D architecture is obligatory. The functional features of spheroid-based models and their resemblance to in vivo tumor microenvironment are shown in Figure 1.18. The active tumor cells are generally present on the spheroid periphery and resemble the cells in contact with capillaries in vivo (Friedrich et al., 2007). As the spheroids grow over 500µm in diameter, they develop hypoxic cores (Vinci et al., 2012). These spheroids also have necrotic centers arising from cell death due to lack of nutrients and oxygen supply and an accumulation of waste products (Mazzoleni et al., 2009).
Over the years several methods have been used to generate spheroids and are listed in Table 1.3. All these methods have their advantages and disadvantages (Mazzoleni et al., 2009). Efforts are being directed towards miniaturising these 3D culture systems for the purpose of drug screening. This has led to the development of 96-well and even 384-well based culture systems, which are being combined with new technologies of high throughput imaging and analysis (Vinci et al., 2012, Lovitt et al., 2013).
Combining 3D cultures with high content imaging and analysis allows for studying cancer cell biology at a higher level of complexity, which more closely resembles that found \textit{in vivo}. These imaging and analysis processes are being automated for a higher throughput screening of a range of biological parameters including cellular morphology, protein expression and cellular interactions in response to drug exposure (Bickle, 2010). This involves management and assessment of a large amount of biological data by employing sophisticated algorithms and software to automatically record differences in biological features upon drug treatment (Zanella \textit{et al.}, 2010).

The matrix-embedded culture systems are most widely used and employ growing cells as multicellular 3D spheroids by culturing them on a reconstituted basement membrane. The spheroids grown on these ECM components possess the complex cell-matrix and cell-cell interactions, found in cells/tissues \textit{in vivo}. These interactions are not only essential for cellular responses to growth factors but are equally important for drug penetration and efficacy (Friedrich \textit{et al.}, 2007). Several studies have demonstrated modified gene expressions between cells cultured as 2D monolayers and as 3D spheroids and have been recently reviewed by Lovitt and colleagues (Lovitt \textit{et al.}, 2014). 2D cells grown in presence of the ECM proteins also exhibit modified sensitivity towards various anti-cancer drugs (Lovitt \textit{et al.}, 2014).

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**Table 1.3: Commonly used 3D spheroid culture techniques** (Friedrich \textit{et al.}, 2007, Vinci \textit{et al.}, 2012).

<table>
<thead>
<tr>
<th>Type of culture system</th>
<th>Example</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Large scale</strong></td>
<td>Spinner flasks, rotary system, roller tubes</td>
<td>(Wartenberg \textit{et al.}, 2001, Mazzoleni \textit{et al.}, 2009)</td>
</tr>
<tr>
<td><strong>Short-term</strong></td>
<td>Soft agar, gel/matrix-based, polymeric scaffolds</td>
<td>(Fischbach \textit{et al.}, 2007, Lee \textit{et al.}, 2007, Friedrich \textit{et al.}, 2009)</td>
</tr>
<tr>
<td><strong>Anchorage-independent</strong></td>
<td>Low-attachment plates, poly-2-hydroxymethyl methacrylate (poly-Hema)-coated plates</td>
<td>(Ivascu and Kubbies, 2007)</td>
</tr>
<tr>
<td><strong>Single spheroid</strong></td>
<td>Hanging drops</td>
<td>(Del Duca \textit{et al.}, 2004)</td>
</tr>
</tbody>
</table>
Drug sensitivity has been shown to be affected by cell-cell and cell-matrix interactions in vivo, which can provide protection to the tumor mass against therapeutics (Ivascu and Kubbies, 2007). Moreover, tumor vasculature plays a critical role in the efficient delivery of drugs to the cells in vivo, particularly those in the hypoxic regions (Tredan et al., 2007). It may be hypothesised that the increased drug resistance observed in 3D spheroid models compared to the 2D monolayer models is due to a delayed and inefficient distribution of the drug, leading to a reduced efficacy (Friedrich et al., 2007). Therefore, considering the tumor microenvironment during drug evaluation and how it may influence the efficacy of drugs in vivo is a pre-requisite for overcoming anti-cancer drug resistance.

1.9 Research Aims

The oxygenation state of the cells has immense importance in cancer biology and both redox- and oxygen-dependent signaling pathways play a crucial role in carcinogenesis. While HIF1 is important for cancer cells to survive under hypoxia, thioredoxin is required to protect them from oxidative damage. Since cancer cells can survive under both hypoxic and oxidative stress, a cross-talk between these two regulatory systems may be expected in tumors. Surprisingly, this interaction is not taken into account while designing and testing anti-cancer drugs, which is done under physiologically irrelevant levels of oxygen.

As described earlier, a previous study performed in our laboratory with MDA-MB-231 cells suggested that preconditioning, a form of intermittent hypoxia, upregulates the thioredoxin protein levels and possibly prepares cells for a future prolonged hypoxic insult (Karlenius et al., 2012). The A549 lung cancer cell line, due to a somatic mutation (Singh et al., 2006), is expected to have higher endogenous levels of the thioredoxin system proteins. This higher expression of thioredoxin could play a role in determining the response of A549 cells under different oxygen conditions. Therefore, this project is aimed at studying the effects of preconditioning on MDA-MB-231 and A549 cell lines with respect to the thioredoxin system. Since auranofin acts by targeting the thioredoxin system, both MDA-MB-231 and A549 cell lines will be examined to
determine if they have different sensitivity levels against auranofin. The effect of auranofin on the thioredoxin system will be assessed under different oxygen conditions. Furthermore, high levels of HIF1α have been observed under intermittent hypoxia. Since thioredoxin regulates HIF1α via Ref-1, a cross-talk may be expected between these two systems, especially under intermittent hypoxia. The effect of auranofin induced inhibition of the thioredoxin system on the expression and activity of HIF1α will also be evaluated.

A role for thioredoxin has been established in enhancing cancer cell invasion by many researchers (Farina et al., 2001, Chaiswing et al., 2007, Ceccarelli et al., 2008, Farina et al., 2011). Extracellular addition of thioredoxin in the culture media was also previously shown to stimulate the migration of MDA-MB-231 cells in our laboratory (King, 2010, Shah, 2011). In this project, the effect of endogenous over-expression of thioredoxin and its redox inactive mutated form on the migration of MDA-MB-231 cells will be studied. Auranofin will also be used as a tool to inhibit the thioredoxin system and study its effect on cancer cell migration.

Since the tumor architecture can influence the efficacy of drugs, the cytotoxic effects of auranofin will also be examined in MDA-MB-231 cells grown as 3D spheroids and compared to the cells grown as 2D monolayers. High throughput imaging will also be employed to examine the morphology of MDA-MB-231 cells in response to auranofin treatment in 2D and 3D models.

Overall, this project is focused on understanding how different environmental conditions influence the performance of auranofin as an anti-cancer drug.
INTRODUCTION

More specifically, the aims undertaken in this research are as follows:

1. To analyse the effect of auranofin induced inhibition of the thioredoxin system on MDA-MB-231 breast and A549 lung cancer cell lines subjected to different oxygen conditions (Chapter 3)

2. To determine the effect of auranofin induced inhibition of the thioredoxin system on the HIF1α system in MDA-MB-231 and A549 cancer cells grown under the different oxygen parameters (Chapter 4)

3. To investigate the migration of MDA-MB-231 cells in response to inhibition of the thioredoxin system by over-expression of thioredoxin’s redox inactive mutated protein and auranofin (Chapter 5)

4. To examine the effect of the 3D microenvironment on the drug activity of auranofin against MDA-MB-231 cells (Chapter 6)
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

2.1.1 Cell Lines

MDA-MB-231 is a highly metastatic and invasive cell line. It is poorly differentiated (Sommers et al., 1994). This cell line (ATCC catalogue number HTB-26™) was a kind gift from the E. Thompson laboratory (Queensland University of Technology, Australia) and has been previously used in our laboratory for preconditioning (Karlenius et al., 2012) and metastasis studies.

A549 is carcinomic human aveolar basal epithelial cell line (Giard et al., 1973). This cell line (ATCC catalogue number CCL-185™) was a kind gift from Dr. Dusan Zencak (Eskitis Institute for Drug Discovery, Griffith University, Australia). These cells have a mutation in Keap1, which is an inhibitor of Nrf2. Therefore, they have a constitutive Nrf2 pathway (Singh et al., 2006).

2.1.2 Vectors and Constructs

2.1.2.1 Plasmid Vectors

Table 2.1: List of plasmid vectors. Vector maps can be found in Appendix I.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Characteristics</th>
<th>Source</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T Easy</td>
<td>f1 origin, LacZ, ori, amp⁵, multiple cloning site, T7 promoter, Sp6 promoter</td>
<td>Promega</td>
<td>Cloning</td>
</tr>
<tr>
<td>pIRES2-EGFP</td>
<td>f1 origin, CMV promoter, IRES, EGFP⁷ gene, multiple cloning site, kan'neo', SV40 promoter</td>
<td>Clontech</td>
<td>Stable transfection of cell lines</td>
</tr>
<tr>
<td>pGL3-Basic</td>
<td>f1 origin, amp⁵, multiple cloning site, ori, SV40 late poly(A) signal, Luc⁷ gene</td>
<td>Promega</td>
<td>Control for promoter reporter assays</td>
</tr>
<tr>
<td>pEF-BOS-CS</td>
<td>EF-1a promoter, amp⁵</td>
<td>Dr Dan Peet, University of Adelaide, Australia</td>
<td>Control for HIF1α-CAD activity assays</td>
</tr>
<tr>
<td>pRL-TK</td>
<td>HSV-TK promoter upstream of the Renilla luciferase (Rluc) gene</td>
<td>Dr Dan Peet, University of Adelaide, Australia</td>
<td>Internal transfection control for the HIF1α-CAD activity assays</td>
</tr>
</tbody>
</table>
2.1.2.2 Plasmid Constructs

**Trx-IRES-EGFP:** This construct was previously generated in our laboratory by Fenil Shah using the pIRES-EGFP vector (Clontech). It contains a CMV promoter that drives the expression of both thioredoxin and GFP genes through the IRES.

**1SS-IRES-EGFP:** This construct was also previously made in our laboratory by Fenil Shah using the pIRES-EGFP vector (Clontech). It contains a CMV promoter that drives the expression of both 1SS (thioredoxin redox-inactive protein) and GFP genes through the IRES.

**pGL3-TrxOREUp:** This construct was previously prepared in our laboratory using the pGL3-Basic vector (Promega) by Simone Osborne (Osborne et al., 2006). It contains the human thioredoxin promoter sequence that drives the luciferase expression.

**pGL3-TrxR:** This construct was also prepared in our laboratory previously by Simone Osborne (Karlenius et al., 2012). It contains the human thioredoxin reductase core promoter sequence cloned into a pGL3-Basic vector (Promega) to drive the expression of the luciferase gene.

**pEF-BOS-CS-HIF1α:** This construct was a kind gift from Dr Dan Peet, University of Adelaide, Australia. It contains the HIF1α DNA binding and CAD domains.

**G5E1bLUC:** This construct was obtained from Dr Dan Peet, University of Adelaide, Australia. It contains the GAL-4 binding site upstream of the firefly luciferase gene (Hsu et al., 1994).
### 2.1.3 Oligonucleotides

Table 2.2: List of oligonucleotides.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Length</th>
<th>Tm °C</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>5´dTAA TAC GAC TCA CTA TAG</td>
<td>18</td>
<td>43</td>
<td>PCR/Sequencing</td>
</tr>
<tr>
<td>Trx.3Eco</td>
<td>5´dGAA TTC TTA GAC TAA TTC ATT AAT GG</td>
<td>26</td>
<td>50</td>
<td>PCR</td>
</tr>
<tr>
<td>IRES-Forward</td>
<td>5´dGTC GTA ACA ACT CCG CCC CAT TGA</td>
<td>24</td>
<td>60.25</td>
<td>PCR</td>
</tr>
<tr>
<td>IRES-Reverse</td>
<td>5´dCCA AGC GGC TTC GGC CAG TAA</td>
<td>21</td>
<td>62</td>
<td>PCR</td>
</tr>
<tr>
<td>SP6</td>
<td>5´dATT TAG GTG ACA CTA TAG</td>
<td>18</td>
<td>41.86</td>
<td>Sequencing</td>
</tr>
<tr>
<td>VEGF-Forward</td>
<td>5´dCAA GGC CAG CCC ATA GGA GAG AT</td>
<td>23</td>
<td>61.02</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>VEGF-Reverse</td>
<td>5´dCGA AAC CCT GAG GGA GGC TCC TT</td>
<td>23</td>
<td>61.88</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>L32-Forward</td>
<td>5´dCAG GGT TCG TAG AAG ATT CAA GGG</td>
<td>24</td>
<td>57</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>L32-Reverse</td>
<td>5´dCTT GGA GGA AAA CAT TGT GAG CTA TC</td>
<td>26</td>
<td>58</td>
<td>RT-PCR</td>
</tr>
</tbody>
</table>

### 2.1.4 Antibiotics

Table 2.3: List of antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 µg/mL</td>
</tr>
<tr>
<td>Geneticin</td>
<td>1250 µg/mL</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100 U/mL</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100 µg/mL</td>
</tr>
</tbody>
</table>
### 2.1.5 Antibodies

Table 2.4: List of antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B3</td>
<td>Anti-human thioredoxin monoclonal antibody raised in mouse</td>
<td>ELISA, as capture antibody</td>
<td>Dr Giovanna Di Trapani, Griffith University (Di Trapani et al., 1998)</td>
</tr>
<tr>
<td>2B1-b</td>
<td>Biotinylated anti-human thioredoxin monoclonal antibody raised in mouse</td>
<td>ELISA, as light-up antibody</td>
<td>Dr Giovanna Di Trapani, Griffith University (Di Trapani et al., 1998)</td>
</tr>
<tr>
<td>Avidin-Horseradish Peroxide</td>
<td>Horseradish-peroxide tagged secondary antibody</td>
<td>ELISA</td>
<td>BioRad (170-6528)</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Primary antibody raised in rabbit</td>
<td>Loading control in Western blots</td>
<td>Abcam (ab6046)</td>
</tr>
<tr>
<td>Lamin B1</td>
<td>Primary antibody raised in rabbit</td>
<td>Loading control in Western blots</td>
<td>Abcam (ab133741)</td>
</tr>
<tr>
<td>5G8</td>
<td>Anti-human thioredoxin monoclonal antibody raised in mouse</td>
<td>Western blots</td>
<td>Giovanna Di Trapani, Griffith University (Karlenius et al., 2012)</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Anti-Nrf2 monoclonal antibody raised in rabbit</td>
<td>Western blots</td>
<td>Abcam (ab62352)</td>
</tr>
<tr>
<td>Heme Oxygenase-1</td>
<td>Primary antibody raised in goat</td>
<td>Western blots</td>
<td>R&amp;D Systems (AF3776)</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Anti-HIF1α monoclonal antibody raised in mouse</td>
<td>Western blots</td>
<td>BD Biosciences (610959)</td>
</tr>
<tr>
<td>Thioredoxin Reductase</td>
<td>Anti-thioredoxin reductase monoclonal antibody raised in mouse</td>
<td>Western blots</td>
<td>R&amp;D Systems (MAB7428)</td>
</tr>
<tr>
<td>Goat-anti Mouse IgG-HRP</td>
<td>Secondary antibody raised against mouse in goat</td>
<td>Western blots</td>
<td>BioRad (170-6516)</td>
</tr>
</tbody>
</table>
### MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Conjugated</th>
<th>Supplier</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat-anti Rabbit IgG-HRP conjugated</td>
<td>Secondary antibody raised against rabbit in goat</td>
<td>Western blots</td>
</tr>
<tr>
<td>Donkey-anti Goat IgG-HRP conjugated</td>
<td>Secondary antibody raised against goat in donkey</td>
<td>Western blots</td>
</tr>
<tr>
<td>6F3</td>
<td>Anti-human thioredoxin monoclonal antibody raised in mouse</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>AlexaFluor 488</td>
<td>Goat anti-mouse secondary antibody</td>
<td>Immunofluorescence</td>
</tr>
</tbody>
</table>

#### 2.1.6 Chemicals and Reagents

**Table 2.5: List of chemicals and reagents**

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG Scientific Inc., CA, USA</td>
<td>Protease Inhibitor Cocktail VI</td>
</tr>
<tr>
<td>Ajax Chemicals, NSW, Australia</td>
<td>Calcium Chloride (CaCl₂), Glycerol</td>
</tr>
<tr>
<td>Bioline</td>
<td>ISOLATE Genomic DNA mini kit, TRIsure™, SensiFAST™ cDNA synthesis kit, SensiFAST™ HRM kit</td>
</tr>
<tr>
<td>BioRad, CA, USA</td>
<td>Agarose, DC Protein Assay Kit, Trypan Blue, Tween 20, Polyvinylidene fluoride (PVDF), 40% Acrylamide/Bis solution (37.5:1), Pre-stained protein molecular weight markers</td>
</tr>
<tr>
<td>Bovogen Biologicals, Australia</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>Chem Supply, SA, Australia</td>
<td>Lithium Chloride (LiCl), Magnesium Chloride (MgCl₂), Sodium Chloride (NaCl), Sodium Hydroxide (NaOH), Formaldehyde</td>
</tr>
<tr>
<td>Cayman Chemicals, MI, USA</td>
<td>Resazurin</td>
</tr>
<tr>
<td>ELISAKit.com, Australia</td>
<td>Human VEGF-A ELISA kits</td>
</tr>
<tr>
<td>Enzo LifeSciences, NY, USA</td>
<td>Zinc Protoporphyrin (ZnPP)</td>
</tr>
<tr>
<td>Fisons, Loughborough, England</td>
<td>Polyethylene Glycol 6000 (PEG 6000)</td>
</tr>
<tr>
<td>ICN Biomedicals, NSW, Australia</td>
<td>Auranofin, MTT</td>
</tr>
<tr>
<td>Company</td>
<td>Products</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Life Technologies, CA, USA</td>
<td>100bp DNA markers (TrackIt), Big-Dye terminator Sequencing Mix v3.1, Lipofectamine™ 2000, Lipofectamine™ 3000, Penicillin/Streptomycin, RPMI-1640, Trypsin, Geneticin, Foetal Bovine Serum, Hoechst, Cell Mask Deep Red, Phalloidin</td>
</tr>
<tr>
<td>Macherey-Nagel</td>
<td>NucleoBond® Xtra Midi Kit</td>
</tr>
<tr>
<td>Merck, VIC, Australia</td>
<td>Acetic Acid, Ethanol, Hydrochloric Acid (HCl)</td>
</tr>
<tr>
<td></td>
<td>Isopropanol, Sodium Acetate (NaAc)</td>
</tr>
<tr>
<td></td>
<td>Sodium Hydrogen Carbonate (NaHCO₃), Methanol</td>
</tr>
<tr>
<td>Molecular Probes, CA, USA</td>
<td>2´,7´-dichlorofluorescein diacetate (H₂DCF-DA)</td>
</tr>
<tr>
<td>MP Biomedicals, VIC, Australia</td>
<td>Sodium Dodecyl Sulphate (SDS)</td>
</tr>
<tr>
<td></td>
<td>Tris (hydroxymethyl) aminomethane (Tris)</td>
</tr>
<tr>
<td>Oxoid, SA, Australia</td>
<td>Agar, Luria-Bertani Broth (L-Broth)</td>
</tr>
<tr>
<td>Promega Corporation, WI, USA</td>
<td>pGEM-T Easy Vector Kit, Passive Lysis Buffer</td>
</tr>
<tr>
<td></td>
<td>Luciferase Assay Kits, DNA Ligation buffer, GoTaq® Green Master Mix</td>
</tr>
<tr>
<td>Sigma Chemical Company, MO, USA</td>
<td>Ethidium Bromide, Bovine Serum Albumin (BSA), Ampicillin, Ammonium persulphate (APS), Bis-Acrylamide, Dimethyl Sulfoxide (DMSO), Dithiothreitol (DTT), 3-(dimethylamino)-propionitrile (DMAPN), DTNB, Phenol/Chloroform</td>
</tr>
<tr>
<td>USB Corporation, Ohio, USA</td>
<td>RNase A</td>
</tr>
<tr>
<td>Univar, NSW, Australia</td>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
</tr>
<tr>
<td></td>
<td>Potassium Dihydrogen Orthophosphate (KH₂PO₄)</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide (H₂O₂)</td>
</tr>
<tr>
<td>Vector Laboratories, CA, USA</td>
<td>VECTASHIELD mounting medium (with DAPI)</td>
</tr>
</tbody>
</table>
### 2.1.7 Solutions

Table 2.6: List of solutions.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Contents</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.25 X Assay mix</strong></td>
<td>0.5M Kpi, pH 7.5; 200mM EDTA; 20mM NADPH; 125mM DTNB</td>
<td>Measuring thioredoxin reductase activity</td>
</tr>
<tr>
<td><strong>5% or 10% Blotto</strong></td>
<td>5% or 10% (w/v) Dutch jug milk powder; 20mM Tris-Cl, pH 7.5; 137mM NaCl</td>
<td>Blocking of protein binding sites in ELISA and Western blots</td>
</tr>
<tr>
<td><strong>BSA/Triton X-100 blocking solution</strong></td>
<td>2% (w/v) BSA; 0.5% (v/v) Triton X-100</td>
<td>Blocking of protein binding sites in immunostaining and permeabilising the cells</td>
</tr>
<tr>
<td><strong>DNA loading buffer</strong></td>
<td>0.25% Bromophenol Blue; 40% (w/v) Sucrose</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td><strong>ELISA coating buffer, pH 9.6</strong></td>
<td>0.05M Na₂CO₃; 0.05M NaHCO₃</td>
<td>Coating ELISA plates with capture antibody</td>
</tr>
<tr>
<td><strong>ELISA light-up solution</strong></td>
<td>0.1M Sodium acetate, pH 4.5; 200µg/mL TMB; 0.001% H₂O₂; made fresh</td>
<td>ELISA</td>
</tr>
<tr>
<td><strong>Freezing solution</strong></td>
<td>50% Serum Containing Media; 40% Foetal Bovine Serum; 10% DMSO</td>
<td>Freezing down cells in liquid nitrogen</td>
</tr>
<tr>
<td><strong>Gene clean wash buffer</strong></td>
<td>10mM Tris-Cl, pH 7.6; 50mM NaCl; 2.5mM EDTA, pH 8; 50% (v/v) ethanol</td>
<td>DNA isolation</td>
</tr>
<tr>
<td><strong>L-plate</strong></td>
<td>7.5g Agar; 7.25g L-Broth in 500mL water; 100µg/mL Ampicillin</td>
<td>Growing bacterial cells</td>
</tr>
<tr>
<td><strong>NP-40 extraction buffer</strong></td>
<td>150mM NaCl; 50mM Tris-Cl, pH 8; 0.5% Nonidet P-40; 0.5mM EDTA; 2mM PMSF; 1µL/mL Protease Inhibitor Cocktail VI</td>
<td>Extraction of proteins from cells</td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SDS extraction buffer</strong></td>
<td>250mM Tris-Cl, pH 7.5; 2% (w/v) SDS; 2mM PMSF; 1µL/mL Protease Inhibitor Cocktail VI</td>
<td>Extraction of proteins from cells</td>
</tr>
<tr>
<td><strong>Phosphate buffered saline (PBS) 10 X stock, pH 6.8</strong></td>
<td>0.1M Na₂HPO₄; 0.02M KH₂PO₄; 1.4M NaCl; 0.03M KCl</td>
<td>Tissue culture</td>
</tr>
<tr>
<td><strong>Plasmid preparation solution</strong></td>
<td>15% (w/v) Sucrose; 25mM Tris, pH 8; 10mM EDTA</td>
<td>Plasmid extraction</td>
</tr>
<tr>
<td><strong>Polyethylene glycol (PEG)</strong></td>
<td>20% (w/v) PEG; 2.5M NaCl</td>
<td>PEG precipitation</td>
</tr>
<tr>
<td><strong>Sample buffer</strong></td>
<td>0.1M Kpi; 20mM EDTA; 0.1mg/mL BSA</td>
<td>Thioredoxin reductase activity assay</td>
</tr>
<tr>
<td><strong>SDS-PAGE Tris-Glycine running buffer</strong></td>
<td>25mM Tris-Cl; 20mM Glycine; 0.1% (w/v) SDS</td>
<td>Running SDS-PAGE gels</td>
</tr>
<tr>
<td><strong>SDS-PAGE 1 X SDS sample buffer</strong></td>
<td>10% (v/v) glycerol; 2% (w/v) SDS; 0.25M Tris-Cl, pH 6.8; 50mM DTT</td>
<td>Loading samples onto SDS-PAGE gels</td>
</tr>
<tr>
<td><strong>SDS-PAGE 5 X SDS sample buffer</strong></td>
<td>40% (v/v) glycerol; 5% (w/v) SDS; 0.25M Tris-Cl, pH 6.8; 50mM DTT</td>
<td>Loading samples onto SDS-PAGE gels</td>
</tr>
<tr>
<td><strong>Silica solution</strong></td>
<td>10g Silica in 3M NaI</td>
<td>DNA isolation</td>
</tr>
<tr>
<td><strong>50 X TAE buffer</strong></td>
<td>2M Tris; 5.71% Acetic acid; 0.05M EDTA, pH 8</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td><strong>TBS-Tween</strong></td>
<td>20mM Tris-Cl; 137mM NaCl; 0.05% Tween-20; Adjusted to pH 7.6</td>
<td>Washing PVDF membranes in Western blots and ELISA plates</td>
</tr>
<tr>
<td><strong>Western blot transfer buffer</strong></td>
<td>25mM Tris-Cl; 192mM Glycine; 10% (v/v) or 20% (v/v) Methanol (HPLC grade)</td>
<td>Western blot</td>
</tr>
<tr>
<td><strong>Crystal violet solution</strong></td>
<td>0.5% (w/v) Crystal violet; 25% (v/v) Methanol</td>
<td>Staining colonies in clonogenic assays</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 DNA Methods

2.2.1.1 Plasmid DNA Extraction

To obtain plasmid DNA from a 50mL bacterial culture

Plasmid DNA extraction was performed using either of the following methods:

2.2.1.1.1 Large-Scale Plasmid Preparation

A colony was selected and grown overnight at 37°C in 50mL L-Broth media containing 100µg/mL ampicillin, on an orbital shaker (180rpm). The cells were collected and centrifuged at 3,100 X g for 5 minutes. The supernatant was discarded and the pellet resuspended in 2.5mL of plasmid preparation solution (2.1.7). 5mL of 0.2M NaOH/1% (w/v) SDS solution was then added to lyse the cells and denature the DNA. The tube was mixed on ice until the solution became clear (approximately 10 minutes). 4mL of 3M sodium acetate (NaAc), pH 4.6 was then added to lower the pH and re-anneal the denatured DNA. The solution was mixed on ice until a white precipitate formed. The solution was then centrifuged at 12,500 X g for 10 minutes. The supernatant was then transferred carefully into a fresh tube and the pellet containing the genomic DNA was discarded.

10mL cold isopropanol was added to the supernatant and the solution stored on ice for at least 10 minutes after which the solution was again centrifuged at 12,500 X g for 10 minutes. The pellet was resuspended in 400µL of sterile water, and transferred to a clean Eppendorf tube. 400µL of 5M LiCl was added to the resuspension to precipitate the unwanted large RNA molecules, and the solution was vortexed and placed on ice for at least 10 minutes. The solution was then centrifuged at 16,200 X g for 10 minutes and the supernatant transferred to a fresh Eppendorf tube. 400µL of cold isopropanol was added to the supernatant and the tube stored on ice until the solution precipitated. The precipitate was centrifuged at 16,200 X g for 10 minutes. The pellet was resuspended in 200µL of sterile water.
2μL of 10mg/mL RNase A was added to the resuspension to digest the remaining small RNA molecules and then incubated at 37°C for 20 minutes. Then 200μL of a buffer saturated phenol/chloroform mix was added to remove the unwanted proteins. The solution was centrifuged at 16,200 X g for 2 minutes and the top layer transferred to a fresh Eppendorf tube. 500μL of cold absolute ethanol and 20μL of 3M NaAc, pH 5.3 were added to precipitate the DNA. This mix was stored at -20°C overnight (or at least 1 hour on ice) and then centrifuged at 16,200 X g for 10 minutes. The supernatant was discarded and the pellet washed with 200μL of 70% (v/v) ethanol. The tube was centrifuged at 16,200 X g for 1 minute and the ethanol removed. The pellet was resuspended in 50μL of sterile water.

2.2.1.1.2 Plasmid DNA Purification using NucleoBond® Xtra Midi Kit (Macherey-Nagel)

This protocol was carried out as per the manufacturer’s instructions using the buffers provided in the kit. A single bacterial colony was selected and grown overnight at 37°C in 50mL L-Broth media containing 100μg/mL ampicillin, on an orbital shaker (180rpm). The cells were collected and centrifuged at 6,000 X g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 8mL Buffer RES containing RNase A avoiding the clumping of the cells. The resuspension was vortexed once and 8mL Buffer LYS was added. The samples were mixed gently by inversion and incubated at room temperature for 5 minutes.

The column was equilibrated by applying 12mL of Buffer EQU onto the outer rim of the column filter, wetting the filter. The column was then allowed to empty by gravitational flow. 8mL of Buffer NEU was then mixed with the lysate by inverting 10-15 times until the solution became clear. The samples were mixed again by inversion three times prior to loading onto the equilibrated NucleoBond® Xtra Column Filter. The column was again allowed to empty by gravitational flow. 5mL of Buffer EQU was then used to wash the column filter. The filter was discarded and the column was washed with 8mL of Buffer WASH.
The plasmid DNA was eluted using 5mL of Buffer ELU into a 15mL collection tube. 3.5mL isopropanol was then added to the eluted plasmid DNA; the samples were vortexed briefly and incubated at room temperature for 2 minutes. The samples were then centrifuged at 5,000 X g for 15 minutes at room temperature, following which the supernatant was discarded. The pellet was washed with 2mL of 70% (v/v) ethanol and centrifuged at 15,000 X g for 5 minutes at room temperature. The pellet was dried completely for 5-10 minutes and resuspended in 50µL sterile water. The concentration of DNA in solution was determined by reading the absorbance at A260 in the Nanodrop ND-1000 (Biolab, Australia). The resuspension was stored at -20°C until required for subsequent experiments.

2.2.1.2 PEG Precipitation of Plasmid DNA

To further purify the DNA obtained from large scale plasmid preparations

To further purify plasmid DNA, 80µL of PEG solution (2.1.7) was added to 25µL plasmid DNA and incubated on ice for 1 hour. The sample was then centrifuged at 16,200 X g for 10 minutes and the supernatant was discarded. The pellet was washed with 1mL of 70% (v/v) ethanol, vortexed, and then centrifuged at 16,200 X g for 5 minutes. The ethanol was removed and the pellet dried completely, and resuspended in 20µL of sterile water. The amount of DNA in solution was determined by reading the absorbance at A260 in the Nanodrop ND-1000 (Biolab, Australia). The resuspension was stored at -20°C until required for subsequent experiments.

2.2.1.3 Preparation of Competent Cells

To prepare bacterial cells to take up plasmid DNA

A culture was set up by selecting a single colony of ED8799 bacteria and growing it overnight at 37°C in 5mL of L-Broth media on an orbital shaker (180rpm). The following day, 1mL of this culture was added to 50mL of L-Broth media and grown at 37°C on an orbital shaker until the culture had reached A600 of 0.4-0.6, corresponding to an early log phase (approximately 2 hours).
CHAPTER 2

After obtaining the desired O.D., the culture was transferred to a sterile tube and centrifuged at 2,800 X g for 5 minutes and the supernatant was discarded. The pellet was resuspended in 30mL 0.1M magnesium chloride (MgCl₂) and centrifuged again at 2,800 X g for 5 minutes. The supernatant was discarded and the pellet resuspended in 1mL of 0.1M calcium chloride (CaCl₂). The cells were then incubated on ice for an hour prior to transformation.

2.2.1.4 Transformation of DNA into Competent Cells

To introduce plasmid DNA into competent cells

5µL of the DNA to be transformed (generally ligation reaction products) was added to 50µL of the cell solution and incubated on ice for 30 minutes. The cells were then given a heat shock by incubating at 42°C for 2 minutes followed by incubation on ice for another 15 minutes. 200µL of L-Broth was added to the solution, which was incubated at 37°C on an orbital shaker (180rpm) for 20 minutes. This solution was then plated out onto an agar L-plate (2.1.7) containing 100µg/mL ampicillin and grown overnight at 37°C. If the vector being transformed contained a LacZ gene (e.g., pGEM-T Easy), 50µL of 0.8% (w/v) X-gal was spread on the agar L-plate for blue/white colour selection.

2.2.1.5 Isolation of Genomic DNA using ISOLATE Genomic DNA Mini Kit (Bioline)

To isolate genomic DNA from tissue culture cells

This protocol was performed using the buffers provided in the kit according to the manufacturer’s instructions. The cell pellets (1 X 10⁶ cells) were resuspended in 400µL Lysis Buffer D. 25µL of Proteinase K was then added to the lysates and mixed by vortexing. The lysates were then incubated at 50°C for approximately 2 hours and were vortexed intermittently. 4µL of 10mg/mL RNase A was added, the lysates were vortexed briefly and incubated at room temperature for 5 minutes. The samples were then centrifuged at 10,000 X g for 30 seconds and the supernatant were transferred to fresh Eppendorf tubes. 400µL of Binding Buffer D was then added to the supernatant
and samples were vortexed for 15 seconds. The suspension was then transferred to Spin Column D placed in 2mL collection tubes and centrifuged at 10,000 X g for 2 minutes. The collection tubes were discarded and the Spin Column D were placed into new collection tubes.

Spin Column D were then washed twice with 700µL of Wash Buffer D by centrifugation at 10,000 X g for 1 minute. The empty columns were centrifuged again at 10,000 X g for 2 minutes to remove all the traces of Wash Buffer D. The columns were finally placed into 1.5mL Elution Tubes and 200µL of Elution Buffer was added directly to the Spin Column membranes and incubated at room temperature for 1 minute. The samples were then centrifuged at 6,000 X g for 1 minute to elute the genomic DNA, which was either used immediately or stored at -20ºC until required.

2.2.1.6 Polymerase Chain Reaction (PCR)

To amplify DNA fragments

The PCR reaction was performed using the C1000™ Thermal Cycler (BioRad). All reactions were made to a final volume of 25µL in PCR tubes using the following reagents: DNA template (250-300ng), 200ng oligonucleotides, and ½ volume of GoTaq® Green Master Mix (Promega). The cycling parameters used were an initial denaturation at 95ºC for 3 minutes, followed by 95ºC for 30 seconds, 55ºC for 1 minute (annealing), and 72ºC for 1 minute (extension). This cycle was repeated 34 times with a final step of 72ºC for 10 minutes.

2.2.1.7 Agarose Gel Electrophoresis

To visually confirm the presence of DNA fragments and approximate their size

1% agarose gels were prepared by dissolving 0.4g of agarose in 40mL 1 X TAE buffer (2.1.7). Then 2µL of 10mg/mL ethidium bromide was added and agarose was poured into the appropriate mould having the appropriate comb. After the gel had set, DNA samples were loaded using 2µL of DNA loading buffer (2.1.7) along with the appropriate molecular weight standards to determine the size of the DNA.
Electrophoresis was performed at 100V for 30-40 minutes. The gel was then viewed and imaged under UV light, using Gene Snap software (PerkinElmer).

2.2.1.8 Gene Clean Procedure

*To excise and purify DNA from an agarose gel*

The desired DNA bands were carefully excised from the agarose gel and transferred to Eppendorf tubes. 2-3 volumes of 6M sodium iodide (NaI) were added and the tubes were heated at 50°C until the agarose had completely melted (approximately 5 minutes). 10µL of well vortexed silica solution (2.1.7) was then added to the tubes; the solutions were mixed well and allowed to stand at room temperature for 5 minutes. The tubes were then centrifuged at 16,200 X g to pellet the silica and the supernatant was discarded. The pellet was resuspended in 200µL of gene clean wash buffer (2.1.7) and centrifuged at 16,200 X g for 5 minutes. This step was repeated twice, after which, the wash buffer was removed. The pellet was centrifuged again for 2 minutes to remove the remaining wash buffer. The pellet was resuspended in 20µL of water and incubated at 37°C for 5 minutes to elute DNA off the silica. The resuspension was centrifuged again at 16,200 X g for 5 minutes; the supernatant containing the eluted DNA was transferred to a fresh tube.

2.2.1.9 Ligation Reaction

*To insert DNA fragments into plasmid vectors*

The DNA inserts were ligated into appropriate vectors via a ligation reaction carried out at 15°C in a water bath overnight. The reactions were carried out in a total volume of 20µL using the pGEM-T Easy Vector system kit (Promega) according to the manufacturer’s protocol. The DNA insert to vector ratio was 5:1 and T4 DNA Ligase (Promega) was used with 2 X Ligation Buffer (Promega).
2.2.1.10 DNA Sequencing

*To confirm the sequence of the DNA inserted into the plasmid*

### 2.2.1.10.1 Sequencing PCR

*To amplify the DNA fragment to be sequenced*

The concentration of the PEG precipitated (2.2.1.2) DNA was determined prior to sequencing. The DNA was diluted to 250ng/µL. The PCR reaction was set up using the following reagents:

- DNA template (250ng) 1µL
- Sequencing primer (3.2pmol) (Table 2.2) 1µL
- Big Dye Terminator Sequencing Mix v3.1 (Life Technologies) 8µL
- Water 10µL

The PCR cycling parameters were an initial step at 96°C for 10 seconds, followed by 50°C for 5 seconds, after which the temperature was raised at 1°C/second until it reached 60°C, where it was held for 4 minutes. The temperature was once again raised to 96°C. This cycle was repeated 25 times.

### 2.2.1.10.2 Ethanol/EDTA/NaAc Precipitation

*To clean-up sequencing reaction products*

2µL of 125mM EDTA and 2µL of 3M NaAc, pH 4.6 were added to the PCR samples. 50µL of absolute ethanol was also added and the tubes inverted several times to mix the samples. The samples were then incubated at room temperature for 15 minutes, centrifuged at 16,200 X g for 20 minutes and the supernatant discarded. 250µL of 70% (v/v) ethanol was then added to the pellets and vortexed. The samples were centrifuged again at 16,200 X g for 5 minutes and the supernatant discarded. The 70% (v/v) ethanol
The wash step was repeated twice. The pellets were dried completely and sent to the Griffith University DNA Sequencing Facility for sequencing.

### 2.2.2 Tissue Culture

All the tissue culture procedures were performed in a Class II Biohazard Laminar Air Flow hood with the exception of cell counting, incubation and centrifugation.

#### 2.2.2.1 RPMI 1640 Media Preparation

*To prepare growth media for tissue culture cells*

2L RPMI 1640 media was prepared by mixing two sachets of RPMI 1640, 20mM sodium bicarbonate, HCl (to adjust pH to 7.2-7.4) and distilled water. The media was filtered into 400mL aliquots and stored at 4°C until required. 45mL of foetal bovine serum (FBS) was added to the media before use to make 10% (v/v) serum containing media (SCM). Penicillin/streptomycin antibiotics (100μg/mL) were also added into each aliquot before use.

#### 2.2.2.2 Passaging of Cells

*To grow and maintain tissue culture cell lines*

The MDA-MB-231 and A549 cell lines were cultured in 10% (v/v) SCM. A portion of cells was passaged into a new culture flask (Corning) once the cells had reached 80-90% confluency. The media was removed and the cells washed twice with 1 X PBS (2.1.7). 800μL of trypsin was added to cover the surface of the flask and incubated at 37°C/5% CO₂ for 2-3 minutes for cells to detach. 7mL of 10% (v/v) SCM was added to rinse the surface of the flask and the cells were transferred to a 10mL tube. The tube was then centrifuged at 265 X g for 5 minutes and the supernatant was poured off. The cell pellet was resuspended in 8mL of fresh 10% (v/v) SCM. An aliquot of the cell suspension was then transferred to a new flask and fresh media added to give a final volume of 12mL. The flask was then incubated at 37°C/5% CO₂. The number of cells
transferred to the new flask depended on the confluency required for subsequent experiments.

### 2.2.2.3 Cell Count Determination

*To count the cells before use in subsequent experiments*

Before being harvested for use in experiments, the cells were counted. 10µL of the cell suspension was mixed with 10µL of trypan blue dye. 10µL of this mixture was applied to the TC10™ dual chamber system sample slide and the cell count per mL was obtained from the TC10™ automated cell counter (BioRad). Total cell number was counted as follows:

\[
\text{Total cell number} = \text{Cells/mL} \times \text{Final volume}
\]

Alternatively, a Haemocytometer was also used to count the cells.

### 2.2.2.4 3D Spheroid Formation (*Lovitt et al., 2013*)

*To grow cells as 3D spheroids*

15µL of 7.6mg/mL growth factor-reduced Matrigel (GFR-Matrigel, BD Biosciences), diluted in ice-cold serum-free media (SFM) was added to the wells of 384-well microtiter plate (PerkinElmer) and incubated at 37°C/ 5% CO₂ for at least 20 minutes allowing the Matrigel to solidify. Cells were harvested and seeded on top of the Matrigel layer at a density of 1000 cells per well in 100µL of SCM. The cells were then incubated at 37°C/ 5% CO₂ for 6 days to allow the formation of 3D spheroids with the culture media being replaced after 3 days.
2.2.2.5 Growing Cells from Liquid Nitrogen Stocks

*To bring up cells from frozen stocks*

Cells were taken out from storage in liquid nitrogen vessels and the tubes immediately placed in warm water until the cells were thawed (approximately 2 minutes). The cells were then transferred into a 10mL centrifuge tube and 5mL of 10% (v/v) SCM was added very slowly into the tube using a transfer pipette. The cells were then centrifuged at 265 X g for 5 minutes. The pellet was resuspended in 5mL of fresh 10% (v/v) SCM. The resuspension was then transferred to a 25cm² flask and incubated at 37°C/ 5% CO₂ until they reached 80-90% confluency, after which they were transferred to a 75cm² flask.

2.2.2.6 Freezing down Cells

*To freeze down cells for later use*

Cells were counted and centrifuged at 265 X g for 5 minutes. The pellets were resuspended in freezing solution (2.1.7) and transferred immediately to sterile 1mL cryo-tubes. The tubes were placed in Styrofoam container and stored at -30°C for 2-3 hours and then transferred to -80°C overnight. The cryo-tubes were then placed in liquid nitrogen for long-term storage.

2.2.3 Oxygen Growth Conditions for Tissue Culture Cell Lines

*To grow tissue culture cells under different oxygen conditions*

Cells were seeded out in tissue culture flasks or appropriate plates and were grown until ~80-85% confluent. Cells were grown under the following conditions:

- **Normoxia**: Cells were grown at 37°C/ 5% CO₂ under atmospheric oxygen conditions.
- **Hypoxia**: Cells were transferred into a C-Chamber hypoxic growth chamber attached to the Proox model C21 (Biospherix, New York) controller that maintained oxygen levels of 0.1% at 37°C/ 5% CO₂.
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- Hypoxia/Re-oxygenation: Cells were grown under 0.1% hypoxic conditions at 37°C/ 5% CO₂ and then re-oxygenated under 37°C/ 5% CO₂ atmospheric oxygen conditions.
- Preconditioning: Cells were incubated under 0.1% hypoxic conditions at 37°C/ 5% CO₂ and then re-oxygenated at 37°C/ 5% CO₂ under atmospheric oxygen conditions. This cycle was repeated four times.

The incubation times under each oxygen conditions will be specified in the appropriate chapters.

2.2.4 Generation of Stable Transfected Cell Lines

*To prepare cell lines over-expressing the desired gene*

2.2.4.1 Transfection of Cells and Geneticin Selection

*To insert the desired DNA into the cells and select for the cells that were positively transfected*

Cells (2 X 10^6) were seeded out in a 6-well plate (Corning) 24 hours prior to transfections and incubated at 37°C/ 5% CO₂ so that they were 80-90% confluent. On the day of transfection, the DNA-Lipofectamine 2000 complex was made by diluting 5μg of DNA in 500μL SFM and 10μL of Lipofectamine 2000 in 500μL of SFM. These dilutions were then mixed and incubated at room temperature for 20 minutes.

Following this incubation, cells were washed twice with 1 X PBS (2.1.7). The DNA-Lipofectamine 2000 complex was further diluted in 2mL SFM, and added to the wells. The transfections were performed in duplicate. Cells were allowed to incubate at 37°C/ 5% CO₂ for 18 hours, following which, the media was replaced with fresh 10% (v/v) SCM. Cells were then incubated for 24 hours at 37°C/ 5% CO₂.
The following day, the media was again replaced with fresh 10% (v/v) SCM supplemented with 1250µg/mL (final concentration) geneticin for selection of the successfully transfected cells. Cells were grown until 80-90% confluent (approximately 2-3 weeks) on geneticin supplemented media, which was replaced every 3-4 days.

2.2.4.2 Single Cell Clonal Isolation (adapted from Corning Life Science)

To select colonies arising from a single transfected cell

Once the geneticin-resistant cells were confluent, they were harvested from the 6-well plate and 96-well plates (Nunc) were set up for clonal selection of the transfected cells. Briefly, 200µL of cell suspension was added to the first well of the 96-well plate, which was serially diluted down the plate. A second set of dilutions was then carried out across the plate. Cells were then grown at 37°C/ 5% CO₂ for a week and observed under the microscope every day. Each well containing a single colony was marked during this period. Once the cells in the marked wells were ~40% confluent, they were harvested and seeded into 24-well plates and gradually scaled up to 6-well plate, 25cm² flasks, and finally to 75cm² flasks.

2.2.4.3 GFP Imaging

To visually verify the expression of GFP in stable transfected cells

Cells (50,000) were seeded onto cover slips in a 4-well plate and allowed to grow at 37°C/ 5% CO₂ until 80-90% confluent. Cells were washed twice with 1 X PBS (2.1.7) and fixed using 0.5mL of freshly prepared 3% (v/v) formaldehyde in 1 X PBS (2.1.7) and incubated at room temperature for 15 minutes.

After two more washes with 1 X PBS (2.1.7), each cover slip was carefully removed from the plate and the back wiped free of PBS before being mounted cell side-down on a glass microscope slide onto a 5µL drop of VECTASHIELD mounting medium (containing DAPI) (Vector laboratories). Slides were stored in the dark at 4°C until being visualised with a Nikon E800 microscope.
2.2.5 Protein Methods

2.2.5.1 Protein Extraction using NP-40 Lysis Buffer

*To lyse cells and extract intracellular proteins*

This method was used to lyse and collect cell extracts for protein estimation, thioredoxin reductase activity assays, western blotting, ROS assays and ELISA. Cells were washed twice with 1 X PBS (2.1.7) and then ice-cold NP-40 cell lysis buffer (2.1.7) was added. Cells were kept on a shaker for 15-30 minutes. For hypoxic samples, cells were scraped using a cell scraper immediately after addition of NP-40 buffer. The lysates were cleared by centrifugation at 12,500 X g for 10 minutes at 4°C. The supernatant was transferred to a new tube and either used immediately or stored at -20°C until required.

2.2.5.2 Protein Extraction using SDS Lysis Buffer

*To lyse cells and extract intracellular proteins*

This method was used to lyse and collect cell extracts for western blotting to detect thioredoxin reductase and Nrf2 protein levels. Cells were washed twice with 1 X PBS (2.1.7) and then SDS cell lysis buffer (2.1.7) was added. Cells were scraped using a cell scraper immediately after addition of the SDS buffer. The lysates were then boiled at 100°C for 5 minutes and mixed with a Hamilton syringe in between. The samples were then centrifuged at 12,500 X g for 10 minutes at 4°C. The supernatant was transferred to a new tube and either used immediately or stored at -20°C until required.

2.2.5.3 Protein Estimation

*To estimate the amount of total proteins extracted from the cells*

The DC protein assay kit (BioRad) was used to determine the concentration of proteins extracted from cells. 20mg/mL BSA stock solution was serially diluted in water to make up 2mg/mL, 1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL, and 0mg/mL BSA. These BSA dilutions were then added onto a 96-well plate (Sarstedt) in triplicate in order to
obtain a standard curve. The cell extracts were then diluted 1/10 in water and added into the 96-well plate in triplicate.

The DC Protein Assay Reagent was prepared by mixing 40μL of DC Protein Assay Reagent S with 2mL of DC Protein Assay Reagent A. 25μL of this mixture was added to each well and the plate was left to stand at room temperature for 1 minute. 200μL of DC Protein Assay Reagent B was then added to each well and the plate was mixed in the SpectraMax M3 plate reader (Molecular Devices) for 5 minutes. The plate was left to stand in the dark for 15 minutes. The absorbance was then measured at 750nm. The protein concentrations of the extracted samples were then calculated from the BSA standard curve using the SOFTmax Pro 2.6 software.

2.2.5.4 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

*To separate and identify proteins on the basis of their size*

SDS-PAGE gels were prepared using either a 10% (v/v) or 15% (v/v) acrylamide separating gel and 4% (v/v) acrylamide stacking gel, using the volumes and solutions specified in Table 2.7.

**Table 2.7: SDS-PAGE gel preparation.**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>10% (v/v)</th>
<th>15% (v/v)</th>
<th>4% (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>5mL</td>
<td>7.5mL</td>
<td>0.5mL</td>
</tr>
<tr>
<td>1.3% Bis-acrylamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75M Tris, pH 8.8</td>
<td>10mL</td>
<td>10mL</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris, pH 6.8</td>
<td>-</td>
<td>-</td>
<td>1.2mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.7mL</td>
<td>2.2mL</td>
<td>3.2mL</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
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<td>0.2mL</td>
<td>0.05mL</td>
</tr>
<tr>
<td>DMAPN</td>
<td>0.025mL</td>
<td>0.025mL</td>
<td>0.03mL</td>
</tr>
<tr>
<td>30% Ammonium persulphate (APS)</td>
<td>0.075mL</td>
<td>0.075mL</td>
<td>0.016mL</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Cell lysates were mixed with either 1 X SDS sample buffer (2.1.7) or 5 X SDS sample buffer (2.1.7) along with 0.1% (w/v) bromophenol blue. The samples were boiled at 100°C for 2 minutes prior to loading onto the gel. The protein concentration of the lysates was determined (2.2.5.3) and equal amounts of cell extracts were applied to each well of the SDS-PAGE gels. Pre-stained molecular weight markers (BioRad) were run in parallel to the samples. The gels were electrophoresed in SDS-PAGE Tris-Glycine running buffer (2.1.7) for approximately 60 minutes at a constant voltage of 200V.

2.2.5.5 Western Blotting

To transfer proteins from SDS-PAGE gels to PVDF membranes for visualisation of specific proteins

The SDS-PAGE gel was equilibrated in ice-cold western blot transfer buffer (2.1.7) along with fibre pads and 3MM chromatography paper (Whatman). The PVDF membrane (Millipore) was prepared for transfer by soaking in methanol (HPLC grade) for 15 seconds followed by washing in mqH2O for 2 minutes and in transfer buffer for 5 minutes. The gel sandwich was then assembled in the following order: cathode, fibre pad, chromatography paper, SDS-PAGE gel, PVDF membrane, chromatography paper, fibre pad and anode. The cassette was placed in the tank chamber along with an ice brick and a magnetic stirrer. The apparatus was filled with transfer buffer and run at 100V for 60 minutes with constant stirring, after which the PVDF membrane was subjected to immunoblotting.

The PVDF membrane was blocked with either 5% (w/v) or 10% (w/v) Blotto (2.1.7) at room temperature for 20-30 minutes and then transferred to 5% (w/v) Blotto (2.1.7) containing the appropriate dilution of the primary antibody (Table 2.4). The membrane was incubated at 4°C overnight on a rocking platform and then washed with TBS-Tween (2.1.7) for 5 minutes. The wash step was performed three times, after which the membrane was transferred to 5% (w/v) Blotto containing the appropriate dilution of the HRP-conjugated secondary antibody (Table 2.4) and incubated at room temperature for an hour, with gentle agitation. The membrane was again washed with TBS-Tween three times for 5 minutes, following which the membrane was developed.
2.2.5.6 Development of Blots

To visualise the probed proteins on the PVDF membranes

The ECL™ Western Blotting Analysis System kit (GE Healthcare) was used according to the manufacturer’s protocol to develop the blots. The washed PVDF membranes were placed on a metal tray and two reagents provided in the kit were mixed (1:1) and applied to cover the membrane. The blots were then visualised using the Fujifilm Las-3000 developer machine.

2.2.6 Promoter Reporter Assay

To measure the promoter activity of the thioredoxin system proteins

2.2.6.1 Transient Transfections

To introduce DNA into cells

The transient transfections were carried out over a period of three days. On the first day, cells were harvested and an appropriate number (115,000) was seeded out in a 24-well plate (Corning) containing 0.5mL of 10% (v/v) SCM and incubated at 37°C/ 5% CO₂ until 80-90% confluency was obtained.

The following day, cells were transfected using either Lipofectamine 2000 or Lipofectamine 3000. The DNA-Lipofectamine 2000 complex was made by diluting 2µg of DNA in 400µL of SFM and 8µL of Lipofectamine 2000 in 400µL of SFM. The diluted DNA and Lipofectamine 2000 were mixed and incubated at room temperature for 30 minutes. Cells were washed twice with 1 X PBS (2.1.7) and 0.5mL of SFM was added to each well. 100µL of the DNA-Lipofectamine 2000 complex was then added to each well. When using Lipofectamine 3000 for transfections, 2µg DNA was diluted in 125µL of SFM containing 2.5µL of P3000. 3.75µL of Lipofectamine 3000 was also diluted in 125µL of SFM. The DNA-Lipofectamine 3000 complex was incubated at room temperature for 10-15 minutes. Cells were washed with 1 X PBS (2.1.7) twice and 0.5mL of SFM was added to each well. 50µL of the DNA-Lipofectamine 3000 complex was then added per well.
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Cells were incubated at 37°C/ 5% CO₂ for 5 hours, after which the media was supplemented with 0.5mL of 20% (v/v) SCM. If the cells were being investigated in response to any stress agent (e.g., auranofin, tBHQ), the agent was applied to the cells at this step. Cells were then incubated at 37°C/ 5% CO₂ for 18 hours either under normoxia or hypoxia. For preconditioning (2.2.3), cells were preconditioned 2.5 hours post transfections and then incubated under hypoxia for 18 hours.

On the third day, cells were washed twice with 1 X PBS (2.1.7) and then lysed at room temperature with 100µL of 1 X Passive Lysis Buffer (Promega) for 30 minutes on a shaker. The cell lysates were analysed immediately by luciferase assays.

2.2.6.2 Luciferase Assay

To measure the luciferase activity produced from the transiently transfected constructs

20µL of each sample was transferred to a 96-well white-walled microtiter plate (Wallac). Luciferase activity in the lysates was then measured using 40µL of Luciferase Assay Reagent as per the manufacturer’s protocol (Promega), using the 1420 Multilabel Counter WallacVictor² (PerkinElmer). The concentration of the proteins in the cells was determined (2.2.5.3) and used to normalise the Luciferase assay results.

2.2.7 HIF1α-CAD Activity Assay (Bracken et al., 2006)

To measure the activity of HIF1α-CAD in cells

2.2.7.1 Transient Transfections

To introduce DNA into cells

Transient transfections were performed for the HIF1α-CAD activity assays, which were carried out over a period of three days. Cells were harvested and appropriate numbers (60,000) were seeded out on Day 1 in a 24-well plate (Corning) containing 0.5mL of 10% (v/v) SCM. Cells were then incubated at 37°C/ 5% CO₂ until 80-90% confluency was obtained.
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The following day, cells were transfected using Lipofectamine 3000. 4.5µg of either pEF-BOS-CS-Empty (2.1.2.1) or pEF-BOS-CS-HIF1α (2.1.2.2) along with 4.5µg of G5E1bLUC (2.1.2.2) and 0.45µg of RL-TK (2.1.2.1) DNA was diluted in 405µL of SFM containing 18µL of P3000. 18µL of Lipofectamine 3000 was also diluted in 432µL of SFM. The DNA-Lipofectamine 3000 complex was prepared and incubated at room temperature for 10-15 minutes. Cells were washed with 1 X PBS (2.1.7) twice and 0.5mL of SFM added to each well. 50µL of the DNA-Lipofectamine 3000 complex was then added per well.

Cells were incubated at 37°C/ 5% CO₂ for 5 hours, after which the media was supplemented with 0.5mL of 20% (v/v) SCM. If cells were being investigated in response to any stress agent (e.g., auranofin), they were applied to cells at this step. Cells were then incubated at 37°C/ 5% CO₂ for 18 hours either under normoxia or hypoxia. For preconditioning studies, cells were preconditioned 2.5 hours post transfections and then incubated under hypoxia for 18 hours.

On the third day, cells were washed twice with 1 X PBS (2.1.7) and then lysed at room temperature with 100µL of 1 X Passive Lysis Buffer (Promega) for 30 minutes on a shaker. The cell lysates were analysed immediately by dual luciferase assays.

2.2.7.2 Dual Luciferase Assay

To measure the luciferase and renilla luciferase activity produced from the transiently transfected constructs

20µL of each sample was transferred to a 96-well white-walled microtiter plate (Wallac). Luciferase activity was then measured from the lysates with 40µL of Luciferase Assay Reagent and 40µL of Stop and Glow as per manufacturer’s protocol (Promega), using the 1420 Multilabel Counter WallacVictor² (PerkinElmer). The luciferase activity was normalised by the renilla luciferase activity for each sample.
2.2.8 Monolayer Scratch Assay
To measure the migration of cells

The monolayer scratch assays, also known as wound healing assays, were performed using either of the following methods:

2.2.8.1 P1000 Tip-based Scratch Assay (Dhanesuan et al., 2002)

This method was adapted from the protocol described by Dhanesuan et al., 2003. Before seeding out cells, a scalpel was used to etch horizontal and vertical lines, resulting in a cross at the centre of each well, on the underside of a 24-well plate (Corning). These lines were used as reference points.

Cells (75,000) were seeded out and incubated at 37°C/5% CO₂ until they were ~80% confluent. Cells were then scratched using a P1000 blue tip to create a scratch wound passing through the reference mark. The media and displaced cells were then removed and replaced with fresh 10% (v/v) SCM containing 10mM thymidine to act as an anti-proliferative agent. Photos of scratches, including the reference points, were taken at 0, 24, and 48 hours to monitor closure of the wound. At the start of each experiment, the distance migrated by cells for each wound-closure was designated as 0. The distance migrated was calculated for 24 and 48 hours based on the reduction of the wound width with respect to the 0 hour time point.

2.2.8.2 Comb-based Scratch Assay

The Cell Comb™ Scratch Assay (Millipore) kit was also used. Before seeding out cells, cross-reference marks were etched at the bottom of the dishes to be used as reference points. Appropriate numbers of cells (2 X 10⁶) were seeded out in a media volume of 15mL and grown until ~80% confluent. A Cell Comb was then used to create multiple scratches passing through the reference marks. The plate was washed twice with 1 X PBS (2.1.7) to get rid of any cells attached loosely near the scratches. 10mL of 10% (v/v) SCM containing 10mM thymidine was then added to the plate. If the migration of
cells was being examined in response to auranofin, appropriate concentrations of auranofin were added at this step. Closure of the wound was monitored by taking photo of the scratches at 0, 24, and 48 hours at the reference marks. The distance migrated was calculated as described in 2.2.8.1.

2.2.9 Enzyme-Linked Immunosorbent Assay

To quantify intracellular thioredoxin levels in cells

2.2.9.1 Coating of Plates with Primary Antibody

A 96-well clear-bottom polystyrene microtiter plate (Sarstedt) was coated with 100µL ELISA coating buffer (2.1.7) containing 20µg/mL primary monoclonal 1B3 antibody (Table 2.4). The first column of the plate was coated with only the coating buffer and was used as a blank. The plate was incubated for one hour at 37°C and then washed twice with TBS-Tween (2.1.7). 150µL of 5% (w/v) Blotto (2.1.7) blocking solution was then applied to the plate to block any remaining protein binding sites. The plate was incubated at 37°C for 30 minutes.

2.2.9.2 Loading Samples

The blocking solution was flicked off and the thioredoxin standard solution and the samples were diluted in 5% (w/v) Blotto solution across the plate. The thioredoxin standards ranged from 50ng/well to 0.098ng/well and were prepared as follows: 4µL of 0.5mg/mL purified recombinant human thioredoxin was reduced with 1µL of 2.5mM DTT at 37°C for 20 minutes. This mixture was then added to 2mL of 5% (w/v) Blotto and a standard curve of thioredoxin in the assay was obtained by adding 100µL (100ng) of this solution to 100µL of 5% (w/v) Blotto in the first well. This was then serially diluted across the plate giving a standard curve of thioredoxin from 50ng/well to 0.098ng/well.
Cell lysates were reduced with 0.8mM DTT at 37ºC for 20 minutes. 2.5µL of the reduced lysates were mixed with 197.5µL of 5% (w/v) Blotto in the first well and a five-step serial dilution was performed. The plate was incubated at 4°C overnight.

2.2.9.3 Light-Up

After the overnight antigen capture step, the plate was washed three times with TBS-Tween (2.1.7). 100µL of the labelled 2B1-b secondary antibody (Table 2.4) in 5% (w/v) Blotto (1 in 500 dilution) were then applied to each well. The secondary antibody was incubated for 2 hours at 37°C. The plate was washed three times with TBS-Tween (2.1.7) before 100µL of ELISA grade Avidin-Horseradish Peroxidase (HRP) (Table 2.4) in 5% (w/v) Blotto (1 in 1000 dilution) was applied. The plate was incubated at 37°C for 1 hour. The plate was then washed again as described above, and 100µL of freshly prepared ELISA ‘light-up’ solution (2.1.7) was added to each well.

The plate was incubated for 15 minutes in the dark at room temperature, before adding 100µL/well of 1M H₂SO₄ to stop the ‘light-up’ reaction. The absorbance of each well was measured at 450nm on the SpectraMax M3 plate reader (Molecular Devices) and the concentration of the unknown samples determined using the thioredoxin standard curve by the SOFTmax PRO 2.6 software. The amount of thioredoxin protein in the lysates was normalised by the total cellular protein content.

2.2.10 VEGF-A ELISA using the Human VEGF-A ELISA Kit (Elisakit)

*To quantitate the levels of hVEGF-A protein secreted by cells*

This protocol was carried out according to the manufacturer’s instructions using the buffers provided in the kit. 96-well strip-well plates pre-coated with human VEGF-A capture antibody were used. The lyophilised standard was reconstituted in 10% (v/v) SCM. The standard was then diluted to 2000pg/mL in 10% (v/v) SCM, which was then serially diluted down to 31.25pg/mL. The samples were also diluted 1 in 10 in 10% (v/v) SCM. 100µL of standards, samples and 10% (v/v) SCM (used as blank) were
added to the appropriate wells in duplicate. The plate was then incubated for 2 hours at room temperature.

The samples were then removed and the plate was washed four times with wash buffer. 100µL of biotin labelled detection antibody was added to the plate, which was then incubated for 1.5 hours at room temperature. The plate was washed again with wash buffer four times. 100µL of freshly diluted streptavidin-HRP conjugate was then added and the plate was incubated at room temperature for 45 minutes.

The plate was washed five times with wash buffer. 100µL of TMB substrate was added next and the plate was incubated at room temperature for 15 minutes in dark. The reaction was stopped using 50µL of stop solution. The absorbance of each well was measured at 450nm on the SpectraMax M3 plate reader (Molecular Devices) and the concentration of the unknown samples determined using the VEGF-A standard curve by the SOFTmax PRO 2.6 software. The amount of VEGF-A protein secreted was calculated per mL of the media.

### 2.2.11 Thioredoxin Reductase Activity Assay

*To measure the specific activity of thioredoxin reductase in tissue culture cells*

Cells (2 X 10^6) were grown under different growth conditions (2.2.7), washed twice with 1 X PBS and lysed into ice-cold NP-40 lysis buffer (2.2.5.1) using a cell scraper. A clear-bottom 96-well plate (Sarstedt) was used for the thioredoxin reductase activity assay. 25µL of water and NP-40 extraction buffer were used as blanks in the first two wells of the plate. 25µL of the lysate was then added to the third well. The samples were added in triplicate. 25µL of sample buffer (2.1.7) was then added to each well. 200µL of freshly prepared assay mix (2.1.7) was finally added to each well and absorbance was measured every 30 seconds at 412nm for 10 minutes on the SpectraMax M3 plate reader (Molecular Devices). Units of thioredoxin activity (U) was calculated as µmole of TNB produced per minute per mL of the cell lysate using an extinction coefficient for TNB at 412nm (13.6 X 10^3 M^-1) and an assay path length of 0.7325cm for the 250µL assay volume in the 96-well plate.
The concentration of the proteins in the cell lysates was also determined as described in 2.2.5.3 and was used to calculate the specific activity of thioredoxin reductase using the following equation:

\[
\text{Specific activity (U/mg)} = \frac{U}{\text{Total protein concentration}}
\]

### 2.2.12 ROS Analysis

*To measure the ROS levels in tissue culture cells*

Cells (1.5 X 10^6) were grown in 6-well plates, after which 5μM H₂DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) (Molecular Probes) was added to cells and incubated for 30 minutes at 37°C/ 5% CO₂. Cells were then lysed in 300μL of ice-cold NP-40 lysis buffer (2.2.5.1). A clear-bottom black-walled 96-well microtiter plate (Corning) was used to read the fluorescence. 100μL of lysates were added to the plate in triplicate. NP-40 lysis buffer was used as blank.

The fluorescence was measured at 495nm excitation and 515nm emission wavelengths with a 495nm cut-off on the SpectraMax M3 plate reader (Molecular Devices). The protein concentration in the lysates was also determined (2.2.5.3). The relative fluorescence units (RFUs) were calculated per mg of the total cellular protein.

### 2.2.13 MTT Proliferation Assay

*To assess cellular proliferation in response to different treatments*

The MTT cell proliferation assay was carried out over a period of 5 days starting from Day -2 to Day2. On Day -2, cells were harvested (2.2.2.2) and counted (2.2.2.3). Appropriate number of cells (30,000-50,000) were seeded out into tissue-culture treated 96-well plates (Nunc) and grown until ~80% confluent. The next day (Day -1), media was replaced by 0.1% (v/v) SCM and cells were incubated at 37°C/ 5% CO₂ for another 24 hours.
The next day (Day 0), the media was replaced again with appropriate concentrations of auranofin diluted in 10% (v/v) SCM and cells were incubated for 24 hours under normoxia or hypoxia. For preconditioning studies, cells were preconditioned prior to addition of auranofin and then incubated under hypoxia for 24 hours.

On Day 1, 20µL of 5mg/mL MTT was added per well and cells were incubated under normoxia or hypoxia for another 3 hours. At this stage 50µL of 20% (w/v) SDS in 0.01M HCl was added to each well and cells were incubated at 37°C/ 5% CO₂ overnight. On Day 2, absorbance was measured at 570nm using the SpectraMax M3 plate reader (Molecular Devices).

2.2.14 Resazurin Metabolic Activity Assay (Tung et al., 2011, Lovitt et al., 2013)

*To assess the metabolic activity of cells in response to drug treatment*

Cells were grown either as a 2D monolayer or as 3D spheroids in 384-well cell carrier plates (PerkinElmer) and treated with appropriate drugs and controls for either 24 or 144 hours. At the end of each treatment, a final concentration of 600µM Resazurin sodium salt was added to the cells. Cells grown as 2D monolayers were then incubated for 4 hours at 37°C/ 5% CO₂ while the 3D spheroids were incubated for 6 hours at 37°C/ 5% CO₂. The total well fluorescence was then measured using an EnVision™ multilabel plate reader (PerkinElmer).

2.2.15 Real-time RT-PCR

*To quantify the mRNA expression levels in cells*

2.2.15.1 Isolation of total RNA using TRIsure™ (Bioline)

*To extract total RNA from cells*

Appropriate number of cells (1.5 X 10⁶) were seeded into 35mm petri dishes and suitable treatments were applied. At the end of each treatment, cells were lysed directly by addition of 1mL TRIsure™ reagent. The lysates were collected from the dishes, transferred to Eppendorf tubes and incubated at room temperature for 5 minutes. 200µL
of chloroform was then added to the lysates, which were mixed vigorously for 15 seconds by shaking. The samples were then incubated at room temperature for 3 minutes and then centrifuged at 12,000 X g for 15 minutes at 4°C. The aqueous phase was very carefully transferred to new Eppendorf tubes without disturbing the interphase.

500µL ice-cold isopropanol was added to the aqueous phase and samples were incubated at room temperature for 10 minutes. The samples were then centrifuged at 12,000 X g for 10 minutes at 4°C. The supernatant was discarded and pellet was washed once with 1mL of 70% (v/v) ethanol, samples were vortexed and centrifuged at 7,500 X g for 5 minutes. All traces of ethanol were removed; the pellets were air-dried and resuspended in 20µL of PCR-grade water. The concentration of the extracted RNA was measured by reading the absorbance at A_{260} in the Nanodrop ND-1000 (Biolab, Australia). The samples were stored at -20°C until required.

### 2.2.15.2 cDNA Synthesis using SensiFAST™ cDNA Synthesis Kit (Bioline)

*To synthesise cDNA from the extracted total RNA*

The reverse-transcriptase PCR reaction was set up on ice as follows:

- **RNA**: 1µg
- **5 X TransAmp Buffer**: 4µL
- **Reverse Transcriptase**: 1µL
- **Water**: up to 20µL

The PCR parameters were an initial primer annealing at 25°C for 10 minutes, reverse transcription at 42°C for 10 minutes, and a final inactivation at 85°C for 5 minutes. After the PCR, the concentration of the cDNA was measured at A_{260} in the Nanodrop ND-1000 (Biolab, Australia). The samples were then stored at -20°C until required.
2.2.15.3 Real-time RT-PCR using SensiFAST™ HRM kit (Bioline)

To measure the mRNA expression

The real-time RT-PCR reaction was set up on ice as follows:

- 2 X SensiFAST™ HRM Mix: 10µL
- 10µM Forward primer (Table 2.2): 0.4µL (200nM)
- 10µM Reverse primer (Table 2.2): 0.4µL (200nM)
- Water: up to 16µL
- cDNA Template: 1µL

The PCR was performed using a Rotor Gene 6000 (Corbett Life Science, QIAGEN). The PCR parameters were an initial polymerase activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 20 seconds. The gene expression was analysed using the comparative cycle threshold algorithm (ΔΔCt method). The mRNA levels were normalised against the expression of the L32 house-keeping gene (Lafleur et al., 2005).

2.2.16 Clonogenic Assay

To study the ability of cells to form colonies

Cells (2 X 10⁶) were grown in 25cm² flasks until confluent and treated with appropriate concentrations of auranofin for 24 hours. Cells were then harvested and counted. 1000 cells per well were seeded into a 12-well plate in drug-free 10% (v/v) SCM and allowed to form colonies for 5 days. At the end of this incubation, cells were washed with 1 X PBS and fixed with ice-cold methanol. Crystal violet solution (2.1.7) was then used to stain the colonies for 10-15 minutes. The staining solution was then taken off and the colonies were washed with mqH₂O three times before being counted.
2.2.17 Immunofluorescence

To visualise proteins within the cells and other cellular structures

Cells were grown either as a 2D monolayer or as 3D spheroids in 384-well cell carrier plates (PerkinElmer) and treated with appropriate drugs and controls. At the end of each treatment, 2D cells were rinsed with 1 X PBS once and 3D spheroids were rinsed twice with 1 X PBS. 4% (v/v) PFA (2.1.7) was then used to fix the 2D cells and 3D spheroids by incubation at room temperature for 10 and 20 minutes respectively with gentle agitation. Cells/spheroids were then washed with 1 X PBS for 5 minutes, three times at room temperature on a shaker.

2D cells were next incubated with the BSA/Triton X-100 blocking solution (2.1.7) overnight at 4°C, after which cells were incubated overnight at 4°C with the primary antibody diluted in the blocking buffer. Cells were then washed six times with 1 X PBS at room temperature for 5 minutes each. Next, cells were incubated with the appropriate secondary antibody along with either Phalloidin or Cell Mask Deep Red and Hoechst, all diluted in the blocking buffer for approximately 2 hours at room temperature, in dark. Cells were again washed with 1 X PBS, six times at room temperature for 5 minutes each. The plate was either imaged immediately or stored at 4°C.

3D spheroids were incubated with Phalloidin and Hoechst in the blocking solution for approximately 2 hours at room temperature, in dark with gentle agitation. At the end, spheroids were washed with 1 X PBS three times, before being imaged.

2.2.18 Statistical Analyses

To statistically analyse the significance of each result

Statistical analysis was performed as defined in each section. To directly compare data from two samples, a Student’s t-test was performed, while to compare multiple samples either a one-way or two-way analysis of variance (ANOVA) was performed. Once a significant difference was obtained from ANOVA, a Sidak’s post test was used to compare data with the control group or a Tukey’s post hoc test was performed to
compare all the data groups to each other. All results were expressed as ‘Mean±SEM’, unless stated otherwise. The statistical analyses were performed with GraphPad Prism Version 6.
CHAPTER 3

THE THIOREDOXIN SYSTEM:
AURANOFIN AND OXYGEN CONDITIONS
3.1 Introduction

The oxygen environment in tumors is unstable due to abnormal vasculature, and cycles between hypoxia and re-oxygenation inducing hypoxic and redox pathways respectively. These redox and hypoxic systems are major contributors to cancer growth and progression. However, this cycling phenomenon, known as intermittent hypoxia, is often neglected while designing and testing anti-cancer drugs and therapies. The overall aim of the work presented in this chapter was to investigate how auranofin, a potential anti-cancer drug, influences the thioredoxin redox system under different oxygen conditions that better mimic the in vivo cancer environment.

As described in Chapter 1, the cycling phenomenon in cancer involves two different timescales (1.2). The one with the shorter durations, caused by fluctuations in the red blood influx, is comparable to the hypoxic preconditioning. Previous work from our laboratory with MDA-MB-231 breast cancer cells (Karlenius et al., 2012) used a preconditioning scheme (Figure 1.12) that mimicked an ischemia/reperfusion study performed in the heart (Turoczi et al., 2003). High levels of thioredoxin were detected in cells preconditioned with short cycles of hypoxia/re-oxygenation followed by a longer exposure to hypoxia and re-oxygenation (Karlenius et al., 2012).

In a separate study by Malec and co-workers, intermittent hypoxia involving 2 hour cycles of normoxia and hypoxia, was shown to increase thioredoxin protein levels in A549 lung cancer cells (Malec et al., 2010). However, preconditioning studies have not been performed with A549 cells. The A549 cell line has a constitutive Nrf2 expression (Singh et al., 2006). Since both thioredoxin and thioredoxin reductase are induced through the Nrf2 pathway (Figure 1.6), A549 cells are expected to have higher endogenous levels of the thioredoxin system proteins. This higher expression of thioredoxin could play a role in determining the response of A549 cells under different oxygen conditions.

Cancer cells often become resistant to anti-cancer therapies. Metal-based chemotherapeutics generate ROS in cells, leading to apoptosis (Desoize, 2002). However, to scavenge ROS cells upregulate their antioxidant defenses and as a result
cancer cells may become resistant to these drugs. High levels of thioredoxin in tumors are correlated with resistance to various chemotherapeutic agents (Sasada et al., 1996, Schiff et al., 2000). Therefore, by enhancing the expression of thioredoxin preconditioning may also affect how anti-cancer drugs perform in vivo.

The importance of the dynamic tumor microenvironment with respect to its oxygenation state in cancer progression has been acknowledged by many researchers (Yuan et al., 2005, Malec et al., 2010, Karlenius et al., 2012). Surprisingly, the variations in the oxygen concentrations in the in vivo tumor environment are not taken into consideration during the in vitro testing of anti-cancer drugs, which are done under physiologically irrelevant normoxic (20% oxygen) conditions. Auranofin is a potential anti-cancer drug that inhibits the thioredoxin system and has been tested only under normoxic conditions. The effect of auranofin on the thioredoxin system under different oxygen parameters was studied in this project using MDA-MB-231 and A549 cancer cells.

Therefore, the aims undertaken in this chapter were as follows:

- To investigate thioredoxin protein levels in A549 cells after exposure to hypoxia, re-oxygenation and hypoxic cycling (as done previously in the laboratory with MDA-MB-231 cells)

- To determine the dose-response for auranofin in MDA-MB-231 and A549 cells grown under different oxygen conditions with respect to cell proliferation and the thioredoxin reductase specific activity

- To study the effect of auranofin treatment on the thioredoxin system promoter activity and protein levels in cancer cells and examine if this is altered by growth under different oxygen conditions

- To evaluate the effect of auranofin induced inhibition of the thioredoxin system on other proteins involved in the cellular redox responses, such as heme oxygenase-1 (HO-1), under the different oxygen growth conditions
3.2 Results

3.2.1 Comparison of Thioredoxin and Thioredoxin Reductase Protein Levels and Thioredoxin Reductase Specific Activity in MDA-MB-231 and A549 Cell Lines

As described earlier, A549 cells have a somatic mutation in Keap1, which prevents it from repressing the constitutively expressed Nrf2 (Singh et al., 2006). As a result, a constitutive expression of thioredoxin system proteins may be expected in these cells since, both thioredoxin and thioredoxin reductase are expressed through the Nrf2 pathway. Thus the first goal of this chapter was to establish a baseline comparison of thioredoxin and thioredoxin reductase protein levels and thioredoxin reductase specific activity in the two cell lines.

Both MDA-MB-231 and A549 cells (at same passage numbers) were grown until confluent. Cells were then lysed with NP-40 lysis buffer (2.2.5.1) and the total protein was estimated (2.2.5.3). Equal amounts of cell lysates were run on a 15% SDS-PAGE gel and western blotting was performed (2.2.5.5). The 5G8 monoclonal antibody specific to thioredoxin (Table 2.4) was used to detect thioredoxin levels (Karlenius et al., 2012). β-tubulin (Table 2.4) was used as a loading control. As shown in Figure 3.1, thioredoxin protein was present in both MDA-MB-231 and A549 cell lysates.

Figure 3.1: Thioredoxin protein in MDA-MB-231 and A549 cell lysates. Equal amounts of cell lysates from both MDA-MB-231 and A549 cell lines were run on a 15% SDS-PAGE gel and probed with an anti-thioredoxin monoclonal antibody. β-tubulin was used as a loading control. Trx = thioredoxin.
A dual-antibody sandwich ELISA (2.2.9) was then employed to quantitate the thioredoxin protein levels in MDA-MB-231 and A549 cells. The thioredoxin levels were normalised by the total protein content in the lysates and were expressed as µg per mg of the total cellular protein. As shown in Figure 3.2, A549 cells had approximately 1.5 fold higher levels of the thioredoxin protein compared to MDA-MB-231 cells.

Figure 3.2: Thioredoxin protein levels in MDA-MB-231 and A549 cells. A dual-antibody sandwich ELISA was employed to determine the thioredoxin protein levels in MDA-MB-231 and A549 cells. A paired t-test was performed and a significant difference was observed in the thioredoxin protein levels between the two cell lines (indicated by an *). Data is presented as Mean±SEM of five independent experiments performed in duplicate. (P<0.001). Trx = thioredoxin.

For the comparison of the thioredoxin reductase protein levels in the two cell lines, cells were grown until confluent and lysed with SDS lysis buffer (2.2.5.2). Equal amounts of cell lysates were run on a 10% SDS-PAGE gel and western blotting was performed (2.2.5.5). A monoclonal antibody against thioredoxin reductase (Table 2.4) was used to detect thioredoxin reductase protein. Lamin B1 (Table 2.4) was used as a loading control. As shown in Figure 3.3, A549 cells had significantly higher levels (approximately 10 fold) of thioredoxin reductase protein as compared to MDA-MB-231 cells.
Figure 3.3: Thioredoxin reductase protein levels in MDA-MB-231 and A549 cells. A) Representative western blot. B) Densitometry analysis of the western blots performed by Image Gauge Ver 4.0 software (Fujifilm). A paired t-test was performed and relative protein levels are presented as Mean±SEM of three independent experiments. A549 cells had significantly higher levels of thioredoxin reductase protein compared to the MDA-MB-231 cells, which is indicated by an *. (P<0.05). TrxR = thioredoxin reductase.

The specific activity of thioredoxin reductase was also determined in MDA-MB-231 and A549 cell lysates using an assay based on the NADPH-dependent reduction of DTNB by thioredoxin reductase (2.2.11). The activity assay results were normalised by the total protein content in the cells and the specific activity was expressed as units per mg of the total protein. A549 cells were observed to have approximately a 3 fold higher specific activity of thioredoxin reductase compared to MDA-MB-231 cells (Figure 3.4).
Figure 3.4: Thioredoxin reductase specific activity in MDA-MB-231 and A549 cells. DTNB reduction assays were used to determine the thioredoxin reductase specific activity in MDA-MB-231 and A549 cells. A paired t-test was employed and the statistical significance is indicated by an *. The specific protein activity is presented as Mean±SEM of five independent experiments performed in triplicate. (P<0.0001). TrxR = thioredoxin reductase.

Therefore, A549 lung cancer cells have increased levels of both thioredoxin and thioredoxin reductase proteins and higher thioredoxin reductase activity as compared to MDA-MB-231 cells. The response of the two cell lines with endogenously different expression of the thioredoxin system proteins and thioredoxin reductase activity to different oxygen conditions and auranofin will be assessed and compared in this chapter.

3.2.2 Expression of Thioredoxin Protein in A549 Lung Cancer Cells in Response to Different Oxygen Conditions

Our laboratory previously used an oxygen scheme (Figure 1.12) to determine if preconditioning MDA-MB-231 cells with intermittent hypoxia affects thioredoxin protein levels. The results obtained in that study suggested that preconditioning provides the breast cancer cells with some protection against the prolonged hypoxic insult by increasing the thioredoxin protein levels (Karlenius et al., 2012).

Since the A549 lung cancer cell line has a deregulated Nrf2 system and therefore, endogenously higher thioredoxin levels, it was examined if this cell line reacts to preconditioning in a manner similar to MDA-MB-231. Moreover, this cell line has not
been previously used for preconditioning studies. A549 cells were grown overnight to form a monolayer, after which they were exposed to the different oxygen conditions shown in Figure 1.12. At the end of each condition, cells were lysed using NP-40 lysis buffer (2.2.5.1) and total protein was estimated (2.2.5.3). The lysates were then run on a 15% SDS-PAGE gel and western blotting was performed (2.2.5.5). To detect the presence of thioredoxin, the 5G8 monoclonal anti-thioredoxin antibody (Table 2.4) was used. β-tubulin (Table 2.4) was used as a loading control. A slight visible difference was observed in the thioredoxin levels in non-preconditioned and preconditioned cells with preconditioned cells having more thioredoxin (Figure 3.5). However, no change was observed when either preconditioned or non-preconditioned cells were re-oxygenated for 2, 4 and 6 hours.

![Figure 3.5: Thioredoxin protein in A549 cell lysates exposed to different oxygen conditions. A549 cells were grown under different oxygen conditions, lysed and run on a 15% SDS-PAGE gel. The proteins were then probed with a monoclonal anti-thioredoxin antibody. β-tubulin was used as a loading control. Trx = thioredoxin, N = Normoxia, H = Hypoxia, HR = Hypoxia-re-oxygenation.](image)

A dual-antibody sandwich ELISA (2.2.9) was also employed to quantitate the thioredoxin protein levels in these samples. The results were expressed as µg per mg of total protein. As shown in Figure 3.6, the thioredoxin protein levels were higher in preconditioned cells grown under 16 hours of hypoxia as compared to their non-preconditioned counterparts. However, this increase was not statistically significant when analysed by a two-way ANOVA followed by Tukey’s post test. Moreover, preconditioning did not increase the thioredoxin protein levels compared to non-preconditioning upon re-oxygenation of 2, 4 and 6 hours. These results were different from those obtained in the study with MDA-MB-231 breast cancer cells (Karlenius et
al., 2012) and highlight that different cell lines may respond differently to preconditioning.

**Figure 3.6**: Thioredoxin protein levels in non-preconditioned and preconditioned A549 cells grown under different oxygen conditions. Cells were grown according to the oxygen scheme described in Figure 1.12, lysed and the thioredoxin protein levels were quantitated by an ELISA. No difference was observed between non-preconditioned and preconditioned samples. A two-way ANOVA followed by Tukey’s post test was employed and results are presented as Mean±SEM of three independent experiments performed in duplicate. Trx = thioredoxin, Non-PC = non-preconditioned, PC = preconditioned, N = Normoxia, H = Hypoxia, HR = Hypoxia-re-oxygenation.

### 3.2.3 Targeting the Thioredoxin System in MDA-MB-231 and A549 Cancer Cells

As discussed in Chapter 1, auranofin is a gold compound that targets thioredoxin reductase and inhibits its activity (Gromer et al., 1998), thereby compromising the thioredoxin system and shifting the cellular redox homeostasis to a more oxidised state (Rigobello et al., 2009). The use of auranofin in anti-cancer studies began in the late 1970s (Kean et al., 1997). The first screening trial with auranofin was performed in 1981 using mouse lymphocytic leukemia (Simon et al., 1981). Since then, auranofin has been studied *in vitro* for almost all types of human cancers. However, all these studies have only been performed under normoxia (20% oxygen), which is physiologically irrelevant. Therefore, it was hypothesised that when grown under different oxygen conditions, the response of cancer cells towards auranofin induced inhibition of the thioredoxin system will be different from those treated under normoxic conditions.
For the subsequent studies performed in this project, a modified oxygen scheme, described in Figure 3.7 was used. Since the effect of these oxygen conditions on the hypoxic HIF1 system was also studied and is described in Chapter 4, the conditions ending in re-oxygenation phases were not used. Moreover, due to technical reasons, the duration of hypoxic incubation during preconditioning was increased to 15 minutes from 10 minutes.

![Oxygen Growth Scheme](image)

**Figure 3.7: Oxygen growth scheme used for studying the effects of auranofin on MDA-MB-231 and A549 cells.** Red indicates growth in 20% oxygen and blue indicates growth in 0.1% oxygen. The grey arrow indicates the time when the treatment was applied. N: normoxia (20% oxygen); H: hypoxia (0.1% oxygen); PCH: preconditioning hypoxia (four preconditioning cycles followed by prolonged hypoxia).

### 3.2.3.1 Cytotoxic Effects of Auranofin

Thioredoxin has been shown to stimulate the proliferation of fibroblasts, lymphoid cells and a number of cell lines derived from human solid tumors. This stimulation was dependent on the reduced state of its highly conserved active site and was lost when thioredoxin redox inactive mutant was used (Powis et al., 1995). Since recycling of thioredoxin back to its active reduced form is impeded when thioredoxin reductase is inhibited by auranofin, this inhibition may also have implications for cancer cell proliferation. Therefore, the effect of auranofin on the proliferation of the two cell lines used in this project was examined.

The effect of auranofin on proliferation of MDA-MB-231 and A549 cancer cells grown under normoxia, hypoxia and preconditioning followed by prolonged hypoxia was determined using an MTT cell proliferation assay (2.2.13). Different concentrations
(0µM to 64µM) of auranofin were selected in accordance to literature (Marzano et al., 2007). Since, auranofin was prepared in DMSO a concentration curve was prepared for DMSO first. A range of DMSO concentrations were tested (0.05% (v/v) to 1.6% (v/v)) and the assays were carried out over a period of 72 hours.

As shown in Figure 3.8, the highest concentration of DMSO (1.6% (v/v)) decreased the growth of cells with a more pronounced effect on MDA-MB-231 cells than A549 cells. Sinha and colleagues in their work with MDA-MB-231 cells suggested that the DMSO concentration with respect to the media should not exceed 0.2% (v/v) (Sinha et al., 2011). Hence, for subsequent experiments, the DMSO concentration was kept constant for each auranofin concentration tested (0.16% (v/v)).

![Figure 3.8: Cell growth at different DMSO concentrations.](image)

**Figure 3.8: Cell growth at different DMSO concentrations.** Growth of MDA-MB-231 cells (A) and A549 cells (B) in response to 0-1.6% (v/v) DMSO concentrations.
The effect of auranofin on the proliferation of MDA-MB-231 and A549 cells was examined next. Cells were grown in 10% (v/v) SCM overnight and then synchronised by replacing the media with 0.1% (v/v) SCM for 24 hours. The media was again replaced by 10% (v/v) SCM containing different concentrations of auranofin and cells were grown for another 24 hours under either normoxia or hypoxia. Cells were also preconditioned prior to auranofin dosing and subsequently grown under prolonged hypoxia. The rate of cell proliferation was measured using the MTT cell proliferation assay.

The dose response curves for both the cell lines are shown in Figure 3.9. The lower concentrations of auranofin did not show any cytotoxic effect on the growth of either cell line. However, after exposure to higher concentrations of auranofin (4µM and higher) for 24 hours, the growth of both cell lines was decreased in a dose-dependent manner. A nonlinear fit to the MTT data was performed using GraphPad Prism6 to calculate the IC$_{50}$ values. For MDA-MB-231 cells the IC$_{50}$ of auranofin induced inhibition of cell growth ranged between 4.5µM and 6.4µM. A549 cells were comparatively more resistant towards auranofin and the IC$_{50}$ was found to be between 9.6µM and 12.6µM (Table 3.1).
Figure 3.9: Growth of MDA-MB-231 and A549 cells in response to auranofin under different oxygen conditions. MDA-MB-231 (A) and A549 (B) cell proliferation was assessed under different oxygen conditions in response to auranofin treatment using MTT cell proliferation assay. A nonlinear fit was performed using GraphPad Prism6 to determine the dose-response curves.

Table 3.1: IC50 values for auranofin induced growth inhibition of MDA-MB-231 and A549 cells under different oxygen conditions.

<table>
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<th>Normoxia</th>
<th>Hypoxia</th>
<th>Preconditioning-Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>5.1µM</td>
<td>4.5µM</td>
<td>6.4µM</td>
</tr>
<tr>
<td>A549</td>
<td>9.6µM</td>
<td>12.6µM</td>
<td>11.5µM</td>
</tr>
</tbody>
</table>

A column graphical representation of the above described results is shown in Figure 3.10. In the MDA-MB-231 cell line, auranofin induced a significant decrease in cell growth at 8µM and higher concentrations under all the oxygen conditions tested (Figure 3.10A). The A549 cell growth under normoxic conditions was also significantly
decreased by 8µM and higher auranofin concentrations while for hypoxic and preconditioned cells, 16µM and higher concentrations significantly decreased the cell proliferation (Figure 3.10B). A one-way ANOVA followed by Sidak’s post test was performed separately for each oxygen condition.

![Figure 3.10](image)

**Figure 3.10: Column graphical representation of growth of MDA-MB-231 and A549 cells under different oxygen conditions upon auranofin treatment.** The MTT cell proliferation assay results were analysed separately for each oxygen condition and are presented as Mean±SEM of at least three independent experiments performed in triplicate. A) MDA-MB-231 cell proliferation was significantly inhibited by auranofin at 8µM and higher concentrations (denoted by an *) under all the oxygen conditions tested. (P<0.0001). B) A549 cell proliferation under normoxia was inhibited by auranofin at 8µM and higher concentrations (denoted by an *) while under hypoxia and preconditioning hypoxia, cell proliferation was inhibited significantly at 16µM and higher auranofin concentrations (denoted by #). (P<0.0001).
3.2.3.2 Inhibition of the Specific Activity of Thioredoxin Reductase by Auranofin

A range of auranofin concentrations were used to find the appropriate concentration to be used in subsequent inhibition experiments. Cells were grown until confluent and appropriate concentrations of auranofin were added after which cells were incubated under normoxia or hypoxia for 24 hours. Preconditioning prior to auranofin dosing was also performed and cells were grown under hypoxia for 24 hours. After 24 hours of growth under different oxygen conditions, the auranofin-treated cells were lysed with NP-40 lysis buffer (2.2.5.1). The NADPH-dependent thioredoxin reductase activity in these lysates was then measured (2.2.11). A nonlinear fit was performed using GraphPad Prism6 to calculate the IC$_{50}$ values, which are summarised in Table 3.2. For MDA-MB-231 cells the IC$_{50}$ of auranofin induced inhibition of the thioredoxin reductase activity ranged between 0.12µM and 0.32µM. A549 cells were comparatively more resistant towards auranofin and the IC$_{50}$ was found to be between 0.73µM and 0.83µM.

Table 3.2: IC$_{50}$ values for auranofin induced inhibition of thioredoxin reductase in MDA-MB-231 and A549 cells under different oxygen conditions.

<table>
<thead>
<tr>
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<th>Normoxia</th>
<th>Hypoxia</th>
<th>Preconditioning-Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>0.32µM</td>
<td>0.12µM</td>
<td>0.31µM</td>
</tr>
<tr>
<td>A549</td>
<td>0.83µM</td>
<td>0.73µM</td>
<td>0.82µM</td>
</tr>
</tbody>
</table>

A one-way ANOVA followed by Sidak’s post test was employed separately for each oxygen condition, to find the auranofin concentration at which the thioredoxin reductase activity was significantly inhibited. In MDA-MB-231 cells grown under normoxia, 0.31µM and higher concentrations of auranofin significantly decreased the thioredoxin reductase activity compared to the control while under hypoxia and preconditioning hypoxia, 0.1µM and higher auranofin concentrations significantly inhibited the thioredoxin reductase activity (Figure 3.11).
Figure 3.11: Thioredoxin reductase specific activity in MDA-MB-231 cells treated with auranofin and grown under different oxygen conditions. A) Cells grown under normoxia. B) Cells grown under hypoxia. C) Cells preconditioned prior to auranofin treatment and grown under hypoxia afterwards (PCHypoxia). A one-way ANOVA followed by Sidak’s post test was employed separately for each oxygen condition and results are presented as Mean±SEM of at least three independent experiments performed in triplicate. The significant decrease in the thioredoxin reductase activity is indicated by an *. (P<0.0001).
In A549 cells grown under normoxia and preconditioning hypoxia, auranofin significantly inhibited the thioredoxin reductase activity at 0.5\(\mu\)M and higher concentrations while in cells grown under hypoxia, 0.31\(\mu\)M was sufficient to significantly decrease the activity of thioredoxin reductase (Figure 3.12).

**Figure 3.12: Thioredoxin reductase specific activity in A549 cells treated with auranofin and grown under different oxygen conditions.** A) Cells grown under normoxia. B) Cells grown under hypoxia. C) Cells preconditioned prior to auranofin treatment and grown under hypoxia afterwards (PCHypoxia). A one-way ANOVA followed by Sidak’s post test was employed separately for each oxygen condition and results are presented as Mean±SEM of at least three independent experiments performed in triplicate. The significant decrease in the thioredoxin reductase activity is indicated by an *, (P<0.0001).
To highlight the effects of the different oxygen conditions on the auranofin induced inhibition of the thioredoxin reductase specific activity, a two-way ANOVA followed by Sidak’s post test was employed to analyse the same data. As shown in Figure 3.13A, lower concentrations of auranofin (0.01µM to 0.31µM) significantly decreased the thioredoxin reductase activity in hypoxic MDA-MB-231 cells compared to normoxic cells while preconditioning the cells prior to auranofin treatment restored the thioredoxin reductase activity to normoxic levels. In A549 cells, auranofin treatment did not affect the thioredoxin reductase activity under hypoxia compared to normoxia while preconditioning prior to auranofin treatment increased the thioredoxin reductase activity even higher than normoxic levels with the increase being statistically significant at 0.31µM and 2µM auranofin concentrations (Figure 3.13B).
CHAPTER 3

Figure 3.13: Thioredoxin reductase specific activity in MDA-MB-231 and A549 cells treated with auranofin and grown under different oxygen conditions. A) Thioredoxin reductase activity in MDA-MB-231 cells under different oxygen conditions upon auranofin treatment. A two-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM of at least three independent experiments performed in triplicate. An * denotes significant difference between hypoxic and normoxic cells while # denotes significant difference between preconditioned and normoxic cells. (P<0.0001). B) Thioredoxin reductase activity in A549 cells under different oxygen conditions upon auranofin treatment. A two-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM of at least three independent experiments performed in triplicate. An * denotes significant difference between preconditioned and normoxic cells. (P<0.0001).
3.2.3.3 Effect of Auranofin on the Cancer Cell Morphology

MDA-MB-231 and A549 cells were also observed under the Olympus CK30 microscope (Olympus Co., Japan) at 10X magnification to study the cell morphology in response to 24 hours of auranofin treatment. The representative images are shown in Figures 3.14 and 3.15.

The MDA-MB-231 cell line contains more than one cell population (Yin, 2011). This cell line has been shown to include a high percentage of the CD44⁺/CD24⁻ sub-population that express mesenchymal stem cell markers giving MDA-MB-231 cells a mesenchymal-like morphology (Sheridan et al., 2006). The untreated control MDA-MB-231 cells exhibited a similar mesenchymal morphology with long thin cell bodies. Upon treatment with higher doses of auranofin, this elongated morphology was lost and cells appeared more rounded in shape (Figure 3.14). At 8µM auranofin treatment, all the cells were morphologically compacted and very loosely attached. Cells were also exposed to 100µM H₂O₂ (an oxidative stress inducer) for 30 and 60 minutes to examine if the morphological changes observed upon auranofin treatment were similar to those induced under oxidative stress. The H₂O₂ treated cells also exhibited an altered morphology comparable to the cells treated with higher concentrations of auranofin (4µM and 8µM).
Figure 3.14: MDA-MB-231 cell morphology 24 hours after auranofin treatment. Cells were treated with different concentrations of auranofin and grown under normoxia for 24 hours. Similar results were obtained for cells under hypoxia and preconditioning hypoxia. Images were taken using a Tucsen TCA 3MP camera at 10X magnification. The scale bar is 50µm.

A549 cells are small triangle-shaped cells which resemble epithelial cells in appearance (Wang et al., 2009). These cells retained their morphology upon treatment with auranofin (Figure 3.15). Cells were also treated with 100µM H₂O₂ and no difference in morphology was observed after 1 hour. When cells were treated with 100µM H₂O₂ for 2 hours, some of the cells appeared stressed by exhibiting a spherical morphology.
Figure 3.15: A549 cell morphology 24 hours after auranofin treatment. Cells were treated with different concentrations of auranofin and grown under normoxia for 24 hours. No difference was observed in cells grown under hypoxia and preconditioning hypoxia. Images were taken using a Tucsen TCA 3MP camera at 10X magnification. The scale bar is 50µm.

These results suggest that at higher concentrations, auranofin induces a cellular stress similar to the oxidative stress induced by H$_2$O$_2$ causing rounding and blebbing of cells. The resistance exhibited by A549 cells towards this stress-induced morphological change may be due to the higher endogenous thioredoxin levels in this cell line.
Absence of an active thioredoxin system is expected to cause a shift in the oxygen homeostasis and result in an accumulation of ROS, thereby driving cells to apoptosis. In fact this is the basis of action of most chemotherapeutic agents (Desoize, 2002). Since the auranofin induced changes in cellular morphology were similar to those induced by H$_2$O$_2$, it was hypothesised that auranofin treatment leads to higher ROS levels in the MDA-MB-231 and A549 cell lines. Based on the results from MTT cell proliferation assays and thioredoxin reductase activity assays, a lower concentration of 0.31µM and a higher concentration of 2µM (at which thioredoxin reductase was inhibited without affecting the cell proliferation) were selected to examine the cellular ROS levels.

ROS assays (2.2.12) were employed to quantitate the intracellular ROS levels based on the measurement of fluorescence of 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) in the presence of ROS. Cells were grown until 80-90% confluent and the appropriate auranofin concentrations were added. After 24 hours of auranofin treatment, cells were incubated with 5µM H$_2$DCF-DA for 30 minutes. H$_2$DCF-DA is cell-permeable and after diffusing into the cells, it is deacetylated by cellular esterases to a non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH). In the presence of ROS, DCFH is oxidised to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were then lysed using the NP-40 lysis buffer and the fluorescence was measured in a Spectramax M3 plate reader (Molecular Devices) using the SOFTmax Pro 2.6 software. Total protein content in the lysates was also determined and used to normalise the relative fluorescent units (RFUs), which were expressed per mg of the total cellular protein. Results from six independent experiments performed in triplicate are presented in Figure 3.16. A dose-dependent increase in the ROS levels was observed in auranofin treated MDA-MB-231 cells, which was statistically significant at 2µM auranofin concentration compared to the untreated cells (P<0.05). The ROS levels in A549 cells were higher than MDA-MB-231 cells, however, auranofin treatment did not cause any change in the ROS levels in A549 cells at either concentration compared to the untreated cells.
3.2.3.5 Effect of Thioredoxin Reductase Inhibition on the Promoter Activity of the Thioredoxin System

In this project, inhibition of the thioredoxin system by auranofin resulted in an accumulation of ROS in MDA-MB-231 cells but not in A549 cells, which had endogenously higher ROS levels. Interestingly, the expression of the thioredoxin system is also induced by oxidative stress. Both thioredoxin and thioredoxin reductase promoters contain ARE elements that are involved in the oxidative stress induced gene expression (Figure 1.6) (Rundlof et al., 2001, Hintze et al., 2003, Osborne et al., 2006).

Preliminary work performed in our laboratory with MDA-MB-231 cells under normoxia showed that when thioredoxin reductase is inhibited by auranofin, cells respond by upregulating the promoter activity of both thioredoxin and thioredoxin reductase. This upregulation was even higher when cells were co-treated with tBHQ, an oxidative stress mimetic (Bhatia, 2011, Karlenius, 2011). Therefore, this was studied further under the oxygen scheme used in the current project. The auranofin

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**Figure 3.16: ROS levels in auranofin treated MDA-MB-231 and A549 cells.** H$_2$DCF-DA was used to perform ROS analysis. Fluorescence was measured at 485nm excitation and 517nm emission and RFUs were calculated per mg of total protein. A one-way ANOVA followed by Sidak's post test was performed separately for each cell line. A statistically significant increase was observed in MDA-MB-231 cells at 2µM auranofin concentration compared to the untreated cells (denoted by an *) (P<0.05). The data is presented as Mean±SEM of six independent experiments performed in triplicate.
concentrations utilised in 3.2.3.4 (0.31µM and 2µM) were used to study the promoter activity of the thioredoxin system.

The thioredoxin and thioredoxin reductase promoter constructs used in this study were generated previously in the laboratory by Simone Osborne (Osborne et al., 2006). Both constructs have a downstream luciferase gene. The plasmids used in this chapter are pGL3-Basic vector (Promega), which does not contain any promoter; pGL3-Basic vector cloned with the thioredoxin promoter (1028bp), designated as Trx-P in this project; pGL3-Basic vector cloned with the thioredoxin reductase promoter (830bp), designated as TrxR-P in this project.

MDA-MB-231 cells were transiently transfected with these plasmids and studied under the following conditions:

- Basal conditions, no stimulus
- Induction by 0.31µM auranofin
- Induction by 2µM auranofin

Cells were transiently transfected (2.2.6.1) with the above mentioned plasmids in antibiotic-free SFM for 5 hours and then treated with auranofin for another 18 hours. Luciferase reporter assays (2.2.6.2) were then performed to measure the luciferase activity, which was normalised by total protein content of the cells. To compare the fold induction between different promoter constructs, the data was expressed as average fold induction in luciferase activity for each promoter relative to its basal levels. A one-way ANOVA followed by Sidak’s post test was employed separately for each auranofin concentration. As shown in Figure 3.17, at the lower concentration of 0.31µM, auranofin significantly induced both the thioredoxin promoter and the thioredoxin reductase promoter (up to 2 fold) compared to pGL3-Basic. At the higher concentration of 2µM, an increase in the induction of both the promoters was observed however, only the induction of thioredoxin reductase promoter was statistically significant (up to 4 fold). These results suggest that when the thioredoxin system is inhibited, to compensate for the loss, cells respond by inducing the expression of both thioredoxin and thioredoxin reductase gene promoters.
The reporter activity of the thioredoxin system promoters upon treatment with auranofin was studied in MDA-MB-231 cells. Results are presented as fold induction of luciferase activity for each reporter construct based on its basal activity. At least six independent experiments were performed in triplicate and are presented as Mean±SEM. A one-way ANOVA followed by Sidak’s post test was performed separately for each auranofin concentration (P<0.01). The significant induction of the promoter activity by 0.31µM auranofin compared to pGL3-Basic is shown by an * while the significant induction by 2µM auranofin is indicated by #.

tBHQ is an oxidative stress mimetic known to induce the expression of the thioredoxin system (Hintze et al., 2003, Kim et al., 2003a). Therefore, tBHQ was also used as a positive control in this study. The concentration of tBHQ (150µM) had been optimised previously in our laboratory for induction of the thioredoxin gene promoter (Karlenius, 2011). The luciferase reporter activity in response to auranofin and tBHQ co-treatment was also measured. As shown in Figure 3.18, simultaneous treatment with auranofin and tBHQ resulted in even higher induction for both the promoters. The lower auranofin concentration of 0.31µM caused approximately a 10 fold induction of both thioredoxin and thioredoxin reductase promoters when co-treated with tBHQ. Co-treatment of 2µM auranofin and tBHQ resulted in an approximate 3 fold increase in the activity of the thioredoxin promoter and approximately a 9 fold increase in the thioredoxin reductase promoter activity. This suggests that under conditions mimicking oxidative stress, cells respond to the auranofin induced inhibition of thioredoxin reductase by upregulating the activity of both thioredoxin and thioredoxin reductase promoters.
CHAPTER 3

Figure 3.18: Induction of the thioredoxin and thioredoxin reductase promoter activity in response to auranofin and tBHQ co-treatment of MDA-MB-231 cells. MDA-MB-231 cells were treated with 150µM tBHQ alone and in conjunction with 0.31µM and 2µM auranofin (AuF). At least five independent experiments were performed in triplicate and are presented as Mean±SEM. A one-way ANOVA followed by Tukey’s Post-Hoc test was performed separately for each promoter construct. For both Trx-P and TrxR-P, maximum induction was observed when samples were co-treated with 0.31µM auranofin and 150µM tBHQ, which was significantly higher than the rest of the treatments except 150µM tBHQ alone (indicated by an *) for Trx-P (P<0.01) and significantly higher than 0.31µM auranofin treatment alone (indicated by #) for TrxR-P (P<0.05).

The effect of the different oxygen conditions on the promoter activity of thioredoxin and thioredoxin reductase in auranofin treated MDA-MB-231 cells was determined next. Cells were transfected as described previously and 5 hours post-transfections, cells were treated with 0.31µM and 2µM auranofin and incubated under hypoxia for another 18 hours. In case of preconditioning, cells were preconditioned 2.5 hours post-transfections, after which auranofin treatment was applied and cells were incubated under hypoxia for 18 hours. Luciferase activity was then measured using luciferase reporter assays and results were normalised by total cellular protein content. The data was expressed as fold induction in the luciferase activity for each promoter relative to its basal levels.

As shown in Figure 3.19, the different oxygen conditions did not have any effect on the activity of either thioredoxin or thioredoxin reductase promoter (compared to normoxia) at the lower concentration of 0.31µM auranofin. However, when treated with 2µM auranofin, both hypoxia and preconditioning hypoxia significantly increased the activity
of the thioredoxin promoter by 2.4 and 3 fold respectively but did not increase the thioredoxin reductase promoter activity. Results were analysed by a one-way ANOVA followed by Sidak’s post test for each promoter separately.

Figure 3.19: Induction of promoter activity under different oxygen conditions in response to auranofin treatment of MDA-MB-231 cells. The reporter activity of the thioredoxin system promoters upon treatment with auranofin and growth under different oxygen conditions was studied in MDA-MB-231 cells. Results are presented as fold induction of luciferase activity for each reporter construct based on its basal activity. At least three independent experiments were performed in triplicate and are presented as Mean±SEM. A one-way ANOVA followed by Sidak’s post test was performed separately for each promoter. The significant induction of the thioredoxin promoter activity by 2µM auranofin in cells non-preconditioned and preconditioned cells grown under hypoxia compared to the normoxic cells is shown by an *. (P<0.05).
The thioredoxin system promoter activity could not be studied in A549 cells due to technical difficulties. Since these cells have endogenously higher expression of both thioredoxin and thioredoxin reductase, the basal activity of both the promoters was very high. As a result, relative luciferase units (RLUs) obtained in this project for the induced samples were saturated and when expressed as the fold change, no difference could be seen compared to the pGL3-Basic vector. Other technical reasons affecting the results obtained with A549 cells could be the transfection and luciferase assay reagents used to perform the reporter assays and selenium concentration in the cell culture serum, which were previously shown to influence the redox-regulated gene expression (Karlenius et al., 2011).

3.2.3.6 Effect of Thioredoxin Reductase Inhibition on the Thioredoxin Protein Levels

The loss of an active thioredoxin system is counteracted by MDA-MB-231 cells by an upregulation of the activity of its promoters. To test if this upregulation also results in an increased expression of thioredoxin protein, western blotting was employed. Cells were grown until confluent and then treated with different concentrations of auranofin and incubated under either normoxia or hypoxia for 24 hours. Cells were also preconditioned prior to auranofin dosing and incubated under hypoxia for 24 hours afterwards. At the end of each treatment, cells were lysed with NP-40 lysis buffer (2.2.5.1) and run on a 15% SDS-PAGE gel and western blotting was performed (2.2.5.5). The anti-thioredoxin monoclonal antibody, 5G8 (Table 2.4) was used to detect thioredoxin and β-tubulin (Table 2.4) was used as a loading control. At least three independent experiments were performed with MDA-MB-231 cells and representative blots and the densitometry results are shown in Figure 3.20. Auranofin treatment did not alter the thioredoxin protein levels in either normoxic or hypoxic cells. However, in cells that were preconditioned prior to addition of auranofin, a dose-dependent increase was observed in the thioredoxin protein levels, which was statistically significant at 4µM auranofin concentration compared to the untreated control.
Figure 3.20: Relative thioredoxin protein levels in MDA-MB-231 cells treated with auranofin and grown under different oxygen conditions. Representative western blots and densitometry analysis for each oxygen condition tested. A one-way ANOVA followed by Sidak’s post test was employed for each oxygen condition separately. At 4µM, auranofin significantly increased the thioredoxin levels in cells that were preconditioned prior to dosing as indicated by an * (P<0.05). Densitometry results are presented as Mean±SEM for at least three independent experiments. Trx = thioredoxin.

A two-way ANOVA was also employed to analyse the same data to compare the effect of the oxygen conditions on the thioredoxin protein levels in MDA-MB-231 cells. No difference was observed between cells grown under normoxia and hypoxia upon auranofin treatment. However, the thioredoxin protein levels observed in cells, which were preconditioned and treated with the higher concentrations of 2µM and 4µM auranofin were significantly higher than their normoxic counterparts (Figure 3.21).
Figure 3.21: Comparison of relative thioredoxin protein levels in auranofin treated MDA-MB-231 cells between different oxygen conditions. A statistically significant increase in the thioredoxin protein levels was observed at 2µM and 4µM auranofin treatment in preconditioned cells compared to the respective normoxic cells (indicated by an *). Data was analysed by a two-way ANOVA followed by Sidak’s post test and is presented as Mean±SEM from at least three independent experiments. (P<0.001).

A549 cells were less sensitive towards auranofin as compared to MDA-MB-231 cells and therefore, could be assayed up to 8µM auranofin. Cells were treated with auranofin, grown under the different oxygen conditions and analysed as stated above for MDA-MB-231 cells. Representative blots and densitometry results are shown in Figure 3.22 for each oxygen condition tested. As observed in MDA-MB-231 cells, A549 cells grown under normoxic and hypoxic conditions did not show any change in the thioredoxin protein levels upon auranofin treatment. However, in cells that were preconditioned, a dose-dependent increase was observed in the thioredoxin levels, which was statistically significant at 8µM auranofin compared to the untreated control.
Figure 3.22: Relative thioredoxin protein levels in A549 cells treated with auranofin and grown under different oxygen conditions. Representative western blots and densitometry analysis for each oxygen condition tested. A one-way ANOVA followed by Sidak’s post test was employed for each oxygen condition separately. No change was observed in the thioredoxin protein levels in A549 cells grown under normoxia and hypoxia. At 8µM, auranofin significantly increased the thioredoxin levels in cells that were preconditioned prior to dosing, denoted by an *. (P<0.05). Densitometry results are presented as Mean±SEM for at least three independent experiments. Trx = thioredoxin.

A two-way ANOVA was also employed to analyse the same data to compare the effect of the oxygen conditions on the thioredoxin protein levels in A549 cells. No difference was observed between cells grown under normoxia and hypoxia upon auranofin treatment. However, the thioredoxin protein levels observed in cells which were preconditioned and treated with the higher concentrations of 2µM, 4µM and 8µM auranofin were significantly higher than their normoxic counterparts (Figure 3.23).
Figure 3.23: Comparison of relative thioredoxin protein levels in auranofin treated A549 cells between different oxygen conditions. A statistically significant increase in the thioredoxin protein levels was observed at 2μM, 4μM and 8μM auranofin treatment in preconditioned cells compared to the respective normoxic cells (indicated by an *). Data was analysed by a two-way ANOVA followed by Sidak’s post test and is represented as Mean±SEM from at least three independent experiments. (P<0.0001).

3.2.3.7 Effect of Auranofin Treatment on the Thioredoxin Reductase Protein Levels

As observed in 3.2.3.5, auranofin treatment resulted in an upregulation of the thioredoxin reductase promoter activity. Western blotting was employed to examine if auranofin induced inhibition of the thioredoxin reductase activity also affects its protein levels.

Cells were grown until confluent and treated with auranofin as in 3.2.3.6. At the end of each treatment, cells were lysed with SDS lysis buffer (2.2.5.2) and run on a 10% SDS-PAGE gel. Western blotting was performed (2.2.5.5) and an anti-thioredoxin reductase monoclonal antibody (Table 2.4) was used to detect thioredoxin reductase protein. Lamin B1 (Table 2.4) was used as the loading control. Three independent experiments were performed with MDA-MB-231 cells and the representative western blots are shown in Figure 3.24A. Contrary to the thioredoxin protein levels, no change was observed in the thioredoxin reductase protein levels upon auranofin treatment under any of the oxygen conditions tested. Similarly auranofin did not modulate the thioredoxin
reductase protein levels in A549 lung cancer cells under any of the oxygen conditions (Figure 3.24B).

![Western Blot Images](image)

**Figure 3.24**: Thioredoxin reductase protein in cells treated with auranofin under different oxygen conditions. Representative western blots for MDA-MB-231 (A) and A549 (B) cells. Three independent experiments were performed. TrxR = thioredoxin reductase.

### 3.2.3.8 Inhibition of the Thioredoxin System and Consequences on Heme Oxygenase-1

As observed in Table 3.3, thioredoxin reductase activity is inhibited by 50% at a very low concentration of auranofin but a 50% decrease in cell proliferation is observed only at higher auranofin concentrations in both the cell lines (Table 3.2). This suggests that there may be other pathways involved that provide protection to cells against the antiproliferative action of auranofin induced inhibition of the thioredoxin system.

Heme oxygenase-1 (HO-1) is an enzyme that plays an important role in oxygen delivery to tissues, cell signalling, detoxification and peroxide metabolism (Ryter and Choi, 2015). The transactivation of HO-1 is regulated by Nrf2 through the ARE element in its promoter region (Kim et al., 2010a). This transactivation of HO-1 was shown to be increased by auranofin in monocytic and synoviocytic cells (Kim et al., 2010a). Several other studies have also implicated that HO-1 is induced upon inhibition of the thioredoxin system (Mostert et al., 2003, Trigona et al., 2006, Ashino et al., 2011). However, all these studies were performed only under normoxia. Therefore, it was
hypothesised that HO-1 compensated for the loss of an active thioredoxin system to provide cytoprotection against auranofin and the HO-1 protein levels were tested in auranofin treated cancer cells under the oxygen scheme used in this project.

Cells treated with the different auranofin concentrations were grown according to the oxygen scheme described in Figure 3.7. At the end of each treatment, cells were lysed with NP-40 lysis buffer (2.2.5.1) and equal amounts of lysates were run on 15% SDS-PAGE gels and western blotting was performed (2.2.5.5). An anti-HO-1 polyclonal antibody (Table 2.4) was used to detect HO-1. β-tubulin (Table 2.4) was used as a loading control. Representative blots are shown in Figure 3.25. In MDA-MB-231 cells, HO-1 was induced only upon auranofin induced inhibition of thioredoxin reductase but in A549 cells, HO-1 was present even in untreated cells. A dose-dependent increase was observed in HO-1 protein levels in both the cell lines irrespective of the oxygen condition tested. The western blot experiments were performed at least two times.

![Figure 3.25: HO-1 protein in cells treated with auranofin under different oxygen conditions.](image)

Representative western blots for MDA-MB-231 (A) and A549 (B) cells treated with auranofin and subjected to different oxygen conditions.

To verify that the auranofin induced HO-1 provides the proliferative protection to cancer cells, a selective chemical inhibitor of HO-1, zinc protoporphyrin (ZnPP) (Maines, 1981) was used. Firstly, a growth curve was prepared with ZnPP alone to assess its effect on cell proliferation. Both cell lines were treated with a range of ZnPP concentrations (1.25µM-40µM) to find a concentration to be used in conjunction with
auranofin, which did not affect cell growth on its own. Cells were grown until confluent and then treated with the different concentrations of ZnPP for 24 hours. MTT assay (2.2.13) was employed to measure the rate of cell proliferation and experiments were performed only under normoxia. As shown in Figure 3.26, none of the ZnPP concentrations tested had an effect on the proliferation of either cell line. Two independent experiments were performed in triplicate. A one-way ANOVA followed by Sidak’s post test was employed for each cell line separately. Based on these results and in accordance to literature (Nowis et al., 2008), 5µM concentration of ZnPP was selected for subsequent experiments.

Figure 3.26: Growth of cancer cells in response to ZnPP. MDA-MB-231 (A) and A549 (B) cell proliferation in response to ZnPP treatment. Results are presented as Mean±SEM of two independent experiments performed in triplicate.
Cancer cells were next treated with the different auranofin concentrations used in 3.2.3.1 along with 5µM ZnPP and MTT assay was performed to analyse the effect of the two agents together on MDA-MB-231 and A549 cell proliferation. As shown in Figure 3.27, co-treatment with auranofin and ZnPP decreased the cell proliferation compared to auranofin alone in both the cell lines. Table 3.3 summarises the IC$_{50}$ values for growth inhibition by auranofin alone and in conjunction with 5µM ZnPP.

![Figure 3.27: Growth of MDA-MB-231 and A549 cells in response to auranofin alone and in conjunction with ZnPP. MDA-MB-231 (A) and A549 (B) cell proliferation upon auranofin and ZnPP treatment. A two-way ANOVA followed by Sidak’s post test was employed for each cell line separately and significant difference in proliferation of cells treated with auranofin alone and those co-treated with auranofin and ZnPP is indicated by an *. (P<0.0001). At least three independent experiments were performed in triplicate and results are presented as Mean±SEM. The dose-response curves are shown in the insets.](image)
Table 3.3: IC\textsubscript{50} values for inhibition of cell proliferation in MDA-MB-231 and A549 cells when treated with auranofin alone and in conjunction with ZnPP.

<table>
<thead>
<tr>
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<th>Auranofin</th>
<th>Auranofin + 5µM ZnPP</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>5.1µM</td>
<td>3.9µM</td>
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<tr>
<td>A549</td>
<td>9.1µM</td>
<td>7.5µM</td>
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3.2.3.9 Effect of Auranofin on the Nrf2 Protein Levels

As discussed previously in Chapter 1, ARE elements are found in the promoter regions of many phase II detoxification enzymes, including both thioredoxin and thioredoxin reductase promoters (Rundlof \textit{et al.}, 2001, Hintze \textit{et al.}, 2003, Osborne \textit{et al.}, 2006) and are regulated by the transcription factor Nrf2 (Hawkes \textit{et al.}, 2014). Under unstimulated conditions, Nrf2 is constantly expressed and bound to its repressor, Keap1 that degrades Nrf2 in the cytosol (Ishii \textit{et al.}, 2000). Keap1, upon sensing oxidative or electrophilic stress, undergoes conformational changes preventing proteosomal degradation of Nrf2 (Figure 1.6). Nrf2 is released and translocates into the nucleus to act on the ARE elements of the proteins required by cells to counteract the oxidative/electrophilic stress (Rushmore \textit{et al.}, 1991).

Auranofin has been previously shown to stabilise Nrf2 protein levels in THP-1 monocytes and synoviocytes derived from rheumatoid arthritis patients. Auranofin was also shown to upregulate Nrf2 mediated induction of HO-1 at both transcript and protein levels (Kim \textit{et al.}, 2010a). This study was performed under normoxic conditions only. In the current project, auranofin upregulated the activity of both thioredoxin and thioredoxin reductase promoters, which are Nrf2 responsive (Figure 3.17). Moreover, the activity of the thioredoxin promoter was further enhanced when hypoxic and preconditioned MDA-MB-231 cells were treated with the higher (2µM) concentration of auranofin (Figure 3.19B). Therefore, the effect of auranofin on the Nrf2 protein levels was examined next in both MDA-MB-231 and A549 cells grown under different oxygen conditions.
Cells were grown until confluent, treated with the different auranofin concentrations and grown under normoxia or hypoxia for 24 hours. Cells were also preconditioned prior to auranofin treatment and incubated under hypoxia for another 24 hours. Cells were lysed with SDS lysis buffer (2.2.5.2) and equal amounts of lysates were run on a 10% SDS-PAGE gel. Western blotting was performed (2.2.5.5) and Nrf2 protein levels were detected using an anti-Nrf2 monoclonal antibody (Table 2.4). Lamin B1 (Table 2.4) was used as a loading control. Three independent experiments were performed with each cell line and representative western blots are shown in Figure 3.28. No change was observed in the Nrf2 protein levels in either cell line at any auranofin concentration tested under any of the oxygen growth conditions used.

![Western Blot](image)

**Figure 3.28:** Nrf2 protein in auranofin treated cells under different oxygen conditions.

Representative western blots for MDA-MB-231 (A) and A549 (B) cells treated with auranofin and grown under different oxygen conditions.
3.3 Discussion

The vital role of hypoxia in solid tumors has been established by numerous experimental and clinical studies and has been correlated with tumor progression and development of resistance against therapies. The intratumoral microenvironment is much more complex and is a consequence of constant cycling between hypoxia and re-oxygenation. Although the concept of intermittent hypoxia was first speculated in the late 1970s (Brown, 1979, Yamaura and Matsuzawa, 1979), its pathophysiological consequences for cancers is still not very well studied. Moreover, its impact is ignored while designing and testing of therapeutics. Therefore, the main goal of this chapter was to determine the effect of intermittent hypoxia on the thioredoxin redox system and to evaluate if this dynamic phenomenon affects the activity of auranofin as an anti-cancer drug.

MDA-MB-231 breast cancer and A549 lung cancer cell lines were used in this project. The MDA-MB-231 cell line was previously used in our laboratory for a hypoxia/re-oxygenation study (Karlenius et al., 2012). A549 cells, as stated previously, have a deregulated Nrf2 system (Singh et al., 2006). It was therefore, hypothesised that these cells have a higher expression of the thioredoxin system proteins compared to MDA-MB-231 cells and may respond differently to the different oxygen conditions and auranofin treatment. A baseline comparison was first performed between the two cell lines to evaluate the expression of thioredoxin and thioredoxin reductase proteins and the enzyme activity of thioredoxin reductase. It was found that A549 cells had significantly higher expression levels of both thioredoxin (Figure 3.2) and thioredoxin reductase (Figure 3.3) proteins than MDA-MB-231 cells. Moreover, the specific activity of thioredoxin reductase was also higher in the lung cancer cell line (Figure 3.4). These results can be correlated to the constitutive Nrf2 pathway in A549 cells since the thioredoxin system proteins are regulated through the Nrf2 system (Kim et al., 2003b).
A hypoxia/re-oxygenation scheme (Figure 1.12) was previously established in our laboratory and its effect on the expression of thioredoxin protein was studied in MDA-MB-231 cells (Karlenius et al., 2012). Since A549 cells have endogenously higher expression of thioredoxin protein compared to MDA-MB-231 cells, the response of A549 cells to the same oxygen scheme was examined in this project. Although in the previous study, preconditioning cells prior to a prolonged hypoxic insult resulted in an increased expression of thioredoxin protein in MDA-MB-231 cells (Karlenius et al., 2012), in the current project preconditioning did not enhance the thioredoxin protein levels in A549 cells (Figure 3.6). Since preconditioning provides protection to cells by increasing thioredoxin levels and A549 cells have upregulated thioredoxin levels, it may be that these cells do not need to express more thioredoxin to seek protection against stresses related to prolonged hypoxia. A study performed in A549 cells by Malec and co-workers showed an upregulation of thioredoxin protein when cells were subjected to an intermittent hypoxia scheme involving two hour cycles of normoxia and hypoxia (Malec et al., 2010). These researchers used 1% oxygen as hypoxia. However, when a similar scheme involving one hour cycles of normoxia and hypoxia (0.1% oxygen) was used in this project (data not presented), no difference was observed in the thioredoxin protein levels in either MDA-MB-231 or A549 cells. These variations in the results from the two studies may be due to the differences in the duration of hypoxic exposures and the concentration of oxygen considered as hypoxia.

For the subsequent studies performed in this chapter, a modified oxygen growth scheme was used (Figure 3.7). Moreover, auranofin, a potential anti-cancer drug, was used as a tool to inhibit thioredoxin reductase. Most of the in vitro studies assessing the effects of auranofin on various cellular pathways have been performed under normoxic conditions (20% oxygen). The effects of auranofin on cancer cells under hypoxia and preconditioning hypoxia was studied for the first time in this project. Different sensitivity levels against auranofin have been observed in different cell lines (Marzano et al., 2007, Cox et al., 2008). Therefore, the cytotoxicity profile of auranofin was prepared first in both the cell lines used in this project under the different oxygen conditions. Auranofin displayed cytotoxic effects by inhibiting cell proliferation at higher concentrations in both the cell lines; however, MDA-MB-231 cells were more sensitive towards auranofin induced growth inhibition compared to A549 cells (Table
The comparatively increased resistance displayed by A549 cells towards auranofin may be linked to the higher endogenous thioredoxin levels in this cell line, which have been shown to stimulate cell proliferation (Powis et al., 1995). Furthermore, the cytotoxic effects of auranofin in MDA-MB-231 cells were not altered by growth under hypoxia or preconditioning hypoxia. On the other hand, hypoxic and preconditioned A549 cells exhibited slight resistance towards auranofin induced growth inhibition compared to their normoxic counterparts (Figure 3.10).

Auranofin was shown to inhibit the activity of thioredoxin reductase purified from human placenta with an IC\textsubscript{50} as low as 20nM (Gromer et al., 1998). Rackham and colleagues observed a complete abolishment of the thioredoxin reductase activity in factor-dependent myeloid cells treated with 10\(\mu\)M auranofin (Rackham et al., 2011). In a separate study, 1\(\mu\)M and higher doses of auranofin resulted in a complete loss of thioredoxin reductase activity in Jurkat T-lymphoma cells within 30 minutes of treatment (Cox et al., 2008). In this project, auranofin inhibited the thioredoxin reductase activity with an IC\textsubscript{50} of 0.3\(\mu\)M in MDA-MB-231 cells (Table 3.2). Similar to the cytotoxic effects of auranofin, A549 cells were comparatively resistant to auranofin induced inhibition of the thioredoxin reductase activity with an IC\textsubscript{50} of 0.8\(\mu\)M (Table 3.2), which may be due to higher endogenous activity of thioredoxin reductase in this cell line. Together with the findings of other researchers described above, these results indicate that auranofin induced inhibition of the thioredoxin system is cell line specific and may be dependent on the endogenous levels of the thioredoxin system proteins.

The auranofin induced inhibition of the thioredoxin reductase activity was found to be influenced by the oxygen conditions. It was found that MDA-MB-231 cells grown under hypoxia were more sensitive towards auranofin induced inhibition of the thioredoxin reductase activity compared to cells grown under normoxia. Moreover, when cells were preconditioned prior to auranofin treatment, this sensitivity was no longer observed (Figure 3.13). In A549 cells, hypoxia did not affect the thioredoxin reductase activity compared to normoxia. However, when these cells were preconditioned before the treatment, they exhibited resistance towards the auranofin induced inhibition of thioredoxin reductase activity in comparison to the cells grown under normoxia and hypoxia (Figure 3.13). As stated before, the \textit{in vitro} activity of
Auranofin as an anti-cancer drug was evaluated under hypoxia and preconditioning for the first time in this project. The results presented in this chapter suggest that the cellular oxygen environment plays an important role in influencing the activity of auranofin.

Rigobello and colleagues showed that auranofin caused mitochondrial swelling and release of cytochrome c by inhibiting the mitochondrial thioredoxin reductase (Rigobello et al., 2002, Rigobello et al., 2004). In a separate study, these researchers showed auranofin induced the accumulation of $\text{H}_2\text{O}_2$ in the mitochondria, which was linked to a decrease of its removal by the thioredoxin system (Rigobello et al., 2005). In yet another study performed by the same group, auranofin was shown to increase ROS levels in human ovarian cancer cells (Rigobello et al., 2009). In the present study, auranofin treatment resulted in morphological changes in MDA-MB-231 cells, which were comparable to the $\text{H}_2\text{O}_2$ induced morphological modifications (Figure 3.14). However, A549 cells did not exhibit any stress-induced morphological differences upon auranofin treatment (Figure 3.15) and this may be due to the higher thioredoxin and thioredoxin reductase proteins in these cells. Moreover, analysis of ROS levels in the two cell lines indicated that auranofin treatment caused an increase in the cellular ROS levels in MDA-MB-231 cells but not in A549 cells. It should be noted that the cellular redox state is not controlled by the thioredoxin system alone. The glutathione system is another important antioxidant system that regulates the redox homeostasis in the cells. In fact, glutathione and glutaredoxin were recently shown to act as a back-up for thioredoxin reductase to reduce thioredoxin (Du et al., 2012). Interestingly, auranofin also inhibits the glutathione system through the active site selenium of glutathione peroxidase (Chaudiere and Tappel, 1984). However, in comparison to thioredoxin reductase, glutathione peroxidase is inhibited at higher concentrations of auranofin (Gandin et al., 2010). Furthermore, glutathione reductase and glutathione peroxidase, which are important components of the glutathione antioxidant system (Filomeni et al., 2002) are also regulated by the Nrf2 pathway (Thimmulappa et al., 2002). Therefore, these proteins may also be expressed at higher levels in A549 cells. The overall upregulation of the antioxidant proteins may be the reason why A549 cells exhibit a resistance towards the inhibitory effects of auranofin that are observed in MDA-MB-231 cells.
As described previously, the expression of the thioredoxin system is induced under oxidative stress conditions through the ARE elements in the promoters of both thioredoxin and thioredoxin reductase (Rundlof et al., 2001, Hintze et al., 2003, Osborne et al., 2006). The effect of auranofin induced inhibition of the thioredoxin system was studied at the gene promoter level in MDA-MB-231 breast cancer cells. The results obtained indicated that when the thioredoxin system is inhibited, cells respond by upregulating the activity of both thioredoxin and thioredoxin promoters to compensate for the loss (Figure 3.17). Auranofin has been previously shown to induce the transactivation of HO-1 gene through the ARE element in its promoter by affecting the Keap1/Nrf2 association (Kim et al., 2010a). It is also known that both thioredoxin and thioredoxin reductase genes are activated by Nrf2 (Kim et al., 2003b). Cells were also treated with tBHQ, which is known to act as an oxidative stress mimetic by directly disrupting the Keap1/Nrf2 interaction that activates the Nrf2/ARE pathway (Abiko et al., 2011) and an upregulation of both thioredoxin and thioredoxin reductase promoter activities was observed. Other studies have also reported that tBHQ induces the activation of thioredoxin through the Nrf2/ARE pathway (Kim et al., 2003b). Co-treatment of MDA-MB-231 cells with auranofin and tBHQ resulted in an even higher increase in the thioredoxin system promoter activities (Figure 3.18) suggesting that auranofin and tBHQ may work co-operatively in inducing the two promoters.

The thioredoxin system promoter activities in response to auranofin treatment were examined under hypoxia and in preconditioned cells for the first time in this project. In comparison to normoxic cells treated with the lower auranofin concentration of 0.31µM, hypoxia and preconditioning did not affect the thioredoxin promoter activity. When cells were treated with the higher concentration of 2µM auranofin, the thioredoxin promoter activity was significantly enhanced in hypoxic and preconditioned cells compared to the normoxic cells (Figure 3.19). This was an interesting observation since 2µM auranofin treatment did not result in a significant change in the thioredoxin promoter activity compared to the empty vector in the normoxic cells (Figure 3.17). These results further strengthen the idea that the performance of auranofin may be influenced by changes in the oxygen environment. However, hypoxia and preconditioning did not alter the promoter activity of thioredoxin reductase, which was comparable to the normoxic levels at both the auranofin concentrations tested. In a
previous study performed in our laboratory with untreated MDA-MB-231 cells, no difference was observed in the activity of either the thioredoxin or thioredoxin reductase promoter when cells were grown under hypoxia or preconditioning hypoxia compared to their normoxic counterparts (Karlenius, 2011).

Although it is an established fact that auranofin exerts most of its cytotoxic effects by inhibiting the thioredoxin reductase activity (Gromer et al., 1998, Omata et al., 2006, Marzano et al., 2007), there are only a few studies, which have assessed the effects of auranofin on thioredoxin and thioredoxin reductase protein levels. When treating A549 cells with 6µM auranofin, Fan and co-workers did not observe any changes in either thioredoxin or thioredoxin reductase protein levels compared to the untreated cells (Fan et al., 2014). In a separate study performed by Liu and colleagues, 4µM auranofin did not affect the thioredoxin reductase protein levels in MCF-7 breast cancer cells (Liu et al., 2013). However, both of these studies were performed under normoxic conditions. Therefore, the thioredoxin and thioredoxin reductase protein levels were also examined in the two cell lines used in the current study upon treatment with auranofin and growth under the different oxygen conditions. In MDA-MB-231 cells, auranofin treatment did not affect the thioredoxin protein levels in either normoxic or hypoxic cells, which were also comparable to each other. However, when cells were preconditioned prior to auranofin treatment, an increase in the thioredoxin protein levels was observed at the higher concentrations of auranofin (Figure 3.21). The re-oxygenation phases of preconditioning are expected to cause oxidative stress and an accumulation of ROS, thereby causing an increase in the antioxidant response. Since auranofin treatment also results in an accumulation of ROS, as shown by others (Rigobello et al., 2009) and in MDA-MB-231 cells in this project (Figure 3.16), the higher thioredoxin levels observed in preconditioned cells treated with higher concentrations of auranofin may be an accumulative effect of preconditioning and auranofin induced inhibition of the thioredoxin system.

Similar results were obtained in A549 cells where auranofin did not modulate the thioredoxin protein levels in cells grown under normoxia and hypoxia but when preconditioned cells were treated with higher concentrations of auranofin, a dose-dependent increase was observed in the thioredoxin protein levels (Figure 3.23). These
results indicate that when the thioredoxin system is inhibited by auranofin, cells respond by upregulating the expression of the thioredoxin protein when subjected to preconditioning. Since preconditioning mimics an aspect of the in vivo tumor conditions, anti-cancer drugs, such as auranofin that are designed and tested only under 20% environmental oxygen levels may not perform as effectively in the patient body as they do in the laboratory testings.

In accordance to the aforementioned studies (Liu et al., 2013, Fan et al., 2014), thioredoxin reductase protein levels were not upregulated upon auranofin treatment in either of the cell line used in the current project (Figure 3.24). Moreover, contrary to the upregulation observed in the thioredoxin protein levels in auranofin treated preconditioned cells, no change was observed in the thioredoxin reductase protein levels under any of the oxygen condition. These results suggest that although auranofin is a potent inhibitor of the enzyme activity of thioredoxin reductase, it does not alter its protein levels.

As stated previously, auranofin inhibited the thioredoxin reductase specific activity at a very low concentration in both the cell lines whereas auranofin’s cytotoxic effect on cell proliferation was observed only at the higher concentrations. This observation led to the hypothesis that another antioxidant pathway may be involved in providing protection to the cells in absence of an active thioredoxin system. It has been shown by Mostert and colleagues that inhibition of thioredoxin reductase by aurothioglucose, a gold compound similar to auranofin, causes the induction of hepatic HO-1 (Mostert et al., 2003). Similar results were observed for selenium-deficient rodents, implying that HO-1 is induced to compensate the loss of the antioxidant activity of thioredoxin reductase (Mostert et al., 2003). Inhibition of thioredoxin reductase by chemical (using DCNB) and siRNA methods also caused the induction of HO-1 in bovine aortic endothelial cells (Trigona et al., 2006). In fact auranofin has been shown to upregulate HO-1 to prevent cocaine induced hepatic injury in mice (Ashino et al., 2011). Auranofin has also been shown to upregulate the HO-1 transcript and protein levels by affecting the Nrf2 signalling pathway (Kim et al., 2010a). These studies suggest a role for HO-1 in protecting the cells from auranofin induced inhibition of cell proliferation. In untreated MDA-MB-231 cells, HO-1 protein was not observed under any of the oxygen
conditions but upon treatment with auranofin, the HO-1 protein levels were visibly increased under all the oxygen conditions (Figure 3.25A). On the other hand, in A549 cells, HO-1 protein was observed even in untreated cells. Kweon and co-workers have previously shown a constitutive expression of HO-1 in A549 cells (Kweon et al., 2006). Upon treatment of A549 cells with auranofin in this project, a dose-dependent increase was observed in the HO-1 protein levels (Figure 3.25B). This increase was consistent under all the oxygen conditions tested.

The results obtained in this chapter along with above stated studies implicate a role for HO-1 in providing protection to cancer cells in absence of an active thioredoxin system. This hypothesis was further strengthened by specifically inhibiting HO-1 in combination with the thioredoxin system and examining the effect of this co-treatment on the proliferation of MDA-MB-231 and A549 cells. A selective inhibitor of HO-1, ZnPP (Maines, 1981) was used in conjunction with auranofin. The co-treatment decreased the proliferation in both the cell lines in comparison to the auranofin treatment alone (Figure 3.27), thus implying that HO-1 upregulation may be protecting cells against the cytotoxic effects of auranofin induced inhibition of thioredoxin reductase. However, as cited previously, the glutathione system may also be serving as a back-up for thioredoxin’s reduction in absence of an active thioredoxin reductase (Du et al., 2012) and therefore, needs to be further assessed.

Nrf2 is considered the master controller of the antioxidant response by regulating the expression of several constitutive and inducible detoxification enzymes (Thimmulappa et al., 2002). In fact, all the protein systems studied and discussed in this chapter are Nrf2 regulated via the ARE elements in their promoter regions. Paradoxically, thioredoxin has been shown to play a role in the transcriptional activation of Nrf2 (Hansen et al., 2004). Additionally, a recently published review article by Cebula and colleagues summarises various studies suggesting that thioredoxin reductase is a potent regulator of Nrf2 and that the inhibition of the thioredoxin system may result in an activation of the Nrf2 pathway (Cebula et al., 2015). This presented Nrf2 as an apparent candidate for evaluation under the oxygen scheme used in this project. Auranofin treatment for 6 hours was earlier shown to increase Nrf2 protein levels in THP-1 monocytic and synoviocytic cells by disrupting the association between Keap1 and Nrf2
However, in the current project, no change was observed in Nrf2 protein levels upon auranofin treatment in either cell line used (Figure 3.28). One reason for the variation observed between the two studies may be the difference in the duration of auranofin exposure. Moreover, with a constitutive expression of Nrf2 pathway in A549 cells, it may be that these cells do not need to express more protein.

The oxygen conditions used in this project also did not have any visible effect on the Nrf2 protein levels in treated or untreated cells. In an earlier study, Nrf2 protein levels were shown to increase when A549 cells were subjected to 2 hour cycles of hypoxia (1%) and re-oxygenation (Malec et al., 2010). As pointed out previously, the current study utilised a different oxygen scheme and cells were subjected to 0.1% oxygen during the hypoxic phases. This may explain the variations in results between the two studies and also highlights the importance of cycling parameters and oxygen concentration in the induction of a redox response.

In summary, the results reported in this chapter support the initial hypothesis that when grown under intermittent hypoxia conditions that mimic an aspect of the in vivo tumor microenvironment, the response of cancer cells towards auranofin induced inhibition of the thioredoxin system is different to those treated under normoxic conditions. These findings lay emphasis on the urgent need to evaluate the effectiveness of various chemotherapeutics under a wide range of oxygen conditions, particularly under intermittent hypoxia.
CHAPTER 4

THE HIF1α SYSTEM:
AURANOFIN AND OXYGEN
CONDITIONS
4.1 Introduction

As solid tumors expand, they outgrow their existing vasculature and develop hypoxic cores. To fulfill their oxygen and nutritional requirements, cancer cells release pro-angiogenic signals to induce the formation of new blood vessels. However, angiogenesis in tumors is aberrant due to sparse arteriolar supply (Dewhirst et al., 1999), low vascular density (Dewhirst et al., 1996), and insufficient orientation of microvessels (Secomb et al., 1993). This makes angiogenesis irregular with respect to both space and time, thereby giving rise to intermittent hypoxia (Dewhirst, 2007).

Obstructive sleep apnea (OSA) is a disorder that results from recurring episodes of complete or partial blockage of the upper airway during sleep resulting in short cycles of intermittent hypoxia (Flemons, 2002). Associations between OSA and a number of disorders, such as cardiovascular diseases, cerebrovascular diseases, and metabolic disorders have been frequently reported through clinical and experimental studies (Cao et al., 2015). It has recently been shown that patients with OSA have a higher likelihood to develop aggressive tumors and have higher cancer-related mortality (Almendros et al., 2014). When melanoma cells were injected into mice exposed to intermittent hypoxia, the tumor volume as well as mass observed in these animals at the end of the study were higher than the control animals subjected to normoxia (Almendros et al., 2012a). Intermittent hypoxia also resulted in enhanced melanoma metastasis in these mice (Almendros et al., 2012b).

Since OSA involves short cycles of hypoxia/re-oxygenation, it can be compared to the preconditioning scheme being used in this project and described in Chapter 3 (Figure 3.7). Several studies have implicated an upregulation of the HIF1α transcription factor under intermittent hypoxia, which supersedes the HIF1α levels found under acute hypoxia (Yuan et al., 2005, Semenza and Prabhakar, 2007). Higher HIF1α levels observed under intermittent hypoxia have been correlated with increased tumor metastasis and development of resistance against radiotherapy (Yao et al., 2005, Martinive et al., 2006). These studies suggest a critical role for HIF1α in cancer prognosis and present HIF1α as an important target for anti-cancer therapeutics.
Inhibition of HIF1α using an antisense knockdown approach has been shown to delay tumor growth following radiation therapy (Moeller et al., 2005b). In a separate study, Martinive and co-workers also showed that siRNA targeting HIF1α abolished radiation resistance observed in intermittent hypoxia exposed endothelial cells (Martinive et al., 2006). HIF1β null tumor lines were also found to be sensitive towards radiotherapy as they prevent the HIF1 response (Williams et al., 2005). Inhibition of thioredoxin using quinol-based compounds and PX-12 has been shown to affect the transcriptional activity of HIF1α resulting in the downregulation of its target gene, VEGF (Baker et al., 2006, Jones et al., 2006). However, the effect of auranofin on the HIF1 system has not yet been studied. In fact, to date there are only two published studies, which have used auranofin and observed a downregulation of VEGF transcription and neither study analysed HIF1α (Kim et al., 2007a, He et al., 2014).

Given its extremely crucial role in angiogenesis and other aspects of tumor progression, targeting HIF1α and its downstream genes may be a valuable anti-cancer approach. The aim of this chapter was to directly examine the effects of auranofin on the HIF1α system under different oxygen conditions in MDA-MB-231 breast and A549 lung cancer cells.

The specific aims of this chapter are summarised below:

- To determine the effect of intermittent hypoxia (preconditioning) on the stabilisation of HIF1α protein in MDA-MB-231 breast and A549 lung cancer cells and to examine if this stabilisation is influenced by auranofin treatment

- To assess if exposure of intermittent hypoxia treated cells to auranofin affects the HIF1α-CAD activity

- To study the effect of auranofin and intermittent hypoxia on the HIF1α target VEGF through its mRNA expression and protein secretion
4.2 Results

4.2.1 Effect of Preconditioning on HIF1α Protein Expression in Cancer Cells

Similar to the involvement of thioredoxin in providing protection to a prolonged hypoxic stimulus (Nakamura et al., 1998, Isowa et al., 2000, Karlenius et al., 2012), hypoxic preconditioning has also been shown to utilise HIF1α for the protection against the detrimental effects of sustained hypoxia (Cai et al., 2003, Zhu et al., 2013, Sheldon et al., 2014). Therefore, the effects of the preconditioning model used in Chapter 3 (Figure 3.7) on the HIF1α protein levels were first examined in MDA-MB-231 and A549 cancer cells.

Cells were grown until confluent and then incubated under normoxia, hypoxia or were preconditioned with intermittent hypoxia using the scheme described in Chapter 3 (Figure 3.7) and then incubated under hypoxia (0.1% oxygen) for 24 hours. At the end of each incubation, cells were scrapped off into the NP-40 lysis buffer (2.2.5.1) and lysed immediately. HIF1α protein levels were then detected in these lysates by western blotting using an anti-HIF1α monoclonal antibody (Table 2.4). β-tubulin (Table 2.4) was used as a loading control. The representative blots are shown in Figure 4.1. The densitometry analysis of the blots was performed using Image Gauge Ver 4.0 software (Fujifilm). As shown in Figure 4.2, preconditioned MDA-MB-231 cells expressed significantly higher levels of HIF1α compared to the non-preconditioned hypoxic cells. In A549 cells, hypoxia significantly increased the expression of the HIF1α protein compared to normoxia when analysed by an unpaired t-test. However, this increase was not statistically significant when analysed by a one-way ANOVA followed by Tukey’s post test. When A549 cells were preconditioned before the longer hypoxic incubation, the HIF1α protein levels were significantly higher than those observed for normoxic cells but were comparable to those observed in non-preconditioned hypoxic cells. Therefore, preconditioning increases the HIF1α protein levels in the MDA-MB-231 cell line as compared to the non-preconditioned cells but not in the A549 cell line.
Figure 4.1: HIF1α protein in cells grown under different oxygen conditions. Representative western blots for MDA-MB-231 (A) and A549 (B) cells subjected to different oxygen conditions. N = Normoxia, H = Hypoxia, PCH = Preconditioning hypoxia.

Figure 4.2: HIF1α protein levels in cancer cells grown under different oxygen conditions. The HIF1α protein levels in MDA-MB-231 and A549 cells grown under different oxygen conditions were determined by western blotting and densitometry analysis. A one-way ANOVA followed by Tukey’s post test was performed separately for each cell line. A significant difference in the HIF1α levels was observed between preconditioned and hypoxic MDA-MB-231 cells (indicated by Δ) and between the preconditioned and hypoxic cells compared to the normoxic cells (indicated by an *) (P<0.0001). In A549 cells, HIF1α levels in preconditioned cells were significantly higher than the normoxic cells (indicated by #) when analysed by a one-way ANOVA followed by Tukey’s post test (P<0.05). HIF1α levels in hypoxic A549 cells were significantly higher than the normoxic cells when analysed by an unpaired t-test (indicated by ^) (P<0.05). Results are presented as Mean±SEM of at least four independent experiments.
4.2.2 Effect of Preconditioning on HIF1α-CAD Activity in Cancer Cells

Under normal oxygen conditions, HIF1α is constantly expressed and degraded in the cytosol (Jaakkola et al., 2001). However, under hypoxic conditions, the oxygen sensors, PHDs and FIH-1, are unable to efficiently hydroxylate HIF1α that is then stabilised. The stabilised HIF1α dimerises with HIF1β resulting in subsequent expression of the target genes (Figure 1.9). Therefore, the accumulation of HIF1α observed in preconditioned cells in 4.2.1 indicates that the protein is stabilised but does not necessarily imply that HIF1α is transcriptionally active.

To study if the oxygen conditions used in this project affect the transcriptional activity of the HIF1α-CAD, reporter assays were employed. The HIF-CAD activity assay (2.2.7) used in this chapter utilises a vector expressing the HIF1α-CAD domain fused to the Gal4-DNA-binding domain (Gal-DBD). This plasmid is co-transfected with GalRE-luciferase reporter plasmid (Gal response element fused with a luciferase gene). The Gal-DBD binds the Gal-RE region under both normoxic and hypoxic conditions. The HIF1α-CAD activity is regulated by FIH-mediated hydroxylation, which under hypoxia drives expression of the downstream luciferase gene (Figure 4.3). Renilla luciferase was used as a transfection control. The plasmids used in this study were obtained from Dr Dan Peet, University of Adelaide, Australia (Bracken et al., 2006) and are as follows:

- Gal-DBD_BOS (empty vector control; Appendix I)
- Gal-DBD_BOS_HIF1α-CAD
- G5E1B (GalRE-luciferase)
- RL-TK (internal transfection control)
Cells were transiently transfected with the above listed plasmids using Lipofectamine 3000 in SFM. Cells were allowed to recover for ~5 hours after which they were grown under normoxia or hypoxia for 18 hours. In the case of preconditioning, cells were preconditioned ~2.5 hours post transfections, and then incubated under hypoxia for 18 hours. At the end of 18 hours, cells were lysed and HIF1α-CAD activity was measured through dual reporter assays. The luciferase activity was normalised by Renilla luciferase activity. Three independent experiments were performed in triplicate and results are presented as average fold induction over the activity of the empty control vector.

As shown in Figure 4.4, in MDA-MB-231 cells, approximately a 3 fold increase in the HIF1α-CAD activity was observed when preconditioned as well as non-preconditioned cells were grown under hypoxia. When analysed by a one-way ANOVA followed by Sidak’s post test, a significant difference was observed in the HIF1α-CAD activity in preconditioned cells compared to the normoxic cells (P<0.05). In A549 cells, an approximate increase of 11 and 9 fold was observed in the activity of HIF1α-CAD in both hypoxic and preconditioned cells respectively compared to the normoxic cells, which was statistically significant when analysed by a one-way ANOVA followed by Sidak’s post test (P<0.0001). However, preconditioning did not affect the HIF1α-CAD activity compared to hypoxia in either cell line.
Figure 4.4: HIF1α-CAD activity in MDA-MB-231 and A549 cells grown under different oxygen conditions. HIF1α-CAD activity assays were performed and fold inductions of the HIF1α-CAD activity was calculated over the activity of the empty vector. The activity of HIF1α-CAD was enhanced in cells subjected to prolonged hypoxia. The significant difference between the hypoxic and normoxic cells is indicated by an * and that between the preconditioned cells and normoxic cells is indicated by #. Results are presented as Mean±SEM for three independent experiments performed in triplicate.

4.2.3 Effect of Auranofin on HIF1α Levels in Cancer Cells Grown under Different Oxygen Conditions

The activity of the HIF1 system is redox regulated by thioredoxin indirectly via Ref-1. Under hypoxic stress conditions, thioredoxin translocates into the nucleus and reduces Ref-1, which in turn reduces a cysteine residue in the CAD domain of HIF1α (Figure 1.7). This reduction enhances the ability of HIF1α to recruit transcriptional co-activators (Ema et al., 1999). Jones and co-workers have shown an upregulation of HIF1α protein levels in hypoxic cancer cells treated with quinol-based inhibitors of thioredoxin (Jones et al., 2006). However, these drugs repressed the HIF1α transcriptional activity and also inhibited the hypoxia induced upregulation of HIF1α targets, carbonic anhydrase IX (CA-IX) and VEGF (Jones et al., 2006). The effect of auranofin on the HIF1 system has only been studied indirectly through its downstream target VEGF, which was found to be downregulated in auranofin-treated zebrafish embryos and HepG2 cells (Kim et al., 2007a, He et al., 2014). Therefore, the HIF1α protein levels were examined in the MDA-MB-231 and A549 cancer cell lines grown under different oxygen conditions upon treatment with auranofin.
Cells were grown until confluent and then treated with different concentrations of auranofin (as used in 3.2.2.5) and grown under either normoxia or hypoxia. Cells were also preconditioned prior to auranofin dosing and then incubated under hypoxia. At the end of each treatment, cells were immediately lysed with NP-40 lysis buffer (2.2.5.1) and western blotting was performed (2.2.5.5) to detect HIF1α protein using a monoclonal anti-HIF1α antibody (Table 2.4). β-tubulin (Table 2.4) was used as a loading control. At least three independent experiments were performed with MDA-MB-231 cells. The representative blots and the densitometry results are shown in Figure 4.5. As found previously (Figure 4.2), untreated preconditioned MDA-MB-231 cells expressed higher HIF1α levels compared to non-preconditioned hypoxic cells. When treated with auranofin, higher HIF1α levels were observed in preconditioned cells compared to the hypoxic cells, but when analysed by a two-way ANOVA followed by Sidak’s post test, this increase was statistically significant only for the cells treated with 2µM auranofin (Figure 4.5B). A decreasing trend was observed in the HIF1α protein levels in both hypoxic and preconditioned cells upon auranofin treatment. However, when analysed by a one-way ANOVA followed by Sidak’s post test performed separately for each oxygen condition, this decrease was not statistically significant.
Figure 4.5: Relative HIF1α protein levels in MDA-MB-231 cells treated with different auranofin concentrations and grown under different oxygen conditions. A) Representative western blot for MDA-MB-231 cells upon auranofin treatment and growth under different oxygen conditions. B) Densitometry of western blots determined by Image Gauge Ver 4.0 software (Fujifilm). The statistically significant increase in the HIF1α protein levels in the preconditioned cells compared to the respective non-preconditioned cells is indicated by an *. Data was analysed by a two-way ANOVA followed by Sidak’s post test and is presented as Mean±SEM from at least three independent experiments. (P<0.001).

As found previously in 4.2.1, preconditioning did not increase the HIF1α protein levels in untreated A549 cells compared to the hypoxic cells. When cells were treated with auranofin, the trend observed was for an increase in the HIF1α levels in preconditioned cells compared to hypoxic cells but this increase was not statistically significant when analysed by a two-way ANOVA followed by Sidak’s post test. Figure 4.6 shows the representative western blot and the relative protein levels determined by the densitometric analysis.
Figure 4.6: Relative HIF1α protein levels in A549 cells treated with auranofin under different oxygen conditions. A) Representative western blot for A549 cells treated with auranofin and subjected to different oxygen conditions. B) Densitometry of western blots is presented as Mean±SEM from four independent experiments. Data was analysed by a two-way ANOVA followed by Sidak’s post test.

4.2.4 Auranofin and HIF1α-CAD Activity

To study if auranofin treatment under the oxygen conditions used in this project modulates the transcriptional activity of HIF1α, HIF-CAD activity assays (2.2.7) were employed. Cells were transiently transfected with the plasmids described in 4.2.2 and the following stimuli were applied:

- Basal conditions, no stimulus
- Induction by 0.31µM auranofin (concentration at which thioredoxin reductase was inhibited)
- Induction by 2µM auranofin (concentration at which cell proliferation was still not affected)
Cells were allowed to recover for ~5 hours post transfections, after which the appropriate concentrations of auranofin were added and cells were grown under normoxia or hypoxia for 18 hours. In the case of preconditioning, cells were preconditioned ~2.5 hours post transfections, treated with auranofin and incubated under hypoxia for 18 hours. At the end of 18 hours, cells were lysed and HIF1α-CAD activity was measured through dual reporter assays. The luciferase activity was normalised by Renilla luciferase activity. Three independent experiments were performed in triplicate and results are presented as average fold induction over the activity of the empty vector.

As shown in Figure 4.7, the hypoxic and preconditioned MDA-MB-231 cells treated with 0.31µM auranofin exhibited significantly higher HIF1α-CAD activity compared to the normoxic cells treated with 0.31µM auranofin when analysed by a two-way ANOVA followed by Sidak’s post test. When the higher concentration of 2µM auranofin was used, the HIF1α-CAD activities in the hypoxic and preconditioned cells were decreased and were no longer statistically significant compared to the normoxic cells treated with 2µM auranofin. However, the overall effect of auranofin on the HIF1α-CAD activity was not statistically significant when analysed by a one-way ANOVA separately for each oxygen condition. Moreover, as observed in Figure 4.4, no difference was obtained in the HIF1α-CAD activity between hypoxic and preconditioned cells at any auranofin concentration tested.
Figure 4.7: HIF1α-CAD activity in response to auranofin treatment in MDA-MB-231 cells grown under different oxygen condition. The activity of HIF1α-CAD was enhanced in cells subjected to prolonged hypoxia. The significant difference between hypoxic and preconditioned cells compared to the normoxic counterparts is indicated by an *. (P<0.001). Data was analysed by a two-way ANOVA followed by Sidak’s post test and results are presented as Mean±SEM for three independent experiments performed in triplicate.

In A549 cells, the HIF1α-CAD activities in both hypoxic and preconditioned cells compared to their normoxic counterparts were significantly higher at all auranofin treatments when analysed by a two-way ANOVA followed by Sidak’s post test (Figure 4.8). However, auranofin treatment did not have any overall statistically significant effect on the HIF1α-CAD activity in A549 cells under any of the oxygen conditions tested when analysed by a one-way ANOVA followed by Sidak’s post test.
Figure 4.8: HIF1α-CAD activity in response to auranofin treatment in A549 cells grown under different oxygen condition. Hypoxic growth after auranofin treatment significantly increased the activity of HIF1α-CAD in A549 cells. A two-way ANOVA followed by Sidak’s post test was performed. The significant difference between hypoxic and preconditioned cells compared to the normoxic counterparts is indicated by an *. (P<0.0001). Results are presented as Mean±SEM for three independent experiments performed in triplicate.

### 4.2.5 Auranofin and VEGF-A mRNA

Tumor angiogenesis is an important aspect of cancer growth and progression and without the process of angiogenesis, tumors cannot grow more than a few mm³ in size (Folkman, 1971). Therefore, targeting the process of angiogenesis may have important implications for cancer progression. VEGF is the most potent and most widely studied angiogenic factor (Jain, 2014). VEGF expression is driven by hypoxia and is regulated by HIF1α (Semenza, 2001). In fact, many studies have focussed on VEGF expression as an indirect measure of the HIF1 activity (Kim et al., 2007a, He et al., 2014).

The anti-angiogenic effect of auranofin was studied by He and co-workers in zebra fish embryos. They found that at a very low concentration of 0.098µM, auranofin upregulated the VEGF-A mRNA levels while at the higher concentrations of 0.39µM and 1.56µM (similar to the auranofin concentrations used in this chapter), auranofin caused a downregulation of the VEGF-A mRNA (He et al., 2014). In another study, HepG2 cells pre-treated with auranofin were shown to have diminished VEGF transcription (Kim et al., 2007a). Therefore, the effect of auranofin on VEGF was
determined in MDA-MB-231 and A549 cancer cells grown under the different oxygen conditions.

There are five different members of the VEGF family. VEGF-A in particular is characterised by hypoxia inducibility and was therefore used in this project. MDA-MB-231 and A549 cells were grown until confluent, treated with either 0.31µM or 2µM auranofin and grown under normoxia or hypoxia. Cells were also preconditioned prior to auranofin treatment and subjected to prolonged hypoxia afterwards. At the end of each treatment, cells were lysed and the total RNA was extracted. Real-time RT-PCR (2.2.14) was performed next to quantify the mRNA expression levels in the cells, which was analysed using the comparative cycle threshold algorithm (ΔΔC_{t} method). The house-keeping gene, L-32 was used to normalise the results, which were expressed as fold change over the untreated normoxic controls (Lafleur et al., 2005).

As shown in Figure 4.9, in the untreated MDA-MB-231 cells hypoxia caused approximately a 1.5 fold increase in the VEGF-A mRNA levels compared to normoxia while preconditioning the cells prior to growth in hypoxia caused approximately a 2.5 fold increase in the VEGF-A mRNA levels compared to normoxia, which was statistically significant when compared by a two-way ANOVA followed by Sidak’s post test. However, auranofin treatment did not have any overall statistically significant effect on VEGF-A expression levels under any of the oxygen conditions tested.
Figure 4.9: Effect of auranofin treatment on the expression of VEGF-A in MDA-MB-231 cells grown under different oxygen conditions. Real-time RT-PCR was used to quantitate VEGF-A gene expression. Results were normalised against the expression of L-32 house-keeping gene and expressed as fold change over the untreated normoxic controls. Data are presented as Mean±SEM of three independent experiments performed in duplicate. A two-way ANOVA followed by Sidak’s post test was employed and the significant increase in VEGF-A expression in the untreated preconditioned cells compared to the untreated normoxic cells is indicated by an *. (P<0.001).

In A549 cells, approximately a 2.5 fold increase in the VEGF-A mRNA levels, which was statistically significant, was observed in hypoxic and preconditioned cells that were untreated or treated with 0.31µM auranofin compared to their normoxic counterparts (Figure 4.10). At the higher concentration of 2µM auranofin, the VEGF-A expression levels in both hypoxic and preconditioned cells were decreased such that these levels were not statistically significantly higher than the normoxic counterparts. However, the overall effect of auranofin was not statistically significant.
CHAPTER 4

Figure 4.10: Effect of auranofin treatment on the expression of VEGF-A in A549 cells grown under different oxygen conditions. Real-time RT-PCR was used to quantitate the expression of VEGF-A. L-32 house-keeping gene was used to normalise the results, which were expressed as fold change over the untreated normoxic controls. Results are presented as Mean±SEM of three independent experiments performed in duplicate. A two-way ANOVA followed by Sidak’s post test was employed and the significant increase in VEGF-A expression in hypoxic and preconditioned cells compared to the respective normoxic cells is indicated by an * . (P<0.001).

4.2.6 Auranofin and VEGF-A Secreted Protein Levels

The VEGF-A secreted protein levels in both cells lines in response to auranofin treatment and growth under different oxygen conditions were also quantitated using ELISA (2.2.10). Cells were grown until confluent, treated with either 0.31µM or 2µM auranofin and grown under normoxia or hypoxia. Cells were also preconditioned prior to auranofin treatment and subjected to prolonged hypoxia afterwards. After 24 hours, the media was collected and cellular debris cleared by centrifugation at 265 X g for 5 minutes and the VEGF-A secreted levels measured through ELISA.

As shown in Figure 4.11, VEGF-A protein levels were higher in hypoxic and preconditioned MDA-MB-231 cells compared to the normoxic cells but not statistically significant at any of the auranofin concentrations tested. There was no statistically significant difference in the VEGF-A secreted protein levels between hypoxic and preconditioned cells, which correlated with the results obtained for the HIF1α-CAD activity upon auranofin treatment in these cells (Figure 4.7). The VEGF-A secreted levels in both hypoxic and preconditioned cells treated with the higher concentration of
2µM auranofin were decreased such that these levels were not statistically significantly higher than the normoxic counterparts when analysed by a two-way ANOVA followed by Sidak’s post test.

Figure 4.11: VEGF-A secreted protein levels secreted by MDA-MB-231 cells grown under different oxygen conditions in response to auranofin treatment. VEGF-A protein levels were measured in the culture media by ELISA. A two-way ANOVA followed by Sidak’s post test was performed and results are presented as Mean±SEM of three independent experiments performed in duplicate. A statistically significant difference was observed between untreated MDA-MB-231 cells grown under hypoxia and preconditioning hypoxia compared to the untreated normoxic cells (as indicated by an *) (P<0.05). Preconditioned cells treated with 0.31µM auranofin also had significantly higher levels of VEGF-A secreted protein compared to their normoxic counterparts (as indicated by *). (P<0.05).

In A549 cells, the overall VEGF-A secreted protein levels were lower than those observed for MDA-MB-231 cells. In these cells, hypoxia or preconditioning did not cause any change in the VEGF-A secreted protein levels compared to normoxia when cells were untreated. However, upon treatment with auranofin, cells that were preconditioned showed an increase in the VEGF-A secreted levels, which was statistically significant at the higher concentration of 2µM compared to the respective normoxic control (Figure 4.12).
Figure 4.12: VEGF-A protein levels secreted by A549 cells grown under different oxygen conditions in response to auranofin treatment. ELISA was employed to quantitate VEGF-A protein levels in the culture media. A two-way ANOVA followed by Sidak’s post test was performed and results are presented as Mean±SEM of three independent experiments performed in duplicate. A statistically significant difference was observed between preconditioned A549 cells treated with 2µM auranofin compared to the normoxic cells (as indicated by an *) (P<0.05).
4.3 Discussion

One of the key hallmarks of cancer is angiogenesis, the process of formation of new blood vessels (Hanahan and Weinberg, 2000). Tumor angiogenesis is a hypoxia-driven process. However, this process is quite aberrant and poorly developed (Secomb et al., 1993, Dewhirst et al., 1996, Dewhirst et al., 1999). As a result, the tumor microenvironment experiences intermittent hypoxia, the effects of which have been underestimated during development and testing of anti-cancer therapies. Therefore, in order to design better therapeutics, it is important to study the complex systems involved in intermittent hypoxia. As stated previously, HIF1α levels observed under intermittent hypoxia supersede those observed under acute hypoxia (Yuan et al., 2005, Semenza and Prabhakar, 2007). Therefore, the effect of the intermittent hypoxia (preconditioning) scheme used in this project on the HIF1α levels was established first in the MDA-MB-231 breast and A549 lung cancer cell lines.

In accordance to the studies reported by others in PC12 cells (Yuan et al., 2005) and lung cancer cells (Liu et al., 2010), preconditioning stabilised the HIF1α protein levels in MDA-MB-231 cells, which were higher than the levels observed in cells subjected to prolonged hypoxia (Figure 4.2). Preconditioning involves re-oxygenation phases, which are expected to cause oxidative stress and an accumulation of ROS. HIF1α has been shown to be stabilised and activated by ROS (Chandel et al., 2000, Moeller et al., 2004). Therefore, the higher levels of HIF1α observed in preconditioned cells may be a result of the ROS generated during the re-oxygenation cycles. Contrary to these findings, no difference was observed in the HIF1α levels in preconditioned A549 cells compared to those grown under hypoxia (Figure 4.2). This suggests that the stabilisation and upregulation of HIF1α under the conditions of acute and intermittent hypoxia may be cell line specific. This hypothesis is supported by the work done by Bracken and co-workers in which they found that the induction of HIF1α with respect to oxygen levels and duration of hypoxic incubation was cell line specific (Bracken et al., 2006). Furthermore, it appears that the intermittent hypoxia schemes used play a part in determining how the hypoxic responses are induced in the cells. For example, the results obtained with A549 cells in this project did not show an increase in the HIF1α protein levels in cells that were preconditioned compared to the hypoxic cells while Liu
and colleagues observed an increase in the HIF1α levels under intermittent hypoxia compared to prolonged hypoxia in the same cell line (Liu et al., 2010). The oxygen scheme utilised by these researchers involved longer durations of hypoxic and re-oxygenation phases and was different from the scheme used in the current project that involved very short cycles of hypoxia/re-oxygenation.

The recruitment of transcriptional activators by HIF1α for its subsequent transcriptional activity is redox regulated by thioredoxin (Ema et al., 1999). Over-expression of thioredoxin enhanced the expression of HIF1α protein levels and the HIF1 DNA binding activity and also increased the activation of HIF1α regulated gene promoters (Welsh et al., 2002). When PX-12 and pleurotin, which are inhibitors of thioredoxin and thioredoxin reductase respectively were used, a dose-dependent decrease was observed in HIF1α protein levels but not the mRNA levels in MCF-7 breast and HT29 colon cancer cells (Welsh et al., 2003). In a separate study, inhibition of thioredoxin with quinol-based inhibitors caused an upregulation of HIF1α protein levels in hypoxic human breast cancer and melanoma cell lines but decreased its transcriptional activity (Jones et al., 2006). In this project, auranofin, an inhibitor of the thioredoxin reductase activity, was used. Although the effects of auranofin on VEGF mRNA expression in specific cell types have been reported previously (Kim et al., 2007a, He et al., 2014), its effect on HIF1α has not yet been established directly. Moreover, the aforementioned studies were performed only under acute hypoxia. Therefore, the effect of auranofin treatment on the HIF1α protein levels was studied for the first time in this project, together with the effect of intermittent hypoxia.

In MDA-MB-231 cells, preconditioning enhanced the stabilisation and accumulation of HIF1α protein in untreated and treated cells compared to their hypoxic counterparts (Figure 4.5). When treated with auranofin, a decreasing trend in HIF1α levels was observed in the cells grown under hypoxia and preconditioning hypoxia, but this decrease was not statistically significant. On the other hand, in A549 cells, although not statistically significant, a slight increase in HIF1α protein levels was observed in preconditioned cells compared to the cells grown under hypoxia alone only when they were treated with auranofin (Figure 4.6). Therefore, auranofin did not have any overall
statistically significant effect on the HIF1α levels in either of the cell lines used in this project.

An accumulation of HIF1α is an indication of its stabilisation, which occurs under hypoxic conditions when the PHDs are unable to hydroxylate HIF1α and its degradation is prevented (Pouyssegur et al., 2006). For HIF1α to be transcriptionally active, it is required to form the HIF1 complex with HIF1β and bind to the HRE of the target genes (Greer et al., 2012). Transcriptional co-activators are then recruited in the absence of an FIH-mediated hydroxylation (Lando et al., 2002b) and following the reduction of Cys800 in the CAD domain of HIF1α by thioredoxin through Ref-1 (Ema et al., 1999). The effect of preconditioning on the activity of the HIF1α-CAD domain compared to hypoxia was examined in this project. The baseline HIF1α protein levels under normoxia were similar in MDA-MB-231 and A549 cells. Moreover, the HIF1α levels induced by hypoxia were also comparable in the two cell lines (Figure 4.2). However, the HIF1α-CAD activity observed in untreated A549 cells under hypoxia was approximately 4 fold higher than that observed in MDA-MB-231 cells (Figure 4.4). Bracken and colleagues also observed the HIF1α-CAD activity to be cell line specific (Bracken et al., 2006). Since the transcriptional activity of HIF1α requires its Cys800 to be reduced by thioredoxin/Ref-1, the higher activity of HIF1α-CAD observed in these cells may also be attributed to the higher thioredoxin expression levels in A549 cells (Figure 3.2).

Auranofin was also used to inhibit the thioredoxin system under all the oxygen conditions tested and study its effect on the transcriptional activity of HIF1α. This was a novel aspect of this study. Preconditioning cells prior to a prolonged incubation under hypoxia did not affect the activity of HIF1α-CAD in either cell line. Treatment of MDA-MB-231 cells with 2µM auranofin decreased the HIF1α-CAD activity in hypoxic and preconditioned cells such that their activity was not statistically significantly higher than the normoxic counterparts (Figure 4.7). However, the HIF1α-CAD activity was not affected by auranofin treatment under any of the oxygen conditions in A549 cells (Figure 4.8). These results suggest that although preconditioning or intermittent hypoxia plays a role in stabilising HIF1α protein, it does not modify its transcriptional activity in the cell lines used in this project. Furthermore, as observed with the HIF1α protein
levels, auranofin had no overall statistically significant effect on the HIF1α-CAD activity under any oxygen conditions tested.

The importance of angiogenesis in tumor formation and progression was established in the early 1970s when Judah Folkman predicted that growing tumors secrete pro-angiogenic factors upon sensing the increasing distance from the existing local vasculatures (Folkman, 1971). Since then many studies have verified this prediction and the findings have been summarised in a review by Liao and Johnson (Liao and Johnson, 2007). VEGF is the most well studied angiogenic factor (Jain, 2014) and its transcription is directly regulated by HIF1 (Forsythe et al., 1996). Increased expression of VEGF has been correlated with highly aggressive and malignant tumors (Jensen et al., 2006). Higher levels of VEGF in patient serum samples are also used as a diagnostic marker for cancer. VEGF has also been implicated to have a critical role in tumor metastasis by facilitating the invasive tumor cells to migrate from the primary tumor site to the secondary site through the blood stream (Liotta et al., 1974). Therefore, anti-angiogenic strategies may play a beneficial role in control of cancer progression.

He and co-workers recently demonstrated the anti-angiogenic effects of auranofin in zebrafish embryos. Treatment of embryos with auranofin caused a downregulation of VEGF-A mRNA (He et al., 2014). In a separate study, IL6 induced transcription of VEGF was shown to be diminished by auranofin in HepG2 cells (Kim et al., 2007a). Therefore, real-time RT-PCR was used to study the fold change in VEGF-A mRNA levels compared between cells treated with auranofin and also under different oxygen parameters (Figure 4.9 and 4.10). The VEGF-A expression is considered to be a representation of the HIF1α-CAD transcriptional activity. The results obtained in this project for VEGF-A mRNA levels were similar to those seen for the HIF1α-CAD activity, where no difference was observed between hypoxia and preconditioning hypoxia in either untreated cell line but were higher than the normoxic counterparts. These results correlated with those obtained by Liu and colleagues in H446 lung cancer cells, where they observed approximately a 5 fold increase in the VEGF-A mRNA levels in both acute and intermittent hypoxia treated cells over the normoxic cells (Liu et al., 2010). Auranofin treatment resulted in slight decrease in the VEGF-A mRNA
levels in both the cell lines when cells were preconditioned prior to the treatment; however this decrease was not statistically significant.

The VEGF-A protein levels secreted into the culture media by cells treated with auranofin and grown under the different oxygen environments were also examined by an ELISA. As with the HIF1α-CAD activity and VEGF-A mRNA levels, preconditioning did not cause any change in the secreted VEGF-A protein levels in MDA-MB-231 cells compared to the cells grown under acute hypoxia (Figure 4.11). In this cell line, a decreasing trend was observed in the secreted VEGF-A levels upon auranofin treatment of hypoxic and preconditioned cells; however this decrease was not statistically significant. In the untreated A549 cells, neither hypoxia nor preconditioning hypoxia caused an increase in the secreted VEGF-A levels compared to the normoxic cells (Figure 4.12). Stress granules are aggregates of protein-mRNA complexes, which are formed under stress conditions. These granules regulate the translation of only the most critical proteins under stressful conditions. Once the stress is alleviated, these granules disassemble allowing the normal translation of the proteins (Kedersha and Anderson, 2002). Moeller and co-workers showed that there was an increase in the VEGF levels when cells were re-oxygenated after a hypoxic incubation (Moeller et al., 2004). As stated above no difference was observed in the VEGF-A secreted protein levels in untreated A549 cells compared to normoxic cells in this project. Since re-oxygenation was not performed and both acute hypoxia and preconditioning hypoxia treatments ended in a hypoxic incubation, this may be due to the formation of stress granules.

Moreover, in this project, auranofin treatment caused an increase in the VEGF-A levels secreted from A549 cells that were preconditioned prior to auranofin treatment. In an earlier study, inhibition of thioredoxin reductase by DNCB was shown to increase the VEGF protein levels in bovine mammary endothelial cells (Streicher et al., 2004). However, that study was performed only under normoxic conditions. It is also possible that the increase in the secreted VEGF-A protein levels observed in this project (Figure 4.12) might have resulted from an inadvertent re-oxygenation step during the collection and processing of the samples and therefore, needs to be further evaluated.
Another observation was that although the unstimulated HIF1α-CAD activity of A549 cells under hypoxia was approximately 4 fold higher than that observed in MDA-MB-231 cells, the VEGF-A protein secreted by untreated A549 cells was approximately 4 fold less than the levels secreted by the untreated MDA-MB-231 cells under hypoxia. These results again highlight the possibility that the hypoxic responses are cell line specific.

The varied results observed by different researchers in studies involving hypoxia and intermittent hypoxia are due to the differences in oxygen concentrations used and the duration of hypoxic and/or re-oxygenation exposures. Most healthy organs reside in an average of 3-6% oxygen (Roy et al., 2003, Carreau et al., 2011) and conditions lower than 3% oxygen are physiologically described as hypoxia. Oxygen levels ranging between 0.05% and 5% have been utilised as hypoxia in studies involving a range of cell lines (Jewell et al., 2001, Jones et al., 2006, Kim et al., 2007b, Malec et al., 2010). However, since most of the in vitro studies are performed under air (20% oxygen), growing cells under 10-11% oxygen can also initiate a hypoxic response in vitro. It has been shown by Bracken and co-workers that different concentrations of oxygen elicit varied levels of HIF1α protein and CAD activity, which are also cell line specific (Bracken et al., 2006). In spite of this knowledge, it is hard to establish which oxygen concentrations would be more physiologically relevant.

The intermittent hypoxia schemes used by researchers also vary significantly. The tumor microenvironment is highly unstable due to inconsistent oxygen supply (Lanzen et al., 2006). This causes cancer cells to be in flux, where they constantly cycle between hypoxia and re-oxygenation. As discussed previously in Chapters 1 and 3, there are two dominant timescales that contribute to the cycling kinetics. One is of the faster frequency with only a few cycles per hour occurring due to fluctuations in red blood cell flux (Lanzen et al., 2006). The slower timescale varies from hours to days and occurs due to vascular remodelling (Nehmeh et al., 2008). As with the oxygen concentrations, it is hard to define which timescales are more physiologically relevant.
Therefore, different cell lines may respond differently to similar stress conditions (oxygen-dependent or chemically induced by drugs) and each cancer cell line should be evaluated for its suitability as a model system for studying oxygen dependent pathways. Previous work done in our laboratory showed that 0.1% oxygen elicited a hypoxic response in MDA-MB-231 cells while 1% oxygen did not have a significant effect (Karlenius, 2011, Bhatia, 2013). Hence it is important to establish appropriate conditions for each cell line since various methodologies are used to generate hypoxia and depending on the methods used, different results can be obtained. As a consequence, it becomes difficult to infer the data obtained from the various hypoxia/intermittent hypoxia studies. This problem is further complicated when results obtained in cell-based models are compared to those obtained from animal models.

The results obtained in this chapter suggest that treatment of MDA-MB-231 cells with a higher dose of auranofin slightly decreased the activity of HIF1α-CAD, VEGF-A mRNA levels and VEGF-A secreted protein levels in hypoxic and preconditioned cells such that these levels were not statistically significantly higher than their corresponding normoxic cells. The response of A549 cells to auranofin treatment under the different oxygen conditions was different to that of the MDA-MB-231 cells. Overall, these results do not provide a definitive answer as to whether auranofin exerts an effect on the hypoxic system in the cell lines tested under the oxygen scheme utilised. Moreover, these results highlight the idea that biological and technical reasons are important determinants of the intermittent hypoxia induced responses. However, since intermittent hypoxia schemes are a better representation of the in vivo cancer microenvironment, they should be taken into consideration while designing and testing of drugs. Hence there is a need to refine the models used in in vitro and in vivo studies for better understanding of the complex systems involved in cancer biology and development of therapeutics against these systems.
CHAPTER 5

ROLE OF THIOREDOXIN IN CANCER CELL MIGRATION
5.1 Introduction

One of the main reasons for the poor prognosis for cancer patients is metastasis, which has already occurred by the time many patients are diagnosed. Metastasis is not a single event but a group of coordinated processes (Figure 1.17). The first stage of metastasis is invasion involving the detachment of cells from the primary tumor mass, degradation of the ECM and the subsequent entry of these cells into the blood stream (Saiki, 1997). These invading cells then migrate through the blood stream and may get arrested in a distant tissue or organ. The next step is called extravasation and involves the degradation of the ECM of the secondary tissue and establishment of the tumor mass in a new location through clonal expansion (Fidler, 1990). During the process of intravasation as well as extravasation, tumor cells are required to migrate through the interstitial matrix towards a blood or lymphatic vessel (Paquette et al., 2005). Therefore, cell migration is an essential aspect of invasion and metastasis.

Cellular redox state has been shown to have important implications on the invasiveness of cancers. The higher thioredoxin expression in many cancers has been linked with highly invasive and metastatic tumors (Lincoln et al., 2003). Highly aggressive tumors have been reported when cell lines over-expressing thioredoxin were injected into immune-deficient mice (Ceccarelli et al., 2008). Elevated thioredoxin levels have also been associated with ECM degradation by preferentially inhibiting TIMPs and enhancing the MMP activity (Farina et al., 2001). In another study, thioredoxin’s redox site was shown to have a role in increasing MMP-9 activity and thereby influencing invasiveness of breast cancer cells in vitro (Farina et al., 2011). These studies implicate an important role for thioredoxin and its redox active site in the growth and spread of cancer.

The redox active site of thioredoxin is highly conserved between species and consists of the Cys-Gly-Pro-Cys consensus sequence. In order to reduce the active site of thioredoxin, the two Cys residues undergo two half reactions by thioredoxin reductase using NADPH (Figure 1.2) (as reviewed by Holmgren, 1985). In the reduced state, thioredoxin exerts its many biological functions including maintaining the cellular redox homeostasis, regulating transcription factors, regulating apoptosis and stimulating
cell proliferation (Figure 1.5). To study the role of thioredoxin’s active site in these functions and its interactions with thioredoxin reductase and other cellular targets, redox inactive mutants have been used by many researchers (Gasdaska et al., 1994, Oblong et al., 1994, Gallegos et al., 1996, Farina et al., 2011). Through site-directed mutagenesis, the active site Cys residues are changed to Ser residues in these mutants (Oblong et al., 1994). Although this Cys to Ser mutation has been shown to induce some changes in the protein structure of the C32S/C35S mutant when compared to the wild type thioredoxin, enough structural information is retained for thioredoxin reductase to recognise it with nearly equivalent affinity as to thioredoxin (Oblong et al., 1994). Therefore, the C32S/C35S mutant acts as a strong competitive inhibitor of thioredoxin. In this project, the C32S/C35S mutant will be referred to as 1SS.

The role of thioredoxin in cancer cell invasion has been previously studied in our laboratory. When recombinant thioredoxin protein was added extracellularly to MDA-MB-231 cells, the invasive capacity of these cells was enhanced (Bloomfield, 2003). Similarly, the migration of MDA-MB-231 cells was increased by the extracellular addition of thioredoxin. However, the addition of the redox inactive thioredoxin protein, 1SS, did not have any effect on the migration (King, 2010, Shah, 2011). Invasion studies were also performed in cells over-expressing thioredoxin and 1SS. Over-expression of thioredoxin caused an increase in the invasion of cells through Matrigel while over-expression of 1SS diminished the invasion (Bloomfield, 2003). These results indicated that thioredoxin’s active site plays a role in stimulating the invasion and migration of cancer cells and that a functional active site is required to carry out these functions. Therefore, the role of thioredoxin and its redox active site was further investigated in the migration of MDA-MB-231 breast cancer cells in this project.
The specific aims of this chapter were as follows:

- To examine if modulation of intracellular thioredoxin levels by over-expressing either thioredoxin or its redox inactive form ISS affects the migration of MDA-MB-231 cells
- To investigate if the inhibition of thioredoxin reductase by auranofin decreases the migration of MDA-MB-231 cells
- To determine the effect of auranofin induced inhibition of the thioredoxin system on the clonogenic capacity of MDA-MB-231 cells
5.2 Results

5.2.1 Generation of Stable Transfected Cell Lines with Modulated Thioredoxin Levels

MDA-MB-231 is a commonly used breast cancer cell line for studying tumor invasion and metastasis (Dhanesuan et al., 2002, Farina et al., 2011, Lin et al., 2011). This cell line has been previously used in our laboratory to generate thioredoxin over-expressing cells, which were used for invasion studies (Bloomfield, 2003). Since migration is an important aspect of metastasis, the role of thioredoxin in cancer cell migration was assessed in this chapter by generating MDA-MB-231 cells stable transfected with thioredoxin and 1SS over-expressing constructs.

The constructs expressing thioredoxin and the redox inactive mutated form of thioredoxin, designated as 1SS were previously prepared in our laboratory by Fenil Shah. These constructs have been cloned into the pIRES-EGFP plasmid (Clontech) (Appendix I). This plasmid contains a strong CMV promoter and an IRES sequence that allows the expression of two genes on the same transcript from the same promoter. In this case, the constructs co-express the green fluorescent protein (GFP) and either thioredoxin or 1SS.

These plasmids along with the parental pIRES-EGFP plasmid (used as a control) were transfected into MDA-MB-231 cells in 6-well plates (2.2.4). The DNA concentrations to be used for transfections had been previously optimised in our laboratory (King, 2010). The constructs used also contain a neomycin resistance gene providing the successfully transfected cells with resistance to geneticin that serves as a selection mechanism. The concentration of geneticin used for selection of the successfully transfected cells had also been previously optimised (King, 2010). For this project, the transfections were carried out in duplicate (Figure 5.1 A).

Post transfections, cells were allowed to recover for 24 hours after which the media was replaced with fresh media containing geneticin. Cells were grown in the 6-well plate for approximately 2-3 weeks until geneticin-resistant colonies appeared. Geneticin was
replaced every 3-4 days. In this initial selection step, only one replicate for pIRES-EGFP-1SS survived.

Once the geneticin-resistant cells were confluent, a single cell clonal isolation technique (2.2.4.2), obtained from Corning Life Sciences (http://csmedia2.corning.com/LifeScience), was employed to select the clones arising from a single colony. Cells were harvested from the 6-well plate and serially diluted into 96-well plates. 200μL of cell suspension was added to the first well of the 96-well plate, which was serially diluted down the plate. A second set of dilutions was then carried out across the plate (Figure 5.1 B). Cells were then grown for a week and observed under the microscope every day. Each well containing a single colony was marked during this period.

Figure 5.1: Schematic for experimental set up for generation and selection of cells with modulated thioredoxin levels. A) Template for transfections. Stable transfections were carried out in duplicate in a 6-well plate. B) Template for single cell clonal isolation using serial dilution technique (obtained with permission from Corning Life Science). Cells were first diluted down the plate (red arrow) and the second set of dilutions was carried out across the plate (blue arrow). C) Example of stained cells after dilution (obtained with permission from Corning Life Science).
CHAPTER 5

Once the colonies in the marked wells were ~40% confluent, they were harvested and transferred to a 24-well plate. Three single cell clones were selected from each 96-well plate. Cells were grown in the 24-well plate until ~60% confluent, after which they were transferred to a 6-well plate and later scaled up to 25cm² flasks and finally to 75cm² flasks. At this stage, a total of 11 clones survived for use in the subsequent experiments (Table 5.1).

Table 5.1: List of stable transfected clones used.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Initial transfections</th>
<th>Final clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>GFP_1</td>
<td>G1.2</td>
</tr>
<tr>
<td>pIRES-EGFP</td>
<td>GFP_2</td>
<td>G1.3 G2.1 G2.2</td>
</tr>
<tr>
<td>Thioredoxin over-expressing</td>
<td>Trx_1</td>
<td>T1.2 T1.3 T2.1</td>
</tr>
<tr>
<td>pGFP-Trx</td>
<td>Trx_2</td>
<td>T2.2</td>
</tr>
<tr>
<td>1SS over-expressing</td>
<td>1SS_1</td>
<td>1SS_1 1SS_2.1</td>
</tr>
<tr>
<td>pGFP-1SS</td>
<td>1SS_2</td>
<td>1SS_2.2 1SS_2.3</td>
</tr>
</tbody>
</table>

5.2.2 Confirmation of the Expression of the Desired Transgene in the Transfected Clones

Once cells were growing in a flask, they were tested for visualisation of GFP (2.2.4.3). The representative images of one clone from each type of transfectants are shown in Figure 5.2 and confirm that the clones were expressing GFP. This indicated that the required transgene was present in all the clones. To further confirm that the transfected clones expressed the correct sequences for thioredoxin and 1SS, the genomic DNA was extracted from each clone (2.2.1.5) and PCR was performed to amplify the thioredoxin
and 1SS gene sequences (2.2.1.6). The PCR products were run on an agarose gel (2.2.1.7) and excised from the gel to elute the DNA (2.2.1.8). The excised DNA was then ligated into pGEM-T Easy vector (2.2.1.9), which was then transformed into ED8799 cells and blue/white colony selection was performed (2.2.1.4). The plasmids containing the thioredoxin and 1SS sequences were then obtained by large scale plasmid preparations (2.2.1.1.1) and sequencing PCR was performed (2.2.1.10). Ethanol/EDTA/NaAc precipitation was then performed and the samples were sequenced at the Griffith University DNA Sequencing Facility. The sequencing results confirmed the presence of the correct transgene sequences in the respective thioredoxin and 1SS over-expressing clones.

![Figure 5.2: GFP expression in the stable transfected cells.](image)

The transfected cells were fixed and visualised for GFP expression. DAPI was used to stain the nucleus. The images were taken at 20X magnification using a Nikon E800 fluorescent microscope. pIRES-EGFP = cells transfected with pIRES-EGFP; pGFP-Trx = cells over-expressing thioredoxin; pGFP-1SS = cells over-expressing 1SS.
5.2.3 Thioredoxin Protein Levels in the Transfected Clones

The thioredoxin protein levels in the stable transfected clones were examined to confirm the over-expression of thioredoxin and 1SS in these clones. One clone of each transfectant was used as a representative. Cells were grown for either 24 or 48 hours and lysed with NP-40 lysis buffer (2.2.5.1). Western blotting (2.2.5.5) was then performed to detect the presence of thioredoxin using the 5G8 monoclonal anti-thioredoxin antibody and β-tubulin as a loading control (Table 2.4). As shown in Figure 5.3, no difference was observed in the thioredoxin protein levels between the thioredoxin and 1SS over-expressing cells compared to the GFP-only controls at either 24 or 48 hours.

![Figure 5.3: Thioredoxin protein in the stable transfected clones. Western blotting was performed using lysates obtained from the stable transfected clones and blots were probed with an anti-thioredoxin monoclonal antibody along with β-tubulin. Trx = thioredoxin; pIRES-EGFP = cells transfected with pIRES-EGFP; pGFP-Trx = cells over-expressing thioredoxin; pGFP-1SS = cells over-expressing 1SS.](image)

5.2.4 Reactive Oxygen Species in the Transfected Clones

The 1SS protein is similar to thioredoxin in structure but with the active site Cys residues mutated to Ser, 1SS cannot reduce other cellular proteins. Oblong and co-workers have shown using circular dichroism spectroscopy that 1SS also acts as a competitive inhibitor of thioredoxin by binding to thioredoxin reductase and preventing it from reducing thioredoxin back to its active form (Oblong et al., 1994). This is expected to increase the cellular oxidative stress in the cells. ROS assays (2.2.12) were employed to assess the levels of ROS in each of the clones. Total protein content in the lysates was also determined and used to normalise the relative fluorescent units (RFUs), which were then expressed as the fold change over the pIRES-EGFP control cells. As expected, 1SS expressing cells had 2.5 fold higher levels of ROS as compared to the
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GFP-only control cells, which was statistically significant when analysed by a one-way ANOVA followed by Sidak’s post test (Figure 5.4). However, thioredoxin over-expression did not result in any change in ROS levels, which were comparable to the levels measured in pIRES-EGFP control cells.

![Figure 5.4: ROS levels in the stable transfected cell lines.](image)

**Figure 5.4: ROS levels in the stable transfected cell lines.** ROS analysis was performed using H$_2$DCF-DA. Results from three independent experiments performed in triplicate are presented as Mean±SEM. A one-way ANOVA followed by Sidak’s post test was employed and the significant difference in the ROS levels between 1SS over-expressing and GFP-only controls cells is indicated by an *. (P<0.05). pIRES-EGFP = cells transfected with pIRES-EGFP; pGFP-Trx = cells over-expressing thioredoxin; pGFP-1SS = cells over-expressing 1SS.

5.2.5 Effect of Thioredoxin Over-Expression on the Migration of MDA-MB-231 Cells

To study the role of thioredoxin in the migration of MDA-MB-231 cells, monolayer scratch assays were employed (2.2.8.1). These assays are based on the principle of wound healing. When skin is injured, the epidermal cells from the damaged area migrate to cover and heal the wounded area. These assays, also known as wound healing or migration assays, have been used by many researchers to study cell proliferation and migration in different culture conditions (Dhanesuan *et al.*, 2002, Adams *et al.*, 2010, Zajac *et al.*, 2011). The technique used in this chapter was adapted from the methods used by Dhanesuan and co-workers (Dhanesuan *et al.*, 2002) and had been used previously in our laboratory to study the effect of extracellular addition of
recombinant thioredoxin and 1SS on the migration of MDA-MB-231 cells (King, 2010, Shah, 2011).

The monolayer scratch assays were performed in 24-well plates and a cross-reference was etched into the underside of the plates. To avoid any misinterpretation of normal cell proliferation as migration, an anti-proliferative agent, thymidine was added to the media at the time of wounding/scratching the cells (Ooi et al., 1993, Dhanesuan et al., 2002). The concentration of thymidine to be used was previously optimised in our laboratory by Mallory King and 10mM was found to be an optimum concentration to block the proliferation of MDA-MB-231 cells.

Each stable transfected clone was used in quadruplicate for the scratch assays (2.2.8.1). The scratches were observed for 48 hours with images being taken of the cross-reference mark at 0, 24, and 48 hours to monitor closing of the ‘wound’ (Figure 5.5). The distance migrated by cells to heal the wound at the start of the experiment (t=0) was designated as 0. The distance migrated by cells at 24 and 48 hours was calculated based on the decrease in the width of the scratch with respect to the 0 hour time point. For each experiment, all the clones listed in Table 5.1 were tested and the distance migrated by each type of transfected cells was expressed as the average distance migrated. At least three independent experiments were performed.
As shown in Figure 5.6, thioredoxin over-expression (pGFP-Trx) did not cause any increase in the migration of cells as compared to the GFP-only control cells (pIRES-EGFP). However, the cells over-expressing 1SS (pGFP-1SS) migrated slower than the GFP-only controls with a significant difference at the 48 hour time point when analysed by a repeated measures two-way ANOVA followed by Sidak’s post test.
Figure 5.6: Effect of over-expression of thioredoxin and its redox inactive form ISS on cell migration. Monolayer scratch assays were used to study the migration of cells over-expressing thioredoxin and ISS. A repeated-measures two-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM of at least three independent experiments performed in quadruplicate. The statistical difference between the distance migrated by ISS over-expressing cells and GFP-only control cells is indicated by an *. (P<0.0001).

5.2.6 Effect of Auranofin on the Migration of MDA-MB-231 Cells

The results obtained in 5.2.5 suggest that the redox-active site of thioredoxin plays an important role in stimulating the migration of cancer cells. Thus inhibiting the redox activity of thioredoxin or thioredoxin reductase may also lead to the selective inhibition of cancer cell migration. The migration of colorectal cells was recently shown to be decreased upon treatment with PX-12, a thioredoxin inhibitor (Wang et al., 2015). However, in a separate study, PX-12 was shown not to be suitable for testing against MDA-MB-231 cells (Park et al., 2014). Therefore, auranofin, a thioredoxin reductase inhibitor was used as a tool to chemically inhibit the thioredoxin system. Both the lower (0.31µM) and the higher (2µM) concentrations of auranofin used in the previous chapters were utilised to study the effect of auranofin on the migration of non-transfected MDA-MB-231 cells.
Migration assays were employed to study the closure of scratches over a period of 48 hours. A major drawback of the monolayer scratch assays used in the studies described in section 5.2.5 is the difficulty of getting scratches of consistent width. The Cell Comb™ Scratch Assay kits (Millipore) (2.2.8.2) were therefore used for the experiments assessing the role of auranofin on the migration of MDA-MB-231 cells. Cells were seeded out in rectangular cell culture plates, with reference marks etched on the underside and grown until ~80% confluent. The cell combs were then used to create multiple scratches across the plate (Figure 5.7). Cells were washed once with 1 X PBS to remove any loosely attached and dead cells. The media was then replaced by fresh 10% (v/v) SCM containing thymidine and the appropriate concentration of auranofin. Cells were then monitored for 48 hours with images taken of multiple scratches (at least 10 scratches per auranofin concentration) across the plate at 0, 24, and 48 hours to monitor the scratch closing (Figure 5.8).

Figure 5.7: Schematic representation of the scratch pattern obtained by using the Cell Comb™ Scratch Assay kits (Millipore). Before use, reference marks were etched on the bottom of the plates horizontally so that the same scratches could be observed at all the time points. This image was obtained with permission from Millipore Cell Comb™ Sctatch Assay (Cat. No. 17-10191) user guide.
Figure 5.8: Representative images of auranofin treated MDA-MB-231 cells used for the monolayer scratch assays. The images are a representative from one experiment performed three times in which MDA-MB-231 cell migration was assessed in response to treatment with 0.31µM and 2µM auranofin. The images were taken at 4X magnification using a Tucsen TCA 3MP camera and the scale bar is 50µm.

The images were analysed as described in 5.2.5 with the distance migrated by cells at the start of the experiment (t=0) designated as 0. The distance migrated by cells at 24 and 48 hours was calculated based on the decrease in the width of the scratch with respect to the 0 hour time point. As shown in Figure 5.9, at the lower concentration of 0.31µM, auranofin had no effect on the migration of MDA-MB-231 cells as compared to the untreated control cells. However, at the higher concentration of 2µM, auranofin significantly decreased the cell migration at both the 24 and 48 hour time points.
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Figure 5.9: Effect of auranofin on MDA-MB-231 cell migration. Monolayer scratch assays were used to study the migration of MDA-MB-231 cells treated with auranofin for up to 48 hours. A repeated-measures two-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM of at least three independent experiments. The statistical difference between the distance migrated by cells treated with 2µM auranofin and the untreated control cells is indicated by an *. (P<0.0001).

5.2.7 Effect of Auranofin on the Migration of Stable Transfected MDA-MB-231 Cells with Modulated Thioredoxin Levels

The effect of auranofin on the migration of stable transfected cells over-expressing thioredoxin and 1SS was also determined. Since auranofin only affected MDA-MB-231 migration at the higher concentration of 2µM (Figure 5.9), the stable transfected cells were treated with 2µM auranofin only and used for scratch assays as described previously. However, at this higher concentration, cells could not survive beyond 24 hours. Therefore, 1µM auranofin was used for subsequent analysis.

All 11 clones described in Table 5.1 were examined. Due to the unavailability of rectangular plates of the correct size, the Cell Comb™ system could not be used for these experiments. Therefore, cells were grown in 24-well plates and scratches were made using P1000 pipette tips (2.2.8.1). The media was then replaced with fresh medium containing 1µM auranofin and thymidine as an anti-proliferative agent. Photos of the wounds were taken at the 0, 24, and 48 hour time points. The images were analysed and the distance migrated was calculated as stated previously.
As shown in Figure 5.10, 1µM auranofin inhibited the migration of the pIRES-EGFP cells as compared to the untreated pIRES-EGFP cells with a statistically significant difference at the 48 hour time point. However, auranofin had no effect on the migration of either thioredoxin or 1SS over-expressing cells compared to their respective untreated counterparts.

Figure 5.10: Effect of auranofin on the migration of MDA-MB-231 cells with modulated thioredoxin levels. MDA-MB-231 cells stable transfected with pIRES-EGFP (A), pGFP-Trx (B), and pGFP-1SS (C) were treated with 1µM auranofin and their migration over a period of 48 hours was studied by monolayer scratch assays. Auranofin inhibited the migration of pIRES-EGFP cells with a significant difference observed at the 48 hour time point as indicated by an *. (P<0.0001). Data was analysed by repeated measures two-way ANOVA followed by Sidak’s post test for each transfectant separately. Results are presented as Mean±SEM of at least three independent experiments performed in quadruplicate.
To highlight that auranofin affects the cell migration in a manner similar to 1SS, the distance migrated was compared between the auranofin treated pIRES-EGFP cells and untreated 1SS over-expressing cells (Figure 5.11). The results obtained suggest that inhibition of the thioredoxin system by either 1SS or auranofin have similar implications for cancer cell migration.

![Image](image.png)

**Figure 5.11: Effect of auranofin treatment of pIRES-EGFP cells compared to untreated pGFP-1SS cells.** A repeated measures two-way ANOVA followed by Sidak’s post test was employed to compare the distance migrated by pIRES-EGFP cells treated with 1µM auranofin to untreated pGFP-1SS cells. No difference was observed in the migration at any time point.

### 5.2.8 Effect of Auranofin on the Clonogenic Capacity of MDA-MB-231 Cells

The effect of auranofin treatment on the ability of MDA-MB-231 cells to form colonies was examined next (2.2.16). Cells were treated with 0.31µM and 2µM auranofin for 24 hours, after which they were harvested and counted. 1000 cells per well were then seeded for each treatment in duplicate in a 12-well plate and incubated for 5 days. Untreated cells were also used as controls. The colonies were then stained with crystal violet and counted. The representative images of the wells taken after 5 days of colony formation are shown in Figure 5.12. The number of colonies obtained for cells treated with 0.31µM auranofin was similar to the number of colonies obtained for untreated cells. However, pre-treatment with 2µM auranofin significantly inhibited clonogenic capacity of MDA-MB-231 cells with approximately 30% survival (Figure 5.13).
Figure 5.12: Representative images of MDA-MB-231 colonies. Auranofin treated cells were allowed to form colonies for 5 days after which colonies were stained with crystal violet and counted.

Figure 5.13: Effect of auranofin pre-treatment on the clonogenic capacity of MDA-MB-231 cells. A) Number of colonies obtained after 5 days. Cells pre-treated with 2µM auranofin had significantly less number of colonies compared to the untreated cells as indicated by an *. A one-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM of two independent experiments performed in duplicate. (P<0.05). B) Survival data is presented as a percentage of the untreated MDA-MB-231 cells.
5.3 Discussion

Breast cancer is a leading cause of mortality among women despite the recent advances in the treatment of primary tumors (WHO, 2015). Metastasis is the most life threatening aspect of breast cancer and a foremost cause of relapse. Metastasis is the process by which cancer cells spread to other tissues/organs to form secondary tumors. It is predicted that 25-50% patients diagnosed with breast cancer will eventually form deadly secondary tumors leading to an average survival rate of only 25% (Lorusso and Ruegg, 2012). Although the primary tumor growth can be controlled by surgery and chemotherapy, cancer relapse is usually inevitable since in most cases the metastasis has already occurred even before the detection of the primary tumor (Fidler and Balch, 1987). These metastases are often difficult to diagnose and remain undetected even decades after the treatment of the primary tumor.

Metastasis is not a single step event and follows a multi-step cascade involving angiogenesis, invasion of cells from the primary tumor into the blood vessels, migration of these cells towards distant tissues/organs, extravasation from the blood vessels at the secondary site, and the subsequent colonisation and growth of the secondary tumor (as reviewed by Lorusso and Ruegg, 2012). Therefore, invasion, migration and colonisation are import aspects of metastasis and this multi-step process requires better understanding in order to target metastasis.

The histological subtypes and molecular markers of breast cancer have strong predictive and prognostic values. For example, triple negative breast cancers (ER−, PR−, HER2−) are associated with an increased risk of metastasis (Acevedo et al., 2015, Bahnassy et al., 2015). MDA-MB-231 is a triple negative breast cancer cell line that is used as a model to study breast cancer metastasis and was therefore, used in this project. This cell line has been previously used in our laboratory to study the effects of thioredoxin on cancer cell invasion (Bloomfield, 2003). Thioredoxin was shown to influence cancer cell invasion through its redox active site.
As stated previously, metastasis is a multi-step process. After invading the blood stream, cancer cells migrate to distant locations to establish secondary tumors. The effect of exogenous addition of recombinant human thioredoxin on the migration of MDA-MB-231 cells was also previously studied in our laboratory (King, 2010; Shah, 2011). However, the effect of over-expression of thioredoxin and 1SS in the intracellular environment on the migration of MDA-MB-231 cells had not been studied previously and was examined in this project.

Stable transfected cell lines were generated using a clone selection technique obtained from Corning Life Sciences. The advantage of using the clone selection technique over batch selection is that it eliminates the chances of having an undefined mixed population of cells. The clone selection technique used in this project involved diluting the cell cultures in a way to achieve single isolated cells that gave rise to homogenous colonies. Although it is an easy to perform technique, its major disadvantage is that it is time consuming and it can take several weeks before the cells are ready to be used for any experiments.

Once the clones were selected, they were subjected to extensive characterisation in order to verify that they express the required transgenes. The first step was to visually inspect the cells for expression of the GFP protein. Since the plasmids used to generate these clones contained an IRES sequence that allows the expression of two genes on the same transcript from the same promoter, the expression of GFP protein implied the transgene was also being expressed. All clones were shown to express GFP (Figure 5.2). Sequencing of the genomic DNA extracted from these clones was also performed that confirmed the presence of the correct gene sequences in these clones.

Western blotting was employed to examine the thioredoxin protein levels in the stable transfected clones and the results showed that in comparison to the GFP-only controls, both thioredoxin and 1SS over-expressing clones expressed similar levels of thioredoxin. Gallegos and co-workers in their work with thioredoxin and 1SS over-expressing MCF-7 cells did not find more than two-fold increase in the intracellular thioredoxin levels and therefore proposed that the over-expressed protein is secreted out of the cells (Gallegos et al., 1996). Thioredoxin has been shown to be released out of
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cells in response to oxidative stress (Rubartelli et al., 1992, Kondo et al., 2004). Extracellular functions of thioredoxin include acting as an autocrine growth factor and a chemoattractant (Schenk et al., 1994, Bertini et al., 1999). The secreted thioredoxin can potentiate cytokines such as interleukins-1 and 2 and also stimulate cell growth and survival on its own (Gasdaska et al., 1995). Tanudji and colleagues showed that the secretion of thioredoxin is independent of its redox active site and no change was observed in the protein secretion when Cys35 was mutated to Ser (Tanudji et al., 2003). A preliminary experiment performed to quantitate the levels of thioredoxin and 1SS proteins secreted from the stable transfected cells showed higher levels of thioredoxin protein in the culture media for the thioredoxin and 1SS over-expressing cells in comparison to the GFP-only control cells (data not presented). However, due to lack of reagents, this experiment could not be repeated. Therefore, it is reasonable to expect that the over-expressed thioredoxin and 1SS proteins are secreted out of the cells and may potentially play a role in the migration of MDA-MB-231 cells.

ROS assays were also performed with the stable transfected clones and it was found that the cells over-expressing thioredoxin had ROS levels comparable to the pIRES-EGFP control cells while over-expression of 1SS resulted in significantly higher levels of ROS (Figure 5.4). As stated before, 1SS acts as a dominant negative inhibitor of thioredoxin by competitively binding to thioredoxin reductase, which then becomes unavailable to reduce thioredoxin to its active form (Oblong et al., 1994). It has been speculated that 1SS may also form heterodimers with the endogenous thioredoxin and thereby lower the thioredoxin monomer concentration (Gallegos et al., 1996). This may result in the inhibition of the reduction of other redox substrates of thioredoxin. It was also suggested that 1SS may additionally bind to these redox substrates of thioredoxin by acting as a competitive inhibitor (Gallegos et al., 1996). Overall, a shift in the cellular oxygenation state leading to an accumulation of ROS may occur. The higher ROS levels observed with the 1SS over-expressing cells in this project correlate with these findings.
To study if modulation of intracellular thioredoxin affected the migration of cancer cells, monolayer scratch assays were employed. It is a very straightforward assay that uses common inexpensive reagents. Its compatibility with microscopy makes it easy to analyse. This method has been validated by many researchers (Dhanesuan et al., 2002, Adams et al., 2010, Zajac et al., 2011) and is therefore a well-developed technique that to some extent mimics the *in vivo* wound healing process. However, being a simple technique, it cannot be used to test the complex cell-cell interactions and the associations between cells and the extracellular matrix. Another disadvantage of this assay is the difficulty in getting consistent and defined scratch gaps, which can result in higher inter-sample variation. To address this problem, care was taken to obtain similar sized scratches every time. Furthermore, any scratches that were too narrow or too wide were discarded from the analysis. In addition, a commercially available Cell Comb™ Scratch assay kit was also used to achieve consistent scratches. However, due to unavailability of appropriate sized plates compatible with the Cell Comb™, this kit could be used only for a part of this project.

In the monolayer scratch assays, thioredoxin over-expression did not enhance the migration of cells, which was comparable to the pIRES-EGFP control cells. It is possible that in the presence of excess thioredoxin, the cells switch off the endogenous thioredoxin expression since the cellular demands are being fulfilled by the expression of the transgene. It may also be that since the endogenous thioredoxin levels are sufficient to stimulate the migration of cells, the protein over-expressed from the transfected constructs does not exhibit any additional effect on the migration of these cells. This could be the reason why the thioredoxin over-expressing cells showed results similar to the pIRES-EGFP control cells with respect to both ROS levels and cell migration. In contrast, the over-expression of 1SS decreased the migration of cells, which was statistically significant at 48 hours (Figure 5.6). Therefore, the results obtained in this project indicate that the active site of thioredoxin is important for the migration of cancer cells.
In a previous study done in our laboratory (King, 2010, Shah, 2011) extracellular addition of thioredoxin into the media enhanced the migration of cells while addition of 1SS did not have any effect compared to the controls. Kondo and co-workers showed that Cys32 of thioredoxin is important for its interactions with its substrates. In that study, 1SS with the Cys32 mutated to Ser was unable to be internalised by the cells since it lacks the capacity to bind thioredoxin’s targets on the cell surface (Kondo et al., 2007). This could be the reason why in the previous study (King, 2010, Shah, 2011), extracellular addition of 1SS did not show any inhibitory effects on the migration of MDA-MB-231 cells and the cells’ endogenous thioredoxin was sufficient to stimulate the migration to levels comparable to the untreated MDA-MB-231 cells. In this project, however, over-expression of intracellular 1SS diminished the cell migration compared to the vector-only pIRES-EGFP control cells. The reason for this downregulation may be that 1SS interferes with reduction of thioredoxin in the intracellular environment, thereby inhibiting the effects of the endogenous thioredoxin.

Auranofin was recently shown to exert anti-angiogenic effects on HUVEC cells in vitro and in zebra fish in vivo. It inhibited the proliferation and migration of HUVECs (He et al., 2014). Migration of endothelial cells is an important aspect of angiogenesis, which in turn is crucial for metastasis. However, the migration of endothelial cells during angiogenesis (Figure 1.13) is different from the migration of tumor cells during metastasis (Martin, 2000). Furthermore, the effect of auranofin has not been previously studied on the migration of tumor cells with respect to metastasis. Therefore, auranofin was used as a tool to chemically inhibit the thioredoxin system and assess its effect on the migration of MDA-MB-231 cells.

Inhibition of thioredoxin reductase by auranofin is also expected to cause an accumulation of ROS in the cells (Rigobello et al., 2009). The two concentrations of auranofin (0.31µM and 2µM) used in the previous chapters were utilised for the monolayer scratch assays. To ensure the wound/scratch sizes were constant, Cell Comb™ Scratch assay kits were used. The results indicated that while at the lower concentration of 0.31µM auranofin had no effect on the migration of MDA-MB-231 cells, at the higher concentration of 2µM, it significantly inhibited the cell migration at both 24 and 48 hour time points (Figure 5.9). When ROS assays were employed for
auranofin treated MDA-MB-231 cells, similar results were obtained where 0.31µM auranofin did not have a statistically significant impact on the ROS levels compared to the untreated cells while at 2µM, auranofin had significantly increased the ROS levels (Figure 3.15). These results correlate with the findings of other researchers that auranofin causes an accumulation of cellular ROS (Kim et al., 2004, Park and Kim, 2005, Marzano et al., 2007, Gandin et al., 2010, Raninga et al., 2015, You et al., 2015), and thereby further strengthens the role of the redox capabilities of the thioredoxin system in cancer cell migration.

The stable transfected cells were also treated with auranofin and its effect studied on the migration of MDA-MB-231 cells with modulated intracellular thioredoxin levels. It was found that at 1µM, auranofin only inhibited the migration of the control pIRES-EGFP cells and not the cells with modulated thioredoxin levels. The reason for this could be that the over-expression of thioredoxin provides a protection to these cells against auranofin and the oxidative stress caused by auranofin induced inhibition of the thioredoxin system is compensated by the thioredoxin over-expression. The results obtained with the 1SS over-expressing cells were unexpected. Mutants lacking functional thioredoxin were previously shown to be hypersensitive to oxidative stress in yeast (Garrido and Grant, 2002, Trotter and Grant, 2002). It was hence expected that when these cells with a compromised thioredoxin system are treated with a drug that further inhibits the system and increases oxidative stress, cells would not be able to survive the accumulated stress. However, the 1SS over-expressing cells did not show any visible signs of being stressed and could survive up to 48 hours. Moreover, their migration was not affected compared to their untreated counterparts. Since 1SS acts as a dominant negative inhibitor of thioredoxin by competing for thioredoxin reductase, it may make thioredoxin reductase unavailable for auranofin to act on, and thereby no further inhibition is observed.

Another reason may be that to seek protection from auranofin, the 1SS transfected cells upregulate their endogenous thioredoxin expression, which provides protection to these cells and their migration, although lower than the pIRES-EGFP controls, is not impacted further. This hypothesis is supported by previous work done in our laboratory, which showed that in 1SS over-expressing cells, the promoter activities of both
thioredoxin and thioredoxin reductase were significantly induced in response to tBHQ treatment (Osborne et al., 2006, Bhatia, 2011). Auranofin treatment decreased the migration of pIRES-EGFP control cells to the levels similar to those of untreated 1SS over-expressing cells (Figure 5.11). These results suggest that both 1SS and auranofin may act on the same pathways through inhibition of thioredoxin reductase to affect the migration of cells. Thus targeting the redox activity of the thioredoxin system may be vital for cancer therapy.

Clonogenic assays are cell survival assays commonly used to determine the long term effects on the growth of culture cells (Qu et al., 2011, Raninga et al., 2015, Wang et al., 2015). With respect to cancer metastasis, colonisation is an important process. Once the tumor cells have metastasised to a distant secondary location, they undergo clonal expansion. If the tumor cells are unable to form these secondary colonies, their fate is death or dormancy. Metastatic dormancy is the process by which these disseminated tumor cells are able to remain viable and silent for long durations of time, thereby escaping detection during the diagnosis and treatment of the primary tumors (Zimmer and Steeg, 2015). Clonogenicity has also been linked to the recurrence of malignancy.

In 1989, Nomura and co-workers established a correlation between the clonogenic capacity of primary breast tumors in vitro and metastasis. They found that cells obtained from biopsied breast tumors that were able to form colonies on soft agar in vitro showed a higher rate of recurrence of malignancy after mastectomy (Nomura et al., 1989). Therefore, the effect of auranofin on the clonogenic abilities of MDA-MB-231 cells was assessed in this project. The traditional clonogenic assays, where the cells are seeded at low densities but are treated for the entire duration of the assay, were modified a little. The cells were treated prior to setting up of the clonogenic assays to study if the treated cells were able to form colonies in a drug-free setting. The results obtained correlated with the migration assay results where the clonogenic capacity was affected by only the higher concentration of 2µM auranofin compared to the untreated control (Figure 5.13). Therefore, auranofin not only affects the migration of MDA-MB-231 cells but also inhibits their ability to form secondary colonies.
The results presented in this chapter highlight the importance of the redox capabilities of the thioredoxin system with respect to cancer cell migration and colonisation, which are crucial steps in the metastatic cascade. Therefore, targeting the function of the thioredoxin system by drugs such as auranofin, or using other thioredoxin system inhibitors (such as 1SS), in combination with other chemotherapeutic agents may offer an opportunity to reduce metastases and enhance survival for breast cancer patients.
CHAPTER 6

EVALUATION OF AURANOFIN IN A 3D CULTURE MODEL
6.1 Introduction

As evident from the work described in the previous chapters, the oxygen environment is an important determinant of cellular responses against auranofin. Apart from the oxygen milieu, tissue architecture and cell-cell as well as cell-matrix interactions also profoundly influence tumor progression and responses to therapeutics \textit{in vivo}. Although monolayer cell-based assays are most widely used for preclinical validation of drugs, their lack of \textit{in vivo} complexity limits their predictive value for clinical efficacy of drugs. As a result of the intricate network of cell-cell and cell-ECM contacts \textit{in vivo}, an increased resistance towards the drug is observed in the clinic. To overcome the clinical drug resistance arising due to the \textit{in vivo} ‘contact effect’, a lot of effort is being directed towards exploring the third dimension of tissue culturing.

Several studies, as reviewed by Lovitt and co-workers, have shown that when cells are grown in three-dimensional (3D) culture systems, they exhibit reduced sensitivity towards drugs as compared to when grown in two-dimensional (2D) cell models (Lovitt \textit{et al.}, 2014). Cell-matrix communication \textit{in vivo} also plays an important role in determining drug sensitivity (Ivascu and Kubbies, 2007). Since 3D culture systems take into account not only the oxygen conditions found \textit{in vivo} but also the tissue architecture, the cytotoxicity of auranofin was evaluated against MDA-MB-231 cells grown as 3D spheroids and compared to 2D monolayer cells.

The 3D cell culture involves growing cells as spheroids that better mimic the avascular microenvironment of solid tumors. The matrix-embedded culture systems are most frequently employed where spheroids are grown on a reconstituted basement membrane. The thin ECMs that separate endothelial or epithelial cells from stromal cells \textit{in vivo} are known as basement membranes (Benton \textit{et al.}, 2011). Matrigel is the most commonly used commercial form of reconstituted basement membrane that is isolated from murine tumors (Benton \textit{et al.}, 2011). It is comprised of several ECM proteins such as laminin, collagen, growth factors and proteases (Kleinman and Martin, 2005, Benton \textit{et al.}, 2011). Normal cells exhibit slower proliferation rates when cultured on Matrigel whereas tumor cells proliferate rapidly with metastatic cells showing invasive features (Kleinman and Martin, 2005).
Due to its animal-origin, Matrigel is expensive and also has inherent production variability. Maintaining well-to-well consistency is another challenge (Justice et al., 2009). Progress is being made towards miniaturising the matrix-based 3D culture systems not only to reduce the amount of Matrigel used and thereby the overall cost but also to increase the extent of screening (Justice et al., 2009). Lovitt and colleagues recently developed a Matrigel-based 384-well 3D cancer model for drug discovery (Figure 6.1) (Lovitt et al., 2013). The 3D model used in this chapter is based on the assay developed by these researchers. In this project, a spheroid was considered as a compact cluster of cells forming a 3D structure, more than 50µm in diameter.

High content imaging and analyses allow for the extraction of multiparametric information of spatio-temporal biological events and employs the use of mathematical algorithms to produce meaningful data from such images (Shariff et al., 2010, Sirenko et al., 2015). Fluorescence microscopy is one of the most powerful tools available to study these spatio-temporal processes (Giuliano, 1997). Functional and morphological parameters can be analysed through high content imaging and analysis in individual
cells in contrast to the traditional cell-based assays where the cellular responses are generally acquired as an average of thousands of cells (Zanella et al., 2010). Another advantage of high throughput screening is the examination of multiple parameters, such as morphological changes, fluorescent intensity modulations, fluorescence distribution and cell movement, on a single cell of interest (Zanella et al., 2010). Moreover, by using highly automated microscopes for imaging and sophisticated computational algorithms for analysis, the risk of human bias is eliminated. The data acquired through automated processes are more robust and the whole process is more time efficient (Bickle, 2010). High content imaging and analysis was also employed to study the effects of auranofin on the morphological features of cells in both 2D and 3D models used in this project.

Doxorubicin is an anthracycline antibiotic with anti-neoplastic activity and is used in the clinic for a broad spectrum of cancers (as reviewed by Tacar et al., 2013). It has been proposed to exert cytotoxicity by two different modes of action. It is oxidised to an unstable metabolite, semiquinone, which gets converted back to doxorubicin while generating free radicals, thereby causing cellular damage (Thorn et al., 2011). Doxorubicin can also enter the nucleus and intercalate into DNA causing disruption to the DNA repair enzyme, topoisomerase II resulting in cellular death (Tewey et al., 1984). In this chapter, doxorubicin was used as a reference standard of care anti-cancer drug along with auranofin, which is still in phase I/II clinical trials.

Upon sensing an accumulation of cellular ROS, thioredoxin1, which is mainly a cytosolic protein, gets translocated into the nucleus (as reviewed by Holmgren, 1985). Auranofin has been shown to cause oxidative stress by increasing an accumulation of ROS, leading to alterations in the mitochondrial functions (Rigobello et al., 2005, Rigobello et al., 2009). Therefore, a translocation of thioredoxin into the nucleus may be expected upon auranofin treatment. Combining immunofluorescence with high throughput imaging and analysis, qualitative as well as quantitative results can be obtained about the localisation of thioredoxin in response to auranofin treatment.
The specific aims undertaken in this chapter were as follows:

- To determine the short (24h) and long (144h) term dose-response profiles for auranofin against MDA-MB-231 cells grown as 2D monolayers and 3D spheroids

- To assess the cellular localisation of thioredoxin following auranofin exposure through high content imaging
6.2 Methodology

In this chapter, MDA-MB-231 breast cancer cells were cultured in 2D and 3D formats to evaluate and compare the drug activity of auranofin and doxorubicin between the two assay systems. In the 2D culture model, cells were grown as monolayers for ~24 hours, after which drugs were added for the times indicated in the appropriate sections. The 3D spheroid model was set up as described previously by Lovitt and colleagues (Lovitt et al., 2013). For this project, Growth Factor-Reduced (GFR) Matrigel (BD Biosciences) was used. Matrigel, at 4°C exists in a liquid form and solidifies under physiological conditions of 25-37°C (Benton et al., 2011). The spheroids were grown (2.2.2.4) for 6 days, with a media change every third day. The pre-formed spheroids were then treated with auranofin and doxorubicin for the times specified in the appropriate sections.

The effect of drug treatment on the size of the cell and the nucleus (in 2D) were analysed using the data analysis package Acapella 4.0 (Figure 6.2). For the size analysis, cells were stained with Cell Mask Deep Red, which stains the entire cell and Hoechst was used for staining the nucleus. A script was prepared using a high content imaging and analysis database, Columbus (PerkinElmer) that employs the Acapella 4.0 analysis package. Figure 6.3 gives an overview of how the script was designed. Briefly, the nuclei, excluding the ones on the edge of the well, were selected for the control well using the appropriate laser channel. The cytoplasm was selected next and the results were defined for the desired parameters. A batch analysis was then run using the prepared script that analysed the whole plate.
Figure 6.2: Screenshot of Columbus high content imaging and analysis tool.
Figure 6.3: Overview of the steps involved in preparing the script for analysis of cell and nucleus size using Columbus/Acapella 4.0. The ‘image analysis’ option was used for designing the script. A) The nuclei were found first using the appropriate laser channel and one of the pre-set methods. B) A population to be analysed was selected (shown in green) avoiding the nuclei on the edges of the field (shown in red). C) The cytoplasm was found next using the appropriate laser channel and selecting one of the pre-set methods. D) Finally the results were defined by selecting the parameters of interest. This script was then saved into the database and a ‘batch analysis’ was performed to analyse all the wells as per the script prepared.
The 3D spheroid size (diameter and area) analyses were performed using the AxioVision LE64 software (Zeiss) and an overview of the protocol followed is depicted in Figure 6.4. Any spheroids that appeared to have merged together (indicated by a white arrow in Figures 6.4A and B) were excluded from the analyses. Moreover, the spheroids that were less than 50µm in diameter (shown by the blue arrows in Figures 6.4A and B) were considered to be a cluster of 2-3 cells rather than actual spheroids and were also excluded from the analyses. At least 30 spheroids were analysed per drug concentration. While measuring the spheroid area, any obvious protrusions (indicated by a white arrow in Figure 6.4C) were considered a part of the spheroid unless they appeared to be single cells disintegrating from the spheroid body (indicated by a blue arrow in Figure 6.4C). The ‘create data table’ tool was then used to generate the size report, which was then imported into Microsoft Excel for further analysis.
Figure 6.4: Overview of spheroid size analysis performed using AxioVision. The images taken at 4X magnification were used and at least 30 spheroids were analysed per drug concentration. The ‘measure length’ tool was used to measure the diameter of the spheroids (A) and the ‘measure outline’ tool was used to draw around the spheroids for the measurement of the spheroid area (B). C) Zoomed in image showing the analysis of spheroid diameter and area.
6.3 Results

MDA-MB-231 breast cancer cell line is one of the most frequently used cell lines in 3D culturing. This cell line has been used in the development of 3D spheroid-based assays (Huyck et al., 2012, Lovitt et al., 2013) as well as a model breast cancer cell line for drug evaluation (Vinci et al., 2012, Ampuja et al., 2013, Lovitt et al., 2015). As stated in the previous section, the 3D spheroid assay used in this project was based on the technique developed by Lovitt and co-workers (Lovitt et al., 2013). This assay has also been optimised for MDA-MB-231 breast cancer cells by this group. Results presented previously in this thesis suggest that the activity of auranofin is influenced by the oxygen environment in which MDA-MB-231 cells were grown. Therefore, in this chapter, MDA-MB-231 cells were used to study the cytotoxic effects of auranofin in a 3D spheroid model compared to a 2D monolayer model.

The metabolic activity of cells following drug treatments in 2D and 3D were analysed using three parameters (Figure 6.5): potency (IC$_{50}$), efficacy (E$_{\text{max}}$), and the area under the curve (AUC), which combines the potency and efficacy of the drug signifying the proportion of cells inhibited by the drug.

![Figure 6.5: Example of drug activity parameters analysed in 2D and 3D cell culture assays. The analyses were performed using GraphPad Prism Version 6.0.](image)
6.3.1 Effect of Auranofin and Doxorubicin on MDA-MB-231 Cells in a 2D Cell Culture Model

The drug activities of auranofin and doxorubicin and their effects on the morphology of MDA-MB-231 cells were examined first in a 2D culture model.

6.3.1.1 Evaluation of Dose-Response for Auranofin and Doxorubicin against MDA-MB-231 Cells Grown as 2D Monolayers

Initially, a 2D monolayer model was set up to evaluate the cytotoxicity of auranofin and doxorubicin against MDA-MB-231 breast cancer cells upon a short term exposure of 24 hours compared to a long term exposure of 144 hours. Since auranofin binds thioredoxin reductase irreversibly (Gromer et al., 1998), it was not re-added during the media change performed for the 144 hour assay. To keep the conditions consistent, doxorubicin was also not re-added. At the conclusion of the assays, resazurin, a metabolic indicator dye, was added to the cells, which were then incubated for 4 hours at 37ºC. The total well fluorescence was measured using an EnVision™ multilabel plate reader (PerkinElmer) (2.2.14).

The auranofin dose-response curves are shown in Figure 6.6. The metabolic activity of cells following auranofin treatment was analysed using the three parameters listed in Table 6.1. A significant difference in the potency of auranofin was observed between the 24 and 144 hour assay time points when analysed by a paired t-test, with auranofin being 17 fold more potent (lower IC₅₀) at the longer time point of 144 hours (P<0.05). No significant difference was observed in the efficacy between the two time points when analysed by a paired t-test. The AUC of auranofin, examined using a paired t-test, was significantly higher when cells were treated for 144 hours compared to 24 hours (P<0.05). Therefore, auranofin exerts its cytotoxic effects towards MDA-MB-231 cells within 24 hours but is more potent after 144 hours.
Figure 6.6: Dose-responses showing the metabolic activity of MDA-MB-231 cells upon auranofin treatment for 24 and 144 hours. The curves are representative of one experiment performed in triplicate.

Table 6.1: Response of MDA-MB-231 cells against auranofin at the 24 and 144 hour time points. Significant difference was observed in the IC_{50} and AUC between the two time points (indicated by an *) when analysed by a paired t-test. Data is presented as Mean±SEM of three independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>IC_{50} (µM)</th>
<th>E_{max} (%)</th>
<th>AUC (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.54±0.3</td>
<td>104.3±0.52</td>
<td>155.1±5.86</td>
</tr>
<tr>
<td>144</td>
<td>0.09±0.02</td>
<td>102.3±0.12</td>
<td>275.8±9.9</td>
</tr>
</tbody>
</table>

The doxorubicin dose-response curves are shown in Figure 6.7. The drug activity parameters evaluated in response to doxorubicin treatment are listed in Table 6.2. When analysed by a paired t-test (performed separately for each parameter), a significant difference was observed for each parameter between the 24 and 144 hour time points. Doxorubicin was approximately 45 fold more potent at the 144 hour time point compared to the 24 hour treatment. Moreover, the efficacy of doxorubicin at the 144 hour time point was two fold higher than that observed after 24 hours of treatment. Therefore, doxorubicin is more potent and more effective against MDA-MB-231 cells after a longer exposure of 144 hours.
Figure 6.7: Dose-responses showing the metabolic activity of MDA-MB-231 cells upon doxorubicin treatment for 24 and 144 hours. The curves are representative of one experiment performed in triplicate.

Table 6.2: Response of MDA-MB-231 cells against doxorubicin at the 24 and 144 hour time points. Significant difference was observed in the IC$_{50}$, $E_{\text{max}}$ and AUC between the two time points (indicated by an *) when analysed by a paired t-test. Data is presented as Mean±SEM of three independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>IC$_{50}$ (µM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>AUC (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>2.35±0.44</td>
<td>50.67±1.25</td>
<td>68.34±3.64</td>
</tr>
<tr>
<td>144</td>
<td>0.05±0.01</td>
<td>100.1±0.03</td>
<td>296.4±7.86</td>
</tr>
</tbody>
</table>

6.3.1.2 Effect of Auranofin and Doxorubicin Treatment on MDA-MB-231 Cell Morphology

After measuring the metabolic activity, cells were fixed with 4% (v/v) paraformaldehyde and stained with Phalloidin-FITC (488) and Hoechst that was used as a nuclear stain (2.2.17). The cells were then imaged using the PerkinElmer Opera™ Confocal High Content Screening System with the aim to examine if auranofin and doxorubicin cause any morphological changes in MDA-MB-231 cells. The representative images for selected auranofin concentrations are shown in Figure 6.8. These results correlate with the dose-response curves for auranofin at 24 and 144 hour time points, for example the immunostained images showed that at 24 hours, MDA-MB-231 cells treated with 1µM auranofin appeared healthy but at 144 hours, only cell
debris was visible. The representative immunostained images for selected doxorubicin concentrations are shown in Figure 6.9. These results also correlate with the dose-response curves obtained for doxorubicin and show that after 24 hours, cells treated with all concentrations of doxorubicin were healthy but after 144 hours, 1µM and higher concentrations of doxorubicin caused cell death. Moreover, auranofin treatment did not cause any visible changes in the cell morphology at either time point however; doxorubicin treatment enhanced the size of the cells in a dose-dependent manner at both time points with a more pronounced effect observed after 144 hours.
Figure 6.8: Morphology of MDA-MB-231 cells exposed to auranofin for 24 and 144 hours. Cells were fixed and stained with Phalloidin-FITC (green) and Hoechst (blue). Cells were imaged using a 20X water objective on the Opera™. The scale bar is 50µm.
Figure 6.9: Morphology of MDA-MB-231 cells in response to doxorubicin treatment for 24 and 144 hours. Fixed cells were stained with Phalloidin-FITC (green) and Hoechst (blue). Imaging was performed using a 20X water objective on the Opera™. The scale bar is 50µm.
The effect of drug treatment on the overall size of MDA-MB-231 cells was examined next using the Acapella 4.0 high content image analysis software as described previously in section 6.2. As shown in Figure 6.10A, the longer auranofin treatment for 144 hours significantly increased the nucleus size in MDA-MB-231 cells compared to the shorter 24 hour treatment when 0.02µM, 0.04µM and 0.1µM auranofin was used. The 144 hour treatment also increased the cell size at 0.04µM and 0.1µM concentrations compared to the 24 hour treatment (Figure 6.10B). However, when analysed by a one-way ANOVA followed by Sidak’s post test separately for each time point, auranofin treatment did not cause any overall change in either the cell or the nucleus size compared to the untreated controls at the 24 hour time point. At the 144 hour time point, the size of the cells treated with 0.04µM and 0.1µM auranofin was significantly increased in comparison with the untreated control (P<0.05). These results indicate that the longer auranofin treatment enhances the size of MDA-MB-231 cells while the shorter exposure does not affect the overall cellular size.

**Figure 6.10: Effect of auranofin treatment for 24 and 144 hours on the MDA-MB-231 nucleus and cell size.** Areas of the nucleus (A) and of the cell (B) were measured using Acapella 4.0. A two-way ANOVA followed by Sidak’s post test was performed and the significant difference in the size between the two time points is indicated by an *. (P<0.0001). Data is presented as Mean±SEM of three independent experiments performed in triplicate.
An increase in the size of both nucleus and cell was observed upon doxorubicin treatment for 24 hours. The longer 144 hour treatment significantly enhanced the nucleus size at 0.02µM and 0.04µM concentrations and cell size at 0.04µM and 0.1µM concentration compared to the shorter 24 hour treatment (Figure 6.11). A one-way ANOVA followed by Sidak’s post test was employed separately to analyse the nucleus and cell size (Appendix II), which was found to be statistically significantly increased at 0.2µM, 0.4µM, 1µM and 2µM doxorubicin concentrations compared to the untreated control (P<0.0001). As observed with metabolic activity (Table 6.2), doxorubicin was more effective at the 144 hour time point; therefore, cells treated with more than 0.1µM doxorubicin could not be used for the size analysis. When analysed by a one-way ANOVA followed by Sidak’s post test, the size of the cells, but not nuclei, was significantly enhanced for the cells treated with 0.04µM and 0.1µM doxorubicin compared to the untreated control cells (P<0.0001). Thus doxorubicin increases the overall size of MDA-MB-231 cells and this increase is greater after 144 hours of drug exposure.

Figure 6.11: Effect of doxorubicin treatment for 24 and 144 hours on the MDA-MB-231 nucleus and cell size. Areas of the nucleus (A) and of the cell (B) were measured using Acapella 4.0. A two-way ANOVA followed by Sidak’s post test was performed and the significant difference in the size between the two time points is indicated by an *. (P<0.0001). Data is presented as Mean±SEM of three independent experiments performed in triplicate.
6.3.2 Effect of Auranofin and Doxorubicin on MDA-MB-231 Cells in a 3D Spheroid Model

The drug activities of auranofin and doxorubicin against MDA-MB-231 cells were examined next in a 3D culture model. The effect of the two drugs on the morphology of the 3D spheroids was also analysed using immunostaining and high content imaging.

6.3.2.1 Evaluation of Dose-Response for Auranofin and Doxorubicin against MDA-MB-231 Cells Grown as 3D Spheroids

The cytotoxicity of auranofin and doxorubicin against the MDA-MB-231 breast cancer cells grown as 3D spheroids upon a short term exposure of 24 hours and a long term exposure of 144 hours was evaluated. As for the 2D assays, drugs were not re-added during the media change performed for the 144 hour treatment. At the end of the assays, resazurin was added to the cells, which were incubated for 6 hours at 37°C. The total well fluorescence was measured using EnVision™ (2.2.14).

The auranofin dose-response curves are shown in Figure 6.12 and the metabolic activity results are listed in Table 6.3. A statistical comparison was not performed between the 24 and 144 hour time points since the 144 hour assay was performed only once in triplicate due to time constraints. The preliminary results suggest that auranofin is more potent at the longer time point of 144 hours compared to the shorter time point of 24 hours.
Figure 6.12: Dose-responses showing the metabolic activity of MDA-MB-231 cells upon auranofin treatment for 24 and 144 hours in a 3D model. The curves are representative of one experiment performed in triplicate.

Table 6.3: Response of MDA-MB-231 cells cultured as 3D spheroids against auranofin at the 24 and 144 hour time points. Data is presented as Mean±SEM of three independent experiments performed in triplicate for the 24 hour assay and as Mean±SD of one experiment performed in triplicate for the 144 hour assay.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>IC$_{50}$ (µM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>AUC (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>19.6±3.4</td>
<td>119.3±1.3$^#$</td>
<td>80.27±10.9</td>
</tr>
<tr>
<td>144</td>
<td>6.81±0.25</td>
<td>100.4±0</td>
<td>80.21±0.65</td>
</tr>
</tbody>
</table>

$^\#$ $E_{\text{max}}$ could not be accurately calculated with GraphPad since at the 24 hour time point, only the highest concentration of auranofin (40µM) resulted in complete cell death. Therefore this $E_{\text{max}}$ value was estimated from the raw data.

The cytotoxicity of doxorubicin against MDA-MB-231 cells cultured as spheroids was examined next upon drug exposure for 24 and 144 hours. For the 24 hour assay, a proper sigmoidal dose-response curve could not be obtained with the concentrations tested since doxorubicin did not cause more than 48% inhibition at the top dose. For the 144 hour assay, the lowest doxorubicin concentration also caused approximately 20% inhibition and therefore, a proper sigmoidal curve was not obtained. Hence, the metabolic activity parameters (IC$_{50}$, $E_{\text{max}}$, and AUC) could not be established for doxorubicin at either time point. The doxorubicin dose-response curves are shown in Figure 6.13.
Figure 6.13: Dose-responsive showing the metabolic activity of MDA-MB-231 spheroids upon doxorubicin treatment for 24 and 144 hours. The curves are representative of one experiment performed in triplicate.

6.3.2.2 Effect of Auranofin and Doxorubicin Treatment on MDA-MB-231 Spheroid Morphology

After measuring the metabolic activity, spheroids were imaged using an Olympus CellR epi-fluorescence microscope at 4X as well as 10X magnification using the bright field setting. Heterogeneity in the size and morphology of the spheroids was observed within the same wells. For example, protrusive structures were observed in some spheroids while others in the same well were round and compact (Figure 6.14A). Other examples depicting the different morphologies are also shown in Figures 6.14B and C.

Figure 6.14: Examples of different morphological features exhibited by the MDA-MB-231 spheroids. A) Protrusive structures in spheroids (white arrows). B) Spheroids forming contacts with each other (blue arrows). C) Disintegration of spheroid (yellow arrows). The images were taken at 10X magnification using the CellR microscope. The scale bar is 200µm.
The images obtained at the 4X magnification using the CellR microscope, were used for determining the size of the spheroids (as described in section 6.2) to analyse if drug treatment in 3D had a similar effect as in 2D. Auranofin treatment up to 10µM concentration for 24 hours did not affect the size of the spheroids compared to the untreated control, which had an average diameter of 100µm and an average area of approximately 7,000µm$^2$. At the highest concentrations of 20µM and 40µM, the spheroids started to disintegrate and were excluded from size analysis (Figure 6.15).

The spheroids were subsequently fixed with 4% (v/v) paraformaldehyde and stained with Phalloidin (635) along with Hoechst as a nuclear stain (2.2.17). The stained spheroids were then imaged with a 10X objective using the Opera™. The representative bright field and fluorescent staining images of auranofin treated MDA-MB-231 cells grown as 3D spheroids are shown in Figure 6.15 for the 24 hour assay. The cell morphology was examined using a central Z-slice through the immunostained spheroids. Auranofin did not affect the spheroid integrity at the lower concentrations (0.04µM - 4µM). Hollow lumen was observed in spheroids treated with 10µM auranofin, although the overall integrity of these spheroids was maintained. As stated above, the highest concentrations of 20µM and 40µM auranofin resulted in disintegration of the spheroids.
Figure 6.15: Morphology of MDA-MB-231 spheroids in response to auranofin exposure for 24 hours. A) Representative bright field (BF) and staining (IF) images of MDA-MB-231 spheroids obtained at 10X magnification using the CellR microscope and the Opera™ respectively. The IF images are a central Z-slice through the spheroids. The scale bar is 100µm. B) Average diameter and C) average area of MDA-MB-231 spheroids treated with auranofin for 24 hours. Results are presented as Mean±SEM of three independent experiments performed in triplicate.
Since the 144 hour assay was carried out for an additional 3 days, the average spheroid size of the untreated control, compared to the 24 hour time point was greater. The representative images for selected auranofin concentrations and spheroid size analysis results are shown in Figure 6.16. The average diameter was observed to be 130µm and the average area was 15,000µm². At the 144 hour time point, the spheroids treated with 10µM and higher concentrations of auranofin appeared as disintegrated cell debris and were thus excluded from the size analysis. Auranofin treatment up to 4µM concentration, did not affect the size of the spheroids compared to the size of the untreated control. However, analysis of the central Z-slice through the spheroids indicated that even at the lower concentrations (0.1µM) auranofin caused the formation of gaps within the spheroid structure.

**Figure 6.16: Morphology of MDA-MB-231 spheroids in response to auranofin exposure for 144 hours.** A) Representative bright field (BF) and staining (IF) images of selected MDA-MB-231 spheroids obtained at 10X magnification using the CellR microscope and the Opera™ respectively. The IF images are a central Z-slice through the 3D spheroids. The scale bar is 100µm. B) Average diameter and C) average area of MDA-MB-231 spheroids treated with auranofin for 144 hours. Results are presented as Mean±SD of one experiment performed in triplicate.
The representative bright field and staining images for doxorubicin treated MDA-MB-231 cells grown as 3D spheroids are shown in Figure 6.17A. The size analysis was performed only for the 24 hour assay and results are shown in Figures 6.17B and C. A decreasing trend was observed for size of spheroids treated with doxorubicin compared to the untreated control, with the difference being statistically significant at 40μM drug concentration for the diameter and 10μM concentration for the spheroid area when analysed separately by a one-way ANOVA followed by Sidak’s post test (P<0.05). The Z-slice analysis through the centre of the spheroids did not show any hollow lumen, contrary to those observed with auranofin treatment. Moreover, these spheroids appeared to be more tightly packed in comparison to those treated with auranofin. Therefore, doxorubicin treatment of the MDA-MB-231 spheroids for 24 hours results in an overall decrease in the size of the spheroids.
Figure 6.17: Morphology of MDA-MB-231 spheroids in response to doxorubicin treatment for 24 hours. A) Representative bright field (BF) and staining (IF) images of MDA-MB-231 spheroids obtained at 10X magnification using the CellIR microscope and the Opera™ respectively. The IF images are a central Z-slice through the spheroid. The scale bar is 100µm. B) Average diameter and C) average area of MDA-MB-231 spheroids treated with doxorubicin for 24 hours. The significant difference between the treated and untreated spheroids is indicated by an * (P<0.05). A one-way ANOVA followed by Sidak’s post test was performed and results are presented as Mean±SEM for three independent experiments performed in triplicate.
6.3.2.3 Comparison of Auranofin Induced Cytotoxicity between the 2D and 3D Culture Models

A direct comparison of auranofin drug activity against MDA-MB-231 cells cultured in 2D and 3D culture models was performed next. The 24 hour dose-response curves are shown in Figure 6.18. Significant differences were observed in 3D culture models with respect to the IC$_{50}$, E$_{max}$ and AUC compared to the 2D model at the 24 hour time point (Table 6.4). The results indicate that auranofin is approximately 13 times less potent against MDA-MB-231 cells grown as 3D spheroids compared to those grown as 2D monolayers.

![Figure 6.18: Dose-responses showing the metabolic activity of MDA-MB-231 cells in a 2D monolayer model vs a 3D spheroid model upon auranofin treatment for 24 hours. The curves are representative of one experiment performed in triplicate.](image)

Table 6.4: Response of MDA-MB-231 cells in a 2D vs 3D model against auranofin at the 24 hour time points. Significant difference was observed in the IC$_{50}$, E$_{max}$ and AUC between the models (indicated by an *) when analysed by a paired t-test. Data is presented as Mean±SEM of three independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Model</th>
<th>IC$_{50}$ ($\mu$M)</th>
<th>E$_{max}$ (%)</th>
<th>AUC (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>* P&lt;0.05</td>
<td>* P&lt;0.05</td>
<td>* P&lt;0.05</td>
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<tr>
<td>2D</td>
<td>1.54±0.3</td>
<td>104.3±0.52</td>
<td>155.1±5.86</td>
</tr>
<tr>
<td>3D</td>
<td>19.57±3.4</td>
<td>119.3±1.3*</td>
<td>80.27±10.97</td>
</tr>
</tbody>
</table>

* This E$_{max}$ value was estimated from the raw data.
The preliminary results obtained from the comparison of auranofin’s cytotoxicity against MDA-MB-231 cells between the two models at the 144 hour time point are shown in Figure 6.19 and the metabolic activity parameters are listed in Table 6.5. These results also suggest that auranofin is more potent against cells grown in 2D compared to those grown in 3D.

![Figure 6.19: Dose-responses showing the metabolic activity of MDA-MB-231 cells in a 2D monolayer model vs a 3D spheroid model upon auranofin treatment for 144 hours. The curves are representative of one experiment performed in triplicate.](image)

<table>
<thead>
<tr>
<th>Model</th>
<th>IC$_{50}$ (µM)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>AUC (units)</th>
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<tr>
<td>2D</td>
<td>0.09±0.02</td>
<td>102.3±0.12</td>
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<td>3D</td>
<td>6.81±0.25</td>
<td>100.4±0</td>
<td>80.21±0.65</td>
</tr>
</tbody>
</table>

A direct comparison of doxorubicin activity in a 2D model compared to a 3D model was not performed since proper sigmoidal curves could not be obtained for doxorubicin in 3D at either the 24 hour or 144 hour time points.
6.3.2.4 Effect of Drug Pre-Treatment on the Ability of MDA-MB-231 Cells to Form 3D Spheroids

As shown in Chapter 5, when treated with 2µM auranofin, the subsequent clonogenic capacity of MDA-MB-231 cells was significantly diminished (5.2.8). In this chapter, the effect of auranofin treatment on the ability of MDA-MB-231 cells to form 3D spheroids was examined. Doxorubicin was also used as a reference drug in this chapter.

MDA-MB-231 cells were grown as 2D monolayers and treated with auranofin and doxorubicin for 24 hours. The pre-treated cells were then harvested and seeded on top of a Matrigel layer for spheroid formation in a drug-free media, which was replaced every three days. Cells were allowed to form spheroids for 144 hours. The metabolic activity of the pre-treated spheroids was measured using EnVision™ as described previously and was calculated based on the metabolic activity of the untreated control. As shown in Figure 6.20, auranofin pre-treatment for 24 hours did not affect the metabolic activity of MDA-MB-231 cells grown subsequently as 3D spheroids for 144 hours.

![Figure 6.20: Metabolic activity of MDA-MB-231 cells pre-treated with auranofin for 24 hours and subsequently grown as 3D spheroids. A one-way ANOVA followed by Sidak’s post test was employed and results are presented as Mean±SEM for three independent experiments performed in duplicate.](image)

200
The effect of doxorubicin pre-treatment for 24 hours on the ability of MDA-MB-231 cells to form 3D spheroids was also examined. As shown in Figure 6.21, doxorubicin pre-treatment for 24 hours significantly decreased the metabolic activity of cells grown as 3D spheroids for 144 hours.

![Figure 6.21: Metabolic activity of MDA-MB-231 grown as 3D spheroids when pre-treated with doxorubicin for 24 hours. A one-way ANOVA followed by Sidak’s post test was employed and significant difference in the spheroid metabolic activity is indicated by an *. (P<0.0001). Results are presented as Mean±SEM for two independent experiments performed in duplicate.](image)

These spheroids were also imaged with the CellR microscope and the size of the spheroids was determined using AxioVision software as described previously in section 6.2. The spheroids were also fixed and stained with Phalloidin (635) and Hoechst (2.2.16) and imaged at 10X magnification using the Opera™ for morphological analysis. The representative bright field and fluorescent staining images of spheroids pre-treated with auranofin are shown in Figure 6.22A. As with the metabolic activity, no difference was observed in the size of the auranofin pre-treated spheroids compared to the untreated control (Figures 6.22B and C). As observed previously, analysis of central Z-slices through the spheroids indicated that auranofin pre-treatment resulted in spheroids with hollow lumen.
Figure 6.22: Morphology of MDA-MB-231 spheroids pre-treated with auranofin for 24 hours. A) Representative bright field (BF) and staining (IF) images obtained at 10X magnification using CellR and the Opera™ respectively. The IF images represent a Z-slice through the centre of the spheroids. The scale bar is 100µm. B) Average diameter and C) average area of the spheroids pre-treated with auranofin for 24 hours. Results are presented as Mean±SEM of three independent experiments performed in duplicate.

The doxorubicin pre-treated spheroids were also imaged with the CellR microscope and the size analysis performed as stated before. Doxorubicin pre-treatment resulted in a significant decrease in the size of the consequent spheroids compared to the untreated controls. The pre-treated spheroids were less than 50µm in diameter and were regarded as a cluster of a few cells rather than spheroids. These spheroids were also fixed and stained with Phalloidin (635) and Hoechst (2.2.16) and imaged at 10X magnification using the Opera™. The representative bright field and fluorescent staining images for doxorubicin pre-treated spheroids are shown in Figure 6.23, which indicate that doxorubicin pre-treatment significantly affected the size of the subsequent spheroids.
CHAPTER 6

Figure 6.23: Morphology of MDA-MB-231 spheroids pre-treated with doxorubicin for 24 hours. A) Representative bright field (BF) and staining (IF) images obtained at 10X magnification using CellR and the Opera™ respectively. The IF images are a central Z-slice through the 3D spheroids. The scale bar is 100µm. B) Average diameter and C) average area of the spheroids pre-treated with doxorubicin for 24 hours. Results are presented as Mean±SEM of two independent experiments performed in duplicate. Significant decrease in spheroid size was observed compared to the untreated controls when analysed by a one-way ANOVA followed by Sidak’s post test and is denoted by an *. (P<0.0001).

6.3.3 Cellular Localisation of Thioredoxin in Response to Auranofin Treatment

Thioredoxin1 is predominantly a cytosolic protein, which under stressful conditions can translocate into the nucleus to regulate the appropriate downstream pathways to counteract the induced stresses (as reviewed by Holmgren, 1985). Treatment of MDA-MB-231 cells with auranofin increased the cellular ROS levels (Chapter 3) in this project and correlated with studies undertaken by other researchers (Rigobello et al., 2009). Therefore, the cellular localisation of thioredoxin was examined in MDA-MB-231 cells in response to auranofin treatment using immunofluorescence with the aim to examine if auranofin induces a translocation of thioredoxin into the nucleus.
6.3.3.1 Thioredoxin Localisation upon Treatment with H$_2$O$_2$- An Oxidative Stress Inducer

Although not a free radical itself, H$_2$O$_2$ forms free radicals as intermediates in a process that leads to formation of the hydroxyl radical (•OH), which is capable of causing more oxidative damage to the cells than any other type of ROS (as reviewed by Nordberg and Arner, 2001). Previous work done in our laboratory showed that 100µM H$_2$O$_2$ caused an approximate 1.8 fold increase in the thioredoxin promoter activity (Hawkes, 2011). Therefore, 100µM H$_2$O$_2$ was used in this chapter to induce oxidative stress and test its effect on the thioredoxin localisation. MDA-MB-231 cells were exposed to 100µM H$_2$O$_2$ for 3, 6 and 24 hours after which the cells were fixed and stained with 6F3 anti-thioredoxin antibody (Table 2.4) along with Cell Mask Deep Red and Hoechst as cytoplasmic and nuclear stains respectively (2.2.17).

The imaging was performed on the Opera™ using a 20X water objective. The representative images are shown in Figure 6.24. The Acapella 4.0 automated data analysis system was then used to calculate the intensity of thioredoxin staining in three cellular locations: nucleus, cytoplasm and membrane. As shown in Figure 6.25, H$_2$O$_2$ treatment of MDA-MB-231 cells caused a time-dependent increase in the intensity of thioredoxin staining in all cellular locations analysed. The overall intensity of thioredoxin staining was higher in the cytoplasm at all time points. However, no clear translocation of thioredoxin was observed at any time point.
Figure 6.24: Representative images of MDA-MB-231 cells treated with H₂O₂ for 3, 6 and 24 hours. Cells treated with H₂O₂ were fixed and stained with an anti-thioredoxin antibody along with Cell Mask Deep Red and Hoechst. Images were taken using a 20X water objective on the Opera™. The scale bar is 50µm. Trx = thioredoxin.
Figure 6.25: Intensity of thioredoxin staining in different cellular locations in response to $H_2O_2$ exposure. The thioredoxin intensity was calculated using the Acapella 4.0 automated data analysis software in the nucleus (A), cytoplasm (B) and membrane (C). A two-way ANOVA followed by Sidak’s post test was performed and the significant difference between $H_2O_2$ treated and untreated cells is indicated by an *. ($P<0.0001$). Results are presented as Mean±SD of one experiment performed in triplicate. Trx = thioredoxin.
6.3.3.2 Thioredoxin Localisation upon Auranofin Treatment

The thioredoxin localisation in response to auranofin treatment was studied at four different time points: 3, 6, 24 and 144 hours. For 3, 6 and 24 hour treatments, MDA-MB-231 cells were treated with 0.1µM to 4µM auranofin whereas for the 144 hour time point, cells were treated with 0.01µM to 0.2µM auranofin. At the conclusion of each treatment, cells were fixed and immunofluorescence (2.2.17) was performed. The cells were then imaged using the Opera™.

While analysing the thioredoxin staining images, cellular protrusions were observed in the MDA-MB-231 cells (Figure 6.26). These protrusive structures were present at all time points but were comparatively less prominent at the 144 hour time point. These structures were mostly present at the cellular edges, which were not in contact with other cells. Thioredoxin staining was co-localised with these protrusions.

![Figure 6.26: MDA-MB-231 cell morphology at different time points of growth.](image)

Figure 6.26: MDA-MB-231 cell morphology at different time points of growth. Representative images of untreated MDA-MB-231 control cells. The presence of cellular protrusions (indicated by white arrows) coincided with thioredoxin staining (green). Cell Mask Deep red was also used to stain the cytoplasm (red) along with Hoechst (blue). Images were taken using a 20X water objective on the Opera™. The scale bar is 50µm. Trx = thioredoxin.
The intensity of thioredoxin staining was calculated next using the Acapella 4.0 automated data analysis system at three cellular locations: nucleus, cytoplasm and membrane. The representative images of MDA-MB-231 cells treated with different auranofin concentrations for 3, 6 and 24 hours are shown in Figure 6.27. The intensity for thioredoxin staining was higher in the cytoplasm at all the time points compared to the nucleus and cell membrane. However, auranofin treatment did not cause any change in thioredoxin intensity in any of the cellular locations at any time point. Moreover, translocation of thioredoxin into the nucleus was not observed upon treatment at any time point. The cellular protrusions were also observed in auranofin treated cells.
Figure 6.27: Thioredoxin localisation in MDA-MB-231 cells upon auranofin treatment. A) Representative images from 3, 6 and 24 hour assays. The scale bar is 50µm. Intensity of thioredoxin staining in the nucleus, cytoplasm and membrane upon treatment with auranofin for 3 (B), 6 (C) and 24 (D) hours. Trx = thioredoxin. Results are presented as Mean±SEM of four independent experiments performed in triplicate.
Thioredoxin localisation was also examined in cells treated with lower concentrations of auranofin (0.01µM to 0.2µM) for 144 hours. An increase in the thioredoxin staining intensity, compared to the respective controls, was observed in the cytoplasm and the nucleus at 0.04µM and higher concentrations of auranofin; however this increase was not statistically significant. Moreover, contrary to the results obtained at the shorter time points of 3, 6, and 24 hours, the thioredoxin intensity in the cytoplasm for the 144 hour time point was comparable to the intensities in the nucleus and the cell membrane (Figure 6.28).

Figure 6.28: Thioredoxin localisation in MDA-MB-231 cells upon auranofin treatment for 144 hours. A) Representative images from a 144 hour assay. The scale bar is 50µm. B) Intensity of thioredoxin staining in the nucleus, cytoplasm and membrane upon treatment with auranofin for 144 hours. Trx = thioredoxin. Results are presented as Mean±SEM of two independent experiments performed in triplicate.
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6.3 Discussion

Most *in vitro* anti-cancer studies are performed using 2D cell-based assays where cells are grown as flat monolayers. However, by growing cells in this format, the *in vivo* cellular complexities, which influence tumor growth and progression as well as development of resistance against chemotherapeutics, cannot be achieved. Therefore, the 2D cell culture models are not a true representative of a human tissue or tumor and this may be the reason for failure of many potent anti-cancer drugs in animal studies and clinical trials (Heylman *et al.*, 2014, Benton *et al.*, 2015).

3D culture systems were introduced in the early 1970s to better reflect the oxygen gradients and cell-cell interactions found *in vivo* (Sutherland *et al.*, 1970). Since then several methods have been developed to culture cells in 3D (Tables 1.2 and 1.3). The scaffold-based systems involving growing multicellular spheroids on the ECM components is one of the most widely used method (Friedrich *et al.*, 2007, Eglen and Randle, 2015). A similar technique of 3D culturing using reconstituted basement membrane was employed in this project and was based on the protocol established by Lovitt and colleagues for breast cancer cell lines including MDA-MB-231 cells (Lovitt *et al.*, 2013).

Since the 3D culture models are able to better recapitulate the tumor microenvironment in comparison to the 2D models, they have several benefits. The 3D culture models encompass not only the *in vivo*-like tissue structure but also the important physiological functions, such as cell proliferation rates (Eglen and Randle, 2015). Several parameters, such as drug diffusion and mechanical interactions between different cell types can only be achieved in a 3D culture model. The 3D model used in this project is a miniaturised culture model and its compatibility with automated analysis systems has made it a cheaper alternative to studying drug responses in animal models (Lovitt *et al.*, 2013).

Several factors determine the response of tumor cells to anti-cancer therapeutics, including the cellular communication: cell-cell as well as cell-matrix (Ivascu and Kubbies, 2007). These cellular interactions may be the reason why a reduced sensitivity is observed in 3D models compared to 2D models when same cell lines are exposed to
same drugs (as reviewed by Lovitt et al., 2014). Therefore, to further study the effect of tumor cellular environment, the response of MDA-MB-231 cells to auranofin was compared between 2D and 3D cell culture systems in this project.

Doxorubicin, an anthracycline antibiotic, which is used as a standard of care chemotherapeutic against breast cancer (Tacar et al., 2013), was used as a reference drug in this project. Initially, a 2D monolayer assay was used to evaluate the drug activity of auranofin and doxorubicin against MDA-MB-231 cells. Auranofin is metabolised quite rapidly and has never been detected intact in the blood (Pormetheus Laboratories Inc., 2007). Therefore, the dose-response assays were conducted for a shorter duration of 24 hours, which also correlated to the lengths of auranofin treatment performed in the previous chapters. As the drug responses were evaluated in a 3D model as well, and it has been previously shown that the spheroid health starts to deteriorate after day 12 of culture due to an increase in the size (Lovitt et al., 2013), the duration of the longer treatment was selected to be 144 hours. End-point assays were employed using resazurin or Alamar Blue as a metabolic activity indicator dye, which has been previously shown by other researchers to accurately indicate the cell/spheroid viability (Tung et al., 2011, Lovitt et al., 2013).

The drug activity was evaluated based on the parameters of potency and efficacy. The medicinal treatment regime used in the clinic are based on a therapeutic index that involves maximising the drug efficacy and potency while minimising the toxicity and therefore, both of these parameters have been suggested to be taken into account while examining anti-cancer drugs (Nirmalanandhan et al., 2010). According to the results obtained from the 2D dose-response experiments, auranofin exhibited similar efficacy at both 24 and 144 hour time points, confirming that it acts very quickly. However, it was significantly more potent when used for the longer duration of 144 hours (Table 6.1). Similarly, doxorubicin was also more potent at the 144 hour time point but was also twice as effective after the 144 hour treatment than the 24 hour treatment (Table 6.2). Moreover, the dose-response curves for doxorubicin displayed different steepness between the two time points, highlighting the differences in drug activity (Figure 6.7). The results obtained for the two drugs suggest that while auranofin acts very quickly and shows a greater effect within the first 24 hours of treatment, doxorubicin requires
more time for exerting its cytotoxicity. Although both auranofin and doxorubicin are known to cause an accumulation of cellular ROS (Rigobello et al., 2009, Thorn et al., 2011), the results obtained in this chapter suggest that the signalling pathways influenced by the two drugs may be different.

High throughput imaging and analysis, which employs fluorescent imaging and mining of multiparametric data from these images using automated analysis systems, was used as a tool to study the effect of auranofin and doxorubicin on the morphology of MDA-MB-231 cells grown in 2D. The results obtained showed an increase in the cellular area upon a long term exposure to higher concentrations of auranofin compared to the short term exposure of 24 hours (Figure 6.10). However, compared to the untreated controls, auranofin treatment did not cause any overall change in the cell/nucleus size at the 24 hour time point but at the 144 hour time point, the higher concentrations of auranofin (0.04µM and 0.1µM) enhanced the cell size. Similarly, doxorubicin treatment for 144 hours at higher concentrations (0.04µM and 0.1µM) also enhanced the nucleus and cell size compared to the shorter 24 hour treatment (Figure 6.11). Moreover, at both the time points tested, at higher concentrations (0.02µM and higher), doxorubicin caused a visible increase in the cell and nucleus size in a dose-dependent manner (Figure 6.9), which was verified by the automated size analysis. Therefore, auranofin and doxorubicin induce different morphological changes in MDA-MB-231 cells indicating that these drugs may be acting on different cellular pathways.

Doxorubicin is known to induce apoptosis by increasing oxidative stress (Thorn et al., 2011), however, the increase in cell size is not a characteristic morphological feature of apoptotic cell death, which includes nuclear condensation and fragmentation along with shrinkage and blebbing of cells (Kerr et al., 1972, Fulda et al., 2010). This phenomenon of increase in cell size has also been observed in doxorubicin treated MDA-MB-231 cells by other researchers (Dr Carrie Lovitt, personal communication). Moreover, exposure of human hepatoma cells with low doses of doxorubicin was earlier shown to induce a senescence-like phenotype along with enlargement of cells (Eom et al., 2005). However, these researchers found that at higher doses, doxorubicin induced apoptosis characterised by a decrease in cell size and apoptotic blebbing (Eom et al., 2005).
Therefore, the increase in cell size observed with doxorubicin treatment could not be explained in the current project and requires further mechanistic research.

The drug activity of auranofin and doxorubicin was next evaluated in a 3D culture system. According to this model, MDA-MB-231 breast cancer cells were seeded on top of a Matrigel layer (reconstituted basement membrane) and allowed to form 3D spheroids for six days (Lovitt et al., 2013). The pre-formed spheroids were then treated with auranofin and doxorubicin for either 24 or 144 hours. The effect of auranofin on a 3D cell culture model was studied for the first time in this project. The preliminary results obtained indicated that auranofin was more potent when used for the longer duration of 144 hours but was more effective within the first 24 hours (Table 6.3). The 144 hour assay was performed only once in triplicate due to time constraints. The cytotoxicity of doxorubicin in the 3D model could not be established at either time point since proper dose-response curves could not be obtained. At the 24 hour time point, the highest concentration (40µM) of doxorubicin tested caused approximately 48% inhibition of the metabolic activity of MDA-MB-231 cells while at the 144 hour time point (experiment performed once in triplicate), the lowest concentration (0.002µM) of doxorubicin caused around 20% inhibition. Hence, the activity of auranofin and doxorubicin could not be compared in the 3D model and the assays need to be repeated to obtain statistically significant results.

A direct comparison between the activity of auranofin in a 2D model and a 3D model revealed that after 24 hours of treatment, auranofin was approximately 13 times more potent against MDA-MB-231 cells grown as 2D monolayers compared to those grown as 3D spheroids (Figure 6.18). Similarly, preliminary results from the 144 hour assay also suggested that auranofin was more potent against 2D cells than 3D spheroids (Figure 6.19). These results correlate with other studies, which have shown altered drug sensitivities when a 3D culture system was employed (Nirmalanandhan et al., 2010, Lovitt et al., 2015). Since the 3D models encompass the complexities of the in vivo tissue architecture, it is possible that specific signals are generated by cell-matrix interactions and are therefore, initiated only in the 3D culture systems. These specific pathways may be responsible for modulating the response of cells to chemotherapeutics.
Moreover, the altered sensitivity may arise due to difficulties in drug diffusion in 3D (Thurber and Wittrup, 2008).

Lovitt and co-workers had previously defined a 3D spheroid as a tightly packed cell mass with minimal shedding of apoptotic cells (Lovitt et al., 2013). This group also found a heterogeneity in the size of spheroids per well with the size distribution following a Gaussian curve (Lovitt et al., 2013). Similar heterogeneity with respect to the spheroid sizes per well was obtained in this chapter and therefore, at least thirty spheroids were examined per treatment group for spheroid size analyses. Moreover, differences were observed in the morphology of spheroids within the same well (Figure 6.14) and were taken into consideration during the analyses. Such differences may be attributed to the heterogeneous population of cells within a spheroid.

MDA-MB-231 cells have been shown to form compact rounded structures when grown in the presence of the ECM components (Vinci et al., 2012, Lovitt et al., 2013, Lovitt et al., 2015). Morphological analyses of the spheroids at different planes indicated that the MDA-MB-231 spheroids had loose cell attachments. These phenotypic features correlate with those observed previously in MDA-MB-231 cells grown as 3D spheroids (Ivascu and Kubbies, 2007, Lovitt et al., 2014). An interesting observation was that even at the highest auranofin concentrations, the disintegrated cells appeared to aggregate together (Figure 6.15). However, it was obvious that these aggregates were reminiscent of cellular debris and were therefore not included in the size analyses. Auranofin treatment did not alter the size of the spheroids at either time point (Figures 6.15 and 6.16), suggesting that auranofin does not cause a complete disintegration of the spheroids and may not be influencing the cell-cell and cell-matrix interactions. These cellular interactions may be responsible for lowering the potency of auranofin in the 3D culture model.

Doxorubicin treatment for 24 hours resulted in an overall decrease in the size of MDA-MB-231 spheroids (Figure 6.17). Interestingly, doxorubicin treatment resulted in an increase in the cellular size in the 2D model while a decrease in the spheroid size was observed in the 3D model. These results strengthen the idea that the cell-cell and cell-
ECM interactions, achieved only through the 3D models, play a crucial role in determining the effects of drugs.

The effect of auranofin pre-treatment on the clonogenic capacity of MDA-MB-231 cells was studied in the previous chapter. In this chapter, the effect of auranofin pre-treatment on the ability of MDA-MB-231 cells to form 3D spheroids was evaluated. MDA-MB-231 cells were treated with auranofin and doxorubicin for 24 hours in 2D. These cells were then seeded on top of Matrigel to form 3D spheroids for 144 hours in a drug-free environment. The metabolic activity of the spheroids, measured at the end of the assay, was used as an indirect measure of the cell viability. Although auranofin pre-treatment with the higher concentration of auranofin (2µM) had resulted in a decrease in the number of subsequent colonies formed in 2D (Figure 5.13), it did not affect the metabolic activity of spheroids formed in 3D (Figure 6.20). Moreover, the size of the spheroids formed was comparable to the untreated controls although the pre-treated spheroids appeared to have hollow lumens (Figure 6.22). These results further strengthen the hypothesis that auranofin may not affect the complex cellular interactions inherent to the 3D culture system. Contrary to the results obtained with auranofin, doxorubicin pre-treatment significantly decreased the metabolic activity as well as the size of the consequent spheroids (Figures 6.21 and 6.23). In fact, the doxorubicin pre-treated spheroids were less than 50µm in diameter and were not regarded as actual spheroids. These results indicate that auranofin and doxorubicin may be exerting their cytotoxic effects through different pathways. These differences between the drugs also suggest that there are mechanistic aspects involved in determining drug-specific responses, which may overlap with the tumor architecture.

The second aim of this chapter was to examine the subcellular localisation of thioredoxin in MDA-MB-231 cells treated with auranofin. Thioredoxin1 is a cytosolic protein, which has downstream targets in all subcellular compartments, including the extracellular space (as reviewed by Holmgren, 1985). Under stressful conditions, both oxidative and hypoxic, thioredoxin gets translocated into the nucleus to regulate several transcription factors, such as NF-κβ (Hirota et al., 1999), AP-1 (Abate et al., 1990) and Ref-1/HIF1α (Ema et al., 1999). As discussed in Chapter 5, thioredoxin is also secreted from the cells (Rubartelli et al., 1992, Di Trapani et al., 1998). Although only a few
extracellular binding partners for thioredoxin are known so far, given its important role in processes such as cell adhesion, invasion and migration, it is reasonable to expect that there are many more cell-surface targets for thioredoxin. Inhibition of the thioredoxin system by auranofin causes accumulation of cellular ROS (Rigobello et al., 2009). As observed in this project (Chapter 3), treatment of MDA-MB-231 cells with auranofin resulted in higher ROS levels, which are expected to cause a translocation of thioredoxin into the nucleus (as reviewed by Holmgren, 1985). Immunofluorescence combined with high throughput imaging and analysis was performed and the intensity of thioredoxin staining was compared between three subcellular locations: nucleus, cytoplasm and cell membrane in an automated manner.

Strong thioredoxin staining was observed at each cellular location with no discernible changes upon auranofin treatment (Figures 6.27 and 6.28). These results correlate with those observed in Chapter 3 where no change in the total thioredoxin protein levels was observed in auranofin treated normoxic MDA-MB-231 cells. The automated analysis of the staining intensities showed that thioredoxin was present at overall higher levels in the cytosol compared to the nucleus and cell membrane in the control as well as the treated cells. Moreover, no difference was observed in the staining pattern between the different time points: 3, 6, 24 and 144 hours. These results suggest that although auranofin treatment of MDA-MB-231 cells increased the cellular ROS levels (Chapter 3), these levels were not sufficient to cause a translocation of thioredoxin from one subcellular location to another. Furthermore, a preliminary experiment showed that treatment of MDA-MB-231 cells with H₂O₂, a known inducer of the oxidative stress response, did not cause any changes in the thioredoxin localisation. Ogata and co-workers previously showed a clear movement of thioredoxin protein into the nucleus in HeLa cells upon a 2 hour treatment with 0.5µM H₂O₂ (Ogata et al., 2013). The differences in the results obtained by these researchers and in the current project may be due to the different cell lines used. Moreover, the concentration of H₂O₂ used in the current project was five times less than the concentration used by Ogata and colleagues. Since the oxidative stress induced responses are very transient in nature, it is reasonable to expect such variations between different studies. H₂O₂ treatment caused an increase in the thioredoxin staining compared to the untreated control cells irrespective of the cellular location (Figure 6.25). However, as stated above, auranofin treatment did not
cause any change in thioredoxin staining compared to the untreated control (as observed in this chapter by immunofluorescence and in Chapter 3 by western blotting). These results suggest that the oxidative stress pathways influenced by H$_2$O$_2$ and auranofin may be different and need to be evaluated further.

An interesting observation that led to studying the thioredoxin intensity on the cell membrane was the presence of protrusive membrane structures co-localising with dense thioredoxin staining (Figure 6.26). These cellular protrusions were observed in the untreated as well as the auranofin treated cells. Moreover, they were present at all the time points examined but were less prominent at the 144 hour time point. Closer examination of these structures indicated that they were present towards the leading edge of the cells. Buccione and colleagues have reviewed such protrusions and invaginations to be a part of highly motile cells and expected to be involved in cell adhesion and migration *in vitro* and matrix remodelling and tissue invasion *in vivo* (Buccione et al., 2004). These cellular processes, known as lamellipodia, involve prominent distortions of the cell membrane and have an enhanced network of actin filaments (Buccione et al., 2004). In addition to lamellipodia, additional transient structures known as podosomes also appear as dot-shaped, F-actin-rich extensions of the plasma membrane, similar to the membrane protrusions observed in this project. These podosomes are expected to be involved in cell adhesion to artificial substrates *in vitro*. In their review article, Buccione and co-workers also proposed that podosomes may be precursor structures that are constantly formed in cells that are normally competent in disintegrating the ECM when cultured in 2D on artificial substrates (Buccione et al., 2004). Moreover, these researchers suggested that podosomes may act through an integrin-based mechanism (Buccione et al., 2004).

Previous work performed in our laboratory showed a similarity in the staining pattern of thioredoxin and β1 integrin on the surface of MDA-MB-231 cells (Shah, 2011). It was also found that this β1 integrin staining was more prominent at the points of contacts between the cell surface and the coverslip on which these cells were grown (Shah, 2011). All β integrin subunits contain highly conserved Cys residues (Yan and Smith, 2000) and may therefore be redox regulated by thioredoxin. Furthermore, actin, which is one of the most important components of the eukaryotic cytoskeleton and is involved
in several crucial processes such as apoptosis, cell division, cellular adhesion and migration, is redox regulated by thioredoxin (Gellert et al., 2015). All these studies implicate that the membrane protrusions observed in this chapter represent the highly dynamic actin-rich podosomes. Furthermore, the localisation of thioredoxin in these regions of the cell membrane suggests a role for thioredoxin in cell migration and invasion, possibly through an actin-dependent pathway, which needs further examination.

In summary, the work described in this chapter further highlights the importance of the tumor microenvironment in determining the response against chemotherapeutics. Auranofin was more potent against MDA-MB-231 breast cancer cells grown as 2D monolayers in comparison to the 3D spheroids, which better mimic the *in vivo* tissue conditions. The differences obtained between the 2D and 3D models suggest that taking the tissue architecture into consideration while developing therapeutics may reduce the risk of failure of potent drugs in clinic. Thus, 3D models may be a potential link between *in vitro* screening and *in vivo* animal models.
CHAPTER 7

FINAL DISCUSSION AND FUTURE DIRECTIONS
7.1 Final Discussion and Future Directions

One of the goals of this project was to study how inhibition of the thioredoxin system is influenced by the tumor microenvironment. The cellular microenvironment is a key player in the development and progression of cancer and the oxygenation state and architecture of the tumor tissue have been identified as important components of this microenvironment (Gilkes et al., 2014). These components influence cell signalling pathways, events like metastasis (Semenza, 2013) and also the response of cancer cells to therapeutics (Ivascu and Kubbies, 2007, Lovitt et al., 2014). Higher levels of thioredoxin are correlated with extremely aggressive and highly metastatic tumors (Chaiswing et al., 2007, Ceccarelli et al., 2008) and also with an overall poor prognosis. Expression of the thioredoxin system proteins is higher in tumor tissues as compared to their normal counterparts. Therefore, the thioredoxin system has been suggested as a potential anti-cancer target (Powis and Kirkpatrick, 2007, Tonissen and Di Trapani, 2009). However, high levels of thioredoxin have also been linked with development of resistance against chemotherapeutics (Sasada et al., 1996, Schiff et al., 2000), which as stated above is also influenced by the tumor microenvironment.

Tumor hypoxia arises when the oxygen demand exceeds its supply due to the uncontrolled proliferation of tumor cells (Carreau et al., 2011). Research focused on tumor oxygenation in the past decade has demonstrated that either ‘physioxia’ or hypoxia in vivo is not static but varies spatially as well temporally (Lanzen et al., 2006). This gives rise to intermittent hypoxia, which is more potent than acute hypoxia in activating redox and hypoxic systems (Nakamura et al., 1998, Malec et al., 2010, Karlenius et al., 2012, Sheldon et al., 2014). Intermittent hypoxia has also been shown to precondition cancer cells towards pro-survival pathways and induce resistance against radiation and chemotherapies (Martinive et al., 2006). Auranofin is an anti-rheumatic agent that has shown potential anti-cancer activity in vitro (Simon et al., 1981, Marzano et al., 2007, Newcombe et al., 2013, Fan et al., 2014, You et al., 2015). However, all these in vitro studies have been performed under physiologically irrelevant normoxic (20% oxygen) conditions. In this project, auranofin was used as a tool to inhibit the thioredoxin system, which was examined under conditions mimicking the in vivo tumor microenvironment.
In this thesis, the influence of the tumor microenvironment on the performance of auranofin was first examined with respect to the oxygen gradient. Preconditioning involving short cycles of hypoxia/re-oxygenation, resembling intermittent hypoxia, was used. The results indicated that although preconditioning did not affect the cytotoxicity of auranofin against MDA-MB-231 and A549 cancer cells, auranofin’s mode of action, which is through inhibition of the activity of thioredoxin reductase, was influenced by preconditioning. Further analysis indicated that MDA-MB-231 cells respond to this inhibition by upregulating the promoter activity of both thioredoxin and thioredoxin reductase. This upregulation was further enhanced under hypoxia and when cells were preconditioned prior to auranofin treatment. Moreover, in both MDA-MB-231 and A549 cells, thioredoxin but not thioredoxin reductase protein levels were increased when preconditioned cells were treated with auranofin. High levels of antioxidant proteins, including thioredoxin, are correlated with development of resistance to chemotherapeutics, such as cisplatin (Sasada et al., 1996), docetaxel (Kim et al., 2005), and tamoxifen (Schiff et al., 2000). Therefore, the upregulation of the thioredoxin system upon auranofin treatment in cells exposed to preconditioning or intermittent hypoxia may have important pathological implications in terms of development of resistance against the drug.

The effect of the tumor microenvironment on the performance of auranofin was also evaluated with respect to the tissue architecture, which is another major factor that influences carcinogenesis. A 3D culture model (Lovitt et al., 2013) was employed to study the cytotoxicity of auranofin against MDA-MB-231 cells. The results obtained indicated that growing cancer cells in 3D, which closely mimics the in vivo tumor microenvironment, significantly decreased the cytotoxicity of auranofin in comparison to cells grown in 2D. This outcome also points towards development of resistance against auranofin due to the cellular environment. However, the mechanisms of resistance are quite complex and may involve an overlap of cell signalling pathways and cell-ECM communications, which needs to be examined in future.
In accordance to previously published reports (Mostert et al., 2003, Trigona et al., 2006, Ashino et al., 2011), HO-1 was observed to be upregulated upon inhibition of the thioredoxin system and provided cytoprotection against auranofin. However, HO-1 may not be the only redox protein that provides protection to cells in absence of an active thioredoxin system. The glutathione system is the most abundant antioxidant system (Nordberg and Arner, 2001), which was recently demonstrated to serve as a back-up for the reduction of thioredoxin in absence of thioredoxin reductase (Du et al., 2012). Therefore, in addition to HO-1, the glutathione system could be providing protection to the cells either directly or by keeping thioredoxin in its reduced form. It will therefore be of interest to establish a role for the glutathione system in this regard under intermittent hypoxia. Moreover, in the future it is important to study the redox state of thioredoxin upon auranofin treatment as well as in response to intermittent hypoxia.

The research undertaken in this thesis suggests that the performance of auranofin is influenced by both the oxygen gradient and the tissue architecture. These factors will be studied together in the future by developing 3D spheroids to contain a hypoxic core and the cytotoxicity of auranofin against this new model will be evaluated. In addition, the avascular nature of the 3D model used in this project makes it not a true representative of the in vivo tumor tissue. In future, fibroblasts may also be used as second cell types to stimulate pro-angiogenic factors in vitro.

Metastasis is one of the hallmarks of cancer (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011) and its most life-threatening aspect. As discussed in Chapter 1, metastasis is a multi-step process involving invasion, migration and colonisation of tumor cells (Lorusso and Ruegg, 2012). Several studies have implicated a role for thioredoxin in cancer invasion through the regulation of proteins involved in the degradation of the ECM (Farina et al., 2001, Farina et al., 2011). The redox state of thioredoxin has been shown to be of importance for the invasion process (Bloomfield, 2003). However, the processes of cancer cell migration and colonisation are less well studied. Extracellular addition of recombinant thioredoxin, into the culture media was previously found to enhance the migration of MDA-MB-231 breast cancer cells (King, 2010, Shah, 2011). In the current project, stable transfected cell lines were generated to over-express thioredoxin and its redox inactive mutated form (1SS) to study the
involvement of thioredoxin in cancer cell migration. Thioredoxin over-expression did not affect the migration of MDA-MB-231 cells but over-expression of 1SS diminished this migration. Inhibition of thioredoxin reductase by auranofin also resulted in a decreased migration of MDA-MB-231 cells. Moreover, over-expression of 1SS and auranofin treatment had similar effects on the migration of MDA-MB-231 cells, thus suggesting that targeting the redox activity of the thioredoxin system may have important implications for cancer cell migration. Since the inhibition of the thioredoxin system by auranofin was shown to be influenced by intermittent hypoxia, it will be of interest to study if different oxygen conditions influence the anti-migration effects of auranofin. Pre-treatment with auranofin decreased the clonogenic capacity of MDA-MB-231 cells indicating that anti-thioredoxin strategies may be a useful tool in targeting cancer metastasis. However, auranofin pre-treatment did not affect the subsequent spheroid formation, highlighting the effect of the ECM components and the 3D architecture on auranofin’s performance.

Emerging research suggests that intratumoral hypoxia and the associated ECM remodelling may have critical consequences for metastasis (Gilkes et al., 2014). Protocols have been established by other researchers to grow 3D breast cancer cells as single spheroids to study the events related to metastasis (Vinci et al., 2012). Further research is required to establish a role for thioredoxin in cancer metastasis in the context of the ECM. The stable transfected cells over-expressing thioredoxin and 1SS may serve as valuable tools in this regard.

The effect of auranofin induced inhibition of the thioredoxin system on the HIF1 system was also studied in this thesis under intermittent hypoxia. HIF1α is a transcription factor, which is indirectly regulated by thioredoxin (Ema et al., 1999) and is considered the master regulator of the hypoxic response. This implicates a cross-talk between the redox and hypoxic systems, which may influence crucial cancer pathways, including metastasis and response towards potential therapies. Auranofin treatment did not have any overall effect on the HIF1 system. Previously, inhibition of the thioredoxin system was shown to downregulate the HIF1α protein levels without affecting its transcriptional activity (Welsh et al., 2003). A separate study demonstrated that inhibition of the thioredoxin system resulted in an increase in the HIF1α protein levels.
but inhibited its transcriptional activity (Jones et al., 2006). Review of the available literature suggested that the variations between different studies arise due to discrepancies in the oxygen concentrations regarded as hypoxia and differences in the duration of hypoxic exposures. Moreover, the hypoxic responses are cell line specific (Bracken et al., 2006). This was also observed in the current project, where the HIF1α protein in MDA-MB-231 cells was stabilised by preconditioning prior to a prolonged hypoxic insult, but not in A549 cells. Furthermore, preconditioning only enhanced the accumulation of HIF1α protein in the MDA-MB-231 cell line but did not affect its activity.

Extremely high levels of HIF1α have also been associated with highly aggressive tumors and development of resistance against therapies (Moeller et al., 2005a, Moeller et al., 2005b). HIF1α has also been linked to multidrug resistance (MDR) by increasing the expression of the transporter protein called P-glycoprotein (Pgp) (Riganti et al., 2009, Doublier et al., 2012). Preconditioning stabilised HIF1α protein in auranofin treated MDA-MB-231 cells in this project. Thioredoxin protein levels were also upregulated when MDA-MB-231 cells were preconditioned prior to auranofin treatment and may have resulted due to an increased demand for the reduction of the stabilised HIF1α. These results also suggest that a single target drug may not be as effective and combination therapies should be evaluated. However, further studies are required to determine the involvement of HIF1α in the regulation of the thioredoxin pathway under intermittent hypoxia.

The effect of an oxygen scheme, previously used in a study performed with MDA-MB-231 breast cancer cells was evaluated in A549 lung cancer cells, which were shown to express higher levels of the thioredoxin system proteins in comparison to MDA-MB-231 cells. Although preconditioning cells prior to a prolonged hypoxic insult increased the thioredoxin protein levels in the previous study (Karlenius et al., 2012), no changes in the thioredoxin levels were observed in the A549 cell line in the current project. These results suggest that the thioredoxin levels are not only influenced by intermittent hypoxia, but these higher thioredoxin levels may also play a role in determining the response of cells towards intermittent hypoxia. The preconditioning scheme used in this project is only one representative of the intermittent hypoxia experienced by cancer
cells *in vivo*. In this study, another scheme involving longer duration cycles of hypoxia/re-oxygenation was also employed but did not yield any usable data. The effect of auranofin on the redox and hypoxic pathways will be repeated in the future under modified oxygen parameters.

The current system of drug discovery and development is an extremely lengthy and expensive process, which has a high failure rate with approximately 70-90% drugs failing in clinical trials (Roder and Thomson, 2015). Drug ‘repurposing’ has been identified as a more affordable alternative. Auranofin is a promising candidate in this category since it is FDA-approved for rheumatoid arthritis (Tiekink, 2002) and hence has an established toxicity profile for humans. Although auranofin is currently in a phase I/II clinical trial against chronic lymphocytic leukemia (CLL) (NCT01419691), it is surprising that all the *in vitro* studies with auranofin have only been performed under normoxic conditions. In the current project, auranofin was examined for the first time under hypoxia and intermittent hypoxia. Moreover, this was the first study to evaluate auranofin’s cytotoxicity in a 3D model.

In conclusion, the results presented in this thesis implicate the importance of the tumor oxygen gradient and tissue architecture in influencing the activity of auranofin as a potential anti-cancer drug. The upregulation of the thioredoxin antioxidant system upon auranofin treatment in combination with intermittent hypoxia and the decrease in auranofin’s cytotoxicity in a 3D culture model suggest that the development of resistance against auranofin may be influenced by the tumor microenvironment. Future studies may refine the overlapping roles of these critical environmental elements (Figure 7.1) in carcinogenesis, which may enable the development of more efficient *in vitro* testing models while remaining cost and time effective.
Figure 7.1: Hypoxia induced ECM remodelling: consequence for metastasis and involvement of thioredoxin. The intratumoral hypoxia can control the ECM remodelling, initiating invasion of the blood stream by the adjacent cancer cells and the subsequent metastasis. ECM remodelling can also recruit fibroblasts that may release pro-angiogenic factors causing the formation of new blood vessels, which may give an oxygen burst to the hypoxic tumor resulting in intermittent hypoxia (IH). The involvement of thioredoxin (Trx) in all these processes is shown in red (figure prepared using in part some of the work reviewed by Gilkes et al., 2014).


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APPENDIX
APPENDIX I

Appendix Ia: pGL3-Basic vector map. This plasmid was used as an empty-vector control in the promoter reporter assays. The thioredoxin and thioredoxin reductase promoter constructs were also cloned into this vector.

Appendix Ib: pEF-BOS plasmid vector map. This plasmid vector was used an empty-vector control in the HIF1α-CAD activity assays. The HIF1α-CAD region was also cloned into this vector.
Appendix Ic: pIRE2-EGFP plasmid vector map. This plasmid vector was used in cloning of the thioredoxin and 1SS genes used in the generation of stable transfected cell lines.

Appendix Id: pGEM-T Easy plasmid vector map. This plasmid was used to sequence the thioredoxin and 1SS genes.
Appendix Ie: **pRL-TK plasmid vector map.** This plasmid was used as an internal transfection control in the HIF1α-CAD activity assays.
Appendix IIa: Effect of doxorubicin treatment of 24 hours on the size of MDA-MB-231 cells. A one-way ANOVA followed by Sidak’s post test was employed to analyse the nucleus and cell size separately. The significant increase upon doxorubicin treatment in comparison to the untreated control is indicated by an *. (P<0.0001).