ANTIOXIDANT PROPERTIES OF NQO2

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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SCHOOL OF HEALTH SCIENCES
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CKI  Cyclin-kinase inhibitor
CMV  Cytomegalovirus
c-Myc Myc
CuZnSOD Copper-zincSOD
CYP  Cytochrome P450
DAPI  4',6-diamidino-2-phenylindole
DBD  DNA binding domain
DCFH-DA  2',7'-dichlorofluorescein diacetate
DMEM  Dulbecco’s modified Eagles’s medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
E2  β-oestradiol
E2-3,4-Q  Oestradiol-3, 4-Quinone
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
eIF2α  Eukaryotic Initiation Factor 2 alpha
EnR  Endoplasmic reticulum
EpRE  Electrophile response element
ER  Oestrogen receptor
ERAD  Endoplasmic reticulum-associated degradation
ERE  Oestrogen response element
ERα  Oestrogen receptor alpha
ERβ  Oestrogen receptor beta
ESR1  Oestrogen receptor 1 gene
ESR2  Oestrogen receptor 2 gene
FAC  Fluorescence-activated cell sorting
FAD  Flavin adenine dinucleotide
FBS  Foetal bovine serum
FSC  Forward-scattered light
GPx  Glutathione peroxidase
GR  Glutathione reductase
GRP 78  Glucose-regulated protein 78
GRP94  Glucose-regulated protein 94
GSH    Glutathione
GSSG   Glutathione disulphide
H$_2$O$_2$ Hydrogen peroxide
HER2   Human epidermal growth factor receptor 2
Hsp70  Heat shock protein 70
Hsp90  Heat shock protein 90
IGF    Insulin-like growth factor-1
IRE1   Inositol-requiring enzyme 1
JC-1   5,5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbocyanine
Keap   Kelch like-ECH-associated protein
LB     Luria broth
LDB    Ligand binding domain
Maf    V-maf musculoaponeurotic fibrosarcoma oncogene homologue
MAPK   Mitogen activated protein kinase
Mcl-1  Myeloid cell leukaemia 1
MDA    Malondialdehyde
MER-25 Ethamoxytriphetol
MnSOD  Manganese SOD
mRNA   Messenger ribonucleic acid
NADH   Nicotinamide adenine dinucleotide
NADPH  Nicotinamide dinucleotide phosphate
NF-κB  Nuclear factor-kappaB
NQO    NAD(P)H: quinone acceptor oxidoreductases
NQO1   Quinone oxidoreductase 1
NQO2   Quinone oxidoreductase 2
Nrf2   Nuclear factor (erythroid-derived 2)-like 2
NRH    Dihydronicotinamide ribose
ONPG   Ortho-Nitrophenyl-β-galactoside
P/S    Penicillin/streptomycin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RAS</td>
<td>G-protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPL 19</td>
<td>Ribosomal Protein L19</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Dodecyl sulphate polyacrylamide</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective oestrogen receptor degrader</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective oestrogen receptor modulators</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scattered light</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferases</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour suppressor p53</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferases</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VB 48</td>
<td>Vitabright48</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic Response Element</td>
</tr>
<tr>
<td>ψMMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
Abstract

Dihydronicotinamide riboside (NRH) quinone oxidoreductase 2 (NQO2) is involved in quinone metabolism reducing quinone to hydroquinone. Quinones are products of oestrogen metabolism and are responsible for the oestrogen-initiated breast carcinogenesis. It has been demonstrated that oestrogen quinones are endogenous biological substrates of NQO2 which acting as a detoxification enzyme catalyses the reduction of oestrogen quinones to hydroquinone. Hydroquinone can then be removed by conjugation to glutathione or glucuronic acid.

In this study, the oestrogen dependent and oestrogen independent effects of NQO2 in a variety of networks implicated in breast tumorigenesis were investigated aiming to understand the potential role of NQO2 overexpression in mammary carcinomas. The use of NRH as a cofactor for NQO2 is being studied in parallel with the β-oestradiol and tamoxifen treatments. The MCF-7, T47D, MDA-MB-231 and MDA-MB-468 breast cancer cells were transfected with increasing amounts of NQO2 and its biological activity in regulating ERα transcriptional activity, reactive oxygen species (ROS) generation, cell cycle control, mitochondrial membrane potential and antioxidant activities including catalase activity, glutathione (GSH) levels and glutathione peroxidase (GPx) activity were studied.

NQO2 overexpression in MDA-MB-231 and T47D cells reduced ROS generation. Increasing amounts of transfected NQO2 induced the ERα transcriptional activity in β-oestradiol treated MCF-7 and T47D cells and decreased cyclin D1 protein levels in these cells treated with β-oestradiol compared to untransfected cells. Reduction of catalase activity was detected in tamoxifen treated T47D cells overexpressing NQO2, an effect that was not evident in β-oestradiol treated cells, whereas NQO2 mediated reduction of GSH levels was detected in these cells treated with β-oestradiol but not with tamoxifen. Finally, NQO2 affected mitochondrial membrane depolarization in β-oestradiol treated MDA-MB-231 cells. Given the fact that NRH is not physiologically synthesized in humans, the results presented in this study are valuable from the fundamental science point of view indicating the existence of a potential link between NQO2 and estrogens affecting a number of biological pathways important for breast carcinogenesis and as such from the clinical angle it could be assumed that NQO2 effects could impact the design of personalised breast cancer treatment of oestrogen receptor positive and negative breast cancers.

Keywords: Quinone oxidoreductase (NQO2), oestrogen receptor, ROS, oxidative stress, ER stress, antioxidant
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Conference Presentation

CHAPTER 1

Introduction
1.0 INTRODUCTION

1.1 Cancer

1.1.1 Development of cancer

Cancer is a group of various diseases, which involves uncontrollable cell growth mediated by multiple pathways (Conda-Sheridan, Marler et al. 2010). Multiple changes in gene expression leading to unbalanced cell proliferation and inhibition of cell death followed by the invasion of neighbouring tissues and metastasis to distant sites are the gradual stages of the development of the disease that eventually if untreated lead to death.

Cells in normal tissues, grow, divide and proliferate following strict rules and precise checkpoint controls that ensure transfer of exact copies of the genetic material from the parental to daughter cells. However, in cancer, these checkpoint control mechanisms are disrupted, and cells divide and proliferate uncontrollably leading to the formation of neoplastic tumours. There are three main stages of carcinogenesis: initiation, promotion and progression. Initiation is the stage where Deoxyribonucleic acid (DNA) is damaged or mutated. Promotion is the second stage that involves the uncontrollable and rapid growth and proliferation of mutated cells, which are ready to continue to the third stage. Progression is the final step where cancer cells invade local tissues and spread throughout the body (Evan and Vousden 2001). More than 6 million people die from cancer each year (Wu, Knox et al. 1997, Siegel, Miller et al. 2017) and it is estimated that 51.2% of cancer patients survive for five or more years after cancer diagnosis.

Cancer arises from numerous factors such as generation and chronic accumulation of DNA-damaging reactive oxygen species (ROS), inherited defects in tumour suppressor genes and adverse everyday lifestyle such as obesity, lack of physical activity and consumption of alcohol (Stein and Colditz 2004). An example of inherited genetic defects in tumour suppressor genes is the mutated breast cancer type 1 (BRCA1) gene (located on chromosome 17) and breast cancer type 2 (BRCA2) genes (located on chromosome 13) in breast and ovarian cancers (Ambrosone 2000). Cancer development is a long multi stage process during which cells acquire characteristic
traits called ‘The hallmarks of cancer’. Six of those characteristics were described in 2000, and four more were added in 2011 (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011).

### 1.1.2 The hallmarks of cancer

The specific features that distinguish cancer from normal cells were initially described by Hanahan and Weinberg and include six characteristics named “The Hallmarks of Cancer” (Hanahan and Weinberg 2000) (Figure 1.1 (A) and (B)). The first characteristic refers to the capability of cancer cells to be self-sufficient as they do not need to receive growth signals to stimulate their growth. Furthermore, cancer cells are insensitive to inhibitory growth signals, and they are capable of evading apoptosis. Also, cancer cells differ from normal cells in that they have limitless replicative potential and they can activate factors, which facilitate the formation of new blood vessels to ensure nutrients and oxygen supply through angiogenesis. Finally, they have the ability to escape from the site of their initial location and invade local as well as distant tissues spreading throughout the body through a process called metastasis.

The first principle in ‘The hallmarks of cancer’ is self-sufficiency in growth signals. Normal cells require stimulatory signals generated by growth factors, which through a cascade of signalling events switch ‘on’ proteins such as cyclin D and cyclin-dependent kinases (CDKs) and result in cell growth and proliferation (Muller and Helin 2000, Malumbres and Barbacid 2009). In contrast, cancer cells are self-sufficient, as they do not require the presence of growth signals to stimulate their growth.

The second principle in ‘The hallmark of cancer’ is insensitivity to antigrowth signals. Cell cycle progression in normal cells is regulated by rigorous and precise mechanisms, which involve the regulation of the phosphorylation of the retinoblastoma protein (pRb) tumour suppressor. This regulation is executed by balancing the levels and activity of kinases such as cyclin D1 and cdk4/6 and inhibitors of these kinases such as p15\(^{INK4b}\), p16\(^{INK4A}\), p21\(^{CIP1/WAF1}\) and p27\(^{Kip1}\). Hyperphosphorylation of pRb by cyclin D1 and cdk4/6 signals cell cycle progression, whereas hypophosphorylation of pRb mediated by the p15\(^{INK4b}\), p16\(^{INK4A}\), p21\(^{CIP1/WAF1}\) and p27\(^{Kip1}\) cyclin/CDK inhibitors prevent cell cycle progression. In cancer cells, the
balance between kinase and kinase inhibitors is deregulated, and signals transmitted by cyclin/CDK inhibitors are ignored. These results in uncontrolled cell cycle progression, cellular proliferation and eventually lead to the formation of neoplastic tissues (Gold 1999, Robson, Gnanapragasam et al. 1999).

The third principle in ‘The hallmarks of cancer’ is the evasion of apoptosis. Normal cells under certain conditions, which include age, the number of division cycles and accumulated mutations, initiate the process of programmed cell death also called apoptosis. This process is vital for cellular physiology as cells that do not exert normal functions are eliminated from the body. Tumour cells, on the other hand, do not respond to apoptotic signals and escape death (Lowe and Lin 2000, Fulda 2009, Fernald and Kurokawa 2013). In addition to the evasion of apoptotic signals, cancer cells also produce excessive amounts of anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w and Mcl-1). Anti-apoptotic proteins suppress the expression of pro-apoptotic proteins (Bax, Bad, Bid, Bok, Bik and Bak) thus favouring the predominance of anti-apoptotic factors and hence cell survival (Amaral, Xavier et al. 2010).

The fourth principle in ‘The hallmarks of cancer’ is limitless replicative potential. Normal cells perform limited number of successive cell growth and division cycles according to ‘Hayflick limit’ which states that when cells have undergone 40-60 division cycles, cell growth slows down and eventually stops (Lopez-Otin, Blasco et al. 2013). Telomere functions in the cells as “counter” of the number of division cycles (Artandi and DePinho 2010) and initiate the process of apoptosis in the case that cells have reached the limit of their division cycles. Cancer cells do not take notice of these signals and continue to divide even when they have passed the limit of the allowed division cycles (Reddel 2003). Cancer cells have the ability to replicate endlessly without breaking generation after generation by destroying the ‘timekeeper’ the telomere thereby accumulating mutations and functional defects.

The fifth principle of ‘The hallmarks of cancer’ is sustained angiogenesis. The need for nutrients and oxygen supply in the case of neoplastic tissues is covered by angiogenesis, which is the process of development of new vessels in the newly formed tumour. Factors such as vascular endothelial growth factor (VEGF), mediate the execution of the process of angiogenesis (Carmeliet 2005).
The final principle of ‘The hallmarks of cancer’ is tissue invasion and metastasis. Normal cells locate in the site where they are initially formed and stay by forming bonds with their neighbouring cells. Cancer cells do not form these bonds with other cells in their vicinity and tend to leave the site where they were initially formed migrating to distant sites in the body through the process of metastasis (Ksiazkiewicz, Markiewicz et al. 2012).

Figure 1.1 (A): The six hallmarks of cancer (Hanahan and Weinberg 2000) (B): The four additional hallmarks of cancer (Hanahan and Weinberg 2011).
1.1.3 Breast cancer

Breast cancer is a type of cancer that can be found in breast tissue, particularly from the inner lining of milk ducts or the lobules that supply the ducts with milk (Bai and Gust 2009). Breast cancer is the second leading cause of death in women. In the United Kingdom in 2014, more than 50,000 women were diagnosed with breast cancer, and 11,433 deaths were recorded (Mazhar, Ang et al. 2006, UK 2017). The highest risk factor for breast cancer development is gender, as women are at higher risk to develop the disease compared to men. However, after gender the strongest risk factor for breast cancer is age. According to Cancer Research UK in 2008, females over 59 years old are at higher risk to develop the disease than younger women due to post-menopausal factors.

Risk factors that eventually lead to breast cancer include non-hormonal and hormonal risk factors. In addition, mutations of breast susceptibility genes such as BRCA1 and BRCA2 also contribute to breast cancer disease. Both genes are tumour suppressor genes which are responsible for limiting the growth of tumour cells in breast and ovarian cancers (Moynahan 2002).

Non-hormonal risk factors include family health background, dietary uptake and environmental factors (Martin and Weber 2000). An individual with family members who have been diagnosed with breast cancer has a higher risk of developing the disease. Unhealthy lifestyle and poor diets such as alcohol consumption (Bowlin, Leske et al. 1997, Ellison, Zhang et al. 2001) and smoking are risk factors contributing to breast cancer (Terry and Rohan 2002). Environmental risk factors, for example, exposure to ultraviolet radiation may cause genetic mutations, increasing the possibility of the development of breast cancer (Tokunaga, Land et al. 1994, Goss and Sierra 1998).

Breast cancer is hormone-related cancer, as oestrogen plays important role in promoting the proliferation of the neoplastic breast epithelium (Hilakivi-Clarke 2000, Sommer and Fuqua 2001, Travis and Key 2003, Botelho, Alves et al. 2017). Increased exposure to oestrogen is known to increase the risk of breast cancer (Pike, Gerkins et al. 1979, Jung, Stanczyk et al. 2015). Oestrogen is responsible for the development of female reproductive systems and secondary sex characteristics such as breasts, pubic
hair and armpit hair (Wang, Eriksson et al. 2000, Morani, Warner et al. 2008). Furthermore, oestrogen plays a major role in promoting the proliferation of neoplastic breast epithelium through several mechanisms. Oestrogen receptor-mediated hormonal activity, genotoxic effects from the DNA mutation caused by oestrogen metabolites such as catechol estrogen-3,4-quinones (CE-3,4-Q) that can cause the induction of ROS and oxidative damage (Cavalieri, Li et al. 2002, Cavalieri, Rogan et al. 2004, Cavalieri and Rogan 2006, Gaikwad, Yang et al. 2009) and induction of aneuploidy (Cavalieri, Frenkel et al. 2000, Russo and Russo 2006). It has been reported that several factors associated with increased oestrogen levels such as early age at menarche, nulliparity, and late onset of menopause (Trichopoulos, MacMahon et al. 1972, Kampert, Whittemore et al. 1988). Therefore, reducing exposure of oestrogen might reduce the risk of the disease. The detailed explanation on the role of oestrogen in breast cancer will be discussed in section 1.3.5.

The third factor involved in breast carcinogenesis is the mutation of breast susceptibility genes (Antoniou and Easton 2006, Apostolou and Fostira 2013). Genetic theories of breast cancer propose that breast cancer is a continuation of the process of development and differentiation, and is a sequence of events taking place into the genome. These events include mutations which activate oncogenes (Varmus 1993) (Grupp Sa 2006) such as human epidermal growth factor receptor 2 (HER2), myc (c-MYC) and G-protein (RAS) oncogenes (von Lintig, Dreilinger et al. 2000, Niemitz 2013). In addition, defects in the function of the tumour suppressor genes such as pRb, tumour suppressor p53 (TP53), phosphatase and tensin homolog (PTEN) and the breast susceptibility genes BRCA1 and BRCA2 can lead to breast cancer development (Hanahan and Weinberg 2000, Vogelstein and Kinzler 2004, Suter and Marcum 2007). According to Lerebours, there are few allelic losses associated with primary breast cancer on chromosome arms 1p, 3p, 6p, 7p, 8p, 9p, 11p, 11q, 16q, 17p and 17q which are possible genetic origins of breast cancer (Lerebours, Bertheau et al. 2002). In fact, at the beginning of the twentieth century, some theories were proposed explaining breast carcinogenesis (Bignold, Coghlan et al. 2006). Boveri’s somatic mutation theory became a predominant theory (Varmus 1993, Boveri 2008). Briefly, in 1914, Theodor Boveri proposed the somatic mutation theory of cancer, which suggested that cancer is the result of genomic instability raised by sporadic and or inheritable genetic
mutations in somatic or germinal cells (Edler and Kopp-Schneider 2005, Grupp Sa 2006, Wunderlich 2007).

However, there is no strong evidence to prove the exact causes of breast cancer because 80% of breast cancer cases are not linked to family history or a mutation in breast cancer susceptibility genes (Martin and Weber 2000). It is evident from the above mentioned that further investigation based on multidisciplinary approaches is necessary to understand breast carcinogenesis.

It has been reported that breast cancer consists of several types based on their prognosis. The first type is known as endocrine hormone receptor-positive breast cancer. 80% of the breast cancer cases are oestrogen receptor positive, and about 65% from these are progesterone receptor positive (Lumachi, Santeufemia et al. 2015). The second type of breast cancer is known as HER2-positive. It has been reported that between 20%-30% of all breast cancers overexpress HER2 (Engel and Kaklamani 2007). The next type of breast cancers is known as triple positive which means that the breast cancer cells are positive for oestrogen receptors, progesterone receptors, and HER2. Moreover, the final type of breast cancer is known as triple negative which means the breast cancer cells do not express oestrogen receptors, progesterone receptor, and HER2. It has been reported that between 10%-15% of all breast cancer cells are triple negative (Chavez, Garimella et al. 2010). Most of the breast cancers are associated with the mutation of BRCA1 and BRCA2 genes (Peshkin, Alabek et al. 2010). The prognosis of breast cancer is important to determine the best treatment for the disease.

1.1.4 Treatments for cancer

Currently, available cancer treatments include surgery, radiation therapy, chemotherapy, targeted therapies – bio reductive drugs and immunotherapy. The treatment used to treat cancer depends on the grade and stage of cancer and other relevant factors such as age and the patient’s general health. The most efficient cancer treatment is to remove a tumour with no impact on healthy tissues and organs. However, aggressive cancers that invade surrounding organs and spread to other sites of the body make the process of treatment more complex and challenging (Portenoy 2011).
1.2 Dihydronicotinamide ribose (NRH) quinone oxidoreductase 2 (NQO2)

1.2.1 Background and history

Quinone reductases also known as NAD(P)H: quinone acceptor oxidoreductases (NQO) is a diverse family of enzymes that includes dihydronicotinamide ribose (NRH) quinone oxidoreductase 2 (NQO2). The known function of the NQO2 enzyme is the detoxification of quinones by catalysing the two-electron reduction of unstable quinones (Vella, Ferry et al. 2005, Shen, Barrios et al. 2010). Other detoxification enzymes such as xanthine oxidoreductase, ubiquinone oxidoreductase, cytochrome P450 reductase, and cytochrome b5 reductase exert similar functions minimizing the production of quinones and ROS and at the same time are responsible for the prevention of tissue degeneration, apoptotic cell death, premature ageing, cellular transformation and neoplasia (Brunmark and Cadenas 1989, Lind, Cadenas et al. 1990, Long and Jaiswal 2000, Fu, Buryanovskyy et al. 2005).

The NQO1 was first identified by Martius and colleagues in 1954 (Martius 1954). It was long forgotten until 1957, when NQO1 was re-discovered by Ernster and Navazio (Ernster and Navazio 1958). NQO1 enzyme was isolated from rat liver in 1962 and was named DT-diaphorase (Ernster, Danielson et al. 1962). The genes encoding quinone oxidoreductase enzymes are conserved in many living organisms such as mammals, birds, amphibians, and bacteria (Vasiliou, Ross et al. 2006). NQO1 and NQO2 enzymes are localised in the cytoplasm of the cells and are typically expressed in breast, lung, liver, skeletal, muscle and kidney tissues (Chomarat, Coge et al. 2007).

NQO2 was first isolated from the bovine kidney by Liao in 1962 (Liao, Dulaney et al. 1962) and rediscovered by Jaiswal and colleagues by screening the human liver cDNA library (Jaiswal, Burnett et al. 1990). Apart from that, NQO2 enzymes were also reported by Talalay and colleagues in 1997 (Zhao, Yang et al. 1997).

Both quinone reductase enzymes belong to the flavoprotein family and are about 49% identical in amino acid sequences (Chen, Wu et al. 2000). Structurally, NQO1 and NQO2 are analogous to each other as shown in Figure 1.2. The major difference between NQO1 and NQO2 is that NQO2 is 43 amino acids shorter than the NQO1 (Long and Jaiswal 2000) being only 231 amino acids long compared (MW=25,956
Da) to NQO1 which is 274 amino acids long (MW=30,880 Da) (Celli, Tran et al.
2006). The other difference between NQO1 and NQO2 is that NRH is the electron
donor for NQO2 whereas NQO1 function is catalysed by the reduction of
nicotinamide adenine dinucleotide (NADH) or nicotinamide dinucleotide phosphate (NADPH)
(Jaiswal 1994, Wu, Knox et al. 1997). The difference in the cofactor binding indicates
that NQO2 and NQO1 have different substrate specificity and inhibitors’ binding
(Ross and Siegel 2010, Antoine, Marcheteau et al. 2012).

![Figure 1.2: Schematic representations of the structures of NQO1 (left) and NQO2 (right) (Bianchet, Faig et al. 2004).](image)

1.2.2 Biological functions of NQO2

Quinones and their derivatives, such as hydroquinone are oxidants leading to DNA
damage and cancer cell proliferation. Since the quinone compounds are oxidants, they
may cause harmful consequences in the human body such as carcinogenesis (Bolton,
Accumulation of quinones leads to the formation of ROS that result in oxidative
damage, apoptotic cell death, premature ageing, cellular transformation and neoplasia
(Li, Ishdorj et al. 2012). Hence, oxidant agents including quinones and its derivatives
need to be metabolised by specialised enzymes known as Phase 2 detoxification
enzymes. Thus, the two-electron reduction of quinones by NQO2 helps to avoid the
generation of toxic electrophilic quinones. The two-electron reduction process
produces a relatively stable hydroquinone that is removed by conjugation with
glutathione, UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) avoiding the formation of free radicals (semiquinones) and high levels of ROS.


Moreover, NQO2 enzyme is activated through the ping-pong mechanism, which will be explained in details in the section 1.2.5. NRH is the reducing agent or co-substrate that occupies the catalytic site of NQO2 thus activating it. NRH is oxidised to NR and is thereby released from the catalytic site allowing quinone to bind to NQO2 as the substrate (Vella, Ferry et al. 2005). This formation is called enzyme-substrate complex accepting hydride from quinone to form hydroquinone. Hydroquinone is a stable compound and will be removed by conjugation of glutathione or glucuronic acid and readily be excreted (Hill, Lo et al. 1991, Chomarat, Coge et al. 2007).

However, under certain circumstances, the hydroquinone produced by NQO2 can autoxidise to generate ROS or directly alkylate DNA leading to toxicity. The NQO2 mediated detoxification of quinone to hydroquinone is shown in Figure 1.3.
It has been reported that NQO2 also controls Cyclin D1 gene expression thereby affecting the cell cycle (Hsieh, Yang et al. 2012). However, the biological functions of NQO2 and its physiological significance and in particular its role in the regulation of the cellular redox state is not very well defined and requires further investigation. In conclusion, NQO2 protects cells from the toxicity of ROS thus indirectly protecting cells against carcinogenesis. Cellular defence mechanisms mediated by NQO2 are shown in Table 1.1

**Table 1.1: The roles of NQO2 in various cellular pathways**

<table>
<thead>
<tr>
<th>NQO2 properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stabilises cyclin D1 and is involved in cell cycle control</td>
<td>(Hsieh, Yang et al. 2012)</td>
</tr>
<tr>
<td>Catalyses oestrogen quinone catabolism especially catechol-oestrogen faster than</td>
<td>(Gaikwad, Yang et al. 2009)</td>
</tr>
<tr>
<td>NQO1 in breast cancer</td>
<td></td>
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</tbody>
</table>
Attenuates quinone-mediated oxidative and endoplasmic reticulum (EnR) stress acting in conjunction with Sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs).

NQO2 enzyme is expressed in the breast cell line MCF-10A and higher levels of NQO2 mRNA have been identified in some types of breast cancer cells.

Elevation of NQO2 induces other protective phase II enzymes including glutathione peroxidase (GPx), catalase (Cat) and superoxide dismutase (SOD) in \textit{in vivo} and \textit{in-vitro} systems thus facilitating cancer chemoprevention.

1.2.3 Expression of NQO2

Cytosolic NQO2 is ubiquitously expressed but the levels of its expression vary between different organs and tissues. According to the human protein atlas website (Uhlen, Fagerberg et al. 2015), NQO2 is highly expressed in the renal kidney tubules, in medium levels in breast and its lowest levels of expression have detected so far in the prostate. NQO2 is undetectable in the placenta. In addition, NQO2 overexpression has been observed in a variety of cancers including breast, skin, and prostate triggering our interest to study the potential involvement of NQO2 in pathways affecting carcinogenesis (Yu, Di et al. 2009). It has been reported that environmental factors can cause induction of NQO2 where the inductions are associated with the response to xenobiotic, oxidants, heavy metals, UV, light and ionising radiation (Cuendet, Oteham et al. 2006). Some studies reported that induction of NQO2, directly and indirectly, activate other antioxidant enzymes stimulating various defensive cellular mechanisms (Cuendet, Oteham et al. 2006).

1.2.4 Regulation of NQO2 gene expression

Induction of NQO2 gene expression is related to the nuclear factor (erythroid-derived 2)-like 2/antioxidant response element (Nrf2/ARE) pathway (Ross and Siegel 2010). NQO2 is induced in response to cellular oxidative, and electrophilic stress through the ARE or electrophile response element (XRE) as shown in Figure 1.4 (Benson, Hunkeler et al. 1980, Jaiswal 1991). Exposure to electrophiles and oxidative stress
leads to the dissociation of Nrf2 from the Nrf2/ Kelch like-ECH-associated protein -1 (Keap-1) inhibitory complex that retains the transcription factor in the cytosol. The disassociation of Nrf2 from Keap-1 allows the translocation of Nrf2 to the nucleus and heterodimerisation with cJun or Maf transcription factors (Itoh, Chiba et al. 1997, Ross and Siegel 2010). In the nucleus, the Nrf2 in complex with these transcription factors binds to specific DNA elements called ARE. The binding of the Nrf2 complex to the ARE consensus sequence leads to induction of NQO2 gene expression (Ross and Siegel 2010). Moreover, NQO2 transcriptional activity is executed through activator protein 1 (AP-1) binding sites present in the promoter of the NQO2 gene.

Other pathways involved in the induction of NQO2 gene expression is the Ahr mediated XRE activation. The electrophile response element (EpRE) has been identified in the regulatory region of the genes encoding quinone reductase (Montano, Jaiswal et al. 1998) and it appears to be activated by antioestrogen such as trans-hydroxytamoxifen and tamoxifen. Compounds activating the aryl hydrocarbon receptor (Ahr) receptor induce NQO2 gene expression by binding of Ahr transcription factor to XRE found in the regulatory region of the promoter of the NQO2 (Wang and Jaiswal 2006).
1.2.5 NQO2 catalytic mechanism

NQO1 and NQO2 enzymes undergo a “ping-pong” mechanism and present identical catalytic mechanisms. The ping-pong mechanism indicates that both enzymes have unique catalytic sites for both co-substrate and substrate binding (Vella, Ferry et al. 2005). The uniqueness of both enzymes is best described in two steps of reduction: first, the reducing agent that is the co-substrate (NRH) occupies the respective site on the NQO2 enzyme and donates electrons to flavin adenine dinucleotide (FAD). After its release, the substrate (quinone) takes over the enzyme’s site as shown in Figure 1.5. NRH is a reducing cofactor for NQO2 and being oxidised to NR\(^+\) and NR\(^+\) is released from the active site to allow the binding of quinone substrate. Quinone binds to the active site of NQO2 and accepts the hydride to form hydroquinone. Hydroquinone is a stable compound and will be removed by conjugation of glutathione or glucuronic acid and readily excreted (Chomarat, Coge et al. 2007).
Figure 1.5: NQO2 utilise cofactor and substrate using “Ping-Pong” mechanism.

1.2.6 Association of NQO2 to cancer

NQO2 overexpression has been observed in a variety of cancers including breast, skin, and prostate (Jaiswal 1994, Gong, Kole et al. 2007). NQO1 and NQO2 are beneficial in detoxifying quinones and are potent antioxidant agents (Wang and Jaiswal 2006). NQO2 knockout mice develop myelogenous hyperplasia of the bone marrow and increased number of granulocytes in the peripheral blood but do not present developmental abnormalities (Iskander and Jaiswal 2005). This allows the hypothesis that NQO1 and NQO2 could function as cellular defensive response mechanisms during tumour formation. However, more experiments need to be carried out to prove this hypothesis.

Previous studies have shown that NQO2 is directly involved in breast cancer (Yu, Di et al. 2009, Hubackova, Vaclavikova et al. 2012). The oestrogen hormones majorly play a key role in breast carcinogenesis. Briefly, oestrogens are metabolised to reactive catechol oestrogen quinone (Cavalieri, Rogan et al. 2004, Cavalieri, Chakravarti et al. 2006, Zahid, Kohli et al. 2006). It has been reported that oestrogen quinones, as well as oestradiol (E2), 3, 4-quinone, produced during oestrogen metabolism, are responsible for oestrogen-initiated carcinogenesis particularly breast cancer (Cavalieri and Rogan 2006, Gaikwad, Yang et al. 2009). There is no clear association between
breast cancer and NQO2. Gaikwad et al. have demonstrated that NQO2 catalyses the reduction of oestrogen quinone endorsing the notion that NQO2 can act as a detoxification enzyme (Gaikwad, Yang et al. 2009). Moreover, it has been demonstrated that oestrogen-3, 4-quinone binds to NQO2 indirectly, implying that oestrogen quinones are endogenous biological substrates of NQO2 (Yu, Di et al. 2009). In addition, it has been reported that NQO2 exhibits faster action in reducing oestrogen compared to its homologue NQO1 (Yu, Di et al. 2009). Previous studies have reported that NQO2 is capable of activating cyclin D1 (Hsieh, Yang et al. 2012) indicating that the biological role of NQO2 in carcinogenesis could be the control of cell cycle.

Quinone reductase enzymatic activity, provide protective effects against the toxicity and mutagenicity caused by quinone (Montano, Jaiswal et al. 1998). However, more studies need to be carried out in finalising the interconnection between breast cancer and NQO2 at the gene and cellular level.

1.3 Oestrogen receptor transcription factor

1.3.1 Background and history

Oestrogen is a term derived from the Greek word oistros, which refers to oestrus, the phase in which females are sexually receptive (Sastre-Serra, Nadal-Serrano et al. 2013). It is estimated that women with active menstrual cycles produce about 70 and 500 micrograms of β-oestradiol (Dean 2005). β-oestradiol concentration increases due to pituitary gland secretion or maturation of oestrogen follicle (Morani, Warner et al. 2008). Studies carried out by Jensen and colleagues in 1972 concluded that the biological effects of oestrogens could only happen when oestrogen receptor is activated (Jensen, Jacobson et al. 1972, Jensen, Jacobson et al. 2010). Briefly, oestrogen receptor is a ligand-inducible transcription factor and a member of the nuclear receptor superfamily (Bai and Gust 2009). There are two types of oestrogen receptor named oestrogen receptor alpha (ERα) (Jensen, Jacobson et al. 2010) and oestrogen receptor beta (ERβ) (Kuiper, Enmark et al. 1996). They can be found in cells (Sommer and Fuqua 2001), each encoded by a separate gene oestrogen receptor gene 1 (ESR1) and oestrogen receptor gene 2 (ESR2) located on the sixth and fourteenth chromosome (6q25.1 and 14q23.2), respectively (Morani, Warner et al. 2008). Under
normal conditions, development and physiology of the mammary glands are under oestrogen control. However, during a women’s lifespan, mammary epithelial will undergo physiological changes and oestrogens play major roles in the changes (Russo, Ao et al. 1999). However, the exact role of oestrogen and ER in mammary epithelial proliferation remains unclear.

1.3.2 Expression of oestrogen receptor

Oestrogen receptor also known as a nuclear hormone receptor, is an important superfamily of transcriptional regulators that are involved in the embryonic development, cell differentiation and homeostasis. Oestrogen receptor is activated by oestrogen hormones namely, β-oestradiol that naturally produced in the ovary (Guo, Wei et al. 2006, Chen, Zeng et al. 2008). Oestrogen receptor was first isolated by Elwood V. Jensen in 1958 who described its importance in breast cancer (Jensen and Jordan 2003, Jensen 2012). The biological effects of oestrogen are mediated by ER using the specific ligand-binding domain present within the receptor. Once ER is activated by oestrogen hormone, it translocates to the nucleus and binds to DNA on a site known as the oestrogen response element (ERE) and regulates the expression of its transcriptional target genes. In fact, long-exposure to oestrogens including the hormone-replacement therapy and oestrogen replacement therapy was reported to contribute to the risk of breast cancer (Sommer and Fuqua 2001, Yager and Davidson 2006). Furthermore, ERα is highly expressed in breast cancer cells, endometrium and hypothalamus (Yaghmaie, Saeed et al. 2005), while ERβ presence has been reported in ovarian cells, kidney, heart (Babiker, De Windt et al. 2002) and bone (Zhao, Dahlman-Wright et al. 2008). ERβ was isolated from rat prostate and ovary by Kuiper and colleague (Kuiper, Enmark et al. 1996). Oestrogen receptor activates gene expression of its transcriptional targets via specific binding of its transcriptional activation domain to DNA sequences present in the regulatory regions of the promoters of its transcription targets (Gruber, Gruber et al. 2004, Guo, Wei et al. 2006). The oestrogen antagonists such as the selective oestrogen receptor modulators (SERMs) including tamoxifen and raloxifene are able to prevent ER activation by inhibiting gene expression of ER target genes (Ottow and Weinmann 2008, Burney 2011).
1.3.3 Structure of the oestrogen receptor

Oestrogen receptor is a ligand-activated transcription factor member of the steroid receptor superfamily. In the presence of oestradiol hormones, oestrogen receptor induces gene expression of genes driving cell proliferation such as cyclin D1 (Yang, Chen et al. 2010). The human oestrogen receptors, ERα and ERβ can be divided into four functional domains as indicated in Figure 1.6. ERα protein is composed of 595 amino acids and ERβ of 530 amino acids (O’Brien, Park et al. 1999, Binder, Winuthayanon et al. 2015). The percentage of homology of domains in ERα and ERβ is shown in Figure 1.6. The location of phosphorylation sites in ERα are also indicated. ERα is activated by kinases that modulate a wide variety of cellular events. The domains A/B are reserved for hormone-independent oestrogen receptor activation function 1 (AF-1), which modulates transcription in a gene- and cell-specific manner. The central and most conserved C domain contains the DNA binding domain (DBD) and it mediates receptor dimerization responsible for oestrogen receptor binding to the oestrogen response element consensus binding. The D domain also called hinge region is a less well-understood region. Hinge regions of ERα and ERβ share 30% similarity in this region (Kuiper, Enmark et al. 1996). Finally, the ligand binding domains (LDB) consist of the E and F domains. LDB is responsible for hormone binding, a homo- or heterodimerization interface and coregulatory (activator and repressor) interaction sites (Kumar, Green et al. 1987, Danielian, White et al. 1992, Montano, Muller et al. 1995).

![Figure 1.6: Structural diagram of oestrogen receptor and the function domains Adapted from (Roman-Blas, Castañeda et al. 2009).](image)
1.3.4 Oestrogen receptor activation and signalling

Oestrogen metabolism plays major roles in oestrogen-induced cancer (Miller 2003, Russo, Hasan Lareef et al. 2003, Santen, Yue et al. 2015). Oestrogen metabolites cause genotoxic effects resulting in DNA damage (Caldon 2014, Yasuda, Sakakibara et al. 2017), mutations (Alluri, Speers et al. 2014) and cell transformation (Russo, Fernandez et al. 2006) and will be further discussed in section 1.3.5. Biological effects of oestrogen are executed through four pathways as shown in Figure 1.7.

In the classical oestrogen genomic pathways, oestrogen receptor activation is induced by agonist resulting in the stimulation of the oestrogen receptor transcriptional activity by direct interaction of the oestrogen receptor with oestrogen response elements (Gruber, Gruber et al. 2004, Huang, Li et al. 2006, Marino, Galluzzo et al. 2006, Heldring, Pike et al. 2007, McDevitt, Glidewell-Kenney et al. 2008). The disassociation of the chaperones results in the association of the coregulatory, either coactivator or corepressor proteins to induce oestrogen receptor transcriptional activity (Chambraud, Berry et al. 1990, McKenna, Lanz et al. 1999, Klinge 2001). The coactivators of oestrogen receptor induce oestrogen receptor transcriptional activity, while corepressors of oestrogen receptor are involved in inhibiting oestrogen receptor transcriptional activity (Chambraud, Berry et al. 1990, McKenna, Lanz et al. 1999, Klinge 2001).

In the non-ERE signalling pathways, regulation of oestrogen receptor can be mediated by the interaction of other DNA-binding proteins such as AP-1, Sp1 and nuclear factor-kappaB (NF-κB) transcription factor, forming complexes that mediate the transcription of genes whose promoters do not harbour EREs. Co-regulator molecules regulate the activity of the transcriptional complexes (Porter, Saville et al. 1997, Cerillo, Rees et al. 1998, McKay and Cidlowski 1998, Webb, Nguyen et al. 1999). However, the interplay between such interactions in mediating the oestrogen response in breast cancer cells remains unclear.

In non-genomic oestrogen signaling pathways, oestrogen activates not only oestrogen receptor but also G protein-coupled estrogen receptor 1 (GPER), also known as G protein-coupled receptor 30 (GPR30) at the cell membrane. These actions result in the activation of the phosphatidylinositol-3/Akt (PI3K/Akt) (Wong and Walker 2013), and
protein kinase C/mitogen activated protein kinase (PKC/MAPK) signal transduction pathways (Marino, Galluzzo et al. 2006).

The ligand-independent pathway oestrogen receptor is activated by growth factors such as insulin-like growth factor (IGF)-1 (Zhang and Yee 2000), epidermal growth factor (EGF) (Flint, Sheldrick et al. 2002), transforming growth factor-β/mothers against decapentaplegic (TGF-β/SMAD) (Li 2014), and the Wnt/β-catenin signaling pathway (Kouzmenko, Takeyama et al. 2004). These signalling pathways occur in the absence of ligands by direct phosphorylation of the oestrogen receptor with MAP or PI3/Akt kinase.
1.3.5 Oestrogen and cancer

The involvement of oestrogen and its receptor in breast carcinogenesis are widely reported where nearly 70% of breast cancer cases are expressing ERα (Rutqvist, Cederman et al. 1989, Miller 2003, Onland-Moret, van Gils et al. 2005). Genetic changes in the oestrogen receptor are one of the causes of carcinogenic effects in breast cancer. Mutation in oestrogen receptor genes contributes to the changes in the activation of oestrogen receptor (Le Goff, Montano et al. 1994) including the interference with co-factor binding (Henttu, Kalkhoven et al. 1997) or changes of agonist/antagonist activity (Montano, Ekena et al. 1996). The changes in the oestrogen
receptor affect the susceptibility to hormone treatments and progression of the disease (Sommer and Fuqua 2001).

Apart from mutations occurring in the oestrogen receptor, oestrogen metabolites via genotoxic pathways can result in DNA damage, mutations due to oxidative stress and cell transformation (Doherty, Weiss et al. 2005, Chen, Zeng et al. 2008). Oestrogen metabolites such as catechol oestrogen, 4-hydroxyE2 (4-OHE2), 2-hydroxy E2 (2-OHE2) and 16a-hydroxy E1 (16a-OHE1) are usually produced during oestrogen metabolism mediated by Cytochrome P450 (CYP450) proteins especially CYP1A1 (Napoli, Villareal et al. 2005, Scornaienchi, Thornton et al. 2010). Catechol oestrogen metabolites undergo further oxidation from catechol oestrogen to the oestrogen o-quinones (Zhou, Wang et al. 2010, Zhou 2016) (Figure 1.8). Accumulation of data show that the oestrogen metabolites, catechol-3, 4-quinones can cause induction of reactive oxygen species and oxidative damage (Cavalieri, Rogan et al. 2004, Gaikwad, Yang et al. 2009). The damage caused by catechol oestrogen quinone becomes worse when the imbalance of oestrogen activating enzymes and deactivating enzymes such as NQO2 antioxidant enzymes, occurs (Cavalieri, Chakravarti et al. 2006, Gaikwad, Yang et al. 2009). A Recent report by Gaikwad and colleagues demonstrated that NQO2 catalyses the reduction of electrophilic oestrogen quinones, suggesting that NQO2 acts as a detoxification enzyme (Gaikwad, Yang et al. 2009).

Figure 1.8: Catechol oestrogen metabolites undergoing further oxidation from catechol oestrogen to the oestrogen o-quinones. The involvement of oestrogen-quinone in breast carcinogenesis pathways.
1.4 Reactive oxygen species (ROS)

1.4.1 Background and history

Reactive oxygen species are composed of several types of molecules and radicals that are reactive chemical species containing oxygen (Cadenas 1989, Riley 1994, Rahman 2007). Reactive oxygen species are categorised into two groups the free radicals and the non-radicals (Rahman 2007, Liou and Storz 2010). Free radicals are reactive chemical species that have a single or more than one unpaired electrons in separate orbitals in their outermost shell. Free radical species are composed of superoxide (O$_2^•$-$)$, hydroxyl radicals (•OH), nitric oxide (NO•), organic radicals (R•), peroxyl radicals (ROO•), alkoxyl radicals (RO•), thiyl radicals (RS•), sulfonyl radicals (ROS•), thiyl peroxyl radicals (RSOO•), and disulfides (RSSR) (Riley 1994). While, non-radical species contain no unpaired electrons but chemically are reactive as they can be converted to radical ROS. Non-radical species are composed of hydrogen peroxide (H$_2$O$_2$), singlet oxygen (¹O$_2$), ozone/trioxygen (O$_3$), organic hydroperoxides (ROOH), hypochloride (HOCl), peroxynitrite (ONO$^-$), nitrosoperoxycarbonate anion (O=NOOCO$_2^-$), nitrocarbonate anion (O$_2$NOOCO$_2^-$), dinitrogen dioxide (N$_2$O$_2$), nitronium (NO$_2^+$), and highly reactive lipid-or carbohydrate-derived carbonyl compounds (Liou and Storz 2010). Superoxide, hydrogen peroxide and hydroxyl radicals are the most extensively studied radical species since they are very toxic and are involved in cancer development (Waris and Ahsan 2006). Elevated ROS levels in the body might lead to the oxidative damage resulting in the initiation of the multistage process of carcinogenesis (Poli, Leonarduzzi et al. 2004, Waris and Ahsan 2006).

ROS are produced endogenously and exogenously as shown in Figure 1.9. The endogenous sources of ROS include the mitochondria where ROS are generated during mitochondrial oxidative phosphorylation (Storz 2005), peroxisome activity (Szatrowski and Nathan 1991), cytochrome P450 (Rahman 2007) and activation of inflammatory cells (mainly phagocytes) (Inoue, Sato et al. 2003). ROS are also produced by exogenous sources such as xenobiotics, environmental agents, chlorinated compounds, pathogens and radiation (Valko, Rhodes et al. 2006, Rahman 2007). The biological activities of ROS have both beneficial and harmful implications. ROS play important roles in regulating various signal transduction pathways by reacting with enzymes, transcription factors, and the DNA. ROS are also involved in
regulating antioxidant enzymes, cell signalling pathways, differentiation, mediating inflammation by stimulating cytokines production and eliminating pathogens and foreign particles (Li 2013, Schieber and Chandel 2014). Adverse effects of ROS are often associated with factors that trigger cancer cells development (Storz 2005, Liou and Storz 2010). Therefore, maintaining the balance of ROS levels is important to ensure homeostasis of the body within certain limit (Ray, Huang et al. 2012).

1.4.2 ROS homeostasis

ROS homeostasis is maintained by the regulation of the cellular redox and antioxidant activity as shown in Figure 1.9 (Trachootham, Alexandre et al. 2009). To maintain the ROS homeostasis, the production of ROS is neutralised by the function of an antioxidant enzyme such as NQO2 (Trachootham, Alexandre et al. 2009), catalase and glutathione peroxidase (Shim and Kim 2013). Production of ROS at low and moderate levels, indicates that redox homeostasis is under control (Valko, Leibfritz et al. 2007, Trachootham, Alexandre et al. 2009). Elevated ROS production initiates damage to lipids, proteins, and DNA (Trachootham, Alexandre et al. 2009, Dayem, Choi et al. 2010).

Antioxidant enzymes play important roles in ROS homeostasis by scavenging molecules and free radicals. Antioxidants enzymes consist of natural dietary antioxidants (Vitamin A, C and E), endogenous antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and antioxidant molecules (bilirubin, coenzyme Q, glutathione and ferritin) (Marengo, Nitti et al. 2016). Superoxide dismutase, catalase and glutathione peroxidase are the most abundant and major enzymatic antioxidant defence systems responsible for scavenging free radicals and nascent oxygen (Manoharan, Kolanjiappan et al. 2005, Li 2013, Ivanova, Zhelev et al. 2016).

The first antioxidant enzyme discovered was superoxide dismutase (SOD) in 1968 by Irwin Fridovich and colleagues (McCord and Fridovich 1969). SOD has the ability to catalyse the breakdown of superoxide anion into hydrogen peroxide (H₂O₂) (Marengo, Nitti et al. 2016). SOD is expressed in human cells and located in the mitochondria or cytoplasm of the cells (Li and Zhou 2011). There are different types of SOD depending on the affinity binding to specific co-factor such as copper-zinc SOD (CuZnSOD)
localised at the cytoplasm (Marengo, Nitti et al. 2016) and manganese SOD (MnSOD), located in the mitochondria and cytoplasm of the cells (Wolfe-Simon, Starovoytov et al. 2006). Catalase is another abundant type of antioxidant enzyme highly expressed in peroxisomes. Catalase is a haem-containing protein which catalyses the conversion of hydrogen peroxides (H$_2$O$_2$) to H$_2$O and O$_2$ (Pazoles, Claggett et al. 1980, Zhou and Kang 2000). Catalase has high binding affinity to Iron or Manganese (Heck, Shakarjian et al. 2010). The details about catalase enzyme are discussed in the section 1.7. Glutathione peroxidase (GPx) is involved in metabolising hydrogen peroxide (H$_2$O$_2$) to H$_2$O using glutathione (GSH) as a substrate and generating glutathione disulphide (GSSG) which is then reduced by the specific enzyme glutathione reductase (GR) (Marengo, Nitti et al. 2016). GPx is localised in the mitochondria and has high binding affinity with selenium (Murakoshi, Osamura et al. 1984, Ribas, García-Ruiz et al. 2014). The details about glutathione peroxidase enzyme are discussed in the section 1.8. Imbalance states between the production of ROS and antioxidants detoxifying the harmful effects of ROS are referred to as oxidative stress (Ambrosone 2000, Baskol, Atmaca et al. 2007). Oxidative stress activates several transcription factors including NF-κB, AP-1, p53, HIF-1α, PPAR-γ, β-catenin/Wnt, and Nrf2 (Reuter, Gupta et al. 2010). Activation of these transcription factors leads to the expression of over 500 different genes, including genes encoding for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory and anti-inflammatory molecules (Reuter, Gupta et al. 2010). The harmful effects of the imbalance-mediated oxidative stress lead to carcinogenesis (Reuter, Gupta et al. 2010).
Figure 1.9: Production of ROS by cell components and external sources that cause oxidative damage, can occur when antioxidant factors are insufficient. Adapted from: (Khalid S Abdel-Lateif, Hany A Eldeab et al. 2016)

1.4.3 Roles of reactive oxygen species (ROS) in cancer

In the biological system, ROS production is steadily generated and eliminated in essential levels required to drive regulatory pathways (Dickinson and Chang 2011) as shown in Figure 1.10. In normal cells, ROS levels are tightly regulated by the balance between the generation of ROS with the ROS elimination mechanism by the scavenging system (Poljsak, uput et al. 2013). Excessive levels of ROS cause cellular dysfunction such as promoting cell proliferation (Zamkova, Khromova et al. 2013), senescence (Davalli, Mitic et al. 2016), cell cycle arrest (Chatterjee, Kundu et al. 2009), and endoplasmic reticulum stress (Malhotra and Kaufman 2007, Reuter, Gupta et al. 2010, Avery 2011). Excessive production of ROS plays important roles in initiating oxidative stress and modulate carcinogenesis (Mates and Sanchez-Jimenez 2000, Boonstra and Post 2004, Son, Cheong et al. 2011). In cancer cells, oxidative stress stimulated by ROS generation increases metabolic activity and mitochondrial malfunction (Cairns, Harris et al. 2011, Cui, Kong et al. 2012). Prolonged period of exposure of cells to high ROS levels causes oxidative stress and damage of DNA, lipid,
and protein (Hemnani and Parihar 1998, Barrera 2012, Jena 2012, Ayala, Mu et al. 2014) and cancer cells survival (Pani, Galeotti et al. 2010). ROS suppress tumour growth through the sustained activation of cell-cycle inhibitors (Poillet-Perez, Despouy et al. 2015). The adaptability of cancer cells to ROS, may also lead to the upregulation of glutathione and other antioxidant molecules (Trachootham, Alexandre et al. 2009).

**Figure 1.10:** Oxidative stress caused by ROS production causes cancer
Adapted from: (Dayem, Choi et al. 2010)

### 1.5 ROS and cell cycle

The second principle of the ‘hallmarks of cancer’ is insensitivity to antigrowth signals that may cause the cell cycle deregulation in cancer. Cell cycle progression in normal cells is regulated by rigorous and precise mechanisms (Hanahan and Weinberg 2011). Generation of ROS produced by external and internal factors play an essential role in cell cycle progression (Vokurkova, Xu et al. 2006, Fehér, Ötvös et al. 2008). The effects of ROS generation on cell cycle progression is dependent upon the amount of ROS and the duration of exposure (Boonstra and Post 2004). Exposure to low levels of ROS may increase cell progression by activating growth factors and the receptor
tyrosine kinases (RTK) (Irani, Xia et al. 1997). Exposure to high levels of ROS may inhibit cell growth through the activation of cell cycle inhibitors and induction of senescence (Ramsey and Sharpless 2006, Takahashi, Ohtani et al. 2006).

The cell cycle is divided into three main stages: Resting phase or G0 phase, interphase that includes the G1, S, and G2 phases and mitotic or M phase (Schafer 1998) as shown in Figure 1.11. G0 phase is the resting period in the cell cycle, during which cells are in a quiescent state and they do not engage in the cell cycle. G1 phase is the phase where protein synthesis facilitates cells’ growth and double their original size. The S or synthesis phase is the phase in which DNA replication occurs. G2 phase is the last phase of interphase where the cells continue growing preparing for the mitosis phase. Mitotic phase is the short period of the cell cycle consisting of four sub phases: prophase, metaphase, anaphase, and telophase. Prophase, is the first stage in mitosis, where chromatin condenses. During metaphase, the chromosomes are lined up along the metaphase plate and during anaphase chromosomes break at centromeres and sister chromatids move to the opposite ends of the cells. The final sub phase in the mitotic phase is the telophase when nuclear membranes reform, nucleoli reappear, and chromosomes unwind into chromatin. In normal cells, the transition from one phase of the cell cycle to the next is strictly controlled by the checkpoint controls. In cancer cells, checkpoints are not functional resulting in uncontrolled cell proliferation (Collins, Jacks et al. 1997, Williams and Stoeber 2012).
Figure 1.11: The cell cycle is executed in four distinctive phases: G1 phase, S phase, G2 phase and M phase.
Adapted from: (Schafer 1998).

The transition from one stage of the cell cycle to the next is regulated by specific CDKs and cyclin (Lim and Kaldis 2013). (Casimiro, Crosariol et al. 2012, Malumbres 2014). Cyclins act as regulatory subunits and CDKs act as catalytic subunits as shown in Figure 1.12. Cyclins and CDKs complexes promote the phosphorylation and activate target proteins (Suryadinata, Sadowski et al. 2010). The activated complexes are required to prepare the cells for the next phase of the cell cycle (Hochegger, Takeda et al. 2008). There are three CDKs involved in the interphase including CDK2, CDK4 and CDK 6, and one involved in mitotic phase the CDK1 (Suryadinata, Sadowski et al. 2010) as shown in Figure 1.13. There are ten different cyclins belonging to four different classes (A-, B-, D- and E- type cyclin) as illustrated in figure 1.13. The cyclin D/CDK4 and cyclin D/CDK6 complexes, and the recently identified cyclin E/CKD3 complex stimulate the initiation of G1 phase and are inhibited by the cyclin-kinase inhibitor (CKI) including P15\textsubscript{inkd}, P16\textsubscript{inkd}, P18\textsubscript{inkd} and P19\textsubscript{inkd} (Reed 1996, Donjerkovic and Scott 2000). The increasing levels of cyclin E/CDK2 trigger the transition from G1 phase to S phase and are inhibited by CKI including P21\textsubscript{Cip1}, P27\textsubscript{Kip1} and P57\textsubscript{Kip2} (Ravitz and Wenner 1997). Increasing levels of cyclin A/CDK2, facilitate
the completion of S phase and cells are ready to enter the G2 phase (Woo and Poon 2003). At the beginning of mitosis, cyclin B1 levels are increased compared to the end of mitosis where the levels of cyclin B1 is diminished (Lindqvist, van Zon et al. 2007). Overall, the cell cycle is tight, regulated by the presence of various cyclin and CDKs complexes. In cancer, the changes in CDK activity due to the mutations result in the dysregulation of the cell cycle (Deshpande, Sicinski et al. 2005). In breast cancer, overexpression of cyclin D1 has been reported and is attributed to defective mechanisms of its degradation (Vermeulen, Van Bockstaele et al. 2003).
Figure 1.12: The activation of CDKs is initiated when they bind to their cyclin partner. Cyclin/cdk complexes then phosphorylate and activate other proteins triggering the transition to the next phase of the cell cycle. The phosphorylation of the cyclin/CDKs targets is inhibited by the cyclin kinase inhibitors (CKI). Adapted from: (Malumbres and Barbacid 2009).
Figure 1.13 Cell cycle phases and the cyclin and cyclin-dependent kinases involved in the regulation of cell cycle progression in each phase
Adapted from: (Malumbres and Barbacid 2009).

1.6 ROS and endoplasmic reticulum stress

Endoplasmic reticulum (EnR) is a eukaryotic cellular organelle responsible for lipid and protein biosynthesis. There are two types of endoplasmic reticulum namely rough EnR and smooth EnR. The rough EnR has ribosomes attached to its outer surface, whereas smooth EnR has no ribosomes. The translation of the mRNA takes place in the rough EnR, and the synthesised proteins then migrate to the smooth EnR where quality control of the protein folding and functionality take place, and functional proteins are then transported to the Golgi apparatus for post-translational modifications before being released in the cytoplasm (Healy, Gorman et al. 2009). To preserve appropriate protein-folding and thus their functionality EnR contains many chaperones which carry out the quality control of newly synthesized proteins and refolding of misfolded proteins in a stable Ca\(^+\) concentration environment (Malumbres and Barbacid 2009). Specific enzymes called protein disulphide isomerasers (PDI) are responsible for facilitating the formation of disulphide bonds involved in the refolding, assembly and post-translational modifications of proteins (Kim, Xu et al. 2008).

ROS promote the imbalance in the redox homeostasis in the EnR lumens, which prevents the formation of the disulphide bonds leading to the generation of misfolded
proteins (Kim, Xu et al. 2008). Accumulation of misfolded proteins in the EnR lumen causes stress in the EnR, which is known as EnR stress. To overcome this stress, EnR responds to the situation by trying to remove the misfolded proteins by a mechanism called unfolded protein response (UPR) (Kim, Xu et al. 2008). There are three main sensitisers/activators of the UPR acting at a different stage of the cellular response namely the Inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase (PERK) as shown in Figure 1.14. Initially, under normal conditions, EnR stress regulators remain inactive by binding to a protein called glucose-regulated protein 78 (GRP78). The accumulation of misfolded proteins stimulates the disassociation of GRP78 from the EnR stress initiators, which become active. The first EnR regulator is IRE1, which carries out X-box binding protein (XBP1) mRNA splicing resulting in the synthesis of the functional XBP1 transcription factor, which later enhances the gene expression of EnR chaperones and genes involved in regulating endoplasmic reticulum-associated degradation (ERAD) (Smith 2014). The second EnR stress regulator is ATF6. After its dissociation from GRP78, ATF6 reaches its active state, translocates to the nucleus and upregulates gene expression of the EnR chaperones including GRP78 and glucose-regulated protein 94 (GRP94) (Shen, Chen et al. 2002) and PDI and inhibits protein synthesis. The final EnR stress regulator is PERK that is the faster regulator to become fully active (Liu, Lv et al. 2015). Activated PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α), which attenuates mRNA translation. Activating transcription factor 4 (ATF4) is upregulated promoting GRP78 and GRP94 activity in the EnR.

Figure 1.14: Mechanisms of endoplasmic reticulum stress
Adapted: (Smith 2014).
1.7 Catalase activity and cancer

To maintain redox homeostasis, cells possess an effective antioxidant system preventing oxidative stress or allowing recovery from oxidative injury (Oberley and Oberley 1997) as shown in Figure 1.15. Antioxidant systems consist of antioxidant molecules such as glutathione, vitamin E, C and A, and enzymatic enzymes such as catalase, superoxide dismutase and glutathione peroxidase (Oberley and Oberley 1997, Birben, Sahiner et al. 2012). Catalase is involved in catalysing the reduction of reactive oxygen species and especially hydrogen peroxide to water and oxygen (Scibior and Czeczot 2006) and plays an important role as a defence mechanism against oxidative stress (Izawa, Inoue et al. 1996). Catalase is a tetrameric protein with molecular weight 250kDa consisting of four similar subunits each one of which contains a heme group (Zamocky and Koller 1999, Glorieux, Dejeans et al. 2011). Previous studies have shown decreased catalase levels in a wide variety of cancer cell lines compared to normal cells (Kwei, Finch et al. 2004, Glorieux, Dejeans et al. 2011). The mechanism by which ROS production affects the expression of catalase protein is not known. It has been reported, that catalase and glutathione activities are down regulated in breast cancer cells treated with β-oestradiol (Mobley and Brueggemeier 2004). The potential effects of NQO2 on catalase activity its activity was monitored in β-oestradiol and tamoxifen treated breast cancer cells in the presence or absence of the NQO2 cofactor NRH.

1.8 Glutathione and cancer

Glutathione is a tripeptide composed of L-γ-glutamyl-L-cysteinyl-glycine and plays an important role as the most abundant antioxidant in cells (Birben, Sahiner et al. 2012, Lushchak 2012). Glutathione is expressed in the liver where the majority of detoxification processes occur (Yuan and Kaplowitz 2009) and dysregulation of its activity leads to defective defence against ROS thereby contributing to the development of diseases such as cancer, ageing and abnormal immunological responses (Richie 1992, Droge and Breitkreutz 2000, Hassan, Hadi et al. 2001, Maher 2005). Glutathione exists in the oxidised glutathione (GSSG) and the reduced glutathione (GSH) forms (Zitka, Skalickova et al. 2012). GSH, the most abundant nonprotein thiols is ROS scavengers and the ratio between GSH and GSSG is used as indication of oxidative stress (Zitka, Skalickova et al. 2012, Lu 2013). In the event of
oxidative stress, GSH is utilised to detoxify reactive oxygen species by the glutathione peroxidase leading to the formation of GSSG (Nakamura, Nakano et al. 1994, Owen and Butterfield 2010) as shown in Figure 1.15. Upon oxidation, GSSG is reduced back to two molecules of GSH by glutathione disulphide reductase, using NADPH as an electron donor (Couto, Malys et al. 2013). The beneficial effects of cycling between GSH and GSSG are mainly to remove free radicals produced and to protect cells from oxidative stress (Gilmore and Kirby, 2004, Neal et al., 2003, Whitekus et al., 2002 and Winiarska et al., 2003). Reduction of the GSH/GSSH ratio affects other cellular events including cell signaling (Filomeni et al., 2002, Goldstone et al., 1996 and Sen, 2000), apoptosis (Pias and Aw, 2002 and Takahashi et al., 2005) cellular growth and differentiation (Kim et al., 2004 and Menon et al., 2003). Elevated levels of glutathione are reported in breast cancer cells compared to normal cells (Ye and Zhang 2001, Balendiran, Dabur et al. 2004). The potential effects of NQO2 on GPx activity, were monitored in β-oestradiol and tamoxifen treated breast cancer cells in the presence or absence of the NQO2 cofactor NRH.

Figure 1.15 shows the functions of the antioxidant enzymes catalase, glutathione peroxidase and quinone oxidoreductase by detoxifying reactive oxygen species hydrogen peroxide. The expression of NQO2 is downregulated by oestrogen and upregulated by tamoxifen (Montano, Bianco et al. 2005).
Figure 1.15: Metabolism of catalase, glutathione peroxidase and NQO2 in detoxifying reactive oxygen species hydrogen peroxide.
Adapted from: (Montano, Bianco et al. 2005).
1.9 Aims and hypothesis

The NQO2 antioxidant enzyme is involved in the detoxification of quinone by reducing quinone to hydroquinone. Quinones are unstable products and are produced from oestrogen metabolism known as catechol oestrogen quinones. The oestrogen metabolites are removed after conjugating with GSH a reaction that is catalyzed by GSH-transferases (GST). The molecular mechanisms mediating the crosstalk between NQO2 and oestrogen hormone are unknown and therefore this project aims to investigate these mechanisms. The biological role of NQO2 in oestrogen receptor positive and oestrogen receptor negative breast cancer cells is explored through the involvement of NQO2 in quinone detoxification and its potential role in mediating cellular redox homeostasis in breast cancer cells treated with β-oestradiol and tamoxifen in the absence and presence of NRH.

The objectives of this research are to explore whether the NQO2 antioxidant effects are direct or indirect through NQO2 mediated regulation of redox responsive transcription factors or antioxidant enzymes, to investigate the potential NQO2 antioxidant effects by determining the effects of NQO2 overexpression on catalase, glutathione levels and glutathione peroxidase in β-oestradiol and tamoxifen treated breast cancer cells and to shed light on the effects of NQO2 on oxidative stress, reactive oxygen species generation, mitochondrial membrane potential and cell cycle control.
CHAPTER 2

Materials and Methods
2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines

The human breast cancer cell lines: MCF-7, MDA-MB-231, MDA-MB-468, T47D and MDA-MB-157 cells were purchased from the European Collection of Cell Culture (ECACC). MCF-7 and T47D cells are ERα-positive and MDA-MB-157, MDA-MB-231 and MDA-MB-468 cells are ERα-negative breast cancer cells. MCF-7 harbour wild-type p53, T47D MDA-MB-231 and MDA-MB-468 express mutated p53 and MDA-MB-157 cells are p53 defective.

The descriptions of the cell lines used in this study are listed in details in Table 2.1.

Table 2.1: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Immunoprofile</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7 (MCF-7) is a human breast adenocarcinoma cell line derived from pleural effusion isolated in 1970 from a 69-years old Caucasian woman with metastatic mammary carcinoma (Soule, Vazquez et al. 1973).</td>
<td>ER+, PR+/−, HER2− (Neve, Chin et al. 2006)</td>
</tr>
<tr>
<td>T47D</td>
<td>Human breast adenocarcinoma cell lines isolated from the pleural effusion of a 54-years old patient (Keydar, Chen et al. 1979).</td>
<td>ER+, PR+/−, HER2− (Neve, Chin et al. 2006)</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>Human breast adenocarcinoma cell lines derived from the pleural effusion of a 44-years old patient (Young, Cailleau et al. 1974).</td>
<td>ER−, PR+, HER2− (Neve, Chin et al. 2006)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human breast adenocarcinoma cell lines isolated from the pleural effusion of a Caucasian patient aged 51-years old (Cailleau, Young et al. 1974).</td>
<td>ER−, PR+, HER2− (Holliday and Speirs 2011)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Human breast adenocarcinoma cell lines isolated in 1977 from the pleural effusion of a patient aged 51-years old (Brinkley, Beall et al. 1980).</td>
<td>ER−, PR−, HER2− (Neve, Chin et al. 2006).</td>
</tr>
</tbody>
</table>
2.1.2 Chemicals and reagents

All chemicals and reagents used in this research were bought from Sigma-Aldrich, Merck Millipore and ThermoFisher Scientific, UK as listed in Table 2.2

Table 2.2: Chemicals and reagents used in this study

<table>
<thead>
<tr>
<th>Chemicals/ Reagents</th>
<th>Catalogue Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-oestradiol</td>
<td>E8875</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>T5648</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>PBS solution</td>
<td>D8537</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Trypan blue solution, 0.4%</td>
<td>15250061</td>
<td>Thermo Fisher Scientific, UK</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>A5354</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>M6250</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Sodium phosphate, 96%</td>
<td>342483</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂)</td>
<td>1374248</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>2-Nitrophenyl-β-D-galactopyranoside</td>
<td>N1127</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>PHR1309</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Dithiothreitol (DTT) ≥99.9%</td>
<td>D0632</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>PMSF-RO</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Protease inhibitors (PI)</td>
<td>P8340</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>β-glycerol phosphate (βGP) ≥99.9%</td>
<td>G9422</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Glycerol ≥99.9%</td>
<td>G5516</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>S6422</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Sodium orthovanadate (Na₃VO₄)</td>
<td>S6508</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Ethanol absolute</td>
<td>107017</td>
<td>Merck Millipore, UK</td>
</tr>
<tr>
<td>Ethylenediaminetetra acetate acid (EDTA)</td>
<td>ED4SS</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Nonidet P40 substrate</td>
<td>74385</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>A3553</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>A3678</td>
<td>Sigma Aldrich, UK</td>
</tr>
</tbody>
</table>
Calcium chloride  C1016  Sigma Aldrich, UK
Methanol  106009  Merck Millipore, UK
Isopropanol  109634  Merck Millipore, UK
Reporter lysis buffer 5x  E3971  Promega, UK
Luciferin reagent  V8921  Promega, UK
Dissociation buffer  1351014  Gibco, UK
Immobilon-P 26.5x3.75m Roll  IPVH00010  Merck Millipore, UK
PVDF .45um
Tetramethylethylenediamine (TEMED)  GE17-1312-01  GE Healthcare, UK
PageRuler™ Plus Prestained Protein Ladder  26619  ThermoFisher Scientific, UK
Propidium iodide (PI)  P4170  Sigma-Aldrich, UK
Ribonuclease A from bovine pancreas  R6513  Sigma-Aldrich, UK
H2DCFDA  D399  Invitrogen, UK
Cycloheximide (CHX)  C7698  Sigma-Aldrich, UK

2.1.3 Media and molecular biology reagents

All the media and molecular biology reagents used in this research were bought from Sigma-Aldrich, ThermoFisher Scientific, Invitrogen, Merck Millipore, UK and Chemometec, Denmark as listed in Table 2.3.

Table 2.3: Culture medium and biological reagents used in this study

<table>
<thead>
<tr>
<th>Media and other biological materials</th>
<th>Catalogue Number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified Eagle medium (DMEM)</td>
<td>D5796</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Leibovitz’s medium (L-15)</td>
<td>11415-049</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>Roswell park memorial institute (RPMI) 1640</td>
<td>R0883</td>
<td>Sigma Aldrich, UK</td>
</tr>
<tr>
<td>Foetal bovine serum (FBS)</td>
<td>F4135</td>
<td>Sigma-Aldrich, UK</td>
</tr>
</tbody>
</table>
Penicillin-streptomycin (P/S) solution  P4333  Sigma-Aldrich, UK
Trypsin-EDTA solution  T4299  Sigma-Aldrich, UK
PolyFect transfection reagent  301107  Qiagen, UK
Plasmid Maxi Kit  12162  Qiagen, UK
RNeasy Plus Mini Kit  74134  Qiagen, UK
SYBR-Green I  204154  Qiagen, UK
Catalase Assay Kit  707002  Cayman chemical, UK
Glutathione Peroxidase Assay Kit  703102  Cayman chemical, UK
Clarity™ Western ECL Substrate  1705060  Bio-Rad, UK
SuperSignal™ WestPico  34080  ThermoFisher
Chemiluminescent Substrate  34080  Scientific, UK
Solution 5 (VB48.PI.AO)  910-3005  Chemometec, Denmark
Solution 7 (JC-1)  910-3007  Chemometec, Denmark
Solution 8 (DAPI.PBS)  910-3008  Chemometec, Denmark
MitoTracker Deep Red FM  M22426  ThermoFisher Scientific, UK
Fluoroshield™ with DAPI  F6057  Sigma-Aldrich, UK

### 2.1.4 Buffers and general solutions

All buffers and general solutions used in this research are listed in Table 2.4

**Table 2.4: Buffers used in this study**

<table>
<thead>
<tr>
<th>Chemicals/ Reagents</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-gal buffer</td>
<td>200mM Sodium phosphate pH: 7.3, 2mM MgCl₂, 100mM β-mercaptoethanol, 4.5mM ONPG.</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10mM Tris-HCL (pH7.5) and 1mM EDTA.</td>
</tr>
<tr>
<td>3x SDS</td>
<td>187mM Tris, 30% Glycerol, 6% SDS, 15% 2-mercaptoethanol and 0.01% bromophenol blue.</td>
</tr>
</tbody>
</table>
High salt lysis buffer 45mM HEPES (pH 7.5), 400nM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 1mM DTT, 1mM PMSF, 1x PI, 2mM NaOV, 20mM β-glycerol phosphate, 5mM NaPi and dH2O

Running buffer 25mM Tris, 190mM Glycine, 35mM SDS and 20% Methanol

Western buffer 25mM Tris, 190mM Glycine and 20% Methanol

Stripping buffer 100 mM 2-Mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl pH 6.7.

2.1.5 Primary antibodies

All primary antibodies used in this research were bought from Abcam, Santa Cruz, ThermoFisher Scientific and BD Biosciences, UK as listed in Table 2.5

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Catalogue number</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-NQO2</td>
<td>Ab137612</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Mouse monoclonal anti-Oestrogen receptor alpha (C-311)</td>
<td>Sc-787</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-Cyclin D1 (Clone SP4)</td>
<td>RM-9104</td>
<td>Thermo Scientific, UK</td>
</tr>
<tr>
<td>Mouse monoclonal anti-GRP 94</td>
<td>Sc-53929</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Mouse monoclonal anti-GRP 78</td>
<td>Sc-166490</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-PDI (H-160)</td>
<td>Sc-20132</td>
<td>Santa Cruz, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-β-actin</td>
<td>Ab8227</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>APC-H7 conjugated CD20 antibody</td>
<td>641396</td>
<td>BD Biosciences, UK</td>
</tr>
</tbody>
</table>
2.1.6 Secondary antibodies

Secondary antibodies used in this research were bought from GE Healthcare and ThermoFisher Scientific, UK as listed in Table 2.6.

Table 2.6: Secondary antibodies used in this study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Species (Origin)</th>
<th>Catalogue number</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECL Anti-Rabbit IgG, HRP-linked whole Ab</td>
<td>Donkey</td>
<td>NA934</td>
<td>GE Healthcare, UK</td>
</tr>
<tr>
<td>ECL Anti-Mouse IgG, HRP-linked whole Ab</td>
<td>Sheep</td>
<td>NXA931</td>
<td>GE Healthcare, UK</td>
</tr>
<tr>
<td>Anti-rabbit IgG, Alexa Flour 488</td>
<td>Goat</td>
<td>A-11034</td>
<td>ThermoFisher, Scientific, UK</td>
</tr>
</tbody>
</table>

2.1.7 Primer used for qRT-PCR

The gene-specific primers used in this project were designed using Primer3 Input software. The primers designed are following the certain characters such as; the primer Tₘ is +/- 60°C, with sequence length 15-18 bases and GC content 40-60%. The primer with these characters, generated a short PCR product of between 60-150 base pairs. The primers used in this project were CCND1, PDIA1 and RPL19 genes. RPL19 was used as the control gene. The PCR products produce from CCND1 is 117 base pairs, from PDIA1 is 101 base pairs and RPL19 is 122 base pairs. The primers design and were purchased from Sigma-Aldrich, UK.

The details of the primer sequences are listed in Table 2.7.

Table 2.7: Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCND1</td>
<td>ATGTGTGCAGAAGGAGGTCC</td>
<td>AGGTagGTCATGGCCAGGC</td>
</tr>
<tr>
<td>PDIA1</td>
<td>GGTGGAGTTCTATGCCCCCTT</td>
<td>ACCTGATCTCGGAACCTTCT</td>
</tr>
</tbody>
</table>
2.1.8 Plasmids

The plasmids used in this research are listed in Table 2.8

Table 2.8: Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCDNA3</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>pCMV6-NQO2</td>
<td>Origene, UK</td>
</tr>
<tr>
<td>ERE-luciferase reporter</td>
<td>Kindly provided by Dr Costas Demonacos</td>
</tr>
<tr>
<td>CMV-β-gal</td>
<td>(Zamanian and La Thangue 1993)</td>
</tr>
<tr>
<td>pCMV-CD 20</td>
<td>(Demonacos, Krstic-Demonacos et al. 2004)</td>
</tr>
</tbody>
</table>

2.2 METHODS

2.2.1 Cell culture

2.2.1.1 Cell thawing

In the thawing process, cells were collected from the liquid nitrogen and thawed out by incubation at 37°C in a water bath. Cells were resuspended in 10ml of complete culture medium and centrifuged at 1,000rpm for three minutes. The supernatant was aspirated and cell pellets were resuspended in 10ml complete medium. The human breast cancer cell lines were suspended with appropriate medium containing foetal bovine serum (FBS) supplemented with 1% penicillin/streptomycin (P/S) and transferred to T25 cm² flask until they reached 70-80% confluency. MCF-7, MDA-MB-231 and MDA-MB-468 cells were cultured in DMEM supplemented with 10% v/v FBS and 1% v/v P/S, the T47D cells were cultured in RPMI supplemented with 10% v/v FBS, 1% v/v L-glutamine and 1% v/v P/S. Finally, the MDA-MB-157 cells were cultured in L-15 supplemented with 15% v/v FBS and 1% v/v P/S. All cell lines were grown in a humidified incubator in the presence 5% CO₂ at 37°C.
2.2.1.2 Cell passaging

Cells in T75 flasks were washed twice with phosphate-buffered saline (PBS) solution and incubated with 3ml of trypsin/ ethylenediaminetetraacetic acid (EDTA) solution for three minutes in the CO₂ incubator. The flask was tapped gently and checked under the microscope to make sure all cells were detached from the flask’s wall. When all cells were detached, 7 ml of complete culture medium was mixed well with cells by pipetting to prevent the formation of cells clumps. The cells mixture was then transferred to a fresh labelled flask. The flask was placed back in the incubator until cells reached 80% confluency when they were passaged again to be maintained.

2.2.1.3 Cell counting

Cell counting was performed using haemocytometer with a light microscope according to the manufacturer’s instruction (Bright-line, Hausser Scientific, USA). Cell counting is an important step in cell culture, ensuring consistent cell numbers and allowing comparisons and accuracy of the experiment (O’Shaughnessy 2015). 100μL of the cells were added to 400μL of 0.4% Trypan blue making the dilution factor is 1:5. Trypan blue solution was used as a stain to dye dead cells which become visible under a light microscope. 10 μl of Trypan blue-treated cell suspensions was loaded in a haemocytometer and projected to the microscope for cell counting. The counting was performed by counting cells individually in four squares (A, B, C and D) in each corner of the haemocytometer ruler as shown in Figure 2.1. An average number of cells were determined as shown below:

\[
\text{Average (N)} = \frac{A+B+C+D}{4}
\]

The average cells counted (N) multiplied by 10,000 \( (10^4) \) and multiply by 5 because of the dilution from the Trypan blue addition.

Total number of cells = \( N \times 10^4 \times 5 \)
Figure 2.1: Haemocytometer grid lines
The Haemocytometer grid lines consist of four set of 16 squares used in the cell counting. (Adapted from: Bright-line, Hausser Scientific).

2.2.1.4 Cell treatments

The β-oestradiol, tamoxifen, dihydronicotinamide riboside (NRH), combination of β-oestradiol and NRH and combination of tamoxifen and NRH were added directly to the cell culture medium. The duration of all treatments was 24 hours and the concentrations are indicated in Table 2.9.

β-oestradiol, is a steroid hormone playing important role in the regulation of menstrual female reproductive cycle. It is produced in the ovary and body tissues including liver, breast and neural. It is known as an agonist of both ERα and ERβ, as it potently binds to and activates these receptors. The activation of the oestrogen receptor modulates the expression of various genes including genes involved in increased cell proliferation (Darbre, Yates et al. 1983). As β-oestradiol induces growth rate, it was used in this project to study the growth of the oestrogen-dependent breast cancer cells and understand the effects of this hormone in the oestrogen-independent breast cancer cells.
Tamoxifen is a selective oestrogen response modifier (SERM), protein kinase inhibitor and anti-angiogenetic factor. It is a prodrug, metabolised by cytochrome P450 isoforms CYP2D6 and CYP3A4 forming the metabolites 4-hydroxytamoxifen (4-OHT) and endoxifen (Smith 2013). In breast cancer, tamoxifen plays major role in blocking oestradiol-stimulated VEGF production in breast tumour cells. It is used in this study to inhibit the oestrogen receptor in breast cancer cells.

The NRH was synthesised in house by Dr Sally Freeman’s medicinal chemistry research group. It was reported that the most compatible co-substrate for NQO2 is NRH (Vella, Ferry et al. 2005).

The compounds’ names, concentrations and incubation times used in this project are shown in the Table 2.9.

Table 2.9 Drugs concentration and incubation time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock concentration</th>
<th>Working concentration</th>
<th>Incubation time</th>
<th>Solvent used to dilute</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-oestradiol</td>
<td>100µM</td>
<td>100nM</td>
<td>24 hours</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>1mM</td>
<td>10µM</td>
<td>24 hours</td>
<td>Ethanol</td>
</tr>
<tr>
<td>NRH</td>
<td>100µM</td>
<td>100nM</td>
<td>24 hours</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

2.2.2 Plasmids preparation

2.2.2.1 Luria Broth and LB plates preparation

Luria Broth (LB) was created by Giuseppe Bertani in 1952 and it was used to enhance bacteria growth due to its high content of nutrients. To prepare LB broth, 20g of LB powder was dissolved in a litre of distilled water and autoclaved at 15 psi, 121°C for 15 minutes. A 50 µg/ml of ampicillin was added to the LB broth when the temperature of the broth solution dropped to room temperature.

The same protocol was used to prepare LB agar plates where, 37g pre-mixed LB-agar powder was dissolved in one litre of distilled water and autoclaved at 15 psi, 121°C for 15 minutes. After the solution was allowed to cool down at room temperature, 50
μg/ml of ampicillin was added to the LB broth. Approximately, 10 ml of the broth was poured into sterile 90 cm Petri dishes and allowed to solidify before it was stored at 4°C for long term storage.

2.2.2.2 Preparation of DH5α competent cells

The DH5α competent cells were prepared using the calcium chloride method. DH5α cells were collected from the frozen glycerol stock by scraping off a portion from the vial using an inoculating loop and were streaked onto an LB agar plate in sterile conditions. The plate was then incubated for 16 hours in an incubator at 37°C. The next day, a single colony was picked and inoculated in 5 ml of LB medium and incubated overnight at 37°C with shaking at 250 rpm. Next day 1 ml of the culture was added to sterile 100 ml LB medium and incubated in a shaker at 37°C for another 2-3 hours or until OD600=0.4. The mixture was placed in an ice bath for 10 minutes before it was centrifuged at 5000 rpm for 10 minutes at 4°C. Later, the cells were centrifuged again at 5000 rpm for 10 minutes at 4°C. 40 ml of ice-cold 100mM CaCl₂ with 15% glycerol was added to the cells pellet and was kept in 4°C for 12 hours. The cells were transferred to the liquid nitrogen and then immediately in a -80°C for longer storage.

2.2.2.3 Transformation of competent bacteria

In order to amplify the yield of the NQO2, CD20 and ERE-luciferase plasmids those plasmids were transformed into DH5α competent cells. 50 ng from each one of the plasmid DNA was mixed with 200 μl suspension of DH5α competent cells using the pipette tip and swirling occasionally. The mixture was then incubated on ice for 30 minutes before being heat shocked at 42°C for 45 seconds. Samples were incubated on ice for 2 minutes. Then, 500 μl of LB medium was added and incubated for 30 minutes at 37°C using shaker at 150 rpm. Next, 100 μl of the transformation mixture was streaked using sterile “hockey stick” onto LB agar plates containing ampicillin. The plates were kept upside down in an incubator for 16 hours at 37°C for the growth of colonies containing the plasmid of interest. The next day, a single colony of the plasmid of interest was picked and inoculated in 100 ml of LB medium and incubated overnight at 37°C with shaking at 250 rpm. Next day the broth mixture containing the
transformation of DNA proceeded with the maxi prep protocol as described in section 2.2.2.5.

2.2.2.4 Plasmids glycerol stock preparation

Glycerol stocks for each one of the plasmids were prepared for long-term storage. Firstly, an individual colony from the transformation plates was inoculated and placed in 2mL of LB broth with ampicillin and incubated at 37°C using shaker at 150 rpm for 5-6 hours. 500 µl of culture was added to 500 µl of LB medium together with 50% of glycerol. The glycerol stocks were stored in -80°C for longer storage.

2.2.2.5 Plasmids purification using Maxiprep protocol

Maxiprep plasmid purification is a process used to purify plasmids expressing the genes of interest. The process was performed according to manufacturer’s instructions (Qiagen, UK). A single colony was picked using sterile inoculation loop from LB agar plate to 150 ml of LB broth containing ampicillin and incubated in a shaker at 37°C overnight or at least 16 hours when bacteria reached the stationary phase of growth curve. This is the phase when the bacterial growth has reached the plateau or steady state, and this time is the most appropriate to harvest the cells and achieve the highest yield. The mixture was centrifuged using SLA 1500 rotor (6000 rpm) at 4°C for 10 minutes. Bacteria pellets were re-suspended in 10 ml of cold buffer P1 containing RNase A solution. 10 ml of buffer P2 was added and mixed well, and the mixture was incubated at room temperature for 5 minutes. Next, 10 ml of chilled buffer P3 was added and mixed immediately by inverting the tube 4-6 times and incubated on ice for 20 minutes. The bacterial debris was precipitated by centrifuging at 12000 rpm for 1 hour.

To collect and purify the DNA, spin columns provided by the manufacturer were used and before that the columns were equilibrated with 10 ml of buffer QBT. The spin columns are equipped with a silica resin that selectively binds to the DNA. The bacterial cell lysate containing the plasmid DNA was loaded on the columns the DNA was retained in the columns, which were washed with 30 ml buffer QC twice. DNA was eluted from the columns with 15 ml of buffer QF in a centrifuge tube and was precipitated by the addition of 10.5 ml isopropanol. To collect the DNA in the pellet,
the QF isopropanol solution was centrifuged at 11,000 rpm for 30 minutes at 4°C. The pellet was washed with 70% ethanol air-dried for 5-10 minutes and re-dissolved with 10mM Tris-Cl, pH 8.5. The concentration of DNA was measured using Nanodrop spectrophotometer ND-1000 at 260nm (ThermoFisher Scientific, UK). The purity of DNA was assessed by observing the 260/280nm ratio. A pure sample of DNA has a A260/A280 ratio of 1.8+/−0.05 and a pure sample of RNA has a A260/A280 ratio of 2+/−0.05.

**Figure 2.2: Typical UV absorbance spectrum of purified nucleic acid**
The purity of DNA and RNA was measured using Nanodrop spectrophotometer ND-1000 by measuring the ratio of absorbance at 260nm to 280nm. The readings for pure DNA solutions give a ratio of ~1.8 and for pure RNA a ratio of ~2.0. (Adapted from: ThermoFisher Scientific).

### 2.2.2.6 Plasmids transient transfection

Transient transfection was carried out using the PolyFect transfection reagent according to manufacturer’s guidelines (Qiagen Inc., UK). Before the transfection, 4 x 10^5 cells were seeded in 6-well plates and incubated at 37°C in the presence of 5% CO₂ until they reached 40-80% confluency. 1.5µg of DNA was dissolved in 100 µl of appropriate media. 6µl of PolyFect transfection reagent was added to the cells and was mixed well before incubated at room temperature for 5-10 minutes to allow complex formation between the PolyFect reagent and plasmid DNA. While incubating the DNA
complex, cells plates were washed once with 4 ml PBS, and the medium was replaced with 1.5 ml of fresh cell growth medium containing FBS and P/S. The complex formation reaction was stopped by adding 0.6 ml of cell medium containing FBS and P/S. Cells were incubated with the complexes at 37°C and 5% CO₂ in an incubator for 24 hours to allow gene expression.

2.2.3 Luciferase reporter assays

Luciferase assay is based on the principle that luciferase enzyme catalyses the oxygenation of D-luciferin to oxyluciferin using ATP and molecular oxygen producing a photon of light which can be detected at 500 to 630nm wavelength using spectrophotometer (Smale 2010). The figure in 2.3 illustrates the luciferin mechanism of action. Transiently transfected and treated cells were washed twice with ice-cold PBS solution and incubated with 100 µl of 1x reporter lysis buffer (Promega, UK) at room temperature for 30 minutes on a shaker to initiate cells lysis. After the incubation, cells were scraped using policeman rubber cell scrapers and transferred to fresh 1.5mL labelled tubes. Cells were centrifuged at 13,000rpm at 4°C for 15 minutes, and the supernatant was collected and used to measure luciferase reporter reading. 10 µl of the samples were transferred to new labelled luminometer tubes, and 100 µl of luciferin reagent (Promega, UK) was added and the luciferase reading was measured using the Lonza luminometer at 562 nm wavelength (Lonza, Switzerland). The β-galactosidase activity was also measured to determine the transfection efficiency (Smale 2010).

β-galactosidase is an enzyme that cleaves lactose to glucose and galactose (Smale 2010, Juers, Matthews et al. 2012). The synthetic compound onitrophenyl-β-D-galactoside (ONPG) is also a β-galactosidase substrate and is cleaved to yield galactose and o-nitrophenol which has a yellow colour whose absorbance can be measured at 420nm. The conversion of ONPG to o-nitrophenol is proportional to the concentration of β-galactosidase enzyme. Transient transfection of the β-galactosidase plasmid together with the ERE-luciferase reporter plasmid was carried out and the β-galactosidase activity was used as a reference to ensure the transfection efficiency was taken into account and did not interfere with the results. 30 µl of cells lysates was mixed with 300 µl of freshly prepared β-galactosidase buffer. The mixture was incubated for 1 hour at 37°C or until yellow colour was visible. The β-galactosidase
reactions were stopped by adding 500 μl of distilled water and the absorbance of the samples was measured at OD 420nm using a spectrophotometer.

![Diagram of luciferase assay](image)

**Figure 2.3: Principle of luciferase assay**

(A) Light is emitted from the reaction of luciferase enzyme with the luciferin in the presence of ATP and oxygen. (B) Model of luciferase reporter assay. (Adapted from: Thermo Fisher Scientific).

2.2.4 Western blot analysis

2.2.4.1 Cell lysate preparation

Cells were grown in 6-well plates and before harvesting the medium was removed and cells were washed with 3 ml of cold PBS. PBS was then aspirated and 100 μl of high
salt lysis buffer (HSL) containing 1M DTT, 100 mM PMSF, 1 mg/ml 1000xPI, 500 mM βGP, 200 mM NaPPi and 1M NaOV were added to lyse the cells. The mixture in the well was scraped gently lysates were collected into labelled eppendorf tubes, and placed on ice. Cells were lysed by rotation for 20 minutes at 4°C and lysates were centrifuged at 4°C for 15 minutes at 12,000 rpm. The supernatants were collected into fresh eppendorf tubes and the pellet was discarded. Supernatants prepared were used to carry out immunoblotting experiments.

2.2.4.2 Determination of protein concentration

Protein concentration was measured using the Bradford assay. A calibration curve was prepared using known concentrations of BSA. Each cuvette contained 800 µl of dH₂O and 200 µl of Bio-Rad reagent. Sample cuvettes were prepared by adding 800 µl of dH₂O, 200 µl of Bio-Rad reagent and 2 µl of the cellular extract. The mixture was incubated at room temperature for 3-5 minutes, and the absorbance at 595 nM was recorded.

Protein concentration recorded as explained above and the lowest absorbance was selected as 40 µl and the rest of the samples were normalised to this calculation to ensure equal loading of protein concentration from each one of the samples; the volume loaded was calculated according to the formula below:

\[
\text{Loading amount of sample X} = \frac{40 \times \text{Absorbance of the lowest value sample}}{\text{Absorbance of X}}
\]

Before loading, 3X SDS buffer was added to the cellular extracts, and the mixture was boiled at 95°C for 3 minutes before loading. After boiling, the mixture was centrifuged at 13,000 rpm for 10 seconds, and then polyacrylamide gel electrophoresis (PAGE) was performed. Electrophoresis was run at 80V the first 20 minutes until the samples entered the resolving gel and the voltage was increased to 120V for the rest of the running.
2.2.4.3 SDS-PAGE gel preparation

Electrophoresis was used to resolve individual proteins in the sample lysates based on their molecular weight. The gel percentages of 7.5% or 10% were used depending on the size of the proteins of interest. Gels were prepared as shown below:

Table 2.10: Table showing the recipes for preparing SDS-PAGE resolving and stacking gels for 7.5% and 10%

<table>
<thead>
<tr>
<th>Solutions</th>
<th>7.5% Resolving</th>
<th>7.5% Stacking</th>
<th>10% Resolving</th>
<th>10% Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>13.3 ml</td>
<td>6.73 ml</td>
<td>10.94 ml</td>
<td>6.73 ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>7 ml</td>
<td>1.67 ml</td>
<td>9.33 ml</td>
<td>1.67 ml</td>
</tr>
<tr>
<td>1.5M Tris pH 8.95</td>
<td>7 ml</td>
<td>-</td>
<td>7 ml</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris pH 6.95</td>
<td>-</td>
<td>1.25 ml</td>
<td>-</td>
<td>1.25 μl</td>
</tr>
<tr>
<td>0.2M EDTA</td>
<td>280 μl</td>
<td>100 μl</td>
<td>280 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>280 μl</td>
<td>100 μl</td>
<td>280 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>157 μl</td>
<td>100 μl</td>
<td>157 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>17 μl</td>
<td>10 μl</td>
<td>17 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

2.2.4.4 Western blot

Proteins were transferred from the polyacrylamide gel to PVDF membrane (Millipore, UK) using transfer cassette by creating a stalk “sandwich” in a tank contained transfer buffer. The cassette contained two faces one black negatively charged (cathode) and one white positively charged (anode). The sandwiches were arranged as shown below:

i. Cassette (Black-end)
ii. Sponge
iii. Blotting paper
iv. Gel
v. PVDF membrane
vi. Blotting paper
vii. Sponge
viii. Cassette (White-end)

The transfer was carried out at 0.4A for 90 minutes with the ice pack and stirrer in the tank. When proteins had been transferred, a membrane with the proteins transferred
was incubated with 5% fat-free dry milk in PBS (v/v) for 1 hour to block non-specific binding of the antibody to the membrane. The membrane was then incubated with primary antibody in 2.5% milk in PBS-0.1% Tween-20 (v/v) overnight at 4°C.

**Figure 2.4**: The arrangement of sandwich in western blotting (Mahmood and Yang 2012).

### 2.2.4.5 Immunoblotting

After incubation with appropriate primary antibody, the membranes were incubated with appropriate secondary antibody either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase in 2.5% milk in PBS-0.1% Tween-20 (v/v) for 1 hour. Membranes were washed with PBS-0.1% Tween-20 three times 10 minutes each time. 3.0 ml of ECL reagent (Bio-rad, UK) was used to visualise the proteins of interest on the membranes using the Chemi Doc MP imaging system (Bio-Rad, UK).

### 2.2.4.6 Densitometric analysis

Image J 1.38e software was used to quantify the intensity of the protein bands of interest. The values obtained from the measurements of the intensity of the bands representing the proteins of interest were normalised to the respective intensity of the actin band.
2.2.4.7 Membrane stripping

To remove the primary antibodies, the membranes were stripped to discard the previous antibody and the membrane was used to detect other protein of interest using specific antibodies recognizing these proteins. Sufficient amount of mild stripping buffer to cover the surface of the membranes was added and incubation at room temperature for 10 minutes twice with slight shaking followed. The membranes were then incubated with PBS twice for 10 minutes at room temperature on a shaker to wash off the stripping buffer. Next, the membrane was incubated twice with 0.05% of PBS/Tween20 (v/v) for 5 minutes and vigorous shaking. Lastly, the polyvinylidene fluoride (PVDF) membranes were washed with PBS for 5 minutes at room temperature on a shaker.

2.2.5 mRNA levels

2.2.5.1 RNA extraction

The treated cells were harvested and total RNAs were extracted using the RNeasy plus mini kit and QIAshredder (Qiagen, UK) following the manufacturer’s instruction. Prior to the treated cells harvesting, cells were trypsinated and cells were isolated by centrifugation at 1,300rpm for 3 minutes. The supernatants were discarded and cells were lysed with 350µl of RLT buffer. The cells were transferred to a QIAshredder spin column and centrifuged for 2 mins at 1200 rpm. The lysed and homogenised cells were transferred into a gDNA Eliminator spin column and centrifuged for 30 seconds at 8000 rpm. 700µl of 70% ethanol was added to the lysates and transferred to the RNeasy spin column tube and centrifuged at 13,000rpm for 15 seconds. Flow-through was discarded and 700µl of RW1 buffer was added to the RNeasy spin column and centrifuged at 13,000rpm for 15 seconds. The flow-through was discarded and 500µl of RPE buffer was added twice to the column and centrifuged at 13,000rpm for first at 15 seconds then at 2 minutes. 50µl RNase-free water was added to the column and RNA was eluted by centrifugation at 13,000 rpm for 1 minute. RNA concentration was measured using Nanodrop spectrophotometer ND-1000 as described in the Section 2.2.2.3.
2.2.5.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

The extracted RNA prepared in the section 2.2.5.1 was used to measure the mRNA level of specific genes using QuantiFast® SYBR® Green one step RT-PCR kit following the manufacturer’s instruction (Qiagen, UK). This method allows both reverse transcription and PCR amplification to take place in the same reaction mix. The one step RT-PCR reactions were prepared in 96-well plates as shown in Table 2.11. The RNA was first converted to cDNA by the reverse transcriptase (Quantifast RT mix) which consists of omniscript and sensiscript. Both enzymes exhibit high RNA affinity. After the reverse-transcription, the DNA polymerase enzyme was activated by initial heating at 95°C for 5 minutes. The heating inactivated the reverse-transcriptase. Denaturation step followed at 95°C for 10 seconds allowing the double-stranded template DNA to be separated into two single strands. Then, annealing and extension steps took place at 60°C for 30 seconds. The annealing step allowed the binding of primers to the template DNA. The steps were repeated for 40 cycles using the real-time PCR system (Applied Biosystem, UK). The PCR thermocycling setting is shown in Table 2.12.

The fluorescence signal produced by SYBR green was detected at excitation and emission 494 nm and 521 nm wavelength, respectively (Bustin and Mueller 2005). (Hunzicker-Dunn and Mayo 2015).

The qRT-PCR amplification curve shows the fluorescence signal versus cycle number, where examples of two samples shown in Figure 2.5. The values are recorded as C_t value. Low fluorescence signals were detected at the initial cycle below the baseline, and increasing copy of PCR products increases the fluorescence signal above the baseline.
Table 2.11 Reaction setup of qPCR was prepared as shown below

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Quantifast SYBR RT-PCR</td>
<td>5</td>
<td>1x</td>
</tr>
<tr>
<td>Master mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.4</td>
<td>1µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.4</td>
<td>1µM</td>
</tr>
<tr>
<td>Quantifast RT mix</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Template RNA</td>
<td>4</td>
<td>100ng</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.12: Thermocycling programme for qPCR reaction

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>50°C</td>
<td>10 min</td>
</tr>
<tr>
<td>PCR initial activation step</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Two-step cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>10s</td>
</tr>
<tr>
<td>Combined annealing/extension</td>
<td>60°C</td>
<td>30s</td>
</tr>
<tr>
<td>Cycles</td>
<td>35-40</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2.5: The principle of qRT-PCR data analysis
Adapted from: Qiagen handbook, UK

Figure 2.6: The qRT-PCR melting curve
Adapted from: Qiagen handbook, UK
2.2.6 Flow cytometry

Fluorescence-activated cell sorting (FACs) was used to measure reactive oxygen species generation and cell cycle profiles in breast cancer cells upon various treatments. The principle of FACs technique is shown in Figure 2.7. Untreated and treated single cells pass through sheath fluid and cells are directed to the light beam system in the flow cytometer which distinguishes single cells in a population and sorts them in different categories by evaluating their specific light scattering and fluorescent emission characteristics. Single cell suspension is injected into sheath and cells are intersected across argon ion laser. Cells characteristics are recorded by signals emitted on a forward light scatter detector and multiple fluorescent emission detectors, which can then be translated into digital format and computationally analysed (Brown and Wittwer, 2000) using the Summit v4.3 software.

![Figure 2.7: Principle of FACs](image)

Adapted from: (Brown and Wittwer 2000)
2.2.6.1 ROS generation measurement

Reactive oxygen species (ROS) in cells treated with β-oestradiol and tamoxifen in the absence and presence of NRH and NRH alone as well as cells transfected with the NQO2 expressing vector were measured using fluorescence activated cells sorting analysis and the 2′,7′–dichlorofluorescein diacetate (DCFH-DA) method. Cells were seeded at density 1x10^6 cells per well in 6-well plates and were allowed to attach overnight. Next day, cells were transfected with the empty vector or NQO2 expressing vector together with CD20 expressing vector and treated with either β-oestradiol or tamoxifen in the absence and presence of NRH or NRH alone. After the treatments, cells were washed once with cold PBS and trypsinized with 500 µL trypsin/EDTA to disassociate adherent cells from culture plates. The 500 µL of culture media was added to the cell suspension to inhibit further tryptic activity that can damage cells.

Cell suspensions were transferred to 1.5 mL eppendorf tubes and centrifuged at 2,000rpm for 3 mins. Supernatants were removed and 1.5ml of PBS was added and centrifuged again at 2,000rpm for 3 mins. Cells were incubated with 1µg/ml Allophycocyanin H7 (BD™ APC-H7) conjugated CD20 antibody in cell culture medium and left rotated for 1 hour at room temperature. After the incubation, cells were washed with PBS and incubated with 100µl H2DCFH-DA in a final concentration of 100µM in the dark for 30 minutes. After the incubation, cells were washed 3 times with PBS. Supernatants were aspirated, 400µl of PBS was added, and samples were subjected to CYAN-ADP flow cytometry (Dako, UK) following the H2DCFH-DA and APC-H7 fluorescent probes.

2′7′-dichlorofluorescein diacetate (DCFH-DA) is a stable, non-fluorescent and cell-permeable compound. Following diffusion into the cells, this compound is deacetylated by intracellular esterase which removes the acetate groups generating DCFH. In the presence of intracellular ROS, DCFH is oxidised into highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Felty 2006). The intensity of fluorescence is proportional to intracellular ROS levels which can be detected by flow cytometry. The excitation and emission of the H2DCFDA fluorescence was measured at 488nm and 530nm respectively.
Figure 2.8: Formation of fluorescent DCF compound by intracellular reactive oxygen species. DCFH-DA is a cell-permeable compound and diffuses into the cell reacting with intracellular esterase which later is being oxidised by intracellular reactive oxidative stress and converted to highly fluorescent DCF that can be measured using FACs (Illustration adapted from Cell Biolabs, Inc).

2.2.6.2 Cell cycle analysis

Cell cycle profiles of breast cancer cells upon treatment with various compounds were determined using fluorescence activated cells sorting (FACs) method. Propidium iodide (PI) dye is widely used to stain DNA as it intercalates with DNA producing a highly fluorescent signal that can be measured at excitation 488nm and emission at 600nm using flow cytometry (Pozarowski and Darzynkiewicz 2004). Propidium iodide-stained cells and forward-scattered light were detected by FACs. Cells were sorted by gating the different cell cycle phases. Cell cycle phases G0/G1, S and G2/M phases were gated as shown in Figure 2.9. The analysis was carried out using Summit v4.3 and Nucleocounter (NC 3000).

Cells were seeded at density $1 \times 10^6$ per well in 6-well plates and were allowed to attach overnight. Next, cells were transiently transfected with the empty vector or NQO2 expressing vector along with CD20 expressing vector, and treated with $\beta$-oestradiol or
tamoxifen, in the absence and presence of NRH and NRH alone. Cells were dissociated from cell culture plates and incubated with 1 ml of APC-H7 conjugated CD20 antibody (1μg/ml, 1:1000) for 1 hour on a rotator. Cells were washed three times with cold PBS by centrifuging at 1,200rpm for 3 minutes. Fixation was carried out using 70% ethanol in PBS solution and adding drop-wise to the cells while vortexing to avoid cells clumps.

Samples were incubated in -20°C for 1 hour to overnight or stored at 4°C for up to one week. Cells were washed with PBS and centrifuged at 1,200rpm for 3 minutes to remove the ethanol. 1 ml of propidium iodide (PI) (50 μg/mL PI, and 0.1 μg/ml RNase in PBS) were added to the samples and incubated in the dark at 37°C for 30 minutes. Cell cycle distribution was analysed by flow cytometry using the CYAN FACS system (Beckman Coulter, USA) and Nucleocounter (NC 3000) (Chemometec, Denmark).

Figure 2.9: Cell cycle analysis profile using PI as stained and measured using FACs machine. Cell gating to analyse the different phases of cell cycle in breast cancer cells. (Adapted from: Beckman Coulter)
2.2.6.3 Mitochondrial membrane potential assay

The mitochondrial transmembrane potential in breast cancer cells was measured using Nucleocounter NC 3000 and the cationic dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbonocyanine iodide). JC-1 is a permeable compound which is able to diffuse through mitochondrial membrane and as it accumulates in the mitochondria it can form aggregates. Formation of JC-1 aggregates in the mitochondria results in the shift of the JC-1 emission spectra from green to red allowing for a semiquantitative assessment of mitochondrial polarization states. Mitochondrial membrane potential was measured using the Nucleocounter (NC 3000) (Chemometec, Denmark) which detects the fluorescence emission shift from green (~529nm) to red (~590nm) as shown in Figure 2.10. Decreased red/green fluorescence ratio indicates mitochondrial depolarization.

Cells were seeded at density 1x10^6 cells per well in 6-well plates and were allowed to attach overnight. Next day, cells were left untreated or treated with various compounds as indicated in the figure legends. Cells were dissociated from the plate using disassociation buffer and transferred to 1.5ml eppendorf tubes. Cells were washed with cold PBS solution and centrifuged at 1,200rpm for 3 minutes. The supernatant was removed and 1ml of PBS solution was added to the pellet and incubated with JC-1 final concentration 200µg/ml for 10 minutes at 37°C. After the incubation, stained cells were washed twice with PBS and supernatants were removed without disturbing the pellets. Cells pellet were suspended in 250µl of 4’,6-diamidino-2-phenylindole (DAPI) (1µg/ml) in PBS solution and subjected to NucleoCounter NC 3000 analysis.
Figure 2.10: Mitochondrial membrane potential was measured using JC-1 dye and Nucleocounter NC3000. (Illustration adapted from Nucleocounter, NC 3000 (Chemometec, Denmark). Cells were left untreated and treated with various treatments and cells were stained with JC-1 and DAPI dye. JC-1 dye was used to measure the polarised and depolarised mitochondria, while DAPI was used to stain DNA. The example above shows the difference between untreated and positive control of mitochondrial disruption after treatment with camptothecin (CPT). (Adapted from: Chemometec).

2.2.6.4 Reduced glutathione (GSH) measurements

Intracellular thiols were measured using the NucleoCounter NC3000 and Solution 5 (VB 48.PI.AO) (Chemometec, Denmark). Solution 5 contains three different reagents. The Acridine orange dye (AO) which stains nucleated cells, Propidium iodide (PI) which stains dead cells, and Vitabright48 (VB 48) which stains viable cells in an intensity-dependent manner reliant on their thiol levels. VB48 after permeating the cellular membrane interacts with thiol groups producing a thioester-coupled fluorescent product as shown in Figure 2.11. Reduced glutathione (GSH) under various treatments was detected in cells using this method. GSH protects cells against oxidative damage (Du, Zhang et al. 2009). During apoptosis, the intracellular concentration of GSH has been found to decrease.

Cells were seeded at density 1x10^6 cells per well in 6-well plates and were allowed to attach overnight. The next day, cells were left untreated and treated with either β-oestradiol or tamoxifen in the absence or presence of NRH or NRH alone. After the treatments, cells were disassociated from the plates using disassociation buffer. Cells
were resuspended in culture medium and transferred to new labelled 1.5ml eppendorf tubes. Cells suspension was centrifuged at 1,200rpm for 3 minutes. The supernatant was aspirated, and cells pellets were resuspended in 190µl of PBS solution. 10µl of solution 5 (VB 48.PI.AO) was added to the cells suspension and samples were loaded on A8 slides (Chemometec, Denmark) and subjected to NucleoCounter NC 3000 (Chemometec, Denmark) analysis to measure intracellular thiol levels. The analysis was carried out using the Nucleocounter (NC 3000) software as shown in Figure 2.12.

Figure 2.11: Reduced glutathione in cells was measured using the solution 5 (VB48.PI.AO) and Nucleocounter (Illustration adapted from Nucleocounter, NC 3000 (Chemometec, Denmark).

(A) A model showing the principle of intracellular detection of thiols using VitaBright stains. VitaBright is added to the cells the dye crosses the cell's membrane and reacts with intracellular thiols, forming a blue fluorescent compound. (B) Example of CHO cells treated with camptothecin (CPT) and etoposide (ETO) and stained with VitaBright-48™ (blue) and a nuclear marker (red) (Adapted from: Chemometec).
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Figure 2.12: Reduced glutathione (GSH) was measured using Solution 5 and Nucleocounter NC3000. Illustration adapted from Nucleocounter, NC 3000 (Chemometec, Denmark). Cells were left untreated or treated with various compounds as indicated in the figure legends and stained with Solution 5 (VB48.PI.AO) and subjected to Nucleocounter analysis. The example above shows the difference between untreated, camptothecin (CPT) and etoposide (ETO) treated cells (Adapted from: Chemometec).

2.2.7 Antioxidant activity measurements

The involvement of NQO2 in regulating the antioxidant activity of catalase and glutathione peroxidase (GPx) was assessed in breast cancer cells under various conditions using the catalase assay kit (Cayman chemical, UK) and the glutathione peroxidase assay kit (Cayman chemical, UK) respectively. The manufacturer’s instructions were followed to extract cellular extract and measure the catalase and glutathione peroxidase (GPx) antioxidant activity (Cayman chemical, UK handbook).

2.2.7.1 Cells lysate preparation

Cells were seeded at density 1x10^6 cells per well and allowed to reach 60% confluency before treatments. Next day, cells were left untreated or treated with either β-oestradiol or tamoxifen in the absence and presence of NRH for 24 hours and transfected with NQO2 or the PCDNA3 empty vector. Cells were washed twice with 500µl of cold
PBS scraped using rubber policeman, and cell lysates were collected into new tubes. Cells were lysed by sonication in an inverted-cup sonicator at 4°C with a pulse-on time 15 second for every 30 seconds (Li and Schellhorn 2007) were then collected and transferred to fresh 1.5 ml labelled tubes and centrifuged at 13,000rpm for 15 minutes at 4°C to isolate the supernatant and cell pellets. The cells supernatants were collected and transferred to new labelled tubes. The samples were stored at -80°C until used for the catalase and glutathione peroxidase assay.

2.2.7.2 Catalase activity measurement

Catalase activity is determined in cell lysates utilizing the peroxidatic function of CAT. The enzyme reacts with methanol in the presence of an optimal concentration of H₂O₂ and the formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color.

Catalase enzyme is a ubiquitous antioxidant enzyme that can be found in most aerobic cells. Catalase plays important role in the detoxification of reactive oxygen species, quinone metabolites and hydrogen peroxide in cells. Catalase converts two molecules of H₂O₂ to one molecule of oxygen and two molecules of water as shown below.

\[
\text{Catalase} \\
2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}
\]

Cells were prepared as mentioned in the section 2.2.8.1. Sample reactions were prepared in 96-wells plate. Formaldehyde standard curve was prepared to determine the efficiency of the assay performed as shown in the Figure 2.13. A positive control well containing bovine liver catalase was also prepared by adding 20µl of catalase to 100µl assay buffer and 30µl of methanol. Sample wells were prepared by adding 20µl of samples to 100µl of assay buffer and 30µl of methanol.

The reactions were initiated with 20µl of H₂O₂ added to each well, and the 96 well plates were placed on the shaker for 20 minutes at room temperature. After the incubation, 30µl of catalase purpald (Chromogen) was added to the reactions, and the
samples were incubated for 10 minutes at room temperature and then their absorbance was read at 540nm in a spectrophotometer plate reader (Biotek, UK).

Data collected were analysed by calculating the formaldehyde produced in each well using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample. The calculation of formaldehyde concentration is shown in the Figure 2.13. After that, the catalase activity of the samples was calculated by using the equation in the Figure 2.14. One unit is defined as the amount of the enzyme forming 1.0 nmol of formaldehyde per minute at 25°C.

![Figure 2.13](image)

**Figure 2.13:** Formaldehyde standard curve in catalase enzyme activity was performed to determine the efficiency of the assay

\[
\text{Formaldehyde (\mu M)} = \left[\frac{\text{sample absorbance} - (y - \text{intercept})}{\text{slope}}\right] \times \frac{0.17 \text{ ml}}{0.02 \text{ ml}}
\]

**Figure 2.14:** The equation to calculate the formaldehyde concentration of the samples under various conditions.

\[
\text{Catalase activity} = \frac{\mu \text{M of sample}}{20 \text{ min}} \times \text{Samples dilution} = \text{ nmol/ min/ml}
\]

**Figure 2.15:** The equation to calculate the catalase activity of the samples under various conditions.
2.2.7.3 Glutathione peroxidase measurement

Glutathione peroxidase activity was measured using the glutathione peroxidase assay kit purchased from Cayman chemical, UK. Glutathione peroxide (GPx) catalyzes the reduction of hydrogen peroxides by reduced glutathione and plays a major role in protecting cells from oxidative damage. Several GPx isozymes encoded by different genes exist and they differ in subcellular localization and substrate specificity. However, all of the GPx enzymes are tetramers that have four identical subunits. Each subunit of the GPx enzymes contains selenocysteine at its active site which is involved in the two-electron reduction of the peroxide substrate. GPx enzymes use glutathione as the electron donor to regenerate the reduced form of the selenocysteine. Oxidised glutathione is recycled to its reduced state by GR and NADPH in a reaction catalyzed by GPx and glutathione reductase GR as shown below. In the second step of the reaction the oxidation of NADPH to NADP$^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A$_{340}$ is directly proportional to the GPx activity in the sample thus allowing the indirect estimation of the GPx activity.

$$\begin{align*}
R-O-O-H + 2 \text{GSH} & \xrightarrow{\text{GPx}} R-O-H + \text{GSSG} + H_2O \\
\text{GSSG} + \text{NADPH} + H^+ & \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+
\end{align*}$$

Cells were prepared as mentioned in the section 2.2.8.1. Sample reactions were prepared in 96-wells plates. A standard curve of bovine erythrocyte GPx was prepared to determine the efficiency of the assay as shown in Figure 2.16. Wells that did not contain enzyme were prepared by adding 120µl of assay buffer with 50µl of the co-substrate mixture. In the wells containing the samples 20µl of samples were added to 100µl of assay buffer and 50µl of the co-substrate mixture. The reactions were initiated with 20µl of cumene hydroperoxide. The 96 well plates were placed in the shaker and incubated for few seconds to mix. The absorbance of the samples was measured using the spectrophotometer plate reader (Biotek, UK) at 340nm for at least five-time points. One-minute measurement was taken for data analysis.
Data collected were analysed by first calculating the change in absorbance ($\Delta A_{340}$) per minute of the standard curve and non-enzymatic wells by using the equation in Figure 2.17. GPx activity of the samples was calculated by the equation shown in Figure 2.18.

**Figure 2.16:** The glutathione peroxidase (GPx) standard curve was performed to determine the efficiency of the assay

$$\Delta A_{340} \text{ (min.)} = \frac{[A_{340} \text{ (Time 2)} - A_{340} \text{ (Time 1)}]}{\text{Time 2 (min) - Time 1 (min)}}$$

**Figure 2.17:** Equation to calculate the change in absorbance ($\Delta A_{340}$) per minute of the standard curve and non-enzymatic wells

$$GPx \text{ activity} = \frac{\Delta A_{340}/\text{min}}{0.00373 \mu M^{-1}} \times \frac{0.19 ml}{0.02 ml} \times \text{sample dilution} = \text{nmol/min/ml}$$

**Figure 2.18:** The equation to calculate the GPx activity of the samples under various conditions.
2.2.8 Immunofluorescence

2.2.8.1 Fluorescence microscopy

Immunostaining was used to determine the subcellular localisation of the proteins of interest. Briefly cells grown on cover slips were incubated with 500nM MitoTracker for 30 minutes. The cells were then washed with 3 ml PBS thrice and then fixed by incubation with 4% paraformaldehyde in PBS for 30 minutes at room temperature. The cells were washed three times with PBS and then permeabilized with 0.5% Triton (X-100) in PBS for 5 minutes. PBS was used again to wash the cells and then were incubated with blocking buffer (10% FBS in PBS) for 10 minutes. The cover slips were then removed from the six well plates and placed cell side up on a wet sponge with PBS. 150µl of appropriate primary antibody either monoclonal-1:1000/PBS or polyclonal-1:200/PBS was added to the cells and incubated for 30 minutes. The cover slips were then washed 20 times each in four universal tubes containing 1% BSA. Then, 150µl of secondary immunofluorescent antibody (1:300/PBS) was added to the cover slips and incubated for 30 minutes. 5µl DAPI was added to the wash buffer containing 1% BSA and cover slips were placed on the slides and a drop of mounting oil was added.

2.2.9 Statistical analysis

All experiments were repeated at least three times independently and the data are presented as mean±SEM. Statistical analysis was carried out using the Graph pad prism software. Analysis of variance (ANOVA) with Tukey’s multiple comparison tests were carried out to determine the statistically significant differences. Significant values were determined and \( p < 0.05 \) was considered as statistically significant.
CHAPTER 3

Regulation of oestrogen receptor transcriptional activity and transcriptional target selectivity mediated by NQO2 in breast cancer cells
3.0 RESULTS

3.1 NQO2 affects ERα transcriptional activity and protein stability

The cellular function of the NQO2 is mainly the detoxification of quinones which are abundant in the environment since quinones’ sources include radiation, chemicals in the smoke originating from automobile exhausts, cigarette smoke and burned organic materials (Begleiter, Leith et al. 1997, Vella, Ferry et al. 2005). The induction of NQO2 expression protects against quinones-induced carcinogenesis in animal models (Boone, Steele et al. 1992, Song, Kosmeder et al. 1999), by inhibiting the initiation of tumorigenesis caused by oestrogen quinone (Gao, Dinkova-Kostova et al. 2001, Gills, Jeffery et al. 2006) and inhibiting certain events associated with later stages of the carcinogenesis (Boone, Steele et al. 1992, Gao, Dinkova-Kostova et al. 2001). Elevation of NQO2 also induces other phase II detoxification enzymes including GST, to detoxify and remove quinones metabolites in in vivo and in vitro systems, protecting cells against oxidative stress (Cuendet, Oteham et al. 2006). Reduction of quinones by NQO2 decreases oxidative stress thereby potentially preventing endoplasmic reticulum stress and unfolded protein response (Cuendet, Oteham et al. 2006, Bock 2012).

Long-term exposure of breast cells to oestrogen hormone induces the initiation of the process known as oestrogen-driven carcinogenesis (Dutta and Pant 2008). Oestrogens are metabolised to toxic metabolites such as catechol oestrogen-3,4-quinones which induce the generation of ROS and oxidative damage (Cavalieri, Rogan et al. 2004, Gaikwad, Yang et al. 2009). The predominance of enzymes metabolising oestrogens to oestrogen-3,4-quinones versus enzymes that metabolise oestrogens to non-toxic metabolites leads to increased ROS production and DNA damage caused by catechol oestrogen quinones (Cavalieri, Chakravarti et al. 2006). NQO2 catalyses the reduction of catechol-oestrogen-quinones in breast cancer cells faster than its homologue NQO1 (Gaikwad, Yang et al. 2009) implying that NQO2 is more efficient in detoxifying quinones.

It has been shown that catechol oestrogens stimulate the neoplastic transformation of breast cells in oestrogen receptor-dependent and oestrogen receptor independent manners (Bolton and Thatcher 2008). In particular, catechol oestrogens modify
cellular ROS levels thereby altering gene expression of ERα regulated genes in ER-positive primary breast cancers (Yau and Benz 2008) as well as in ER-negative breast cancer cell lines (Creighton, Cordero et al. 2006). Induction of ROS levels in breast cancer cells exposed to catechol oestrogen quinones elevates NQO2 levels by pathways mediated by AhR and NRF2 transcription factors (Shelton and Jaiswal 2013). In addition, in an oestrogen receptor-independent manner, catechol oestrogens stimulate the neoplastic transformation of the ERα negative MCF-10 mammary epithelial cells and induce differential gene expression of redox responsive genes in these cells (Bolton and Thatcher 2008). Studies carried out by Hsieh et al. showed that NQO2 activity is upregulated in MCF-7 cells and downregulated in MDA-MB-231 cells treated with tocotrienols, (Hsieh, Elangovan et al. 2010) confirming the differential function of NQO2 in ERα-positive and ERα-negative breast cancer cells.

Apart from AhR and NRF2, NQO2 has been shown to interfere with the function of other transcription factors such as the regulation of the protein stability of the p53 tumour suppressor (Gong, Kole et al. 2007) and the gene expression of ERα transcription targets such as the cell cycle regulator cyclin D1 gene (Hsieh, Yang et al. 2012) suggesting that NQO2 is implicated in different signalling pathways in ERα-positive and ERα-negative breast cancer cells.

Given the link between NQO2, oestrogens’ metabolism and regulation of cellular redox balance to ERα dependent and ERα independent neoplastic transformation (Gaikwad, Yang et al. 2009, Paugh, Stocco et al. 2010) and the fact that the interplay between NQO2 and oestrogen receptor has not been extensively investigated the effects of NQO2 on ERα protein stability and transcriptional activity were explored in this thesis.

### 3.1.1 Expression of NQO2 in human breast cancer cells

To investigate the cross-talk between NQO2 and ERα-mediated transcriptional activity in breast cancer cells, the protein levels of NQO2 in five different breast cancer cell lines were determined first. The ERα-positive: MCF-7 and T47D, and ERα-negative: MDA-MB-231, MDA-MB-468 and MDA-MB-157 breast cancer cells were used for this purpose (Figure 3.1). NQO2 and ERα protein levels were determined using western blot analysis and actin was used as loading control. As expected, ERα
protein was detected only in MCF-7 and T47D cells and was not detectable in MDA-MB-157, MDA-MB-231 and MDA-MB-468 cells. The highest NQO2 protein levels were detected in MDA-MB-468 followed by MDA-MB-231 breast cancer cells whereas very low NQO2 protein levels were identified in the ERα positive MCF-7 and T47D cells. There was no NQO2 protein detected in MDA-MB-157 cells (Figure 3.1). The MDA-MB-468 breast cancer cells showed the highest expression of NQO2 and these results are consistent with those reported by Nolan et.al (Nolan, Dunstan et al. 2012).

Figure 3.1: NQO2 protein levels in human breast cancer cells
Cellular extracts from MCF-7, T478D, MDA-MB-157, MDA-MB-231 and MDA-MB-468 untreated breast cancer cells were submitted to western blotting following endogenous NQO2 protein levels. Actin was used as loading control. One representative out of two independent experiments is shown.

3.1.2 Involvement of NQO2 in the regulation of the ERα transcriptional activity in ERα-positive human breast cancer cells

Results shown in Figure 3.1 indicating low NQO2 protein levels in the ERα-positive breast cancer cells stimulated our interest to investigate the crosstalk between ERα and NQO2 in these cells. In particular, the role of NQO2 in the ERα-mediated transcriptional regulation of gene expression was followed using an oestrogen responsive luciferase reporter and luciferase assay. The ERα responsive reporter was transiently transfected in MCF-7 and T47D cells together with increasing amounts of an NQO2 expression vector in the presence of the NQO2 inducer NRH and in the presence or absence of β-oestradiol as indicated in Figure 3.2. The ERα mediated
transcriptional regulation of gene expression is executed through binding of this transcription factor to specific DNA sequences called oestrogen responsive elements (Gruber, Gruber et al. 2004) present within the regulatory region of the promoter of its transcriptional targets. (Montano, Jaiswal et al. 1998).

In untreated MCF-7 cells, transfection of low amounts of NQO2 did not affect the ERα transcriptional activity (Figure 3.2, compare bars 2 and 3 to bar 1). Increased ERα-reporter luciferase activity was evident when higher amounts of NQO2 were transfected (Figure 3.2, compare bar 4 to bar 1). Treatment of MCF-7 cells with β-oestradiol resulted in 4-fold increase of the ERE luciferase activity compared to the untreated cells (Figure 3.2, compare bar 5 to bar 1). Increasing amounts of transfected NQO2 in the presence of β-oestradiol stimulated further the ERα transcriptional activity (Figure 3.2, compare bars 6, 7 and 8 to bar 5).

Figure 3.2: Effects of NQO2 on ERα transcriptional activity in MCF-7 breast cancer cells
(A) MCF-7 cells were transfected with increasing amounts of NQO2 (0.25μg, 0.5μg and 1μg), treated with β-oestradiol or left untreated as indicated and luciferase reporter assay was carried out using an ERα responsive reporter as described in Materials and Methods. Data shown are the mean of three independent experiments performed in triplicates. [#] indicates significant difference compared to bar 5, p<0.05. (B) Protein levels of ectopically expressed NQO2.
A similar trend to that observed in MCF-7 cells was recorded in the T47D cells. Increasing amounts of ectopically expressed NQO2 in these cells increased the ERα-reporter luciferase activity in both the absence and presence of β-oestradiol (Figure 3.3(A)). In untreated T47D cells, transfection of increasing amounts of NQO2 increased ERα-reporter luciferase activity (Figure 3.3, compare bars 2, 3 and 4 to bar 1). Treatment with β-oestradiol stimulated the ERα-reporter luciferase activity by approximately 2-fold compared to the untreated T47D cells (Figure 3.3, compare bar 5 to bar 1). In β-oestradiol treated T47D cells increasing amounts of ectopically expressed NQO2 induced the ERα-reporter luciferase activity to a higher level compared to that observed in untreated cells (Figure 3.3, compare bars 2, 3 and 4 to bars 6, 7 and 8 respectively).

Figure 3.3: Effects of increasing amounts of NQO2 on the ERα transcriptional activity in T47D ERα-positive breast cancer cells
(A) T47D cells were transfected with increasing amounts of NQO2 (0.25µg, 0.5µg and 1µg), treated with β-oestradiol or left untreated as indicated and luciferase reporter assay was carried out using an ERα responsive reporter as described in Materials and Methods. Data shown are the mean of three independent experiments performed in triplicates. (B) Protein levels of ectopically expressed NQO2.

Results presented in Figures 3.2 and 3.3 indicate that increasing amounts of ectopically expressed NQO2 increased the ERα reporter luciferase activity in both MCF-7 and T47D cells implying that NQO2 might play a role in the regulation of ERα-mediated transcriptional activity.
3.1.3 NQO2 is potentially involved in the regulation of ERα protein stability

Results shown in Figure 3.2 and 3.3 indicating that overexpression of NQO2 in ERα positive MCF-7 and T47D breast cancer cells increased ERα transcriptional activity in both the absence and presence of β-oestradiol. These results triggered our interest to determine the effect of NQO2 in the ERα protein levels in MCF-7 cells. To investigate the involvement of NQO2 in the regulation of ERα protein stability, western blot analysis was employed to monitor the ERα protein levels in MCF-7 cells transfected with increasing amounts of NQO2 in the presence of β-oestradiol (Figure 3.4).

Low amounts of NQO2 increased ERα protein levels (Figure 3.4 A and B, compare bars 2 and 3 to bar 1) whereas in cells expressing higher NQO2 amounts ERα protein levels were significantly downregulated (Figure 3.4 A and B, compare bars 4 and 5 to bars 2 and 3). The most pronounced effect was observed in cells transfected with 1µg of NQO2 expression vector where the ERα protein levels were reduced by more than 50% compared to untransfected cells (Figure 3.4 A and B, compare bar 5 to bar 1). Results shown in Figure 3.4 indicate that NQO2 might play a role in the regulation of ERα protein stability in ERα positive breast cancer cells.
Figure 3.4: Effects of NQO2 on ERα protein stability
(A) Endogenous ERα protein levels were monitored in MCF-7 cells transfected with increasing amounts of NQO2 (0.25µg, 0.5µg, 1µg and 1.5µg) and treated with β-oestradiol using western blot analysis as described in Materials and Methods. (B) and (C) Densitometric analysis of the western blot shown in (A) measuring the intensity of ERα (B) and NQO2 (C) proteins. Data shown are the mean of three independent experiments performed in triplicates. Asterisks [*] indicate significant difference compared to untransfected cells (bar 1) and [#] indicate significant differences compared to the lower amount of transfected NQO2 (0.25µg) (bar 2). One [#] indicates $p<0.05$, two [##] indicate $p<0.01$, three [###] indicate $p<0.001$ and four [####] indicate $p<0.0001$. 
3.2 NQO2 is potentially involved in the regulation of cyclin D1 cellular levels

Many studies have shown that gene expression of the cyclin D1 subunit of the holoenzyme that phosphorylates the retinoblastoma protein thereby regulating cell cycle progression is under the control of ERα transcriptional activity (Ogba, Chaplin et al. 2008). Cyclin D1 on the other side has been shown to regulate ERα transactivation for the majority of the ERα transcriptional targets (Casimiro, Wang et al. 2013). Cyclin D1 overexpression has been detected in many cancers, and it is strongly related to ERα positive breast cancers (Arnold and Papanikolaou 2005) and correlates with early onset and tumour progression (Diehl 2002). The fact that NQO2 was shown to induce ERα transcriptional activity (Figures 3.2 and 3.3) together with published observations reporting that deregulation of NQO2 is correlated with resistance to hormone therapy (Kenny, Hui et al. 1999, Hui, Finney et al. 2002, Hodges, Cook et al. 2003) triggered our interest to investigate the effects of NQO2 on cyclin D1 cellular levels in the ERα positive MCF-7 and T47D cells.

NQO2 stabilises cyclin D1 in prostate cancer cells (Hsieh, Yang et al. 2012) but the effects of NQO2 on cyclin D1 cellular levels in breast cancer cells have not been investigated. MCF-7 and T47D breast cancer cells were transiently transfected with either the empty vector PCDNA3 or an NQO2 expressing vector and treated with β-oestradiol, tamoxifen, or left untreated in the presence or absence of the NQO2 activator NRH as indicated in Figures 3.5 and 3.6.

In untransfected MCF-7 cells, an increase in cyclin D1 protein levels was detected in β-oestradiol treated MCF-7 cells in the absence of NRH compared to untreated cells (Figure 3.5, compare lane 2 to lane 1). Tamoxifen treatment in the absence of NRH resulted in no changes in cyclin D1 protein levels when compared to the untreated (Figure 3.5, compare lane 3 to lane 1). Increased cyclin D1 protein levels were observed in MCF-7 cells in the presence of NRH compared to the MCF-7 cells in the absence of NRH (Figure 3.5, compare lane 4 to lane 1). No difference in the cyclin D1 protein levels was observed between the β-oestradiol and tamoxifen-treated MCF-7 cells in the presence of NRH compared to those in the absence of NRH (Figure 3.5, compare lanes 5 and 6 to lanes 2 and 3 respectively).
NQO2 overexpressing MCF-7 cells in the absence of NRH exhibited higher but not significant cyclin D1 protein levels compared to the untransfected cells in the absence of NRH (Figure 3.5, compare lane 7 to lane 1). No difference in the cyclin D1 protein levels was evident in the NQO2 transfected and β-oestradiol or tamoxifen-treated MCF-7 cells in the absence of NRH compared to those in the transfected with the empty vector cells under the same conditions (Figure 3.5, compare lanes 8 and 9 to lanes 2 and 3 respectively). The combination of NQO2 transfection and β-oestradiol treatment in the presence of NRH significantly reduced the cyclin D1 protein levels compared to NQO2 transfected cells treated with β-oestradiol in the absence of NRH (Figure 3.5, compare lane 11 to lane 8). The treatment with a combination of β-oestradiol and NRH significantly reduced cyclin D1 protein levels in NQO2 transfected MCF-7 cells when compared to the same treatment in untransfected MCF-7 cells (Figure 3.5, compare lane 11 to lane 5). The presence of NRH did not have any effect on the cyclin D1 protein levels in NQO2 transfected MCF-7 cells treated with tamoxifen (Figure 3.5, compare lane 12 with lane 9).
Figure 3.5: Effects of NQO2 overexpression on cyclin D1 protein levels in MCF-7 breast cancer cells

(A) MCF-7 human breast cancer cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected and western blot analysis was carried out as described in Materials and Methods. Cyclin D1 and NQO2 protein levels were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK).

(B) Graph showing the quantification of the intensity of the cyclin D1 bands in PCDNA3 and NQO2 transfected MCF-7 cells normalised to beta-actin using the Image J software. Data shown are the mean of three independent experiments. [*] significant difference ($p<0.05$) between the intensity of cyclin D1 band in β-oestradiol treated and NQO2 transfected MCF-7 cells in the absence of NRH (bar 8) and β-oestradiol treated and NQO2 transfected MCF-7 cells in the presence of NRH (bar 11). [#] significant difference $p<0.05$ between the intensity of cyclin D1 band in untransfected β-oestradiol treated MCF-7 cells in the presence of NRH (bar 5) and NQO2 transfected β-oestradiol treated MCF-7 cells in the presence of NRH (bar 11).

In line with the results observed in MCF-7 cells in T47D cells transfected with the empty vector, β-oestradiol treatment in the presence of NRH significantly increased cyclin D1 protein levels compared to untreated cells (Figure 3.6, compare lane 5 to lane 4). The combination of NRH and β-oestradiol significantly increased cyclin D1 protein levels in untransfected T47D compared to those recorded in cells treated with β-oestradiol alone (Figure 3.6, compare lane 5 to lane 2). Tamoxifen treatment of T47D transfected with the empty vector significantly decreased cyclin D1 protein levels in the absence and presence of NRH compared to untransfected and non-treated cells (Figure 3.6, compare lanes 3 and 6 to lanes 1 and 4 respectively).
Similar trends of cyclin D1 protein levels with those in untransfected cells were observed in T47D cells ectopically expressing NQO2 and treated with β-oestradiol in the absence of NRH. In particular, combination of NQO2 overexpression with β-oestradiol treatment in the absence of NRH increased the cyclin D1 protein levels (Figure 3.6, compare lane 8 to lane 7) whereas in the presence of NRH under the same conditions T47D cells exhibited significantly lower cyclin D1 protein levels (Figure 3.6, compare lane 11 to lane 10). Tamoxifen treatment of T47D cells overexpressing NQO2 irrespectively of the presence of NRH reduced the cyclin D1 protein levels compared to NQO2 transfected and non-treated cells (Figure 3.6, compare lanes 9 and 12 to lanes 7 and 10 respectively).
Figure 3.6: Effects of NQO2 overexpression on cyclin D1 protein levels in T47D human breast cancer cells

(A) T47D human breast cancer cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected and western blot analysis was carried out as described in Materials and Methods. Cyclin D1 and NQO2 protein levels were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK). (B) Graph showing the quantification of the intensity of the cyclin D1 bands in PCDNA3 and NQO2 transfected T47D cells normalised to beta-actin using the ImageJ software. Data shown are the mean of three independent experiments performed in duplicate. [*] significant difference (p<0.05) between the intensity of cyclin D1 bands in untransfected β-oestradiol (bars 2 and 5) and tamoxifen (bars 3 and 6) treated and untreated (bars 1 and 4) T47D cells in the presence (bars 4, 5, 6) and absence (bars 1, 2, 3) of NRH. [#] significant difference (p<0.05) between the intensity of cyclin D1 bands in NQO2 transfected β-oestradiol (bars 8 and 11) and tamoxifen (bars 9 and 12) treated and untreated (bars 7 and 10) T47D cells in the absence of NRH (bars 7, 8 and 9) and presence (bars 10, 11, 12) of NRH. [a] significant difference (p<0.05) between the intensity of cyclin D1 bands in untransfected β-oestradiol treated (bars 2 and 5) T47D cells in the presence (bar 5) and absence (bar 2) of NRH.[^] significant difference (p<0.05) between the intensity of cyclin D1 bands in NQO2 transfected T47D cells NRH treated (bars 4, 5, 6) in the absence (bar 4) and presence of β-oestradiol (bar 5) and in the absence (bar 4) and presence of tamoxifen (bar 6). [^] significant difference (p<0.05) between the intensity of cyclin D1 bands in NQO2 transfected T47D cells NRH treated (bars 10, 11, 12) in the absence (bar 10) and presence of β-oestradiol (bar 11) and in the absence (bar 10) and presence of tamoxifen (bar 12). [$] significant difference (p<0.05) between the intensity of cyclin D1 bands in untransfected β-oestradiol and NRH treated (bar 5) and NQO2 transfected β-oestradiol and NRH treated (bar 11) T47D cells. Two symbols indicate p<0.01, three symbols p<0.001 and four symbols p<0.0001.
Taken together results shown in Figures 3.5 and 3.6 indicate that in the ERα positive MCF-7 and T47D cells combination of NQO2 overexpression with β-oestradiol treatment decreased the cyclin D1 protein levels compared to untreated cells under the same conditions (Figures 3.5 and 3.6, compare lanes 11 to lanes 10). Furthermore, tamoxifen treatment reduced cyclin D1 protein levels in all conditions tested compared to untreated cells (Figures 3.5 and 3.6, compare lanes 3, 6, 9 and 12 to lanes 1, 4, 7 and 10 respectively).

3.2.1 Effects of NQO2 on cyclin D1 gene expression

To investigate whether the effects of NQO2 overexpression in the β-oestradiol treated cells in the presence of NRH on cyclin D1 protein levels were determined at the level of transcription, the cyclin D1 mRNA levels were assessed in the ERα positive MCF-7 and the ERα negative MDA-MB-231 cells using q-RTPCR. Cells were treated with β-oestradiol or tamoxifen or left untreated and total mRNA was isolated as described in the Materials and Methods.

Increased but not statistically significant cyclin D1 mRNA levels were recorded in MCF-7 cells treated with β-oestradiol in the absence of NRH compared to untreated cells (Figure 3.7, compare bar 2 to bar 1) whereas no changes in the cyclin D1 mRNA levels were evident in the tamoxifen-treated MCF-7 cells in the absence and presence of NRH compared to untreated cells (Figure 3.7, compare bar 3 to bar 1 and bar 6 to bar 4). MCF-7 cells treated with β-oestradiol or tamoxifen in the presence of NRH exhibited reduced but not statistically significant cyclin D1 mRNA levels compared to the cells treated with the two hormones in the absence of NRH (Figure 3.7, compare bars 5 and 6 to bars 2 and 3 respectively).
Cells were treated with β-oestradiol (bars 2 and 5) or tamoxifen (bars 3 and 6) or left untreated (bars 1 and 4) in the absence (bars 1, 2 and 3) and presence (bars 4, 5 and 6) of NRH. Total mRNA was extracted from cells, and cyclin D1 mRNA expression was measured using qRT-PCR. Data were analysed using the StepOne software. Data shown are the mean of two independent experiments performed in duplicates.

To confirm that the effects of β-oestradiol observed in MCF-7 cells were mediated by the ERα transcriptional activity, the cyclin D1 mRNA levels were assessed in β-oestradiol or tamoxifen-treated ERα negative MDA-MB-231 cells in the presence or absence of NRH. Results shown in Figure 3.8 indicate that β-oestradiol or tamoxifen treatments increased but not statistically significant cyclin D1 mRNA levels in the absence of NRH (Figure 3.8, compare bars 2 and 3 to bar 1). NRH alone did not have any significant effect on the cyclin D1 mRNA levels in MDA-MB-231 cells (Figure 3.8, compare bar 4 to bar 1). Not statistically significant reduction in cyclin D1 mRNA levels was observed in MDA-MB-231 cells treated with β-oestradiol or tamoxifen in the presence of NRH compared to the same treatments in the absence of NRH (Figure 3.8, compare bars 5 and 6 to bars 2 and 3 respectively).
Cells were treated with β-oestradiol (bars 2 and 5) or tamoxifen (bars 3 and 6) or left untreated (bars 1 and 4) in the absence (bars 1, 2 and 3) and presence (bars 4, 5 and 6) of NRH. Total mRNA was extracted from cells and cyclin D1 mRNA expression was measured using qRT-PCR. Data were analysed using the StepOne software. The results shown are from three independent experiments performed in duplicates.

3.2.2 NQO2 affects cyclin D1 protein stability in β-oestradiol treated cells

Results shown in Figures 3.7 and 3.8 indicate that NQO2 potentially regulates the cyclin D1 cellular levels in a manner independent of ERα transactivation and possibly at the level of protein stability. To test this hypothesis the cyclin D1 protein levels were monitored in untreated ERα positive MCF-7 cells (Figure 3.9, top panel) or treated with β-oestradiol (Figure 3.9, lower panel) in the presence of cycloheximide (CHX) for 0, 1, 3 and 5 hours to inhibit protein synthesis (Figure 3.9). Cells were transfected with either the empty vector PCDNA3 (Figure 3.9, lanes 1, 2, 3 and 4) or an NQO2 expression vector (Figure 3.9, lanes 5, 6, 7 and 8). Pulse-chase assay with CHX was performed for the indicated times, and cell lysate was submitted to western blot analysis using actin as loading control.

The cyclin D1 half-life in untreated cells transfected with the PCDNA3 empty vector was found to be approximately 1.5h (Figures 3.9, upper left panel and 3.10). The cyclin
D1 half-life increased to approximately 3h in the untreated NQO2 transfected MCF-7 cells (Figures 3.9 upper right panel and 3.10). In contrast, in the untreated NQO2 overexpressing MCF-7 cells, the cyclin D1 half-life was found to be approximately 2h (Figures 3.9 lower left panel and 3.11) and in the β-oestradiol treated and NQO2 overexpressing MCF-7 cells cyclin D1 half-life was reduced to approximately 45mins (Figures 3.9 lower right panel and 3.11) implying that β-oestradiol treatment in combination with NQO2 overexpression destabilise cyclin D1 protein in ERα positive MCF-7 breast cancer cells.

Figure 3.9: Cyclin D1 protein stability in MCF-7 cells overexpressing NQO2
MCF-7 human breast cancer cells were seeded in 6-well plates and either left untreated (top panel) or treated with β-oestradiol (lower panel) and transfected with either the empty vector PCDNA3 (lanes 1, 2, 3 and 4) or an NQO2 expression plasmid (lanes 5, 6, 7 and 8). Cycloheximide (CHX) was added for 1h (lanes 2 and 6), 3h (lanes 3 and 7) or 5h (lanes 4 and 8) or was not added (lanes 1 and 5). Cell lysates were collected and western blot analysis was carried out following cyclin D1, NQO2 and actin using specific antibodies recognising these proteins. The results shown are from one representative out of three independent experiments.
Figure 3.10: Cyclin D1 protein stability is affected by β-oestradiol in MCF-7 cells
Cyclin D1 protein stability was monitored in untreated or β-oestradiol treated MCF-7 cells pulse-chased with CHX for 0, 1, 3 and 5 hours as indicated. The intensity of the cyclin D1 protein bands was measured densitometrically using Image J and normalised to the intensity of actin. The results shown above are the average of three independent experiments. [*] Significant difference of the intensity of the cyclin D1 bands in cells treated with CHX for 1, 3 and 5 hours compared to that at 0 hours. Four asterisks indicate \( p < 0.0001 \) compared to 0 hours.

Figure 3.11: Cyclin D1 protein stability is affected by NQO2 overexpression in MCF-7 cells
Cyclin D1 protein stability was monitored in untreated or β-oestradiol treated MCF-7 cells pulse-chased with CHX for 0, 1, 3 and 5 hours as indicated. The intensity of the cyclin D1 protein bands was measured densitometrically using Image J and normalised to the intensity of actin. The results shown above are the average of three independent experiments. [*] Significant difference of the intensity of the cyclin D1 bands in cells treated with CHX for 1, 3 and 5 hours compared to that at 0 hours. Four asterisks indicate \( p < 0.0001 \) compared to 0 hours.
3.3 Chapter discussion

3.3.1 Regulation of NQO2 on oestrogen receptor transcriptional activity and the transcriptional target selectivity in breast cancer cells treated with β-oestradiol and tamoxifen

Given that ROS affect a broad range of cell signalling pathways including transcription (Law, Leung et al. 2013) one possibility could be that NQO2 affects the ERα transcriptional activity. The regulation of the ERα transcriptional activity is a complex process that involves post-translational modifications of the receptor and protein-protein interactions with transcriptional cofactors and cross talk with other transcription factors (Frasor, Weaver et al. 2009, Renoir, Marsaud et al. 2013). ROS generated during the process of endogenous oestrogens’ metabolism and the formation of semiquinone metabolites trigger the induction of the transcriptional activity of the redox responsive transcription factors p53, NF-κB, activator protein 1 (AP-1), hypoxia inducible factor 1α (HIF-1α), and SP1, which then stimulate the expression of genes involved in redox homeostasis (catalase, peroxiredoxins, glutathione peroxidases), cell cycle control (cyclin D1, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKIs) p21cip1/waf1 and p27kip1), senescence (p16INK4a), DNA damage repair genes (apurinic/apyrimidinic endonuclease (APE1, APEX, REF-1), endonuclease VIII-like 1 (NEIL1), Proliferating Cell Nuclear Antigen (PCNA), endoplasmic reticulum stress (protein disulphide isomerases (PDI)s and cell death (Bcl-2, inhibitors of apoptosis (IAPs) (Marinho, Real et al. 2014, Espinosa-Diez, Miguel et al. 2015).

To investigate the potential involvement of the NQO2 mediated alterations of ROS levels on the ERα transcriptional activity, ERE luciferase reporter assays were performed in ectopically expressing NQO2 MCF-7 and T47D cells untreated or treated with β-oestradiol. NQO2 overexpression significantly increased the ERE luciferase activity in both ERα positive breast cancer cells implying that NQO2 directly or indirectly modulates ERα transcriptional activity. This implies that NQO2 directly or indirectly modulates the oestrogen receptor transcriptional activity. A recent report showed that other antioxidant enzymes localised at the mitochondria known as Cu/Zn...
superoxide dismutase interact with oestrogen receptor and influence oestrogen-mediated transcription and are involved in the regulation of oxidative stress in human breast cancer cells (Rao, Ziegler et al. 2008). This justifies the rationale to study the potential effects of NQO2 on the ERα transcriptional activity as well as protein stability in human breast cancer cells. The effects of NQO2 on ERα protein levels were determined indicating that NQO2 potentially regulates ERα protein levels in the presence of oestrogen hormone in ERα-positive cells. Overexpression of NQO2 downregulated ERα protein levels in MCF-7 cells but there was direct correlation between ERα protein and mRNA levels in NQO2 transfected MCF-7 cells, suggesting that this downregulation was an effect of NQO2 on the ERα protein stability (Ghigna, Cartegni et al. 2015).

Genes relevant to tumorigenesis encoding proteins involved in cell proliferation such as cyclin D1 are regulated directly or indirectly by ERα (Benz 1998). Cyclin D1 is the key regulator in cell cycle signalling and in particular in the G1-S phase transition and is highly overexpressed in cancer cells especially breast cancer cells (Wilcken, Prall et al. 1997). Previous studies have shown that NQO2 modulates the transcriptional activity of NF-κB and upregulates gene expression of the NF-κB transcription target CCND1 (Ahn, Gong et al. 2007). Induction of cyclin D1 gene expression by oestrogen usually appears during the early stages of breast carcinogenesis inducing cell proliferation (Singh, Rajendran et al. 2016). Deregulation of cyclin D1 is a major reason for cancer progression, and this kinase has been targeted to prevent breast cancer. Antioestrogen agents such as tamoxifen inhibit cyclin D1 gene expression by repressing ERα-mediated transcription (Musgrove, Caldon et al. 2011). ERα is a known regulator of the cyclin D1 gene expression and protein levels (Eeckhoute, Carroll et al. 2006). NQO2 has been reported to stabilise cyclin D1 at the protein level (Hsieh, Yang et al. 2012) and its gene expression at the transcriptional level in breast cancer cells (Hsieh, Elangovan et al. 2010) and prostate cancer cells (Hsieh, Yang et al. 2012).

NQO2 overexpression upregulated cyclin D1 protein levels in the untreated MCF-7 and T47D cells compared to the cyclin D1 protein levels in the untransfected cells. In contrast, NQO2 overexpression in the β-oestradiol treated MCF-7, and T47D cells decreased cyclin D1 protein levels compared to untransfected cells under these
conditions. No effect of transfected NQO2 was observed in tamoxifen treated MCF-7 and T47D cells.

Oestrogen and tamoxifen treatments downregulated cyclin D1 mRNA expression in MCF-7 and MDA-MB-231 cells in the presence of NRH suggesting that NRH activates NQO2 to inhibit cyclin D1 gene expression. The potential involvement of NQO2 in the regulation of on the cyclin D1 protein stability was investigated in MCF-7 cells (harbouring wild-type p53) treated with oestrogen hormone. This experiment was carried out in order to understand the role of NQO2 bioactivation in the regulation of the cyclin D1 half-life. NQO2 decreased cyclin D1 half-life from ~2h in the untreated to ~45min in the oestrogen hormone treated MCF-7 cells implying a potential role of NQO2 in regulating cyclin D1 protein stability in a complex manner involving oestrogen activated ERα in the MCF-7 breast cancer cells.
CHAPTER 4

Regulation of endoplasmic reticulum stress
by NQO2 in breast cancer cells
4.0 RESULTS

4.1 Potential role of NQO2 in regulating endoplasmic reticulum stress in human breast cancer cells

Several reports converge to the view that there is an association between oestrogens and carcinogenesis in tissues which are oestrogen hormone sensitive such as the breast, the endometrium and the cervical tissue (Brake and Lambert 2005, Bolton and Thatcher 2008, den Boon, Pyeon et al. 2015). The biotransformation of oestrogens to catechol is mediated by the phase I cytochrome P450 1A1 and CYP1B1 enzymes. The subsequent oxidation of these catechols to quinones is one of the main pathways leading to breast carcinogenesis (Shanle and Xu 2011) as quinones are highly reactive species that form adducts in the DNA (Bolton and Thatcher 2008) and induce excessive ROS generation and oxidative stress (Roy, Cai et al. 2007, Fussell, Udasin et al. 2011, Santos, Nabeebaccus et al. 2014). High ROS levels and oxidative stress induce endoplasmic reticulum stress in cells, which are chronically exposed to these stressors (Cao and Kaufman 2014, Zeeshan, Lee et al. 2016). Since quinone detoxification is mediated by several enzymes including NQO2 (Gaikwad, Yang et al. 2009), we reasoned that altered redox status of breast cancer cells resulting from β-estradiol or tamoxifen treatment and/or NQO2 overexpression would potentially have differential effects on the induction of the endoplasmic reticulum stress in the ROS-resistant MCF-7 and other ROS-sensitive breast cancer cells. To test this hypothesis, the induction of the endoplasmic reticulum was assessed by following the protein levels of the ER stress markers GRP94 and GRP78 in MCF-7 and T47D cells.

To determine the effects of NQO2 on the endoplasmic reticulum stress and unfolded protein response, western blotting was performed using actin as loading control. GRP94 and GRP78 were used as markers of endoplasmic reticulum stress, and PDI was used as marker of unfolded protein response (Andreu, Woehlbier et al. 2012, Grek and Townsend 2014). GRP94 and GRP78 are well known as endoplasmic reticulum chaperones involved in the regulation of several processes in cells such as protein folding and assembly, protein quality control, Ca^{2+} binding and endoplasmic reticulum signalling. These chaperones also act as anti-apoptotic proteins and play critical roles in cell surviving, cells progression, metastasis and resistance to therapy (Ni, Zhang et al. 2011). PDI is a chaperone involved in oxidative stress events and plays a role in
mediating oxidative protein folding to maintain cellular homoeostasis. It has recently been shown that quinone-quinol redox cycle plays critical roles in regulating oxidative stress and activate endoplasmic reticulum stress (Pang, Xie et al. 2012). We investigated the effects of NQO2 in regulating endoplasmic reticulum stress and unfolded protein response in ERα-positive human breast cancer cells. Cells were transiently transfected with PCDNA3 and NQO2 and treated with oestrogen or antioestrogen in the presence of the NQO2 inducer NRH in MCF-7 and T47D cells as shown in Figure 4.1 and Figure 4.2.

Increased but not statistically significant GRP94 protein levels were detected in β-oestradiol treated MCF-7 cells in the absence of NRH compared to untreated cells (Figure 4.1A and B, compare lane 2 to lane 1). GRP94 was significantly decreased in β-oestradiol treated MCF-7 cells in the presence of NRH compared to the same treatment in the absence of NRH (Figure 4.1A and B, compare lane 5 to lane 2). Tamoxifen treatment in the absence of NRH did not affect the GRP94 protein levels (Figure 4.1A and B, compare lane 3 to lane 1). GRP94 protein levels were significantly decreased in tamoxifen treated MCF-7 cells in the presence of NRH compared to untreated cells (Figure 4.1A and B, compare lane 6 to lane 1). GRP94 protein levels were decreased in MCF-7 cells in which NRH was added alone compared to those in which NRH was not added but this effect was not statistically significant (Figure 4.1A and B, compare lane 4 to lane 1).

NQO2 overexpressing MCF-7 cells treated with β-oestradiol or tamoxifen in the absence of NRH exhibited significantly decreased GRP94 protein levels compared to to the untreated cells (Figure 4.1A and B, compare lanes 8 and 9 to lane 7). Statistically significant decreased of the GRP94 protein levels was observed in NQO2 overexpressing and β-oestradiol and tamoxifen treated MCF-7 cells in the absence of NRH compared to untreated cells overexpressing NQO2 in the absence of NRH (Figure 4.1A and B, compare lanes 8 and 9 to lane 7). Increased GRP94 protein levels were observed in the NQO2 transfected and β-oestradiol treated MCF-7 cells in the presence of NRH compared to the NQO2 transfected and β-oestradiol treated cells in the absence of NRH (Figure 4.1A and B compare lane 11 to lane 8).

Apart from GRP94, the protein levels of the other endoplasmic reticulum stress chaperone GRP78 were also determined. Significant decreased in GRP78 protein...
levels were detected in β-oestradiol treated MCF-7 cells in the absence of NRH compared to untreated cells (Figure 4.1A and C, compare lane 2 to lane 1). A small but statistically significant increased in GRP78 protein levels was observed in tamoxifen treated MCF-7 cells in the absence of NRH compared to untreated cells (Figure 4.1A and C, compare lane 3 to lane 1). GRP78 protein levels were decreased in untransfected MCF-7 cells in the presence of NRH compared to the MCF-7 cells in the absence of NRH but this effect was not statistically significant (Figure 4.1A and C, compare lane 4 to lane 1). Significant increased of GRP78 protein levels was observed in β-oestradiol treated MCF-7 cells in the presence of NRH compared to cells treated with β-oestradiol in the absence of NRH (Figure 4.1A and C, compare lane 5 to lane 2) or not treated in the presence of NRH (Figure 4.1A and C, compare lane 5 to lane 4). GRP78 protein levels were significantly increased in tamoxifen treated MCF-7 cells in the presence of NRH compared to those recorded in cells treated with NRH alone (Figure 4.1A and C, compare lane 6 to lane 4).

GRP78 protein levels were significantly decreased in the NQO2 transfected and β-oestradiol-treated MCF-7 cells in the absence of NRH compared to NQO2 transfected and untreated cells in the absence of NRH (Figure 4.1A and C, compare lane 8 to lane 7). Statistically significant increase in GRP78 protein levels were observed in NQO2 transfected MCF-7 cells and treated with β-oestradiol in the presence of NRH compared to NQO2 transfected MCF-7 cells and treated with β-oestradiol in the absence of NRH (Figure 4.1A and C, compare lane 11 to lane 8). Small but statistically significant decreased GRP78 protein levels were recorded in NQO2 transfected MCF-7 cells treated with β-oestradiol and tamoxifen in the presence of NRH compared to non treated NQO2 overexpressing cells in the absence of NRH (Figure 4.1A and C, compare lanes 11 and 12 to lane 7).
Figure 4.1: Effects of NQO2 on the endoplasmic reticulum stress biomarkers GRP94 and GRP78 in MCF 7 human breast cancer cells

(A) MCF 7 human breast cancer cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected and western blot analysis was carried out as described in Materials and Methods. GRP94, GRP78 and NQO2 proteins were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK). A representative experiment out of three repeats is presented in the figure. (B) and (C) Graph showing the quantification of the intensity of the GRP94 and GRP78 protein bands normalised to that of actin using the Image J software. [*] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in untransfected MCF-7 cells treated with β-oestradiol or tamoxifen compared to untransfected and non-treated cells (bar 1). [#] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in NQO2 transfected cells and treated with β-oestradiol or tamoxifen compared to NQO2 overexpressing and non-treated cells (bar 7). [a] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in β-oestradiol treated cells in the absence of NRH (bars 2 and 8) compared to β-oestradiol treated cells in the presence of NRH (bars 5 and 11). [^] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in β-oestradiol or tamoxifen treated cells in the presence of NRH (bars 5 and 6) compared to non-treated cells in the presence of NRH (bar 4). [$] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in NQO2 transfected cells (bars 7-12) compared to the respective untransfected cells (bars 1-6). One symbol indicates p<0.05, two symbols indicate p<0.01, three symbols indicate p<0.001 and four symbols indicate p<0.0001.

In line with the results observed in MCF-7 cells, in T47D cells transfected with the empty vector, similar trends were observed, namely increment of GRP94 protein levels in most of the treatments compared to those in non-treated cells. Increased GRP94 protein levels were evident in T47D cells in the presence of NRH compared to those in the absence of NRH (Figure 4.2A and B, compare lane 4 to lane 1). NQO2 transfected and β-oestradiol treated T47D cells in the presence of NRH exhibited
increased GRP94 protein levels compared to NQO2 transfected non-treated cells in the absence of NRH (Figure 4.2A and B, compare lane 1 to lane 7). NQO2 transfected and β-oestradiol treated T47D cells in the presence of NRH showed increased GRP94 protein levels compared to NQO2 transfected β-oestradiol treated cells in the absence of NRH (Figure 4.2A and B, compare lane 11 to lane 8).

The other endoplasmic reticulum stress chaperone GRP78 protein levels were also investigated in T47D cells. Statistically significant increased of GRP78 protein levels in β-oestradiol or tamoxifen treated T47D cells in both the absence and presence of NRH compared to the untreated cells were recorded (Figure 4.2A and C, compare lanes 2-6 to lane 1). The GRP78 protein levels were statistically significant increased in T47D cells treated with β-oestradiol in the presence of NRH compared to those in cells treated with β-oestradiol in the absence of NRH (Figure 4.2A and C, compare lane 5 to lane 2).

NQO2 transfected T47D cells in the absence or presence of NRH exhibited significantly decreased GRP78 protein levels compared to the untransfected cells in the absence or presence of NRH (Figure 4.2A and C, compare lanes 7-11 to the respective lanes 1-6). Significantly increased GRP78 protein levels were observed in the NQO2 transfected and tamoxifen-treated T47D cells in the absence of NRH compared to those in NQO2 transfected and non-treated cells (Figure 4.2A and C, compare lane 9 to lane 7). Significantly increased GRP78 protein levels were observed in NQO2 transfected untreated T47D cells in the presence of NRH compared to those in NQO2 transfected untreated cells in the absence of NRH (Figure 4.2A and C, compare lane 10 to lane 7). Significantly increased GRP78 protein levels were observed in NQO2 transfected and β-oestradiol or tamoxifen-treated T47D cells in the presence of NRH compared to those in NQO2 transfected and untreated cells in absence of NRH (Figure 4.2A and C, compare lanes 11 and 12 to lane 7). Statistically significant differences in GRP78 protein levels were also observed between the NQO2 transfected and β-oestradiol treated cells in the presence of NRH compared to those in the NQO2 transfected and β-oestradiol treated in the absence of NRH (Figure 4.2A and C, compare lane 11 to lane 8). Decreased GRP78 protein levels were observed in NQO2 transfected cells treated with tamoxifen in the presence of NRH compared to
untreated and NQO2 transfected cells in the presence of NRH (Figure 4.2A and C, compare lane 12 to lane 10).
Figure 4.2: Effects of NQO2 on the endoplasmic reticulum stress biomarkers GRP94 and GRP78 in T47D human breast cancer cells

(A) T47D human breast cancer cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected and western blot analysis was carried out as described in Materials and Methods. GRP94, GRP78 and NQO2 protein levels were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK). A representative experiment out of three repeats is presented in the figure. (B) and (C) Graph showing the quantification of the intensity of the GRP94 and GRP78 bands normalised to that of actin using the Image J software. [*] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in untransfected MCF-7 cells treated with β-oestradiol or tamoxifen compared to untransfected and non-treated cells (bar 1) [#] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in NQO2 transfected cells and treated with β-oestradiol or tamoxifen compared to NQO2 overexpressing and non-treated cells (bar 7). [a] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in β-oestradiol treated cells in the absence of NRH (bars 2 and 8) compared to β-oestradiol treated cells in the presence of NRH (bars 5 and 11) [^] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in NQO2 transfected and tamoxifen treated cells in the presence of NRH (bar 11) compared to non-treated and NQO2 transfected cells in the presence of NRH (bar 10) and [$] significant difference (p<0.05) between the intensity of GRP94 or GRP78 bands in NQO2 transfected cells (bars 7-12) compared to the respective untransfected cells (bars 1-6). One symbol indicates p<0.05, two symbols indicate p<0.01, three symbols indicate p<0.001 and four symbols indicate p<0.0001.

Taken together results shown in Figures 4.1 and 4.2 suggest that in the ERα positive MCF-7 and T47D cells, a combination of NQO2 overexpression with β-oestradiol treatment increased GRP94 protein levels in the presence of NRH compared to those identified in cells under the same conditions in the absence of NRH (Figure 4.1A and
4.2A, compare lane 11 and lane 12 to lane 8 and lane 9 respectively). Furthermore, tamoxifen treatments in the presence of NRH decreased GRP78 protein levels compared to the levels of this protein detected in untransfected cells in the presence of NRH (Figure 4.1A and Figure 4.2A, compare lane 11 and lane 12 to lane 5 and lane 6 respectively).

4.1.1 Effects of NQO2 on the unfolded protein response in human breast cancer cells

The imbalance between oxidant and antioxidant cellular metabolites results in the accumulation of intracellular reactive oxygen species which induce oxidative stress. Among the cellular functions stimulated by oxidative stress is endoplasmic reticulum stress and unfolded protein response (Clarke and Cook 2015). Protein disulphide isomerases are a family of redox responsive proteins localised in the endoplasmic reticulum mainly involved in the quality control of newly synthesised proteins (Grek and Townsend 2014). PDIs have chaperone, isomerase and oxidoreductase activities (Hong and Soong 2008). Their isomerase activity catalyses refolding of misfolded proteins through a cycle of oxidation and reduction reactions. Many studies have shown a connection between PDI and oestrogen receptor (Schultz-Norton, McDonald et al. 2006). PDI has been demonstrated to act as an oestrogen receptor coregulatory factor modulating its transcriptional activity (Schultz-Norton, McDonald et al. 2006) and is present in the endoplasmic reticulum and nuclei of breast cancer cells (Schultz-Norton, McDonald et al. 2006).

Results described in section 3.1.2 and 3.1.3 indicating the potential role of NQO2 in regulating ERα transcriptional activity and protein stability together with its role in metabolising quinones thereby affecting the cellular redox state stimulated our interest to study the possibility of the existence of a crosstalk between NQO2 and PDI. To this direction, the effects of β-oestradiol or tamoxifen in the absence and presence of NRH on the PDI protein levels were investigated in ERα negative and ERα positive breast cancer cells.

The effects of NQO2 on UPR were investigated employing western blot analysis probing for PDI and using actin as a loading control in untransfected or overexpressing NQO2 breast cancer cells. No difference in the PDI protein levels was detected in
untransfected MCF-7 cells treated with either β-oestradiol, tamoxifen or NRH compared to untreated cells (Figure 4.3A and B, compare lanes 2, 3 and 4 to lane 1). Statistically significant decrease of PDI protein levels was detected in MCF-7 cells treated with β-oestradiol in the presence of NRH compared to cells treated with β-oestradiol in the absence of NRH (Figure 4.3A and B, compare lane 5 to lane 2).

In NQO2 transfected MCF-7 cells, significantly decreased PDI protein levels were recorded in the absence of NRH compared to untransfected cells under the same conditions (Figure 4.3A and B, compare lane 7 to lane 1). Significantly decreased PDI protein levels were observed in NQO2 transfected and treated with either β-oestradiol or tamoxifen MCF-7 cells in the absence of NRH compared to untransfected cells treated with either β-oestradiol or tamoxifen in the absence of NRH (Figure 4.3A and B, compare lanes 7, 8 and 9 to lanes 1, 2 and 3 respectively).
Figure 4.3: Effects of NQO2 on PDI protein levels in MCF-7 human breast cancer cells
(A) MCF-7 cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected, and western blot analysis was carried out as described in Materials and Methods. PDI and NQO2 protein levels were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK). One representative out of two independently repeated experiments is shown (B) Graph showing the quantification of the intensity of the PDI bands in cells transfected with the empty vector and cells transfected with NQO2 normalised to that of actin using the Image J software. [*] significant difference ($p<0.05$) between the intensity of PDI bands in untransfected MCF-7 cells treated with β-oestradiol or tamoxifen compared to untransfected and non-treated cells (bar 1). [a] significant difference ($p<0.05$) between the intensity of PDI bands in β-oestradiol treated cells in the absence of NRH (bar 2) compared to β-oestradiol treated cells in the presence of NRH (bar 5). [$S$] significant difference ($p<0.05$) between the intensity of PDI bands in NQO2 transfected cells (bars 7-9) compared to the respective untransfected cells (bars 1-3). One symbol indicates $p<0.05$ and two symbols indicate $p<0.01$.

In untransfected T47D cells, no differences in PDI protein levels were detected in β-oestradiol treatment in the absence and presence of NRH compared to the untreated (Figure 4.4A and B, compare lanes 2 and 5 to lane 1 and 2 respectively). PDI protein levels showed no differences in untransfected and tamoxifen treated cells compared to those in untransfected and untreated (Figure 4.4A and B, compare lane 3 to lane 1). There were no differences in PDI protein levels in the absence of NRH compared to those in the presence of NRH (Figure 4.4A and B, compare lane 4 to lane 1). No differences in PDI protein levels in untransfected tamoxifen treated-T47D cells in the
presence of NRH compared to the untransfected tamoxifen treated-T47D cells in the absence of NRH (Figure 4.4A and B, compare lane 6 to lane 3).

Significantly decreased PDI protein levels were recorded in NQO2 overexpressing T47D cells in the absence of NRH compared to untransfected cells under the same conditions (Figure 4.4A and B, compare lane 7 to lane 1). Significantly increased PDI protein levels were observed in NQO2 transfected and NRH treated T47D cells compared to those detected in NQO2 transfected and untreated T47D cells (Figure 4.4A and B, compare lane 10 to lane 7). Significantly decreased PDI protein levels were recorded in NQO2 transfected and tamoxifen treated T47D cells in the presence of NRH compared to NQO2 transfected and tamoxifen treated cells in the absence of NRH (Figure 4.4A and B, compare lane 12 to lane 9).
Figure 4.4: Effects of NQO2 on PDI protein levels in T47D human breast cancer cells

(A) T47D human breast cancer cells were seeded in 6-well plates and transfected with either the empty vector PCDNA3 or NQO2 expression vector and treated with β-oestradiol, tamoxifen or left untreated in the presence or absence of NRH as indicated. Cell lysates were collected and western blot analysis was carried out as described in Materials and Methods. PDI and NQO2 protein levels were detected using specific antibodies and visualised using the Chemi Doc MP imaging system (Bio-Rad, UK). One representative out of two independently repeated experiments is shown (B) Graph showing the quantification of the intensity of the PDI band in cells transfected with the empty vector and cells transfected with NQO2 normalised to that of actin using the Image J software. [#] significant difference (p<0.05) between the intensity of PDI bands in NQO2 transfected cells and treated with β-oestradiol in the presence of NRH to NQO2 overexpressing and non-treated cells (bar 7). [a] significant difference (p<0.05) between the intensity of PDI bands in NQO2 transfected and tamoxifen treated cells in the absence of NRH (bar 9) compared to NQO2 overexpressing and tamoxifen treated cells in the presence of NRH (bar 12). [\^] significant difference (p<0.05) between the intensity of PDI bands in NQO2 transfected and tamoxifen treated cells in the presence of NRH (bar 12) compared to this in untreated and NQO2 transfected in the presence of NRH (bar 10) and [\$] significant difference (p<0.05) between the intensity of PDI bands in NQO2 transfected cells (bar 7) compared to this in the respective untransfected cells (bar 1). One symbol indicates p<0.05 and two symbols indicate p<0.01.

Taken together results shown in Figure 4.3 and 4.4 indicate that in ERα positive MCF-7 and T47D cells, a combination of NQO2 overexpression with tamoxifen treatment decreased PDI protein levels in the presence of NRH compared to those identified in cells under the same conditions in the absence of NRH (Figure 4.3A and 4.4A, compare lane 12 to lane 6). PDI protein levels were decreased in NQO2 transfected and β-oestradiol-treated MCF-7 cells in the presence of NRH compared to NQO2
transfected MCF-7 cells in the absence of NRH (Figure 4.3A, compare lane 11 to lane 2).

4.1.2 Effects of NQO2 on PDI gene expression

To investigate whether the effects of NQO2 overexpression on PDI protein levels were regulated at the level of transcription the PDI mRNA levels were measured in the ERα positive MCF-7 cells treated with β-oestradiol or tamoxifen in the presence or absence of NRH using qRT-PCR. Cells were treated with β-oestradiol, tamoxifen or NRH alone or a combination of the hormones with NRH or left untreated as indicated and total mRNA was isolated as described in the Materials and Methods section.

Decreased PDI mRNA levels were detected in MCF-7 cells treated with β-oestradiol in the absence of NRH compared to the untreated cells (Figure 4.5, compare lane 2 to lane 1). In the presence of NRH, PDI mRNA levels were decreased in β-oestradiol treated MCF-7 cells compared to those detected in cells treated with β-oestradiol alone (Figure 4.5, compare lane 5 to lane 2). On the other side, slightly increased PDI mRNA levels were recorded in tamoxifen treated MCF-7 cells in the absence of NRH compared to the untreated cells (Figure 4.5, compare lane 3 to lane 1). There were no differences in PDI mRNA levels in MCF-7 cells treated with tamoxifen in the presence of NRH compared to the untreated cells (Figure 4.5, compare lane 6 to lane 1).
MCF-7 cells were treated with β-oestradiol (bars 2 and 5) or tamoxifen (bars 3 and 6) in the absence (bars 1, 2 and 3) and presence (bars 4, 5 and 6) of NRH. Total mRNA was extracted and the PDI mRNA levels were measured using qRT-PCR as described in the Materials and Methods section. Data were analysed using the StepOne software. The results shown are the average of three independent experiments performed in duplicates.

To test whether the effects of β-oestradiol or tamoxifen treatment observed in MCF-7 cells were mediated by the ERα transcriptional activity, the PDI mRNA levels were accessed in β-oestradiol or tamoxifen-treated ERα negative MDA-MB-231 cells in the absence or presence of NRH as indicated. Results shown in Figure 4.6 suggest that β-oestradiol increased PDI mRNA levels in the absence of NRH (Figure 4.6, compare lane 2 to lane 1). Tamoxifen treatment did not affect PDI mRNA level compared to untreated cells (Figure 4.6, compare lane 3 to lane 1). Significantly decreased PDI mRNA levels were observed in β-oestradiol-treated MDA-MB-231 in the presence of NRH compared to the β-oestradiol-treated MDA-MB-231 in the absence of NRH (Figure 4.6, compare lane 5 to lane 2). No significant differences were observed in tamoxifen-treated MDA-MB-231 in the presence of NRH compared to the tamoxifen-treated MDA-MB-231 in the absence of NRH (Figure 4.6, compare lane 6 to lane 3).
MDA-MB-231 cells were treated with β-oestradiol (bars 2 and 5) or tamoxifen (bars 3 and 6) in the absence (bars 1, 2 and 3) and presence (bars 4, 5 and 6) of NRH. Total mRNA was extracted and the PDI mRNA levels were measured using qRT-PCR as described in the Materials and Methods section. Data were analysed using the StepOne software. The results shown are the average of three independent experiments performed in duplicates. [**] significant difference (p<0.01) between the PDI mRNA levels detected in β-oestradiol treated cells in the absence of NRH (bar 2) compared to those detected in the β-oestradiol treated cells in the presence of NRH (bar 5).

Taken together results shown in the Figures 4.5 and 4.6 suggest that β-oestradiol or tamoxifen treatments in the absence of NRH regulate PDI mRNA levels differently in the ERα positive MCF-7 cells versus the ERα negative MDA-MB-231 cells. In particular, slightly decreased PDI mRNA levels were detected in the β-oestradiol-treated MCF-7 cells whereas increased PDI mRNA levels were recorded in the β-oestradiol-treated MDA-MB-231 cells compared to those measured in untreated cells (compare lane 2 to lane 1 in Figures 4.5 and 4.6). Tamoxifen treatment, on the other hand, increased PDI mRNA levels in MCF-7 cells and decreased PDI mRNA levels in MDA-MB-231 cells compared to those measured in untreated cells (compare lane 3 to lane 1 in Figures 4.5 and 4.6). Decreased PDI mRNA levels were recorded in both MCF-7 and MDA-MB-231 cells treated with NRH alone or a combination of NRH with β-oestradiol compared to untreated cells (compare bars 4 and 5 to bar l in Figures 4.5 and 4.6). No difference in PDI mRNA levels was evident in both MCF-7 and
MDA-MB-231 cells treated with NRH alone or combination of NRH with tamoxifen compared to untreated cells (compare bar 6 to bar 1 in Figures 4.5 and 4.6). Results shown in Figures 4.5 and 4.6 lent support to the conclusion that PDI is an ERα transcriptional target (Schultz-Norton, McDonald et al. 2006) and NRH potentially through induction of NQO2 downregulates the PDI protein levels. PDI has oncogenic activities (Higa, Mulot et al. 2011) therefore a physiological NQO2 substrate (since NRH is known not to be synthesised in human cells) could be beneficial for the treatment of ERα positive and ERα negative breast cancer patients.

4.2 Chapter discussion

Endoplasmic reticulum stress and unfolded protein response (UPR) are induced by several factors including oxidative stress to maintain cellular homoeostasis or promote apoptosis (Zeeshan, Lee et al. 2016). Several reports have indicated the relation between endoplasmic reticulum stress and unfolded protein response with breast cancer progression (Clarke, Cook et al. 2012, Wang, Groenendyk et al. 2014). Quinones’ metabolism as a potential source of ROS and hence oxidative stress might be involved in the induction of endoplasmic reticulum stress and unfolded protein response (UPR) in breast cancer cells (Bock 2012). In breast cancer cells, accumulation of oestrogen induces production of catechol oestrogens quinones by cytochrome P450 enzymes and in particular the CYP1B1 family member that induces the production of ROS and genotoxicity by metabolising oestrogen to the genotoxic 4-hydroxyl catechol oestrogens (Bolton and Thatcher 2008). NQO2 detoxifies catechol oestrogens quinones to maintain redox homoeostasis in breast cancer cells (Bock 2012). Endoplasmic reticulum stress chaperones such as GRP94 and GRP78 are highly expressed in breast cancer cell (Brown and Naidoo 2012). Overexpression of GRP94 in breast cancer cells is related to cell aggressiveness and play a role in the initiation, proliferation and metastasis as well as cell migration (Bini, Magi et al. 1997, Dejeans, Glorieux et al. 2012, Gutiérrez and Simmen 2014).

There is a potential correlation between NQO1, the NQO2 homologue and other antioxidant enzymes including catalase, glutathione peroxidase, superoxide dismutase in regulating endoplasmic reticulum stress and unfolded protein response (UPR) in human breast cancer cells (Dejeans, Glorieux et al. 2012). Overexpression of endoplasmic reticulum stress and unfolded protein response chaperones such as
GRP94 and GRP78 upregulates the antioxidant activity of NQO1 and other antioxidant enzymes in breast cancer cells (Dejeans, Glorieux et al. 2012). Accumulation of evidence suggests that there is a connection between ER stress and ROS with redox signalling mediators such as protein disulfide isomerase (PDI)-endoplasmic reticulum oxidoreductin (ERO)-1, glutathione (GSH)/glutathione disulfide (GSSG), NADPH oxidase 4 (Nox4), NADPH-P450 reductase (NPR), and calcium (Zeeshan, Lee et al. 2016).

To this direction, GRP78, GRP94 and PDI gene expression were determined under various treatments in untransfected and NQO2 transfected breast cancer cells. Endoplasmic reticulum stress chaperones, GRP94 and GRP78 protein levels were measured in ERα-positive breast cancer cells, MCF-7 and T47D treated with different hormones. NQO2 upregulated GRP94 in cells treated with oestrogen and antioestrogen hormones in the presence of NRH. Recent reports have suggested that there is a correlation between oestrogen signalling and GRP94 in gastric cancer (Fu, Zhen et al. 2014) but there is no clear evidence of this correlation in breast cancer cells. The opposite trend occurred in GRP78 expression, where NQO2 significantly downregulated GRP78 in MCF-7 and T47D cells. The induction of GRP78 is important to maintain cellular homoeostasis. GRP78 plays important roles in regulating endoplasmic reticulum homoeostasis, protein folding and calcium concentration in the endoplasmic reticulum (Eeckhoute, Carroll et al. 2006). The physiological role of GRP78 in antioxidant activity is largely unknown, but endoplasmic reticulum stress induction by GRP78 can be the main factor of the pro survival arm of the unfolded protein response (Eeckhoute, Carroll et al. 2006). Results obtained in this study showing NQO2 mediated upregulation of GRP94 allow the hypothesis that NQO2 induces a cytoprotective ER-stress response (Pizzo, Scapin et al. 2010).

Protein disulphide isomerase is an enzyme involved in unfolded protein response (UPR) in endoplasmic reticulum stress metabolism (Wang, Shi et al. 2012). NQO2 significantly decreased PDI protein levels in β-oestradiol and tamoxifen-treated MCF-7 and T47D cells in the presence of NRH. To confirm the involvement of NQO2 in unfolded protein response, we examined the PDI mRNA expression in MCF-7 and MDA-MB-231 cells under various treatments. The combination of NRH and β-
oestradiol and tamoxifen treatment decreased PDI mRNA expression in MCF-7 and MDA-MB-231 cells indicating that NQO2 might be involved in regulating endoplasmic reticulum stress and unfolded protein response chaperones in β-oestradiol and tamoxifen-treated breast cancer cells.
CHAPTER 5

Regulation of cell cycle progression and mitochondrial membrane potential by NQO2 in breast cancer cells
5.0 RESULTS

5.1 NQO2 in the control of cell cycle progression and mitochondrial membrane potential in breast cancer cells

The role of NAD(P)H quinone oxidoreductases in providing antioxidant action against stress induced by ROS through the catalysis of quinone metabolism and thereby alteration of the redox state of cancer cells is well documented (Nioi and Hayes 2004, Zhuang, Ma et al. 2012). ROS are essential players in the regulation of cell cycle progression since alterations in the cellular redox balance mediate post-translational modifications such as ubiquitination and phosphorylation which change the activity of factors involved in the control of cell proliferation such as cyclins and cyclin-dependent kinases (CDKs) (Verbon, Post et al. 2012). Apart from altering ROS, NQO1 has been shown to affect cell cycle progression by modulating the activity of transcription factors such as the NF-κB p50 subunit thereby upregulating the expression of cyclins A2, B1 and D1 in melanoma cells (Garate, Wani et al. 2010). In breast cancer cells, oestrogen receptor plays vital role in the regulation of cell cycle progression by inducing gene expression of cyclin-dependent kinases (CDK) (Caldon, Daly et al. 2006, Malumbres 2014) and the cyclin D1, CDK inhibitors p21\textsuperscript{Waf1/Cip1}, and p27\textsuperscript{Kip1} thereby contributing to mammary oncogenesis (Sutherland and Musgrove 2004).

Although many studies have explored the role of the NQO1 in the cell cycle control and carcinogenesis in several types of cancer (Cao and Kaufman 2014, Yang, Zhang et al. 2014, Madajewski, Boatman et al. 2016). The impact of NQO2 overexpression in cancer pathogenesis remains largely unknown. NQO2 overexpression downregulated cyclin D1 protein levels in the ERα-positive breast cancer cells MCF7 and T47D treated with β-oestradiol (Figure 3.5 and 3.6). To investigate whether the NQO2 mediated downregulation of cyclin D1 protein levels in β-oestradiol treated MCF7 and T47D cells affected their cell proliferation the cell cycle profile of these cells was followed using flow cytometry.
5.1.1 Cell cycle profile of ERα positive and ERα negative breast cancer cells treated with β-oestradiol or tamoxifen in the absence and presence of NRH

The cell cycle profiles of the ERα-positive MCF7 and T47D and the ERα-negative MDA-MB-231 and MDA-MB-468 breast cancer cells were determined in β-oestradiol or tamoxifen treated cells in the absence or presence of NRH as shown in Figures 5.1, 5.2, 5.3 and 5.4. Cell cycle profiles were created using propidium iodide and the Nucleocounter, NC-3000 (Chemometec, Denmark) as described in the Materials and Methods. PI intercalates in DNA within the cells and produces a fluorescent signal that can be measured at excitation 488nm and emission at 600nm using flow cytometry (Pozarowski and Darzynkiewicz 2004).

The effects of ectopically expressed NQO2 in regulating the cell cycle of the ERα-positive T47D and the ERα-negative MDA-MB-231 cells were also determined using FACs analysis as described in the Materials and Methods. The NQO2 overexpressing T47D and MDA-MB-231 cells were co-transfected with CD20 expressing vector, which was used as an indicator of the transfection efficiency. CD20 is the gene encoding the B-lymphocytes antigen 20 (Henry, Deschamps et al. 2010). CD20 surface marker was detected with an anti-CD20 antibody tagged to FITC (Cat no: 555622, BD Bioscience, UK). The effects of NQO2 on cell cycle profile in β-oestradiol-treated T47D and MDA-MB-231 cells in the absence and presence of NRH were detected by FACs analysis.

The ERα positive MCF-7 cells were treated with β-oestradiol, tamoxifen or NRH alone or combination of β-oestradiol with NRH or tamoxifen with NRH as indicated in Figure 5.1. Treatment of MCF-7 cells with β-oestradiol did not have any effect on the cell cycle profile in these cells compared to untreated (Figures 5.1A and B compare bar β-oestradiol with bar UT). Tamoxifen-treated MCF-7 cells displayed a higher percentage of the cellular population arrested in the G1 phase compared to untreated cells (untreated 78.0%, tamoxifen-treated 80.25%) (Figures 5.1A and B, compare bar tamoxifen with bar UT). AHigher percentage of MCF-7 cells treated with NRH alone arrested in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 78.0%, NRH treated 83.27%) (Figures 5.1A and B, compare bar NRH to bar UT). Combined β-oestradiol with NRH treatment slightly increased the percentage
of the population of cells arrested in the G1 phase (untreated 78.0%, \(\beta\)-oestradiol+NRH treated 78.8%) (Figure 5.1A and B, compare bar \(\beta\)-oestradiol+NRH to bar UT). Combined tamoxifen with NRH on the other side increased significantly the percentage of the population of cells in both the G1 (untreated 78.0%, tamoxifen+NRH treated 84.16%) and the G2/M phases (untreated 8.59%, tamoxifen+NRH treated 9.17%) (Figures 5.1A and B, compare bar tamoxifen+NRH to bar UT).

(A)
Figure 5.1: Cell cycle analysis of the ERα positive MCF-7 breast cancer cells under different conditions

MCF-7 cells were seeded in 6-well plates and left untreated or treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. Cells were fixed with 50% ethanol/PBS, stained with PI, and subjected to Nucleocounter, NC-3000 (Chemometec, Denmark) analysis. (A) Cell cycle profiles of MCF-7 cells treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. (B) Diagram indicating the percentage of the population of cells distributed in different phases of the cell cycle upon treatment with the indicated chemicals.

The ERα positive T47D breast cancer cells were treated with β-oestradiol, tamoxifen or NRH individually or combination of β-oestradiol with NRH or tamoxifen with NRH as indicated in Figure 5.2. Increased percentage of T47D cells treated with β-oestradiol arrested in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 80.34%, β-oestradiol-treated 85.36%) (Figures 5.2A and B, compare bar β-oestradiol with bar UT). Similar effect to that of β-oestradiol was observed upon treatment of T47D cells with tamoxifen, namely higher percentage of the tamoxifen-treated T47D cells accumulated in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 80.34%, tamoxifen-treated 84.99%) (Figures 5.2A and B, compare bar tamoxifen with bar UT). Individual NRH treatment of T47D cells resulted in similar increase in the percentage of cells arrested in the G1 phase of the cell cycle (untreated 80.34%, NRH treated 82.69%) (Figures 5.2A and B, compare bar NRH with bar UT).

T47D cells treated with combination of β-oestradiol with NRH exhibited a higher number of cells in G1 phase compared to untreated (untreated 80.34%, β-
oestradiol+NRH treated 83.75%) (Figures 5.2A and B, compare bar β-
oestradiol+NRH with bar UT). Combined tamoxifen with NRH treatment of T47D
cells resulted in an increase in the percentage of the cellular population in G1
(untreated 80.33%, tamoxifen+NRH treated 81.94%) and more significant in the G2/M
phase of the cell cycle compared to that of the untreated (untreated 9.96%,
tamoxifen+NRH treated 11.39%) (Figures 5.2A and B, compare bar tamoxifen+NRH
to bar UT).

(A)
Figure 5.2: Cell cycle analysis of the ERα positive T47D breast cancer cells under different conditions

T47D cells were seeded in 6-well plates and left untreated or treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. Cells were fixed with 50% ethanol/PBS, stained with PI, and subjected to Nucleocounter, NC-3000 (Chemometec, Denmark) analysis. (A) Cell cycle profiles of T47D cells treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. (B) Diagram indicating the percentage of the population of cells distributed in different phases of the cell cycle upon treatment with the indicated chemicals.

MDA-MB-231 cells treated with β-oestradiol displayed decreased percentage of population in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 85.84% β-oestradiol treated 83.24%) and increased in the G2/M phase of the cell cycle (untreated 4.73% β-oestradiol treated 5.59%) (Figures 5.3A and B, compare bar β-oestradiol with bar UT). Tamoxifen-treated MDA-MB-231 cells exhibited increased percentage of cellular population accumulated in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 85.84% tamoxifen-treated 87.98%) (Figures 5.3A and B, compare bar tamoxifen with bar UT).

NRH individual treatment of MDA-MB-231 cells decreased the percentage of these cells in the G1 phase of the cell cycle (untreated 85.84%, NRH treated 80.60%) and increased it in the G2/M phase of the cell cycle (untreated 4.73% NRH treated 7.09%) compared to that of the untreated cells (Figures 5.3A and B, compare bar NRH with bar UT). Combined NRH and β-oestradiol treatment increased the percentage of the proliferating population as the number of cells arrested in the G1 phase of the cell cycle under these conditions decreased compared to that of the untreated cells (untreated
85.84% NRH+β-oestradiol treated 79.82%). Furthermore, the percentage of the population of these cells in the G2/M phase increased under these conditions (untreated 4.73% NRH treated 6.78%) (Figures 5.3A and B, compare bar β-oestradiol+NRH with bar UT). MDA-MB-231 cells treated with a combination of tamoxifen with NRH exhibited increased number of cells accumulating in the G1 phase of the cell cycle (untreated 85.84% tamoxifen+NRH treated 87.34%) (Figures 5.3A and B, compare bar tamoxifen+NRH with bar UT).
MDA-MB-231 cells were seeded in 6-well plates and left untreated or treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. Cells were fixed with 50% ethanol/PBS, stained with PI, and subjected to Nucleocounter, NC-3000 (Chemometec, Denmark) analysis. (A) Cell cycle profiles of MDA-MB-231 cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH. (B) Diagram indicating the percentage of the population of cells distributed in different phases of the cell cycle upon treatment with the indicated chemicals.

MDA-MB-468 cells treated with β-oestradiol displayed increased percentage of the population in the G1 phase of the cell cycle compared to that of the untreated cells (untreated 73.22% β-oestradiol treated 84.07%) (Figures 5.4A and B, compare bar β-oestradiol with bar UT). A similar effect was observed in MDA-MB-468 cells treated with tamoxifen where the percentage of the cellular population in the G1 phase of the cell cycle was higher compared to that recorded in the untreated cells (untreated 73.22% tamoxifen-treated 82.76%) (Figures 5.4A and B, compare bar tamoxifen to bar UT). NRH treatment of MDA-MB-468 cells also increased the number of cells arrested in the G1 phase of the cell cycle but to a lesser extent compared to that observed in the β-oestradiol or tamoxifen-treated cells, (untreated 73.22% NRH treated 77.97%) (Figures 5.4A and B, compare bar NRH to bar UT).

The combination of β-oestradiol with NRH treatment increased the percentage of the cellular population in the G1 phase of the cell cycle (untreated 73.22% β-oestradiol+NRH treated 87.60%) (Figures 5.4A and B, compare bar β-oestradiol+NRH to bar UT). Increase of the cellular population in the G1 phase of the cell cycle was also observed in MDA-MB-468 cells treated with combination of NRH
with tamoxifen but not to the same extent as that observed in the β-oestradiol+NRH treated cells (untreated 73.22% tamoxifen+NRH treated 78.09%) (Figures 5.4A and B, compare bar tamoxifen+NRH to bar UT).

Figure 5.4: Cell cycle analysis of the ERα negative MDA-MB-468 breast cancer cells under different conditions.
MDA-MB-468 cells were seeded in 6-well plates and left untreated or treated with either β-oestradiol or tamoxifen in the absence or presence of NRH. Cells were fixed with 50% ethanol/PBS, stained with PI, and subjected to Nucleocounter, NC-3000 (Chemometec, Denmark) analysis. (A) Cell cycle profiles of MDA-MB-468 cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH. (B) Diagram indicating the percentage of the population of cells distributed in different phases of the cell cycle upon treatment with the indicated chemicals.

Comparative analysis of the cell cycle profiles in G1 phase of breast cancer cells treated with β-oestradiol in the absence or presence of NRH was analysed (Figure 5.5A and B).

The population of MCF-7 cells arrested in the G1 phase of the cell cycle after treatment with β-oestradiol alone or in combination with NRH was similar to that of the untreated MCF-7 cells (Figure 5.5A, compare bar 1 to bars 2 and 3). An increased percentage of MCF-7 cells arrested in the G1 phase of the cell cycle was observed upon treatment with NRH compared to untreated cells (Figure 5.5B, compare bar 2 to bar 1).

T47D cells treated with β-oestradiol exhibited approximately 5% increase in the population of cells arrested in G1 phase compared to the untreated cells (Figure 5.5A, compare bar 5 to bar 4). No difference in the number of T47D cells arrested in the G1 phase of the cell cycle was observed upon combined treatment of β-oestradiol with NRH compared to those treated with β-oestradiol alone (Figure 5.5A, compare bar 6 to bar 5). A slightly increased population of T47D cells arrested in the G1 phase of the cell cycle was detected in the presence of NRH compared to untreated cells (Figure 5.5B, compare lane 4 to lane 3).

Decreased percentage of MDA-MB-231 cells arrested in the G1 phase of the cell cycle was measured in those cells that were treated with β-oestradiol in the absence and in the presence of NRH compared to the untreated cells (Figure 5.5A, compare bars 8 and 9 to bar 7). Approximately 9% decrease in the population of MDA-MB-231 cells was counted in the NRH treated compared to untreated cells (Figure 5.5B, compare bar 6 to bar 5).

MDA-MB-468 cells treated with β-oestradiol exhibited approximately 13% increase in the population arrested in the G1 phase of the cell cycle in the absence of NRH compared to the untreated cells (Figure 5.5A, compared bar 11 to bar 10) and even
further increase in the presence of NRH compared to untreated cells (Figure 5.5A, compare bar 12 to bar 10). Increased accumulation of MDA-MB-468 cells population in the G1 phase of the cell cycle was also evident upon NRH treatment compared to untreated cells (Figure 5.5B, compare bar 8 to bar 7).

Taken together results shown in Figures 5.5A and B indicate that β-oestradiol in both the presence and the absence of NRH did not affect the G1 phase of the cell cycle in the ERα positive MCF-7 and T47D cells whereas the ERα negative MDA-MB-231 and MDA-MB-468 cells exhibited opposite trends. In particular, lower number of MDA-MB-231 cells accumulated in the G1 phase of the cell cycle was detected in the β-oestradiol-treated cells in the absence and the presence of NRH compared to untreated cells whereas higher number of MDA-MB-468 in the G1 phase of the cell cycle was measured in the cells treated with β-oestradiol alone or in combination with NRH compared to untreated cells.
Figure 5.5: Comparison of the G1 phase of the cell cycle of breast cancer cells treated with β-oestradiol and NRH

Cells were seeded in 6-well plates and left untreated or treated with β-oestradiol and NRH individually or in combination as indicated. Cells were collected, fixed with 50% ethanol/PBS, and stained with Propidium Iodide (PI) and subjected to cell cycle analysis as described in the Materials and Methods section. (A) Comparison of the G1 phase of the cell cycle of breast cancer cells treated with β-oestradiol alone or in combination with NRH (B) Comparison of the G1 phase of the cell cycle of breast cancer cells treated with NRH versus untreated cells.

Analysis of the population of cells accumulated in the G1 phase of the cell cycle of breast cancer cells treated with tamoxifen in the presence or absence of NRH indicated an increased population of tamoxifen-treated MCF-7 cells in the absence and presence of NRH compared to untreated cells (Figure 5.6, compare bars 2 and 3 to bar 1) and no effect of individual tamoxifen or combined tamoxifen and NRH treatment in the G1 phase of the cycle in the other breast cancer cells.

Taken together results shown in Figure 5.6 indicate increased number of tamoxifen-treated MCF-7 cells in the presence and absence of NRH compared to untreated cells and no effect of tamoxifen in the G1 phase of the cell cycle in the presence or absence of NRH in the T47D, MDA-MB-231 and MDA-MB-468 cells.
Figure 5.6: Comparison of the G1 phase of the cell cycle of breast cancer cells treated with tamoxifen

Cells were seeded in 6-well plates and left untreated or treated with tamoxifen and NRH individually or in combination as indicated. Cells were collected, fixed with 50% ethanol/PBS, and stained with Propidium Iodide (PI) and subjected to cell cycle analysis as described in the Materials and Methods section.

5.1.2 Roles of NQO2 in regulating cell cycle in breast cancer cells

The potential involvement of NQO2 in regulating the cell cycle progression in breast cancer cells was investigated by analysing the cell cycle profiles of NQO2 overexpressing T47D and MDA-MB-231 cells using CYAN ADP flow cytometer. T47D and MDA-MB-231 cells were transfected with either the PCDNA3 empty vector or an NQO2 expression vector along with CD20 expressing vector and treated with β-oestradiol or NRH individually or in combination as indicated in Figure 5.7A. Data were analysed using the Beckman Coulter Summit 4.1 software and plotted in Figure 5.7B.

NQO2 overexpressing and untreated T47D cells displayed decreased number of cells accumulated in the G1 phase (73.58%) and S phase (9.45% compared to cells transfected with the PCDNA3 empty vector (76.33% and 9.50% respectively). Increased number of cells in the G2/M phase of the cell cycle was observed in NQO2 overexpressing T47D cells (16.97%) compared to PCDNA3 transfected (14.18%) (Figures 5.7A and B, compare bar 5 to bar 1).
Overexpression of NQO2 in β-oestradiol-treated T47D cells resulted in the reduction of the number of cells accumulated in the G1 (74.79%) and S (6.46%) phases of the cell cycle compared to PCDNA3 transfected and β-oestradiol-treated cells (G1 81% and S 7.19%) (Figures 5.7A and B, compare bar 6 to bar 2). NQO2 overexpression under these conditions increased the number of T47D cells accumulating in the G2/M phase of the cell cycle (18.76%) compared to PCDNA3 transfected and β-oestradiol-treated (11.81%) cells.

T47D cells overexpressing NQO2 and NRH treated showed decreased number of cells arrested in the G1 phase (67.62%) compared to PCDNA3 transfected and NRH treated cells (79.8%). Increased number of cells arrested in S (7%) and G2/M (25.58%) phases of the cell cycle were recorded in T47D cells overexpressing NQO2 compared to PCDNA3 transfected (S 6.27%) and (G2/M 13.93%) cells (Figure 5.7A and B, compare bar 7 to bar 3).

NQO2 overexpressing T47D cells treated with a combination of β-oestradiol and NRH displayed reduced G1 (77.90%) and S (7.16%) phase arrest compared to PCDNA3 transfected cells under the same conditions (G1 78.86% and S 9.29%). In contrast, overexpression of NQO2 increased G2/M phase arrest (14.94%) compared to PCDNA3 transfected cells (11.85%) (Figure 5.7A and B, compare bar 8 to bar 4).

Analysis of the cell cycle profile of T47D cells indicated that NQO2 overexpression decreased G1 phase arrest in β-oestradiol, NRH and combination of β-oestradiol and NRH treatments compared to PCDNA3 transfected cells under the same conditions. On the other side, overexpression of NQO2 increased G2/M phase arrest in untreated, β-oestradiol, NRH and combination of β-oestradiol and NRH treatments compared to PCDNA3 transfected cells under the same conditions.
Figure 5.7: Effects of NQO2 overexpression on the regulation of cell cycle of T47D breast cancer cells

(A) T47D cells were transfected with either PCDNA3 empty or NQO2 expressing vectors along with CD20 expressing vector and treated with β-oestradiol or NRH alone or combination of β-oestradiol and NRH. After the treatments, cells were fixed with 50% ethanol/PBS, stained with PI and FITC CD20-antibody and measured using CYAN ADP flow cytometer detecting fluorescent emission at 613nm. Black curves indicate PCDNA3 transfect ed and green curves NQO2 transfect ed cells. One representative profile out of two independent experiments is shown.

(B) Data from two independent experiments were analysed using Beckman Coulter Summit 4.1 software and plotted in the graph shown.
The cell cycle profiles of the ERα-negative MDA-MB-231 breast cancer cells overexpressing NQO2 were also studied. Cells were transfected with either PCDNA3 empty or NQO2 expression vectors and treated with β-oestradiol, NRH alone, or a combination of β-oestradiol and NRH.

NQO2 overexpression in untreated MDA-MB-231 cells increased G1 phase arrest (82.65%) compared to untreated PCDNA3 transfected cells (70.16%). Decreased number of untreated NQO2 overexpressing cells was detected in the S (14.66%) and G2/M (2.69%) phases of the cell cycle compared to untreated PCDNA3 transfected cells (S 16.04% and G2/M 13.80%) (Figure 5.8A and B, compare bar 5 to bar 1).

Treatment with β-oestradiol of MDA-MB-231 cells overexpressing NQO2 increased the number of cells arresting in the G1 phase (87.41%) and decreased it in S (10.59%) and G2/M (1.99%) phases compared to cells transfected with PCDNA3 in the same conditions (G1 66.09%, S 17.28% and G2/M 16.63%) (Figure 5.8A and B, compare bar 6 to bar 2).

NRH treated and NQO2 overexpressing MDA-MB-231 cells displayed increased G1 phase arrest (84.20%) and decreased S (13.25%) and G2/M (2.55%) phases compared to cells transfected with PCDNA3 under the same conditions (G1 69.09%, S 16.45% and G2/M 14.46%) (Figure 5.8A and B, compare bar 7 to bar 3).

NQO2 overexpression in MDA-MB-231 cells treated with combination of β-oestradiol and NRH, displayed increased G1 phase arrest (86.58%) and decreased S (11.83%) and G2/M (1.59%) phases compared to PCDNA3 transfected and treated the same way (G1 69.59%, S 16.11% and G2/M 14.50%) (Figure 5.8A and B, compare bar 8 to bar 4).

Analysis of cell cycle profile in MDA-MB-231 cells showed that NQO2 overexpression increased G1 phase arrest in β-oestradiol, NRH and combination of β-oestradiol and NRH treatments and decreased accumulation in S and G2/M phases in untreated, β-oestradiol, NRH and combination of β-oestradiol and NRH treatments compared to cells transfected with PCDNA3 under the same conditions.
Figure 5.8: Effects of NQO2 in the cell cycle regulation of MDA-MB-231 breast cancer cells

(A) MDA-MB-231 cells were transfected with the empty PCDNA3 or NQO2 expressing vectors and left untreated or exposed to different treatments including β-oestradiol, NRH and the combination of β-oestradiol and NRH as indicated. After the treatments, cells were disassociated from the six-well plate, fixed with 50% ethanol/PBS, stained with PI and FITC CD20-antibody and cell cycle profiles were created using the CYAN ADP flow cytometer detecting fluorescent emission at 613nm. Black curves indicate PCDNA3 transfection and green curves NQO2 transfection. (B) Data were analysed using Beckman Coulter Summit 4.1 software.
5.1.3 NQO2 as a potential regulatory factor of the mitochondrial membrane potential (ΔψMMP) in human breast cancer cells

The role of NQO2 in maintaining cellular homoeostasis and preventing redox cycling especially in reducing quinone-induced ROS carcinogenesis and oxidative stress has been reported in several studies (Gaikwad, Yang et al. 2009). ROS generated as by-products of the mitochondrial respiration reduce O₂ to superoxide anion which mediates a chain of oxidative reactions (Miettinen and Björklund 2014, Zorov, Juhaszova et al. 2014). Mitochondrial generated ROS affect various cellular functions including mitochondrial membrane potential. Given that NQO2 modulates oxidative stress its effects on the mitochondrial membrane potential were studied in β-oestradiol, tamoxifen and NRH treated breast cancer cells using the Nucleocounter (NC 3000) (Chemometec, Denmark) as described in the Materials and Methods section.

ERα- positive (MCF-7 and T47D) and ERα-negative (MDA-MB-231 and MDA-MB-468) cells were treated with β-oestradiol, tamoxifen and NRH and their effects on mitochondrial membrane potential were followed using the JC-1 dye. JC-1 is a permeable membrane dye that as a monomer (polarised state) is detected as green fluorescent colour at 530nm and as an aggregate polymer (depolarised state) as red fluorescent colour at 590nm (Wen, Zhu et al. 2013). Higher depolarised cell values indicate low ΔψMMP.

No significant ΔψMMP differences were observed in β-oestradiol or tamoxifen-treated MCF-7 in the absence of NRH compared to the untreated cells (Figures 5.9A and B, compare bars 3 and 5 to bar 1). NRH treatment increased MCF-7 ΔψMMP compared to untreated cells (Figures 5.9A and B, compare bar 2 to bar 1). MCF-7 cells treated with combination of β-oestradiol with NRH exhibited increased ΔψMMP compared to cells treated with NRH alone (Figures 5.9A and B, compare bar 4 to bar 2) whereas decreased ΔψMMP was detected in MCF-7 cells treated with combination of tamoxifen with NRH compared to cells treated with NRH alone (Figures 5.9A and B, compare bar 6 to bar 2).
Figure 5.9: Effects of β-oestradiol, tamoxifen and NRH on the mitochondrial membrane potential (ΔψMMP) of MCF-7 cells

(A) MCF-7 cells were left untreated or treated with β-oestradiol, tamoxifen or NRH alone or in combination as indicated stained with JC-1 and subjected to ΔψMMP analysis using the Nucleocounter (NC3000) (Chemometec, Denmark). Representative ΔψMMP profiles of one out of three independent experiments performed in duplicate is shown in the figure. (B) Graph representing the effect of β-oestradiol, tamoxifen or NRH treatments on ΔψMMP of MCF-7 cells. The untreated samples of all cell lines were arbitrarily set to 1, and the ΔψMMP values obtained from β-oestradiol, tamoxifen or NRH treated cells were calculated accordingly.

Treatment of T47D cells with β-oestradiol, tamoxifen or NRH alone did not have any impact on the ΔψMMP compared to the untreated cells (Figures 5.10A and B, compare lanes 2, 3 and 5 to lane 1). On the other hand, β-oestradiol or tamoxifen-treated T47D cells in the presence of NRH significantly decreased ΔψMMP compared to NRH treatment alone (Figures 5.10A and B, compare bars 4 and 6 to bar 2). Significantly decreased ΔψMMP was observed in β-oestradiol-treated T47D in the presence of NRH compared to the β-oestradiol-treated T47D in the absence of NRH (Figures 5.10A and B, compare bar 3 to bar 4).
Figure 5.10: Effects of β-oestradiol, tamoxifen and NRH on the mitochondrial membrane potential (ΔψMMP) of T47D cells
(A) T47D cells were left untreated or treated with β-oestradiol, tamoxifen or NRH alone or in combination as indicated and stained with JC-1 and subjected to ΔψMMP analysis using the Nucleocounter (NC3000) (Chemometec, Denmark). Representative ΔψMMP profiles of one out of three independent experiments performed in duplicate is shown in the figure. (B) Graph representing the effect of β-oestradiol, tamoxifen or NRH treatments on ΔψMMP of T47D cells. The untreated samples of all cell lines were arbitrarily set to 1, and the ΔψMMP values obtained from β-oestradiol, tamoxifen or NRH treated cells were calculated accordingly [*] significant ΔψMMP difference between cells treated with β-oestradiol alone or combination of β-oestradiol and NRH. [#] significant ΔψMMP difference between cells treated with NRH alone (bar 2) or combination of NRH with β-oestradiol (bar 4) or with tamoxifen (bar 6). One symbol indicates p<0.05.

No significant differences of ΔψMMP were observed in β-oestradiol, tamoxifen or NRH alone-treated MDA-MB-231 cells compared to the untreated cells (Figures 5.11A and B, compare bars 2, 3 and 5 to bar 1). MDA-MB-231 cells treated with a combination of β-oestradiol with NRH exhibited decrease ΔψMMP compared to cells treated with NRH alone (Figures 5.11A and B, compare bar 4 to bar 2). No difference in ΔψMMP was detected in MDA-MB-231 cells treated with combination of tamoxifen with NRH compared to cells treated with NRH alone (Figures 5.11A and B, compare bar 6 to bar 2).
Figure 5.11: Effects of β-oestradiol, tamoxifen and NRH on the mitochondrial membrane potential (ΔψMMP) of MDA-MB-231 cells

(A) MDA-MB-231 cells were left untreated or treated with β-oestradiol, tamoxifen or NRH alone or in combination as indicated stained with JC-1 and subjected to ΔψMMP analysis using the Nucleocounter (NC3000) (Chemometec, Denmark). Representative ΔψMMP profiles of one out of three independent experiments performed in duplicate is shown in the figure. (B) Graph representing the effect of β-oestradiol, tamoxifen or
NRH treatments on ΔѱMMP of MDA-MB-231 cells. The untreated samples of all cell lines were arbitrarily set to 1, and the ΔѱMMP values obtained from β-oestradiol, tamoxifen or NRH treated cells were calculated accordingly.

MDA-MB-468 cells treated with β-oestradiol did not show any differences in the ΔѱMMP compared to untreated cells (Figures 5.12A and B, compare bar 3 to bar 1). The combination of β-oestradiol with NRH increased ΔѱMMP in MDA-MB-468 cells compared to untreated cells but to a greater extent compared to cells treated with NRH alone (Figures 5.12A and B, compare bar 4 to bars 1 and 2). Increased ΔѱMMP was observed in MDA-MB-468 cells treated with NRH alone compared to untreated cells (Figure 5.12A and B, compare bar 2 to bar 1). Tamoxifen treatment of the MDA-MB-468 cells in the absence and presence of NRH significantly reduced ΔψMMP compared to the untreated or NRH-treated cells (Figures 5.12A and B, compare bars 5 and 6 to bars 1 and 2 respectively).
Figure 5.12: Effects of β-oestradiol, tamoxifen and NRH on the mitochondrial membrane potential (ΔψMMP) of MDA-MB-468 cells

(A) MDA-MB-468 cells were kept untreated or treated with β-oestradiol, tamoxifen or NRH alone or in combination as indicated stained with JC-1 and subjected to ΔψMMP analysis using the Nucleocounter (NC3000) (Chemometec, Denmark). Representative ΔψMMP profiles of one out of three independent experiments performed in duplicate is shown in the figure. (B) Graph representing the effect of β-oestradiol, tamoxifen or NRH treatments on ΔψMMP of MDA-MB-468 cells. The untreated samples of all cell lines were arbitrarily set to 1, and the ΔψMMP values obtained from β-oestradiol, tamoxifen or NRH treated cells were calculated accordingly. [*] significant ΔψMMP difference between cells treated with NRH alone (bar 2) and NRH in combination with tamoxifen. One asterisk indicates p<0.05.

5.1.4 Subcellular localisation of NQO2 in breast cancer cells treated with β-oestradiol or NRH

Results shown in section 3.1.2 indicated that NQO2 is a potential modulator of the ERα transcriptional activity implying that this oxidoreductase under certain conditions might be localized in the nucleus functioning as a transcriptional cofactor. To investigate the NQO2 subcellular localisation, the T47D and MDA-MB-468 breast cancer cells were transfected with an NQO2 expression vector and treated with β-oestradiol or NRH, and the subcellular localisation of NQO2 was followed using immunofluorescence microscopy.

Mainly cytoplasmic localisation and weak mitochondrial staining of NQO2 were observed in the untreated T47D cells (Figure 5.13A, UT, NQO2 green fluorescence and MitoTracker staining red fluorescence). More intense nuclear and mitochondrial
NQO2 staining was observed in T47D cells treated with β-oestradiol (Figure 5.13A, β-oestradiol). Several reports have shown that β-oestradiol induces translocation of ERα in the mitochondria (Borrás, Gambini et al. 2010) and ERα under these conditions plays an important role in the modulation of mitochondrial gene expression (Borrás, Gambini et al. 2010, Sanchez, Shearwood et al. 2015). In NQO2 overexpressing and NRH treated T47D cells strong nuclear and mitochondrial NQO2 staining was observed (Figure 5.13A, NRH).

In untreated MDA-MB-468 cells, overexpressing NQO2 intense cytoplasmic and weak mitochondrial staining of NQO2 was detected (Figure 5.13B, UT). Weak nuclear and intense mitochondrial NQO2 staining was observed in MDA-MB-468 cells treated with either β-oestradiol or NRH (Figure 5.13B, β-oestradiol and NRH respectively).
Figure 5.13A: NQO2 subcellular localisation in untreated, β-oestradiol and NRH treated T47D cells
T47D cells overexpressing NQO2 were left untreated or treated with β-oestradiol or NRH as indicated and stained with the NQO2 antibody (green), Mito tracker (red) and DAPI (blue). One representative out of two independent experiments is shown.
Figure 5.13B: NQO2 subcellular localisation in untreated, β-oestradiol and NRH treated MDA-MB-468 cells

MDA-MB-468 overexpressing NQO2 were left untreated or treated with β-oestradiol or NRH as indicated and stained with the NQO2 antibody (green), Mito tracker (red) and DAPI (blue). One representative out of two independent experiments is shown.
5.2 Chapter discussion

Increased generation of ROS due to the redox cycling of the oestrogen catechols regulate cell cycle progression in breast cancer cells (Fussell, Udasin et al. 2011). Recent report has shown that oestrogen independently regulates the expression of cMyc and cyclin D1 in cell cycle pathways (Doisneau-Sixou, Sergio et al. 2003). The inhibition of active cyclin E–Cdk2 complexes are blocked by the cyclin dependent kinase (CDK) inhibitor p21WAF1/CIP1 (Stewart, Leach et al. 1999). Tamoxifen treatment in breast cancer cells leads to an acute decrease of cMyc and cyclin D1 expression, inhibition of DNA synthesis and ultimately arrest of cells in a state with features characteristic of quiescence (Doisneau-Sixou, Sergio et al. 2003). Tamoxifen causes G1 cell cycle arrest by reducing the G1/S specific kinase cyclin-D1 expression and increasing the activity of the CDK inhibitors p21WAF1/CIP1 (Thiantanawat, Long et al. 2003) and p27Kip1 (Planas-Silva and Weinberg 1997) and thus decrease of the cyclin-E1 and CDK-2 activity (Beelen, Zwart et al. 2012). Taking these observations into consideration the cell cycle profiles of breast cancer cells treated with β-oestradiol or tamoxifen were followed in the absence or presence of NRH. Our data showed that the NRH treatment caused G1 phase arrest in MCF-7, T47D and MDA-MB-468 cells compared to untreated cells. In contrast, NRH-treated MDA-MB-231 cells induced G2/M arrest compared to untreated cells. These results imply that NRH might play a role in the regulation of the cell cycle progression in a manner dependent on the type of breast cancer cells. The combination of β-oestradiol and NRH induced G1 phase arrest in MCF-7 and MDA-MB-468 cells whereas the combination of tamoxifen with NRH treatment induced G1 phase arrest only in MCF-7 cells.

The effects of ectopically expressed NQO2 on cell cycle in ERα-positive (T47D) and ERα-negative cells (MDA-MB-231) were determined. The cells were co-transfected with an NQO2 encoding plasmid together with the cell surface marker protein CD20 and stained with propidium iodide and FITC as described in the Materials and Methods. There was no significant difference in NQO2 transfected and β-oestradiol-treated T47D cells in the absence and presence of NRH compared to the untransfadiol-treated cells. NQO2 overexpression induced G2/M phase arrest in T47D cells with the highest percentage shown in cells treated with NRH alone compared to cells transfected with PCDNA3. In contrast, NQO2 overexpression induced G1 phase arrest in MDA-MB-
231 cells compared to PCNDA3 transfected cells. The different effects of NQO2 in cell cycle phase arrest are possibly due to the difference in the ERα protein levels in the two cell lines.

The effects of oestrogens and oestrogen receptors on mitochondrial biogenesis and respiration have been well documented (Chen, Cammarata et al. 2009). Energy production in mitochondria depends on the electrochemical gradient of the mitochondrial membrane. In particular, the respiratory rate is faster when the mitochondrial membrane is depolarised as the energy required to drive protons outside of the mitochondria in these conditions is reduced and slower when the mitochondrial membrane is polarised (Duchen 2004). The investigation of the impact of NQO2 on the mitochondrial membrane potential of breast cancer cells was reasoned based on the fact that oestrogens induce ANT-1 gene expression (Too, Giles et al. 1999) and tamoxifen decreases ANT mitochondrial content (Chen, Brown et al. 2009) in combination with the known role of ANT in mitochondrial energy generation (Kwong and Molkentin 2015). To evaluate the effects of NQO2 on ΔψMMP in breast cancer cells, the JC-1 dye method was used in four-breast cancer cell lines. NRH caused mitochondrial membrane depolarisation in MCF-7 and MDA-MB-468 cells when compared to the untreated allowing the hypothesis that NQO2 is involved in the regulation of mitochondrial energy production in cell type dependent manners. Mitochondrial membrane depolarization in β-oestradiol treatment in the absence and presence of NRH leads to cell death in MCF-7 and MDA-MB-468 cells (Kroemer, Galluzzi et al. 2007). Tamoxifen treatments showed reduced mitochondrial membrane depolarisation in MCF-7 and MDA-MB-468 cells where imply that tamoxifen induced rapid death of breast cancer cells in cell type dependent manners. This is associated with an increase in ROS production, release of mitochondrial cytochrome C and decrease of mitochondrial membrane depolarisation (Kallio, Zheng et al. 2005).

In order to explore the potential interplay between NQO2 and ERα, the localisation of NQO2 was investigated in β-oestradiol and NRH treated T47D and MDA-MB-468 cells ectopically expressing NQO2. Mitochondrial localisation of NQO2 was determined by staining the cells with NQO2 immunofluorescence antibody and dyes staining mitochondria (MitoTracker FM) or the nuclei (DAPI) and analysed with immunofluorescence microscope. Oestrogen receptors ERα and ERβ are localised in
the nucleus, plasma membrane and mitochondria (Chen, Delannoy et al. 2004, Chen, Russo et al. 2007). Accumulating evidence indicates that the subcellular localisation of ERs is different in diverse types of cancer cells. Different subcellular localisation of ERs is influenced by β-oestradiol in a dose and time-dependent manners (Chen and Yager 2004). ERs are localised in the mitochondria through activation of oestrogen-mediated Akt pathways in breast cancer cells (Guo, Wei et al. 2006). Several studies have reported the detection of ERα and ERβ in mitochondria of rabbit ovarian and uterine tissue (Chen, Delannoy et al. 2004). Treatment with β-oestradiol induces the transcription of several mitochondrial DNA (mtDNA)-encoded genes involved in the mitochondrial respiratory chain (MRC) (Chen, Delannoy et al. 2004). Mitochondrial ERs are activated by β-oestradiol and bind to oestrogen response elements (ERE) or ERE-like sites located in the mitochondrial genome (Demonacos, Karayanni et al. 1996, Enmark and Gustafsson 1999, Chen and Yager 2004). Thus, targeting mitochondrial ERα and ERβ is rapidly emerging as a novel therapeutic strategy (Lee, Sharma et al. 2008).

Results obtained in this study showed that in untreated T47D and MDA-MB-468 cells, NQO2 was localised in the cytoplasm and weakly in mitochondria in untreated breast cancer cells. More intense nuclear and mitochondrial NQO2 staining was observed in T47D cells treated with β-oestradiol. In NQO2, overexpressing and NRH treated T47D cells strong nuclear and mitochondrial NQO2 staining was observed. Weak nuclear and intense mitochondrial NQO2 staining was observed in MDA-MB-468 cells treated with either β-oestradiol or NRH. It is known that NQO2 is activated through an electron donating cofactor by using ping-pong mechanisms. The physiological NQO2 electron donor is unknown and the conditions and mechanisms by which NQO2 is activated in vivo have not been elucidated yet.

β-oestradiol treatment activates the migration of NQO2 to mitochondria in T47D and MDA-MB-468 cells. It is possible that NQO2 associates with ERs in β-oestradiol treated cells and localises in the mitochondria.
CHAPTER 6

Regulation of reactive oxygen species
generation by NQO2 in breast cancer cells
6.0 RESULTS

6.1 Reactive oxygen species (ROS) generation in breast cancer cells treated with β-oestradiol, tamoxifen and NRH

In order to determine the effects of NQO2 on ROS generation in β-oestradiol or tamoxifen treated breast cancer cells in the absence and presence of NRH, the ROS levels were determined as shown in Figure 6.1, 6.2, 6.3 and 6.4. ROS generation was measured using the Nucleocounter, NC-3000 (Chemometec, Denmark) as explained in details in the Materials and Methods section.

The effects of ectopically expressed NQO2 in regulating ROS generation of the ERα-positive T47D and the ERα-negative MDA-MB-231 cells were also determined using the CYAN flow cytometry (Beckman Coulter, UK) as described in the Materials and Methods. T47D and MDA-MB-231 cells were co-transfected with CD20 expressing vector together with NQO2 plasmid and CD20 surface marker was detected with a tagged APC-H7-conjugated CD20 antibody (BD Bioscience, UK). The effects of NQO2 on ROS generation in β-oestradiol-treated T47D and MDA-MB-231 cells in the absence and presence of NRH were detected by FACs analysis.

In MCF-7 cells, ROS production was measured with the treatments of β-oestradiol, or tamoxifen in the absence and presence of NRH as shown in the Figures 6.1A and B. In particular, MCF-7 cells treated with β-oestradiol alone exhibited increased ROS levels compared to untreated cells (Figures 6.1A and B, compare bar 3 to bar 1). The combination of NRH with β-oestradiol decreased ROS production in MCF-7 cells compared to the same treatment in the absence of NRH (Figures 6.1A and B, compare bar 4 to bar 3) suggesting that NRH antagonises the effects of β-oestradiol on ROS productions in these cells.

Similarly, to β-oestradiol treatment, tamoxifen treatment alone increased ROS production compared to the untreated cells (Figures 6.1A and B, compare bar 5 to bar 1) and the combination of tamoxifen with NRH reduced ROS production compared to the same treatment in the absence of NRH (Figures 6.1A and B, compare bar 6 to bar 5).
Treatment of MCF-7 cells with NRH alone increased ROS production compared to untreated cells (Figure 6.1A and B, compare bar 2 to bar 1).

Overall ROS generation in MCF-7 cells treated with β-oestradiol or tamoxifen in the absence and presences of NRH, indicated that the higher ROS levels were detected in MCF-7 cells treated with tamoxifen compared to the other treatments, whereas the MCF-7 cells treated with a combination of β-oestradiol with NRH displayed the lowest ROS levels compared to other treatments. Furthermore, results shown in 6.1B indicate that although NRH alone increased ROS levels compared to untreated cells combination of NRH with either β-oestradiol or tamoxifen decreased ROS levels compared to cells treated with the hormones alone.
Figure 6.1: ROS production in MCF-7 breast cancer cells

(A) Cells were treated with \(\beta\)-oestradiol, tamoxifen or NRH either alone or in combination or left untreated as indicated. Following the treatments, cells were incubated with the H\(_2\)DCFDA fluorescent dye, and ROS production was measured and analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark). In the profiles shown, the black curves indicate untreated, and the green curves indicate treated cells. One representative profile out of three independent experiments performed in duplicate is shown. (B) The profiles shown in 6.1(A) were analysed using the Nucleocounter, NC-3000 software (Chemometec, Denmark) and plotted in the diagram.

ROS levels were determined in other types of ER\(\alpha\)-positive, T47D cells treated with either \(\beta\)-oestradiol or tamoxifen in the absence and presence of NRH as indicated in Figure 6.2A and B.

Increased ROS production was observed in T47D cells treated with \(\beta\)-oestradiol compared to untreated cells (Figures 6.2A and B compare bar 3 to bar 1). The combination of NRH with \(\beta\)-oestradiol significantly reduced ROS production compared to the same treatment in the absence of NRH (Figure 6.2A and B, compare bar 4 to bar 3).

Increased ROS production was observed in tamoxifen-treated T47D cells compared to untreated cells (Figure 6.2A and B, compare bar 5 to bar 1). In the presence of NRH, ROS production was decreased significantly in tamoxifen-treated T47D cells compared to untreated, NRH or tamoxifen-treated T47D cells in the absence of NRH (Figure 6.2A and B, compare bar 6 to bars 1, 2 and 5 respectively).
Treatment of T47D cells with NRH alone did not change the ROS levels in these cells compared to untreated (Figures 6.2A and B compare bar 2 to bar 1).

In summary, higher ROS production was detected in β-oestradiol treated T47D cells compared to the other treatments. T47D cells treated with a combination of tamoxifen and NRH displayed the lowest ROS production compared to other treatments.

Taken together results shown in the Figures 6.1 and 6.2 support the conclusion that treatment of the ERα positive MCF-7 and T47D cells, with either β-oestradiol or tamoxifen in the absence of NRH, increased ROS production (Figure 6.1 and 6.2, compare bars 3 and 5 to bar 1). Furthermore, combination of NRH with either β-oestradiol or tamoxifen decreases ROS levels compared to cells treated with the β-oestradiol or tamoxifen alone suggesting that, NRH antagonises the effects of both β-oestradiol and tamoxifen on ROS generation in the ERα positive cells (Figures 6.1 and 6.2, compare bars  and 6 to bars 3 and 5 respectively).
Figure 6.2: ROS production in T47D cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH

(A) Cells were treated with β-oestradiol, tamoxifen or NRH either alone or in combination or left untreated as indicated. Following the treatments, cells were incubated with the H$_2$DCFDA fluorescent dye, and ROS production was measured and analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark). In the profiles shown, the black curves indicate untreated and the green curves indicate treated cells. One representative profile out of three independent experiments performed in duplicate is shown. (B) The profiles shown in 6.2(A) were analysed using the Nucleocounter, NC-3000 software (Chemometec, Denmark) and plotted in the diagram. [*] significant difference ($p<0.05$) in ROS levels measured in the treated cells compared to those measured in the untreated (bar 1). [#] significant difference ($p<0.05$) in ROS levels measured in the cells treated with combination of NRH with β-oestradiol or tamoxifen compared to those measured in cells treated with NRH alone. [a] significant difference ($p<0.05$) in ROS levels measured in the cells treated with combination of β-oestradiol with NRH (bar 4) compared to those measured in cells treated with β-oestradiol alone (bar 3) and [^] significant difference ($p<0.05$) in ROS levels measured in the cells treated with combination of tamoxifen with NRH (bar 6) compared to those measured in cells treated with tamoxifen alone (bar 5). One symbol indicates $p<0.05$ and two symbols indicate $p<0.01$.

ROS production was assessed in the ERα-negative MDA-MB-231 cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH as shown in Figures 6.3A and B.

Reduction in ROS production was detected in MDA-MB-231 cells treated with β-oestradiol in the absence and presence of NRH compared to untreated or NRH alone treated cells (Figures 6.3A and B, compare bars 3 and 4 to bars 1 and 2 respectively).
Increase but not statistically significant in the ROS production was evident in the MDA-MB-231 cells treated with tamoxifen alone compared to the untreated cells (Figures 6.3A and B, compare bar 5 to bar 1). On the other hand, the combination of tamoxifen with NRH decreased ROS production compared to cells treated with tamoxifen alone (Figures 6.3A and B, compare bar 6 to bar 5).

MDA-MB-231 cells treated with NRH alone did not show any statistically significant changes in the ROS levels compared to the untreated cells (Figures 6.3A and B, compare bar 2 to bar 1).

Assessment of ROS production in MDA-MB-231 cells indicated that the higher ROS production was detected in the cells treated with tamoxifen compared to the other treatments (Figure 6.3B, bar 5), whereas the β-oestradiol treated MDA-MB-231 cells in the presence of NRH exhibited the lowest ROS levels compared to the other treatments (Figure 6.3B, bar 4).
Figure 6.3: ROS generation in MDA-MB-231 cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH

(A) Cells were treated with β-oestradiol, tamoxifen or NRH either alone or in combination or left untreated as indicated. Following the treatments, cells were incubated with the H$_2$DCFDA fluorescent dye, and ROS production was measured and analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark). In the profiles shown, the black curves indicate untreated, and the green curves indicate treated cells. One representative profile out of three independent experiments performed in duplicates is shown. (B) The profiles shown in 6.3(A) were analysed using the Nucleocounter, NC-3000 software (Chemometec, Denmark) and plotted in the diagram.

ROS production was evaluated in the ERα-negative MDA-MB-468 cells treated with β-oestradiol, tamoxifen or the NQO2 cofactor, NRH alone or in combination with β-oestradiol or tamoxifen as indicated in the Figures 6.4A and B. The analysis of ROS production was carried out using the Nucleocounter, NC 3000 software (Chemometec, Denmark) and is shown in the Figure 6.4B.

Increased but not statistically significant ROS production was identified in MDA-MB-468 cells treated with β-oestradiol compared to untreated cells (Figures 6.4A and B, compare bar 3 to bar 1). The combination of β-oestradiol with NRH decreased ROS production compared to the cells treated with β-oestradiol alone (Figures 6.4A and B, compare bar 4 to bar 3).

Alike β-oestradiol, tamoxifen treatment of MDA-MB-468 cells alone increased ROS production compared to untreated cells but this effect was not statistically significant (Figures 6.4A and B, compare bar 5 to bar 1). MDA-MB-468 cells treated with a
combination of tamoxifen with NRH did not show any significant effect on ROS production compared to tamoxifen treatment alone (Figures 6.4A and B, compare bar 6 to bar 5).

Treatment of MDA-MB-468 cells with NRH alone did not have any significant effect on ROS levels in these cells compared to untreated (Figures 6.4A and B compare bar 2 to bar 1).

In conclusion, NRH reversed the effects of both β-oestradiol and tamoxifen reducing ROS levels in MDA-MB-468 cells treated with a combination of NRH with hormone and antioestrogen (Figures 6.4A and B, compare bars 2, 4 and 6 to bars 1, 3 and 5).
Figure 6.4: ROS generation in MDA-MB-468 cells treated with either β-oestradiol or tamoxifen in the absence and presence of NRH

(A) Cells were treated with β-oestradiol, tamoxifen or NRH either alone or in combination or left untreated as indicated. Following the treatments, cells were incubated with the H2DCFDA fluorescent dye, and ROS production was measured and analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark). In the profiles shown, the black curves indicate untreated, and the green curves indicate treated cells. One representative profile out of three independent experiments performed in duplicates is shown. (B) The profiles shown in 6.4(A) were analysed using the Nucleocounter, NC-3000 software (Chemometec, Denmark) and plotted in the diagram.

Results shown in Figures 6.3 and 6.4 indicate a difference in ROS production in MDA-MB-231 and MDA-MB-468 cells treated with β-oestradiol alone. In particular, MDA-MB-231 cells treated with β-oestradiol exhibited 50% reduction in ROS production compared to untreated cells (Figure 6.3B bar 3 to bar 1). On the other hand, approximately 2-fold increase in ROS production was detected in β-oestradiol-treated MDA-MB-468 cells compared to untreated cells (Figure 6.4B, compare bar 3 to bar 1).

Comparative analysis of the ROS levels measured in MCF-7, T47D, MDA-MB-231 and MDA-MB-468 breast cancer cells treated with β-oestradiol or tamoxifen alone or combination of β-oestradiol or tamoxifen with NRH is shown in Figure 6.5.

MDA-MB-468 treated with β-oestradiol exhibited the highest ROS production compared to the other cell lines (Figure 6.5, MDA-MB-468) whereas the β-oestradiol-treated MDA-MB-231 displayed the lowest ROS production compared to other cell
Tamoxifen-treated MDA-MB-468 cells, displayed the highest ROS levels compared to other cell lines (Figure 6.5, MDA-MB-468) whereas, under these conditions, T47D cells exhibited the lowest ROS levels compared to other cell lines (Figure 6.5, T47D). MCF-7 cells demonstrated the highest ROS production upon treatment with a combination of tamoxifen with NRH (Figure 6.5, MCF-7) whereas the lowest ROS production under these conditions was observed in T47D breast cancer cells compared to other cell lines (Figure 6.5, T47D).

Treatment with NRH alone resulted in inducing the highest ROS production in MCF-7 cells compared to other cell lines (Figure 6.5, MCF-7) and under these conditions, the lowest ROS production was observed in MDA-MB-231 cells compared to other cell lines (Figure 6.5, MDA-MB-231).

Figure 6.5: Comparison of ROS levels in breast cancer cells treated with β-oestradiol or tamoxifen in the absence or presence of NRH

Cells were left untreated or treated with either β-oestradiol or tamoxifen in the absence or presence of NRH as indicated. Following the treatments, cells were incubated with the H2DCFDA fluorescent dye and ROS production was measured and analysed using the Nucleocounter, NC-3000 software (Chemometec, Denmark) and plotted in the diagram. Error bars represent SEM of three independent experiments.
6.1.1 Effects of NQO2 overexpression on ROS production in breast cancer cells

To shed light on the role of NQO2 in the regulation of ROS generation in breast cancer cells, ROS levels were determined in the ERα-positive T47D cells and the ERα-negative MDA-MB-231 cells as described in the Materials and Methods section. Cells were transfected with NQO2 expression plasmids or empty vector along with CD20 expressing vector and treated with β-oestradiol in the absence and presence of NRH. Following the treatment, cells were incubated with H2DCFDA fluorescent dye and tagged with FITC CD20 antibody (BD Bioscience, UK). β-oestradiol has been shown to induce ROS production and mammary tumorigenesis (Okoh, Felty et al. 2013). Given that, β-oestradiol treatment induced ROS levels in MCF-7 (Figure 6.1B), T47D (Figure 6.2B) and MDA-MB-468 cells (Figure 6.4B) but caused slight reduction of ROS in MDA-MB-231 cells (Figure 6.3B) the effects of NQO2 on ROS levels generated in ERα-positive T47D cells and the ERα-negative MDA-MB-231 treated with β-oestradiol in the absence and presence of NRH were followed (Figure 6.6 and 6.7).

Significantly increased ROS levels were identified in T47D cells transfected with the empty vector and treated with β-oestradiol compared to untreated (Figures 6.6A and B, compare bar 2 to bar 1). In contrast, combination of β-oestradiol with NRH resulted in the production of lower ROS levels in these cells compared to cells treated with β-oestradiol alone (Figures 6.6A and B, compare bar 4 to bar 2), suggesting that NRH inhibited ROS production effectively possibly by activating the antioxidant activity of the endogenous NQO2 or other antioxidant enzymes given that the endogenous NQO2 levels in T47D cells were found to be low (Figure 3.1).

Ectopic expression of NQO2 in T47D cells reduced the levels of ROS compared to those measured in cells transfected with the empty vector irrespectively of the treatment (Figure 6.6A and B, compare bars 5-8 to bars 1-4). In the β-oestradiol-treated and NQO2 overexpressing T47D cells, ROS levels were significantly reduced compared to the untransfected cells under the same conditions (Figure 6.6A and B, compare bar 6 to bar 2). T47D cells ectopically expressing NQO2 and treated with NRH displayed similar results namely reduction of ROS levels by approximately 50% compared to NQO2 overexpressing untreated cells (Figures 6.6A and B, compare lane
Results shown in the Figure 6.6 suggest that NQO2 overexpression is sufficient to inhibit ROS generation in T47D human breast cancer cells.

**Figure 6.6: ROS production in NQO2 overexpressing T47D breast cancer cells**

(A) T47D cells were co-transfected with empty or NQO2 expression vectors together with CD20 expressing vector and treated with β-oestradiol or NRH alone or combination of β-oestradiol with NRH or left untreated as indicated. Cells were then incubated with the H$_2$DCFDA fluorescent dye and the APC-H7-conjugated CD20 antibody (BD Bioscience, UK) and ROS levels were measured using the CYAN ADP flow cytometer (530 nm). Black curves indicate ROS levels in cells transfected with empty vector whereas green curves indicate ROS levels in cells overexpressing NQO2. Data are representative of two independent experiments performed in duplicates. (B) ROS levels in PCDNA3 and NQO2 transfected T47D cells were analysed using Beckman Coulter Summit 4.1 software. [*] significant difference ($p<0.05$) in ROS
levels measured in treated compared to those measured in untreated cells. [a] significant difference \( p<0.05 \) in ROS levels measured in the cells treated with combination of NRH with \( \beta \)-oestradiol compared to those measured in cells treated with \( \beta \)-oestradiol alone [\(^\wedge\)] significant difference \( p<0.05 \) in ROS levels measured in the cells treated with combination of \( \beta \)-oestradiol with NRH (bar 4) compared to those measured in cells treated with NRH alone (bar 3) and [\(^\sharp\)] significant difference \( p<0.05 \) in ROS levels measured in the cells transfected with NQO2 expression vector treated with \( \beta \)-oestradiol (bar 6) and NRH (bar 7) compared to those measured in untreated cells transfected with the empty vector treated with \( \beta \)-oestradiol (bar 2) and NRH (bar 3). One symbol indicates \( p<0.05 \), two symbols indicate \( p<0.01 \), three symbols indicate \( p<0.001 \) and four symbols indicate \( p<0.0001 \).

ROS levels were also measured in the ER\(\alpha\)-negative MDA-MB-231 breast cancer cells overexpressing NQO2 and treated with \( \beta \)-oestradiol or NRH individually or in combination or left untreated as shown in Figure 6.7A and B. No significant differences in the ROS levels were evident between treatments in cells transfected with the empty vector (Figures 6.7A and B, compare bars 2, 3 and 4 to bar 1).

Statistically significant decrease in ROS levels was recorded in MDA-MB-231 cells transfected with NQO2 compared to the ROS levels measured in cells transfected with the empty vector (Figure 6.7A and B, compare bars 5-8 to bars 1-4). MDA-MB-231 cells ectopically expressing NQO2 and treated with \( \beta \)-oestradiol or NRH individually displayed significantly decreased ROS levels compared to those detected in untransfected cells (Figures 6.7A and B, compare bars 6 and 7 to bars 2 and 3 respectively). NQO2 overexpressing MDA-MB-231 cells treated with \( \beta \)-oestradiol and NRH exhibited significantly decreased ROS levels compared to those measured in cells transfected with the empty vector (Figures 6.7A and B, compare bar 8 to bar 4) possibly reflecting the fact that endogenous NQO2 levels in MDA-MB-231 cells are high (Hsieh, Elangovan et al. 2010) thereby masking the effects of the excess of exogenously expressed NQO2.
Figure 6.7: ROS production in NQO2 overexpressing MDA-MB-231 breast cancer cells

(A) MDA-MB-231 cells were co-transfected with empty or NQO2 expression vectors together with CD20 expressing vector and treated with β-oestradiol or NRH alone or combination of β-oestradiol with NRH or left untreated as indicated. Cells were then incubated with the H₂DCFDA fluorescent dye and the APC-H7-conjugated CD20 antibody (BD Bioscience, UK) and ROS levels were measured using the CYAN ADP flow cytometer (530 nm). Black curves indicate ROS levels in cells transfected with empty vector whereas green curves indicate ROS levels in cells overexpressing NQO2. Data are representative of two independent experiments performed in duplicates. (B) ROS levels in PCDNA3 and NQO2 transfected MDA-MB-231 cells were analysed using Beckman Coulter Summit 4.1 software. [$] significant difference (p<0.05) in ROS levels measured in the cells transfected with NQO2 expression vector (bars 5-8) compared to those measured in untreated cells transfected with the empty vector (bars 1-4). One symbol indicates $p<0.05$ and two symbols indicate $p<0.01$. 

Data are representative of two independent experiments performed in duplicates.
Taken together with results shown in the Figures 6.6 and 6.7 support the notion that NQO2 overexpression in T47D and MDA-MB-231 breast cancer cells inhibits ROS production. Furthermore, combination of β-oestradiol and NRH treatment in the T47D overexpressing NQO2 cells decreased ROS generation whereas under the same conditions MDA-MB-231 cells exhibited increased ROS levels compared to untreated cells ectopically expressing NQO2.

6.2 Chapter discussion

ROS play important roles in cell survival and cell death (Orrenius 2007, Trachootham, Lu et al. 2008, Liou and Storz 2010). Increased reactive oxygen species levels have been reported in almost all cancer cells (Liou and Storz 2010, Liu and Wang 2015). NQO2 exerts a protective role by reducing ROS levels (Wu, Knox et al. 1997) but several reports have indicated that NQO2 overexpression could also increase cellular ROS levels (Wang, Le et al. 2008, Cassagnes, Perio et al. 2015). To understand the effects of NQO2 on ROS production, ROS generation was measured in four breast cells lines treated with β-oestradiol, tamoxifen, or NRH alone or combination of β-oestradiol or tamoxifen with NRH. Tamoxifen-induced ROS production in MCF-7, T47D, MDA-MB-231 and MDA-MB-468 cells. These results are consistent with those reported by Khalio and colleagues indicating increased ROS generation in tamoxifen treated MCF-7 and MDA-MB-231 cells leading to cell death by mechanisms associated with the release of mitochondrial cytochrome c, and decrease of the mitochondrial membrane potential proportional to the induction of ROS levels (Kallio, Zheng et al. 2005). NRH treated breast cancer cells increased ROS level in all breast cancer cells except in MDA-MB-231 cells (Figure 6.1, 6.2, 6.3 and 6.4). The combination of NRH and oestrogen or tamoxifen treated breast cancer cells showed no changes in the ROS production when compared to the untreated.

To reveal the impact of NQO2 on ROS generation in breast cancer cells, ROS generation was measured in NQO2 transfected and β-oestradiol-treated T47D and MDA-MB-231 cells in the absence and presence of NRH. Reduction of ROS generation was observed in NQO2 transfected T47D and MDA-MB-231 cells treated with β-oestradiol in the absence and presence of NRH compared to the untransfected cells. NQO2 ectopic expression in MDA-MB-231 and T47D cells, which
demonstrated moderate endogenous NQO2 protein levels, resulted in the reduction of ROS generation when transfected with NQO2 suggesting that NQO2 might contribute to the determination of the endogenous oxidative stress levels in these cells. NQO2 may be able to reduce ROS in breast cancer cells such as those produced by the catechol oestrogen-3, 4- metabolism which is known to be involved in initiating breast cancer (Cavalieri, Chakravarti et al. 2006). Catechol oestrogen-3, 4- is removed by conjugation of catechol oestrogens with glutathione (Cao, Devanesan et al. 1998). Published evidence has reported the significant role of NQO2 in catalysing the reduction of oestrogen quinones (Gaikwad, Yang et al. 2009). This could be the explanation of reduced levels of ROS production observed in the NQO2 overexpressing breast cancer cells. To investigate the NQO2 protein levels and examine their potential association to ROS levels in breast cancer cells the NQO2 protein levels were studied in five breast cancer cell lines: ERα-positive (MCF-7 and T47D) and ERα-negative (MDA-MB-231, MDA-MB-468 and MDA-MB-157) as shown in Figure 3.1. High NQO2 protein levels were detected in MDA-MB-468 cells, moderately high in MCF-7 and MDA-MB-231 cells, and undetectable in T47D and MDA-MB-157 cells.
CHAPTER 7

Effects of NQO2 on the function of antioxidant enzymes in breast cancer cells
7.0 RESULTS

7.1 Assessment of the catalase enzymatic activity in NQO2 overexpressing breast cancer cells

The contribution of ROS generated by the estrogen metabolism in breast carcinogenesis is well documented (Okoh, Deoraj et al. 2011). Redox homeostasis is maintained by the function of antioxidant enzymes including catalase and glutathione peroxidase (GPx). Results shown in Figure 6.1, 6.2, 6.3 and 6.4 provide evidence to support the view that NQO2 is at least in part involved in the regulation of ROS levels in breast cancer cells, therefore, it was hypothesized that this reductase might modulate the activity of antioxidant enzymes in response to the accumulation of cellular ROS in breast cancer. To test this hypothesis, the catalase activity was assessed in breast cancer cells treated with estradiol or tamoxifen alone or in combination with NRH in untransfected or ectopically expressing NQO2 cells.

To gain further insight into the antioxidant mechanisms modulated by NQO2, the enzymatic activity of the antioxidant enzyme catalase was measured in the Erα-positive cells (MCF-7, T47D) and the Erα-negative cells (MDA-MB-231, MDA-MB-468) transfected with increasing amounts of an NQO2 expression vector.

Statistically significant decrease of catalase enzymatic activity was detected in MCF-7 cells transfected with minimum amounts of an NQO2 expression vector compared to untransfected cells (Figure 7.1, compare bar 2 to bar 1). The addition of NRH increased the enzymatic activity of catalase in untransfected and NQO2 transfected MCF-7 cells compared to untreated MCF-7 cells under the same conditions (Figure 7.1, compare bars 10, 11 and 12 to bars 1, 2 and 3 respectively).

Increased catalase activity was observed in the β-oestradiol-treated MCF-7 cells transfected with increasing amounts of NQO2 expression vector compared to untreated cells (Figure 7.1, compare bars 4, 5 and 6 to bars 1, 2 and 3 respectively). Statistically significant decrease of catalase enzymatic activity was detected in β-oestradiol-treated MCF-7 cells transfected with minimum amounts of an NQO2 expression vector compared to untransfected cells under the same condition (Figure 7.1, compare bar 5 to bar 4).
Statistically significant reduction of catalase activity was recorded in β-oestradiol-treated MCF-7 cells in the presence of NRH compared to untreated or cells treated with NRH alone (Figure 7.1, compare bar 13 to bars 1 and 10 respectively). MCF-7 transfected with increasing amounts of the NQO2 expression vector and treated with a combination of β-oestradiol with NRH displayed significant decrease of catalase activity compared to that detected in MCF-7 cells treated with NRH and transfected with increasing amounts of NQO2 expression vector (Figure 7.1, compare bars 14 and 15 to bars 11 and 12 respectively).

There were no significant differences detected in the catalase activity of the tamoxifen-treated MCF-7 cells compared to untreated cells (Figure 7.1, compare bar 7 to bar 1). MCF-7 cells transfected with increasing amounts of the NQO2 expression vector and treated with tamoxifen did not show any significant differences in catalase activity compared to untreated cells (Figure 7.1, compare bars 8 and 9 to bars 2 and 3 respectively).

Transfection of MCF-7 cells with increasing amounts of NQO2 expression vector and treatment with a combination of tamoxifen with NRH significantly decreased the catalase activity in these cells compared to the untransfected cells under the same conditions (Figure 7.1, compare bars 17 and 18 to bar 16 respectively). Statistically significant decreases of catalase activity were observed in MCF-7 cells transfected with increasing amounts of NQO2 and treated with a combination of tamoxifen and NRH compared to MCF-7 cells transfected with increasing amounts of NQO2 and treated with NRH only (Figure 7.1, compare bars 17 and 18 to bars 11 and 12 respectively).
Figure 7.1: Assessment of the catalase enzymatic activity in NQO2 transfected MCF-7 breast cancer cells

MCF-7 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cellular lysates were collected, incubated initially with methanol as the hydrogen donor in the presence of H₂O₂ for 20 minutes at room temperature and subsequently with purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chromogen for 10 minutes at room temperature. The absorbance of the samples was measured at 540nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates. [*] significant difference ($p<0.05$) of catalase activity detected in untransfected and treated cells compared to that measured in untransfected and non-treated cells. [#] significant difference ($p<0.05$) of catalase activity detected in cells transfected with NQO2 expression vector compared to that measured in cells transfected with the empty vector. [a] significant difference ($p<0.05$) of catalase activity detected in cells treated with combination of NRH with β-oestradiol or NRH with tamoxifen compared to that measured in cells treated with NRH alone. Two symbols indicate $p<0.01$ and four symbols indicate $p<0.0001$.

Next, the effects of increasing amounts of transfected NQO2 expression vector on the catalase activity in the ERα-positive cell line T47D were examined as shown in Figure 7.2.

Increasing amounts of transfected NQO2 significantly decreased catalase activity in non-treated T47D cells (Figure 7.2, compare bars 2 and 3 to bar 1). In the presence of NRH, catalase activity was significantly decreased compared to the untreated cells.
Catalase activity was significantly decreased in NRH-treated T47D cells transfected with increasing amounts of NQO2 expression vector compared to the untransfected cells and treated with NRH (Figure 7.2, compare bars 11 and 12 to bar 10).

Statistically significant decrease of catalase enzymatic activity was observed in β-oestradiol or tamoxifen-treated T47D cells compared to untreated cells (Figure 7.2, compare bars 4 and 7 to bar 1). The T47D cells transfected with increasing amounts of the NQO2 expression vector and treated with β-oestradiol exhibited lower catalase activity compared to untransfected cells treated with β-oestradiol (Figure 7.2, compare bar 5 to bar 4). The T47D cells transfected with increasing amounts of the NQO2 expression vector and treated with tamoxifen exhibited lower catalase activity compared to untransfected cells treated with tamoxifen (Figure 7.2, compare bars 8 and 9 to bar 7).

Statistically significant decrease of catalase activity was observed in β-oestradiol-treated T47D in the presence of NRH compared to untreated or cells treated with NRH alone (Figure 7.2, compare bar 13 to bars 1 and 10). Further reduction of catalase activity was evident in T47D cells transfected with increasing amounts of the NQO2 expression vector and treated with β-oestradiol in the presence of NRH compared to that recorded in cells treated with β-oestradiol alone under the same conditions (Figure 7.2, compare bars 14 and 15 to bars 5 and 6 respectively).

Treatment of T47D cells with combination of tamoxifen with NRH and transfected with the empty vector increased significantly the catalase enzymatic activity compared to the untreated (Figure 7.2, compare bar 16 to bar 1). Cells transfected with the empty vector and treated with combination of tamoxifen with NRH showed increased catalase enzymatic activity compared to transfected with the empty vector cells and treated with tamoxifen alone (Figure 7.2, compare bar 16 to bar 7). Reduced catalase enzymatic activity was measured in T47D cells transfected with increasing amounts of the NQO2 expression vector and treated with a combination of tamoxifen and NRH compared to untransfected cells under the same conditions (Figure 7.2 compare bars 17 and 18 to bar 16).
Figure 7.2: Assessment of the catalase enzymatic activity in NQO2 transfected T47D breast cancer cells

T47D cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cellular lysates were collected, incubated initially with methanol as the hydrogen donor in the presence of H₂O₂ for 20 minutes at room temperature and subsequently with purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chromogen for 10 minutes at room temperature. The absorbance of the samples was measured at 540nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates. [*] significant difference (p<0.05) of catalase activity detected in untransfected and treated cells compared to that measured in untransfected and non-treated cells. [#] significant difference (p<0.05) of catalase activity detected in cells transfected with NQO2 expression vector compared to that measured in cells transfected with the empty vector and [a] significant difference (p<0.05) of catalase activity detected in cells treated with combination of NRH with β-oestradiol or NRH with tamoxifen compared to that measured in cells treated with NRH alone. One symbol indicates p<0.05, two symbols indicate p<0.01, three symbols indicate p<0.001 and four symbols indicate p<0.0001.

The effects of increasing amounts of transfected NQO2 expression vector on the catalase activity were also investigated in the ERα-negative cells, MDA-MB-231 and MDA-MB-468 cell lines shown in Figure 7.3 and 7.4.

Increasing amounts of transfected NQO2 expression vector decreased catalase activity in MDA-MB-231 untreated cells (Figure 7.3, compare bar 3 to bar 1). NRH treatment
of cells transfected with the empty vector increased the enzymatic activity of catalase compared to non-treated cells (Figure 7.3 compare bar 10 to bar 1).

MDA-MB-231 transfected with the NQO2 expression vector and treated with β-oestradiol exhibited increased catalase enzymatic activity compared to non-treated cells transfected with the same amounts of NQO2 expression vector (Figure 7.3, compare bar 6 to bar 3). Statistically significant increase of catalase activity was also observed in MDA-MB-231 cells transfected with the NQO2 expression vector and treated with combination of β-oestradiol with NRH compared to NRH-treated MDA-MB-231 cells under the same conditions (Figure 7.3, compare bar 15 to bar 12).

Tamoxifen treatment of MDA-MB-231 cells did not affect the catalase activity irrespectively of the presence of NRH or whether these cells were transfected with NQO2 expression vector or not compared to non-treated (Figure 7.3, compare bars 7 and 8 to bars 1 and 2).

Figure 7.3: Assessment of the catalase enzymatic activity in NQO2 transfected MDA-MB-231 breast cancer cells
MDA-MB-231 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cellular lysates were collected, incubated initially with methanol as the hydrogen donor in the presence of H2O2 for 20 minutes at room temperature and
subsequently with purpald (4-Amino-3-hydrazino-5-mercaptop-1,2,4-triazole) as a chromogen for 10 minutes at room temperature. The absorbance of the samples was measured at 540nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates. [#] significant difference ($p<0.05$) of catalase activity detected in cells transfected with NQO2 expression vector compared to that measured in cells transfected with the empty vector and [a] significant difference ($p<0.05$) of catalase activity detected in cells treated with combination of NRH with β-oestradiol compared to that measured in cells treated with NRH alone. One symbol indicates $p<0.05$.

MDA-MB-468 transfected with a low amount of an NQO2 expression vector showed statistically significant increased catalase activity compared to untransfected cells (Figure 7.4 compare bar 2 to bar 1). A similar pattern of catalase activity to that observed in untreated MDA-MB-468 cells was evident in the cells treated with NRH (Figure 7.4, compare bars 10, 11 and 12 to bars 1, 2 and 3 respectively).

No differences in the catalase activity were observed in MDA-MB-468 cells treated with β-oestradiol and transfected with high amount of an NQO2 expression vector compared to the untreated cells in the absence (Figure 7.4, compare bars 4, and 6 to bars 1 and 3 respectively) or the presence of NRH (Figure 7.4, compare bars 13, and 15 to bars 1 and 3 respectively).

Increased catalase activity but not significant was recorded in MDA-MB-468 cells in the untransfected and the transfected with high amount of NQO2 expression vector and treated with tamoxifen alone compared to untreated cells (Figure 7.4, compare bars 7 and 9 to bars 1 and 3 respectively) or combination of tamoxifen with NRH compared to untreated cells (Figure 7.4, compare bars 16 and 18 to bars 1 and 3 respectively). Statistically significant increase in catalase activity was observed in NQO2 overexpressing MDA-MB-468 cells treated with combination of tamoxifen with NRH compared to that detected in NQO2 overexpressing MDA-MB-468 cells treated with NRH alone (Figure 7.4, compare bar 18 to bar 12).
Figure 7.4: Assessment of the catalase enzymatic activity in NQO2 transfected MDA-MB-468 breast cancer cells

MDA-MB-468 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cellular lysates were collected, incubated initially with methanol as the hydrogen donor in the presence of H$_2$O$_2$ for 20 minutes at room temperature and subsequently with purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chromogen for 10 minutes at room temperature. The absorbance of the samples was measured at 540nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates. [#] significant difference ($p$<0.05) of catalase activity detected in cells transfected with NQO2 expression vector compared to that measured in cells transfected with the empty vector and [a] significant difference ($p$<0.05) of catalase activity detected in cells treated with combination of NRH with tamoxifen compared to that measured in cells treated with NRH alone. Two symbols indicate $p$<0.01.

7.2 Estimation of glutathione (GSH) levels in breast cancer cells

Non-enzymatic low-molecular-weight antioxidant compounds represented mainly by glutathione (GSH) provide antioxidant protection to the cells. These molecules are very sensitive to the redox state of the cells, and their intracellular levels correspond to their antioxidant activity (Valko, Leibfritz et al. 2007). Because of these properties, GSH cellular content is used as a physiological marker of quinone-induced stress (Watanabe, Dickinson et al. 2004). For these reasons, the effects of the activity of NQO2 on the GSH content were studied in MCF-7, T47D, MDA-MB-231 and MDA-
MB-468 cells treated with estradiol or tamoxifen alone or in the presence of NRH as shown in Figure 7.5, 7.6, 7.7 and 7.8.

In order to investigate the effects of NQO2 on reduced glutathione (GSH) synthesis in the ERα-positive (MCF-7 and T47D) and the ERα-negative (MDA-MB-231 and MDA-MB-468) breast cancer cells, GSH was measured using the Solution 5 (VB48.PI.AO) and the Nucleocounter (NC 3000) (Chemometec, Denmark) following the manufacturer’s instructions as described in the Materials and Methods section. Declining GSH levels are indicative of apoptotic cells (Coppola and Ghibelli 2000).

MCF-7 cells did not display a significant difference in the GSH levels irrespectively of the treatment in the absence (Figures 7.5A and B, compare bars 3 and 5 to bar 1) or the presence (Figures 7.5A and B, compare bars 4 and 6 to bar 2) of NRH compared to untreated cells.
Figure 7.5: Estimation of GSH levels in MCF-7 breast cancer cells

MCF-7 cells were left untreated or treated with β-oestradiol, tamoxifen, NRH, β-oestradiol and NRH, and tamoxifen and NRH as indicated, stained with VB48.TM, Acridine Orange and Propidium Iodide (PI) and GSH levels were analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark) as described in the Materials and Methods section. (A) Histogram representing different cellular populations as indicated in the Materials and Methods section. Data are representative of three independent experiments performed in duplicates. (B) Graph representing GSH levels in MCF-7 breast cancer cells treated as indicated.

T47D cells treated with β-oestradiol alone exhibited higher GSH levels compared to the GSH levels measured in the untreated cells (Figures 7.6A and B, compare bar 3 to bar 1). Statistically significant decreased of GSH levels were observed in β-oestradiol-treated T47D cells in the presence of NRH compared to T47D cells treated with β-oestradiol alone (Figures 7.6A and B, compare bar 4 to bar 3). A significant increase in GSH levels was detected in tamoxifen-treated T47D cells in the presence of NRH compared to those detected in T47D cells treated with NRH alone (Figures 7.6A and B, compare bar 6 to bar 2).
Figure 7.6: Estimation of GSH levels in T47D breast cancer cells
T47D cells were left untreated or treated with β-oestradiol, tamoxifen, NRH, β-oestradiol and NRH, and tamoxifen and NRH as indicated, stained with VB48.TM, Acridine Orange and Propidium Iodide (PI) and GSH levels were analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark) as described in the Materials and Methods section. (A) Histogram representing different cellular populations as indicated in the Materials and Methods section. Data are representative of three independent experiments performed in duplicates. (B) Graph representing GSH levels in T47D breast cancer cells treated as indicated. [*] Significant difference (*p<0.05) of GSH levels detected in cells treated with combination of NRH with tamoxifen.
compared to those measured in cells treated with NRH alone and [?] significant difference \( (p<0.05) \) of GSH levels detected in cells treated with combination of NRH with β-oestradiol compared to those measured in cells treated with β-oestradiol alone. One symbol indicates \( p<0.05 \).

Statistically significant reduction of GSH levels was detected in MDA-MB-231 cells treated with NRH alone (Figures 7.7A and B, bar 2 to bar 1), β-oestradiol alone (Figures 7.7A and B, compare bar 3 to bar 1) and combination of β-oestradiol with NRH (Figures 7.7A and B, compare bar 4 to bar 1) compared to untreated cells.

Statistically significant increase in the GSH levels was observed on the other side in MDA-MB-231 cells treated with combination of tamoxifen with NRH compared to the GSH levels detected in cells treated with NRH alone (Figures 7.7A and B, compare bar 6 to bar 2).

Lower GSH levels were detected in MDA-MB-231 cells treated with NRH and β-oestradiol alone or combination of β-oestradiol and NRH (Figures 7.7A and B, compare bars 2, 3 and 4 to bar 1) whereas higher GSH levels were measured in these cells treated with combination of tamoxifen with NRH compared to untreated cells (Figures 7.7A and B, compare bar 6 to bar 1).
Figure 7.7: Estimation of GSH levels in MDA-MB-231 breast cancer cells

MDA-MB-231 cells were left untreated or treated with β-oestradiol, tamoxifen, NRH, β-oestradiol and NRH, and tamoxifen and NRH as indicated, stained with VB48.TM, Acridine Orange and Propidium Iodide (PI) and GSH levels were analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark) as described in the Materials and Methods section. (A) Histogram representing different cellular populations as indicated in the Materials and Methods section. Data are representative of three independent experiments performed in duplicates. (B) Graph representing GSH levels MDA-MB-231 breast cancer cells treated as indicated. [*] significant difference ($p<0.05$) of GSH levels detected in treated compared to untreated cells and [#] significant difference ($p<0.05$) of GSH levels detected in cells treated with combination of NRH with tamoxifen compared to those measured in cells treated with NRH alone. Two symbols indicate $p<0.01$, three symbols indicate $p<0.001$ and four symbols indicate $P<0.0001$.

In MDA-MB-468 cells, statistically significant reduction of the GSH levels was observed with NRH alone compared to the GSH levels measured in non-treated cells (Figures 7.8A and B, compare bar 2 to bar 1).

Statistically significant increase of GSH levels on the other hand was evident in MDA-MB-468 cells treated with β-oestradiol alone or combination of β-oestradiol with NRH compared to the GSH levels measured in untreated cells (Figures 7.8A and B, compare bars 3 and 4 to bar 1). Statistically significant increase of GSH levels was also detected in MDA-MB-468 cells treated with combination of NRH with β-oestradiol compared
to those measured in cells treated with NRH alone (Figures 7.8A and B, compare bar 4 to bar 2).

Tamoxifen treatment of MDA-MB-468 cells significantly increased the GSH levels in these cells in the absence of NRH compared to non-treated cells (Figures 7.8A and B, compare bar 5 to bar 1). Statistically significant decrease of GSH levels was observed in tamoxifen-treated MDA-MB-468 cells in the presence of NRH compared to those measured in cells treated with tamoxifen alone (Figures 7.8A and B, compare bar 6 to bar 5). Statistically significant increase of GSH levels was observed in cells treated with combination of tamoxifen with NRH compared to those measured in cells treated with NRH alone (Figures 7.8A and B, compare bar 6 to bar 2).

The lowest GSH levels were detected in the NRH treated MDA-MB-468 cells, and the highest GSH levels were measured in these cells treated with β-oestradiol alone or combination of β-oestradiol and NRH compared to untreated cells.
Figure 7.8: Estimation of GSH levels in MDA-MB-468 breast cancer cells
MDA-MB-468 cells were left untreated or treated with β-oestradiol, tamoxifen, NRH, β-oestradiol and NRH, and tamoxifen and NRH as indicated, stained with VB48.TM, Acridine Orange and Propidium Iodide (PI) and GSH levels were analysed using the Nucleocounter (NC 3000) (Chemometec, Denmark) as described in the Materials and Methods section. (A) Histogram representing different cellular populations as indicated in the Materials and Methods section. Data are representative of three independent experiments performed in duplicates. (B) Graph representing GSH levels MDA-MB-468 breast cancer cells treated as indicated. [*] significant difference ($p<0.05$) of GSH levels detected in treated compared to untreated cells. [#] significant difference ($p<0.05$) of GSH levels detected in cells treated with combination of NRH with β-oestradiol or NRH with tamoxifen compared to those measured in cells treated with NRH alone. [a] significant difference ($p<0.05$) of GSH levels detected in cells treated with combination of tamoxifen with NRH compared to that measured in cells treated with tamoxifen alone. One symbol indicates $p<0.05$, two symbols indicate $p<0.01$, three symbols indicate $p<0.001$ and four symbols indicate $p<0.0001$.

Comparative analysis of GSH levels in MCF-7, T47D, MDA-MB-231 and MDA-MB-468 breast cancer cells treated with either β-oestradiol, tamoxifen, or NRH in the absence and presence of NRH as shown in Figure 7.5, 7.6, 7.7 and 7.8 indicated there were no large changes in the MCF-7 and T47D cells irrespectively of the treatment. Low GSH levels on the other side were detected in β-oestradiol-treated MDA-MB-231 cells in the presence and absence of NRH whereas MDA-MB-468 cells treated with β-oestradiol alone or combination of β-oestradiol with NRH exhibited high GSH levels compared to those measured in the untreated cells.
7.3 Assessment of the glutathione peroxidase (GPx) enzymatic activity in breast cancer cells overexpressing NQO2

There are several GPx isozymes (GPx1-8) encoded by different genes, but all are tetramers carrying four identical subunits. Each subunit in GPx enzyme contains selenocysteine at its active site involved directly in the two-electron reduction of the peroxide substrate. GPx enzymes use glutathione as the electron donor to regenerate the reduced form of selenocysteine. Oxidized glutathione is recycled to its reduced state by glutathione reductase (GR) and NADPH.

Considering the importance of the estrogenic compounds in the gene expression and activity of various antioxidant enzymes (Vina, Borras et al. 2005) it was hypothesized that the enzymatic activity of the glutathione peroxidase (GPx) antioxidant enzymes would be affected in breast cancer cells treated with β-oestradiol or tamoxifen in a manner involving the NQO2 activity. To test this hypothesis the GPx enzymatic activity was followed in untransfected or ectopically expressing NQO2 MCF-7, T47D, MDA-MB-231 and MDA-MB-468 cells treated with β-oestradiol or tamoxifen in the presence or absence of NRH as shown in Figures 7.9, 7.10, 7.11 and 7.12.

Increasing amounts of NQO2 expression vector transfected in untreated MCF-7 cells significantly increased the enzymatic activity of GPx compared to untransfected cells (Figure 7.9, compare bar 2 to bar 1).

Increased GPx enzymatic activity was observed in MCF-7 cells transfected with the PCDNA3 empty vector and treated with β-oestradiol compared to untreated cells (Figure 7.9, compare bar 4 to bar 1), tamoxifen (Figure 7.9, compare bar 7 to bar 1), NRH (Figure 7.9, compare bar 10 to bar 1), the combination of β-oestradiol with NRH (Figure 7.9, compare bar 13 to bar 1) and combination of tamoxifen with NRH (Figure 7.9, compare bar 16 to bar 1) compared to PCDNA3 transfected and untreated cells.

Increasing amounts of transfected NQO2 expression vector significantly decreased the GPx enzymatic activity in MCF-7 cells treated with β-oestradiol (Figure 7.9, compare bar 5 to bar 4), tamoxifen (Figure 7.9, compare bars 8 and 9 to bar 7), NRH (Figure 7.9, compare bars 11 and 12 to bar 10), combination of β-oestradiol with NRH (Figure 7.9, compare bars 14 and 15 to bar 13) and combination of tamoxifen with NRH.
(Figure 7.9, compare bars 17 and 18 to bar 16) compared to PCDNA3 transfected cells under the same conditions.

**Figure 7.9: Assessment of the glutathione peroxidase (GPx) enzymatic activity in NQO2 transfected MCF-7 breast cancer cells**

MCF-7 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cell lysates were collected, incubated with GPx co-substrate mixture containing NADPH, glutathione and glutathione reductase and the reaction was initiated with the addition of GPx hydperoxide (2-hydroperoxypropan-2-ylbenzene). The absorbance of the samples was read at 340nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates. [#] significant difference (p<0.05) of GPx enzymatic activity detected in NQO2 overexpressing MCF-7 cells compared to that measured in cells transfected with the PCDNA3 empty vector. One # indicates p<0.05 and two ## indicate p<0.01.

There were no differences in the GPx enzymatic activity recorded in T47D cells transfected with the empty vector PCDNA3 and treated with β-oestradiol (Figure 7.10, compare bar 4 to bar 1), tamoxifen (Figure 7.10, compare bar 7 to bar 1), NRH (Figure 7.10, compare bar 10 to bar 1), combination of β-oestradiol with NRH (Figure 7.10, compare bar 13 to bar 1) and combination of tamoxifen with NRH (Figure 7.10, compare bar 16 to bar 1) compared to PCDNA3 transfected and untreated cells.
Transfection of increasing amounts of NQO2 expression vector did not affect the enzymatic activity of GPx in T47D cells treated with β-oestradiol (Figure 7.10, compare bars 5 and 6 to bar 4), tamoxifen (Figure 7.10, compare bars 8 and 9 to bar 7), and NRH (Figure 7.10, compare bars 11 and 12 to bar 10) compared to PCDNA3 transfected under the same conditions.

Increased GPx enzymatic activity but not statistically significant was observed in T47D cells transfected with increasing amounts of NQO2 expression vector and treated with combination of β-oestradiol with NRH (Figure 7.10, compare bars 14 and 15 to bar 13) and combination of tamoxifen with NRH (Figure 7.10, compare bars 17 and 18 to bar 16) compared to PCDNA3 transfected under the same conditions.

Figure 7.10: Assessment of the glutathione peroxidase (GPx) activity in NQO2 transfected T47D breast cancer cells
T47D cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cell lysates were collected, incubated with GPx co-substrate mixture containing NADPH, glutathione and glutathione reductase and the reaction was initiated with the addition of GPx hydperoxide (2-hydroperoxypropan-2-ylbenzene). The absorbance of the samples was read at 340nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates.
There were no significant differences in the GPx enzymatic activity evident in the MDA-MB-231 cells transfected with the empty vector PCDNA3 and treated with β-oestradiol (Figure 7.11, compare bar 4 to bar 1), tamoxifen (Figure 7.11, compare bar 7 to bar 1), NRH (Figure 7.11, compare bar 10 to bar 1), combination of β-oestradiol with NRH (Figure 7.11, compare bar 13 to bar 1) and combination of tamoxifen with NRH (Figure 7.11, compare bar 16 to bar 1) compared to PCDNA3 transfected and untreated cells.

No significant differences of the GPx enzymatic activity were evident in these cells transfected with increasing amounts of NQO2 and treated with β-oestradiol (Figure 7.11, compare bars 5 and 6 to bar 4), tamoxifen (Figure 7.11, compare bars 8 and 9 to bar 7), NRH (Figure 7.11, compare bars 11 and 12 to bar 10), combination of β-oestradiol with NRH (Figure 7.11, compare bars 14 and 15 to bar 13) and combination of tamoxifen with NRH (Figure 7.11, compare bars 17 and 18 to bar 16) compared to PCDNA3 transfected under the same conditions.

Figure 7.11: Assessment of the glutathione peroxidase (GPx) activity in NQO2 transfected MDA-MB-231 breast cancer cells
MDA-MB-231 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated
treatment cell lysates were collected, incubated with GPx co-substrate mixture containing NADPH, glutathione and glutathione reductase and the reaction was initiated with the addition of GPx hydroperoxide (2-hydroperoxypropan-2-ylbenzene). The absorbance of the samples was read at 340nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates.

No differences in the GPx enzymatic activity were evident in the MDA-MB-468 cells transfected with the empty vector PCDNA3 and treated with β-oestradiol (Figure 7.12, compare bar 4 to bar 1), tamoxifen (Figure 7.12, compare bar 7 to bar 1), NRH (Figure 7.12, compare bar 10 to bar 1), combination of β-oestradiol with NRH (Figure 7.12, compare bar 13 to bar 1) and combination of tamoxifen with NRH (Figure 7.12, compare bar 16 to bar 1) compared to PCDNA3 transfected and untreated cells.

No significant differences in the GPx enzymatic activity were evident in these cells transfected with increasing amounts of NQO2 and treated with β-oestradiol (Figure 7.12, compare bars 5 and 6 to bar 4), tamoxifen (Figure 7.12, compare bars 8 and 9 to bar 7), NRH (Figure 7.12, compare bars 11 and 12 to bar 10), combination of β-oestradiol with NRH (Figure 7.12, compare bar 14 and 15 to bar 13) and combination of tamoxifen with NRH (Figure 7.12, compare bars 17 and 18 to bar 16) compared to PCDNA3 transfected and under the same conditions.

Figure 7.12: Assessment of the glutathione peroxidase (GPx) activity in NQO2 transfected MDA-MB-468 breast cancer cells
MDA-MB-468 cells were transfected with increasing amounts of NQO2 expression vector and treated with β-oestradiol (bars 4, 5 and 6), tamoxifen (bars 7, 8 and 9), NRH (bars 10, 11 and 12), β-oestradiol and NRH (13, 14 and 15), and tamoxifen and NRH (bars 16, 17 and 18), or left untreated (bars 1, 2 and 3) as indicated. After the indicated treatment cell lysates were collected, incubated with GPx co-substrate mixture containing NADPH, glutathione and glutathione reductase and the reaction was initiated with the addition of GPx hydroperoxide (2-hydroperoxypropan-2-ylbenzene). The absorbance of the samples was read at 340nm using the spectrophotometer plate reader (Biotek, UK). Data are representative of two independent experiments performed in duplicates.

Comparative analysis of GPx activity in MCF-7, T47D, MDA-MB-231 and MDA-MB-468 breast cancer cells treated with either β-oestradiol, tamoxifen, or NRH in the absence and presence of NRH as shown in Figure 7.9, 7.10, 7.11 and 7.12 indicated that overexpression of NQO2 decreased GPx activity in all treatments in MCF-7 cells. No significant differences in GPx enzymatic activity were observed in NQO2 transfected T47D, MDA-MB-231 and MDA-MB-468 cells treated with either β-oestradiol, tamoxifen, NRH alone or combination of β-oestradiol with NRH and tamoxifen with NRH.

7.4 Chapter discussion

The intracellular environment is maintained in the reduced state by several antioxidant enzymes including catalase (Valko, Rhodes et al. 2006) and GPx (Brigelius-Flohe and Kipp 2009, Brigelius-Flohe and Maiorino 2013). It has been reported that NQO2 gene expression is induced by activation and binding of Nrf2 transcription factor at the ARE sites present in the regulatory region of the NQO2 promoter (Nguyen, Nioi et al. 2009). Nrf2 activates antioxidant biological response by inducing the expression of antioxidant enzymes and detoxify chemicals producing reactive oxygen species in cells. Oestrogens have been reported to stimulate the enzymatic activity of catalase and GPx in endothelial cells (Yen, Hsieh et al. 2001) and more recent findings have described the potential molecular mechanism involved in the induction of detoxifying enzymes such as GPx in MCF-7 cells through the induction of NF-κB transcriptional activity (Borras, Gambini et al. 2005). Taking into account these observations, the effects of NQO2 in catalase and GPx enzymatic activities were explored. NQO2 overexpression exerted more pronounced effects on the catalase activity in the tamoxifen treated T47D cells, in which endogenous NQO2 protein levels are not
detectable. Furthermore, NQO2 ectopic expression reduced the GPx enzymatic activity in MCF-7 cells, which express relatively low endogenous NQO2, treated with β-oestradiol or tamoxifen in the presence or absence of NRH. The catalase or GPx enzymatic activities were not affected by NQO2 overexpression in the ERα negative MDA-MB-231 and MDA-MB-468 breast cancer cells.

Quinones alter cellular redox status by modulating the ratio of the reduced GSH versus its oxidized form (GSSG) signifying the link of the metabolic fates of quinones and GSH (Watanabe, Dickinson et al. 2004). In addition, quinones are implicated in the regulation of gene expression of the rate-limiting step in GSH synthesis governed by the glutamate cysteine ligase (GCL) (Watanabe, Dickinson et al. 2004, Mattingly, Ivanova et al. 2008). Taken together these observations prompted our interest to explore the possibility that NQO2 might affect the cellular GSH content in breast cancer cells treated with β-oestradiol or tamoxifen. Significant reduction of the GSH content was observed in T47D cells treated with a combination of β-oestradiol with NRH compared to cells treated with β-oestradiol alone and in the NRH treated MDA-MB-231 and MDA-MB-468 cells compared to non-treated cells, implying that GSH synthesis in the T47D cells involves the transcriptional activity of ERα whereas in the ERα negative cells, NADPH cycling is the major regulator of the cellular GSH levels.
CHAPTER 8

Conclusions and future work
8.0 CONCLUSIONS

8.0.1 Significance of studies

Dihydronicotinamide riboside (NRH) quinone oxidoreductase 2 (NQO2) is a phase II metabolising enzyme involved in the two-electron reduction of quinones to the stable hydroquinones (Wu, Knox et al. 1997). Quinones’ metabolism including endogenous and exogenous oestrogens is an important mechanism of cellular ROS generation leading to DNA damage (Fussell, Udasin et al. 2011, Sharma, Jha et al. 2012) due to the formation of the unstable semiquinones formed during quinones’ metabolism mediated by other reductases (cytochrome b5 reductase, cytochrome P-450 reductase and ubiquinone oxidoreductase) (Chesis, Levin et al. 1984). The product of the NQO2 mediated metabolism of quinones is the stable hydroquinones thereby circumventing the production of unstable intermediates (Wu, Knox et al. 1997). Quinones are abundant in nature found in foods, cigarette smoke and burned organic materials (Bolton, Trush et al. 2000), but their accumulation in cells can lead to the generation of reactive oxygen species (ROS) and consequent oxidative damage to proteins, lipids and DNA (Monks, Hanzlik et al. 1992). Quinones are also formed as products of oestrogen metabolism (Cavalieri, Chakravarti et al. 2006) and chronic exposure of women to oestrogen and oestrogen-quinone metabolites results in ROS production which is an important contributing factor responsible for oestrogen-initiated breast carcinogenesis (Gaikwad, Yang et al. 2009).

Although NQO2 mediated detoxification of quinones protects cells from oxidative and electrophilic stress, NQO2 overexpression has been observed in a variety of cancers including breast, skin, and prostate (Jaiswal 1994) associated with cancer development and proliferation through unknown molecular mechanisms (Vella, Ferry et al. 2005). This implies that in breast cells overexpressing NQO2, endogenous oestrogens could be metabolised to non-reactive intermediates whereas its absence or low expression might lead to the production of reactive oxygen species and hence elevated oxidative stress.

ROS induce the transcriptional activity of redox responsive transcription factors. Given the fact that NQO2 overexpression alters the cellular redox state in combination with reports indicating that the Nrf2 signalling is critical for oestrogen stimulation
(Yao, Brodie et al. 2010) and is inhibited by ERα in MCF-7 breast cancer cells (Lo and Matthews 2013) it was reasoned that NQO2 and oestrogen receptor might crosstalk. Support to this hypothesis is lent by published observations indicating that antioestrogens induce NQO1 gene expression in an oestrogen receptor alpha (ERα) dependent manner, and β-oestradiol represses this induction (Montano, Jaiswal et al. 1998, Katzenellenbogen and Katzenellenbogen 2000). Given the structural similarity of NQO1 and NQO2 genes (Jaiswal 1994), it is possible that NQO2 gene expression is under the control of a similar ERα dependent mechanism of gene expression.

Oestrogen signalling is mediated through the oestrogen receptors (Gao and Dahlman-Wright 2011). Oestrogen receptor alpha upon binding to oestrogens changes its conformation, dissociates from the complex with heat shock proteins and translocates into the nucleus where it induces or represses the expression of its transcription target genes (Morani, Warner et al. 2008). The fact therefore that MCF-7 and T47D are ERα positive and MDA-MB-231, MDA-MB-468 and MDA-MB-468 are ERα negative cells is a possible justification for the different NQO2 cellular levels observed in these cells.

8.1 Conclusion

In an attempt to determine the biological role of NQO2 in oestrogen receptor positive and oestrogen receptor negative breast cancer cells, we studied the involvement of NQO2 in regulating ROS generation, cell cycle progression, mitochondrial membrane potential and catalase and glutathione peroxidase enzyme activity. Results presented in this study suggest that NQO2 affects the ERα transcriptional activity in MCF-7 and T47D cells in the presence or absence of β-oestradiol. NQO2 has been shown to regulate gene expression of the ERα transcriptional target cyclin D1 and PDI acting as cell cycle regulator and unfolded protein response (UPR) respectively. NQO2 overexpression decreased cyclin D1 and PDI mRNA levels in the presence of its cofactor NRH. Consistent with that, the addition of NRH reduced the cyclin D1 and PDI protein levels. These findings indicated that NQO2 might be involved in the regulation of the cell cycle progression, the endoplasmic reticulum stress and the unfolded protein response. Consistent with the reduction of cyclin D1 protein levels in NQO2 overexpressing breast cancer cells G2/M cell cycle arrest was observed in T47D cells and G1 arrest in MDA-MB-231 cells ectopically expressing NQO2.
Investigation of the effects of NQO2 on mitochondrial membrane depolarization in breast cancer cells, showed that NRH caused mitochondrial membrane depolarization in β-oestradiol-treated MCF-7 and MDA-MB-468 cells and predominantly mitochondrial NQO2 subcellular localization in β-oestradiol treated-T47D and MDA-MB-468 cells.

Investigation of the effects of NQO2 in redox signalling in breast cancer cells, showed that NRH reduced ROS in oestrogen and tamoxifen treated breast cancer cells. It has been reported that induction of ROS generation activates antioxidant enzymes to make sure the redox signalling homoeostasis is maintained. However, results presented in this study showed that overexpression of NQO2 reduced significantly ROS levels in both oestrogen receptor positive and oestrogen receptor negative cells. This can be explained, by taking into account that the reduction of quinones to hydroquinones by NQO2 is completed when hydroquinones are being excreted by GST or SULTs. This mechanism can reduce the toxicity of cells produced by the redox signalling of quinones to hydroquinones.

Results presented in this study suggest there is an interplay between NQO2 and other Phase II antioxidants enzymes such as catalase and glutathione peroxidase (GPx). Catalase activity was increased in tamoxifen-treated MCF-7, T47D, MDA-MB-231 and MDA-MB-468 cells in the presence of NRH. Overexpression of NQO2 reduced catalase activity in tamoxifen-treated MCF-7, T47D and MDA-MB-231 cells in the presence of NRH. Reduced GPx activity was identified in β-oestradiol and tamoxifen-treated ERα-positive cells in an NQO2 dependent manner since overexpression of NQO2 increased GPx activity in β-oestradiol and tamoxifen-treated T47D cells in the presence of NRH whereas NQO2 did not exert any effects on GPx activity in the β-oestradiol and tamoxifen-treated MCF-7, MDA-MB-231 and MDA-MB-468 cells in the presence of NRH.

Reduced GSH levels were detected in NRH-treated MCF-7, T47D, MDA-MB-231 and MDA-MB-468 cells. Reduced GSH levels were also detected in the β-oestradiol treated MCF-7, T47D, MDA-MB-231 and MDA-MB-468 cells in the presence of NRH whereas increased GSH levels were recorded in tamoxifen-treated MCF-7, T47D, and MDA-MB-231 cells.
In conclusion, this study has displayed NQO2’s involvement in breast cancer shedding light on the role of this enzyme in the regulation of intracellular ROS generation, cell cycle control, cellular redox state and mitochondrial energy metabolism. The findings described in this report are consistent with the hypothesis that NQO2 cellular levels play a crucial role in determining the outcome of anti-estrogenic therapies and should be taken into consideration in the design of personalised treatment of breast cancer patients. Further investigation is required to unravel other NQO2 functions and determine whether NQO2 could be used as a target for the development of novel anti-cancer therapeutic strategies.

8.2 Future work

Further investigation is required to understand the functions of NQO2 in the process of carcinogenesis.

Firstly, it would be beneficial to indicate the effects of NQO2 in redox signalling pathways in others hormone inducible cancers such as ovarian cancer, prostate cancer, and the normal breast cancer cells.

The involvement of NQO2 in endoplasmic reticulum stress should be further studied. Production of ROS has been linked to ER stress and UPR. ROS play a critical role in carcinogenesis and can be produced in the cytosol and several organelles including the ER and mitochondria (Cao and Kaufman 2014). Several reports have shown that altered redox homeostasis in the ER causes ER stress and UPR. Data shown in this study imply that NQO2 regulates ER stress and UPR signalling in breast cancer cells. Investigation of potential NQO2 subcellular localization would unravel possible direct endoplasmic reticulum functions of NQO2. The effects of NQO2 in regulating apoptosis and autophagy are worth investigating given the fact that NQO2 affects ROS production and autophagy is induced by ROS (Azad, Chen et al. 2009).

The lack of knowledge regarding the physiological NQO2 cofactor is a significant obstacle in the NQO2 research. Therefore, studies towards unravelling the physiological NQO2 cofactor would benefit future developments in the field. Until the physiological NQO2 cofactor is revealed studies using various NQO2 cofactors apart from NRH to confirm consistency of the results would benefit this research field.
Lastly, it would be interesting to determine potential interplay between NQO2 and the other type of oestrogen receptor the ERβ.
CHAPTER 9

References
9.0 REFERENCE


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