Peroxiredoxin III: A candidate for drug resistance to chemotherapy

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To my Mo – this one's for you.

Abstract

The development of drug resistance to chemotherapeutic drugs is a serious obstacle in the successful treatment of cancer. New cancer drugs are continually being developed with the goal of increasing the effectiveness of chemotherapy. However, new mechanisms of drug resistance are also continually being identified. Understanding the mechanisms of drug resistance is a vital step in identifying new drug targets which may prevent or reduce the development of drug resistance. A recent unpublished study identified peroxiredoxin III (prx III) as being up-regulated in breast cancer cells in culture following exposure to the commonly used anti-cancer drug doxorubicin.

Doxorubicin and the almost identical drug epirubicin have multiple mechanisms of activity. One function of these drugs is to increase intracellular hydrogen peroxide (H_2O_2) concentrations to induce cell death. As prx III is a mitochondrial protein which reduces H_2O_2, it has been suggested that increased expression of prx III may contribute to the development of drug resistance to doxorubicin or epirubicin. However, before such a role for prx III in the development of drug resistance can be further investigated, prx III expression needs to be examined in patients undergoing chemotherapy.

The aim of this study was to examine prx III expression in the white blood cells of patients undergoing chemotherapy with epirubicin, and in healthy control subjects. Additionally, as the activity of a number of peroxiredoxins has been shown to be modulated through the formation of complexes and over-oxidation, complex formation and over-oxidation in response to treatment with doxorubicin or epirubicin was also examined. The results of this study could identify a new target for preventing or reducing the development of drug resistance. While the sample sizes were too small to draw conclusions, some patients showed a change in the expression of peroxiredoxin III following chemotherapy with epirubicin, suggesting that further investigation into the expression of peroxiredoxin III following chemotherapy would be worthwhile.

Abbreviations

2-cys	2-cysteine
2DE	Two-dimensional electrophoresis
ABC	ATP-binding cassette transporter
APS	Ammonium persulfate
ARE	Antioxidant response element
ATP	Adenosine triphosphate
bp	Base pairs (DNA)
BSA	Bovine serum albumin
cDNA	Complimentary DNA
c-Myc	Proto-oncogene
CS	Citrate synthase
C_T	Threshold cycle
DCF	Dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescin diacetate
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleoside triphosphate (dATP, dTTP, dGTP, dCTP)
Dox	Doxorubicin
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
Epi	Epirubicin
ER	Estrogen receptor
ERRα	Estrogen-related receptor α
FBS	Fetal bovine serum
G1	Gap 1

HeLa	Human cervical carinoma cells
Hi95	Human sestrin
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
K562	Human leukemia cell line
kDa	Kilodaltons
MCF10A	Normal human breast epithelial cell line
MCF7	Human breast carcinoma cell line
MEM	Minimum essential medium
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
$O_2^{\cdot-}$	Superoxide anion
p53	Tumour suppressor protein
PA26	Human sestrin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Pen-Strep	Penicillin-streptomycin
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
P-gp	P-glycoprotein
pI	Isoelectric point
Prx	Peroxiredoxin
Prx III	Peroxiredoxin III
prxs	Peroxiredoxins
PVDF	Polyvinylidine fluoride
RBC	Red blood cell
RIPA	Radio-immuno precipitation
RNA	Ribonucleic acid

RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal RNA
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
Srx1	Sulfiredoxin
TAE	Tris acetate EDTA buffer
TBST	Tris-buffered saline-TWEEN 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEN	Tris-EDTA-Sodium chloride buffer
Thr	Threonine
TNF-α	Tumour necrosis factor α
tRNA	Transfer RNA
TrypLE	Express stable trypsin-like enzyme plus phenol red
UV	Ultraviolet
WBC	White blood cell

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Chapter 1: Introduction

1.1 Cancer

Cancer is a leading cause of death and disease in many (Ministry of Health, 2007). Cancer, or the development of tumours, appears to be the result of a number of genetic changes in a cell. Hanahan and Weinberg (2000) outlined six "hallmarks" of cancer – changes that are seen in most, if not all, tumours.

The first is the ability of cells to mimic normal growth signals. Normal cells require signals from their environment to grow. Without these signals, normal cells do not proliferate. Cancer cells develop the ability to grow without these signals, through a number of mechanisms. Some cells appear to develop the ability to synthesise their own growth factors, while other cells over-express growth factor receptors allowing stronger responses to background levels of growth factor, or express mutant receptors which are constitutively active, mimicking constant stimulation with growth factor (Faivre and Lange, 2007).

Cells must also develop insensitivity to the anti-growth signals received from their environment. Similarly to growth factors, anti-growth signals are received by cell-surface receptors. If these cell-surface receptors are lost or mutated, cells become unresponsive to anti-growth signals. This in turn allows cells to grow when they otherwise would not.

As well as being able to proliferate inappropriately, it appears that cells also need to be able to avoid programmed cell death (apoptosis) if a tumour is to develop. Sensors continually monitor both the intracellular and extracellular status for any signs of abnormality, such as DNA damage or hypoxia. Signals suggesting an abnormality or a lack of signals indicating the normal situation trigger apoptosis. For example, a major protein involved in sensing DNA damage and triggering apoptosis is p53. Loss of p53 is seen in a large percentage of cancers and appears to represent an important mechanism for avoiding apoptosis (Harris, 1996).

Normal cells in culture appear to be able to double only 60 to 70 times, while tumour cells are able to double indefinitely (Hayflick, 1965). The ends of the chromosomes, called telomeres, appear to be responsible for this limit in the number of doublings a normal cell can undergo. Telomeres consist of many copies of a short repeat sequence. Due to the inability of the replication machinery to reach the ends of the chromosomes, 50-100 base pairs (bp) are lost from the end of the telomeres at each cycle of replication. Eventually the telomeres become too short, chromosomes become unstable and cell death occurs (Counter *et al.*, 1992). Cancer cells however are able to prevent telomere shortening by up-regulating expression of telomerase, the telomere maintenance enzyme (Shay and Bacchetti, 1997).

A large network of capillaries supplies oxygen and essential nutrients to all cells of the body; without this blood supply cells die. The same is true for cancer cells. Therefore if tumours are to continue to grow, new blood vessels are required. Not surprisingly, many tumours develop the ability to induce angiogenesis (the growth of new blood vessels). A number of changes in gene expression appear to be involved in the ability to induce angiogenesis however, these are not yet fully understood (Bouck *et al.*, 1996).

Finally, many tumours eventually develop the ability to invade surrounding tissue and form tumours at new sites, a process known as metastasis. This is responsible for approximately 90% of cancer deaths (Sporn, 1996). Once tumours metastasise, often the only practical treatment available is chemotherapy (Sauna *et al.*, 2007), a treatment which presents its own problems as will be discussed later in this chapter.

The development of cancer is clearly a complicated process, involving numerous alterations of the genome and gene expression patterns. Not surprisingly, the treatment of cancer is also complicated. Currently a combination of surgery, chemotherapy, and in some cases radiotherapy is used to target cancer cells. There are numerous anti-cancer drugs available for use, and new drugs are continually being developed. Two drugs routinely used to treat a variety of tumours are doxorubicin (adriamycin), and the almost identical drug epirubicin. Doxorubicin (dox) and epirubicin (epi) differ only in the orientation of one hydroxyl group, which appears to be responsible for the slightly decreased cardiotoxicity of epirubicin

(Salvatorelli *et al.*, 2006). Dox binds DNA by intercalating between base pairs, inhibiting both RNA synthesis and DNA replication. This prevents production of proteins and replication of cells (Blum and Carter, 1974). Dox also associates with the topoisomerase II-DNA complex, inhibiting DNA re-ligation which results in the accumulation of double-stranded DNA breaks (Lothstein *et al.*, 2001). Finally, doxorubicin has been associated with an increase in the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which in turn have been linked to the induction of apoptosis (Lothstein *et al.*, 2001). Epirubicin functions through the same mechanisms as doxorubicin (Salvatorelli *et al.*, 2006).

Chemotherapy is usually administered in three week cycles, giving patients time to recover between doses. Optimal doses of anti-cancer drugs are designed to produce maximal death of cancer cells, without being toxic to the patient. However, the side-effects associated with anti-cancer drugs often prevent use of the optimal dose, or prevent patients from receiving all doses of chemotherapy, which reduces the effectiveness of the treatment. Individual variations in the rate of absorption, metabolism and excretion of anti-cancer drugs can also influence the efficacy of treatment (Ralph *et al.*, 2003). However, the ability of cells to develop resistance to anti-cancer drugs poses the biggest challenge to chemotherapy and the treatment of cancer.

1.2 Mechanisms of drug resistance

A number of mechanisms of drug resistance have been characterized. These mechanisms may be present before treatment, or develop as a response to chemotherapy, and usually involve up-regulation of resistance mechanisms or down-regulation of drug targets (Di Nicolantonio *et al.*, 2005). One of the most extensively studied mechanisms of drug resistance involves up-regulation of P-glycoprotein (P-gp). P-gp is expressed in several cell lines which are resistant to a number of different chemotherapeutic agents. P-gp is an ATP-binding cassette (ABC) transporter protein found in the plasma membrane, and is capable of pumping drugs such as doxorubicin and epirubicin out of cells. P-gp expression has been shown to quickly increase in some patients in response to a variety of chemotherapeutic

drugs (Di Nicolantonio *et al.*, 2005). Increased expression of P-gp leads to increased efflux of drugs from the cells, reducing their ability to induce cell death.

Changes that result in a decrease in the uptake of certain drugs may also play a role in the development of drug resistance. For example in the case of methotrexate, a toxic folate analog, resistance occurs through the mutation of one or both of the folate transporters (Gottesman, 2002). Reduced uptake decreases the effectiveness of a drug in mediating cell death and preventing cellular proliferation.

Expression of topoisomerase II (topo II) appears to be down-regulated in response to a number of topo II-targeting drugs, including doxorubicin and epirubicin (Gottesman, 2002; Allen *et al.*, 2004). Down-regulation of topo II results in decreased accumulation of double-stranded DNA breaks. This allows DNA replication and transcription to occur, and prevents activation of cell death pathways usually activated in response to extensive DNA damage. Increased DNA repair has also been associated with the development of drug resistance, as increased repair of DNA damage allows cells to continue to proliferate (Gottesman, 2002).

As with the development of cancer, the development of drug resistance appears to involve changes in the expression of multiple genes. While many mechanisms involved in drug resistance have been partially characterized, new mechanisms are continually being identified. Understanding the mechanisms involved in drug resistance is a vital step in the development of new and more effective treatments for cancer.

1.3 Searching for new mechanisms of resistance

As drug resistance plays such an important role in decreasing the effectiveness of anti-cancer drugs, a great deal of work is being carried out to identify new target proteins involved in its development. Recent developments in both microarray technology and two-dimensional electrophoresis have provided researchers with new ways to compare gene expression in drug-sensitive and drug-resistant cells. For example, complimentary DNA (cDNA) microarrays have been used to examine camptothecin resistance in glioblastoma cell lines (Morandi *et al.*, 2006), and two-dimensional electrophoresis has been used to

examine doxorubicin resistance in MCF7 breast cancer cells (Liu *et al.*, 2006). An unpublished study which used cDNA microarrays to examine changes in the expression patterns of cells in culture following doxorubicin exposure identified peroxiredoxin III (prx III) as one protein which is up-regulated in response to treatment with doxorubicin (Williams *et al.*, unpublished). In this study, MDAMB231, MCF12A and MCF7 cells were exposed to 3 μM doxorubicin for 2 hours, 24 hours or 48 hours before RNA was extracted, first strand cDNA was synthesized and microarray experiments were performed. Expression of peroxiredoxin III was found to be highest 24 hours after exposure to doxorubicin. MDAMB231, MCF12A and MCF7 cells showed 4-fold, 2.5-fold and 2-fold increases in peroxiredoxin III expression respectively, 24 hours following treatment with doxorubicin. Prx III may therefore play a role in the development of resistance to doxorubicin, or epirubicin.

1.4 Peroxiredoxins

The peroxiredoxins (prxs) are a family of small, ubiquitously expressed peroxidases, which are highly conserved in both prokaryotes and eukaryotes (Kim *et al.*, 2005). There are six known human isoforms, which localize to different regions of the cell. Prx I and II are found in the cytoplasm and nucleus, and prx III localizes to mitochondria, while prx IV is found in the endoplasmic reticulum and extracellular space. Prx V exists in two forms, long and short. The long form localizes to mitochondria while the short form is found in peroxisomes (Kinnula *et al.*, 2002). Prx VI is found in the cytosol, mitochondria and peroxisomes (Knoops *et al.*, 1999). Based on the number of conserved cysteine residues present and the mechanism of activity, the peroxiredoxins have been divided into three separate classes – 2-cys (2-cysteine), atypical 2-cys and 1-cys (1-cysteine) peroxiredoxins. Peroxiredoxins I to IV are 2-cys proteins as they contain two conserved cysteine residues and form a homodimer during catalysis (Jeong *et al.*, 2006). Prx V is an atypical 2-cys peroxiredoxin as it forms an intramolecular rather than an intermolecular disulfide bond during catalysis (Jang *et al.*, 2006). Prx VI only contains one of the conserved cysteine residues, and is classed as a 1-cys peroxiredoxin, however other cysteine residues may play a role in catalysis (Kinnula *et al.*, 2002).

The mechanism of the 2-cys peroxiredoxins has been the most extensively examined, and an outline is presented in figure 1.1. H_2O_2 oxidises the conserved amino-terminal cysteine residue to produce an unstable cysteine-sulfinic acid intermediate. This unstable residue forms an intermolecular disulfide bond with the carboxyl terminal cysteine residue of a second prx molecule, to produce a head-to-tail homodimer. Thioredoxin then reduces the disulfide bond, to restore prx to an active state. Thioredoxin reductase then reduces thioredoxin at the expense of a molecule of nicotinamide adenine dinucleotide phosphate (NADPH) (Seo *et al.*, 2000; Chang *et al.*, 2004). In the case of prx III, thioredoxin-2 the mitochondrial thioredoxin, is the electron donor, and thioredoxin reductase-2 restores active thioredoxin-2 (Chang *et al.*, 2004).

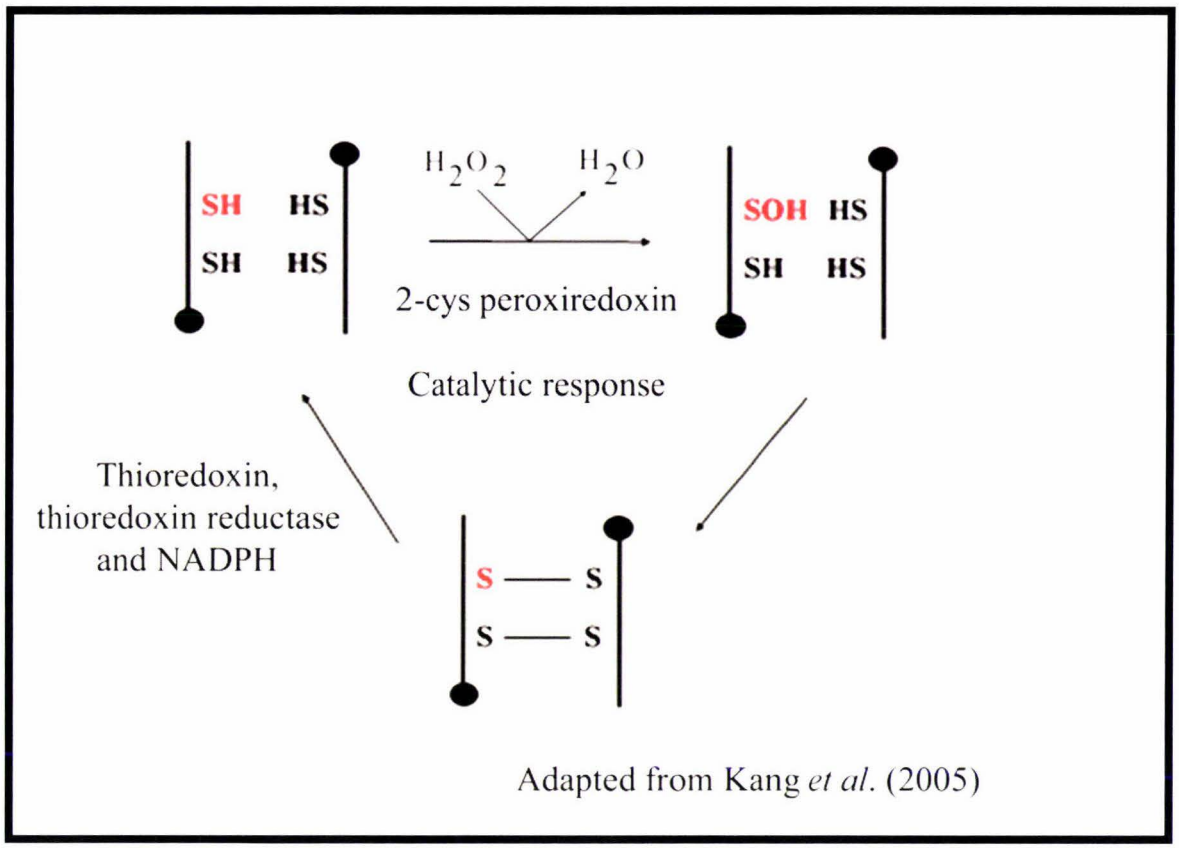

Figure 1.1 Mechanism of H_2O_2 reduction by 2-cys peroxiredoxins

H_2O_2 oxidises the conserved amino-terminal cysteine residue to produce an unstable cysteine-sulfinic acid intermediate (SOH). This unstable residue forms an intramolecular disulfide bond with the carboxyl terminal cysteine residue of a second prx molecule, to produce a head-to-tail homodimer. Thioredoxin reduces the disulfide bond, to restore prx to an active state and thioredoxin reductase reduces thioredoxin at the expense of a molecule of nicotinamide adenine dinucleotide phosphate (NADPH).

1.5 Peroxiredoxins and Reactive Oxygen Species

Reactive oxygen species such as H_2O_2 and the superoxide anion ($O_2^{\cdot-}$) are generated through the activity of the electron transport chain within mitochondria, and through cellular processes such as inflammation, the immune response and removal of foreign compounds (Apel and Hirt, 2004). The superoxide anion can be converted to H_2O_2 spontaneously, or through the activity of a number of superoxide dismutases. H_2O_2 is removed from cells through the activity of a number of enzymes, including the

peroxiredoxins, catalase and glutathione peroxidases (Rhee *et al.*, 2003). It is generally accepted that reactive oxygen species (ROS) such as H_2O_2 play an important role in oxidative damage and apoptosis. ROS are associated with damage to molecules such as DNA, proteins, carbohydrates and lipids (Rhee *et al.*, 2003). Furthermore, generation of H_2O_2 in response to apoptotic signals, such as those received in response to serious DNA damage, appears to lead to the release of cytochrome *c* into the cytoplasm, which in turn triggers the activation of caspases and eventually leads to cell death (Ragu *et al.*, 2007; Tsang *et al.*, 2003).

A number of studies have shown that the peroxiredoxins are able to remove H_2O_2 from cells, and protect cells from H_2O_2-induced apoptosis when over-expressed. Bae *et al.* (2007) found that the normal breast cell line (MCF10A) transfected with prx I and II allowed cells to resist H_2O_2-induced cell death, when non-transfected cells could not. Depletion of prx III sensitized HeLa cells, a human cervical carcinoma cell line, to staurosporine- and TNF-α (tumour necrosis factor α)-induced apoptosis, and also resulted in increased intracellular H_2O_2 levels (Chang *et al.*, 2004).

ROS have also been implicated in a number of non-apoptotic signaling pathways. Signals from both platelet derived growth factor (PDGF) and epidermal growth factor (EGF) result in transient increases in intracellular H_2O_2 concentration. Inhibition of the increase in H_2O_2 prevents protein tyrosine phosphorylation normally seen in response to PDGF or EGF (Sundaresan *et al.*, 1995; Bae *et al.*, 1997). Stimulation of these cells with PDGF or EGF results in an increase in intracellular H_2O_2. Over-expression of prx I or II prevented this increase in H_2O_2 in a number of different cell lines (Kang *et al.*, 1998). Furthermore, extracellular H_2O_2 activates the transcription factor NFκB, which is then able to bind to NFκB binding sites to modulate expression of a number of genes. Over-expression of prx II in HeLa cells reduced NFκB binding to NFκB binding sites and reduced expression from a reporter construct under the control of NFκB binding sites (Kang *et al.*, 1998). Over-expression of prx III has also been shown to result in decreased cell proliferation, providing further evidence in support of a role for peroxiredoxins in regulating or terminating signals from growth factors (Nonn *et al.*, 2003). Therefore, ROS appear to be important in a

number of cellular signaling pathways, and peroxiredoxins are capable of terminating the signals generated by these pathways.

1.6 Peroxiredoxins as molecular chaperones

In 2002, Rabilloud *et al.* (2002) used two-dimensional electrophoresis and tandem mass spectrometry to examine the cellular response to oxidative stress. Their results showed that under oxidative stress a second, more acidic, form of several of the peroxiredoxins was present in cells. This more acidic form was most prominent for peroxiredoxin II, possibly because it is one of the most abundant peroxiredoxins in the Jurkat cells used in the study. These more acidic forms were not seen in response to other types of stress, therefore it is unlikely that this acidic form is associated solely with cell death. The more acidic form of prx appears to be caused by over-oxidation of the acidic site cysteine residue. The authors also report that cell death correlated with a decrease in the levels of normal prx under oxidative stress, suggesting that without functional peroxiredoxins a cell is more susceptible to oxidative stress-induced cell death.

Moon *et al.* (2005) examined prx II structural changes in response to oxidative stress, and found that over-oxidised prx II forms much higher molecular weight complexes than the dimer formed by active prx II. To test whether the higher molecular weight complexes showed chaperone activity, the aggregation of several proteins in the presence and absence of prx II was measured. The first protein to be examined was citrate synthase (CS), a protein known to aggregate at high temperatures. In the presence of prx II, thermal aggregation was inhibited. The insulin β chain was also found to be protected from dithiothreitol-induced precipitation in the presence of prx II. Finally α-synuclein aggregation in response to oxidative stress was found to be inhibited by the presence of prx II. These higher molecular weight complexes were found to possess little peroxidase activity. In contrast, prx II dimers showed high levels of peroxidase activity, and did not exhibit any molecular chaperone activity. These results suggest that prx II is able to prevent aggregation and unfolding of a wide variety of proteins, rather than a small sub-set of proteins. Therefore, prx II may function as a peroxidase in dimer form and as a molecular chaperone, preventing protein unfolding and aggregation, when over-oxidised. In contrast

to the results presented by Rabilloud *et al.* (2002), Moon *et al.* (2005) report that the over-oxidised forms of prx may increase resistance to H_2O_2-induced cell death. Kang *et al.*, (2005) suggest that when H_2O_2 concentrations increase to higher than normal levels as a result of a "death signal" the prxs are over-oxidised to prevent termination of this signal. The prxs then take on the role of molecular chaperone, protecting proteins present in the cell. If the cell survives, the prxs may be reduced and return to functioning as peroxidases.

Prx I also appears to act as a molecular chaperone after inactivation of its peroxidase activity. Jang *et al.* (2006) report that inactivation of prx I can occur through the phosphorylation of a threonine residue (Thr^{90}). Prx I phosphorylated at Thr^{90}, or mutated to aspartate at this residue to mimic phosphorylation, forms higher molecular weight complexes which exhibit negligible levels of peroxidase activity. Instead these complexes were found to possess chaperone activity, inhibiting thermal aggregation of malate dehydrogenase. Phosphorylation of Thr^{90} was found to be mediated by several cyclin-dependent kinases *in vitro*. *In vivo*, prx I phosphorylation levels cycled in parallel with cyclin-dependent kinase 2 (cdc2), suggesting that cdc2 is likely to play the most important role in prx I phosphorylation *in vivo* (Chang *et al.*, 2002). The authors suggest that phosphorylation of prx I is likely to occur after disintegration of the nuclear envelope, and that the subsequent increase in H_2O_2 levels may play an important role in cell cycle progression.

While there is evidence of molecular chaperone activity in higher molecular weight forms of prx I and II, formed following over-oxidation of the active cysteine residues, similar studies do not appear to have been carried out with other peroxiredoxins despite evidence for similar over-oxidation of these proteins.

Recently Cao *et al.* (2007) examined the structures of oxidised and reduced prx III *in vitro*. The results suggest that reduced prx III spontaneously forms into a decameric ring structure, while oxidised prx III forms homodimers. Oxidised prx III was only found to form decameric rings at high protein concentrations (10 mg/mL), which may not be physiologically relevant. Whether these complexes also acted as molecular chaperones was

not examined. However, if the results obtained with the high molecular weight complexes formed by prx I and II apply to the structures formed by prx III these results suggest that prx III may have dual functions within the cell and that adopting the role of molecular chaperone may not be dependent on enzyme inactivation. Further work on the higher molecular weight structures formed by the 2-cys prxs clearly need to be carried out to determine the exact role these structures play within the normal cellular environment.

1.7 Regeneration of active peroxiredoxin

As peroxiredoxins appear to play an important role in signal transduction through the removal of H_2O_2 from cells, it was necessary to determine whether over-oxidised peroxiredoxin could be returned to its active form. Woo *et al.* (2003) used extracts from cells incubated with $^{[35]}$S-labelled amino acids, followed by washing and incubation with un-labelled amino acids, to examine whether re-generation of active prx I was due to *de novo* synthesis or reduction of the over-oxidised form. The results indicate that the active form of prx I can be regenerated through reduction of the over-oxidised form. Regeneration of other prx isoforms has since been shown. Sulfiredoxin (Srx1), is a *Saccharomyces cerevisiae* protein encoded by a gene induced by high H_2O_2 levels. Biteau *et al.* (2003) discovered this protein was able to reduce the over-oxidised form of peroxiredoxins in yeast (a previously unknown role for Srx1). Following this discovery, the human sestrins Hi95 and PA26 were also found to reduce over-oxidised peroxiredoxins. Over-expression of Hi95 or PA26 was found to significantly increase the regeneration rate of active prx I (Budanov *et al.*, 2004) however further work is required to determine the mechanism of prx regeneration, and to identify proteins involved in regeneration of other prxs.

1.8 Peroxiredoxins and disease

Altered expression of sub-sets of the six peroxiredoxin isoforms has been linked to several diseases. Prx I expression appears to be enhanced in thyroid cancer (Yanagawa *et al.*, 1999), and lung cancer (Chang *et al.*, 2001). Prxs I, II and III appear to be over-expressed in breast cancer (Noh *et al.*, 2001). Prxs I, II, III, V and VI show increased expression compared to normal tissue in malignant mesothelioma (Kinnula *et al.*, 2002). Furthermore, prxs I and II have been found to be over-expressed, and prx III down-regulated in both

Alzheimer's disease and Down's syndrome (Kim *et al.*, 2001; Sanchez-Font *et al.*, 2003). Prxs III to VI have been shown to be down-regulated in failing human myocardium (Brixius *et al.*, 2007), while prx V appears to be up-regulated in cells of the central nervous system in multiple sclerosis patients (Holley *et al.*, 2007). This list is far from exhaustive as changes in prx expression patterns are continually being associated with new diseases.

Cancer cells are generally dividing rapidly, and as a result of constant stimulation by growth factors may have higher than normal intracellular H_2O_2 levels. The increased expression of peroxiredoxins in cancer cells may therefore be an attempt by cells to adapt to increased oxidative stress (Park *et al.*, 2006). The expression of different peroxiredoxins in different disease states suggests that while they all remove H_2O_2, the peroxiredoxins have distinct functions within the cell.

1.9 Peroxiredoxin III

Recent unpublished work suggested that peroxiredoxin III (AOP-1, MER5, PRDX3) may be up-regulated in cells exposed to doxorubicin. The exact role of prx III within the cell remains to be elucidated, however experimental results suggest that prx III plays a role in the regulation of H_2O_2 levels. Depletion of prx III from HeLa cells results in increased intracellular levels of H_2O_2, and sensitizes cells to induction of apoptosis by TNF-α (Chang *et al.*, 2004). These cells showed increased rates of mitochondrial membrane potential collapse, cytochrome *c* release and caspase activation, which suggests that prx III plays a role in mitochondria-mediated apoptosis by regulating intracellular H_2O_2 levels. Furthermore, prx III (MER5) knockout mice showed increased susceptibility to lipopolysaccharide-induced oxidative stress, and 1.5 to 2.0 fold higher intracellular H_2O_2 levels in macrophages compared to normal mice (Li *et al.*, 2007). Lee *et al.* (2007) examined prx III expression in cultured human lens epithelial cells, and cultured whole lenses and found that prx III mRNA transcript levels significantly increased following exposure to H_2O_2 concentrations as low as 2 μM. These results further support the suggestion that prx III plays an important role in the removal of H_2O_2 *in vivo*.

There is some evidence that prx III plays a role in a number of signaling pathways within the cell. Nonn *et al.* (2003) reported reduced cell proliferation in cells over-expressing prx III, which may be due to increased removal of H_2O_2 produced during growth factor signaling. Yang *et al.* (2007) also report a role for prx III in proerythrocyte differentiation. Prx III expression was found to fluctuate in human leukemia K562 cells following chemical induction of differentiation into erythrocytes. Furthermore, a K562 cell line over-expressing prx III was found to arrest in the G1 phase of the cell cycle. These results suggest that prx III plays an important role in proerythrocyte differentiation, possibly by regulating intracellular H_2O_2.

As both doxorubicin and epirubicin function to increase intracellular H_2O_2 levels (Lothstein *et al.*, 2001; Tsang *et al.*, 2003) this suggests a possible role for prx III in the development of drug resistance. Increased expression of prx III following chemotherapy with dox or epi may make cells more tolerant to the increases in H_2O_2 caused by these drugs, increasing cell survival and reducing the effectiveness of chemotherapy. Such a role for prx III is supported by the observation that prx III over-expression in WEHI7.2 mouse thymoma cells protected cells against apoptosis induced by the anti-cancer drug imexon, which also functions to increase intracellular H_2O_2 (Nonn *et al.*, 2003).

As increased or reduced peroxiredoxin expression has been shown to have consequences for a number of cellular processes and signaling pathways, it is likely that the expression of prx III and other peroxiredoxins is carefully regulated. Currently little is known about the regulation of prx III expression however recent work has identified some possible mechanisms of prx III regulation. Baker *et al.* (2007) found that prx III mRNA transcripts are up-regulated in the peripheral blood mononuclear cells (PBMC) of some patients treated with imexon. This up-regulation was seen within three hours of treatment; however sample sizes were very small. The authors also identified a putative antioxidant response element (ARE) in the prx III promoter which may play a role in this up-regulation. Specific binding of transcription factors to the ARE in the prx III promoter was not examined.

Prx III expression also appears to be partly regulated by c-Myc, and is required for Myc-mediated transformation. Wonsey *et al.* (2002) examined prx III expression using a cell system where c-Myc was fused to the hormone binding domain of the estrogen receptor. This retains c-Myc in the cytoplasm until tamoxifen, an estrogen analog, is added. Cyclohexamide was used to inhibit protein synthesis. Prx III expression was found to increase following treatment with tamoxifen and cyclohexamide, which provides indirect support for a link to c-Myc. Chromatin immunoprecipitation experiments coupled with real time PCR identified a number of regions within the prx III gene which are bound by c-Myc following the addition of serum to serum-starved cells (Haggerty *et al.*, 2003). Together, these results suggest that c-Myc directly regulates prx III expression. Logarithmically growing c-*myc*-null fibroblasts showed a reduction, but not a complete loss, in prx III expression (Wonsey *et al.*, 2002), suggesting that while c-Myc plays a role in regulating expression of prx III, other factors are likely to be involved.

Rangwala *et al.* (2007) report a further mechanism of regulation of prx III in mouse embryonic fibroblasts. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) appears to induce prx III expression in an estrogen-related receptor α (ERRα)-dependent manner. ERRα regulates transcription of a number of genes by binding to estrogen response elements, however it does not bind estrogens. Prx III mRNA expression also appears to be up-regulated in mouse uterine cells in response to estradiol. This up-regulation appears to be dependent on the presence of the estrogen receptor (ER) (Deroo *et al.*, 2004), a ligand-activated transcription factor. Therefore, while prx III expression appears to be ubiquitous, there may be tissue-specific differences in the levels of prx III expression.

The regulation of prx III expression appears to be a complicated process, and remains to be fully elucidated. However, a number of studies suggest that regulation of prx III expression may be further complicated by regulation of the process of protein synthesis, or degradation. A number of studies have reported that prx III mRNA transcripts do not increase significantly, with increases above three-fold rarely being reported (Lehtonen *et al.*, 2005; Baker *et al.*, 2007; Williams *et al.*, unpublished) . Instead, larger changes are seen

in protein expression. Therefore, examining both prx III mRNA and protein levels is important when studying and drug-induced changes in prx III expression.

1.10 Research aims

Prx III was identified as being up-regulated in response to doxorubicin treatment in cultured cells, which has led to the suggestion that prx III may play a role in the development of resistance to doxorubicin and epirubicin. However, before such a role can be investigated further, the expression of prx III needs to be examined in patients undergoing chemotherapy with doxorubicin or epirubicin. White blood cells were collected from patients immediately before and three weeks after their first dose of chemotherapy with epirubicin. Samples were also collected from control subjects at three week intervals. Real time reverse transcription PCR was used to examine prx III mRNA expression, while sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) coupled with western blotting was used to examine prx III protein levels. Furthermore, as prx III has been shown to form higher molecular weight structures, native polyacrylamide gel electrophoresis (PAGE) coupled with western blotting was used to examine complex formation by prx III in cultured MCF7 cells and white blood cells. Finally, prx III is known to be inactivated by over-oxidation of the active cysteine residue. Therefore two-dimensional electrophoresis was used to examine over-oxidation of prx III in MCF7 cells following exposure to doxorubicin.

The specific objectives of this work were:

- To examine peroxiredoxin III mRNA and protein expression in patients undergoing chemotherapy with epirubicin using real time RT-PCR and SDS-PAGE coupled with western blotting.
- To examine peroxiredoxin III mRNA and protein expression in control subjects using real time RT-PCR and SDS-PAGE coupled with western blotting.

- To examine prx III complex formation in MCF7 cells in response to doxorubicin exposure, and the white blood cells of patients and control subjects using native-PAGE coupled with western blotting.
- To examine over-oxidation of prx III in MCF7 cells following exposure to doxorubicin using two-dimensional electrophoresis coupled with western blotting.