The Role of NQO2 in Tumour Growth and Response to Therapeutic Drugs

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<td>Adriamycin</td>
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<tr>
<td>NQO1</td>
<td>NAD(P): quinone oxidoreductase 1</td>
</tr>
<tr>
<td>NQO2</td>
<td>NRH: quinone oxidoreductase 2</td>
</tr>
<tr>
<td>NQO2-OE</td>
<td>Overexpressed NRH: quinone oxidoreductase 2</td>
</tr>
<tr>
<td>NRC</td>
<td>Dihydronicotinamide riboside</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-targeting control</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OVC</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline tween-20</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>P-cyclin D1</td>
<td>Phospho-cyclin D1</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>P-gp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>PI-3 Kinase</td>
<td>Phosphatidylinostiol-3 kinase</td>
</tr>
<tr>
<td>P-Rb</td>
<td>Phospho-Retinoblastoma</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatidylinostiol-3 kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>QR1</td>
<td>Quinone reductase 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>QR2</td>
<td>Quinone reductase 2</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescence protein</td>
</tr>
<tr>
<td>rhNQO1</td>
<td>Recombinant human NQO1</td>
</tr>
<tr>
<td>rhNQO2</td>
<td>Recombinant human NQO2</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell park memorial institute-1640</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SF</td>
<td>Survival fraction</td>
</tr>
<tr>
<td>ShRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>Sh27</td>
<td>Small hairpin RNA 27-NQO2</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline tween-20</td>
</tr>
<tr>
<td>Tc</td>
<td>Doubling time</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</tbody>
</table>
NRH quinone oxidoreductase 2 (NQO2) is regarded as a mammalian Phase I detoxifying enzyme responsible for reducing quinones to hydroquinones. NQO2 is highly expressed in different types of cancer such as breast and prostate cancer suggesting its participatory role in the progression of these diseases. A potential reason for this is that NQO2 has the ability to modulate the stability of cyclin D1 and activity of NF-κB and it has been shown that inhibition of NQO2, either genetically or pharmacologically, can alter the pattern of proliferation of cancer cells. However, the biological roles of NQO2 in cancer progression are still ambiguous and need further investigation.

A panel of seven ovarian cancer cell lines (OVCs) were screened for the presence and functionality of NQO2. SKOV-3 and TOV-112D cells expressing comparatively the highest and lowest levels of NQO2 were stably transduced to silence and overexpress NQO2 respectively. Pharmacological inhibition was achieved using resveratrol or a series of novel 4-aminoquinolines synthesised in-house. Cell proliferation was monitored by cell counting and clonogenic assays. Flow cytometric analysis was used to determine cell cycle distribution and levels of ROS following modulation of NQO2 function. The expression of cell cycle regulatory markers was determined by Western blot. The contributory roles of NQO2 in determining the cytotoxicity of Adriamycin (ADR) towards OVCs was investigated using MTT assay together with evaluation of P-gp expression and basal ROS levels.

In the OVCs panel, NQO2 protein levels and enzymatic activity showed an excellent correlation; with activity varying 36-fold between the cell lines. The sensitivity of OVCs to CB1954 was significantly increased when combined with the NRH-like co-factor, EP0152R. This supports the notion that NQO2 mediates the toxicity of CB1954, which is further confirmed by the strong correlation between cellular NQO2 activity and the responsiveness of the OVC cell lines to CB1954. Hydrazone quinolines showed the highest inhibitory potency against NQO2 in SKOV-3 when compared to the typical and in-house synthesised quinolines inhibitors.

NQO2-overexpressing TOV-112D cells showed more aggressive growth pattern and higher capacity to form colonies than wild-type cells. This was consistently associated with an enhancement in the progression of cells through cell cycle phases and significant reduction in Rb expression. A reduction in ROS levels in NQO2-OE cells may also explain this enhancement in cell growth. Overexpressing NQO2 also resulted in destabilisation of CDK4 and cyclin D1 with significant reduction in their expression levels, and concomitant increase in p-cyclin D1 (Thr286). The involvement of NQO2 in controlling cyclin D1 turnover is also confirmed in SKOV-3 cells when genetic silencing of NQO2 was accompanied by significant reduction in p-cyclin D1 and subsequent stabilisation of cyclin D1 levels. In spite of this, no alterations in the growth pattern of SKOV-3 cells were observed highlighting the impact of cell type on the variations in cellular responses.

The role of NQO2 in determining the toxicity of ADR treatment was not proved in OVC cells. This was despite that modulation of NQO2 levels caused significant changes in P-gp expression. The intracellular basal levels of ROS was found to affect the responsiveness of OVCs to ADR as demonstrated when treating SKOV-3 with resveratrol was accompanied by significant increase in ROS levels and concomitant enhancement in the cells’ response to ADR. In conclusion, NQO2 can profoundly alter the proliferation characteristics of OVCs and is a potential therapeutic target for the treatment of this disease. However, the biological functions of NQO2 and its contributory roles in particular pathways are varied among different types of cancer -in other words- are highly dependent on cancer type.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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I dedicate this thesis to my wonderful parents
Chapter 1

Introduction
1 Introduction

1.1 Cancer

Cancer is a malignant disease which is characterised by the persistent and uncontrolled growth and proliferation of damaged cells, forming masses of cancerous cells called tumours [1]. The development of cancer is a multistage process through which the cells gain new capabilities for sustainable survival and proliferation. These capabilities were first categorised by Hanahan and Weinberg in 2000 as six hallmarks shared by all tumours; these were independence on exogenous growth signals for proliferation, unresponsiveness to growth-inhibitory signals, escape from apoptosis, unlimited replicative potential, encouraging the growth of new blood vessels (angiogenesis) and inducing the cells invasion and metastasis [2]. In 2011, two emerging hallmarks of cancer have been added to the previous list. The first of these being the capability of cancer cells to modify cellular energy metabolism to be only restricted to the aerobic glycolysis, avoiding the oxidative phosphorylation; this was termed the Warburg-like metabolism. The cells take advantage of this modification and consequent reduction in energy production to activate the oncogenes, thereby supporting their proliferation [3]. Moreover, these cells deal with the high production of glycolytic metabolites by converting them to biosynthetic precursors, in order to constitute the new macromolecules needed to generating new cells [3]. The second hallmark concerns the capability of cancer cells to resist destructive immune responses [3]. The involvement of inflammation in the acquisition of hallmark capabilities in cancer cells has also been suggested; this can be mediated by chemicals such as bioactive factors (e.g. survival and growth factors) and reactive oxygen species (ROS) which are released from inflammatory cells. Such ROS cause mutagenic alterations in the genome of neighbouring cancer cells, thereby enhancing their malignancy [3, 4].

1.2 Ovarian cancer

Ovarian cancer (OVC) is a major health problem which affects women across the globe [5], and is the most frequent cause of cancer-related death in Europe, with an estimated 65,697 new cases
and 41,448 female deaths annually [6]. In 2012, the incidence and mortality rates of OVC in the UK were estimated to be ninth and 16th highest respectively, in Europe [7]. In 2014, Cancer Research UK’s constitution registered approximately 7,378 new diagnoses of OVC in addition to 4,128 deaths [7]. Carcinoma is the most common type of OVC, accounting for more than 90% of all cases [8], and is divided into five histological subtypes, including serous, endometrioid, clear, mucinous and transitional [9]. Serous carcinoma is the most common subtype followed equally by endometrioid and clear-cell carcinoma [8, 10]. The identification of biomarkers that characterise each subtype is important, in order to distinguish one from another, and to subsequently personalise the treatment for maximal efficacy [11, 12]. The lack of symptoms in the initial stage of OVC, in addition to the lack of effective diagnostic methods, are the main reasons for the late-stage diagnosis of OVC, and consequent high mortality rate among patients. The chemoresistance developed among relapsed OVC patients also interferes with successful treatment, thereby increasing the mortality rate [6, 9]. Fully understanding the underlying pathways that contribute to tumourgenesis is important for the identification of therapeutic targets, the development of treatments and, ultimately, the prevention of ovarian carcinoma progression [8, 13]. Several factors have been reported to be involved in increasing the risk of developing OVC, including late-age, excessive inflammation, starting menstruation at an early age, late menopause and nulliparity [14].

1.3 Carcinogenesis

Carcinogenesis is a multistage process by which normal cells are transformed to malignant cells with invasive traits. It comprises of three distinct stages, including initiation, promotion and progression [15], **Figure (1.1)**. Initiation occurs due to irreversible genetic alterations caused by multiple mutations in the crucial genes which are involved in controlling normal cell proliferation and apoptosis. These mutations lead to the activation of proto-oncogenes to oncogenes, and the inactivation of the tumour-suppressor genes [16]. Proto-oncogenes such as Ras are central to the stimulation of normal cell growth, proliferation and survival. Upon mutational activation, these become oncogenes, leading to uncontrolled cell growth. In contrast, tumour suppressor genes such as Rb are central to the inhibition of unrestrained cell proliferation, repairing DNA damage and activation of cell cycle checkpoints. These lose their normal functions upon mutational inactivation.
Mutational changes occur as a result of DNA damage induced by endogenous cellular processes, such as defects in the DNA replication process or attacks mediated by free radicals produced through cellular metabolism. DNA damage can also be induced by interactions between DNA and exogenous factors, including ionising radiation and carcinogenic chemicals [16]. Promotion occurs when the initiated cells are exposed to external, non-mutagenic stimuli that promote the clonal expansion and consequent formation of benign colonies. The progression of benign tumours to malignant ones requires some additional, heritable changes in the cellular genome that ultimately generate cells with new traits such as being highly invasive, metastatic, unlimitedly proliferative and adaptive to harsh environmental conditions, such as high acidity and hypoxia, where competent non-invasive cells cannot survive [15].
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Figure 1.1 Stages of the development of cancer

1. Initiation of cancer occurs due to cumulative exposure to exogenous factors such as ionising radiation and/or endogenous factors such as ROS, which ultimately cause DNA damage and subsequent mutations in crucial genes involved in cell proliferation. Inheriting the mutations leads to irreversible genetic alterations. 2. Initial cells are promoted to expand and form benign colonies upon chronic exposure to non-mutagenic stimuli such as inflammation. The density of blood vessels and blood flow to the affected region is also enhanced during the promotion stage. 3. The progression of benign tumours to malignancy requires more heritable alterations in the cellular genome that allow the cells to gain malignant traits and become aggressive [15]. Adapted from (Vincent et al., 2008).

1.4 Reactive oxygen species

ROS are unstable chemical molecules containing oxygen, which have important functions in biological systems. They can be classified into radical and non-radical ROS. Radical ROS are very chemically reactive molecules that have one or more unpaired electrons in their outer molecular
shell. Non-radical ROS are still considered to be highly reactive molecules, despite them not having any unpaired electrons. This is because they can become free-radical ROS when undergoing chemical interactions [18]. Examples of non-radical ROS include hydroxide (OH), hydrogen peroxide (H$_2$O$_2$) and peroxynitrate (ONOO). Radical ROS include superoxide anion (O$_2^-$), hydroxyl (OH) and nitric oxide (NO) radicals; these are the most commonly observed in the biological system [18]. ROS play regulatory roles in controlling vital pathways in the cells, such as cell growth and differentiation. They also regulate enzymatic activities and inflammatory processes [18]. Maintaining the balance between ROS production and scavenging is central to cell survival and normal growth. Moderate increases in endogenous ROS levels lead to enhancing cell proliferation and differentiation, while substantial increases in their levels cause extensive oxidative damage to biomolecules (such as lipid, protein and DNA) [19].

1.4.1 Sources of ROS

1.4.1.1 Endogenous sources of ROS

The generation of ROS is a normal cellular process, as these species are natural by-products of pivotal biological processes in the cells, such as oxidative phosphorylation [1], acute or chronic inflammation [19] and biochemical reactions [18].

Oxidative phosphorylation is a metabolic process that takes place in mitochondria to provide the cells with the fuel molecules, namely ATP, used for their vital processes. The production of ATP occurs as a result of electron transport chain (ETC) reactions during which electrons are transferred through a series of electron carriers called respiratory complexes, to ultimately reduce oxygen molecules to form water [20]. The reduction of oxygen results in releasing large amount of energy, which is used to reform ATP molecules. ETC in mitochondria is considered to be the major source of ROS production [20]. The leakage of electrons from respiratory complexes during ETC allows them to react with oxygen molecules, thereby generating superoxide [1, 21]. Superoxide rapidly undergoes a dismutation reaction by mitochondrial superoxide dismutase (Mn-SOD) to generate hydrogen peroxide [21]. The high reactivity of superoxide makes it undetectable,
therefore, hydrogen peroxide is used as a measure of mitochondrial superoxide production instead [21]. Hydrogen peroxide is either fully reduced by catalase (CAT) or glutathione peroxidise (GPX) to water, or partially reduced to hydroxyl radical [22]. Hydrogen peroxide is less reactive in its nature than superoxide, but it can be converted to one of the most reactive oxidant, namely hydroxyl radical, when it undergoes a one-electron reduction reaction (otherwise called Fenton reaction). This reaction is catalysed by reduced iron ion (Fe$^{2+}$, ferrous ion). Thus, it is very important to keep the reduction reaction of hydrogen peroxide under control; this can be achieved by inducing the enzymes responsible for fully reducing hydrogen peroxide [23]. The propagation of the Fenton reaction depends on the availability of reduced metal ions, which can be induced by superoxides; the latter re-reduce the oxidised metal ion resulting from the Fenton reaction to its original reduced form in a positive feedback loop [22]. The production of superoxide as a primary precursor of other forms of ROS is illustrated in Figure (1.2).
Figure 1.2 Illustration of stepwise generation of ROS

The stepwise one-electron reduction reactions of oxygen molecules lead to the generation of different forms of ROS. The first step of the monovalent reduction of oxygen results in the production of superoxide. Superoxide anion, which is a precursor of the other ROS radicals, undergoes a dismutation reaction induced by SOD dismutase, generating hydrogen peroxide. The presence of reduced transition iron ion, in its ferrous status (Fe$^{2+}$), which is a source of hydride, allows hydrogen peroxide to be partially reduced to highly reactive hydroxyl radicals. Hydrogen peroxide can also be fully reduced by catalase (CAT) and glutathione peroxidase (GPX) to water molecules [24]. Adapted from (Sharma et al., 2012).

Other sources of intracellular ROS are phagocytic immune cells such as macrophages and neutrophils [19] in addition to some cancer cells [18]. The immune cells possess a superoxide generator system that relies on NAD(P)H-oxidase complexes to catalyse the generation of superoxide toxic species to kill microbial pathogens and neoplastic cells. This system becomes active during acute and chronic inflammation [19, 25].

Examples of biochemical reactions that result in the production of ROS are detoxification reactions catalysed by cytochrome P450 [1, 18] and the β-oxidation of long chain fatty acids which occurs in peroxisomes [19].
1.4.1.2 Exogenous sources of ROS

ROS cannot only be generated by endogenous sources but also by exogenous sources. ROS can be found in plant-based phenolic-rich diets, tobacco smoke and environmental pollutants. They are also formed as a result of exposure to radiation [19].

1.4.2 Oxidative stress

Mammalian cells can protect themselves against oxidative damage by maintaining intracellular redox homeostasis and by limiting the concentration of ROS to ensure they are within the harmless range. The cells can achieve this by using their antioxidant defence system which consists of ROS-detoxifying enzymes (e.g. SOD and CAT) and ROS scavengers (e.g. alpha-tocopherol (vitamin E), ascorbic acid (vitamin C) and glutathione (GSH)) [1].

Oxidative stress occurs due to the loss of redox homeostasis between oxidants and anti-oxidants, resulting in the oxidative damage of cellular macromolecules such as DNA, RNA, lipids and proteins [1]. This imbalance occurs as a result of excessive production of ROS or a decline in the antioxidant defence system [26]. Oxidative damage of DNA leads to modifications in nitrogenous bases of nucleotides and breakage of DNA strands. As polyunsaturated lipids in cell membranes are vulnerable targets for ROS [19], increased ROS levels cause lipid peroxidation of cell membranes, and this may result in increasing cell permeability and consequently cell death [27]. Additionally, proteins are sensitive to ROS-induced oxidative modification, which causes alterations in their structure and, consequently, causes changes to or loss of their biological functions [1].

1.4.2.1 Oxidative stress in cancer cells

In comparison to normal cells, cancer cells show much higher levels of ROS [1]. This might be explained by their high proliferative ability and metabolic activity increasing their ATP demand. Thus, mitochondria are forced to synthesise more ATP and this consequently results in an increase in superoxide production [1].
Elevated oxidative stress in cancer cells during cancer development and progression results from an increase in ROS generation. This increase has been suggested to be mediated by some intrinsic pathways, including the activation of ROS-producing enzymes, the activation of oncogenes and defects in mitochondrial functions [18, 28, 29]. Oxidative damage of mitochondrial DNA results in mutational alterations in members of the electron transport chain process; this consequently impairs their functions, thereby increasing the leakage of electrons and subsequent production of ROS [1, 18]. Oxidative damage of mitochondria also includes the proteins that constitute the mitochondrial membrane, leading to more ROS being produced [1]. The dysregulation in redox homeostasis in cancer cells is manifested by an increase in the amount of oxidative damage products, such as are produced during lipid peroxidation and DNA base oxidation. There also appear to be substantial alterations in the expression levels of ROS-detoxifying enzymes (e.g. GPX and SOD) in those cells [18]. Despite the elevated intrinsic oxidative stress, cancer cells have been demonstrated to be functional in vitro and in vivo [18, 30].

It has been postulated that elevated oxidative stress contributes to the cancer cells’ acquisition of new capabilities and traits, such as sustained cell proliferation and survival, and unresponsiveness to growth-inhibitory signals [18]. It has also been shown that increased ROS concentrations might be involved in the initiation and progression of cancer [18]. Direct correlations have been established between the degree of ROS production and aggressiveness of cancer and poor prognosis [31]. ROS can also act as signalling molecules to control vital processes in the cells. Low concentrations of ROS have been found to contribute to enhancing cell proliferation and cell cycle transition [32].

On the other hand, elevated oxidative stress can be toxic to cancer cells, as it makes them more sensitive to oxidative damage induced by exogenous ROS-generating agents [18]. This is because the ROS-scavenging capacity of cancer cells cannot overwhelm the substantial increase in ROS levels, eventually bringing about cell death [1, 33]. Therefore, the manipulation of ROS levels in cancer cells is a strategy that can be exploited to selectively eradicate cancer cells without hurting normal cells, taking into account the natural differences in the endogenous ROS levels between normal and malignant cells [18]. It is worth mentioning that manipulating ROS levels in cancer cells
is not always associated with cell death; there is a plethora of factors and pathways regulating redox balance and affecting cellular response to stress [18].

1.4.2.2 Oncogenic consequences of adaptation to oxidative stress in cancer cells

Although cancer cells consistently generate high ROS levels and are persistently exposed to high intrinsic oxidative stress, they show a high capacity to adapt to such stress and even to exogenous stress [18, 33, 34]. This well-adaptation allows the cells to overcome severe oxidative damage and survive under high intrinsic oxidative stress. In addition, it is suggested that this ability is involved in the development of cancer and chemotherapy resistance [18, 34]. In other words, redox adaptation is induced upon persistent exposure to intrinsic oxidative stress, and includes several mechanisms, such as the activation of redox-sensitive transcription factors and the increase in the anti-apoptotic factors (e.g. Bcl2 family proteins). The activation, for example, of nuclear factor-kappa B (NF-κB) and Nrf2 redox transcription factors promotes the expression of ROS-detoxifying enzymes, allowing the cancer cells to overcome the destructive elevation of ROS levels, and to subsequently maintain redox balance. Furthermore, the activation of redox-sensitive transcription factors that promote AKT survival pathway may confer cancer cells' resistance against chemotherapeutic agents [18]. Some of these transcription factors have also been found to contribute to regulating the expression of several genes that encode proteins required for cell proliferation and metastasis, conferring additional growth and survival advantages for cancer cells [18, 35], Figure (1.3).
Cumulative exposure to ROS either from exogenous or endogenous sources results in elevated oxidative stress. The cells adapt to this increase by inducing the redox-sensitive transcription factors that in turn modulate the expression of multiple factors involved in cell death, cell survival and antioxidant enzymes (e.g. GPX and SOD). This consequently gives the cells the opportunity to survive under elevated intrinsic oxidative stress and to gain new traits for cancer development. In addition to increased cell survival, the enhanced ROS-scavenging capacity of cells allows them to quickly detoxify the chemotherapeutics, and these alterations confer resistance to chemotherapy [18]. Adapted from (Trachootham et al., 2009).

1.4.2.3 Exploiting redox alteration in cancer therapy

As aforementioned, cancer cells with elevated intrinsic oxidative stress are dependent on an endogenous antioxidant defence system to survive, thus, exposing these cells to exogenous ROS-producing agents or to compounds that decline their antioxidant capacity might enhance their
sensitivity to oxidative damage associated with elevated ROS levels [18]. Consequently, exploiting the difference in redox status between normal and cancer cells to cause preferential cancer-cell death through the elevation of ROS levels is a promising strategy which has recently regained importance [18, 36].

1.5 Treatment of cancer

The main purposes of treating cancer patients are to prolong life, control symptoms and decrease cancer-related complications [37]. The conventional approaches used for treating cancer patients are surgery, radiation and chemotherapy [1]. Surgery is the oldest modality of cancer treatment and is still used as first-line therapy for some types of solid tumours [38]. It is the most effective approach for treating patients with localised tumours [1]. However, this approach has several disadvantages; it can cause damage to the normal tissues surrounding debulked tumours, and has limited effectiveness against metastasised cancer cells that can proliferate and form new tumours of cancer cells elsewhere. Radiotherapy is used to shrink the tumour size. It is given to the patients in the forms of X-ray and γ-ray [1]. It is preferably used as the adjuvant therapy to surgery, in order to kill the peripheral tumour cells that cannot be removed surgically, in this way reducing local recurrence rates [39]. However, radiotherapy has a disadvantageous effect on normal tissues, leaving them damaged [1]. Chemotherapy has systematic effects on cancer patients’ bodies, as anticancer drugs are carried by the circulatory system and distributed throughout the patient’s body, reaching the site of action. Therefore, chemotherapy can exert its deleterious effects on localised as well as metastasised cancer cells within the body of cancer patients [1]. However, the resistance which has developed against chemotherapy is a major obstacle to successful treatment [40].

The selection of a proper approach to the treatment of cancer patients is dependent on the stage and type of the cancer, as well as the risk of recurrence [41]. For some early-stage cancers, it might be beneficial to select surgery alone or in combination with either radiotherapy or chemotherapy, while for some advanced cancer, a combination therapy of three treatments might be most suitable. Radiotherapy or chemotherapy can also be used as neoadjuvant therapies
before surgery to reduce tumour size and therefore facilitate complete surgical removal. While surgery or radiotherapy is limited to treating local cancer, chemotherapy is preferred option to treat metastatic cancer [41].

1.5.1 Chemotherapy

Chemotherapy includes cytotoxic agents that induce irreversible damage to the cells by interfering with some vital cellular processes such as DNA synthesis, replication, repair, mitosis, etc., resulting in cell death. The cytotoxicity of these agents can be used as an indicator of their efficacy [42]. Anticancer drugs can be administered as a single or combination therapy. The use of combination therapy has been in effect since 1960, and the results have demonstrated that it is more efficient in increasing the response rate in cancer patients than monotherapy [43]. Chemotherapy agents can be classified into different groups according to the mechanism of action that they use to attack cancer cells, Table (1.1).

There are several factors that can influence the decision to use a particular anticancer drug, including the type and location of the cancer, its stage, whether other approaches need to be used, the severity of the side effects accompanied by the drug [44] and the responses of some of the cancer patients who were previously treated with this drug [45].
### Table 1.1 The main classes of anticancer drugs with their mechanism of actions

<table>
<thead>
<tr>
<th>Classification</th>
<th>Example of treatment</th>
<th>Mechanism of action</th>
<th>Type of tumour</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Cisplatin (Planitol®)</td>
<td>Forms DNA adducts and consequently activates signalling pathways involved in apoptosis (e.g. p53).</td>
<td>Head and neck, testicular, ovarian and bladder cancer.</td>
<td>[46, 47]</td>
</tr>
<tr>
<td></td>
<td>Melphalan (Aspen®)</td>
<td>Mustard nitrogen DNA interstrand crosslink.</td>
<td>Multiple myeloma, ovarian and breast cancer.</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>Treosulfan (Ovastat®)</td>
<td>DNA interstrand crosslink.</td>
<td>Ovarian cancer.</td>
<td>[48]</td>
</tr>
<tr>
<td>Topoisomerase I (top I) inhibitors</td>
<td>Topotecan (Hycamtin®)</td>
<td>Binds with top I resulting in accumulation of Top I-DNA cleavable complexes during DNA replication and this subsequently activates cell death.</td>
<td>Ovarian, cervical and small cell lung cancers.</td>
<td>[49]</td>
</tr>
<tr>
<td>Topoisomerase II (top II) inhibitors</td>
<td>Etoposide (Vepesid®)</td>
<td>Binds with top II resulting in accumulation of Top II-DNA cleavable complexes and this subsequently activates the cells apoptosis event.</td>
<td>Leukaemia, lymphoma, testicular, small cell lung cancers.</td>
<td>[50, 51]</td>
</tr>
<tr>
<td>Anti-metabolites</td>
<td>5-fluorouracil (5-FU) (Adrucil®)</td>
<td>Works by inhibiting vital synthetic process of biomolecules (DNA and RNA) or by substituting for the normal molecular building-block of DNA and RNA blocking thereby their normal functions.</td>
<td>Colorectal, breast and head and neck cancers.</td>
<td>[52]</td>
</tr>
<tr>
<td>Signal transduction inhibitor</td>
<td>Imatinib mesylate (Gleevec®)</td>
<td>Inhibits abnormal constitutive form of tyrosine kinase (called BCR-ABL tyrosine kinase) to suppress cell proliferation.</td>
<td>Chronic myeloid leukaemia (CML).</td>
<td>[53]</td>
</tr>
</tbody>
</table>

Anticancer drugs are grouped into different classes according to their mechanism of action. Example(s) of the well-known drugs with their brand name(s) from each group and the types of cancer that are effectively treated by these drugs are also demonstrated in this table.

The use of chemotherapeutic agents is limited by two main factors; the lack of specificity toward tumour cells and the chemoresistance problem [54]. Conventional anticancer drugs are designed to interfere with the cell division process by targeting molecular mediators involved in controlling
subsequent events within this process [55]. As these drugs cannot differentiate between rapidly-dividing malignant cells and actively-multiplying normal cells such as hair-follicle and bone marrow cells, they can be highly toxic to these normal cells, causing severe side effects including alopecia (hair loss) and bone marrow suppression (including neutropenia, anemia, and thrombocytopenia) [56].

1.5.2 Chemoresistance

Chemoresistance is a major cause of treatment failure in cancer patients, whereby the cancer cells fail to respond to a broad range of anticancer drugs. The resistance can be divided into two types; intrinsic (inherent) or acquired. Intrinsic resistance occurs due to the presence of a subpopulation of cancer cells with distinct expression patterns of particular genes involved in controlling survival and cell cycle, which makes the tumour inherently resistant to the treatment from the beginning. These tumours are eventually able to re-grow [57]. In contrast, acquired resistance develops during or following treatment, as a result of biochemical changes occurring within cancer cells which leads to a decrease in drug uptake, an increase in its efflux or an increase in the cells’ capacity to repair damaged DNA [58].

1.5.2.1 Mechanisms of drug resistance

1.5.2.1.1 Alterations in expression of redox proteins

It has been demonstrated that drug-resistant cancer cells possess high levels of endogenous antioxidant enzymes which have the ability to scavenge oxidants that induce apoptosis and DNA damage [59]. As previously mentioned, the increased levels of detoxifying enzymes and antioxidants are adaptive mechanisms that are induced within cancer cells to survive elevated intrinsic oxidative stress [60]; this consequently renders the cells resistant to further exogenous stress inducers such as anticancer drugs [61]. Moreover, the aberrant expression of antioxidant enzymes has been shown to significantly affect the metabolism of alkylating agents; this might also be involved in the development of drug resistance [18], as demonstrated in Figure (1.3). For
example, the resistance conferred to oxidative stress inducers such as doxorubicin and platinum-based anticancer drugs has been suggested to be due to an elevation in the capacity of antioxidant systems. This elevation renders the cells insensitive to further exogenous stress, as well as attenuates the cells’ metabolic capacity to activate these drugs [18].

1.5.2.1.2 Overexpression of P-gp transmembrane protein

Alterations in the expression of cell-membrane transporter proteins such as permeability glycoprotein (P-gp) result in alterations in the drug uptake and efflux, consequently affecting the therapeutic concentration of drugs inside cells, and therefore cancer cells’ responsiveness to anticancer drugs such as doxorubicin [62]. P-gp is a member of the ATP-dependent ABC transporters superfamily [1], and the product of multi-drug resistance-1 gene (MDR-1) [63]. P-gp was first discovered in rodent cells in 1976 by Juliano and Ling. These cells were already known to have high resistance against anticancer agents [64]. By the 1980s, it was demonstrated that P-gp was also expressed in numerous types of cancer cell lines exhibiting high resistance against a wide range of anticancer drugs with various structures and functions [65]. P-gp has been found to be overexpressed in ovarian [1, 66], testicular, hepatic, kidney and pancreatic cancer; this is responsible for conferring high resistance to a broad range of anticancer drugs by pumping them out of the cancer cells [1]. This consequently prevents sufficient accumulation of these drugs inside the cells, thereby attenuating their cell cytotoxicity [1, 64].

The overexpression of P-gp in cancer cells can be either an intrinsic or acquired event [64]. Many factors are involved in regulating the expression of P-gp in cancer cells, including hypoxia inducible factor 1α (HIF-1α) [67], glucose deficiency [68] and the production of ROS [69] which are all actually derived from the tumour microenvironment. ROS can act as a positive regulator of P-gp expression, so that the presence of high ROS concentrations leads to the upregulation of the P-gp expression, and vice versa [70]. On the other hand, recent studies have demonstrated that ROS can also act as a negative regulator, so that the presence of high ROS levels leads to the downregulation of P-gp [71-73]. This negative correlation has been proved when HepG2 cancer cells treated with increasing concentrations of CAT (antioxidant enzyme) showed dose-dependent
reductions in the ROS levels, accompanied by an upregulation in P-gp expression [72]. The upregulation of P-gp induced by ROS occurs at different levels such as protein and functional levels as efflux transporter [70]. It is worth mentioning that both factors including ROS concentration and exposure time affect the cellular levels of P-gp in cancer cells [70].

1.5.2.2 Strategies for overcoming chemoresistance

1.5.2.2.1 Modulation of intrinsic oxidative stress

As cancer cells strongly rely on elevated antioxidant defence systems to overcome increased ROS levels, antioxidant enzymes represent potential therapeutic targets for a novel anticancer approach [61]. Disabling the antioxidant defence system in cancer cells raises the intrinsic oxidative stress to critical, non-tolerable levels, leading to severe cellular damage and consequent cell death for malignant cells rather than normal cells, which have naturally lower basal levels of ROS [60]. Therefore, this strategy is effective in selectively killing cancer cells and sparing the normal ones [60]. It has been suggested that abrogating the ROS-antioxidant mechanism prior to treating the cells with anticancer drugs (oxidative stress inducers) might be a promising therapeutic strategy for the treatment of drug-resistant cancer cells [60]. For example, the administration of a combination therapy of antioxidant inhibitors (e.g. GSH inhibitors) and anticancer drugs that induce cell death by increasing endogenous ROS levels might be beneficial in potentiating cancer cell death mediated by elevated ROS levels [61].

1.5.2.2.2 Modulation of P-gp activity or expression levels

To overcome the drug efflux-mediated chemoresistance problem (particularly the P-gp problem), general approaches have been followed to develop novel compounds that act either as direct inhibitors of P-gp or as P-gp substrates, that can be co-administered with anticancer drugs to compete with them for transport through the P-gp pump [64]. The general aim of these approaches is to enhance intracellular concentrations of anticancer drugs inside cancer cells, consequently enhancing their cytotoxic effects. Although these compounds were able to exert their activity
successfully \textit{in vitro}, none of them can be used clinically. This is due to several reasons related to their high toxicity and to adverse drug reactions which result from their poor potency and their ability to bind with both P-gp and drug metabolising enzymes respectively [64]. The first P-gp inhibitor or modulator detected was verapamil, which also acts as a calcium channel blocker. Verapamil has shown high efficacy in attenuating the multidrug resistance action of P-gp, but this was achieved with micromolar concentrations, which is one million times more than the concentration required to block calcium channels in the cardiac cells, leading to severe cardiotoxicity side effects [74]. Despite the general approaches’ failure to overcome chemoresistance, the urgent need to use anticancer drugs as a curative or palliative treatment drives researchers to develop novel strategies. The use of the liposomal encapsulation strategy as a novel route to deliver doxorubicin (encapsulated within liposome) to the cells holds several advantages over the traditional delivery system. This strategy allows the drug to enter the cells by endocytosis rather than through cells membrane diffusion, in this way reducing the likelihood of being pumped out from the cytoplasm. Additionally, the incorporation of polyethylene glycol (PEG) in the formulation of liposome allows the drug to be slowly released and therefore to reside for a prolonged duration in the circulatory system, thereby decreasing the need to increase the therapeutic dose [74].

The exposure of cancer cells to anticancer drugs inducing stress has been observed to be associated with an increase in the production of P-gp, as a protective mechanism to remove the toxins. Thus, altering intracellular oxidative stress levels in cancer cells might be a promising strategy to modulate the expression of P-gp [64]. On the other hand, it has been demonstrated that treating HepG2 cancer cells with antioxidants results in a reduction in the intracellular ROS levels and upregulation of P-gp levels. Therefore, the elevation of stress levels in the cells through reversing the positive effects of antioxidant enzymes may result in the downregulation of P-gp levels [72].

Moreover, it has been reported that signalling pathways which contribute to controlling cell proliferation, such as phosphatidylinositol-3 kinase (PI-3K), are also involved in regulating the expression and function of P-gp. Therefore, genetic silencing or pharmacological inhibition of the
enzymes mediating these pathways has been found to alter P-gp expression. Examples of these inhibitors are phenethyl isothiocyanate (PEITC), which targets the PI-3K/AKT pathway [75] and procyanidin, which targets nuclear factor-kappa B (NF-κB) and MAPK/ERK pathways [76].

1.5.3 Exploiting the reductive capacity of cancer cells for cancer treatment with bioreductive prodrugs

Bioreductive prodrugs are chemotherapeutic agents specially designed to counteract one of the most common limitations associated with using systemic anticancer drugs - the lack of selectivity toward the cancer cells that consequently leads to developing severe side effects [54]. Bioreductive drugs are inactive under a broad range of physiological conditions. However, in the presence of reductive environments such as reductive enzymes or low-oxygen condition (hypoxia), they are reductively bioactivated generating cytotoxic active species [77]. Therefore, only cancer cells with adequate levels of reductive enzymes will be selectively targeted by anticancer prodrugs, which execute their cytotoxic effects on them following bioactivation [54]. It is worth mentioning that the expression levels of reductive enzymes affect the sensitivity of cancer cells to the anticancer prodrugs. Thus, it is of utmost importance to gain knowledge about the key enzymes that are responsible for the activation of individual prodrugs and the extent of their affinity to them [77].

These prodrugs can be targeted by either endogenous or exogenous reductive enzymes [54]. Examples of endogenous enzymes are NADP(H): quinone oxidoreductase 1 (NQO1) which is a two-electron reductive enzyme and NADPH: cytochrome c (P450) reductase, a one-electron reductive enzyme [77]. Exogenous enzymes can be incorporated into cancer cells through different delivery approaches such as antibodies, genes or virus-directed approaches; of these is nitroreductase [54], which is implicated in activating prodrugs containing nitro groups [77].

Bioreductive prodrugs can be grouped into four classes: quinones, nitroaromatic heterocyclic compounds, aliphatic N-oxide and aromatic N-oxides, which are exemplified by the following leading compounds: mitomycin C (MMC), CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide), AQ4N and tirapazamine respectively [78].
Chapter 1

1.5.3.1 Quinones

Quinones are ubiquitously distributed in nature and are found in plants [79], animals and microorganisms [1]. Human bodies are exposed to exogenous quinones on a daily basis through diet, air as air-borne pollutants or medicine [79], as well as to endogenous quinones such as catecholamine, which may contribute to the degeneration of neuronal tissue [79].

Quinones are reductively metabolised by either one-electron or two-electron reduction reactions. The one-electron reduction of quinones is catalysed by cytochrome P450 reductase or cytochrome b5 reductase, and results in the generation of semiquinones. Under aerobic conditions, semiquinones can be back-oxidised to parent quinones, thereby reducing molecular oxygen during redox-cycling reaction; this consequently results in the formation of ROS [54]. Resultant ROS and semiquinones are responsible for the deleterious effects of quinones through the induction of vital macromolecules’ oxidative damage, including DNA [80]. However, under certain conditions, semiquinones might be further reduced, generating hydroquinones [54]. In comparison, the two-electron reduction is catalysed by Phase I detoxifying enzymes, among these are nicotinamide adenine dinucleotide (NADH): quinone oxidoreductase 1 (NQO1) and dihydronicotinamide riboside (NRH): quinone oxidoreductase 2 (NQO2). The two-electron reduction results in the generation of more stable metabolites called hydroquinones. Hydroquinones subsequently undergo a conjugation reaction with glutathione or glucuronic acid to be excreted out of the biological system as a pathway to detoxify quinones [81], Scheme (1.1). In competition with one-electron reductive enzymes, NQO1 and NQO2 enzymes catalyse the metabolism of quinones to fully-reduced hydroquinones [80]. Notably, it has been found that the predominant level of either group of enzymes is also involved in this competition, and affects the direction of reactions in cells [80]. For example, in cancer cells expressing adequate levels of NQO1, the two-electron reduction reaction of quinones has been observed to be predominant, thereby protecting the cells from the toxic effects of ROS catalysed by cytochrome P450 reductase, and allowing them to proliferate. In contrast, in the normal cells, cytochrome P450 reductase has been found to be predominant, thereby inducing the formation of unstable semiquinones.
On the other hand, two-electron reduction reactions induced by NQO1 might be accompanied by the autoxidation of hydroquinones, resulting in the generation of ROS. The occurrence of autoxidation is dependent on parent quinones’ innate properties and the resultant hydroquinones’ chemical reactivity, which is affected by the nature of their substituent [82]. This indicates that NQO1 may also act as an activating enzyme rather than a detoxifying enzyme. NQO2 has also been shown to exert an activating function in the experiment using menadione as demonstrated in the cellular system where NQO2 gene expression had been silenced [81]. As little is known about NQO2 functions and its endogenous substrates, there is a need to synthesise and evaluate novel inhibitors. These inhibitors can then be used as facilitator tools to identify the nature of NQO2’s catalytic functions [81].
Scheme 1.1 Schematic illustration of quinone metabolism

Quinones undergo reductive metabolism through either one-electron or two-electron reduction reactions. A one-electron reduction is catalysed by cytochrome P450 (CYP450) reductase and results in semiquinone which, upon redox-cycling, produces ROS. A two-electron reduction is catalysed by either NQO1 or NQO2 and, in contrast, results in hydroquinones. Depending on the stability of the resultant hydroquinones, they may either undergo conjugation and consequent excretion or further autoxidation accompanied by the production of ROS. ROS are harmful species, due to their ability to attack the essential biomolecules, including DNA, thus causing oxidative damage which may ultimately lead to the development of cancer. Illustration adapted from (Hussein B. thesis, 2016) [83].

Characterisation studies have been conducted on quinones and have demonstrated their biological activities as anticancer compounds. Quinones derived from natural or artificial sources can be used as cytotoxic compounds for cancer therapy. Among these are MMC and anthracyline-derived doxorubicin [1]. Quinones-containing alkylating agents are a subclass of quinones that consists of two elements; quinone and alkylating groups. The reduction of quinone moiety is crucial for the activation of the alkylating activity of some of these agents, such as MMC and EO9. Although redox
cycling of quinones and resultant ROS might also contribute to the toxicity of these compounds, the alkylation of cellular macromolecules, particularly DNA mediated by alkylating groups, is considered as the main contributor to the compounds' toxicity [84].

1.5.3.1.1 MMC

MMC is a natural antibiotic first isolated by Wakaki et al. from the Streptomyces caespitosus bacteria in 1958 [84]. MMC has been also proved to be an efficient bioreductive alkylating anticancer drug for the treatment of a variety of solid tumours, particularly the following ones: breast [85], superficial bladder cancer [86], pancreatic [87], esophageal [88] and gastric tumours [84]. It is also used in combination with doxorubicin and 5-FU as a relieving treatment for patients with late-stage cancers. [85]. MMC’s alkylating activity is only activated following the reduction of quinones moiety [84] through either a one-electron reduction reaction catalysed by cytochrome P450 reductase, or a two-electron reduction reaction catalysed by NQO1 [85]. Following the reduction activation of MMC, alkylating groups can bind with DNA and form DNA crosslinks [84]. A one-electron reduction results in the generation of reactive semiquinones which can also function as alkylating agent forming DNA cross-links. This reaction confers selectivity to MMC to be active only under hypoxic conditions, as in the well-oxygenated conditions, the semiquinones undergo redox cycling to its parent MMC compound, thereby detoxifying MMC and protecting the normal cells. In contrast, as NQO1 is an oxygen-independent reductase enzyme, MMC can be activated through the two-electron reductive mechanism under different oxygen levels, thereby reducing the hypoxia selectivity of MMC, and allowing the development of systemic side-effects [85].

Notably, it has been demonstrated that NQO2, the second member of the quinone oxidoreductase family, is also implicated in the reductive activation of MMC. This was proved when Chinese hamster ovary cancer cells overexpressing NQO2 showed much higher sensitivity to MMC compared to that of expressing wild-type (WT) basal levels; this sensitivity increases much more when overexpressing cells have been concomitantly treated with NRH to support NQO2 activity. Furthermore, it has been shown that knocking out NQO2 in mouse keratinocyte reduces their responsiveness to MMC significantly, as demonstrated by cytotoxicity assay [89].
1.5.3.1.2 EO9

EO9 is a synthetic bioreductive alkylating agent. Despite its structural similarity to MMC, EO9 reveals a distinct anticancer profile and activation system [90]. Preclinical studies of its effectiveness as an anticancer drug have been conducted on a panel of National Cancer Institute (NCI) human cancer cell lines, and the results demonstrate that EO9 is an efficient treatment for a wide variety of cancer cell lines, and is most effective against those derived from solid tumours such as colon, renal and non-small lung tumours [84, 91]. However, EO9 shows weak antitumour activity in leukaemia [90] and no activity against breast, pancreatic and gastric tumours [92]. Interestingly, treating mice with EO9 has not been associated with myelosuppression [84].

EO9’s DNA-alkylating activity is only activated following the reduction of quinones through one-electron and two-electron reduction reactions [84], and results in the formation of DNA single-strand breaks and cross-links [93]. NQO1 is the primary enzyme responsible for the two-electron reductive activation of EO9 under well-oxygenated conditions. The importance of NQO1 in mediating the activation of EO9 stems from the activation extent of EO9 and subsequent toxicity, which strongly correlate with the intracellular activity levels of NQO1 [84]. Other reductive enzymes that have been shown to be implicated in the two-electron reduction activation of EO9 are xanthine oxidase [93] and NQO2 [89]. High toxicity in Chinese hamster ovary cancer cells (WT and overexpressing-NQO2) has also been detected following treatment with EO9, thought this was to a lesser magnitude when compared to MMC [89].

1.5.3.1.3 Doxorubicin

Doxorubicin (otherwise called adriamycin (ADR) [94]) is a quinone-containing anticancer drug. It is used for the treatment of haematological cancers (e.g. aggressive lymphomas [95] and leukaemia) as well as various types of solid tumours (e.g. breast, ovarian and lung tumours) [1, 96]. However, the cardiotoxicity (e.g. heart failure) associated with chronic administration of ADR limits its utility [96]. It is suggested that the reason behind the development of cardiotoxicity is due to increased oxidative stress caused by ROS [96]. ADR undergoes a one-electron reduction reaction to
generate semiquinones, which subsequently undergo redox cycling resulting in the formation of ROS such as superoxide and hydrogen peroxide [1, 94]. Cardiocytes have been demonstrated to have several detoxifying enzymes involved in scavenging ROS generated during the redox activation of ADR, such as SOD and CAT [96]. NQO1 also participates in protecting cells against ADR cytotoxicity through catalysing the two-electron reduction reaction of ADR, thereby generating less reactive hydroquinones and preventing ADR-induced oxidative stress [96].

ADR’s anticancer activity has been mediated through multiple mechanisms, including the alkylation of DNA, cross-linking of DNA, inhibition of topoisomerase II leading to DNA damage [95], and the formation of ROS resulting in DNA damage and lipid peroxidation [78]. Cytotoxicity associated with reductive ADR metabolism is developed from the formation of semiquinones and ROS. Under hypoxic conditions, semiquinones can exert their cytotoxicity through alkylating cellular macromolecules. Under aerobic conditions, semiquinones undergo redox-cycling to form ROS, which execute their cytotoxicity through oxidative damage [78]. It is worth mentioning that ROS have dual roles in mediating ADR toxicity [95], and these are affected by the intracellular concentrations of ROS. Low ROS concentrations allows them to act as signalling molecules to activate kinases and transcription factors involved in controlling cell cycle progression and pro-apoptotic pathways. In the presence of a high concentration, ROS can act as an oxidising agent, thereby inducing oxidative damage [95]. Although NQO1’s detoxifying activity has been shown to be important in cytoprotection against ADR, it also confers resistance to ADR, thereby reducing its anticancer activity. The pharmacological inhibition of NQO1 via dicoumarol has been proved to be beneficial in enhancing ADR’s cytotoxicity [97]. Furthermore, NQO2 -an NQO1 analogue- is also involved in conferring resistance to ADR, but through altering P-gp expression levels and ADR uptake via an unknown mechanism. The inhibition of NQO2 either genetically or pharmacologically has been associated with a reduction in P-gp expression levels, and an increase in the uptake of ADR [98, 99].
1.5.3.2 Nitroaromatic

1.5.3.2.1 CB1954

CB1954 is an anticancer prodrug that demonstrates a substantial increase in its cytotoxicity upon enzymatic bioactivation, to generate a bifunctional alkylating agent that is able to produce DNA-interstrand crosslinks [100]. CB1954 was first found to be a highly effective treatment for rat Walker 256 tumours. The cytotoxicity of CB1954 in rat tumours is induced by rat NQO1, which catalyses its bioreductive activation. Despite its high efficacy in treating rat tumours, CB1954 shows much less toxicity in human cancer cells expressing high levels of human NQO1 [100]. This difference in catalytic activity between rat and human NQO1 has been attributed to the difference in amino acid at position 104; tyrosine in the rat enzyme but glutamine in the human enzyme [101]. Interestingly, human NQO2 has been found to be highly efficient at catalysing the bioreductive activation of CB1954, through a four-electron nitroreduction mechanism. This only occurs in the presence of exogenous NRH which is required to support the latent catalytic activity of NQO2 [100], see Figure (1.4). The catalytic activity of NQO2 and activation of CB1954 has been demonstrated to be 3000-fold higher than that of human NQO1 [100, 102, 103]. This is attributed to the difference in the amino acid residue at position 104 which is tyrosine in human NQO2, and glutamine in human NQO1, indicating that the human NQO2 is completely identical to rat NQO1 in this particular region [100]. Resolving the crystal structures of NQO1 and NQO2 demonstrates that NQO2 has a unique metal binding site which is absent in NQO1. The presence of metal has been suggested to be central and supportive for the redox activity of NQO2, thereby making NQO2 more efficient at reducing CB1954 than NQO1 [100].

CB1954 has been recently renamed as tretazicar [104]. Tretazicar is efficiently activated by NQO2 when it is co-administrated with caricotamide (NRH analogue). Caricotamide is the trade name of EP0152R. This combination therapy has been used in Phase I clinical trials within the United Kingdom. The results drawn from it emphasise the importance of the co-administration of the drug with the NRH analogue for maximum bioreductive activation and subsequent toxicity [104].
Tretazicar plus caricotamide therapy can be used as a selective treatment to target cancer cells with high expression levels of NQO2, as found in bladder and ovarian tumours [104].

CB1954 has been used as a reliable approach to evaluate the functional activity of novel compounds inhibiting NQO2 in vitro. The reduction extent in the cytotoxicity of CB1954 in the presence of inhibitors can be used as an indirect measure of their inhibitory potency against cellular NQO2 [105]; pharmacological inhibition of NQO2’s catalytic activity suppresses the bioactivation of CB1954 and its subsequent cytotoxicity.

**Figure 1.4 Nitroreduction activation of CB1954**

CB1954 prodrug is inactive in its original form. After the 4-nitroreduction reaction, the 4-nitro group is converted to a 4-nitrohydroxyl bifunctional alkylating group. This reaction is induced by EP0152R which in turn supports the nitroreductive activity of NQO2 to catalyse the activation of the CB1954 [106]. Adapted from (Middleton et al., 2010).
1.6 NAD(P)H: quinone oxidoreductase gene family

The history of NAD(P)H: quinone oxidoreductase (NQO) gene family can be traced back to billions of years ago, when the appearance of this family was necessary to protect unicellular organisms against the destructive effects of quinones [107]. The human NQO gene family comprises of two genes that encode two flavoenzymes, including NQO1 and NQO2. These enzymes catalyse two-electron reduction of quinones protecting the living cells from deleterious effects of ROS [107].

1.6.1 NQO1

NQO1 was first detected in 1958 by Ernster and co-workers [108]. It is considered to be “the first member of the NQO family”. NQO1 was previously known as DT-diaphorase and quinone reductase 1 (QR1), and has recently been identified as vitamin K reductase [107].

The human NQO1 gene is located on chromosome 16q22 and encodes a cytosolic flavoprotein [80, 107]. This flavoprotein comprises of two identical monomers, each one is composed of 273 amino acids and one FAD. Each monomer is divided into two parts: the N-terminus (1-220 amino acid residues) which is the main catalytic part, and the C-terminus (221-273 amino acid residues) [109]. Figure (1.5) illustrates the crystal structure of NQO1.

Figure 1.5 The crystal structure of human NQO1 homodimer (taken from Asher et al., 2006) [110].
NQO1 constitutes part of the oxidative stress-induced cellular defence system. It is upregulated in the presence of various inducers such as xenobiotics, oxidants and radiation. NQO1 acts as a detoxifying enzyme that reduces quinones and structurally related compounds via the two-electron transfer mechanism, leading to the formation of nonreactive hydroquinones instead of semiquinones and ROS [80]. Thus, NQO1 plays important roles in protecting the cells from oxidative damage accompanied by the metabolism of quinones and other carcinogens [81]. NQO1 is involved in the reduction of menadione (vitamin K3) to hydroquinone, thereby protecting liver cells from hepatotoxicity induced by the vitamin K3 semiquinone and ROS [111]. Furthermore, it has been suggested that the detoxifying role of NQO1 has potential in protecting the dopaminergic neurons against the destructive effects of dopamine quinone metabolites, therefore preventing the development of neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases (PD and AD respectively). NQO1 expression levels have been found to be upregulated in patients with the aforementioned diseases. This occurs as an adaptive mechanism to the increase levels of dopamine toxic metabolites, in order to prevent the degeneration of neurons [112].

The cytoprotective roles of NQO1 are more than that of just being a detoxifying enzyme; NQO1 can protect cells from oxidative stress through the stabilisation of tumour suppressor proteins, including p53. The physical binding of NQO1 to p53, protects p53 from degradation mediated by the 20S proteasome [112].

The presence of high levels of NQO1 in cancer cells along with its reductive ability for quinone-containing compounds make NQO1 a potential target in developing highly selective bioreductive anticancer drugs [113]. Examples of NQO1 activated prodrugs are MMC and B-lapachone. B-lapachone is a naturally-occurring derivative of quinone which exerts high cytotoxicity in breast and prostate cancer cells overexpressing NQO1. NQO1 mediates the anticancer activity of B-lapachone by inducing the production of ROS, through generating unstable hydroquinones that undergo redox-cycling, producing ROS [113].
1.6.2 NQO2

NQO2 was first identified in 1961 by Liao and Williams-Ashman as a cytosolic FAD-containing protein that catalyses the oxidation of N-ribosyl and N-alkyl dihydronicotinamides instead of NAD(P)H, NADH or NMNH, to induce its reductive activity [81]. NQO2 has been described as the “long-forgotten flavoenzyme” [114], since it was rediscovered after more than 30 years by Jaiswal et al. while cloning and sequencing human NQO1 genes [81]. They found that NQO2 is an analogue of NQO1 [115], and named quinone reductase 2 (QR2) [81].

1.6.3 General features of NQO2 in comparison with NQO1

1.6.3.1 Structure of NQO2

NQO2 is a homodimer metalloflavoprotein [114]. Each monomer is composed of 230 amino acid residues and one FAD. Each monomer consists of two parts: the N-terminal catalytic part (1-220 amino acid residues) and the C-terminal part (221-230 amino acid residues) [81, 116]. Figure (1.6) represents a three-dimensional model of NQO2. NQO2 is known as a truncated homologue of NQO1 [81]; NQO2’s C-terminus is shorter than that of NQO1 by 43 amino acid residues [102, 107]. The remaining 10 amino acid residues constituting NQO2’s C-terminus are completely different from NQO1’s corresponding amino acids [114, 116]. Despite these differences, human NQO2 cDNA and protein show 54% and 49% similarity to that of human NQO1 respectively [80, 89]. Therefore, NQO2 is believed to be an isoenzyme of NQO1 [102, 103].
Figure 1.6 The crystal structure of human NQO2 homodimer

The overall structure of the NQO2 homodimer with imatinib is represented in this three-dimensional structure. The monomers are illustrated in orange and green, while the yellow colour indicates the FAD. Imatinib is represented as a blue chain and shown as imatinib-bound NQO2 [117]. Adapted from (Winger et al., 2009).

The NQO2 dimer includes two identical catalytic binding sites. Each catalytic site is a large cavity built from one FAD isolloxazine ring that constitutes the bottom, in addition to this are amino acid residues that line its internal surface. In comparison with NQO1, the NQO2 binding cavity is larger and more lipophilic [81, 114, 118]. This can be explained by the noticeable differences in particular amino acid residues lining their cavities; the Phe$^{126}$, Ile$^{128}$ and Phe$^{131}$ amino acids in NQO2 are replaced by Tyr$^{126}$, Tyr$^{128}$ and Met$^{131}$ in NQO1. These changes ultimately affect the substrate and inhibitor specificity towards both enzymes [114].

NQO2 selectively utilises N-ribosyl and N-alkyl dihydronicotinamides as the electron donors instead of NAD(P)H or NADH, which are preferably used by NQO1 [81, 109, 116]. Figure (1.7) illustrates the structures of NRH and NAD(P)H, the typical co-factors utilised by NQO2 and NQO1 respectively. It has been observed that the amino acid residues involved in the interactions between NQO1 and co-substrates (electron donors) are either mutated in the N-terminus or truncated from the C-terminus of NQO2 [81]. This may explain NQO2’s inability to interact with NQO1’s electron donors [81, 116].
In contrast to NQO1, NQO2 has been found to have a metal binding site that might be involved in its redox activity, explaining in part the differences in the catalytic activity between these enzymes (NQO1 and 2) [100]. The location of the metal site on the surface of NQO2 suggests that it is involved in the electron transfer mechanism rather than playing a role in structural stability [114].

![Figure 1.7 The structure of (A) NRH and (B) NAD(P)H](image)

1.6.3.2 Expression and distribution of NQO2

Unlike NQO1 which is expressed in almost all types of tissue, NQO2 expression is limited to specific tissues at widely varying levels. This indicates that NQO2 functions according to tissue type [114]. NQO2 expression is highest in skeletal muscle but is completely absent in placenta. NQO2 is also expressed in other tissues, including the liver and lung but at moderate levels [80]. Interestingly, red blood cells show modest levels of NQO2 whereas NQO1 is absent [81]. Moreover, NQO2 has also been detected in various types of tumour cells but at different levels [104-106]. Xenobiotics and antioxidants have been found to be among the inducers of NQO2 expression [80].

1.6.3.3 Genetic polymorphism of NQO2

The locus of the human NQO2 gene on chromosome 6p25 is highly polymorphic [80]. The polymorphism of NQO2 includes the insertion or deletion of 29 base pair of nucleotides within the
promoter region. The nature of that sequences affects the expression and subsequent activity of NQO2, and consequently may result in the development of diseases such as neurodegenerative diseases [81]. Several studies have hypothesised that the expression of the NQO2 gene with a 29 base pair insertion (I29) at the promoter might be regulated by both Sp1 and Sp3 transcription factors [119]. The former factor is an activator of NQO2 gene expression, whereas the latter is a repressor [120]. The presence of these factors generates a state of equilibrium between the activation and repression of NQO2 gene transcription [119]. The expression of NQO2 is upregulated when the NQO2 gene contains deletions (D) at the allele promoter site. The upregulation is attributable to the deletion of the Sp3 binding site from the NQO2’s D allele promoter, leading to the reduction in the repression of the NQO2 gene expression, subsequently increasing NQO2 levels [119]. In the same manner, it has been speculated that the deletion of sequences related to Sp1 binding sites from the NQO2 gene results in decreased expression of the NQO2 gene [121]. The NQO2 polymorphism in the form of I29 occurs when the Sp3 binding site is introduced into the NQO2 promoter region. This has been demonstrated to be implicated in the development of PD [81, 120] and enhances susceptibility to breast carcinogenesis [122].

1.6.3.4 Reaction mechanism of NQO1 and NQO2 enzymes

Since NQO1 utilises the same binding site for substrate and electron donors, it has been suggested that NQO1 relies on a Ping-Pong mechanism to catalyse the reductive metabolism of its own substrates [114], Figure (1.8). As NQO2 shares this characteristic with NQO1, with their binding sites being very similar, it has been proposed that NQO2 also uses the Ping-Pong mechanism to mediate its catalytic activity [114, 123]. The mechanism includes two steps of direct transfer of hydrides. Initially, the hydrides move from the electron donors, namely co-factors (e.g. NADH or NRH) to FAD. Thereafter, then they move from FADH2 to the electron acceptor substrates (e.g. quinones) [118]. The binding sites of NQO1 and NQO2 are occupied alternatively by co-factor and substrates; the co-factor should first release to allow the substrate to bind, and allow the reduction reaction to proceed to form reductive-related metabolites [118].
Figure 1.8 Schematic representation of the Ping-Pong mechanism mediated by NQO2

NQO2 relies on the Ping-Pong mechanism for its reductive activity; NQO2 utilizes the same catalytic site for binding with co-factor (e.g. NRH) and substrate (e.g. quinones). NRH binds and leaves the site in its oxidised form. The binding site is then sequentially occupied by quinones (the NQO2 substrate) which accept the hydrides from reduced FAD, and become fully reduced (hydroquinone form) before leaving the site [118]. Scheme is adapted from (Scott K. thesis, 2010, [124]).

These enzymes have the unique ability to generate fully reduced metabolites of quinones (hydroquinones) by transferring two-electrons simultaneously to quinones, thereby avoiding the formation of semiquinones [118]. Although the transfer of one electron may occur in two sequential steps, there is no evidence indicating the presence of a reactive intermediate [116]. This ability facilitates the understanding of the biological function of NQO1 and NQO2 as detoxifying enzymes that protect the cells from the deleterious effects of ROS [118]. It is worth mentioning that the amino acids residues contributing to the electron transfer process in NQO1 are Gly\textsuperscript{149}, Tyr\textsuperscript{155} and His\textsuperscript{161}. These residues are also conserved in NQO2 with the exception of His\textsuperscript{161} which is replaced by Asn\textsuperscript{161} in NQO2 [81].

1.6.3.5 NQO2 inhibitors

The role of NQO2 in the development of various human diseases such as cancer, malaria and neurodegeneration has been suggested, and has attracted attention due to findings that demonstrate the ability of certain drugs used for the treatment of malaria, leukaemia and PD to inhibit NQO2 activity. Examples of these drugs are chloroquine (anti-malarial), imatinib (anti-leukaemia) and melatonin (anti-oxidant neurohormone) [125]. Furthermore, the importance of
NQO2 as a therapeutic target was highlighted when resveratrol, which is a known anticancer and chemopreventive agent targeting NQO2 [126]. Thus, NQO2 inhibitors can be used as molecular tools to understand and identify the nature of the relationship between NQO2 activity and the pathogenesis of different diseases [115].

Notably, NQO2 shows high resistance to the conventional inhibitors of NQO1 such as dicoumarol, cibacron blue and phenindone [80, 81, 103]. Benz(a)anthracene and its derivatives were among the first discovered inhibitors of NQO2 [127]. More recently, a wide range of novel NQO2 inhibitors have been discovered, namely flavones (e.g. quercetin and benzo(a)pyrene [80, 89]), indolequinones [125], acridines (e.g. 9-aminoacridine), quinolines, elipticines, polyaromatic and furanylamidines (e.g. NSC17602) compounds [105]. The structural diversity of NQO2 inhibitors demonstrates the flexibility of NQO2’s catalytic site to bind and occupy a variety of compounds. This also indicates the presence of many different biological pathways wherein NQO2 can act as a mediator.

1.6.3.5.1 Resveratrol

Resveratrol is a natural compound present in red grapes, peanuts and blueberries [128]. It has many diverse biological attributes including anti-oxidative, anti-inflammatory, anti-cancer and neuro-protective activity [128, 129], Figure (1.9). The diversity in its biological properties has been attributed to its ability to interact with cellular receptors including estrogen receptors, as well as having interactions with enzymes including DNA polymerases [126].

The anti-cancer function of resveratrol has been suggested to result from its ability to block multiple stages of the carcinogenesis process. Resveratrol’s exact mechanisms of action have not yet been fully understood. However, multiple hypotheses have emerged which suggest that resveratrol can function as an antioxidant, apoptotic inducer, cell cycle regulator and inhibitor of several kinases [130]. In order to identify the resveratrol target proteins that possibly mediate its biological functions, affinity chromatography has been used. Among the proteins detected, NQO2 has been purified and confirmed to be one of the resveratrol target proteins with high binding affinity [126].
Resveratrol is recognised as a classical inhibitor of NQO2 [105]. As the reductive activity of NQO2 may cause the generation of toxic metabolites, the inhibition of NQO2 can confer protection against carcinogenesis [131]. It has been proposed that the antioxidant activity of resveratrol might be mediated by the inhibition of the NQO2 toxifying capacity, thereby protecting the human body from increasing levels of ROS [81]. Increased resistance to the cytotoxicity of quinones and a significant reduction in cell proliferation has been observed after treating K562 cells possessing high basal levels of NQO2 with resveratrol. The same observations were found after silencing the NQO2 gene in K562 cells, indicating the involvement of NQO2 in anticancer properties of resveratrol [130]. However, the low selectivity of resveratrol in the presence of multiple targets makes it a non-powerful tool to identify the physiological roles of NQO2 [105].

![Figure 1.9 The structure of resveratrol, a classical NQO2 inhibitor.](image)

### 1.6.3.5.2 Quercetin

Quercetin is a natural compound belonging to the flavone family [81] and can be found in all products made from plants [132]. Figure (1.10). According to enzymatic activity assays, quercetin has been demonstrated to be the most potent inhibitor of NQO2 when compared to other flavones (e.g. galangin and chrysin), with an IC$_{50}$ value of 80nM [102]. Quercetin can exert its inhibitory activity against NQO2 by competing with NRH to occupy the catalytic site [102]. Quercetin shows weak inhibitory potency to NQO1 [133]. This flavone has anticancer activity and results in growth suppression for ovarian cancer cells due to its ability to arrest the cell cycle at different phases [14].
Figure 1.10 The structure of quercetin

1.6.3.5.3 Melatonin

Melatonin is a neurohormone synthesised in and secreted from the pineal gland [134]. It can exert its activities on the body using an unknown number of receptors. MT1, MT2 and MT3 receptors exhibit high binding affinity for melatonin [123, 134]. The Melatonin-binding site 3 (MT3) has been found to be a homologue of NQO2 [134]. This hypothesis has been supported by several pieces of evidence: Firstly, 57 out of 71 amino acids of MT3 are similar to that of NQO2. Secondly, the association and dissociation kinetics of MT3 ligands resemble the ligand exchange kinetics of enzymes [134]. Thirdly, it has been noticed that NQO2 deficient mice show no response to \(^{125}\text{I}\)-iodo-5-methoxycarbonylamo-N-acetyltryptamine \((^{125}\text{I})\text{-MCA-NAT})\), which is a highly specific radio-ligand of MT3 [123]. Lastly, MT3 has been found to be absent in the tissue of NQO2 deficient mice. These facts support the notion that NQO2 is actually MT3 [81]. Melatonin has been demonstrated to be a functionally active inhibitor of NQO2 at a 1µM concentration [135]. This indicates that NQO2 may play a role in the antioxidant activity of melatonin [107].

It is worth mentioning that it is less than ideal to use melatonin as a molecular tool to identify the physiological functions of NQO2, since melatonin has different pharmacological activities [136].
1.6.3.5.4 9-aminoacridine

9-aminoacridine (9AA) is a synthetic compound which belongs to the acridine family, Figure (1.12). 9AA shows strong inhibitory activity against cellular NQO2 at a concentration of less than 1µM [105]. 9AA can also function as an inhibitor of NF-κB transcriptional activity. The activity of NF-κB induced by TNF and NRH (NQO2 co-factor) has been demonstrated to be attenuated in MDA-MB-468 cells upon exposure to 9AA, proving the functional activity of 9AA as a modulator of NF-κB activity. Thus, it has been suggested that there might be a relationship between NQO2 inhibition and NF-κB activity. Genetic silencing of NQO2 in MDA-MB-468 is also accompanied by a significant reduction in the NF-κB activity, supporting the notion that NQO2 may play a potential role in regulating NF-κB activity. This finding also indicates that the alterations in NF-κB activity accompanied by 9AA treatment might be mediated through the pharmacological modulation of NQO2 activity [105].

As modulating NF-κB activity has therapeutic benefits against cancer progression, targeting NQO2 may be an efficient approach in fulfilling this and consequently eradicating the cancer [105]. 9AA's anticancer activity has been also demonstrated in renal cell carcinoma. This occurs through its simultaneous action at inhibiting basal and inducible activities of NF-κB, and activating p53 function, thereby inducing cell death [137]. The involvement of NQO2 in mediating the activity of 9AA has also been proposed in renal cell carcinoma if they can demonstrate that NQO2 level in these cells is high [105].
According to virtual screening studies carried out on NCI compounds, furan-amidines have been identified as novel ligands for NQO2 with some inhibitory activity [105]. Series of furan-amidine analogues have been synthesised and evaluated as functional inhibitors of NQO2. Among these analogues are non-symmetrical furan-amidines that show high inhibitory potency against NQO2, and this has been achieved within nanomolar concentrations. The non-symmetrical furan-amidines possessing meta and para-nitro substituents on the aromatic ring are amongst the most potent inhibitors, with IC$_{50}$ values of around 15nM, see Figure (1.13). Although the non-symmetrical furan-amidines derivatives show low binding affinity toward DNA and therefore low DNA intercalation, they have high toxic effects on breast cancer cell lines, whereby NQO2 levels are not involved in their cytotoxicity [138].

In addition to the inhibition of NQO2, non-symmetrical furan-amidines derivatives also demonstrate high toxicity against the growth of the *Plasmodium* parasite, this also occurs within nanomolar range. Therefore, these compounds have been suggested as an effective treatment for malarial disease once further optimisation has been carried out. The molecular targets of the non-symmetrical furan-amidines derivatives in the parasite cells are still unknown [138].
Figure 1.13 The structure of non-symmetrical p-nitrofuran amidine analogue

1.6.3.6 NQO2 in association with cancer

The biological roles of NQO2 in carcinogenesis are still ambiguous as several controversial assumptions regarding it have been made. It has been suggested that NQO2 plays important roles in providing protection for mice against the development of myeloid hyperplasia of bone marrow [139], skin carcinogenesis [140] and lymphoma upon exposure to carcinogens [141]. The down-regulation of NQO2 in mice has been found to be associated with the development of myeloid hyperplasia, as a result of the substantial reduction in the apoptosis of myeloid cells [139]. Also, the lack of NQO2 in mice enhances their susceptibility to skin carcinogens including benzo(a)pyrene and 7,12 dimethylbenz(a)athracene, thereby promoting the development of skin cancer [140]. Furthermore, it has been observed that exposing mice deficient in NQO2 to γ-radiation results in the development of multiple lymphomas in different tissue, including the bone marrow, spleen, thymus and lymph nodes [141]. These findings therefore emphasise the potential role of NQO2 as a protective enzyme against cancer development. In contrast, it has been demonstrated that the genetic silencing of NQO2 in CWR22RV1 prostate cancer cells leads to a significant reduction in cell proliferation and cell cycle progression. The same finding has also been obtained upon the pharmacological inhibition of NQO2 in these cells, indicating the possible role of NQO2 in mediating the progression of prostate cancer instead [142]. The association between NQO2 and cancer development has also been demonstrated in other types of solid tumours, including breast and papillary thyroid tumours. The association between NQO2 and cancer might be linked to the modulation of cellular redox status, the modulation of kinases that are responsible for controlling cell proliferation, or the modulation of other factors important for regulating immune responses.
However, the exact mechanisms through which NQO2 mediates carcinogenesis still need to be addressed.

1.6.3.6.1 The role of NQO2 in regulating cellular oxidative stress

NQO2’s potential roles in breast cancer pathogenesis have been suggested to be linked to its function of detoxifying estrogens and their metabolites [143]. It has been hypothesised that estrogens and their derived quinone or semiquinone metabolites are carcinogenic substances for breast tissues [143], and that cumulative exposure to them enhances susceptibility to developing breast cancer [144]. Increases in the levels of estrogens and their metabolites in the female body occur through reductive metabolism [143]. The metabolism of estrogens and their derived quinones is catalysed by Phase I and Phase II enzymes. The activity levels of Phase I enzymes are important in protecting the breast tissues from oxidative damage induced by estrogen toxic metabolites [143]. NQO1 and NQO2 are considered as Phase I detoxification enzymes which are involved in the reduction of quinones derivatives [143]. The role of NQO1 in providing protection against oxidative stress and preventing breast carcinogenesis has been reported [145]. The involvement of NQO2 in breast cancer has also been demonstrated through its participatory role in mediating the reduction of estrogen quinones at an interestingly higher rate than that of NQO1 [143]. Gaikwad et al. demonstrate that estrogen-derived quinones are endogenous biological substrates of NQO2 [146].

Furthermore, the role of NQO2 as a detoxifying enzyme in thyroid glands has also been explained. NQO2 confers protection to the follicle cells of thyroid glands from oxidative stress induced by iodine and irradiation, thereby preventing the development of papillary thyroid cancer [122].

On the other hand, several studies have indicated the role of NQO2 as an activating enzyme for oxidative stress rather than as a detoxifying enzyme [81, 140, 147]. NQO2 has been found to act as vitamin K reductase [102]. NQO2 catalyses the reductive activation of menadione causing hepatotoxicity, as demonstrated in mice-expressing WT of NQO2 [120]. However, NQO2-null mice show less hepatic damage following exposure to menadione [89, 139]. This was not the case with
NQO1-null mice which become more sensitive to menadione inducing hepatic damage with the suppression of NQO1 levels [130]. Similarly, the pharmacological inhibition of NQO2 in mice using resveratrol has also been associated with a reduction in menadione toxicity [81]. These findings therefore support the idea regarding the function of NQO2 as an activator of oxidative stress [81]. As NQO2 has been demonstrated to have a melatonin binding site, melatonin’s antioxidant activity has been suggested to be mediated by NQO2 [107].

1.6.3.6.2 The role of NQO2 in regulating cyclin D1 turnover

1.6.3.6.2.1 Cell cycle and its key regulators

The cell-division cycle in mammalian cells includes cascades of events that ultimately result in the division of parental cells into two daughter cells having identical genetic composition [148]. It comprises of two major phases termed interphase and mitotic (M). The interphase is divided into three distinct phases, namely gap1 (G1), synthesis (S) and gap2 (G2) phases [149], as depicted in Figure (1.14).
The cell cycle is divided into two major phases: the interphase and mitotic (M). Cells spend a long time in interphase to prepare themselves for cell division. Interphase is divided into three distinct phases: gap1 (G1), synthesis (S) and gap2 (G2). During G1, cells grow and synthesize critical proteins and enzymes for the S phase, where DNA is duplicated. During the G2 phase, the cells prepare essential proteins required for mitosis. The progression through the cell cycle phases is regulated by multiple factors called cyclins and their binding partners called CDKs. CDKs remain inactive until they form complexes with cyclins. Cyclin D1/CDK4 or CDK6 complexes are required to induce the progression of cells through the G1 phase, and for the synthesis of important proteins involved in S phase initiation. The formation of cyclin E/CDK2 complexes facilitates the entry to the S phase, while the formation of cyclin A/CDK2 is required to induce S phase progression. To initiate mitosis, cyclin B/CDK1 complexes are formed, thereby inducing cell division [150]. Adapted from (Dubravka et al., 2000)

The cell cycle is a tightly regulated process that requires the sequential events of formation, activation and inactivation of critical regulators including cyclins and their binding partner, cyclin-dependent kinases (CDKs) [151]. Cyclins are a family of proteins that contribute to controlling cell cycle progression through the activation of a family of protein kinases called CDKs [152]. During the G1 phase, the cells grow and synthesize proteins in preparation for the S phase [153]. The cells’ entry and progression through the G1/S phase requires the formation of cyclin D1/CDK4/6 complexes, which in turn phosphorylates Rb [151] at serine and threonine amino acid residues [150]. Figure (1.15). The status of Rb phosphorylation influences the activity of E2F and
subsequently, the cells’ ability to progress through the G1/S phase. Rb acts as a growth suppressor protein if it is present in an active hypophosphorylated state. This state sequesters the transcriptional activator E2F. Once Rb becomes hyperphosphorylated (inactive), E2F releases and activates the transcription of several proteins such as cyclin E, cyclin A and CDK2, which are essential for entry into the S phase and for DNA synthesis [150]. Binding cyclin E with the G1 phase CDK2 is important in facilitating the transition of cells from the G1 to S phase (i.e. for S phase initiation), where DNA synthesis occurs. This complex is no longer needed when the cells enter into S phase [150]. Therefore, new complexes of cyclin A and S phase CDK2 are formed to promote the progression of cells through the S phase [150, 151]. During the G2 phase, the cells grow and synthesise proteins in preparation for the M phase [153]. The progression of cells from the G2 to M phase is triggered by the activation of cyclin B/CDK1, which is a critical complex in initiating mitosis [152]. During M phase, the nucleus divides, followed by cell division [153]. Aberrant activation and deregulated expression of cell cycle regulators occur in cancer, leading to uncontrolled cell proliferation [151].

Figure 1.15 Phosphorylation inactivation of Rb induces cell cycle progression through the G1/S phase

To induce the transition of cells from G1 to S phase, active CDK4/6, in complex with cyclin D1, phosphorylates Rb to release E2F transcription factor. E2F therefore becomes active to induce the transcription of crucial proteins for S phase initiation and progression, such as cyclin E and CDK2 [154]. Adapted from (Pérez-Galán et al., 2011).
Cyclin D1 proto-oncogene is a critical regulator for the cells to progress through the cell cycle phase (G1/S phase) via the activation of CDK4/6 [155, 156]. It has been reported that cyclin D1 has other biological functions in the cells which are unrelated to its catalytic activity. It can, for example, act as a transcriptional regulator of several genes via its interactions with transcription factors. These genes are involved in promoting cell proliferation, migration and invasion as well as in DNA replication and DNA damage response [156].

1.6.3.6.2.3 Regulation of cyclin D1 levels via the PI-3K/AKT/GSK-3β pathway

It has been demonstrated that cyclin D1’s expression levels and activity show a pattern of fluctuation through cell cycle phases, and this is essential for the progression of active cell cycles [157]. The regulation of cyclin D1 expression occurs at three basic levels; transcription, translation and protein stability. Protein stability is regulated by a sequential signalling cascade mediated by the PI-3K/AKT/GSK-3β (glycogen synthase kinase-3β) pathway [158]. The cyclin D1 stability is triggered when PI-3K and its downstream target AKT are activated to mediate the inhibitory phosphorylation of GSK-3β, Figure (1.16). As GSK-3β is responsible for the proteolytic phosphorylation of cyclin D1 at Thr286, the inhibition of GSK-3β catalytic activity results in the stabilisation of cyclin D1 during the G1 phase [158]. In other words, the state of GSK-3β affects its activity and subsequently the levels of cyclin D1; GSK-3β is active when non-phosphorylated, but becomes inactive when phosphorylated [157]. Cyclin D1 degradation mediated by GSK-3β is a tightly regulated process, as it affects cells’ ability to re-enter and progress through the cell cycle [158, 159].
Regulation of cyclin D1 turnover via the PI-3K/AKT/GSK-3β pathway

GSK-3β kinase is responsible for the degradation of cyclin D1 levels during cell cycle progression. The activity of GSK-3β is regulated by upstream factors including AKT and PI-3K. PI-3K is responsible for the phosphorylation activation of AKT and subsequent inactivation of GSK-3β. The phosphorylation state of GSK-3β affects its activity. GSK-3β when non-phosphorylated is able to phosphorylate cyclin D1 at Thr286, and induce its proteolysis. Nuclear cyclin D1 either in free or bound form with CDK4/CDK6 undergoes proteolytic phosphorylation by GSK-3β. This process induces the exportation of cyclin D1 from the nucleus to the cytoplasm. In the cytoplasm, phosphorylated cyclin D1 is degraded by 26S proteasome [154]. Adapted from (Pérez-Galán et al., 2011).

Cyclin D1 levels fluctuate while cells progress through the G1 phase; these levels are low at the start, and continuously increase to reach maximal levels at the G1/S phase boundary, where they then begin to decline. The degradation of cyclin D1 during the G1 phase impairs the progression of cells through the G1/S phase, but becomes imperative in late G1 phase, in allowing DNA synthesis and S phase initiation [150, 160]. It has been reported that the increased levels of cyclin D1 in fibroblast cells suppress their entry into the S phase. This is because cyclin D1 inhibits DNA synthesis when it stays bound to the proliferating cell nuclear antigen (PCNA) and CDK2, the main participants of this process [160]. Cyclin D1 turnover during S phase is also mediated by GSK-3β, which in turn phosphorylates cyclin D1 at Thr286 and consequently triggers proteasomal degradation [155, 157, 159]. The activity of GSK-3β during the S phase has been reported to also be regulated by PI-3K/AKT. Thus, it is expected that, during S phase, the inactivation...
phosphorylation activity of PI-3K/AKT for GSK-3β is suppressed, leading to activating the GSK-3β activity-mediated proteolytic phosphorylation of cyclin D1, thereby declining cyclin D1 levels and allowing the synthesis of DNA [157]. GSK-3β action is also involved in redistributing cyclin D1 from the nucleus to the cytoplasm. The exportation of cyclin D1 and subsequent accumulation in the cytoplasm occurs during the cyclin D1 phosphorylation process in order to induce its proteolysis degradation. In contrast, GSK-3β levels have been detected to be low in the cytoplasm but high in the nucleus [159].

1.6.3.6.2.4 Cyclin D1 as a therapeutic target in cancer

The overexpression of cyclin D1 and the consequent aberrant activation of CDK4/6, have been widely documented to be involved in uncontrolled cell proliferation that is independent from mitogenic signals—the most common hallmark of cancer [156]. Multiple mechanisms have been shown to be implicated in the overexpression of cyclin D1 and consequently in the development of cancer. These mechanisms are genetic mutations, post-transcriptional regulation and post-translational regulation of protein expression and stability, respectively [158]. The dysregulation in cyclin D1 degradation results in increased cyclin D1 levels, which contributes to the development and progression of cancer [155, 159]. Several pathways and factors have been reported to be involved in cyclin D1 dysregulation. The occurrence of mutations or deletion on cyclin D1 and other genes mediating its degradation (such as E3 ligase) has been found to impair the phosphorylation of cyclin D1 and proteolysis efficiency, resulting in increased cyclin D1 stability, as demonstrated in different types of cancers such as esophageal and endometrial cancer [158]. Moreover, the constitutive activation of the PI-3K/AKT pathway associated with loss of PTEN (phosphatase and tensin homologue), a negative PI-3K regulator, has also been found to contribute to increasing cyclin D1’s stability in mantle cells lymphoma and prostate cancer cells, thereby enhancing cell proliferation [161, 162]. Therefore, inducing cyclin D1 degradation might be key to preventing and treating cancer; this can be achieved via pharmacological intervention [155].
1.6.3.6.2.5 Drugs induced cyclin D1 turnover

As the overexpression of cyclin D1 is responsible for the development and progression of cancer, cyclin D1 becomes an attractive target for therapeutic intervention [155]. It is difficult to directly target cyclin D1 due to its short half-life. Thus, there is a strong need to establish novel therapeutic agents with alternative mechanisms of action to mediate cyclin D1 turnover. The action of these agents is selectively directed to modulate the catalytic activity of GSK-3β. This is because GSK-3β is responsible for producing a degradable, phosphorylated form of cyclin D1. Thus, GSK-3β is also a promising therapeutic target that enables the impairment of increased cyclin D1 levels’ oncogenic capacity, particularly in the cells overexpressing cyclin D1 upon modulating its activity [157, 163].

Other approaches have also been used to induce cyclin D1 degradation; these rely not only on targeting the phosphorylation of cyclin D1 at Thr286, but also on modulating pathways and factors-mediating the control of cyclin D1 levels [155]. There appear to be several conventional and natural anticancer drugs that rely on these alternative strategies to induce cyclin D1 turnover in cancer cells. For example, resveratrol, which is known as an effective antiproliferative agent in vitro, has shown high efficacy in reducing cyclin D1 levels in SW480 colon cancer cells through the induction of proteasomal degradation [155, 164]. Curcumin is also used as an effective treatment of prostate and breast cancer cells, and has been demonstrated to exert high efficacy in inducing cyclin D1 clearance and subsequent reduction in its levels, through modulating the activity of 26S proteasome as well as decreasing the expression of cyclin D1 mRNA [165].

1.6.3.6.2.6 NQO2’s role in regulating cyclin D1 levels via modulating the AKT/GSK-3β pathway

NQO2 plays a potential role in regulating the stability of cyclin D1 in cancer cells. Genetic silencing of NQO2 in CWR22Rv1 prostate cancer cells has been demonstrated to maintain the stability of cyclin D1 through the suppression of cyclin D1 phosphorylation at Thr286, and consequent degradation via proteasome. NQO2 has been found to be involved in regulating the activity of GSK-3β-mediating cyclin D1 proteolysis [166]. However, the exact mechanisms through which
NQO2 facilitates this pathway needs to be investigated. Hsieh et al. (2014) have demonstrated that NQO2 can directly interact with AKT, thereby inhibiting its phosphorylation activation mediated by PIP3. This inhibition protects GSK-3β from inactivation phosphorylation and consequently suppresses the phosphorylation proteolysis of cyclin D1 [162]. Therefore, silencing the NQO2 gene allows for the phosphorylation activation of AKT, which in turn suppresses the activity of GSK-3β, in this way protecting cyclin D1 from proteasomal degradation. As NQO2 shows therapeutic benefits through the inhibition of AKT, it can be exploited as a potential target to overcome the resistance problem to AKT-inhibitors in cells overexpressing NQO2 [162].

Multiple evidence has emphasised the involvement of NQO2 in the prostate cancer progression. Genetic silencing of NQO2 has been found to be accompanied by a significant reduction in cell growth and arrest of cell cycle at the G1 phase [166]. Moreover, the anti-prostatic cancer activity of resveratrol has been suggested to be mediated through the inhibition of NQO2 activity in the cells. This was further supported when the genetic silencing of NQO2 in prostate cancer cells was seen to cause attenuation in the growth suppression activity of resveratrol, therefore outlining the role of NQO2 as a mediator of chemoprevention and resveratrol’s anticancer action. The reduction in cyclin D1 levels associated with resveratrol treatment has been found to also be abrogated in the absence of NQO2, supporting the notion of NQO2’s integral role in cyclin D1 turnover [166]. Hsieh et al. (2014) also demonstrated that NQO2 participates in the anticancer activity of resveratrol by regulating cyclin D1 levels via the same mechanism-mediated AKT/GSK-3β pathway [162].

1.6.3.6.3 The role of NQO2 in mediating inflammatory responses

TNF is an essential proinflammatory cytokine for normal immune system functioning [167]. The cellular effects of TNF are induced through the activation of various signalling pathways, including the activation of NF-κB [167]. The dysregulation of TNF results in aberrations in cell survival, and the proliferation and metastasis of cancer cells [167].

NF-κB is an inducible transcription factor [168]. It is responsible for the regulation of cell proliferation, apoptotic cell death and inflammatory and immune responses through inducing the
expression of a plethora of gene involved in these cellular events [168]. NF-κB is activated in response to several signals including oxidative stress [169, 170], inflammatory cytokines and administrated drugs [170]. Interestingly, NF-κB activity can also be induced by NRH, in a way that is reliant on NQO2 [105].

The aberrant activation of NF-κB has been shown to be implicated in the development of cancer as a result of the increased levels of nuclear NF-κB; this appears in a wide variety of cancers including ovarian, breast and colon cancers [168]. The inhibition of NF-κB causes an increase in cellular death and consequently tumour regression [105], validating the participatory role of NF-κB in tumourgenesis, upon its constitutive activation [170].

The putative roles of NQO2 in TNF-dependent cell signalling pathways have been investigated following the exposure of NQO2-null keratinocytes to TNF. It has been found that the presence of NQO2 is essential to the signalling activity of TNF [167]. NQO2 may act as a TNF activity mediator; the lack of NQO2 attenuates TNF’s ability to activate IκBα kinase (IKK) and this consequently inhibits the phosphorylation degradation of IκBα, thereby blocking NF-κB activation [167]. The suppression of NF-κB activity has been associated with a significant reduction in the expression levels of its anti-apoptotic gene products including cyclin D1, thereby enhancing cell apoptosis. In other words, the lack of NQO2 abolishes the activity of TNF-inducing cell proliferation and potentiates apoptosis. Remarkably, NQO2 may have a potential role in tumourgenesis [167].

Furthermore, resveratrol, the classical NQO2 inhibitor, also causes the inhibition of TNF activity-induced NF-κB activation [167]. Nolan et al. have demonstrated that some of the NQO2 inhibitors that exhibit anticancer activity are able to attenuate NF-κB activity; this occurs via interference with the NQO2/NF-κB pathway [105]. These findings collectively support the idea of considering NQO2 as a potential therapeutic target in cancer.
1.6.3.7 NQO2 in association with malaria

Malaria is a life-threatening disease for 50% of the world’s population, with approximately two million deaths recorded from it annually. It is widely rampant in Africa, Southeast Asia and India [171]. This fatal disease is caused by *Plasmodium* parasites, which degrade haemoglobin to heme products. Heme undergoes Fenton reaction to produce ROS, and this consequently results in increased oxidative stress levels. It has been demonstrated that malaria parasites are highly sensitive to oxidative stress, and therefore use the hosts’ antioxidant defence system to protect themselves from oxidative damage. Thus, inducing the host’s oxidative mechanisms can be exploited as a therapeutic approach to killing the parasites [171]. Quinolines, including chloroquine, primaquine and quinacrine, have long been used as effective treatments for malaria [172]. The mechanism of quinolines’ antimalarial action has been generally accepted to be through its inhibitory action for heme detoxification mechanisms in the red blood cells, thereby increasing oxidative stress levels [172]. Quinolines have been found to be potent NQO2 inhibitors, but at the same time inactive against NQO1 [172]. As NQO2 acts as an antioxidant enzyme, it has been suggested that its inhibition in red blood cells by quinolines leads to a high increase in oxidative stress levels, in this way creating an inhospitable environment for the malaria parasites [81]. Therefore, NQO2 can be exploited as a therapeutic target to develop novel antimalarial drugs, taking into account the high expression levels of NQO2 in red blood cells.

1.6.3.8 NQO2 in association with neurodegenerative diseases

1.6.3.8.1 General introduction to neurodegenerative diseases

Neurodegenerative diseases are associated with the progressive and irreversible death of neurons [135]. Multiple factors are implicated in neurodegeneration, including increased oxidative stress levels which consequently cause oxidative injury to the neurons [135, 173]. The brain has been found to be a highly sensitive organ to oxidative stress, due to several reasons:
1. The brain consumes large amounts of oxygen for its metabolic processes (approximately 20% of total oxygen in the body), although its weight constitutes only 2% of total body weight. The more oxygen is consumed, the higher the ROS levels produced, consequently increasing the probability of oxidative injury to neurons [174].

2. The brain is enriched with polyunsaturated fatty acids that are highly vulnerable to peroxidation [175].

3. The levels of antioxidants in the brain are low [175].

4. The brain contains high amounts of iron that catalyses the production of hydroxyl radical [174].

The antioxidant defence system is crucial to the scavenging of ROS and/or the prevention of ROS formation, thereby protecting the human tissue from oxidative injury and consequent cell death [174]. The antioxidant system comprises of non-enzymatic antioxidants (e.g. melatonin and resveratrol) and enzymatic antioxidants (e.g. NQO1 and NQO2) [176]. The detoxification activity of those enzymes for xenobiotics and toxic radicals prevents the formation of electrophilic metabolites and ROS, in this way protecting the neural cells from oxidative stress-induced neurotoxicity. Any impairment in the function of antioxidant enzymes has been demonstrated to be associated with the development of neurodegenerative diseases such as PD and AD [176].

1.6.3.8.2 Alzheimer’s disease

Alzheimer’s disease (AD) is an aging-associated disease which shows a significant loss of neurons in the hippocampal region [177]. AD is a multifactorial disease; a lot of evidence highlights the contributory role of increased oxidative stress levels in the hippocampal region in AD’s pathogenesis [173, 175]. The hippocampus has been reported to be a part of the brain which is highly sensitive to oxidative stress [177]. Therefore, the enzymes that detoxify ROS and/or inhibit their generation may provide protection against stress-induced neurotoxicity and may thereby prevent the progression of AD [178]. Of these enzymes is NQO2, which is believed to be involved in the detoxification of quinones and the prevention of oxidative damage [177]. Several studies
have demonstrated that the overexpression of NQO2 in the hippocampus of AD patients occurs in response to an increase in oxidative stress. The overexpression of NQO2, however, might be implicated in the pathogenesis of AD; enhancing NQO2 activity in the hippocampus is associated with an increase in the production of hydroquinones and deleterious semiquinones. The latter metabolites are deleterious for the neurons and can cause neuronal cell death. This means that NQO2 might be a causative agent for the observed damage to the hippocampal neurons in AD patients [177, 179]. It has been shown that the genetic deletion or pharmacological inhibition of NQO2 in mice can improve their learning capabilities [177, 179]. Resveratrol and quercetin, which are known as NQO2 inhibitors, have been demonstrated to have neuroprotective functions which might be mediated by inhibiting toxifying NQO2 activity [177]. Therefore, targeting NQO2 might be used as a new therapeutic approach to treat diseases associated with cognitive deficits [179].

1.6.3.8.3 Parkinson’s disease

Parkinson’s disease (PD) is a neurodegenerative movement disease manifested by bradykinesia and postural instability. The incidence of PD has been reported to increase with age [173]. PD is caused by progressive loss of dopaminergic neurons in the substantia nigra region of the brain [135, 180]. Several studies have demonstrated that the levels of oxidative stress in the substantia nigra of patients with Parkinson’s are very high, supporting the possible role of increased oxidative stress levels in inducing dopaminergic neuronal loss [119, 173]. Dopaminergic neurons are very sensitive to exogenous ROS. This is attributed to the excessive usage of dopamine as a neurotransmitter [180]. The oxidative metabolism of dopamine results in the generation of quinones, which is the major source of ROS [181].

NQO2 has been suggested to play contributory roles in the pathogenesis of PD. It has been hypothesised that individuals with the D allele in the NQO2 gene promotor region are highly susceptible to PD [119, 121]. This is because the deletion of 29-nucleotide-containing sequences related to Sp3 binding sited from the NQO2 gene promoter region leads to enhanced NQO2 expression levels. Overexpression of NQO2 is associated with increased NQO2 activity, and this can ultimately result in a substantial enhancement in ROS levels, particularly upon exposure to
exogenous dopamine and their derivatives [119]. Increased ROS levels have been suggested to be implicated in dopaminergic oxidative damage and subsequent development of PD [119]. The involvement of NQO2 in the development of PD was further validated when resveratrol and melatonin showed high efficiency in preventing neuronal degeneration and offered protection from PD. This has been suggested to occur through the inhibition of NQO2 activity [119].

On the other hand, it has been proposed that the deletion of 29-bp nucleotides from the NQO2 gene promoter region may remove Sp1 binding site sequences, ultimately resulting in the reduction of NQO2 expression. The downregulation of NQO2 levels might be accompanied by impairments in the detoxification mechanism of catecholamine-derived quinones, which are neurotoxic compounds, resulting in the development of PD. However, this hypothesis has to be confirmed by using large samples of different ethnic groups [121]. Contrastingly, the study conducted by Okada et al. (2005) has demonstrated that there is no significant association between NQO2 I/D variant genotype and PD pathogenesis, in the Seattle population [181]. Furthermore, Benoit et al. (2010) have suggested that polymorphism in the NQO2 gene promoter region is a weak risk factor in the development of PD [179].

1.7 Background and aims of the project

NQO2 was first discovered in 1960 by Liao and Williams-Ashman as a flavoprotein that catalyses the oxidation of dihydronicotinamide to induce its reductive activity. NQO2 was forgotten for more than 30 years until Jaiswal et al. rediscovered it while sequencing NQO1 in the early 1990s. The high similarity in the structures and catalytic properties of NQO2 and NQO1 allows for the consideration of NQO2 as an analogue of NQO1. NQO2 is an oxidoreductase enzyme that, along with NQO1, belongs to the NAD(P)H: quinone oxidoreductase family.

Nevertheless, in contrast to NQO1, the biological roles of NQO2 are still not well understood. NQO2 has been found to be expressed in different types of cancer tissue at much higher levels comparing with their corresponding normal cells [106]. As such, it has been proposed that NQO2 could be implicated in cancer progression; this is linked to its identified functions first as a
detoxifying enzyme-mediated regulation of redox status of the cells, second as a regulator of TNF-signalling pathways involved in activation of NF-κB and its subsequent pivotal processes including cell proliferation and death, and third as a regulator of cyclin D1 turnover in cancer cells through controlling AKT/GSK-3β pathway. Furthermore, it has been speculated that NQO2 is also responsible for the resistance of cancer cells to chemotherapy; this has been linked to its contributory role in modulating the redox status, the expression levels of P-gp transporter and the uptake levels of anticancer drug via unknown mechanisms.

The potential roles of NQO2 in carcinogenesis have been defined in several types of cancers including breast, prostate and leukaemia. In intraperitoneal ovarian metastases cancer, the high activity levels of NQO2 have been exploited to selectively target the cells with tretazicar:caricotamide chemotherapy [104]. However, the biological roles of NQO2 in ovarian cancer and their possible implication in ovarian cancer progression and chemotherapy resistance have not yet been studied. Thus, this study has two main aims. First it seeks to investigate the contributory roles of NQO2 in ovarian cancer growth. Second it aims to explore the impact of modulating NQO2 on toxicity of adriamycin anticancer drug in ovarian cancer. To achieve these aims, genetic and pharmacological approaches will be established and tested for their efficiency at targeting NQO2 in ovarian cancer cells. The availability of poor inhibitors with low potency and high toxicity raises the need to find novel inhibitors with better properties in order to generate reliable findings regarding the actual roles of NQO2 in cancer cells. Recently, Nolan et al. (2012) have conducted virtual screening study on NCI compounds with diverse structures in order to identify novel ligand scaffolds that have inhibitory activity against NQO2. Of these, quinoline class has led Dr Freeman and her team to design and synthesise more derivatives. Based on the molecular docking findings, the novel quinoline derivatives show high inhibitory potency against NQO2. As such, it is crucial to further investigate the compounds’ properties and functionality as NQO2 inhibitors in different systems, and this is what the project aims to do.

The aims of this project can be divided into four-folded aims. Firstly, to screen a panel of seven ovarian cancer cell lines for expression and activity levels of NQO2 and subsequently for the distribution extent of NQO2. The screening study facilitates the selection of appropriate cell lines to
generate NQO2 genetically modified cell lines with silenced and overexpressed levels of NQO2. Secondly, to identify which of the novel quinoline derivatives have the highest potency and/or lowest toxicity. Evaluation of inhibitory potency of the novel compounds will be conducted in both a cell free system and *in vitro* using CB1954 approach. These inhibitors along with the modified cells will be used as molecular tools for studying the cellular functions of NQO2. Thirdly, to investigate the contributory roles of NQO2 in mediating ovarian cancer cell growth and proliferation, and to identify the underlying pathways through which NQO2 may generate alterations in the cells’ growth behaviour. Three pathways are intended to be investigated in NQO2 genetically modified cell lines, which are selected based on the profound roles of NQO2 in intracellular ROS levels, cell cycle progression pattern, and cyclin D1 stability. Fourthly, to explore the contributory roles of NQO2 in determining responsiveness levels of ovarian cancer cells to ADR through evaluating the cytotoxicity of adriamycin upon modulating NQO2 levels genetically and/or pharmacologically. The last chapter also aims to identify the underlying pathways through which NQO2 may alter the ADR toxicity; P-gp expression and intracellular basal ROS levels will be the main pathways investigated.

Identification of the actual roles of NQO2 in ovarian cancer whether they mediate cancer progression or repression allows us to decide the most proper treatments to be used for targeting NQO2 and consequently managing cancer disease. Also, the characterisation of novel compounds enables us to determine whether they are valuable tools to be used for the future investigation of biological functions of NQO2.
Chapter 2

Materials and methods
2 Materials and methods

2.1 Suppliers of materials

All chemicals and reagents were obtained from Sigma Aldrich (Poole, Dorset, UK), unless otherwise mentioned. For cell culture, heat inactivated foetal bovine serum (FBS) was supplied by GIBCO, Invitrogen (GIBCO BRL, Paisley, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (Hampshire, UK). Dimethyl sulfoxide (DMSO) was supplied by Fisher Scientific (Leicestershire, UK). 1-(carbamoylmethyl)-3-carbamoylpiridinium iodide (EP0152R, abbreviated as EPR) was kindly provided by Professor Gavin Halbert from the University of Strathclyde.

2.2 Cell culture

2.2.1 Cell lines

A panel of human OVC cell lines was chosen for various experimental purposes. These cell lines were provided by St Mary’s Hospital, and authenticated at the facility department of the University of Manchester. Further details about the cells are illustrated in Table (2.1). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, except CaOV-3 cells which were maintained in Dulbecco's Modified Eagle Medium (DMEM) instead. Both media were supplemented with 10% FBS and L-glutamine (2mM). To generate pure cultures of NQO2 genetically modified cells of TOV-112D and SKOV-3 cells sub-lines, the cultures were maintained in a selective RPMI medium containing 1μg/ml of puromycin for a specific period of time before regrowing them in a fresh medium that is free of antibiotic. For induction of NQO2 silencing in SKOV-3 cells, doxycycline hyclate (dox) was added to the culture medium at a concentration of 1μg/ml on an alternate day basis for five days before seeding for the experiment. To ensure that the silencing levels of NQO2 are stable throughout the experiment, dox was also added directly to the culture on an alternate day basis for the experiment duration. This was carried out in all experiments where NQO2-silencing SKOV-3 cells were used for studying NQO2 functions. Concentration of dox and
duration of treatment were determined following the protocols described in section (2.12.7). The cells were incubated in all experiments in a humidified atmosphere with 5% carbon dioxide (CO$_2$) at 37°C. The cells were handled under aseptic conditions using class II laminar flow cabinet.

Table 2.1 General information about the panel of cancer cell lines used in the study

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Tissue of Origin</th>
<th>P53 State</th>
<th>P-gp State</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Ovarian carcinoma</td>
<td>WT p53 [14, 182]</td>
<td>P-gp negative [63]</td>
</tr>
<tr>
<td>CaOV-3</td>
<td>Ovarian adenocarcinoma</td>
<td>Null p53 [183]</td>
<td>P-gp positive [184]</td>
</tr>
<tr>
<td>OV-90</td>
<td>Papillary serous ovarian</td>
<td>Mutant p53 (S215R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adenocarcinoma</td>
<td>[183, 185]</td>
<td></td>
</tr>
<tr>
<td>OVC-AR3</td>
<td>Ovarian adenocarcinoma</td>
<td>Mutant p53 (R248Q)</td>
<td>P-gp positive [186]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[183]</td>
<td></td>
</tr>
<tr>
<td>SKOV-3</td>
<td>Ovarian serous adenocarcinoma</td>
<td>Null p53 [14, 185, 187, 188]</td>
<td>P-gp positive [186]</td>
</tr>
<tr>
<td>TOV-112D</td>
<td>Ovarian endometroid</td>
<td>Mutant p53 (R175H)</td>
<td>P-gp negative [186]</td>
</tr>
<tr>
<td></td>
<td>adenocarcinoma</td>
<td>[183, 185, 189]</td>
<td></td>
</tr>
<tr>
<td>TOV-21G</td>
<td>Clear cells ovarian adenocarcinoma</td>
<td>WT p53 [183]</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Human breast adenocarcinoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Subculturing adherent cell lines

Cells were cultured in tissue culture flasks and maintained in 5% CO$_2$ humidified incubator at 37°C. When the adherent cells covered 70-80% of the flask substrate, the cells were harvested and split into a new flask. The previous medium was briefly aspirated and the cell monolayer was washed with sterile PBS. Thereafter, the cells were placed in the incubator with 2-3ml of 0.5% trypsin/ 0.2% ethylenediaminetetraacetic acid (EDTA) for 2-5min until they were completely detached. A volume of complete growth medium, which is four times larger than that used for dissociation agent, was added to the cells to stop the trypsin activity. The cells were then re-seeded in a fresh complete medium at the desired splitting ratio.
2.2.3 Cryopreservation of cell lines

The early passaged cells were preserved at -196°C as a frozen seeding stock for further experimental studies. After harvesting the cells, as stated in section (2.2.2), the cell suspension was centrifuged at 1500 rotations per minute (rpm) for 5min at room temperature (RT). The supernatant was discarded and the cell pellet was re-suspended in a freezing medium consisting of 50% (v/v) complete growth medium, 40% (v/v) FBS and 10% (v/v) DMSO. 1ml aliquots of this mixture were transferred into 1.8ml cryogenic vials (Greiner Bio-One, Germany). These vials were preserved at -80°C for 24hr in a cryofreeze container containing isopropanol (Nalgene, Milton Keynes, UK) before being transferred to the liquid nitrogen tank (-196°C) for long-term storage.

2.2.4 Thawing cryopreserved cell lines

Cells were quickly thawed in a pre-warmed water bath and resuspended in a fresh growth medium. Cell suspension was centrifuged at 1200rpm for 5min at RT to eliminate the freezing medium. Cell pellet was then suspended in a fresh medium and cultured until the cells became confluent. Once this happened, the cells were transferred into new flasks for experimental studies and/or refreezing. Cells had to be split at least twice before using them for the experiments.

2.2.5 Trypan blue exclusion assay

Determination of cell concentration using manual hemocytometer was the preliminary step in many of the experiments conducted in this study. The hemocytometer consists of a thick glass microscope slide with two rectangular counting chambers. Each chamber is divided by a counting grid into nine squares, as illustrated in Figure (2.1). The cells located at corner squares were counted excluding the ones that lie on perimeter lines of these squares. A mixture of equal volumes of cell suspension and 0.4% (w/v) trypan blue stain was prepared. The stain was used to assess cell viability through distinguishing blue-stained dead cells from the transparent viable cells [190]. 10µl of this mixture was dispensed between glass coverslip and counting chamber. The slide was examined under the microscope at 10x magnification and only the unstained viable cells were
counted. The total of viable cells was averaged and then multiplied by dilution factor to give a cell number x10000 per ml of the suspension.

![Counting chamber of hemocytometer](image)

**Figure 2.1 Counting chamber of hemocytometer**

Only the cells located on the corner squared were counted excluding the ones located on their perimeter lines and blue-coloured dead ones.

To monitor the growth rate of NQO2 genetically modified cells, the cells were counted manually on a day-to-day basis for eight days using the hemocytometer. TOV-112D and SKOV-3 cells sub-lines were seeded at a density of 50,000 and 35,000 cells, respectively, per well in 6-well plates. Following a 4hr incubation, the cells were treated with 100µM EPR and incubated along with untreated control till the first collection time point. At 24hr time intervals, the cell suspension was prepared for counting as mentioned earlier. The experiment was performed in duplicate and repeated three independent times. Cell counts were normalised at logarithmic scale and plotted on semi-log plot against time (in hours). Doubling time (Tc) of cell population was calculated in hours from exponential growth of cells **Figure (2.2)**, using this **Equation (2.0.1)**: 

\[ T_c = 0.3 \times \frac{T}{ \log(A/A_0) } \]

where T is time when cells are in the exponential growth phase, A is cell number at the end of this
time (when the exponential growth terminates) and $A_0$ is cell number at the beginning of this time (when the exponential growth initiates) [191, 192]. $T_c$ is defined as the number of hours needed to generate a double number of cells population. This occurs when exponentially growing cells enter log phase [190].

![Cell growth curve](image)

**Figure 2.2 Cell growth curve**

### 2.3 Preparation of cell lysate

Cells were seeded in 10cm cell culture dishes and incubated in 5% CO$_2$ humidified atmosphere at 37°C until the exponentially growing cells reached 70-80% confluence. For best results, all the subsequent steps were carried out on ice to protect cellular contents from degradation. The growth medium was aspirated and cell monolayer was washed twice using ice-cold PBS. The adherent cells were scrapped and collected in an ice-cold lysis buffer. The cell lysate was left on ice for 30min and then sonicated twice on ice for 3sec each at 10 Hertz (Soniprep 150 ultrasonic disintegrator MSE, Sanyo, UK). Thereafter, the lysate was centrifuged at 13,000×g for 10min at 4°C to separate supernatant containing solubilised target proteins from cell debris. Supernatant was then transferred to a fresh cold Eppendrof tubes, and stored at -80°C until further experiments, or kept on ice for immediate use.
Different lysis buffers were prepared and selected depending on the purpose of the experiment and the site of target proteins. NonidetP-40 (NP-40) lysis buffer was used to extract cytoplasmic proteins while radioimmunoprecipitation assay (RIPA) buffer was preferred to extract the whole cell content, including nuclear proteins. Both lysis buffers were used to prepare samples for Western blot analysis. NQO lysis buffer was used to prepare cell lysate and reconstitute recombinant human NQO1 or NQO2 (rhNQO1 or rhNQO2 respectively) powder for enzyme activity assay.

2.4 Quantitative determination of protein concentration

Total protein concentration in cell lysates was determined using bicinchoninic acid (BCA) assay. The biochemical basis of this assay relies on Biuret reaction whereby copper sulfate (II) Cu$^{+2}$ is reduced at a basic pH to Cu$^{+1}$ by peptide bonds of the protein. Cu$^{+1}$ can then form a 1:2 complex with BCA generating a purple colour solution [193]. Depth of purple colour was proportional to the protein levels. Bovine serum albumin (BSA) was used as a standard protein to prepare a set of protein standard solutions of known concentrations dissolved in lysis buffer. These preparations covered a wide range of protein concentration (0.0, 0.3, 0.6, 1.25, 2.5 and 5.0 mg/ml). A working solution of colorimetric reagents was prepared by mixing 50 parts of BCA with one part of 4% (w/v) of Cu$^{2+}$ reagent. Equal volumes (10µl) of blank (lysis buffer used as diluent), protein standard solutions, and unknown samples were dispensed in duplicate into a 96-well plate. The reactions started when 200µl of the working solution was loaded to the wells. The plate was incubated at 37ºC for 30min prior to measuring absorbance at 562nm wavelength using a multi-well scanning spectrophotometer. The OD values of BSA standard solutions were corrected by subtracting them from OD of blank containing diluent only, and then plotted against known concentrations of BSA solutions to generate a standard curve with a straight line equation using Microsoft Excel Software. This equation was used to calculate the protein concentrations of unknown samples.
2.5 NQO2 and NQO1 enzymes activity assay

2.5.1 Chemicals and reagents

Phosphate buffer (50mM, pH 7.4) was made by mixing 50mM dipotassium hydrogen orthophosphate solution (K$_2$HPO$_4$; VWR, UK) with 50mM potassium dihydrogen orthophosphate solution (KH$_2$PO$_4$; VWR, UK) at a specific ratio to reach the required pH of 7.4. To make NQO lysis buffer, flavine adenine dinucleotide (FAD) and sucrose (VWR, UK) were dissolved in the phosphate buffer (pH 7.4) at final concentrations of 5µM and 250mM respectively. NQO buffer was stored in aliquots at -20ºC for further usages. Mixtures of either rhNQO1 or rhNQO2 were prepared by reconstituting 1mg lyophilised powder of the enzymes in 200µl NQO buffer, making mixtures at (5mg/ml) stock concentration. The mixtures were then aliquoted and stored at -80ºC until use. Typical inhibitors of NQO2 (e.g. resveratrol) and NQO1 (e.g. dicoumarol) were made at 10mM stock concentration in DMSO and 0.13M NaOH respectively.

2.5.2 Measuring cellular NQO2 and NQO1 activity using cytochrome c-based spectrophotometric assay

NQO2 and NQO1 activity in OVC cell lines was determined using cytochrome c spectrophotometric assay. The methodology used in this study was adapted from the original method established by Ernster et al. [194, 195]. The rate of cytochrome c reduction catalysed by NQO2 and NQO1 enzymes was measured as changes in the absorbance values over a minute of reaction. Since cytochrome c is a weak substrate of NQO1 enzyme, menadione (NQO1 and NQO2 substrate [120]) was also added to the reaction mixture as an initial electron acceptor [196]. Menadione is first reduced by active enzymes to menadiol which in turn reduces the terminal electron acceptor, cytochrome c, changing the colour from orange to deep red. The depth of red colour gives an indication about the catalytic activity of the enzymes in the cells [197, 198]. The protocol used to prepare cell lysate for enzyme activity assay was slightly adjusted from that described in section (2.3). These modifications were useful for maintaining the original activity of the enzymes stable over long-term storage. Cells were collected by scraping in protease inhibitors containing PBS.
and then centrifuged at 500rpm for 5min. After that, the supernatant was discarded and pellets were snap-frozen and stored at -80ºC. On the same day of assay, the pellets were defrosted on ice and then mixed with NQO buffer by pipetting several times. The lysate was sonicated twice on ice and then centrifuged at 13,000×g for 10min at 4ºC. The total protein concentration in the supernatant was measured as described in section (2.4). Stock solutions of 140µM cytochrome c, 10mM EPR and 10mM reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) were prepared in phosphate buffer whereas a solution of 10mM menadione was made in DMSO. All of these solutions were freshly prepared on the same day of assay. Reaction mixture was made in a 1ml final volume by sequential mixing of 50mM phosphate buffer (pH 7.4) with cytochrome c (70µM), menadione (20µM) and enzyme cofactor (200µM) (either EPR for NQO2 or NAD(P)H for NQO1), Figure (2.3). The reaction was initiated upon the addition of cell lysate. The changes in absorbance values were recorded over 1min at 550nm wavelength using Beckman DU 7400 spectrophotometer. As cell lysate containing a variety of proteins that may also participate in the reduction reaction of the substrates, a selective inhibitor of NQO2 (e.g. resveratrol, 100µM) and NQO1 (e.g. dicoumarol, 100µM) was added to a parallel reaction. As these compounds are able to inhibit the enzymes’ activity to a high extent, any change in absorbance values is expected to be attributed to the reductive activity of the other components present in the reaction mixture. To exclude their reductive activity, OD of control was subtracted from that of the treated sample. Thus, the resultant OD would indicate those changes in the absorbance mediated by enzymes’ activity. This value is then converted to activity using Equation (2.0.2) wherein 21.1 refers to the molar absorbance value of cytochrome c [199]. The volume of reaction mixture was 1ml. Volume of cell lysate loaded to the reaction was different among cell lines and dependent on the enzymes’ intracellular activity levels. The volume that caused 1.0 unit change in the absorbance over a minute of reaction was chosen. The activity of enzyme was measured relative to the total protein concentration, and expressed as nmole of cytochrome c reduced per mg of protein per minute of reaction. Each sample was analysed three times and the assay was independently performed at least three times.
Equation (2.0.2): Enzyme activity (nmole cyto c/min/mg) = change in absorbance at 550nm/min x (1 / 21.1mM⁻¹cm⁻¹) x (total cuvette volume (ml)/volume of suspension (ml)) / protein concentration (mg/ml)

![Diagram of reaction](image)

Figure 2.3 Reduction reaction of cytochrome c induced by cellular NQO2 and NQO1 enzymes

1ml of reaction mixture was made by mixing 50mM phosphate buffer with cytochrome c (70µM), menadione (20µM) and either EPR or NAD(P)H, the NQO2 and NQO1 co-factor respectively, at a 200µM concentration. Enzymatic reduction reaction begins upon the addition of cell lysate which catalyses the conversion of menadione to menadiol. Menadiol is responsible for non-enzymatic reduction of cytochrome c, changing the solution colour from orange to deep red. Enzyme activity referred to that fraction inhibited by 100µM concentration of resveratrol or dicoumarol, the NQO2 or NQO1 inhibitor respectively. The activity was then measured relative to the total protein concentration.

2.5.3 Measuring recombinant human NQO2 and NQO1 activity using DCPIP-based spectrophotometric assay

Dichlorophenolindophenol (DCPIP) spectrophotometric assay was used to evaluate extracellular inhibitory potency of novel compounds against rhNQO2 and rhNQO1 in a cell free system. DCPIP is also an electron acceptor substrate of NQO2 and NQO1 enzymes [115], which was used in this assay in place of menadione/cytochrome c as it is a cost-efficient reagent. The reduction of DCPIP is directly mediated by active enzyme converting its original blue colour (oxidised form) to colourless (reduced form). The rate of DCPIP reduction was measured as changes in the absorbance over a minute of reaction, and was directly proportional with the enzymatic activity levels.
The method used herein was taken from Nolan et al. [105, 200]. Aliquot of either rhNQO2 or rhNQO1 was thawed on ice and diluted using NQO buffer to a concentration that gave a change in the absorbance falling between 0.85-1.25 units per min of reaction. DCPIP was prepared in distilled water at 2mM stock concentration. EPR and NAD(P)H solutions were prepared in phosphate buffer at 10mM stock concentration. Working solutions of each compound were prepared through diluting the stock solutions serially at 1:10 dilution ratio to cover these ranges of concentration (0.001-1000μM) for NQO2 evaluation while (0.001-10000μM) for NQO1 evaluation. 10μl of each of these working solutions was dispensed to 1ml reaction mixtures containing DCPIP (40μM) and either co-factor (200μM), covering ranges of final concentration of (0.00001-10μM) for NQO2 evaluation while (0.00001-1000μM) for NQO1 evaluation. For solvent control, a 10μl of DMSO was used instead. 10μl of enzyme was added to the reaction mixture to start reduction reaction of DCPIP, Figure (2.4). The changes in the absorbance values of DCPIP were recorded over 1min or reaction at 600nm wavelength using Beckman DU 7400 spectrophotometer. The activity of enzymes that remained unaffected by inhibitory activity of the compound was calculated as percentage relative to the control enzyme activity and then plotted against concentration ranges to generate dose-response curves. Nonlinear regression analysis was used to fit the data with the best line. These curves were used to determine IC50 for extracellular inhibitory potency of the compounds, which is defined as a concentration that inhibits enzyme activity by 50% relative to control enzyme activity.
Figure 2.4 Reduction reaction of DCPIP induced by rhNQO2 and rhNQO1 enzymes

1ml of reaction mixture was made by mixing 50mM phosphate buffer with DCPIP (40µM) and EPR or \( \text{NAD(P)H} \) (200µM). By the addition of either rhNQO2 or rhNQO1 to the mixture, the reduction reaction of DCPIP began changing the solution colour from blue (oxidised DCPIP) to colourless (reduced DCPIP). This happened in the solvent control where DMSO or NaOH was used. However, in the sample reaction, the solvent was replaced by a series of inhibitors that prohibited the enzymatic reduction of DCPIP and therefore reduced the rate of DCPIP reduction.

2.6 Western blot analysis

Western blot analysis was performed to measure the expression levels of endogenous and ectopically expressed proteins.

2.6.1 Chemicals and reagents

2.6.1.1 Lysis buffers

NP-40 buffer was made by mixing triton-X-100 (1%), NP-40 (0.5%), Tris-HCl (50mM, pH 7.5; VWR, UK), sodium chloride (NaCl, 150mM), sodium fluoride (NaF, 50mM) and protease inhibitor cocktail (PIC) (1 tablet per 10ml lysis buffer).

RIPA buffer was made by mixing Tris-HCl (pH 7.5, 50mM), NaCl (150mM), NP-40 (1%), EDTA (1mM) and PIC. It was also supplemented with several protease and phosphatase inhibitors, e.g. phenylmethylsulphonyl fluoride (PMSF, 1mM), NaF (1mM) and sodium orthovanadate (\( \text{Na}_3\text{OV}_4 \), 1mM). These inhibitors were added to the buffer just prior to using it for preparation of cell lysate. The lysis buffers were then stored in aliquots at -20°C until use.
2.6.1.2 Sample buffer

2x concentrate of sample buffer was made from 0.15g Tris-HCl (pH 6.8), 3ml glycerol (Invitrogen, UK), 4ml sodium dodecyl sulfate (SDS) (10% w/v) and bromophenol blue (0.5% w/v), which were diluted up to 20ml using distilled water. This mixture was divided into 950µl aliquots and stored at -20°C. 50µl of β-mercaptoethanol was added to the mixture immediately before usage.

2.6.1.3 Running buffer (10x)

10x running buffer solution was prepared by dissolving 30.2g Tris (0.025M), 144g glycine (0.192M; Fisher Scientific, UK) and 10g SDS (1% w/v) in a volume of distilled water completed up to 1L. 1x concentrate was made by diluting 100ml of the 10x running buffer in 900ml distilled water.

2.6.1.4 Transfer buffer (10x)

10x transfer buffer solution was prepared by dissolving 30.2g Tris (0.025 M) and 144g glycine (0.192M) in a volume of distilled water completed up to 1L. To make a 1x concentrate of working solution, a 100ml of 10x transfer buffer was mixed with 200ml of 100% methanol and then the volume was completed up to 1000ml using distilled water.

2.6.1.5 PBST and TBST

PBS solution was made by dissolving 5 tablets of PBS in 500ml of distilled water to get pH 7.6 while Tris-buffered saline (TBS) solution (50mM, pH 7.6) was prepared by dissolving one tablet of TBS in 500ml of distilled water. To make PBS-tween-20 (PBST) and TBS-tween-20 (TBST), tween-20 was added to the buffer solutions at a final concentration of 0.1% (v/v).

2.6.2 Preparation of samples

The samples were prepared by mixing a volume of cell lysate containing the required amount of protein with sample buffer. Then, this mixture was heated for 5min at 100°C and placed
immediately on ice to prevent its aggregation. Before loading the samples into gel, it was recommended to spin them down for few seconds at 13,000×g to ensure that the entire amount of protein will be loaded later into the gel.

2.6.3 SDS-PAGE gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate multiple proteins on the basis of their molecular weights (MW) to facilitate the detection of target proteins immunogenically using specific antibodies.

Gel electrophoresis was executed using Mini-Protean (II) cell system apparatus (Bio-Rad, Hemel Hempstead, UK). The gel was freshly prepared as described in Table (2.2). The gel components were sequentially mixed in 50ml falcon tube. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added just prior to casting the gels. The separating gel mixture was poured promptly into two glass plates and overlaid with distilled water to speed up the polymerisation process. Once it became solid, distilled water was decanted and stacking gel was poured. Desired 1.5mm comb was inserted between spacer plates to form lanes for loading the samples. The casted gel was then inserted into the electrophoresis tank. The 1x working solution of running buffer was used to fill the inner and outer chambers of the tank before loading the samples. 10µl of pre-stained molecular weight ladder (Thermo Scientific, PageRuler, prestained protein ladder, Lithuania) was loaded along with other samples into gel lanes. The gel was then run at 100 volts (V) until the sample buffer dye front reached the bottom of separating gel.
Table 2.2 Reagents and volumes used to prepare separating and stacking gels

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7%</td>
<td>10%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.1ml</td>
<td>4.1ml</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>0.5M Tris (pH 6.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20% SDS</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>Acrylamide (AA)/Bis-Acrylamide (Bio-Rad, UK)</td>
<td>2.3ml</td>
<td>3.3ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
<td>5µl</td>
</tr>
</tbody>
</table>

This table includes list of reagents and volumes required to prepare one separating and one stacking gels. Choosing the percentage of separating gel depended on the size of target proteins. Separating gels with 7%, 10% and 12% acrylamide were used to separate the proteins possessing MW located within these ranges (50kDa - 500kDa), (20kDa- 300kDa) and (10kDa - 200kDa) respectively. These ranges were taken from Assay-Protocol-Home. ([http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page](http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page))

### 2.6.4 Electroblotting

Electroblotting was performed to transfer the separated proteins from SDS-PAGE gel to polyvinylidene fluoride (PVDF) membrane (0.2µm, Bio-Rad, UK) using either a semi-dry or a wet transfer technique. The former technique is efficient for transferring the low MW protein while the latter is preferable for the transfer of the large MW proteins [201].

#### 2.6.4.1 Semi-dry protein blotting

PVDF membrane was first activated in 100% methanol. The gel was dismantled and soaked along with blotting filter papers (Whatman Plc, Brentford, UK), sponges and activated membrane in freshly prepared 1x transfer buffer. Thereafter, these components were assembled in specific order as shown in Figure (2.5) to constitute a gel sandwich. The sandwich was placed in a correct orientation in the cassette of the transfer machine (Bio-Rad, Trans-Blot Turbo Transfer System,
Singapore), so that the gel and membrane were in the side of cathode and anode plates respectively. The proteins were transferred at 25V and 1ampere (A) for 30min. Once the transfer was completed, the sandwich was disassembled and the membrane was immersed in Ponceau S solution to check the efficiency of the protein transfer process. The membrane was washed with PBST to remove the stain.

![Figure 2.5 Components of gel sandwich](image)

**2.6.4.2 Wet protein blotting**

The components of gel sandwich used for wet transfer were the same as those of semidry. They were also assembled in the same manner as shown in Figure (2.5). The cassette was then inserted in the tank of transfer apparatus in a position that allowed the gel to be close to the black cathode side while the membrane to be close to red anode side. Transfer tank was filled with ice-cold 1x transfer buffer and placed on ice-filled box to keep the buffer cool during the transfer process protecting the protein from heat-degradation. The transfer was performed at 100V for 1.5hr. The membranes were handled in the same way mentioned earlier in section (2.6.4.1).
2.6.5 Immunodetection of target proteins

Before probing PVDF membrane with antibodies, it was first necessary to immerse it in blocking buffer for 1hr at RT with gentle shaking using a rotating gyrorocker mixer (Staurt Scientific, Staffordshire, UK). Blocking step was important to avoid nonspecific binding of the antibodies and consequent high background problem on the membrane. Blocking buffer was made either from non-fat dried milk (Café Blanco, Douwe Egberts, France) or BSA dissolved in TBST at 5% (w/v) final concentration. The membrane was briefly rinsed with PBST and then incubated with a primary antibody overnight at 4°C with shaking. The primary antibody was prepared in TBST (pH 7.6) containing 5% (w/v) non-fat dried milk or BSA. To remove excess and nonspecific bound antibody, the membrane was washed three times with PBST; 15min per rinse at RT with shaking. Thereafter, the membrane was incubated with a secondary antibody for 2hr at RT on shaker. The same procedures were used to prepare the antibody and wash the membrane as that of the primary antibody. The secondary antibody is conjugated with a horseradish peroxidase enzyme that facilitates the chemiluminescent detection of target proteins when the membrane is developed using special detecting reagents. The prepared antibodies were stored at -20°C for multiple uses. Details about antibodies used are stated in Table (2.3).
Table 2.3 Primary and secondary antibodies used for immunodetection

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
<th>MW (kDa)</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO2</td>
<td>Rabbit polyclonal</td>
<td>Abcam (UK)</td>
<td>1:1000</td>
<td>26</td>
<td>ab137612</td>
</tr>
<tr>
<td>NQO1</td>
<td>Mouse monoclonal</td>
<td>Cell Signaling (UK)</td>
<td>1:1000</td>
<td>29</td>
<td>A180</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz (UK)</td>
<td>1:1000</td>
<td>37</td>
<td>M-20: sc-718</td>
</tr>
<tr>
<td>Phospho-Cyclin D1 (Thr286)</td>
<td>Rabbit monoclonal</td>
<td>Cell Signaling</td>
<td>1:500</td>
<td>36</td>
<td>D29B3 (XP)</td>
</tr>
<tr>
<td>CDK4</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz (UK)</td>
<td>1:1000</td>
<td>34</td>
<td>C-22: sc-260</td>
</tr>
<tr>
<td>Rb</td>
<td>Rabbit monoclonal</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>110</td>
<td>D20: 9313</td>
</tr>
<tr>
<td>Phospho-Rb (Ser807/811)</td>
<td>Rabbit polyclonal</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>110</td>
<td>9308</td>
</tr>
<tr>
<td>P-gp</td>
<td>Rabbit polyclonal</td>
<td>Abcam (UK)</td>
<td>1:1000</td>
<td>141</td>
<td>ab129450</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse monoclonal</td>
<td>Sigma-Aldrich (UK)</td>
<td>1:10000</td>
<td>42</td>
<td>A1978</td>
</tr>
<tr>
<td>HRP-linked anti- rabbit IgG</td>
<td>Secondary</td>
<td>Sigma-Aldrich (UK)</td>
<td>1:5000</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HRP-linked anti- mouse IgG</td>
<td>Secondary</td>
<td>Cell Signaling</td>
<td>1:2500</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

2.6.6 Chemiluminescent detection

1ml of both ECL substrate and ECL enhancer (Bio-Rad, UK) were mixed at 1:1 ratio and dispensed on the membrane surface for few seconds prior to inserting the membrane into the charge coupled device imager (ChemiDoc MP Imaging system, Bio-Rad, USA). ECL was oxidised immediately by horseradish peroxidase enzyme-conjugated with secondary antibody, generating light as by-product. The light was visualised and analysed using image lab software connected with imager. The membrane was then rinsed with PBST and stored at 4°C for further immunological detection.
2.7 Immunofluorescence assay

Immunofluorescence (IF) assay was used to evaluate the expression levels of target proteins by tracking the fluorescence signals using fluorescence microscope. The intensity of signals was directly proportional with protein levels in the cells. It was also useful to investigate the efficiency of lentiviral transduction process of OVC cells stably expressing shRNA sequence by tracking red fluorescence protein (RFP) emitted upon dox induction.

2.7.1 Preparation of monolayer cover slip

Cells were seeded in sterile coverslip in 6-well plates at a seeding density of 100,000 cells. Once the cells reached 60-70% confluence, they were washed with PBS and fixed with 10% (v/v) formalin for 10 min at RT. This was followed by three times washing with PBS (5 min per wash).

2.7.2 Antibody labeling

To facilitate antibodies penetration, cells were permeabilised with triton X-100 (0.1% v/v) for 15 min and then washed three times with PBS. Non-specific binding of antibodies was prohibited by incubating the cells with PBST blocking buffer for an hour at RT. The blocking buffer was composed of 10% goat serum, 1% BSA, and 22.52 mg/ml glycine. It was preferable to block the cells with serum raised in the same species of secondary antibody. Glycine was used to quench autofluorescence signals produced by formalin. A plastic box covered with both water-soaked filter paper and parafilm was prepared to make a humidified chamber for coverslips protecting the cell monolayer from dehydration. Coverslips were briefly washed with PBS and then the cell side was placed on the top of 100 µl anti-NQO2 primary antibody blob and incubated overnight at 4°C. Negative control coverslip was also run with other samples where the cells were incubated with PBST (1% (w/v)) buffer in place of primary antibody in order to investigate the specificity of secondary antibody. The following day, coverslips were transferred to their respective places in 6-well plates for washing with PBS. They were then incubated in the dark with 100 µl blobs of secondary antibody conjugated with Alexa Fluor 488 (green) for an hour at 37°C. The details about
the antibody used for IF assay are shown in Table (2.4). The coverslips were washed again to remove excess unbound antibody. One spot of prolong diamond anti-fade mounting medium already mixed with DAPI (ThermoFisher, UK) was applied onto microscopic slides. DAPI is a blue counterstain used to visualise nuclear morphology and determine subcellular location of protein of interest. The cell side was then inverted and placed on the top of spot. The coverslips were sealed by nail polish and stored at 4°C in dark for snapshot imaging.

Table 2.4 Primary and secondary antibodies used for immunofluorescent analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution Ratio</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO2</td>
<td>Rabbit polyclonal</td>
<td>Proteintech</td>
<td>1:50</td>
<td>15767-1-AP</td>
</tr>
<tr>
<td>Anti-rabbit Alexa Fluoro-488</td>
<td>Goat polyclonal</td>
<td>Invitrogen</td>
<td>1:1000</td>
<td>A-11008</td>
</tr>
</tbody>
</table>

2.7.3 Fluorescence microscopic analysis

Olympus BX51 upright wide-field fluorescence microscope was used to visualise the fluorescent cells. The images were acquired through a Coolsnap EV camera (Photometric) by using Meta Vue software (Molecular Devices, Berkshire, UK) which was also used to set up the image settings. Special bandpass filters for DAPI (blue), FITC (green) and TXRD (red) fluorophores were used to selectively isolate fluorescence emission wavelengths

2.8 Assessment of drug toxicity using MTT tetrazolium assay

2.8.1 MTT assay concept

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay is a cell-based colorimetric assay used in this project to measure cytotoxicity of a broad spectrum of compounds. In viable cells, yellow MTT dye is reductively metabolised to purple formazan crystals that absorb maximally at 540nm wavelength. The intensity of purple colour is directly proportional to the
number of viable cells. Thus, formation of purple colour is an effective marker of cell viability and can also be used to indicate the cytotoxic effects of the compounds [202].

2.8.2 Cell seeding

The optimal seeding density of each cell line was first determined in a preliminary experiment to ensure that the cell populations were in exponential growth throughout the assay, Table (2.5).

After harvesting the cells and determining the cell concentration, 200µl of cell suspension containing the optimum cell number was seeded in triplicates into 96-well plates. The plates were placed overnight in a humidified incubator, allowing the cells to recover and attach before starting the treatment.

Table 2.5 Recommended seeding density of OVC cells used for MTT assay

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Seeding Density (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>3750</td>
</tr>
<tr>
<td>SKOV-3 (WT)</td>
<td>7500</td>
</tr>
<tr>
<td>SKOV-3 (NTC)</td>
<td>7500</td>
</tr>
<tr>
<td>SKOV-3 (sh27)</td>
<td>7500</td>
</tr>
<tr>
<td>CaOV-3</td>
<td>10000</td>
</tr>
<tr>
<td>OV-90</td>
<td>15000</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>15000</td>
</tr>
<tr>
<td>TOV-21G</td>
<td>10000</td>
</tr>
<tr>
<td>TOV-112D (WT)</td>
<td>5000</td>
</tr>
<tr>
<td>TOV-112D (NTC)</td>
<td>5000</td>
</tr>
<tr>
<td>TOV-112D (NQO2-OE)</td>
<td>5000</td>
</tr>
</tbody>
</table>

2.8.3 Cell treating

Stock solutions of all tested compounds were prepared in DMSO or sterile PBS, Table (2.6). DMSO was mostly utilised to dissolve the hydrophobic compounds. DMSO tolerance assay was
performed to determine non-toxic range of concentration of DMSO at which the cells remained survival, and it was found to be between 0.2% and 0.5% (v/v) depending on the cell type. Solvent control wells, where the cells were incubated with solvent alone, were also run along with treated wells. Medium control wells with no cells were included to eliminate the background effect of medium.

Serial dilutions of the compounds were freshly prepared in the growth medium at concentrations five times higher than the desired final concentrations; 50µl of each dilution was dispensed to the wells at 1:5 dilution ratio. Each compound was tested at a different range of concentrations selected based on the findings of preliminary experiment for the compounds’ cytotoxicity, Table (2.6). The exposure period of the cells to the compounds varied from 3hr to 96hr. For the brief exposure periods, the medium containing treatments was aspirated after 3hr or 24hr and the cells re-nourished with 250µl fresh growth medium for the remainder of 96hr period. For the long exposure period, the cells were continuously exposed to the treatments for the whole period of 96hr.
Table 2.6 Selection of compounds which were studied in this project at particular ranges of concentrations

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Solvent</th>
<th>Stock Concentration Range</th>
<th>Final Concentrations Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Typical inhibitors of NQO2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>DMSO</td>
<td>(10µM-20mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>DMSO</td>
<td>(10µM-20mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td>(NSC13000 or 9AA)</td>
<td>DMSO</td>
<td>(10µM-20mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td><strong>Novel compounds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4, 8, 10, 11, 13, 20, 21, 24, 25 and 27)</td>
<td>DMSO</td>
<td>(10µM-25mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td>(9 and 14)</td>
<td>DMSO</td>
<td>(10µM-20mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td>16</td>
<td>DMSO</td>
<td>(10µM-25mM)</td>
<td>(0.1µM-100µM)</td>
</tr>
<tr>
<td>26</td>
<td>DMSO</td>
<td>(10µM-15mM)</td>
<td>(0.1µM-100µM)</td>
</tr>
<tr>
<td><strong>CB1954</strong></td>
<td>DMSO</td>
<td>(10µM-50mM)</td>
<td>(0.001µM-500µM)</td>
</tr>
<tr>
<td><strong>Carboplatin</strong></td>
<td>PBS</td>
<td>(10µM-10mM)</td>
<td>(0.001µM-100µM)</td>
</tr>
<tr>
<td><strong>Doxorubicin or adriamycin (ADR)</strong></td>
<td>PBS</td>
<td>(50µM-10mM)</td>
<td>(0.001µM-20µM)</td>
</tr>
<tr>
<td><strong>Puromycin</strong></td>
<td>PBS</td>
<td>(500µg/ml-25mg/ml)</td>
<td>(0.25µg/ml-7µg/ml)</td>
</tr>
<tr>
<td><strong>EPR</strong></td>
<td>PBS</td>
<td>(500µM-10mM)</td>
<td>(1µM-100µM)</td>
</tr>
</tbody>
</table>

The compounds investigated in this study were found to be completely soluble at these ranges in the presence of a proper solvent. The compounds were serially diluted in growth medium and then tested at a wide range of final concentrations. Selection of these ranges was based on the toxicity results of preliminary toxicity experiments in which different ranges were tested.

### 2.8.4 Terminating the actions of the compounds

MTT solution (2.5mg/ml) was prepared by dissolving 1.250g MTT powder in 500ml PBS. It was stored in a foil-wrapped bottle at 4°C. Following 96hr incubation, 50µl MTT solution was added into the cells and incubated for 4hr at 37°C. The medium was then aspirated and the formed formazan
crystals were dissolved in 200µl DMSO. The plate was agitated on the plate shaker for 5min before reading the absorbance using the plate reader.

2.8.5 Calculating IC₅₀ values

The ODs of the cells were measured on the multi-well scanning spectrophotometer at 540nm by using the Gen 5 Software package (BioTek). They were used to calculate the fractions of survival cells, which remained unaffected by the treatment, as percentages relative to the solvent control where cell survival is considered as 100%. Dose-response curve was generated by plotting cell survival (%) against the final concentrations of drug (µM) using the GraphPad Prism 7 Software. Nonlinear regression analysis was used to fit the data with the best line. IC₅₀ values were determined from the curve. It was defined as the concentration of drug required to decrease cell survival by 50% relative to solvent control [203]. IC₅₀ values were calculated from at least three independent experiments.

2.9 Inhibition of cellular NQO2

To evaluate functional activity of novel compounds as inhibitors of cellular NQO2, cytotoxicity of CB1954 in conjugation with those compounds were evaluated in cells possessing high levels of NQO2 using MTT assay. As NQO2 has a unique ability to activate CB1954 to cytotoxin, subsequent toxicity developed after CB1954 activation can be used as an indirect measure of the compounds’ potency against NQO2 activity.

Cell suspension containing the optimum number of cell shown in Table (2.5) was loaded in 96-well plates at a volume of 150µl per well and incubated overnight. The next day, cells were treated with a range of concentration of CB1954 combined with 100µM EPR in concomitant with a fixed concentration of the compounds. CB1954 stock solutions were serially diluted in growth medium and 50µl of each dilution was loaded in sextuplicate across the plate covering this range of final concentration (0.001-10µM). One half of the CB1954 treated plate was co-treated with 50µl working solution containing a fixed dose of compounds whereas the second half was loaded with
the same volume containing fresh medium-free of treatment. Cells were then incubated with the combination therapy for 24hr and 96hr. For a short exposure period, 24hr, the medium was replaced with 250µl of fresh medium and the cells were re-incubated for a further 72hr. CB1954 cytotoxicity was evaluated using MTT assay as described in section (2.8.4). The control treated with inhibitors only was also included and used as a reference control to calculate the relative percentage of survival cells that remained unaffected by CB1954. This allowed for the exclusion any marginal toxicity developed by inhibitors themselves although they were used at non-toxic and sub-IC\textsubscript{50} concentration.

### 2.9.1 Evaluation of intracellular NQO2 inhibitory potency of the compounds

Cells were seeded in 96-well plates (150µl/well), and following overnight incubation they were concomitantly treated with a fixed concentration of CB1954 plus 100µM EPR and a non-toxic range of concentration of each compound. Serial dilutions of the compounds were made in the growth medium covering varying ranges of concentrations which were selected depending on the compounds’ toxicity. 50µl of each dilution was pipetted in sextuplicate across the plates. This was followed by the addition of 50µl of medium containing CB1954 treatment into one half of the plates at a final concentration of 1µM. This concentration reduces SKOV-3 cells survival by 60%. Inhibitory potency of those compounds was estimated by measuring percentages of survival cells which were protected from cytotoxic effect of 1µM concentration of CB1954 after 24hr and 96hr incubations. After 24hr treatment, the cells were re-nourished with fresh medium-free of treatment and incubated for a further 72hr. The experiment was terminated upon the addition of MTT solution. Dose-response curves illustrated the levels of protection provided by the compounds against CB1954 toxicity, and were used to extract the compounds’ IC\textsubscript{50} of intracellular NQO2. IC\textsubscript{50}, in this context, is defined as the concentration of inhibitors that protect the cells from cytotoxicity of CB1954 by 50% relative to control as a result of inhibition of 50% of NQO2 enzymatic activity in the cells.
2.10 Colony survival assay

The principle of clonogenic assay is based on the ability of an individual cell to grow and proliferate persistently, originating a single colony consisting of 50 cells or more [204]. The assay was used in this project to investigate the impact of modulating NQO2 levels on the capacity of cells to form colonies. The protocol used herein was according to the original method of Puck and Marcus (1956) with some modifications previously described by Franken et al. [204, 205].

2.10.1 Cell preparation and treatment

Exponentially growing cells were harvested and passed twice through a 21 gauge needle to make a single cell suspension. Cells were counted twice before preparing serial dilutions of cell suspension. A preliminary experiment was conducted to optimise cell seeding density required for each treatment condition to produce a number of colonies ranging between 50 and 100. Seeding densities used in this study were from 100 to 6000 cells depending on experimental conditions. The cells were plated in 6cm culture dishes and incubated overnight before treating the cells with EPR. After a week of treatment, untreated control dishes were examined under the microscope to check whether the cells had started to generate colonies. The optimal seeding duration for SKOV-3 and TOV-112D cell lines was 14 days to produce proper colonies.

2.10.2 Methylene blue cell colony staining

After 14 days incubation, colonies were fixed with 2ml of 70% (v/v) ethanol for 15min and then stained with 0.5% (w/v) methylene blue dissolved in 70% (v/v) methanol for 20min. Thereafter, the dishes were washed carefully with tap water to remove unbound stain. Dishes were left to air-dry before counting the colonies.

2.10.3 Colony analysis

A colony consisting of at least 50 cells was counted and documented to measure other parameters. Plating efficiency (PE) was calculated by dividing final numbers of colonies by the initial numbers of
Chapter 2

cells, Equation (2.0.3). It was used to indicate the percentages of surviving colonies. Survival fraction (SF) was calculated by dividing averaged PE of treated cells by that of untreated control in order to evaluate the effect of treatment on the ability of cells to survive and form colonies, Equation (2.0.4). The experiment was independently repeated three times and results were plotted on a log scale using GraphPad Prism version 7.0 Software.

Equation (2.0.3): Plating efficiency (PE) = (final number of colonies at each condition/ the initial cell seeding density) x 100%.

Equation (2.0.4): Survival fraction (SF) = (averaged PE of treated wells/ averaged PE of untreated wells)

2.11 Generation of stably NQO2-overexpressing TOV-112D cells

TOV-112D (NQO2-OE) cells sub-line stably overexpressing NQO2 was generated using HIV-based lentiviral vector system (GeneCopoeia, USA). In this system lentiviral transfer vector transduces and integrates the gene of interest (such as NQO2 overexpression construct) into the DNA genome of TOV-112D (WT) cells, generating stable NQO2 overexpressing cells. The procedure is described in Scheme (2.1). To produce lentiviral vector titer, Lenti-Pac HIV expression packaging system and lentiviral constructs were co-transfected into HEK293-FT packaging cells. The Lenti-Pac system is composed of lentiviral packaging plasmid mix, EndoFectin transfection reagent and Titer Boost concentration reagent. Lenti-pac plasmid mix is a mixture of plasmids that encode structural and regulatory elements required to make highly infectious lentiviral pseudo-typed particles. Lentiviral constructs of interest, including plasmid-expressing NQO2 (Cat. No. EX-C0280-Lv105-10) and negative control pReceiverLv105 (Cat. No. EX-Neg-LV105) were purchased from GeneCopoeia. pReceiver Lv105 is a puromycin resistance vector that carries either NQO2 expression or negative control construct. 293-FT cells are highly transfectable and high-titer lentivirus producer cells derived from embryonal kidney cells. The protocol used to make these cells was developed by the manufacturer of the kits and described in details in the following sections.
HIV-based lentiviral vector system from GeneCopoeia was used to prepare stably gene-expressing cells. Briefly, (1) lentiviral DNA plasmid constructs and lentiviral packaging plasmid mix were mixed at 1:1 ratio in a falcon tube containing Opti-MEM medium. In a separate tube, EndoFectinLenti transfection reagent was diluted in Opti-MEM medium at 1:13 ratio. (2) Diluted transfection reagent was then added drop by drop to the diluted DNA tube and the mixture left for 20min at RT to form complexes. (3) 293-FT cells were transfected with DNA/EndoFectinLenti complexes. 8hr later, the medium was replaced with DMEM containing 5% FBS and TiterBroost and the cells were further incubated until 48hr post-transfection. (4) Culture medium containing lentiviral particles were harvested and filtered. (5) Supernatant was then aliquoted and stored at -80°C. (6) To transduce target cells, lentivirus was diluted at 1:4 ratio in culture medium containing 10% FBS and polybrene (5μg/ml) and incubated with the cells for 48hr. (7) To purify the culture from non-transduced cells, the culture were incubated in conditioned medium containing puromycin for selection purpose. Adapted from GeneCopoeia’s manual.

Scheme 2.1 Preparation of genetically modified cells stably expressing sequence of interest
2.11.1 Production of pseudolentiviral particles

293-FT cells were plated at a plating density of 1,500,000 cells in 10cm culture dishes preloaded with 10ml DMEM supplemented with 10% FBS. Following overnight incubation, the cells should be evenly distributed and covered 70-80% of the dishes surface. DNA/EndoFectinLenti complexes were prepared in multiple steps. 2.5µg lentiviral plasmid and 5µl Lenti-Pac plasmid mix were diluted into a sterile falcon tube preloaded with 200µl Opti-MEM medium. In another sterile falcon tube, 15µl EndoFectinLenti transfection reagent was diluted into 200µl Opti-MEM medium. The diluted EndoFectin solution was added drop wise into the DNA plasmids containing tube whilst gently vortexing. This mixture was left for 20min at RT to allow the formation of lentiviral complexes. To transfect the plated packaging cells, DNA/EndoFectinLenti complexes were added directly and in a drop wise manner to the cells whilst swirling the dishes for equal distribution. Cells were incubated for 8hr for efficient transfection before replacing the medium with fresh DMEM containing 5% FBS and TiterBoost reagent (diluted at 1:500 volume). This reagent was used to enhance the titer production by 5-10 folds. The plates were further incubated until 48hr post-transfection.

2.11.2 Harvesting lentiviral particles

Pseudovirus-containing culture medium was collected 48hr post-transduction and centrifuged at 500xg for 10min to pellet cell debris. Supernatant was then filtered using syringe bound with 0.45µm polyethersulfone (PES) low-protein binding filters. To preserve lentiviral particles, the lentiviral stock was aliquoted in 1ml cryovials and stored at -80ºC until further uses for transduction.

2.11.3 Lentiviral transduction of target cells

Low passage, mycoplasmic free cells were plated in 6cm dishes at 250,000 plating density per dish and incubated for 24hr. The next day, the medium was replaced with 3ml fresh culture medium containing 10% FBS, 2mM glutamax and 5µg/ml polybrene. Polybrene was used to decrease repulsion between lentiviral particles and target cells thereby enhancing the transduction efficiency.
1ml lentiviral supernatant containing the construct of interest was added directly and in a drop wise manner to the target cells, diluting lentiviral particles to 1:4 ratio. The cells were incubated for 48hr for optimal transduction. They were then cultured in selection medium containing optimised concentration of puromycin. The conditioned medium was replaced every three days with a fresh one containing puromycin for up to three weeks. Once the cells became confluent, a part of cell suspension was split into bigger flask while the rest was frozen at -80°C. The cells were handled in the same manner used for non-transduced cells.

2.11.4 Puromycin selection

To get purified culture containing only the transduced cells, puromycin was added to the cell culture at a concentration sufficient to kill WT non-transduced cells. To determine the optimal concentration of puromycin, MTT toxicity assay was performed as described in section (2.8). The WT cells were seeded in 96-well plates. Serial dilutions of puromycin stock solution (25mg/ml) were prepared in culture medium covering this range of concentration (0.25-7µg/ml). The cells were incubated with the conditioned medium for 48hr. Thereafter, the medium was replaced with a fresh one containing the same range of concentration of puromycin for a further 48hr before terminating the experiment by adding MTT solution. Puromycin-kill curve was generated by plotting percentages of survival cells against concentration range of puromycin. The optimal concentration selected to purify the culture was that lowest concentration that killed almost 100% of non-transduced WT cells.

2.12 Generation of stably expressing shNQO2 SKOV-3 cells

SKOV-3 cells sub-lines stably expressing doxycycline-inducible shRNA silencing sequence against NQO2 were generated using inducible TRIPZ lentiviral shRNA system (Dharmacon, UK). This system is composed of pTRIPZ lentiviral inducible vector-containing specific antisense sequence and Dharmacon Trans-Lentiviral packaging system. pTRIPZ transfer vector is designed to be Tet-on as depicted in Scheme (2.2). It consists of various components, including: First, tetracycline response element (TRE) promoter which drives the expression of shRNA and RFP sequences.
Second, reverse tetracycline transactivator which binds to TRE promoter in the presence of dox to induce sequences expression. Third, TurboRFP reporter used to estimate the transduction efficiency. Last, puromycin resistance marker which provides protection to the cells from puromycin. The following protocol was amended from the original protocol suggested by manufacturer.

Scheme 2.2 Schematic map of TRIPZ lentiviral carrying shRNA of interest (Dharmacon)

2.12.1 Chemicals and reagents

Lennox broth (LB) is a growth medium used for culturing E.coli bacterial cells. It was prepared at 2% (w/v) and promptly autoclaved at 125°C to inhibit unwanted growth. Polyethylenimine (PEI) is a transfection reagent used as a replacement of calcium phosphate transfection reagent received with the kit. PEI was prepared in distilled water at 7.5mM stock concentration and the pH of the solution was adjusted to pH 7.4 using 5N NaOH. NaCl was also prepared in distilled water at 150mM stock concentration. Both solutions were filtered and then stored at 4°C for further use. Concentrated lentiviral particles were preserved for long time in formulation buffer which was prepared by dissolving 1mg/ml human serum albumin, 5µg/ml protamine sulphate, and 40mg/ml lactose (pH 7.2) in 50ml sterile PBS.

Four different shRNA plasmids were received in four glycerol stocks, and of these were shRNA22, shRNA27 and shRNA29 which all worked against NQO2, in addition to non-targeting control (NTC) sequence. The nucleotide sequences of each antisense strand are illustrated in Figure (2.6, A)
They were prepared and introduced into the genome of target cells separately. These antisense strands target different regions of human NQO2 gene, as shown in Figure (2.6, B).

(A)

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Mature Antisense Sequence</th>
<th>Sense sequence of NQO2 gene targeted by shRNA antisense strands</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2THS_131222 shRNA22</td>
<td>TGCAATTTCAGGAGCAAGG</td>
<td>ACGTTAAAATGTCCTTCGCCCTC</td>
<td></td>
</tr>
<tr>
<td>V3THS_369427 shRNA27</td>
<td>TGCTTGTAGGCTTCGATGGG</td>
<td>ACGAAGATTGCAGAGCCAGGCC</td>
<td></td>
</tr>
<tr>
<td>V3THS_369429 shRNA29</td>
<td>CTTCGTGTACATCTCGGCC</td>
<td>GAAGCAGACATGAGACGCCG</td>
<td></td>
</tr>
</tbody>
</table>

(B)

Gggccgaggagctggattagggcctcggtgatagcccggcaggctgctgtaagagagccctgtgccgaggggcaggtgattcggctttgaatcatcactgggacctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc

(C)

Figure 2.6 Nucleotide sequences of shRNA constructs used for silencing human NQO2 gene with their complementary strands (continue on next page)
A. The antisense strands were purchased from Dharmacon Life technology. They were used to silence NQO2 in SKOV-3 cells by targeting their complementary sense strands located at different regions in human NQO2 gene. B. This map of nucleotide sequences is for human NQO2 gene. It was obtained from Pubmed with an Accession Number of NM_001290222 and National Center for Biotechnology Information (NCBI) Reference Sequence of NM_001290222.1. The underlined sequences in text highlighted bold are the sense strands targeted by different shRNA antisense constructs C. Nucleotide sequence of a particular region of human NQO2 gene targeted by shRNA29 was compared with that of NQO1 gene to show the high similarity of these genes in this particular region. Because of this similarity, it was highly possible that NQO1 will be misleadingly targeted by shRNA29 as well. The blank areas are those positions where the sequences of both genes match. This particular region of NQO2 gene nucleotide sequence was obtained from Pubmed with NCBI Reference number of NM_001290222.1 while NQO1 nucleotide sequence was got from NQO1 gene map sequenced by Jaiswal et al. [206].

2.12.2 Preparation of lentiviral plasmids

Glycerol stocks of E-coli-containing shRNA plasmid construct were thawed from -80°C on ice. Four falcon tubes were prepared for each construct. Each tube was preloaded with 5ml LB medium containing carbenicillin (100µg/ml) used to enhance the plasmids stability. Inoculum from each glycerol stock was dispensed into four tubes (10µl per tube). The tubes were then incubated for 18hr at 37°C with vigorous shaking. To harvest E-coli cells, the culture was centrifuged at 6800xg for 2min. The plasmid DNA of E-coli cells was then extracted using GeneJET plasmid miniprep kit (Thermo Scientific, USA). To do this, purification using centrifuge method was selected as suggested by manufacturer. NanoDropLite spectrophotometer (Thermo Scientific, USA) was used to measure DNA concentration in (ng/µl) at A260nm wavelength, and to evaluate DNA purity at 260/280nm ratio which should be between 1.8 and 2.1.

2.12.3 Production of lentiviral particles

HEK293-T cells were plated at a plating density of 12,000,000 cells in 15cm culture dishes preloaded with 16ml DMEM supplemented with 10% FBS to generate 70% confluence culture in the following day. DNA/PEI complex was prepared as following: First, 9µg lentiviral shRNA plasmid and 7µl Trans-lentiviral packaging mix were diluted into 2ml NaCl (150mM). This was carried out for each shRNA plasmid. The mixture was then left for 10min at RT. Second, in another sterile falcon tube, PEI was mixed with NaCl at 1:15 dilution ratio by mixing 65.52µl of 7.5mM PEI with
934.48µl of 150mM NaCl. The mixture was also left for 10min at RT. Third, PEI mix was added in a drop wise manner to shRNA plasmids whilst gently vortexing and the mixture was left for 10min at RT to allow the formation of complexes. To transfect HEK293-T packaging cells, 2ml of each shRNA/PEI complexes-containing distinct sequence was directly added to the cells in a drop wise manner whilst swirling the dishes for equal distribution. Cells were incubated for 18hr at 37°C before replacing the medium with 16ml fresh DMEM supplemented with 10% FBS. The plates were further incubated until the first time point of harvesting viral particles.

2.12.4 Harvesting lentiviral particles

Lentiviral particles were harvested twice; 48hr and 72hr post-transfection using the same protocol. In the first harvest, the medium containing lentiviral particle was collected into a 50ml falcon tube. The cells were then re-nourished with a fresh complete medium and incubated for a further 24hr (until the next harvest). The collected medium was centrifuged at 1000rpm for 5min in the bench top centrifuge to pellet cell debris. Furthermore, supernatant was filtered to remove any remaining cell debris, using a vacuum pump connected with 0.45µm cellulose acetate 1000ml, low-protein binding filters. The filtered supernatant was transferred to 50ml ultracentrifuge falcon tubes that can withstand the high centrifuge speed, and centrifuged at 13,500rpm for 2.5hr at 4°C using ultracentrifuge, 2169 rotor. Once the centrifugation process was completed, the supernatant was decanted inside the hood and the pellet location was highlighted as the pellet size is very small and unnoticeable. 100µl of formulation buffer was added to the pellet and mixed 10 to 25 times. The mixture was left on ice for an hour with periodic pipetting every 10min. This was performed to dissociate the lentiviral particle from the serum protein that came from the culture medium used for transfection. Thereafter, the mixtures were transferred to eppendorf tubes and centrifuged at 8,000rpm for a minute to get rid of pelleted debris and serum protein. The concentrated supernatant containing lentivirus was aliquoted and stored at -80°C until transducing the cells.
2.12.5 Determination of lentiviral titer via quantitative PCR (qPCR)

2.12.5.1 Preparation of DNA samples

To assess the transducibility and concentration of lentiviral particles, EL4s cells, which are mouse lymphoma suspension cells, were seeded in 12-well plates at a seeding density of 200,000 cells per well in RPMI medium supplemented with 10% FBS. The cells were transduced afterward with a series of 10-fold dilutions of lentiviral stock, with the exception of the cells in the first well which were transduced with 1µl neat vector from lentiviral stock ($10^{-2}$) as shown in Figure (2.7). Lentiviral stock ($10^{-3}$) was made as mentioned previously by re-suspending pelleted lentiviral in 100µl formulation buffer. Another 1µl neat vector was taken from the same stock ($10^{-3}$) and diluted in 9µl culture medium making ($10^{-4}$) dilution. 2µl was taken from this mixture ($10^{-4}$); the first 1µl was transferred to the second well labeled as ($10^{-3}$) while the second 1µl was further diluted in 9µl culture medium making ($10^{-5}$) dilution. In the same manner, 2µl was taken from ($10^{-5}$) mixture; the first 1µl was dispensed to the third well while the second 1µl was further diluted to make ($10^{-5}$) dilution used latter to transduce the fourth well. The same steps were followed with each preparation of lentiviral vector. The cells were transduced for 72hr before extracting the DNA using GenElute mammalian genomic DNA miniprep kit.
To measure the transducibility and concentration of lentiviral vectors, suspension EL4 cells were plated in 12-well plates and directly transduced with a series of 10-fold dilutions prepared from ($10^2$) stock solution containing neat vector. Cells left to be transduced for 72hr before extracting DNA for further analysis.

### 2.12.5.2 Preparation of calibration curves via qPCR technique

To measure the number of lentiviral transducing unit per ml (TU/ml) of lentiviral stocks, calibration curves of common transgenes (e.g. WPRE and UCR1 which are present in EL4 cells) were created using qPCR technique. UCR1 is a reference gene in the cells while WPRE gene is a lentiviral specific gene that is stably expressed in EL4 cells and constitutes part of the shRNA lentiviral vector used in this study; see **Scheme (2.2)**. By comparing the amount of WPRE sequence amplified in our samples with that of calibration curves, the lentivirus concentration (otherwise...
called titer) of the stock can be determined. TU/ml values of all lentiviral stocks carrying the shRNA of interest were kindly provided by Dr Stuart Ellison (University of Manchester). The method used is briefly explained herein. Specific volumes of DNA unknown samples and DNA standard samples were dispensed into 96-well optical reaction plates. DNA standard samples have known numbers of WPRE and UCR1 genomic copies ranging from 1 to 10000. WPRE and UCRI primers were transferred to all wells and the qPCR was run using BioRad real-time PCR system. After that, Ct values were determined from the amplification curve for unknown and standard samples. Ct value is defined as the number of cycles needed by amplification curves to reach the detectable threshold absorbance values. Ct values of standard samples were plotted against genomic copies number creating the standard curves with linear equations using Microsoft Excel Software.

2.12.5.3 Analysis

The number of genomic copies of both WPRE and UCR1 was determined for the unknown samples using linear equations of standard curves and then WPRE copy numbers were normalised to that of UCR1 reference gene. The normalised copy numbers were multiplied by two to calculate the copy number for the diploid cells. To measure the number of copies in the original stocks, copy numbers of those mixtures transduced with \((10^{-4})\) and \((10^{-5})\) lentivirus were multiplied by 10 and 100 dilution factors respectively. Mean values of all dilutions were first calculated and then applied in Equation (2.0.5), wherein number of cells seeded was 200,000 cells. Equation (2.0.5): 

\[
\text{TU/ml} = \text{number of cells on day of transduction} \times \text{genomes copy numbers} \times 1000\mu l
\]

2.12.6 Lentiviral transduction of target cells

Determination of the lentiviral titer enabled us to estimate the multiplicity of infection (MOI) of SKOV-3 cells. MOI is defined as the number of lentiviral transducing units required to transduce one cell. MOI is affected by several factors such as the cells nature—in other words, whether they are actively growing and dividing, the cells’ transduction efficiency, and the nature of inserted gene. SKOV-3 cells were transduced with shRNA-NQO2 at varying MOIs for subsequent selection. The
volume of vector was calculated using the following Equation (2.0.6): Volume of vector = (MOI x number of cells)/ transducing units (TU/ml)

SKOV-3 cells were plated at a seeding density of 200,000 cells per 25cm² flask. Two 25cm² flasks were prepared for each sequence to test the sequence’s efficiency to transduce the cells at two different MOIs (1 and 5). One extra flask was seeded with the same number of cells to determine the cell number on the transduction day. The flasks were incubated overnight at 37°C. The following day, the cells in the extra flask were counted to determine the required volume of lentiviral vector. The culture medium in the other flasks was substituted with 2ml of serum free RPMI medium containing a specific volume of lentiviral vectors. Following four hours of incubation, 2ml of RPMI medium supplemented with 20% FBS was loaded gently to the cells. The flasks were left for 72hr before replacing the medium with a fresh complete medium containing 1µg/ml puromycin to start the cell selection process. The cells were split into bigger flasks once they became confluent. They were cultured with the conditioned medium for approximately three weeks with periodic addition of puromycin every three days.

2.12.7 Doxycycline-inducing shRNA expression

To select the optimal concentration of dox and exposure time that generated a maximum silencing of NQO2 with minimal effect on NQO1 levels, SKOV-3 cells sub-line were exposed to varying concentrations of dox, including 0.5, 1, and 2µg/ml for 48, 72, 96, and 120hr. The activity and protein levels of both NQO2 and NQO1 were evaluated as described in sections (2.5.2) and (2.6) respectively.

2.13 Flow cytometer

2.13.1 Cell cycle analysis

Cell cycle distribution of OVC cells expressing genetically modified levels of NQO2 was examined using the protocol adapted by Charoenfuprasert et al. [207].
2.13.1.1 Sample preparation and propidium iodide staining

Cells were seeded in 6cm cell culture dishes at seeding density formerly optimised to generate 70% confluence culture of exponentially growing cells at the collection time point. Following a 4hr incubation, cells were treated with 100µM EPR or incubated with medium only in case of untreated control cells. In SKOV-3 transduced cells, the expression of shRNA-NQO2 silencing sequence was induced by treating the cells with dox (1µg/ml) for five days before seeding for the experiments. Dox was also added directly to the cells every other day until the collection time point. Cells were harvested by trypsinisation at 24hr time intervals until 96hr. They were collected in 3ml complete medium and pelleted by centrifugation at 500xg for 5min. Cell pellets were washed once with cold PBS and centrifuged at 500xg for 5min. They were resuspended in a small volume of PBS and fixed by a drop wise addition of 2ml of ice-cold 70% (v/v) ethanol whilst vortexing. Samples were stored for a maximum of one week at 4ºC. The collected samples were then centrifuged at 1000xg for 5min to remove ethanol. Pellets were resuspended in 300µl PBS followed by the addition of 50µl RNase A (125µg/ml) and 50µl propidium iodide (PI, 50µg/ml). The mixtures were then incubated at 37ºC in dark for 30min. Samples were kept on ice until subsequent analysis using flow cytometer. Since PI intercalates double stranded nucleic acid including RNA, RNase should be added to cell suspension along with PI in order to hydrolyse RNA and consequently block its interference effects on fluorescence signals of DNA. RNase solution was reconstituted in 10mM Tris-HCl (pH 7.5) containing 15mM NaCl. The solution was heated at 100ºC for 15min to hydrolyse contaminating DNase, and then left to cool at RT before storage at -20ºC.

2.13.1.2 Cell cycle data analysis

PI is a DNA-binding fluorescent dye with an excitation of 488nm and emission of 617nm wavelengths. The intensity of fluorescent signals emitted by PI-labelled cells is proportional to cellular DNA content (i.e. cells in G2-phase are two times brighter than cells in G1-phase [208]). LSR Fortessa fluorescence activated cell sorting (FACS) machine supplied with FL-2 channel was used to measure fluorescence intensity of PI-labelled cells. Fluorescence intensity of cell debris and aggregates were excluded from analysis by gating the single cells using forward-side scatter
plot, **Figure (2.8, A)**. Fluorescence intensity of single cell populations was then plotted against cell accounts to generate DNA histogram profile of the cells, **Figure (2.8, B)**. DNA histogram enabled us to differentiate the distinct cell cycle phases for a cell population consisting of 10,000 cells, which underwent further analysis using FlowJo Software version 10.2 to mathematically estimate the percentage of cells within each phase. The samples were run and histograms were analysed under the supervision of Mr Michael Jackson.

![DNA histogram profile](image)

**Figure 2.8 Gating of cells for flow cytometric analysis**

A. Forward-side scatter plot was used to gate cell population of single living cells for further analysis. B. DNA-histogram profile shows distribution of cells through cell cycle phases (G1, S and G2/M) which was analysed with FlowJo Software in order to determine the percentage of cells within each distinct phase.

### 2.13.2 Flow cytometric measurement of ROS levels in OVC cells

The intracellular basal levels of ROS in particular Hydrogen peroxide (H$_2$O$_2$) were measured in NQO2 genetically modified OVC cells using cell permeable pro-fluorescent 5-(and-6)-carboxy-2’7’-dichlorofluorescein diacetate dye (carboxy-DCFDA, invitrogen). Inside the cells, carboxy-DCFDA undergoes deacetylation by esterase converting it into non-fluorescent dihydroH$_2$DCF intermediary molecules that are subsequently oxidised by ROS to dichlorofluorescein (DCF) fluorescent
molecules [209, 210]. DCF emits green fluorescence that was collected by Fortessa machine through band pass filter 530/30. The intensity of fluorescent signal was directly proportional to the ROS levels in the cells. Fluorescence of intact single cells gated as shown in Figure (2.9, A) was only measured. FlowJo Software version 10.2 was also used to analyse the data collected from 10,000 events. Mean values were chosen to indicate the fluorescence intensity emitted from the samples and therefore to the levels of ROS. The DCF of the samples were first normalised to their respective samples with no carboxy-DCFDA dye Figure (2.9, B) and then the fold changes were calculated relative to dye-treated control, Figure (2.9, B).

![Figure 2.9](https://via.placeholder.com/150)

**Figure 2.9** Representative image of forward-side scatter plot and histograms for ROS analysis

**A.** Scatter plot was used to gate population of intact single cells which underwent further analysis to measure ROS levels. **B.** A histogram example of the cells incubated without carboxy-DCFDA dye. The background fluorescence intensity of these cells was used as a reference point to measure the fold increase in the samples’ fluorescence signal upon addition of the dye. **C.** A histogram example of cells incubated with carboxy-DCFDA dye. The fluorescence intensity is represented on x-axis of both histograms **B** and **C.** ROS enhances fluorescence intensity levels and consequently shifts the histogram to the right. The DCF intensity of treated cells was first normalised to their respective treated cells incubated with no dye and then fold change was measure relative to control.

Cells were plated in 6cm dishes and left to recover in the incubator for 24hr. The effect of EPR supporting NQO2 activity on basal levels of ROS was examined by treating the cells with EPR (100µM) for 24hr. The effects of the genetic and/or pharmacological inhibition of NQO2 on the ROS levels were also investigated by treating the cells for 24hr with NQO2 inhibitors either alone or in
the presence of dox. Actually, the cells had to be treated with dox (1µg/ml) five days prior to and throughout seeding for the experiment. Following a 24hr treatment, the cells were harvested and centrifuged at 13,000rpm for five minutes. Cell pellets were washed with PBS before incubation with carboxy-DCFDA dye for 30 min at 37°C. Carboxy-DCFDA was prepared at 10mM stock solution in DMSO and used at a working concentration of 10µM prepared in PBS. Dye was removed by spinning the cell suspension at 13,000rpm for five minutes. Cells were washed with PBS and resultant pellets were re-suspended in 500µl PBS for analysis. Samples were left on ice until flow cytometric analysis.

2.14 NRH synthesis

NRH was used in the assays to support the reductive activity of NQO2. The synthesis of NRH was carried out following the method established by Long et al. [139]. NADH was used as a starting material to synthesise NRH. NADH was analysed sequentially by phosphodiesterase 1 type IV and alkaline phosphatase VII-S enzymes. The former enzyme is responsible for the cleavage of phosphodiester bond of NADH whereas the latter enzyme works to remove the phosphate group from the intermediate product, forming NRH [211], Scheme (2.3).

Initially, 0.5g of NADH was dissolved in 20ml of sodium carbonate/bicarbonate buffer solution (0.4M, pH 10.0), followed by the addition of 0.01unit of phosphodiesterase and 500units of alkaline phosphatase. This mixture was incubated at 37°C for 16hr to ensure the complete digestion of NADH. The NRH was purified using preparative high performance liquid chromatography (HPLC) and then concentrated by using freeze dryer since it is a thermolabile product. The collected powder was stored at 4°C in a tightly sealed bottle. Nuclear magnetic resonance $^1$H-NMR $^{13}$C-NMR was also performed by the assistance of Dr Asma Belgath from Dr Freeman’s lab to check the purity of NRH. It is worth mentioning that NRH was used at the beginning of this project, but was later replaced by EPR, which is one of the NRH derivatives, because of its high availability.
2.15 Statistical analysis

The primary analysis of data was performed on Microsoft Excel software (2007) and then transferred to GraphPad Prism (version 7.0 for Windows, La Jolla California, USA, www.graphpad.com) for further statistical analysis. Student t-test was used to study the effect of one factor on one type of cells. One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons, test were applied to investigate the levels of significance effect of one factor on two different groups while two-way repeated-measures ANOVA was used to study the effects of two or more factors on two unrelated groups. Post hoc tests such as Tukey’s and Sidak’s multiple comparisons test were also selected to compare all samples with the control or with each other respectively. The number of asterisks indicates the significance levels (*\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\) and ****\(p \leq 0.0001\)). Non-linear regression analysis for survival data was also conducted to get the best fit line facilitating the extraction of the IC\(_{50}\) values.
Chapter 3

Evaluation of NQO2 and NQO1 protein expression and activity levels in a panel of OVC cell lines; generation of NQO2 genetically modified OVC cell lines
3 Evaluation of NQO2 and NQO1 protein expression and activity levels in a panel of OVC cell lines; generation of NQO2 genetically modified OVC cell lines

3.1 Introduction

Few studies are interested in measuring the expression levels of NQO2 in human tissue [212]. NQO2 has been found to be highly expressed in human skeletal muscle while moderately expressed in the kidney, heart, liver and human blood cells [80, 81]. The brain and pancreas have shown minimal expression of NQO2, whereas placental cells have shown no expression of NQO2 at all [81]. NQO2 expression has also been detected in different types of solid tumours. In breast cancer, a high expression level of NQO2 has been observed, which may confer protection against breast carcinogenesis through its roles in detoxifying estrogen-containing quinones [143, 213]. NQO2 is also expressed in human superficial bladder tumours [104], PC3 and CWR22Rv1 prostate and LoVo colorectal cancer cell lines. Furthermore, hepatocellular tumour tissue [106] and K562 myelogenous leukaemia cells have shown extremely high levels of NQO2 protein and enzymatic activity [105, 133]. Therefore, it was suggested that NQO2 may play a potential role in cancer, and that its inhibition may have therapeutic benefits on the disease. It seems that the association between NQO2 levels and OVC progression has not yet been studied. To address this, it was first necessary to assess the NQO2 levels in OVC cells. This chapter has two primary aims; firstly, to screen a panel of seven OVC cell lines for NQO2 protein and activity levels, and secondly, to generate NQO2 genetically modified cell lines.

3.2 Results

3.2.1 Basal expression levels of NQO2 and NQO1 in a panel of OVC cell lines

Western blot analysis was performed in order to determine the basal protein levels of NQO2 and NQO1 enzymes in a panel of seven OVC cell lines. Cell lysates were prepared and then quantified for the total protein concentration using a BCA assay. Volumes of the cell lysates containing an equal amount of 20µg protein were taken to analyse the enzymes expression levels, as explained
in section (2.6). Figure (3.1, A) represents an example of the resultant blot. The same blot was sequentially developed against NQO2, NQO1 and β-actin proteins. In order to facilitate the comparison of protein levels among cell lines, densitometric analysis using image J software was performed on the whole Western blot, measuring the intensity of the different bands. Then, the results related to NQO2 and NQO1 bands were normalised to their respective β-actin bands. The normalised data is represented graphically as shown in Figure (3.1, B). β-actin was used as a loading control to correct inconsistencies in the amounts of proteins stemming from technical errors [214].

Considerable variations in the protein levels of both NQO2 and NQO1 were detected among OVC cell lines. This variation was more pronounced in NQO1 than NQO2, whilst the overall protein expression level of NQO2 was lower than NQO1. The latter was detected mostly in all cell lines, except for TOV-112D cells, whereas NQO2 was only detected in four out of seven cell lines. NQO2 protein expression was highest in SKOV-3 cells, followed by OV-90, A2780 and TOV-21G which all (the latter three cells) had nearly comparable protein levels of NQO2. The SKOV-3 cell line is uniquely characterised by having higher protein levels of NQO2 than NQO1. Interestingly, TOV-112D was the only cell line that showed non-detectable levels of both NQO2 and NQO1, whereas other cell lines manifested high levels of either NQO1 or NQO2. The MDA-MB-468 breast cancer cell line was used in this study as a positive control for making comparisons with this panel of OVC cell line. Caraher M. (2012) has demonstrated that the NQO2 level in MDA-MB-468 cells is high, as also shown in Figure (3.1, A) [215]. It is obvious that the NQO2 protein level in SKOV-3 is significantly higher than that in MDA-MB-468 cells, Figure (3.1, B). Further characterisation of NQO2 and NQO1 levels in OVC cell lines should be made to select appropriate models for this study.
Figure 3.1 Basal NQO2 and NQO1 protein expression levels in a panel of cell lines

A. Cell lysates were prepared and analysed via the Western blot technique for measuring the protein levels. The same blot was probed with antibodies against NQO2, NQO1 and β-actin. This blot is representative of three independent experiments. B. Densitometric analysis was performed on three independent blots to measure the intensity of all bands. NQO2 and NQO1 bands were first normalised to β-actin, the mean of these values were then represented graphically with their standard errors (± SEM). The bars represent the standard errors. An unpaired t-test was used to analyse the data. The asterisk (*) indicates the level of significance between SKOV-3 and MDA-MB-468 cell lines, *p value<0.05. The plus signs (+++) refer to the positive control, MDA-MB-468 cells, used for comparison purposes.

3.2.2 Basal enzymatic activity levels of NQO2 and NQO1 in the panel OVC cell lines

The basal activity levels of both enzymes in OVC cell lines were measured using a cytochrome c-based spectrophotometric assay. This was done in order to assess whether the expressed NQO2 and NQO1 enzymes were metabolically active, and the activity directly correlated with the protein expression level. The correlation between enzyme activity and protein level was determined by measuring the strength of the relationship between these variables. To do this, the data regarding enzyme activity and protein levels was plotted on a scatter graph and Pearson’s correlation coefficient (r) was calculated using the Excel Correl function in Microsoft Excel 2007. The r value was used as a measure of the strength of correlation.

Cell lysates were prepared from the culture used previously for the Western blot and analysed for NQO2 and NQO1 activity; see section (2.5.2). To measure the activity of a specific enzyme in cell lysates consisting of a mixture of proteins, a selective inhibitor had to be added to the parallel
reaction mixture. Therefore, resveratrol and dicoumarol were used as NQO2 and NQO1 inhibitors respectively, to determine the amount of inhibited NQO2 and NQO1 enzyme activity. The lysates’ total protein concentration was quantified, so as to be able to measure the rate of enzyme activity, which was expressed as nmole of cytochrome c reduced by a metabolically active enzyme per minute of reaction relative to the protein concentration. An unpaired t-test was used to analyse the data.

Table 3.1 Basal activity levels of NQO2 and NQO1 in a panel of cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Enzyme Activity ± SEM (nmol/min/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NQO2</td>
</tr>
<tr>
<td>A2780</td>
<td>63.43 ± 8.3</td>
</tr>
<tr>
<td>* SKOV-3</td>
<td>153.29 ± 14.9</td>
</tr>
<tr>
<td>CaOV-3</td>
<td>15.56 ± 3.9</td>
</tr>
<tr>
<td>OV-90</td>
<td>49.40 ± 5.5</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>4.20 ± 0.4</td>
</tr>
<tr>
<td>TOV-21G</td>
<td>65.97 ± 7.8</td>
</tr>
<tr>
<td>TOV-112D</td>
<td>5.07 ± 0.7</td>
</tr>
<tr>
<td>++ MDA-MB-468</td>
<td>91.22 ± 8.2</td>
</tr>
</tbody>
</table>

Lysates prepared from OVC cell lines were analysed via a spectrophotometric assay to measure the enzymes activity. The NQO2 and NQO1 activity was attributed to the fraction that was inhibited by resveratrol and dicoumarol respectively, and measured relative to total protein concentration. It is expressed as nmole of cytochrome c reduced by active enzyme per minute per mg of protein. The tabulated data represents the mean values of enzyme activity calculated from three independent inter- and intra-experiments with their standard error (± SEM). The plus signs (++) refer to the positive control, MDA-MB-468 cells. The asterisk (*) indicates the level of significance between SKOV-3 and MDA-MB-468 cell lines, *p value<0.05. An unpaired t-test was used for data analysis.
Table (3.1) demonstrates a wide variation in the activity of NQO2 among OVC cell lines, with approximately a 36-fold range. NQO1 activity was also found to vary within this panel, but at a slightly higher range of 49-fold. The overall NQO1 activity level across the cell lines was greater than that of NQO2. NQO2 activity was the highest in SKOV-3, followed by A2780 and TOV-21G cells, while it was the lowest in both OVCAR-3 and TOV-112D cells. SKOV-3 cells also showed a much higher level of NQO2 activity than MDA-MB-468 cells (p value<0.05). All of these findings are consistent with the NQO2 protein levels measured in the same panel of cell lines. Thus, a very good correlation between the NQO2 protein level and enzymatic activity is evident in this panel of OVC cell lines, with an r value of 0.96, Figure (3.2, A). Similarly, the correlation between the NQO1 protein level and enzymatic activity is also shown to be good, although to a lesser extent than that observed with NQO2, with an r value of 0.7, Figure (3.2, B). Although NQO1’s protein level was very high in OV-90 compared to in TOV-21G cells, its activity level was interestingly higher in TOV-21G than in OV-90 cells. Similarly, NQO1 activity in A2780 cells was five-fold higher than in OVCAR-3 cells, although both cell lines had comparable NQO1 protein levels. Interestingly, it was found that NQO1 was active in TOV-112D cells, despite the detection of very low NQO1 protein levels. It is worth pointing out that the aforementioned interpretations for r values were suggested by Evan J., 1996 [216].
Figure 3.2 Correlation between protein level and enzyme activity of NQO2 and NQO1 enzymes

Scatter graphs were generated by plotting NQO2 and NQO1 activity (expressed as nmole/ min/ mg of protein) against their protein levels (measured by densitometric analysis). They were used to determine the strength of correlation between these variables by determining Pearson’s correlation coefficient (r) value. The closer the value is to 1, the stronger the correlation between the data is, as suggested by Evan J., 1996 [216].

3.2.3 Generation of stably NQO2-overexpressing TOV-112D cells

The TOV-112D cell line was selected to overexpress NQO2 in a stable manner, as it has several advantageous attributes compared to other cell lines. Firstly, the protein and enzymatic activity levels of NQO2 and NQO1 were much lower in TOV-112D than any other OVC cell lines, so the possibility of confusing NQO2 related findings with NQO1 is low. Secondly, the null state of P-gp allows for it to be used as a negative control to investigate the correlation between NQO2 levels and P-gp expression-mediating chemotherapy resistance. A HIV-based lentiviral vector system was used to generate isogenically cell-derived TOV-112D cell sub-lines; please refer to section (2.11) for more details about the protocol used. The genetic composition in these cell sub-lines is identical to that of its parent wild-type cells, with the only difference relating to the presence of an extra sequence. Within this text, cells expressing wild-type basal levels of NQO2 are referred to as WT, cells expressing the wild-type basal level of NQO2 and carrying an empty vector are termed EV, whilst cells overexpressing NQO2 are described as NQO2-OE.
3.2.3.1 Protein and enzyme activity levels of NQO2 and NQO1 in stably NQO2-overexpressing TOV-112D cells

Several assays were used to evaluate the protein and activity levels of NQO2 and NQO1 enzymes in TOV-112D cell sub-lines. The investigation’s primary aims were to find out whether NQO2 was successfully overexpressed, the resultant protein was also active, and whether NQO1 level was affected by modifying NQO2 levels in the cells. NQO2 and NQO1 protein levels were measured using Western blot and IF staining techniques. Samples containing 20µg protein were taken from prepared cell lysates and analysed for protein expression using the Western blot technique. The blot in Figure (3.3, A) represents three independent experiments. The intensity of bands was first quantified using densitometric analysis. Thereafter, the bands related to NQO2 and NQO1 protein were normalised to their respective β-actin. Fold differences in the enzymes protein levels in EV and NQO2-OE cells were determined relative to WT cells, which were used as a reference control. The average of fold differences which was calculated from three independent experiments is represented graphically with its standard error (± SEM), as shown in Figure (3.3, B). Regarding IF staining, TOV-112D cell sub-lines fixed on coverslips were incubated with an anti-NQO2 secondary antibody conjugated with fluorescence staining and with DAPI counterstain. IF signals of DAPI (blue) and NQO2 (green) were visualised and recorded using an Olympus wide-field fluorescence microscope; please refer to section (2.7) for further details regarding the protocol. The activity levels of NQO2 and NQO1 enzymes in the cell lysates prepared from the same culture were also measured using a cytochrome c spectrophotometric assay. The rate of enzymatic activity was calculated from three independent experiments and listed in Table (3.2) as mean ± SEM. It was expressed as nmole of cytochrome c reduced per minute per mg of protein.

Figure (3.3, B) illustrates the enormous increase in the expression level of NQO2 in the NQO2-OE sample, with more than a 400-fold difference compared to EV, (p value <0.005). Notably, the protein level of NQO2 in TOV-112D (NQO2-OE) cells was highly comparable to that which is naturally present in SKOV-3 cells, which is used in this section as a positive control. Similar levels of NQO2 were evident in both EV and WT cells, indicating that the transduction process had no impact on the basal levels of NQO2. This means that the significant increase in the cellular levels...
of NQO2 was mainly related to the ectopic overexpression of NQO2, rather than to the cells’ natural response to exogenous stress. Similarly, there was no detectable difference in NQO1 protein levels between WT and EV. However, a marginal reduction in NQO1 protein levels was noticed upon overexpressing NQO2 in NQO2-OE cells.

IF staining of TOV-112D cell sub-lines also proves that NQO2 protein levels are substantially increased in NQO2-OE cells, with the presence of a relatively very high-intensity of green signals compared to those detected in WT and EV cells, Figure (3.3, C). These findings support the Western blot data obtained. It is also worth noting that the location of IF signals within the cells indicated that NQO2 is not only a cytoplasmic protein but also nuclear protein.
Figure 3.3 Protein expression levels of NQO2 and NQO1 in cell-derived TOV-112D sub-lines (continue to the next page)
A. Cell lysates prepared from TOV-112D (WT) and its derived cell sub-lines (EV and NQO2-OE) as well as SKOV-3 cells were analysed via the Western blot technique for measuring NQO2 and NQO1 protein levels. The plus signs (++) refer to SKOV-3 cells, which were used here as a positive control. The same blot was developed against NQO2, NQO1 and β-actin. This blot is representative of three independent experiments. B. A densitometric analysis was performed on three independent blots. The NQO2 and NQO1 bands were first normalised to β-actin. Then, the fold differences were determined relative to WT, which was used as a reference control for comparison purposes. This figure represents the mean of these cells’ fold differences, with their standard errors (± SEM) represented by the bars. The three asterisks centered over the bar graph indicate the significant differences in the protein levels of NQO2 in NQO2-OE cells compared to EV cells (***p value<0.005). An unpaired t-test was used to analyse the data. C. IF staining of NQO2 protein (green) and DAPI nuclear counterstain (blue) observed in different cell sub-lines was viewed using a wide-field fluorescence microscope at 20x magnification.

NQO2 overexpression in NQO2-OE cells resulted in a significant enhancement in the activity levels of NQO2, with a 25-fold difference compared to EV. There was no detectable difference in the activity level of NQO2 in both EV and WT cells, supporting the idea that the transduction process itself did not influence the basal activity levels of NQO2 in the cells. The overexpression of NQO2 was, however, accompanied by a slight reduction in NQO1 activity, as observed in NQO2-OE cells compared to EV cells. All of these results were consistent with the Western blot analysis and therefore indicated the presence of a strong correlation between these variables.
Table 3.2 Activity levels of NQO2 and NQO1 in cell-derived TOV-112D sub-lines

<table>
<thead>
<tr>
<th>TOV-112D Cell Sub-lines</th>
<th>Enzyme Activity ± SEM (nmol/ min/ mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NQO2</td>
</tr>
<tr>
<td>WT</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>EV</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>** NQO2-OE</td>
<td>128.3 ± 4.9</td>
</tr>
</tbody>
</table>

The activity levels of NQO2 and NQO1 enzymes were measured in TOV-112D (WT, EV and NQO2-OE) cells using a cytochrome c spectrophotometric assay. NQO2 and NQO1 activity were attributed to that fraction inhibited by resveratrol and dicoumarol respectively and measured relative to total protein concentration. It is expressed as nmole of cytochrome c reduced per minute per mg of protein. The tabulated data represents the mean values of enzyme activity calculated from three independent experiments with their standard errors (± SEM). An unpaired t-test was also used to analyse the data, which showed a significant enhancement in NQO2 activity in NQO2-OE cells compared to EV cells; this is represented with asterisks (**), p value<0.005.

3.2.4 Generation of stably expressing shNQO2 SKOV-3 cells

SKOV-3 was the preferred selection to generate a stable NQO2-silencing cell line for the following reasons: firstly, the NQO2 protein and activity levels were not only the highest in this cell line when compared to the rest of the tested OVC cell lines, but were also predominant over NQO1 levels. Secondly, the state of P-gp is positive, thus suited to the requirements of this study. An inducible TRIPZ lentiviral shRNA system was used to make lentiviral particles carrying either a non-targeting control sequence or shRNA-NQO2 targeting sequences (namely shRNA22, 27 and 29). SKOV-3 cells were transduced with these sequences separately at MOI 1 and 5. The cells transduced with MOI 5 were selected for further analysis, as they survived under the selection condition of puromycin treatment at (1µg/ml) concentration. Further details about the protocol can be found in section (2.12). Non-targeting control sequences, shRNA22, shRNA27 and shRNA29, were termed in the text as NTC, sh22, sh27 and sh29 respectively. The same terms were also used to describe the cells transduced with these sequences. These cell-derived SKOV-3 sub-lines are isogenic; (i.e.
they have identical genetic makeup with the exception of the sequences inserted with the aim of silencing NQO2). The cells untreated or treated with dox are denoted as (-dox) or (+dox) respectively within the text.

3.2.4.1 The efficiency and specificity of several shRNA sequences at silencing the NQO2 gene expression

Several constructs (sh22, sh27 and sh29) targeting different regions within the NQO2 gene were tested for their efficiency and specificity at silencing the gene. The construct that causes optimal silencing of NQO2 with minimal interference to the basal levels of cellular NQO1 was selected to study the biological roles of NQO2. As this system relies on dox to induce NQO2 silencing, the concentration of dox and incubation time of cells with dox were optimised for maximal silencing. Initially, a Western blot analysis was carried out on many samples prepared from these SKOV-3 derived sub-lines under different treatment conditions. SKOV-3 cells expressing sh22, sh27 and sh29 sequences were seeded separately in 10cm dishes, and treated with varying concentrations of dox (0.5, 1 and 2µg/ml) on an alternate day basis. The samples were then prepared at 24hr time intervals starting from 48hr till 120hr, and analysed via Western blot in parallel.

As shown in Figure (3.4, A), there is no noticeable reduction in NQO2 protein levels in sh22 samples exposed to different treatment conditions, even upon exposure to the highest dose of dox for the longest duration (e.g. 2µg/ml for 120hr), compared to untreated. This indicates that the sh22 sequence is not efficient at silencing NQO2 and therefore, the cells-expressing this sequence were excluded from this study. On the other hand, the sh27 and sh29 sequences were found to be effective at reducing NQO2 protein levels to similar extents following induction via dox. The reduction in NQO2 levels was more pronounced when the cells possessing these sequences were treated with dox for the extended durations of 96hr and 120hr. However, there appear to be no significant differences in the protein levels of NQO2 among samples of either sh27 or sh29 following exposure to increasing concentrations of dox, as shown at each time interval in Figure (3.4, A). This indicates that the silencing of NQO2 was dependent mainly on incubation time with dox rather than dox concentration. Thus, a dose of 1µg/ml of dox was selected for future studies. It
is worth mentioning that prior to seeding for the experiments, NQO2 expression had to be silenced to the maximum by treating the cells with dox (1µg/ml) for 120hr. It was also recommended for dox to be continuously added to the cells throughout the experiment on an alternate day basis, so as to avoid any fluctuations in the silenced levels.

Following the selection of the most efficient sequence targeting NQO2 expression product and the optimal concentration and exposure time to dox, further analysis using other assays was carried out on the sequences. The cells, including WT and NTC control cells as well as sh27 and sh29, were seeded in both the presence and absence of dox (1µg/ml) for 120hr and then prepared for Western blot analysis. The band intensity of NQO1, NQO2 and β-actin were quantified via densitometric analysis. Fold differences in the band intensity between (+dox) and (-dox) samples of each cell sub-line were then calculated, in order to measure the reduction levels of NQO2 and NQO1 induced by dox. The mean values of fold differences were calculated from three independent experiments, and are represented graphically, Figure (3.4, C). Since dox caused slight alterations in the levels of both enzymes in NTC control cells, further comparisons were made relative to the NTC control (+dox) sample.

The expression level of NQO1 in these cells was analysed in parallel with NQO2. Both NQO2 and NQO1 are members of the same family, and show a high degree of similarity in the cDNA sequences. Thus, it was imperative to investigate whether the NQO1 gene product is also targeted by any of these sequences, or upregulated to compensate NQO2 silencing. From the data shown in Figure (3.4, C), it is apparent that the addition of dox to sh27 and sh29 samples causes a significant reduction (p<0.0001) in the protein levels of NQO2 when compared to NTC/+dox. Interestingly, NQO1 was found to be also targeted by the sh29 sequence, resulting in a moderate reduction in NQO1 protein levels. Additionally, NQO1 was observed to be slightly targeted by the non-targeting sequence of NTC cells.

The sh27 sequence’s efficiency in silencing NQO2 expression was also evaluated using IF staining. The intensity levels of red fluorescence signals induced by dox and green fluorescence signal, which indicated basal NQO2 levels, were measured in the cell sub-lines (NTC and sh27).
following exposure to different treatment conditions. The cells were first treated with dox (1µg/ml) for five days before being seeded onto coverslips for IF analysis. The fixed cells were then incubated with an anti-NQO2 secondary antibody conjugated with green fluorophore and preserved in mounting medium containing DAPI (blue) counterstain. IF signals were visualised and recorded using an Olympus wide-field fluorescence microscope, Figure (3.4, D). In the absence of dox, NTC and sh27 cells emitted green fluorescence; please refer to Figure (9.1) in the appendix chapter, which shows that the secondary antibody is specifically-bound; therefore, the emitted green fluorescence represents the basal levels of NQO2 in the cells, which also indicates that NQO2 is located in both subcellular compartments including cytoplasm and nucleus. A similar intensity of green fluorescence was also emitted from NTC cells treated with dox, which was however abrogated significantly in sh27, as a result of inducing shRNA expression against NQO2. Red fluorescence signals were detected in NTC and sh27 cells exposed to dox. The intensity of red fluorescence can be used as a measure of the efficiency of dox at inducing the expression of the fused sequences.
Figure 3.4 Assessment of efficiency and specificity of several shRNA constructs against NQO2 in SKOV-3 cells

A. Cell-derived SKOV-3 sub-lines transduced with three different shRNA sequences, namely sh22, sh27 and sh29, were seeded for Western blot analysis. The cell sub-lines were treated with varying concentrations of dox (0.5, 1 and 2µg/ml) on an alternate day basis up to 120hr. The samples were collected at 24hr time intervals starting from 48hr to 120hr. NQO2 protein levels were then evaluated in these samples to investigate which dose and exposure time to dox were optimal for the maximal silencing of NQO2. These blots are representative of two independent experiments. B. This blot shows the protein levels of both NQO2 and NQO1 in the samples prepared from SKOV-3 derived sub-lines, including non-targeting control (NTC) cells, sh27 and sh29 cells. These cells were seeded in the absence and presence of dox (1µg/ml) for 120hr, before a Western blot analysis was carried out. C. A densitometric analysis was performed on three independent blots to measure the intensity of bands. The NQO2 and NQO1 bands were first normalised to β-actin. Then, the fold changes in their intensity between (-dox) and (+dox) treated samples for each cell sub-line were determined. The mean values of these changes are represented graphically with their standard errors (±SEM). An unpaired t-test was used to analyse the data, and found that NQO2 was significantly silenced upon dox exposure in both sh27 and sh29 cells, relative to dox-treated NTC control cells, (****p value<0.0001). Asterisks refer to the samples that showed significant levels of difference compared to the control. D. NTC control cells and sh27 cells treated with 1µg/ml dox (+dox) for 120hr were fixed for IF staining, and incubated with an anti-NQO2 secondary antibody conjugated with green fluorophore. Untreated cultures were also included. Green fluorescence refers to the basal levels of NQO2 which upon dox exposure became faint. DAPI was used as a nuclear counterstain. RFP was expressed following exposure to dox, which however was not detected in its absence as illustrated in the image as a black background.
A cytochrome c spectrophotometric assay was also conducted to investigate reduction levels in NQO2 and NQO1 activity upon exposure to dox, and to determine whether these are directly correlated to the reduction observed in NQO2 protein levels. NTC, sh27 and sh29 samples prepared from culture treated with and without dox (1µg/ml) for 120hr were analysed for activity, as described in section (2.5.2). It can be seen from the data in Table (3.3) that the induction of sh27 and sh29 sequences expression by dox was accompanied by a significant reduction in NQO2 activity levels, with a 2.9- and 2.7-fold difference respectively, relative to NTC/+dox. Interestingly, some measurable activity of NQO2 was detected in sh27 and sh29 samples following exposure to dox. No significant differences in the activity of either NQO2 or NQO1 were found among all samples in the absence of dox. However, a slight reduction in NQO1 activity levels was noticed in NTC and sh29 samples following exposure to dox. These results were consistent with the Western blot findings. As the sh27 sequence showed the highest specificity and efficiency at silencing NQO2 when compared to other sequences, sh27 cells possessing this sequence were selected for the investigation of the hypothesis regarding NQO2 roles in OVC.

**Table 3.3 Activity levels of NQO2 and NQO1 in cell-derived SKOV-3 sub-lines**

<table>
<thead>
<tr>
<th>SKOV-3 Cell Sub-lines</th>
<th>Enzyme Activity ± SEM (nmol/ min/ mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NQO2</td>
</tr>
<tr>
<td></td>
<td>-Dox</td>
</tr>
<tr>
<td>NTC</td>
<td>155.5 ± 9.7</td>
</tr>
<tr>
<td>***sh27</td>
<td>129.8 ± 22.5</td>
</tr>
<tr>
<td>***sh29</td>
<td>130.4 ± 20.9</td>
</tr>
</tbody>
</table>

Samples were prepared from NTC, sh27 and sh29 cultures treated with and without dox (1µg/ml) for 120hr. The activity of both NQO2 and NQO1 was measured in these samples using the cytochrome c spectrophotometric assay. The rate of enzyme activity was expressed as nmole of cytochrome c reduced per min per mg of protein. It was represented as a mean value of three independent experiments with their standard errors (± SEM). An unpaired t-test was also used to analyse the data. Asterisks refer to the cells that showed significant difference compared to NTC/+dox, (***p value<0.001).
3.3 Discussion

NQO2 activity levels were evaluated in intraperitoneal ovarian metastases and found to be high. Therefore, NQO2 has been suggested as a potential therapeutic target for directing chemotherapy such as tretazicar:caricotamide for ovarian tumours [104]. The association between NQO2 levels and ovarian cancer progression has not yet been studied. A panel of OVC cell lines were screened for NQO2 expression and activity levels. The assessment of NQO2 levels in OVC cells allowed to determine the expression intensity as well as the distribution extent, and to decide which cell lines can be used to generate NQO2 genetically modified cells. A cytochrome c spectrophotometric assay was used to evaluate NQO2 and NQO1 activity levels. The findings were consistent with those obtained from an NCI screening study conducted on 69 human tumour cell lines to evaluate NQO1 activity levels. The NCI findings showed that NQO1 activity is extremely low in SKOV-3 and OVCAR-3 ovarian cancer cells [217]. In agreement with the previous study, the NQO1 activity levels were very low in SKOV-3, OVCAR-3 and TOV-112D cells compared to the rest of the cell lines in the tested panel. The consistency in the findings supports the reliability of using cytochrome c assay for screening NQO2 in the whole panel.

Notably, the NQO2 protein and activity levels through the tested panel of OVC cell lines were indirectly correlated with those of NQO1. It was apparent that the cell lines with low NQO1 expression or activity level had high levels of NQO2 and vice versa. This observation suggests that both enzymes might be responsible for the regulation of the same pathways in the cells that require high-level control. Consistently, Caraher M. (2012) also demonstrates negative correlations between NQO2 and NQO1 protein and activity levels in other types of cancers such as breast, leukaemia, lymphoma, colon, lung and cervical cancer [215].

As expression levels of protein are not always reflective of the protein's actual activity level in cells, it was imperative to then assess the expression along with the activity levels of NQO2 and NQO1, and estimate the correlation strength between these variables. A very good correlation ($r = 0.96$) was found between NQO2 protein levels and enzyme activity in this panel of OVC cell lines. This means that differences observed in the expression levels of NQO2 among OVC cell lines were
consistent with the differences in its enzymatic activity. The high correlation strength between NQO2 expression and activity levels was further proved when the overexpression of NQO2 in TOV-112D cells was accompanied by a substantial increase in its activity levels. Interestingly, some residual NQO2 activity was observed in sh27 samples following dox treatment, in spite of a significant reduction of almost 98% in its expression level. This result is consistent with that of another study, and suggests that the noted activity may stem from NQO2-associated proteins rather than free NQO2 [139]. The associated NQO2 remained non-targeted by the silencing sequence, and was responsible for the residual activity observed.

On the other hand, the correlation strength between NQO1 protein levels and activity was found to be weaker than that with NQO2. Although OV-90 cells expressed four-fold more NQO1 than TOV-21G cells, NQO1 activity levels were found to be comparable in these cell lines. Furthermore, TOV-112D cells showing undetectable NQO1 expression levels had highly active NQO1 compared to OVCAR-3 cells that expressed higher levels of less active NQO1. This is not unique, as Caraher M. found that NQO2 activity was substantially high in MDA-MB-231 and T47D breast cancer cell lines, despite very low expression levels of NQO2 [215]. All of these findings therefore highlight the importance of evaluating enzymatic activity along with protein levels, as they are not always representative of each other.

The generation of NQO2 genetically modified OVC cells provides useful tools for the study of the biological and therapeutic roles of NQO2 in vitro. The lentiviral system was the preferred option used to generate NQO2 overexpressing and NQO2 silencing cells, as it has distinct advantages over retroviral and non-viral systems. Namely, it is highly-efficient at infecting the dividing and non-dividing cells [218], and is also able to introduce the sequences of interest into the genome of target cells in a stable manner, resulting in long-term expression of the transgene. This aided in the generation of consistent and reliable data [219].

From all OVC cells, the TOV-112D cell line was the most suitable model for the overexpression of NQO2. Introducing an NQO2 overexpression construct into the cell genome was successfully performed, and this was demonstrated by the substantial upregulation of NQO2 expression and
increased activity levels. The earlier findings suggested a negative correlation between NQO2 and NQO1 enzymes. Thus, it was necessary to evaluate whether overexpressing NQO2 level in TOV-112D cells also influences the basal levels of NQO1. It is clear from Figure (3.3, B) that the upregulation of NQO2 in these cells results in a marginal reduction in NQO1 level, thereby supporting the notion of the presence the negative correlation between both enzymes.

To establish a representative model for NQO2 silencing, SKOV-3 cells expressing high, predominantly basal levels of NQO2 were selected. A TRIPZ lentiviral system was chosen, as it is engineered to be Tet-on, which expresses shRNA silencing sequence upon dox induction. This characteristic lends more advantages to the lentiviral system. It was found that the level of NQO2 silencing could be easily modified according to the requirements of the study by adjusting exposure time to dox. Modulating the silencing levels of NQO2 allowed for the study of varying NQO2 levels’ effect on particular pathways. It is worth noting that some target genes have vital roles in the cells; therefore silencing them to a high extent might be harmful to the cells. The possibility of modulating the silencing levels of target proteins through an adjustment of the dox exposure time demonstrates the benefits of the TRIPZ lentiviral system’s Tet-on feature in protecting the cells from the toxicity of maximal silencing.

To generate reliable results from NQO2-silencing cells, it was first necessary to find out the most appropriate sequence that causes maximal reduction in NQO2 levels without altering NQO1 basal levels. Thus, four cell conditions were prepared using four sequences; three of these sequences (including sh22, sh27 and sh29) targeted the NQO2 gene at different regions, while the last one did not target any gene in the cells, and was therefore used as a negative control. Each sequence was introduced into the cells at the MOI of 1 and 5. The cells transduced with a higher concentration of lentivirus (MOI 5) were selected for this study, as they were able to survive under the puromycin selection condition. A possible explanation for this is that these cells expressed more copies of puromycin resistant gene products, thereby protecting them from the cytotoxicity of puromycin. Each sequence was investigated for its efficiency in silencing NQO2. Sh22 was found to be inefficient, even upon exposure to a high concentration of dox (2µg/ml) for 120hr. This was attributed to several reasons, some of them more biological than experimental. The simplest
reason concerns a particular mutation in the recognition sequence of the RNA secondary structure that masks the shRNA recognition site, restricting accessibility to the mRNA target site [220, 221], or the presence of multiple mRNA variants of the NQO2 gene, which were not all targeted by the construct. Alternatively, the integration of the sh22 construct may have taken place in the heterochromatin region, leading to a very low expression of the transgene. However, it was difficult to identify experimental reasons behind the failure of the sh22 construct in silencing NQO2, as the same protocol was followed with all constructs. The titers of lentiviral particles carrying the tested sequences were first determined, and accordingly, the volumes of lentiviral suspension required from each sequence in order to get the same MOI were determined. The importance of measuring the lentiviral titer lies in the fact that not all sequences are packaged into lentivirus in the same efficiency.

In contrast, both sh27 and sh29 constructs caused maximal NQO2 silencing after long term induction via dox. This might be due to these constructs’ nucleotide sequences, which were completely aligned against the NQO2 gene sequence, as illustrated in Figure (2.6, A). NQO1 levels were also measured in these samples. It was found that NQO2-targeting sh29 interfered markedly with the NQO1 gene, causing a significant reduction in its protein and activity levels. This could be due to the complementarity of sh29 nucleotide sequences to both NQO2 and NQO1. Figure (2.6, C) supports this suggestion, as it is clear that the nucleotide sequences of the NQO2 gene targeted by sh29 highly match those of NQO1. In accordance with the present result, a previous study on CWR22Rv1 prostate cancer cells has found that targeting NQO2 via shRNA28 is also associated with a significant reduction in NQO1 level. This may be attributed to the high degree of similarity in their nucleotide sequences targeted by shRNA28 [166]. Therefore, sh27 cells were deemed the most suitable cell condition to use for further analysis.

In conclusion, NQO2 is heterogeneously expressed in the OVC cell line. A strong correlation was detected between NQO2 protein level and its enzymatic activity. NQO2 levels in a selection of TOV-112D and SKOV-3 cell lines were successfully genetically modified, and were used for the further study of NQO2’s potential roles in OVC.
Chapter 4

Biochemical evaluation of novel quinoline inhibitors of NQO2
4 Biochemical evaluation of novel quinoline inhibitors of NQO2

4.1 Introduction

After the discovery of NQO2 in 1961 by Liao and Williams-Ashman, several experimental analyses have been performed on NQO2 in order to identify its characteristics and properties. NQO2 is able to oxidise N-alkyl derivatives of dihydronicotinamides (NRH) rather than their phosphorylated derivatives (e.g. NADH), and only weakly responds to NQO1 inhibitors such as dicoumarol. These properties distinguish NQO2 from NQO1 [127, 222]. Polycyclic aromatic hydrocarbons including benz(a)anthracene and its derivatives are amongst earlier inhibitors of NQO2 that show the highest potency [222]. Research on NQO2 was forgotten for over 30 years, until Jaiswal and his colleagues re-discovered human NQO2 while cloning and sequencing human NQO1 [81, 206]. Since then, structurally diverse compounds have been recognised as NQO2 inhibitors. Polyphenol compounds such as resveratrol (now considered to be the classical NQO2 inhibitor) and quercetin [130], in addition to benzo(a)pyrene [223], melatonin [224] and imatinib [225] have shown to have inhibitory activity against NQO2. The potential roles of NQO2 in cancer are still undefined, with the existence of several conflicting theories regarding its functional activity in cells. Unfortunately, none of the previous inhibitors are ideal tools for studying the cellular functions of NQO2 as they have multiple functions in cells, making it difficult to decide whether these functions are relevant to NQO2 or other targets. Thus, the identification of novel inhibitors with high potency and specificity is crucial.

Recently, Nolan et al. conducted a virtual screening study on NCI compounds with diverse structures in order to identify novel ligand scaffolds for NQO2. The identified scaffolds were categorised into five classes: quinolines, acridines, ellipticines, polyaromatic and furanylamidines, which afterwards were tested for their inhibitory activity against NQO2 [105]. These scaffolds have been used as a guidance to synthesise further derivatives with functional activity as NQO2 inhibitors. From the acridine class, 9AA has been reported to be a highly potent inhibitor. Also from the quinoline class, seven derivatives have been identified as NQO2 inhibitors with various levels of potency and toxicity, and among these inhibitors compound (NSC617933) shows, comparatively, the highest potency with low binding affinity to other proteins [105]. Indeed, previous studies have
encouraged synthesising a broad spectrum of quinoline derivatives, as they possess a remarkable ability to bind and inhibit NQO2 activity at a low concentration of 1µM [130, 226]. The quinoline class has drawn Dr Whitehead and co-workers’ attention to the design and synthesis of more derivatives using the 4-aminoquinoline scaffold. They have also assessed their inhibitory potency and found hydrazone derivatives to be the most potent NQO2 inhibitors [227]. This work has been accomplished by Dr Freeman and her team, who designed and synthesised further novel derivatives of hydrazone, hydrazide and carboxamide-quinolines with several structural modifications, in an attempt to enhance potency and reduce cytotoxicity of the novel compounds [83]. This chapter aims to characterise the potential inhibitors which can be valuably used as pharmacological tools to investigate the functional activity of NQO2 in the cells.

4.2 Results

4.2.1 Extracellular inhibitory potency of quinoline analogues against recombinant human NQO2

Dr. Buthaina Hussein synthesised a series of compounds detailed in Table (4.1, 4.2 and 4.3), and went on to evaluate their ability to inhibit the enzymatic activity of NQO2 in a cell free system. To do this, DCPIP-based spectrophotometric assay was used (described in detail in section 2.5.3). Various concentrations of the compounds ranging from 0.01nM to 10000nM were added to the reaction mixtures containing rhNQO2, EPR and DCPIP. EPR, the novel derivative of NRH, has been demonstrated to be effective at catalysing rhNQO2 enzymatic activity [99]. DCPIP’s rate of reduction was measured over a minute in all reaction mixtures. Then, the proportion of remaining enzyme activity at varying concentrations of compounds was calculated as a percentage relative to the control enzyme activity. RhNQO2 enzyme activity (%) was plotted against a range of concentrations to generate the compounds’ dose-response curves. These curves were used to determine IC$_{50}$ values, which is defined as the concentration of compound required to decrease the enzymatic activity by 50%, relative to the control enzyme activity. IC$_{50}$ values were calculated from three independent experiments, and represented as the mean ± SEM, as shown in Table (4.1). Resveratrol and 9AA (also known as typical inhibitors of NQO2) are commonly used in NQO2
studies, hence their use as positive controls here allowed for the comparison of NQO2 inhibitory potency for the novel compounds.

**Table (4.1)** demonstrates that all 4-hydrazone-quinoline derivatives (except compound 21) are highly effective at inhibiting rhNQO2 activity at nano-molar concentrations. In contrast, compound 21 is inactive and ineffective in the inhibition of rhNQO2, even when used at a high concentration of 10000nM. The IC\textsubscript{50} values of hydrazone derivatives ranged from 10 ± 0.7nM to 1420 ± 204nM. Introducing different \textbf{R1} (at position 2 of quinoline ring) and \textbf{R2} (on the side chain of quinoline ring) functional groups to the quinoline ring may contribute to this wide variation in the activity of hydrazones, **Table (4.1)**. Changing the \textbf{R1} group from phenyl to methyl converts some compounds from less potent to highly potent. This has been marked in compounds 20, 22, 23 and 24, which are structurally identical to compounds 12, 17, 13 and 14 respectively, with the exception of the \textbf{R1} substitution, which is phenyl in the former compounds but methyl in the latter ones. This slight structural modification results in, for example, a 46-fold enhancement in the potency of compound 14 compared to its corresponding compound 24, with IC\textsubscript{50} values of 13 ± 3.1nM and 595 ± 178nM respectively. On the other hand, using different aromatic substitution (\textbf{R2}) on hydrazone moiety, which is attached to the quinoline ring at position 4, results in remarkable changes in the potency of the compounds. It has been shown that having para-fluorine substituents on the phenyl ring (\textbf{R2}) enhances inhibitory potency of compound 8 approximately 5-fold, compared to compound 9 that has an unsubstituted phenyl ring, with IC\textsubscript{50} values of 103 ± 16.3nM and 519 ± 14.5nM respectively. Alternatively, having hydrophilic substituents on the phenyl ring as shown in compounds 11, 14, 16, 18 and 19 greatly improves the inhibitory activity of these compounds compared to compound 9 also. For example, the IC\textsubscript{50} value of compound 14 is 40-fold less than that of compound 9. Moreover, changing the phenyl ring (\textbf{R2}) to heterocycles like imidazoyl, pyridinyl and nitrofuranyl considerably enhances the inhibitory potency of hydrazone derivatives. This is clear in compounds 10, 12 and 15 which are much more potent than compound 9, with IC\textsubscript{50} values of 71 ± 14.9nM, 10 ± 0.7nM and 18 ± 1.2nM respectively. **Figure (4.1)** illustrates 4-hydrazine-quinoline (compound 4), which has been used as a starting material to synthesise the novel hydrazone and hydrazide
derivatives. Notably, a big difference in the IC$_{50}$ values has been noticed between compound 4 (2047 ± 132nM) and hydrazone, as well as hydrazide derivatives.

It has also been found that changing the hydrazone moiety to hydrazide reduces the potency of the novel derivatives. This has been observed in compound 27, which is 28-fold less potent than its corresponding compound 8, Table (4.2 and 4.1). Interestingly, the lack of the compounds’ activity as NQO2 inhibitors is also evident when hydrazone moiety has been replaced with carboxamide. This leads to more than an 80-fold difference in the inhibitory activity between compound 32 and its corresponding compound 23, Table (4.3 and 4.1). Moreover, hydrazones have shown much higher potency than the typical NQO2 inhibitors (resveratrol and 9AA), with an estimation of 9-fold and 4-fold differences in the IC$_{50}$ values, since the IC$_{50}$ of most hydrazones is around 100nM, while the IC$_{50}$s of typical inhibitors are 900nM and 440 ± 90nM respectively.

In conclusion, the novel derivatives of hydrazone-quinoline were recognised as the most potent NQO2 inhibitors in comparison with hydrazide and carboxamide derivatives. Therefore, most of the compounds selected for further investigation are from the hydrazone group. However, compounds 12, 15 and 18 were excluded from this study as they showed poor solubility in different solvent systems.

![Figure 4.1 Structure of 4-hyadrazine-quinoline (compound 4)](image)

Figure 4.1 Structure of 4-hyadrazine-quinoline (compound 4)
Table 4.1 Structures of 4-hydrazone-quinoline derivatives with averaged IC\textsubscript{50} values representing their inhibitory potency against rhNQO2 [83]. Asterisks (**) were used to mark the inactive compounds.

<table>
<thead>
<tr>
<th>Compound Number/Name</th>
<th>R1</th>
<th>R2</th>
<th>NQO2 IC\textsubscript{50} (nM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>CH\textsubscript{3}</td>
<td>4-Fluorophenyl</td>
<td>103 ± 16.3</td>
</tr>
<tr>
<td>9</td>
<td>CH\textsubscript{3}</td>
<td>Phenyl</td>
<td>519 ± 14.5</td>
</tr>
<tr>
<td>10</td>
<td>CH\textsubscript{3}</td>
<td>4-Imidazoyl</td>
<td>71 ± 14.9</td>
</tr>
<tr>
<td>11</td>
<td>CH\textsubscript{3}</td>
<td>4-Hydroxyphenyl</td>
<td>55 ± 6.6</td>
</tr>
<tr>
<td>12</td>
<td>CH\textsubscript{3}</td>
<td>3-Pyridiny</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>13</td>
<td>CH\textsubscript{3}</td>
<td>Benzyl</td>
<td>15 ± 4.4</td>
</tr>
<tr>
<td>14</td>
<td>CH\textsubscript{3}</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>13 ± 3.1</td>
</tr>
<tr>
<td>15</td>
<td>CH\textsubscript{3}</td>
<td>2-Nitrofuranyl</td>
<td>18 ± 1.2</td>
</tr>
<tr>
<td>16</td>
<td>CH\textsubscript{3}</td>
<td>4-Benzoic acid</td>
<td>64 ± 5.9</td>
</tr>
<tr>
<td>17</td>
<td>CH\textsubscript{3}</td>
<td>4-Nitrophenyl</td>
<td>83 ± 8.0</td>
</tr>
<tr>
<td>18</td>
<td>CH\textsubscript{3}</td>
<td>3,5-Dihydroxyphenyl</td>
<td>46 ± 8.6</td>
</tr>
<tr>
<td>19</td>
<td>CH\textsubscript{3}</td>
<td>3-Hydroxyphenyl</td>
<td>137 ± 15.9</td>
</tr>
<tr>
<td>20</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>3-Pyridinyl</td>
<td>372 ± 109.4</td>
</tr>
<tr>
<td>21</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>4-N,N-dimethylaniline</td>
<td>**</td>
</tr>
<tr>
<td>22</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>4-Nitrophenyl</td>
<td>1420 ± 204</td>
</tr>
<tr>
<td>23</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>Benzyl</td>
<td>125 ± 2.8</td>
</tr>
<tr>
<td>24</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>595 ± 178</td>
</tr>
<tr>
<td>25</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>4-Imidazoyl</td>
<td>90 ± 10.7</td>
</tr>
<tr>
<td>Resveratrol</td>
<td></td>
<td></td>
<td>900</td>
</tr>
<tr>
<td>9AA</td>
<td></td>
<td></td>
<td>440.0 ± 90.0</td>
</tr>
</tbody>
</table>
Table 4.2 Structures of 4-hydrazone-quinoline derivatives with averaged IC₅₀ values representing their inhibitory potency against rhNQO2 [83]

Table 4.3 Structure of 4-carboxamide-quinoline derivative with averaged IC₅₀ values representing their inhibitory potency against rhNQO2 [83]. Asterisks (**) were used to mark the inactive compounds.

4.2.2 Influence of putative NQO2 inhibitors on the activity of recombinant human NQO1

Due to the structural similarities between NQO2 and NQO1 [80, 89], and to eliminate the potential confounding influence of NQO1 expression and activity in subsequent cellular studies, the ability of
the compounds listed in Tables (4.1, 4.2 and 4.3) to inhibit recombinant NQO1 was evaluated. To do this, the compounds’ inhibitory potency was measured over the micro-molar range of concentration (0.00001µM-100µM). DCPIP assay was again conducted following the same protocol described in section (4.2.1), with the exception of using rhNQO1 in place of rhNQO2, and NADH instead of EPR. Dicoumarol (typical inhibitor of NQO1 [228]) was used here as a positive control to allow for the comparison of potency between the novel compounds. Because of its high potency, the concentration range prepared was different, ranging from 0.00001µM to 1µM. To facilitate addressing differences in the compounds’ potency against NQO1 activity, dose-response curves were generated by plotting NQO1’s remaining activity against the compounds’ concentration range, as shown in Figure (4.2). IC₅₀ values were determined from these curves, as listed in Table (4.4).

Figure 4.2 Assessing the NQO1 inhibitory potency of the compounds

Representative curves showing the dose-response curves of dicoumarol (●), the typical inhibitor of NQO1, hydrazones compounds (including 10 (▲), 11 (▼), 20 (★) and 25 (■)) and hydrazide compound 27 (◆). They were plotted on the same figure to investigate whether these novel compounds have better or worse inhibitory potency against NQO1 than dicoumarol. The points on the curves were calculated from at least three independent experiments. Nonlinear regression analysis was used to fit the data with the best line to generate the dose-response curves.
Dicoumarol shows the highest inhibitory potency against NQO1, at a very low micro-molar concentration of $0.0048 \pm 0.001\mu M$, as illustrated in Table (4.4). Neither hydrazone nor hydrazide-quinoline analogues showed activity against NQO1 within the tested range of concentration. This is displayed in Figure (4.2) as linear curves, indicating that the activity of NQO1 is almost similar to that of the untreated control, with 100% reductive efficiency of NQO1. Exceptionally, compounds 10, 14 and 25 reported some activity against NQO1. Although compound 25 ($IC_{50}=0.064 \pm 0.08\mu M$) was demonstrated to be the most potent inhibitor of NQO1 compared to the rest of quinoline analogues, it was still 133-fold less potent than dicoumarol. Furthermore, the concentration of compound 25 required to inhibit 50% of NQO2 activity was found to be seven-fold less than that used to get the same level of inhibition of NQO1. In comparison with Table (4.1), it can be seen that the $IC_{50}$ values of compounds 10 ($71 \pm 14.9nM$) and 14 ($13 \pm 3.1nM$) required to inhibit 50% of NQO2 are approximately 1,760-fold and 1,190-fold, respectively, less than that used to produce similar levels of inhibition for NQO1 with $IC_{50}$ values of $85,000 \pm 5,000nM$ and $22,900 \pm 7,400nM$ respectively, Table (4.4). This means that these novel compounds are poor inhibitors of NQO1, where high concentrations are needed to generate measurable levels of NQO1 inhibition.
Table 4.4 NQO1 inhibitory potency of the compounds

<table>
<thead>
<tr>
<th>Compound Name/ Number</th>
<th>NQO1 (IC₅₀ (µM) ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicoumarol</td>
<td>0.0048 ± 0.001</td>
</tr>
<tr>
<td>10</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>11</td>
<td>**</td>
</tr>
<tr>
<td>13</td>
<td>**</td>
</tr>
<tr>
<td>14</td>
<td>22.9 ± 7.4</td>
</tr>
<tr>
<td>16</td>
<td>**</td>
</tr>
<tr>
<td>20</td>
<td>**</td>
</tr>
<tr>
<td>24</td>
<td>**</td>
</tr>
<tr>
<td>25</td>
<td>0.64 ± 0.08</td>
</tr>
<tr>
<td>27</td>
<td>**</td>
</tr>
</tbody>
</table>

IC₅₀ values of the compounds were calculated from the dose-response curves represented in Figure (4.2), and tabulated as mean values calculated from three independent experiments with their standard error (± SEM). These values were used as an indicator of the compounds’ inhibitory potency. Asterisks (**) were used to mark the inactive compounds which were unable to inhibit rhNQO1 at the tested range of concentration.

4.2.3 Evaluation of the compounds’ cytotoxicity

Carrying out an in vitro evaluation of the novel 4-aminoquinoline analogues’ cytotoxic effects was important, as it assisted in studying the effect of hydrazone and hydrazide substitutions on the cytotoxicity of quinoline derivatives. It was also useful in assessing the stability of hydrazone derivatives in cell environments, since the N=C double bond of hydrazone moiety was found to be labile and hydrolysed easily, producing the starting compound 4 [227, 229]. Typical inhibitors of NQO2 including quercetin, resveratrol and 9AA were used as positive controls to allow for comparisons between the cytotoxicity of the novel compounds and to determine whether they were less or more toxic.
MTT colorimetric assay was selected to assess the compounds’ cytotoxicity. The OD of the treated cells was used to calculate the fraction of survival cells, which remained unaffected by treatment, as a percentage relative to the solvent controls (100%, DMSO). To generate a dose-response curve, the cell survival (%) was plotted against the concentrations range, as displayed in Figure (4.3), thereby facilitating the extraction of the compounds’ IC\textsubscript{50} values.

SKOV-3 was selected as a suitable cell line to evaluate the cytotoxicity of the compounds. The presence of comparatively high activity levels of NQO2 in SKOV-3 cells allowed to determine whether there was a correlation between the extent of NQO2 inhibition and cell death. The cells were seeded in 96-well plates and treated with various concentrations of the compounds, ranging from 0.001\(\mu\)M to 100\(\mu\)M, for 24hr and 96hr. To terminate the experiments after the 96hr incubation, MTT solution was added to the cells as a read out of cell survival. IC\textsubscript{50} values were determined from the dose-response curves of the compounds, Figure (4.3) and listed in Table (4.5) as the mean values of four independent experiments with their standard error (± SEM).

It can be seen that most compounds’ cytotoxicity increases in a dose-dependent manner, Figure (4.3). However, non-significant enhancement in the compounds’ toxicity was detected upon extending treatment duration to 96hr. As Table (4.5) shows, there is a strong similarity in the IC\textsubscript{50} values between 24hr and 96hr durations for most compounds, with the exception of compound 10, resveratrol and 9AA. These compounds showed much more toxicity when incubated with the cells for an extended period of 96hr compared to 24hr. It can be also seen from the data in Table (4.5) that hydrazones are the most toxic compounds when compared to hydrazides and typical inhibitors, with IC\textsubscript{50} values of around 2\(\mu\)M. Contrastingly, both compounds 13 and 16 from the hydrazone series showed the lowest cytotoxicity, even after 96hr exposure, with IC\textsubscript{50} values of 40.67 ± 2.3\(\mu\)M and 70.0 ± 4.93\(\mu\)M respectively.
The Cytotoxicity of novel analogues of 4-hydrazine-quinoline along with resveratrol was assessed using MTT assay. SKOV-3 cells expressing high levels of NQO2 were selected to be treated with the compounds at this range of concentrations (0.001-100µM) for 96hr. After that time, MTT was added to the cells to estimate cell survival by measuring the ODs of the treated cells which survived. The ODs were then calculated as percentages relative to the solvent control cells. The percentages of cell survival were plotted against the concentrations to generate the dose-response curve of starting compound 4 ( ), hydrazones (including compound 11 ( ), 13 ( ) and 16 ( )) and hydrazide compound 27 ( ) and resveratrol ( ) as shown in this figure, using GraphPad Prism 7 software. These curves were used to determine IC50 values. The points on the curve represent the mean values of cell survival (%) of four independent experiments with their error bars (± SEM), while the curves are the best fit lines for these points. A horizontal line was used to indicate the differences in compounds’ cytotoxicity.

It is noteworthy to mention that there were huge differences between the IC50 values of hydrazone derivatives and their starting compound 4. For instance, a 24hr exposure of SKOV-3 cells to compound 14 generated much greater cytotoxicity compared to that of compound 4, with a 40-fold difference in the IC50 values, 2.1 ± 0.25µM and 84 ± 16µM respectively. Interestingly, changing the hydrazone moiety to hydrazide was also seen to have the beneficial effect of reducing hydrazones’ cytotoxicity to a high extent, resulting in a significant increase in the IC50 values of the hydrazide derivatives (compounds 26 and 27). It is apparent from the table below that the IC50 value of compound 26 (68.3 ± 4.06µM) is 39-fold higher than that of its corresponding compound 9, from hydrazone groups (IC50=1.77 ± 0.09µM), (i.e. the former compound is less toxic than the latter). In
the same manner, it was also found that compound 27 was not only much less toxic than its corresponding compound 8, but also less toxic than its partner from the same group, compound 26. Compound 27 developed toxicity (around 20%) only when used at the highest concentration of 100µM, as demonstrated in Figure (4.3) with a linear curve over the tested range. In comparison with the typical inhibitors, compounds 16 and 27 showed much less cytotoxicity than resveratrol, whereas most hydrazones showed similar cytotoxicity to 9AA, with the exception of compounds 10, 13 and 26, which were much less toxic than 9AA.

Table 4.5 Toxicity extent of the compounds in SKOV-3 cells

<table>
<thead>
<tr>
<th>Compound Number/ Name</th>
<th>Toxicity (IC₅₀ (µM) ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKOV-3 (WT)</td>
</tr>
<tr>
<td></td>
<td>24hr</td>
</tr>
<tr>
<td>4</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>8</td>
<td>2.3 ± 0.18</td>
</tr>
<tr>
<td>9</td>
<td>1.77 ± 0.09</td>
</tr>
<tr>
<td>10</td>
<td>32.0 ± 2.08</td>
</tr>
<tr>
<td>11</td>
<td>2.75 ± 0.26</td>
</tr>
<tr>
<td>13</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>2.1 ± 0.25</td>
</tr>
<tr>
<td>16</td>
<td>**</td>
</tr>
<tr>
<td>20</td>
<td>2.8 ± 0.36</td>
</tr>
<tr>
<td>21</td>
<td>1.57 ± 0.42</td>
</tr>
<tr>
<td>24</td>
<td>2.88 ± 0.22</td>
</tr>
<tr>
<td>25</td>
<td>2.47 ± 0.30</td>
</tr>
<tr>
<td>26</td>
<td>68.3 ± 4.06</td>
</tr>
<tr>
<td>27</td>
<td>**</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>91.67 ± 4.4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50.3 ± 0.67</td>
</tr>
<tr>
<td>9AA</td>
<td>5.43 ± 0.47</td>
</tr>
</tbody>
</table>

Toxic effects of the compounds were evaluated in SKOV-3 cells following 24hr and 96hr treatment durations by using MTT assay as an experimental end point. The compounds’ IC₅₀ values were determined from dose-response curves and tabulated as a mean calculated from four independent experiments with its standard error (± SEM); this was carried out for each compound. These values were used as measurements for cytotoxicity of the compounds. Asterisks (**) refer to the compounds that did not show cytotoxicity even at the maximum concentration (100µM) of tested range.
4.2.3.1 Cytotoxicity in NQO2 genetically modified cells

It was speculated from the previous findings that there was no a correlation between the NQO2 inhibitory potency of the compounds and their cytotoxicity. This is because the compounds with low inhibitory potency developed high toxicity in SKOV-3 while others that showed similar potency levels developed in contrast low cytotoxicity. The very weak correlation is demonstrated in Figure (4.4) where the compounds’ IC₅₀'s representing their inhibitory potency against rhNQO2 were plotted against their IC₅₀'s for cytotoxicity, with r value of 0.03. These values were taken from Table ((4.1 and 4.2) and 4.5)) respectively. The lack of association between the NQO2 inhibition level and the compounds’ cytotoxicity was further proved using isogenic pairs of cell lines differing in their NQO2 state. Of these pairs are TOV-112D (EV and NQO2-OE cells sub-lines), which have low endogenous and modified high levels of NQO2 respectively, in addition to SKOV-3 (NTC and sh27), which have high endogenous and modified low levels of NQO2 respectively. These cells were successfully prepared in this project, as shown in section (3.2.3.1) and (3.2.4.1). Groups of compounds with diverse structures were suggested to be assessed for cytotoxicity to investigate whether the hypothesis was only applicable to a particular scaffold or whether it can be generalised to include others. Therefore, hydrazide (e.g. compound 27), resveratrol and 9AA were all selected for investigation along with hydrazone derivatives.

The same protocol mentioned earlier in section (4.2.3) was followed for the evaluation of cytotoxicity of the compounds, with one more additional preparatory step. Sh27 cell sub-line was exposed to dox (1µg/ml) for five days before being seeded for the experiment, to ensure the maximum silencing of NQO2. Then, all cell types (TOV-112D and SKOV-3 and their derived cell sub-lines) were seeded in parallel in 96-well plates and left overnight, before being treated with the compounds at a range of concentrations (0.001-100µM) for 96hr. Dose-response curves for each compound were established from three independent experiments, and used to determine IC₅₀ values. These values are represented in Table (4.6) as mean ± SEM.

The compounds’ cytotoxicity was firstly compared between NTC and EV cells expressing different endogenous levels of NQO2. It became apparent that each compound developed similar levels of
toxicity to these cells. However, compound 27 and resveratrol showed less toxicity in NTC than EV cells, whereas compound 16 (from a different group) was more toxic in NTC compared to EV cells. For further validation, the compounds’ cytotoxicity was compared in each pair of isogenic cells with genetically modified levels of NQO2. There appeared to be no significant differences in the IC$_{50}$ values for cellular toxicity of any compound in both pairs of isogenic cell lines, as demonstrated in Table (4.6). The cytotoxicity levels of all tested compounds were similar between SKOV-3 cells (NTC (+ dox) and sh27 (-dox)) expressing basal levels of NQO2 and its derived cell sub-line sh27 treated with dox (+dox) where NQO2 levels were substantially silenced. The similarity in the compounds’ toxicity was also noticed in TOV-112D cell sub-lines.

![Figure 4.4 Correlation between extracellular inhibitory potency of a subset of the novel quinolines analogues and their cytotoxicity](image)

A subset of the novel inhibitors including compound 8, 9, 10, 11, 13, 14, 16, 20, 24, 25, 26 along with typical inhibitor (e.g. resveratrol and 9AA) were investigated for the strength of correlation between their NQO2 inhibitory potency and cytotoxicity. To do this, compounds’ IC$_{50}$ extracellular inhibitory potency was plotted against their IC$_{50}$ for cytotoxicity on a scatter graph. The former IC$_{50}$ values represented in Table (4.1 and 4.2) were measured in cell free system using DCPIP spectrophotometric assay while the latter values represented in Table (4.5) were measured in vitro using MTT cytotoxicity assay. This plot shows that extracellular inhibitory potency of these compounds was weakly correlated with their cytotoxicity, as demonstrated by the very low value of Pearson’s correlation coefficient ($r = 0.03$), which was calculated using the Excel Correl function in Microsoft Excel 2007.
To assess whether the inhibition of NQO2 was responsible for the cytotoxicity of the compounds, the levels of cellular NQO2 were genetically modulated prior to re-evaluating the compounds’ toxicity. This was conducted by using isogenically paired cell lines such as SKOV-3 (NTC control and sh27 cells sub-lines), as well as TOV-112D (EV control and NQO2-OE cells sub-lines). To investigate the effect of silencing NQO2 on the sensitivity of SKOV-3 cells to the compounds’ toxicity, it was first necessary to induce NQO2 silencing before initiating the experiment. Therefore, the sh27 cells were exposed to dox (+dox) for five days. The cells were then seeded and, following overnight incubation, were treated with the compounds at this concentration range (0.001-100µM) for 96hr. The possible role of NQO2 in mediating the compounds’ toxicity was also evaluated in TOV-112D overexpressing NQO2, and compared with EV. The compounds’ IC\textsubscript{50} values were determined from dose-response curves generated from three independent experiments. They are represented in this table as mean ± SEM, and used for comparative purposes. Asterisks (**) indicate the compounds that did not show any cytotoxicity at the tested range of concentration.

### Table 4.6 Comparing toxicity extent of the compounds in pairs of cell lines expressing varying levels of NQO2

<table>
<thead>
<tr>
<th>Compound Number/ Name</th>
<th>NTC -Dox</th>
<th>+Dox</th>
<th>Sh27 -Dox</th>
<th>+Dox</th>
<th>EV</th>
<th>NQO2-OE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.98 ± 0.66</td>
<td>6.9 ± 0.30</td>
<td>5.03 ± 0.07</td>
<td>6.73 ± 0.41</td>
<td>3.65 ± 0.29</td>
<td>3.72 ± 0.24</td>
</tr>
<tr>
<td>10</td>
<td>2.52 ± 0.16</td>
<td>2.87 ± 0.15</td>
<td>2.47 ± 0.03</td>
<td>2.58 ± 0.10</td>
<td>3.17 ± 0.22</td>
<td>2.98 ± 0.38</td>
</tr>
<tr>
<td>11</td>
<td>45.3 ± 2.60</td>
<td>44.67 ± 6.9</td>
<td>44.17 ± 4.64</td>
<td>49.67 ± 0.88</td>
<td>42.5 ± 2.57</td>
<td>41.0 ± 5.0</td>
</tr>
<tr>
<td>13</td>
<td>3.05 ± 0.43</td>
<td>2.7 ± 0.1</td>
<td>2.78 ± 0.23</td>
<td>2.78 ± 0.17</td>
<td>3.4 ± 0.10</td>
<td>3.77 ± 0.19</td>
</tr>
<tr>
<td>14</td>
<td>72.3 ± 3.84</td>
<td>65.0 ± 2.89</td>
<td>64.67 ± 2.6</td>
<td>61.3 ± 3.48</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>16</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>27</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>61.3 ± 3.3</td>
<td>57.3 ± 2.9</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>50.3 ± 0.88</td>
<td>29.0 ± 5.51</td>
<td>49.67 ± 0.33</td>
<td>39.67 ± 2.91</td>
<td>10.67 ± 0.6</td>
<td>11.0 ± 0.58</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>2.45 ± 0.48</td>
<td>2.53 ± 0.32</td>
<td>2.17 ± 0.34</td>
<td>2.57 ± 0.20</td>
<td>3.6 ± 0.21</td>
<td>3.15 ± 0.22</td>
</tr>
</tbody>
</table>

To assess whether the inhibition of NQO2 was responsible for the cytotoxicity of the compounds, the levels of cellular NQO2 were genetically modulated prior to re-evaluating the compounds’ toxicity. This was conducted by using isogenically paired cell lines such as SKOV-3 (NTC control and sh27 cells sub-lines), as well as TOV-112D (EV control and NQO2-OE cells sub-lines). To investigate the effect of silencing NQO2 on the sensitivity of SKOV-3 cells to the compounds’ toxicity, it was first necessary to induce NQO2 silencing before initiating the experiment. Therefore, the sh27 cells were exposed to dox (+dox) for five days. The cells were then seeded and, following overnight incubation, were treated with the compounds at this concentration range (0.001-100µM) for 96hr. The possible role of NQO2 in mediating the compounds’ toxicity was also evaluated in TOV-112D overexpressing NQO2, and compared with EV. The compounds’ IC\textsubscript{50} values were determined from dose-response curves generated from three independent experiments. They are represented in this table as mean ± SEM, and used for comparative purposes. Asterisks (**) indicate the compounds that did not show any cytotoxicity at the tested range of concentration.

#### 4.2.4 Using cytotoxicity levels of CB1954 as a measure of functional activity of the novel compounds against cellular NQO2

The intracellular inhibitory activity of the novel compounds against NQO2 should be evaluated in order to determine the most potent inhibitors to be used for the future study of NQO2 functions in the cells. To do this, a subset of novel compounds with the highest extracellular inhibitory potency and/or low cytotoxicity was selected according to the previous findings. Furthermore, cell lines with high activity levels of NQO2 were utilised, and assays relying on the compound targeted mainly by
NQO2 were also designed. The CB1954 chemotherapeutic agent was selected for this purpose, as it has been found that it is bioactivated primarily by NQO2 giving potent cytotoxins [100]. This means that the bioactivation extent and subsequent toxicity of CB1954 may rely, in the first place, on the NQO2 activity levels in the cells. This point was exploited to evaluate the intracellular NQO2 inhibitory potency of the compounds, which may proportionally influence the cytotoxicity extent of CB1954. The toxicity of CB1954 in SKOV-3 cells expressing high level of NQO2 was measured in the presence of these inhibitors and used as an indirect measure of the compounds’ potency against NQO2 activity in the cells. To validate the CB1954 approach, it was first necessary to confirm that the NQO2 activity level in OVC cell lines was the major determinant of CB1954 bioactivation and subsequent toxicity; this was done via the use of several methods.

4.2.4.1 Effect of EPR on NQO2-mediated CB1954 bioactivation in SKOV-3 cells

As CB1954 has been found to be bioactivated only in the presence of NQO2 co-factor [100], the EPR function as an activating co-factor of NQO2 enzymatic activity in OVC cells was evaluated by measuring the cytotoxicity of CB1954 in the presence of various doses of EPR. To do this, SKOV-3 cells were seeded in 96-well plates and, following overnight incubation, were treated with various concentrations of CB1954 (0.001µM-100µM) alone and in combination with fixed concentrations of EPR (1, 10, 50 and 100µM) for 24hr. Thereafter, the treatment was replaced with fresh medium for 96hr. To terminate the experiment after this time, the MTT solution was added to the cells as a read out of cell survival. The fraction of viable cells unaffected by the treatment was calculated as a percentage of solvent control, and plotted against the concentrations range of CB1954, generating dose-response curves for each treatment condition. Figure (4.4) shows that treating the cells with CB1954 alone, even at the highest concentration of 100µM, only generates a 25% cytotoxicity, which is substantially enhanced to 70% with EPR (100µM) presence. Clearly, there appears to be a direct correlation between EPR concentration and CB1954 cytotoxicity caused by NQO2-mediated CB1954 bioactivation. Increased EPR concentrations were accompanied by consistent enhancements of CB1954 cytotoxicity. In other words, EPR induced NQO2-mediated CB1954 bioactivation and subsequent cytotoxicity in a dose-dependent manner. Notably, EPR at these concentrations showed negligible cellular toxicity, as demonstrated in Figure (9.2) in the appendix
chapter. The concentration of EPR (100µM) was selected for future works for the maximum induction of NQO2. The combination therapy of CB1954 and EPR is termed as CB1954/EPR within the text.

Figure 4.5 Effects of EPR-supporting NQO2 activity on the CB1954 cytotoxicity in SKOV-3 cells

The role of EPR as a supportive co-factor of NQO2 enzymatic activity in SKOV-3 cells was evaluated by measuring CB1954 cytotoxicity using MTT assay. The cells were treated with a range of concentration of CB1954 (0.001-100µM); alone (▲) and in combination with several fixed doses of EPR 1µM (●), 10µM (◇), 50µM (■) and 100µM (★) for 24hr before re-incubating the cells with treatment-free medium for 96hr. Each point on the curve is representative of the mean of three independent experiments with its standard error (± SEM), and the curves are the lines for the best fit for these points.

4.2.4.2 Effect of varying basal levels of NQO2 on CB1954 bioactivation

To investigate the effect of NQO2 cellular activity levels on CB1954 bioactivation and subsequent toxicity, the toxicity of CB1954 was measured in OVC cell lines possessing heterogeneous levels of NQO2, and then examined for its correlation with the NQO2 activity. The cells were seeded in parallel in 96-well plates, and treated with this range of concentration (0.001–500µM) of CB1954, both alone and in combination with EPR (100µM), for 24hr. Thereafter, the treatment was replaced with fresh medium for 96hr before adding MTT solution to the cells. The fraction of survival cells unaffected by CB1954 treatment was calculated as a percentage of solvent control. The percentage of cell survival is plotted against the CB1954 concentrations range to create a dose-
response curve for each treatment condition (CB1954 alone and CB1954/EPR), Figure (4.5). The IC$_{50}$ values of both treatment conditions are determined from these dose-response curves, and listed in Table (4.7). Since the EPR concentration was fixed in this experiment, any change in the extent of CB1954 bioactivation and subsequent cytotoxicity appearing among cell lines can be attributed to the differences in the NQO2’s cellular activity levels. To facilitate addressing these changes and their correlation with NQO2 activity, the toxicity enhancement ratio of CB1954 in each cell line was determined using this formula; (toxicity ratio= IC$_{50}$ of CB1954 alone/ IC$_{50}$ of CB1954/EPR). These ratios refer to relative folds increase in the cytotoxicity of CB1954 upon the addition of EPR as a result of catalysing NQO2-mediated CB1954 bioactivation.

It was proved previously that EPR mediated the bioactivation of CB1954 and subsequent cytotoxicity by supporting the activity of NQO2. Therefore, the toxicity ratio might be affected by the activity levels of NQO2 in the cells. To better understand the correlation between NQO2 activity and CB1954 toxicity ratio, the Pearson’s correlation coefficient (r) was determined from the scatter graph generated from plotting these variables, as shown in Figure (4.6). The activity levels of NQO2 in these cell lines have already been evaluated in chapter 3 section (3.2.2), using the cytochrome c spectrophotometric assay, and are re-shown here in Table (4.7). This table demonstrates a 36-fold range in the NQO2 activity among the OVC cell lines.
Figure 4.6 Sensitivity of OVC cell lines to CB1954 (continue on next page)
CB1954 cytotoxicity in OVC cell lines (possessing heterogeneous levels of NQO2) was measured in order to address the effect of varying NQO2 activity levels on the cytotoxicity of CB1954 bioactivation. The cells were treated with the range of concentration (0.001-500µM) of CB1954, both alone (▲) and in combination with EPR (100µM) (●) for 24hr prior to re-incubating them with fresh medium for 96hr. MTT assay was used to be able to measure the percentages of survival cells unaffected by treatment relative to solvent control. The average of survival percentages at various concentrations was calculated from three independent experiments and used to generate the best fit curves.

It is apparent from Figure (4.5) that the cytotoxicity of CB1954 in all tested cell lines increases in a dose-dependent manner. Markedly, this cytotoxicity was significantly increased in the presence of EPR in several cell lines, including SKOV-3, A2780, OV-90 and TOV-112D. As can be seen from Table (4.7), there is a relatively small variation of only a 181-fold range in the responsiveness of OVC cell lines to CB1954 alone. This, however, enhances enormously to 1,385-fold range upon the addition of EPR.

Compared to other cell lines, SKOV-3 was found to be the most resistant cell line to CB1954 in the absence of EPR. Interestingly, the addition of EPR to the CB1954 treatment rendered SKOV-3 cells much more sensitive, with more than a 200-fold increase in the toxicity ratio as compared to CB1954 alone. This enhancement in responsiveness to the combination therapy (CB1954 plus EPR) was not only restricted to the resistant cells, but was also present in the most sensitive cell line A2780, which responded to CB1954 treatment much better in the presence of EPR, with a 32-fold increase in the CB1954 toxicity ratio. On the other hand, exposing CaOV-3 and OVCAR-3 cells to the combination therapy of CB1954 plus EPR resulted in a marginal increase in their sensitivity as compared to CB1954 alone, with approximately a 4-fold change. Interestingly, there was no substantial increase in the sensitivity of TOV-21G cells to CB1954 upon the addition of EPR, although their characteristics (e.g. sensitivity level to CB1954 alone and activity level of NQO2) were similar to that of A2780 cells. Additionally, treating TOV-112D cells with CB1954 plus EPR resulted in a significant increase in toxicity ratio (around 31-fold difference), although the activity levels of NQO2 was similar to that low levels of OVCAR-3 cells.

A potential correlation emerged between the toxicity ratio of CB1954 and NQO2 activity levels ($r = 0.87$), Figure (4.6). This verified that the extent of CB1954 bioactivation was strongly dependent on
the activity levels of NQO2 in the cells: the higher the NQO2 activity levels in the cells, the more bioactivation of CB1954 to cytotoxins is catalysed.

Table 4.7 Sensitivity of OVC cell lines to CB1954 alone and in combination with EPR

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Cellular NQO2 Activity (nmol/ min/ mg of protein)</th>
<th>IC\textsubscript{50} (µM) ± SEM at 24hr</th>
<th>Toxicity Ratio ((-/+) EPR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CB1954 Alone</td>
<td>CB1954+ 100µM EPR</td>
</tr>
<tr>
<td>A2780</td>
<td>63.43 ± 8.3</td>
<td>0.84 ± 0.21</td>
<td>0.026 ± 0.004</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>153.29 ± 14.9</td>
<td>152.3 ± 35.71</td>
<td>0.70 ± 0.15</td>
</tr>
<tr>
<td>CaOV-3</td>
<td>15.56 ± 3.9</td>
<td>65.67 ± 22.84</td>
<td>17.0 ± 3.79</td>
</tr>
<tr>
<td>OV-90</td>
<td>49.40 ± 5.5</td>
<td>33.67 ± 4.1</td>
<td>1.0 ± 0.29</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>4.20 ± 0.4</td>
<td>135.0 ± 5.0</td>
<td>36.0 ± 9.85</td>
</tr>
<tr>
<td>TOV-21G</td>
<td>65.97 ± 7.8</td>
<td>3.63 ± 1.41</td>
<td>0.93 ± 0.49</td>
</tr>
<tr>
<td>TOV-112D</td>
<td>5.07 ± 0.7</td>
<td>42.67 ± 8.19</td>
<td>1.37 ± 0.27</td>
</tr>
</tbody>
</table>

The above table lists the activity levels of NQO2 in OVC cell lines which were measured using the cytochrome c-based spectrophotometric assay, and expressed as nmole of cytochrome c reduced per min per mg of protein. It also demonstrates the IC\textsubscript{50} values of both treatment conditions (CB1954 alone and CB1954/EPR) in OVC cell lines following 24hr exposure, which were determined from the dose-response curves displayed in Figure (4.5). These values were represented as the means of three independent experiments with their standard error (±SEM). Toxicity ratios were calculated following the formula (IC\textsubscript{50} of CB1954 alone/ IC\textsubscript{50} of CB1954/EPR) to indicate the levels of enhancement in the toxicity of CB1954 upon the addition of EPR.
Figure 4.7 Correlating the sensitivity of OVC cell lines to CB1954 with the intracellular activity levels of NQO2

NQO2 activity levels in OVC cell lines were plotted against the toxicity ratios of CB1954 on a scatter graph, so as to define the strength of the correlation between these variables. Enzyme activity was determined using the spectrophotometric assay, while the toxicity ratio was calculated by dividing IC_{50} values of CB1954 alone by CB1954 plus EPR. This plot shows that the cytotoxicity of CB1954 is potentially correlated with the NQO2 activity levels, as demonstrated by the high value of Pearson’s correlation coefficient (r = 0.87), which was calculated using the Excel Correl function in Microsoft Excel 2007.

4.2.4.3 Effect of genetic modifications of the NQO2 levels on CB1954 bioactivation

To further validate the previous findings regarding the participatory role of NQO2 in mediating cytotoxicity of CB1954 bioactivation, the isogenically paired cell lines possessing different NQO2 activity levels were tested for their sensitivity to CB1954/EPR. CB1954 alone treatment was excluded from this experiment as the focus here is on studying the effect of modifying (removing/inserting) one component of the CB1954 bioactivation system on CB1954 cytotoxicity, which was the NQO2 in this part of the study. Also by doing this, it can be proved that EPR itself had no impact on CB1954 bioactivation, and its action was actually mediated by NQO2 activity.

4.2.4.3.1 Effect of genetic silencing of NQO2 on CB1954 bioactivation in SKOV-3 cells

Initially, SKOV-3 derived cell sub-line expressing sh27 silencing sequence against NQO2 (sh27) needed to be incubated with (1µg/ml) of dox for five days prior to conducting the experiment, in
order to induce the maximum silencing of NQO2. Then, all cell types (including NTC (± dox) and sh27 (± dox)) were re-seeded in parallel in 96-well plates. The following day, they were treated with a range of concentrations of CB1954 (0.001-100µM) combined with EPR (100µM) for 24hr, after which the treatment was replaced with fresh medium for 96hr. The proportions of survival cells were assessed using MTT assay after 96hr, and calculated as percentages relative to the solvent control. Figure (4.7) is representative of the dose-response curves of all cell types.

From the data in Figure (4.7), it is evident that although all cells respond to the increasing concentrations of CB1954/EPR, they do so to different extents. For example, treating NTC/+dox cells with 0.2µM CB1954/EPR caused a 50% reduction in cell survival compared to only a 20% reduction in the case of sh27/+dox cells. This means that inducing NQO2 silencing in sh27/+dox cells rendered the cell resistant to CB1954/EPR treatment compared to NTC/+dox cells (expressing endogenous high level of NQO2), with a 4-fold difference in the IC_{50} values; IC_{50} values significantly increased from 0.24 ± 0.03µM (in NTC/+dox cells) to 0.94 ± 0.09µM (in shN27/+dox cells). This result is significant at the **p value <0.005 level. These findings also verify the lack of direct activity of EPR on CB1954 cytotoxicity, as sh27/+dox cells were unable to respond to CB1954/EPR to a similar extent as that of NTC/+dox cells, even though they both underwent the same treatment conditions.
Figure 4.8 Sensitivity of SKOV-3 cells to CB1954 toxicity after silencing the NQO2 gene expression

SKOV-3 control (NTC) cells and their derived cell sub-line sh27 were used to verify the participatory role of NQO2 in the cytotoxicity of CB1954 bioactivation. This was conducted by measuring and comparing CB1954 toxicity between NTC expressing high levels of NQO2 and sh27 possessing silenced NQO2. NQO2 silencing is induced by treating sh27 cells with dox (1µg/ml) for five days prior to conducting the experiment. The cells were re-seeded and treated with the range of concentration (0.001-100µM) of CB1954 in conjunction with EPR (100µM) for 24hr. The cell survival was analysed following a 96hr incubation using MTT assay. This figure is representative of the dose-response curves of the CB1954/EPR therapy generated from three independent experiments for NTC control cells, both untreated (∙) and treated (∙) with dox, in addition to sh27 cells both untreated (▲) and treated (▲) with dox. The black perpendicular lines indicate the IC$_{50}$ values on the x-axis. An unpaired t-test was used to determine the significance level for the difference in sensitivity of NTC/+dox and sh27/+dox cells to CB1954/EPR. Asterisks on the sh27/+dox curve were used to indicate that the IC$_{50}$ value was significantly higher than that of NTC/+dox (p value<0.005).

4.2.4.3.2 Effect of overexpression of NQO2 on CB1954 bioactivation in TOV-112D cells

Having validated the effect of silencing the functional activity of NQO2 on the bioactivation of CB1954 in SKOV-3 cells, it was worth proving this again through the use of a completely different system of NQO2-overexpressing TOV-112D cells.
Interestingly, as noted in Table (4.7) in section (4.2.4.2), the wild type cells of TOV-112D become more sensitive to CB1954 upon the addition of EPR, as reflected by the high value of the toxicity ratio, despite the presence of low NQO2 activity levels. To verify that the bioactivation of CB1954 in these cells was primarily mediated by NQO2, the cytotoxicity of CB1954/EPR combination therapy was re-evaluated in TOV-112D derived cell sub-lines (controls (WT and EV) and NQO2-overexpressing (NQO2-OE) cells). To do this, the cells were seeded in parallel in 96-well plates. The following day, they were treated with a range of concentration (0.001-100µM) of CB1954 in conjunction with EPR (100µM) for 24hr, before being re-incubated with fresh medium (free of treatment) for 96hr. To analyse cell survival after this time, the MTT assay was used. The percentages were calculated relative to the solvent control and represented against the concentration range of CB1954, as shown in Figure (4.8).

In fact, overexpressing NQO2 in TOV-112D cells shifts the dose-response curve downwards, as illustrated in Figure (4.8), as a result of substantial enhancement of the cell sensitivity to CB1954/EPR treatment. By contrast, the EV curve remained in the same place, close to the WT curve. This indicates that EV cells responded to the treatment to the same extent that WT did, although not as much as the NQO2-OE cells. The IC$_{50}$ value of CB1954/EPR in NQO2-OE was found to be significantly 21-fold less than that of EV, with 0.09 ± 0.003µM and 1.88 ± 0.41µM respectively, (**p value<0.05).
To elucidate the effect of overexpressing NQO2 on CB1954 bioactivation, TOV-112D isogenically paired cells, including EV control cells and NQO2-overexpressing (NQO2-OE) cells, were selected. The cells were treated with the range of concentration (0.001-100µM) of CB1954 combined with EPR (100µM) for 24hr. MTT assay was used to measure cell survival after 96hr and percentages were calculated from three independent experiments. This figure represents the dose-response curves of WT (▲), EV (●) and NQO2-OE (◆) cells. Asterisks indicate significant difference in the IC₅₀ values of CB1954/EPR treatment between NQO2-OE and EV, as determined from the unpaired t-test (p value<0.05). The red perpendicular lines indicate the IC₅₀ values on the x-axis.

4.2.5 Sensitivity of OVC cell lines to carboplatin alkylation agent

Weedon et al. have suggested that the cell lines that show resistance to platinum-based drugs might have collateral resistance to other alkylation agents [230]. In light of this, an investigation was carried out to determine whether the resistance mechanisms developed in OVC cell lines against platinum drugs does impair the bioreductive activity of NQO2 to activate CB1954, or interferes with the responsiveness of the cells to CB1954 cytotoxicity. In other words, does the toxicity profile of OVC to CB1954 essentially rely on the intracellular activity of NQO2? To answer this question, the sensitivity profiles of the same panel of cell lines for both alkylation agents (CB1954 and platinum-based drugs such as carboplatin) were examined for comparison purposes. The cells were treated,
following overnight incubation, with a range of concentration of carboplatin (0.001-100µM) for 24hr. Cell survival was estimated again with MTT assay to ensure that both agents were evaluated for cytotoxicity by the same method. The percentage of survival cells unaffected by the carboplatin treatment is represented by curve fitting, as shown in Figure (4.9). The IC\textsubscript{50} values were determined from these dose-response curves and listed in Table (4.8). They were used as indicator values for cell sensitivity to carboplatin.

Figure 4.10 Sensitivity of OVC cell lines to carboplatin

The 24hr cytotoxicity of carboplatin was evaluated in this panel of OVC cell lines over a range of concentrations of (0.001-100µM). The survival cell proportions at these concentrations were estimated using MTT assay following 96hr incubation, and were calculated as percentages relative to the solvent control. Points on the graphs are representative of mean values of cell survival percentages calculated from three independent experiments, which are connected with best fit lines. Representative curves are dose-response curves for A2780 (△), SKOV-3 (●), CaOV-3 (◆), OV-90 (■), OVCAR-3 (▼), TOV-21G (★) and TOV-112D (●).

Table (4.8) represents the sensitivity profile of OVC cell lines for carboplatin and CB1954/EPR alkylating agents. Markedly, this panel of OVC cells showed a 25-fold difference in its sensitivity ranges between carboplatin (55-fold) and CB1954/EPR (1,385-fold). SKOV-3 followed by OVCAR-3 cells were found to be the most resistant cell lines to carboplatin, with IC\textsubscript{50} values of 73.3 ± 7.7µM and 59.67 ± 12.9µM respectively, while TOV-21G cells were the least resistant, with an IC\textsubscript{50} of 1.32
± 0.22µM. Despite the resistance of SKOV-3 cells to carboplatin, they were amongst the most sensitive cell line to CB1954/EPR treatment, with a 105-fold difference in IC₅₀s between both treatments. In contrast, OVCAR-3 cells showed comparable resistance levels for carboplatin and CB1954/EPR with a very small 1.7-fold difference in IC₅₀ values. Another pair of cell lines with similar sensitivity profiles for carboplatin was selected to investigate whether their sensitivity to CB1954/EPR correlates to that of carboplatin. Different patterns of responsiveness were also found between OV-90 and CaOV-3 cell lines towards CB1954/EPR, despite the high similarity in their sensitivity to carboplatin. It became evident that OV-90 cells were much more sensitive (by 29-folds) to CB1954/EPR than carboplatin, whereas there was little difference in the sensitivity of CaOV-3 cells to both alkylating agents, with IC₅₀ values close to 17µM. Interestingly, the carboplatin-sensitive A2780 cell line also responded much better to CB1954/EPR than carboplatin, with a more than 100-fold difference in the IC₅₀ values.

It is important to note that these cell lines possess various NQO2 activity levels; the highest levels appeared in SKOV-3, A2780 and OV-90 cells while the lowest were in OVCAR-3 and CaOV-3 cells, as shown in Table (4.7).
Table 4.8 Sensitivity profiles of OVC cell lines to carboplatin and CB1954/ EPR alkylating agents

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Carboplatin IC_{50} (µM) ± SEM</th>
<th>CB1954/ EPR IC_{50} (µM) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr</td>
<td>24hr</td>
</tr>
<tr>
<td>A2780</td>
<td>2.67 ± 0.28</td>
<td>0.026 ± 0.004</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>73.3 ± 7.7</td>
<td>0.70 ± 0.15</td>
</tr>
<tr>
<td>CaOV-3</td>
<td>17.3 ± 1.2</td>
<td>17.0 ± 3.79</td>
</tr>
<tr>
<td>OV-90</td>
<td>28.5 ± 2.5</td>
<td>1.0 ± 0.29</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>59.67 ± 12.9</td>
<td>36.0 ± 9.85</td>
</tr>
<tr>
<td>TOV-21G</td>
<td>1.32 ± 0.22</td>
<td>0.93 ± 0.49</td>
</tr>
<tr>
<td>TOV-112D</td>
<td>22.3 ± 1.67</td>
<td>1.37 ± 0.27</td>
</tr>
</tbody>
</table>

This table lists the IC_{50} values of carboplatin and CB1954/EPR for OVC cell lines following 24hr exposure, which were determined from the dose-response curves displayed in Figures (4.9) and (4.5) respectively. These values were represented as the mean of three independent experiments with their standard error (± SEM). They were used as indicator values for the sensitivity of cells to carboplatin and CB1954/EPR.

4.2.6 Assessing the intracellular inhibitory potency of putative NQO2 inhibitors in SKOV-3 cells

4.2.6.1 Effect of putative inhibitors of NQO2 on CB1954 bioactivation in SKOV-3 cells

All of the previous findings provided a strong platform for the evaluation of novel compounds' functional activity as NQO2 inhibitors, and their intracellular inhibitory potency. A subset of the compounds that showed high extracellular inhibitory potency against rhNQO2 was selected to undergo further evaluation in the cellular system. As with the earlier experiment which studied the impact of silencing of NQO2 gene on the cytotoxicity of CB1954 bioactivation, SKOV-3 cells were also used to assess the effect of combining CB1954 with NQO2 inhibitors on the enzymatic bioactivation and subsequent cytotoxicity of CB1954. To do this, the cells were treated with the combination therapy of varying concentrations of CB1954, ranging from 0.001µM to 10µM plus EPR, and concomitantly with fixed concentrations of the inhibitors. The cells were incubated with
the treatment for 24hr and 96hr, after which the experiment was terminated via the use of MTT assay to evaluate cell viability. Percentages of survival cells unaffected by treatments (CB1954/EPR and CB1954/EPR/inhibitor) were determined and used to generate dose-response curves, as shown in Figure (4.10) (please refer to the appendix to find the curves of the remaining compounds, Figure (9.3)). These curves were useful in examining the effect of the pharmacological inhibition of NQO2 on the cytotoxicity of CB1954 bioactivation, which indirectly pointed to the inhibitory activity of the compounds against cellular NQO2 activity. Concomitant treatment of the cells with inhibitors was observed to protect the cells effectively against CB1954 cytotoxicity in a dose-dependent manner, as reflected by the upward shifting of the curves. For instance, the toxicity developed by the highest concentration of CB1954 is substantially reduced by the concomitant treatment with compound 11, resulting in a significant enhancement in the cell viability, which ranged from 5% in the presence of 0.05µM concentration of compound 11, to 50% upon increasing the concentration to 1µM, Figure (4.10, B*). It was also noted that this protective effect caused by the inhibitors against CB1954 toxicity became more pronounced when the cells were treated for the longer duration of 96hr with CB1954/EPR/inhibitor. Since these inhibitors generated a similar pattern of CB1954 cytotoxicity reduction as that observed by the genetic silencing of NQO2, it can be argued that these compounds were functionally active as inhibitors of cellular NQO2.
Figure 4.11 Sensitivity of SKOV-3 cells to CB1954 toxicity in the presence of putative inhibitors of NQO2 (continue on next page)
The protective effects of NQO2 inhibitors against enzymatic bioactivation and subsequent cytotoxicity of CB1954 were measured using MTT assay. SKOV-3 cells were treated with a range of concentration of CB1954 (0.001-10µM) plus EPR, as well as in combination with compound 10 (A/A*), 11 (B/B*), 13 (C/C*), 27 (D/D*), 9AA (E/E*) and quercetin (F/F*) for (24hr and 96hr*). CB1954 (-) (●) means that the cells were treated with CB1954/EPR only, without the inhibitor. Dashed curves and solid curves represent the best fit lines for the average points calculated from three independent experiments; these were generated after 24hr and 96hr exposure to treatment respectively. The bars represent SEM. Each inhibitor was tested at varying concentrations selected depending on their cytotoxicity: 0.05µM (○), 0.1µM (○), 0.5µM (●), 1µM (●), 2µM (●), 5µM (●), 10µM (●), 20µM (●), 30µM (●), 50µM (●) and 100µM (●). It is evident that these inhibitors protect the cells from CB1954 cytotoxicity in a dose-dependent manner via NQO2 inhibition, as demonstrated by the upward shifting of the curves.

After validating these inhibitors’ effectiveness in inhibiting NQO2 in the cells, their intracellular inhibitory potency needed to be measured for comparison and selection purposes. These measurements can be also used as indicators of the protein binding affinity of the compounds, which might be directly correlated to their potency in the cellular system. To measure this, a fixed concentration of 1µM of CB1954, which caused a 60% reduction in cell survival, was chosen to be combined with increasing concentrations of selected inhibitors. It was necessary to begin with low concentrations range of inhibitors, in order to determine whether the inhibition of NQO2 activity-mediated CB1954 bioactivation began from a lower range of concentration. SKOV-3 cells were treated with these preparations for 24hr and 96hr. The viability of cells was assessed after 96hr using MTT assay to generate dose-response curves. These curves displayed the percentages of survival cells that were protected from the cytotoxicity caused by the bioactivation of 1µM CB1954. This protection was mediated by the novel inhibitors which in turn inhibits catalytic activity of NQO2-mediated CB1954 bioactivation and subsequent toxicity. Therefore, the concentration of inhibitor that reduces CB1954 cytotoxicity by 50% can be used to effectively measure the compounds’ IC₅₀ of intracellular NQO2 activity. These values were determined from dose-response curves generated from at least three independent experiments, Figure (4.11). This figure illustrates that the structural diversity of compounds provides generally different levels of protection against CB1954, which begin from the low concentrations range of most compounds, and are enhanced substantially with the presence of higher concentrations.
The inhibitory potency of the compounds to the cellular NQO2 was evaluated by measuring their protection levels against the cytotoxicity of CB1954 bioactivation using MTT assay. SKOV-3 cells were concomitantly treated with a fixed concentration of 1µM of CB1954 plus EPR and varying concentrations of NQO2 inhibitors for 24hr and 96hr. Cell viability was assessed after 96hr to generate dose-response curves. Red dashed curves and black solid curves represent the best fit lines for the average points calculated from three independent experiments, which were generated after 24hr (●) and 96hr ( ○) exposure to combination therapy respectively. The bars represent SEM. These curves show that the cytotoxicity of CB1954 (1µM) decreases while the inhibitor concentrations increase, which in turn enhances the inhibition of NQO2 activity-mediated CB1954 bioactivation in a dose-dependent manner. Figures (A-F) illustrate concomitant treatments with compound 10, 11, 13, 14, 27 and 9AA respectively.
As seen in Table (4.9), the highest intracellular inhibitory potency appears among the hydrazone derivatives. Compound 11 was the most potent inhibitor from this group, with an IC$_{50}$ value of 0.053 ± 0.007µM. It was approximately 9-fold even more potent than 9AA, the typical potent NQO2 inhibitor (IC$_{50}$ value is 0.45 ± 0.14µM). Compound 10 also showed slightly higher potency than 9AA. Furthermore, all novel compounds were observed to be much more potent than resveratrol and quercetin, (please refer to the appendix to find the survival curves of CB1954 in the presence of these compounds and others, Figure (9.4)). Although hydrazide compound 27 was the least potent inhibitor compared to the novel hydrazone derivatives, it was still effective at inhibiting NQO2 activity at low non-toxic concentrations. The very low toxicity of compound 27 therefore allows for the use of high concentrations in order to achieve required levels of NQO2 inhibition. It is worth noting that the concentrations of inhibitors that showed high inhibition levels of cellular NQO2 were decisively far from their cytotoxic ranges. This supports the aforementioned conclusion regarding the non-involvement of NQO2 inhibition in the compounds’ cytotoxicity.

### Table 4.9 Potency of NQO2 inhibitors as represented by IC$_{50}$ values of NQO2 in SKOV-3 cells

<table>
<thead>
<tr>
<th>Compound Number/ Name</th>
<th>24hr (IC$_{50}$ (µM) ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibitor + CB1954/ EPR</td>
</tr>
<tr>
<td>10</td>
<td>2.07 ± 0.5</td>
</tr>
<tr>
<td>11</td>
<td>0.053 ± 0.007</td>
</tr>
<tr>
<td>13</td>
<td>0.98 ± 0.22</td>
</tr>
<tr>
<td>14</td>
<td>0.44 ± 0.22</td>
</tr>
<tr>
<td>16</td>
<td>22.7 ± 8.9</td>
</tr>
<tr>
<td>27</td>
<td>74 ± 11.4</td>
</tr>
<tr>
<td>9AA</td>
<td>0.45 ± 0.14</td>
</tr>
</tbody>
</table>

SKOV-3 cells were concomitantly treated with CB1954 (1µM)/EPR and a range of concentration of inhibitors for 24hr. Different ranges of concentrations of inhibitors were selected, depending on the compounds’ cytotoxicity. Cell viability was estimated using MTT assay to generate dose-response curves Figure (4.11), from which IC$_{50}$ values were determined. The definition of IC$_{50}$ in this context is the concentration of inhibitors required to protect the cells from the toxicity of CB1954 (1µM)/EPR by 50% relative to control, as a result of inhibition of 50% of NQO2-mediated CB1954 bioactivation. Therefore, this term can also be used to refer to the IC$_{50}$ of intracellular NQO2 which indicates to the compounds’ potency.
To investigate whether the novel compounds’ extracellular inhibitory potency is consistent with their intracellular inhibitory potency, comparisons were made between the compounds’ IC₅₀ in the extracellular system listed in Table (4.1 and 4.2), and the intracellular system listed in Table (4.9). It was observed that the inhibitors’ intracellular potency levels were potentially correlated with their extracellular potency, with \( r = 0.9 \), Figure (4.12).

![Figure 4.13 Correlation between intra- and extra-cellular inhibitory potency of a subset of the novel quinolines analogues](image)

A subset of the novel inhibitors including compound 10, 11, 13, 14, 16 and 27 along with typical inhibitor (9AA) were investigated for the strength of correlation between their NQO2 inhibitory potency in different systems. To do this, compounds’ IC₅₀ extracellular inhibitory potency was plotted against their IC₅₀ intracellular inhibitory potency on a scatter graph. The former values represented in Table (4.1 and 4.2) were measured in cell free system using DCPIP spectrophotometric assay while the latter values represented in Table (4.9) were measured in vitro using CB1954 approach. This plot shows that extracellular inhibitory potency of these compounds was potentially correlated with their intracellular inhibitory potency, as demonstrated by the high value of Pearson’s correlation coefficient \( r = 0.93 \), which was calculated using the Excel Correl function in Microsoft Excel 2007.

### 4.2.6.2 Effect of the concomitant inhibition of NQO2 activity pharmacologically and genetically on CB1954 bioactivation in SKOV-3 cells

Since the silencing of NQO2 gene resulted in only a 65% reduction in the enzymatic activity, it was worth investigating whether the integration of pharmacological inhibition with shRNA-27-NQO2...
gene silencing could maximise the inhibition levels of NQO2. To do this, the cytotoxicity of CB1954/EPR was evaluated again in the presence of compound 11 and 9AA (the most potent inhibitors of NQO2), but in the cells-possessing basal and silenced levels of NQO2. NQO2 activity had to be silenced prior to and throughout the experiment, so sh27 cells were exposed to dox (1µg/ml) for five days and then re-seeded with dox in 96-well plates. They were treated with a range of concentration of CB1954 (0.001-10µM) combined with EPR, concomitantly with fixed concentrations of inhibitors for 24hr. Thereafter, the cells were re-incubated with fresh medium for 96hr. Cell viability was assessed using MTT assay to generate dose-response curves. Figure (4.13). Figure (4.13, A-B) demonstrates that each inhibitor reduces the cytotoxicity of CB1954 in a dose-dependent manner and at a similar efficacy in NTC/-dox and sh27/-dox cells expressing endogenous high basal levels of NQO2 (before inducing shRNA-NQO2 silencing via dox). However, the levels of protection provided by each inhibitor against CB1954 toxicity was significantly different between NTC/+dox cells (expressing endogenous NQO2) and sh27/+dox cells (possessing silencing NQO2). For example, the cell death caused by CB1954 (1µM) in NTC/+dox was reduced by 57% in the presence of 1µM concentration of compound 11, compared to a 40% reduction in sh27/+dox cells. In the same manner, in the presence of 2µM of 9AA, the cell death caused by CB1954 (1µM) was significantly reduced in NTC/+dox by over 50% compared to a 30% reduction in sh27/+dox, when both were compared to their corresponding cell sub-lines treated with only CB1954 (i.e. in the absence of inhibitor). These findings supported the previous notion that the novel inhibitors are highly potent and selective. Although NQO2 levels were downregulated in sh27/+dox, compound 11 and 9AA were still efficient at inhibiting the remaining NQO2 activity in the cells, but to a lesser extent when compared to that inhibition levels provided in the entire existence of NQO2 in sh27 cells untreated with dox.

Interestingly, analysing the results from different perspectives allowed for the observation of a massive reduction in the cytotoxicity of CB1954 when NQO2 activity was inhibited genetically as well as pharmacologically. Figure (4.13, C-D) shows that the cell death caused by the highest CB1954 concentration (10µM) is reduced greatly in sh27/+dox cells upon targeting the remaining activity of NQO2 by 1µM of compound 11, with more than a 53% increase in cell viability when
compared to sh27/+dox treated with (CB1954 (-) alone). This level of protection is comparatively higher than that provided by either NQO2 gene silencing (as in sh27) alone or by NQO2 pharmacological inhibition using potent inhibitors alone (as in NTC/+dox/+inhibitor).

Figure 4.14 Efficiency of NQO2 inhibitors at protecting SKOV-3 cells possessing silencing NQO2 against CB1954 toxicity

In order to examine the effect of using a combination therapy of dox and potent inhibitors on the cellular activity of NQO2, the toxicity of CB1954 was assessed using MTT assay after 96hr. This allowed to investigate whether the usage of such combination could maximise the NQO2 inhibition levels compared to that of each individual treatment. SKOV-3-derived cells sub-lines (NTC and sh27) were treated with dox (1µg/ml) for five days prior to the experiment, and were seeded along with untreated cells in 96-well plates. The cells were then exposed to a range of concentrations of CB1954 (0.001-10µM) plus EPR, and also in combination with compound 11 (A and C) and 9AA (B and D) for 24hr. Compound 11 was tested at 0.05µM (○) and 1µM (●) while 9AA at 0.5µM (●) and 2µM (○). CB1954 (-) (●) refers to the cells which were treated with CB1954/EPR only (i.e. in the absence of inhibitors). Figures (A and B) represent the CB1954 dose-response curves of the cells treated with inhibitors only, while blue highlighted Figures (C and D) represent the curves of the cells treated with a combination of dox and inhibitors. Dashed curves and solid curves represent the best fit lines for the average points calculated from three independent experiments, which were also referred to sh27 and NTC cells respectively. The bars represent SEM.
The biological roles of NQO2 in cancer are still not well understood; there exist several controversial assumptions regarding its cellular activity in cancer. The availability of less effective NQO2 inhibitors with poor specificity, low potency or high toxic properties made the discernment regarding the actual functions of NQO2 in cells difficult. To overcome this obstacle, a virtual screening study has been conducted on NCI compounds in order to identify novel ligands with an inhibitory activity against NQO2. These ligands can afterward be used as lead compounds to synthesise further NQO2 inhibitors with better properties, such as high potency. Among these compounds, NCI quinolines, which showed high inhibitory potency properties, were selected by Dr Buthaina Hussein from Dr Freemans’ lab, and used as a lead scaffold to synthesise novel analogues of hydrazone, hydrazide and carboxamide quinolines with different substitutions. These substitutions helped to get highly potent, specific and less toxic NQO2 inhibitors. Thereafter, Dr Freeman’s group computationally evaluated the compounds’ affinity to NQO2 using the molecular docking method. This method is routinely used in the rational drug design to predict the binding mode of the ligand to protein [231, 232], which in this case is the inhibitor to NQO2. The binding affinity of the ligand to protein can then be estimated by a molecular docking programme and called “score function” [233]. The score value is used as a measure of compounds’ affinity and potency. The majority of novel compounds tested in this study have shown higher score values than that of resveratrol. However, molecular docking is still a computational method, so these measures cannot be used to make a final decision regarding the compounds’ potency. Therefore, further investigations into the compounds’ properties in different systems needed to be conducted.

The novel compounds’ inhibitory potency was first evaluated in a cell-free system to identify the most potent inhibitors for further investigation in a cell system. Hydrazone-quinoline derivatives have shown the highest potency compared to other derivatives (starting compound 4, hydrazide and carboxamide). Based on the molecular docking findings (Hussein B. thesis, 2016) [83], the binding mode of the compounds within NQO2’s active sites affects their affinity and subsequent potency. It has been observed that the orientation and conformation (pose) of hydrazone derivatives within the active site of NQO2 allow them to form better interactions with the
The isoalloxazine ring of FAD molecules, which constitute the bottom of the active site, as well as with other amino acid residues, which line the active site cavity [83]. The presence of aromatic ring substitution (R2) at the hydrazone moiety enables the compounds to participate, along with the quinoline ring, in π-π staking hydrophobic interactions, not only with the FAD ring but also with lipophilic amino acids such as PheB408 and Trp105. Furthermore, the presence of hydrophilic substitutions at the side chain ring (R2) allows the hydrazones to form hydrogen bonds with hydrophilic amino acids such as Asn161. These interactions collectively enhance the binding affinity of the hydrazone derivatives to the NQO2, and subsequently enhance their inhibitory potency [83].

On the other hand, changing the hydrazone moiety attached to the position 4 of quinoline ring to hydrazine, hydrazide or carboxamide moieties has shown to cause a significant reduction in the inhibitory potency of the novel derivatives. For example, the IC50 values of compound 27 (hydrazide) and 32 (carboxamide) were 28-fold and >80-fold higher than that of their corresponding compounds 8 and 23 (from the hydrazone group) respectively. Again, the molecular docking study attributes this significant difference in the compounds' inhibitory potency to their chemical structure containing hydrazide and amine moieties, which affect their pose inside the NQO2 active sites. This pose allows only for the quinoline ring to form hydrophobic interactions with the FAD ring. It also does not allow for any of the substitutions at the side chain (R2) to form hydrophobic interactions or hydrogen bonds with amino acid residues [83]. In other words, the weak interactions and binding affinity of hydrazide and carboxamide is the reason behind their low inhibitory potency.

The same explanation can be used to interpret the low potency of resveratrol in comparison with hydrazones, which demonstrate a strong ability to create better interactions with the NQO2 active sites.

Interestingly, a wide variation in the inhibitory potency has also been found among hydrazone derivatives, with a 142-fold difference. This may be attributed to R1 and R2 functional groups, which exhibit appreciable effects on the enzyme activity. The hydrazone derivatives with methyl group (R1) substitution and with either a substituted phenyl ring or heterocycles (R2) show higher potency than their corresponding derivatives with phenyl substitution (R1) and with an unsubstituted phenyl ring (R2). According to the molecular docking findings, the high potency is
attributed to these former derivatives’ ability to form additional hydrophobic interactions with the FAD ring, as well as hydrogen bonds with the residues in the NQO2 active sites, thereby strengthening their binding with NQO2 [83]. The previous interpretations are consistent with the findings of the molecular docking study on NCI quinoline analogues, which refer the high potency of compound NSC617933 to its ability to form additional interactions with the NQO2 binding site. These include hydrophobic interactions with the FAD ring and Trp$^{105}$, Phe$^{126}$ and Phe$^{178}$ residues, and hydrogen bond interactions with Asn$^{161}$, Asp$^{117}$ and Gly$^{174}$ residues [215]. In summary, the inhibitory potency of the novel compounds is likely dependent on their binding affinity to the NQO2, and the hydrazone derivatives are amongst the most potent novel inhibitors.

The structural similarity between NQO2 and NQO1 and the presence of noticeable levels of NQO1 in SKOV-3 cells raised the necessity to estimate the efficacy of hydrazones and hydrazide compounds in inhibiting NQO1 activity. This allowed to determine the specificity of these inhibitors toward NQO2, and to eliminate the possibility of misinterpreting subsequent cellular data. Most of the tested compounds are unable to generate any NQO1 inhibition, even within a micro-molar concentration range, with the exception of compounds 10 and 25. The molecular docking study attributes these two compounds’ extraordinary ability to their exceptional binding modes inside the enzymes’ active sites, which allow them to create additional interactions, enabling them to bind with both NQO1 and NQO2 enzymes. It is worth mentioning that these compounds are still considered very potent inhibitors of NQO2, since small nano-molar concentrations sufficed to generate high NQO2 inhibition levels. In contrast, 1000-fold higher concentrations of these compounds were required to inhibit NQO1 in comparison to the concentration needed to obtain similar levels of inhibition against NQO2. This means that the safety margin between IC$_{50}$ values of the same compound for inhibiting NQO1 and NQO2 is very large.

In vitro evaluation was also conducted on a wide range of quinoline derivatives prior to selecting the best pharmacological tools for studying NQO2 functions in the cells. Toxicity and stability of the novel compounds along with the typical NQO2 inhibitors in the cellular system were first evaluated using SKOV-3 WT cells (expressing highly active NQO2) at short (24hr) and long (96hr) exposure times. The toxicity results in Table (4.4) show that the majority of hydrazones generate 50% toxicity
at a similar concentration of around 2µM. The similarity in the toxicity of structurally diverse hydrazones indicates that the hydrazone moiety might be the main participant in the compounds' toxicity. It is worth noting that changing hydrazone substitution to hydrazide reduced the toxicity of the compounds, as seen between these pairs of compounds (compounds 8 and 9) and their corresponding (compounds 26 and 27) from hydrazone and hydrazide classes respectively. As the only difference between these pairs of compounds ((26 and 9) and (27 and 8)) is in the linker (moiety), it seems that the hydrazone moiety is more toxic than hydrazide, causing this significant difference in cytotoxicity. Interestingly, it was found that compounds 13 and 16 from the hydrazone group demonstrated no toxicity over a wide range of concentration after the 24hr treatment. This can be attributed to the large size of substituents attached to the hydrazone moiety, such as benzyl in compound 13 and benzoic acid in 16, which hinder the activity of hydrazone to exert its toxicity in the cells. In comparison with the typical inhibitors, the cytotoxicity of hydrazones is apparently higher than that of resveratrol and quercetin, but at the same time similar to the 9AA toxicity. Notably, the concentrations of hydrazones that showed 50% cytotoxicity are within micro-molar concentrations, (i.e. they are thousands fold more than the concentrations required to inhibit NQO2 activity, which are within nano-molar range). Thus, the concentration of hydrazones required to inhibit NQO2 can be much less than that used for resveratrol to generate a similar level of NQO2 inhibition, thereby producing very low cytotoxicity.

It has been demonstrated that the N=C bond constituting hydrazone moiety is unstable and labile to hydrolysis [229], thereby producing 4-hydrazine-quinoline (compound 4); the likely hydrolysis product of the 4-hydrazone-quinoline analogues [227]. The hydrolysis of the double bond in hydrazone moiety can be catalysed under mildly acidic condition [234]. It was therefore worth evaluating the stability of hydrazone derivatives in vitro. The significant difference in the IC{sub 50} values for cellular toxicity between hydrazones and compound 4 indicates that hydrazone derivatives are stable in a cell environment. This finding has been further supported by the IC{sub 50} values for hydrazones' extracellular inhibitory potency, which is observed to be significantly different from that of hydrazine compound 4, with an approximately 20-fold difference; hydrazones' IC{sub 50} values are around 100nM compared to compound 4's 2047nM. All of these findings therefore support the
notion that the novel derivatives of hydrazone-quinoline are stable both extra- and intra-cellularly. A possible explanation of hydrazones’ stability is attributed to resonance delocalisation around nitrogen and carbon (N=C), which contributes to the high negativity of the carbon atom and consequent decrease in the electrophilicity, precluding them from hydrolysis [229].

To test whether there was a correlation between NQO2 inhibitory potency and the cytotoxicity of the hydrazone compounds, the toxicity of structurally diverse compounds was again measured in SKOV-3 (NTC) and TOV-112D (EV) with varying endogenous levels of NQO2. The similarity in the pattern of toxicity of all compounds (except compounds 16, 27 and resveratrol) between these cell lines allowed to conclude that NQO2 is not involved in the compounds’ cytotoxicity, and that there exists no correlation between NQO2 inhibition levels and cytotoxicity. To investigate the reason behind this exception from the general conclusion, and determine whether it is related to the presence of varying NQO2 levels or to other distinguished cell characteristics, isogenically paired cells from each cell line were then treated with this subset of the compounds. The lack of difference in the compounds’ toxicity between the isogenically paired cells allowed to conclude that the usage of different cell types with distinct characteristics wa...
was established based on the unique ability of NQO2 in the human cells to bioactivate CB1954 to a potent cytotoxin [100, 105]. The cytotoxicity of CB1954 was used as an indicator of the bioactivation extent of CB1954, which particularly relies on the functional activity of NQO2 in the cells. Therefore, CB1954 cytotoxicity levels can be used as indirect indicators of the NQO2 activity levels in the cells. The reduction in CB1954 cytotoxicity occurs when the enzymatic activities of NQO2 are inhibited genetically and/or pharmacologically and this can be used as a measure of the compounds’ inhibitory potency. A similar approach has been formerly used to evaluate the inhibitory potency of novel coumarin-based compounds against NQO1, taking advantage of NQO1’s ability to bioactivate chemotherapeutic agent EO9 [235]. Recently, the CB1954 approach has been used to assess the intracellular inhibitory potency of NCI compounds against NQO2 [105]. Several assays were designed in the current study to validate this approach and prove its reliability for further evaluation of the novel compounds.

It has been noted that the reductive activity of NQO2 can be induced only in the presence of a proper co-factor called NRH. Therefore, CB1954 is efficiently bioactivated if it is applied simultaneously with NRH, and the absence of any components from the CB1954 system including NRH or NQO2 may lead to loss of activation of CB1954, which appears as an extensive reduction in the subsequent cytotoxicity of CB1954 [100]. NRH is not amenable to synthesis and its stability is weak in the aqueous solution. Thus, it has been necessary to synthesise a series of reduced pyridinium derivatives that still act as NQO2 co-factors and have superior pharmaceutical properties over NRH [100]. EPR is one of the pyridinium derivatives, which has been shown to be synthetically accessible and much more stable and selective than NRH [100]. Consequently, EPR was used as an alternative to NRH in this study. Increasing CB1954 cytotoxicity by increasing the concentrations of EPR indicates that the activity of NQO2 mediated CB1954 bioactivation and subsequent toxicity is enhanced in a dose-dependent manner. At the highest concentration of CB1954, the cell survival was reduced substantially from 70% in the presence of 1µM of EPR, to 30% by the addition of 100µM. This is in agreement with another study, in which increasing the concentrations of NRH has been associated with a sequential increase in CB1954 cytotoxicity [105]. It is worth mentioning that all concentrations of EPR show little toxicity (please refer to the
appendix chapter 7, Figure (9.2)), (i.e. a high degree of cytotoxicity is actually a result of CB1954 bioactivation catalysed by NQO2 rather than EPR itself). The strong relationship between the NQO2 activity levels and EPR concentrations supports the notion that the activity of cellular NQO2 is hidden, and needs to be induced by an exogenous source of a proper co-factor [100]. It has been suggested that the reason behind this is due to the presence of very small concentrations of an endogenous co-factor for NQO2 in the cells [100, 105]. Thus, an EPR concentration of 100µM was selected to be used in this study, as it showed high efficacy at inducing around 70% of latent activity of cellular NQO2, with negligible toxicity. This concentration of EPR has also been reported in other studies [89, 100]. EPR has been used in combination with CB1954 as a treatment of xenografts of human prostate (PC3) and colorectal (LoVo) cancer cell lines expressing NQO2, and manifested high efficiency in inducing CB1954 cytotoxicity [106].

The potential correlation between NQO2 activity levels and CB1954 cytotoxicity has been verified in the panel of OVC cell lines, as demonstrated by the high Pearson’s value, and indicates that CB1954 toxicity is dependent primarily on NQO2 activity levels. High responsiveness levels to CB1954 treatment were observed among SKOV-3, A2780 and OV-90 cells expressing relatively high activity levels of NQO2, which was reduced significantly in Ca-OV3 and OVCAR-3 cells expressing very low levels of NQO2. This pattern of sensitivity also accords with that of Nolan et al.’s findings, which demonstrated the presence of a potential correlation between the cytotoxicity of CB1954 in MDA-MB-468 and BT474 breast cancer cell lines and the activity levels of NQO2 in these cells. Upon the addition of NRH, CB1954 cytotoxicity was substantially potentiated in the former cells expressing relatively high levels of NQO2, while comparatively much less in the latter cells expressing very low levels [105].

Interestingly, different response patterns to CB1954 were detected in both TOV-112D and TOV-21G cell lines. This can be attributed to differences in OVC cell lines characteristics, which interfere with either the bioactivation or subsequent cytotoxicity of CB1954. Thus, further proofs were required to validate this approach. To do this, isogenically paired cell lines of TOV-112D and SKOV-3 cells were selected to be treated with the same combination therapy. Clearly, silencing 65% of the NQO2 activity in SKOV-3 (sh27) cells, as shown in Table (3.3), renders the cells highly
resistant to CB1954/EPR treatment, with a significant, 4-fold reduction in CB1954 cytotoxicity compared to NTC control cells. This finding is in agreement with those of Nolan et al., which show that transient down-regulation of NQO2 activity in MDA-MB-468 also reduced the toxicity of CB1954/NRH to a high extent [105]. It is important to note that the significant difference in the responsiveness of sh27 and NTC cells to CB1954 also confirms that EPR activity has no direct impact on the cytotoxicity of CB1954, and is actually mediated by NQO2. On the other hand, a 25-fold up-regulation of NQO2 activity in TOV-112D (NQO2-OE) cells, as shown in Table (3.2), leads to a substantial increase of 21-fold in the cytotoxicity of CB1954/EPR, compared to EV, with IC₅₀ values of 0.09 ± 0.003µM and 1.88 ± 0.41µM respectively. These findings therefore support the notion regarding the participatory role of NQO2 in the bioactivation of CB1954, and suggest that the aberrant observations seen in TOVs cells can be attributed to biological differences among the OVC cell lines.

The high expression levels of NQO2 in some OVC cell lines as well as its unique ability to activate the CB1954 anticancer drug re-emphasise the potential of using NQO2 in the enzyme-directed tumour approach for CB1954 therapy of human malignant diseases. This study showed that catalysing the latent activity of NQO2 in SKOV-3 cells through the use of EPR resulted in a 200-fold increase in the sensitivity of the cells to CB1954. Moreover, overexpression of NQO2 in TOV-112D (NQO2-OE) cells enhanced their sensitivity to CB1954 treatment by more than 470-fold when EPR was added. Despite NQO2's effectiveness at catalysing CB1954 bioactivation, there were surprisingly few studies interested in NQO2 as a prodrug therapeutic target. Knox et al. (2000) have found that prostate and brain cancer cell lines expressing high endogenous levels of NQO2 are highly sensitive to CB1954 in the presence of EPR [100]. Additionally, overexpressing NQO2 in Chinese hamster V79 cells potentiates the toxicity of CB1954 up to 3000-fold upon the addition of NRH [100]. Substantial delay in tumour growth has also been observed when xenografts of PC-3 prostate and LoVo colorectal cell lines (expressing endogenous NQO2) are treated with CB1954 in combination with EPR [236]. This study therefore reopens the potential role of NQO2 as the CB1954 prodrug therapeutic target, and encourages the use of NQO2 in an enzyme-directed tumour approach.
Knowing that OVC cells, which develop resistant mechanisms against platinum-based drugs (e.g. cisplatin), might be collaterally resistant to other alkylating agents raised doubts about the sensitivity profile of OVC cell lines to CB1954/EPR, notably whether it is mainly dependent on NQO2 activity levels or is affected by platinum-resistance mechanisms [230, 237]. It is evident that the susceptibility of each cell line for both alkylating agents (carboplatin and CB1954) is completely different, pointing to the lack of collateral resistance between these agents. For instance, the susceptibility of each pair of cell lines (SKOV-3 and OVCAR-3) and (OV-90 and CaOV-3) is similar towards carboplatin but completely different towards CB1954/EPR. SKOV-3 and OV-90 expressing relatively high levels of NQO2 show higher sensitivity to CB1954/EPR, as opposed to OVCAR-3 and CaOV-3 expressing low level of NQO2. This demonstrates that NQO2 activity is still the major determinant of the cells’ sensitivity to CB1954, and is not precluded by carboplatin-associated resistant mechanisms in OVC cell lines. Another important conclusion which can be drawn is that CB1954 can be used as an alternative therapy to carboplatin in platinum-resistant tumours. This is highly beneficial in SKOV-3 and A2780 cells that show more than a 100-fold sensitivity to CB1954/EPR, relative to that of carboplatin. Furthermore, overexpressing NQO2 in TOV-112D cells increases their sensitivity to CB1954/EPR by 248-fold, also relative to that of carboplatin. Although SKOV-3 has been reported to be cisplatin-resistant [238], using alternative chemotherapeutic agents with different mechanisms of action can overcome the resistance problem, and improve the sensitivity of the cells to chemotherapy.

A representative selection of hydrazo and hydrazide derivatives along with the typical NQO2 inhibitors was chosen to investigate whether they were functionally active as NQO2 inhibitors in the cells using the CB1954 approach. Increasing the concentrations of the compounds was accompanied by significant reductions in CB1954 bioactivation and subsequent toxicity. As inducing NQO2 silencing via dox causes substantial reduction in the cytotoxicity of CB1954/EPR in SKOV-3 (sh27) cells, the reduction in CB1954 cytotoxicity observed in the presence of these compounds is strongly thought to be due to the pharmacological inhibition of the functional activity of NQO2-mediated CB1954 bioactivation in the cells. To analyse the intracellular inhibitory potency of these putative NQO2 inhibitors, SKOV-3 cells were co-treated with a concentration of CB1954
(1µM) plus EPR and a range of concentration of the compounds. Increased cell survival was observed to begin from low ranges of inhibitors’ concentrations, indicating that they are highly effective at inhibiting NQO2 activity-mediated CB1954 bioactivation. Amongst these inhibitors that clearly showed this observation were compounds 10, 11, 13, 14 and 9AA. The concentrations of inhibitors that protected 50% of the cells from the cytotoxicity of CB1954 (1µM) is also the concentration that inhibited 50% of NQO2’s catalytic activities. Among all tested compounds, hydrazone derivatives are still the most potent inhibitors compared to hydrazide, resveratrol and quercetin. These findings are consistent with the extracellular inhibitory potency findings, which also directly correlate with molecular docking results. Thus, there is strong evidence backing the notion that hydrazone derivatives’ high potency is due to their high affinity to the cellular NQO2. A possible explanation of hydrazone compounds 11 and 14 being the most potent inhibitors in vitro can be attributed to the presence of hydroxyl substitution on the phenyl ring (R2), which allows them to form additional hydrogen bonds interactions with hydrophilic amino acids lining the NQO2 active sites. By contrast, a much lower potency is apparent with hydrazone compound 25 compared to its corresponding compound 10, and other hydrazones. This is possibly due to a substitution (R1) on the quinoline ring, which is phenyl in compound 25 instead of methyl as in the rest of tested hydrazones. The methyl substitution, as mentioned earlier, allows the compounds to form additional hydrophobic interactions with the FAD ring, and this subsequently enhances the binding affinity with the enzyme. Hydrazone quinolines’ high potency allows for them to be used at low concentrations to produce the required levels of NQO2 inhibition, minimising the possibility of getting non-specific off-target binding. These compounds might therefore be used in place of resveratrol, which is known to have multiple actions in cells. High concentrations of resveratrol are required to effectively inhibit NQO2 in the cells, resulting in non-specific bindings and multiple cellular activities [130].

It was found in chapter 3 that silencing the NQO2 gene was effective in targeting only 65% of NQO2 activity in the cells. To investigate whether the concomitant use of the potent inhibitors with dox in sh27 cells would maximise the NQO2 inhibition levels (via targeting the remaining activity of NQO2), CB1954 cytotoxicity was measured in the presence of compound 11 and 9AA (the most
potent inhibitors) in SKOV-3 cells sub-lines NTC and sh27 possessing endogenous and silencing NQO2 respectively. The difference in the levels of protection provided by each inhibitor to either cell lines is mainly due to the varying intracellular NQO2 activity levels. This indicates that the inhibitors’ activity against CB1954 toxicity is mediated primarily by NQO2, and therefore, any downregulation of NQO2 can negatively affect their efficacy in targeting NQO2-mediated CB1954 cytotoxicity. The interesting finding that the addition of NQO2 inhibitors to the sh27 (+dox) produces much higher levels of protection against CB1954 indicates that the concomitant use of NQO2 genetic and pharmacological inhibition is useful in maximising NQO2 inhibition levels comparing with each of individual treatment. It also indicates that compound 11 and 9AA are very potent inhibitors, as they enable to target even the small remaining NQO2 activity.

In summary, the novel derivatives of hydrazone and hydrazide are functionally active as inhibitors of NQO2 in the cells, but at different inhibitory potency levels. These results are consistent with the extracellular inhibitory potency, as well as with the molecular docking findings. The potency of hydrazone (compounds 10, 11, 13, 14 and 16) and hydrazide (compound 27) is clearly much higher than that of resveratrol and quercetin. However, these novel compounds show more toxicity than resveratrol and quercetin, which fortunately is non-observable at the low concentrations used for NQO2 inhibition. Therefore, this subset of hydrazone derivatives is valuable pharmacological tools for the intracellular investigation of NQO2’s biological roles in OVC cells.
Chapter 5

Investigating the contributory roles of NQO2 in controlling cell growth and proliferation in ovarian cancer
Chapter 5

5 Investigating the contributory roles of NQO2 in controlling cell growth and proliferation in ovarian cancer

5.1 Introduction

Uncontrolled cell proliferation is the fundamental cause behind the development and progression of cancer, which arises from the accumulation of abnormalities in cells’ regulatory systems [239]. Ovarian serous carcinoma is “the most lethal gynaecological malignancy” with a high proliferative ability [152, 240]. Studies have demonstrated that OVC cells display deregulations in the pathways involved in cell signalling transduction (e.g. RAS-RAF-MEK-ERK and PI-3K/AKT pathways) [241-243], in addition to abnormalities in the cell cycle regulatory markers [244-246]. However, the nature of the early molecular events behind ovarian carcinogenesis is still unclear [241, 245]. Inflammation and resultant oxidative damage associated with the ovulation process are risk factors for the development of OVC. Therefore, the dysfunction of antioxidant enzymes involved in controlling oxidative stress might increase the risk of developing OVC [247]. Cancer cells’ inexorable demand for ATP as fuel for their persistent proliferation results in increased cellular ROS levels, and consequently, oxidative stress [61]. This increase in cellular stress promotes scavenging mechanisms, to overcome the high toxicity of ROS. It has been found that the induction of a detoxification process against ROS in cancer cells can enhance cancer aggressiveness [248]. Also, the presence of low ROS levels has been shown to be important in supporting cancer cell growth and proliferation [61]. Fully understanding the underlying pathways contributing to ovarian tumourigenesis is useful in identifying the potential targets for chemotherapy development and for preventing its initiation and progression [8].

NQO2 has been detected in different types of cancer, and its potential roles in carcinogenesis are still debated. Several in vivo studies have shown that the knock-out of NQO2 expression enhances the susceptibility of mice to developing myeloid hyperplasia of bone marrow [139], B-cell lymphomas [141], and skin carcinogenesis [140]. Conversely, other in vitro studies have found that silencing the basal level of NQO2 using siRNA, or inhibiting its activity using resveratrol, suppressed the progression and proliferation of CWR22Rv1 human prostate cancer cells [142].
NQO2 plays central roles in controlling the activation of the AKT/GSK-3β/cyclin D1 pathway [162, 166]. NQO2's two-electron reductive activity is found to also play the critical role of protecting the cells from quinones' harmful effects [80], as demonstrated in breast cells through catalysing estrogen reduction to estradiol thereby preventing the generation of reactive intermediary metabolites [146]. All of these findings emphasise the importance of studying NQO2 as a potential therapeutic target of cancer [167]. Thus, in the work described in this chapter, we used two OVC cell lines with modified NQO2 levels to:

1. Investigate alterations in their proliferative ability and clonogenicity.
2. Identify the molecular events behind these alterations by analysing the progression of cells through cell cycle phases.
3. Evaluate the stability and function of G1/S phase regulators.
4. Relate any of these molecular and cellular changes to cellular ROS production and investigate whether they correlate with the NQO2 levels.

5.2 Results

5.2.1 Investigating the influence of overexpressing NQO2 on TOV-112D cells growth and proliferation

5.2.1.1 Proliferation rates of NQO2-overexpressing TOV-112D cells

The effects of upregulating NQO2 levels on the growth rates of ovarian cancer cells were studied in TOV-112D cells. To do this, growth curves for TOV-112D cell sub-lines overexpressing NQO2 (NQO2-OE) and control EV cells were established, using a trypan blue exclusion assay; see section (2.2.5). Cell suspensions were prepared for counting at every 24hr intervals during the 192hr test. Cells were counted manually using a haemocytometer. Trypan blue dye was used as a read out of cell death. Living cell counts were then plotted on a semi-log graph against collection time points, generating cell growth curves, Figure (5.1, A). These curves gave an overview of the cells' growth characteristics. They were also used to determine Tc, as described in section (2.2.5).
To address changes in the growth characteristics of TOV-112D sub-lines associated with EPR-induced NQO2 catalytic activity, the growth curves of NQO2-OE and EV cells treated with EPR were also established in a similar manner, Figure (5.1, B). Following seeding, the cells were incubated for four hours to allow for recovery and proper adherence and then treated with EPR (100µM). This concentration showed high efficiency in catalysing the latent catalytic activities of NQO2 in the cells, as demonstrated in chapter 4, Figure (4.8). EPR was left with the cells for the duration of the experiment (192hr). NQO2-OE and EV cells treated with EPR are denoted as NQO2-OE/EPR and EV/EPR respectively within the text.

![Figure 5.1 Growth curves of TOV-112D cell sub-lines possessing modified high levels of NQO2](image)

**Figure 5.1 Growth curves of TOV-112D cell sub-lines possessing modified high levels of NQO2**

**A.** This figure shows the effects of overexpressing NQO2 on the proliferation rates of NQO2-OE cells (▲) in comparison with control EV cells expressing low basal levels NQO2 (●). **B.** Changes in the cell proliferation rates associated with the addition of EPR (100µM) were also assessed in EV/EPR (●) and NQO2-OE/EPR (▲) cells. Cell numbers were scored on a 24hr time interval during the 192hr test. Counts of living cells only were plotted against time on semi-log graphs. Points represent the mean values of three independent experiments performed in duplicate, while bars represent SEM.

**Figure (5.1, A)** shows that EV and NQO2-OE cells grow exponentially for approximately 96hr (24-120hr) before entering a plateau phase (120-192hr). Despite this similarity in the proliferative
patterns, NQO2-OE cells were found to be more proliferative than EV during the exponential phase (especially over 72hr) and the plateau phase, with a 1.3-fold increase in the cell numbers. NQO2-OE cells’ high proliferative ability allowed them to produce the double number of cells (Tc) in a shorter time compared to EV, as demonstrated in Table (5.1).

These results raised the question as to what happened to the growth characteristics of TOV-112D cell sub-lines upon the addition of EPR. It can be seen from Figure (5.1, B) that treating EV cells with EPR causes a slight reduction in the number of viable cells, and expands the duration of exponential growth to 144hr, resulting in a three-hour increase in Tc (23.7hr) compared to untreated EV (20.3hr), Table (5.1). As EV cells have low levels of NQO2, the changes associated with EPR treatment seemed to stem from EPR itself rather than from catalysing NQO2 activity. NQO2-OE/EPR cells were also more proliferative and fast-growing, producing double the number of cells in three hours less than EV/EPR. In fact, there were no alterations in NQO2-OE growth characteristics upon the addition of EPR when compared to its absence.

Table 5.1 Doubling times of TOV-112D cell sub-lines expressing varying levels of NQO2

<table>
<thead>
<tr>
<th>TOV-112D Cell Sub-lines Conditions</th>
<th>Tc (hr) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>20.3 ± 0.3</td>
</tr>
<tr>
<td>NQO2-OE</td>
<td>18.0 ± 0.3</td>
</tr>
<tr>
<td>EV/EPR</td>
<td>23.7 ± 1.5</td>
</tr>
<tr>
<td>NQO2-OE/EPR</td>
<td>20.7 ± 0.9</td>
</tr>
</tbody>
</table>

Tc refers to the period of time (in hours) that the cells require to grow and duplicate their numbers. The Tc of TOV-112D cells at different conditions were calculated from the exponential growth phases of the growth curves shown in Figure (5.1). The way used to calculate the Tc was described in section (2.2.5). Tc represents the mean of three independent experiments ± SEM.
5.2.1.2 Clonogenic capacity of NQO2-overexpressing TOV-112D cells

It is evident from the previous findings that upregulating NQO2 levels in TOV-112D cells elevated their proliferation rate compared to control EV cells. To confirm the impact of upregulating NQO2 in OVC cell proliferation, the reproductive viability of TOV-112D cells to form colonies in long-term cultures was investigated using a clonogenic assay.

The colony formation capacity (or clonogenicity) of TOV-112D (NQO2-OE) cells overexpressing NQO2 was determined and compared with control EV cells. The effects of EPR-induced NQO2 catalytic activity on the cells’ clonogenicity were also investigated at different concentrations of 50µM and 100µM. To do this, a low number of cells were seeded in 6cm dishes. Following overnight incubation, they were treated with EPR and incubated along with untreated cells for 14 days. At the end of the incubation period, colonies were fixed and then stained with methylene blue, as illustrated in Figure (5.2, A). Colonies were counted macroscopically and the counts were used to determine the PE relative to the initial cell numbers. PE can be used as a measure of the capacity of individual plated cells to survive and undergo unlimited divisions forming colonies. PE was also used to calculate the survival fractions of EPR-treated cells relative to the untreated control, as presented in Figure (5.2, C).

From the data in Figure (5.2, B), there appears to be no difference in the PE between NQO2-OE and EV, indicating that overexpressing NQO2 did not alter the clonogenicity of TOV-112D cancerous cells. Interestingly, inducing NQO2 activity in NQO2-OE/EPR cells via EPR using these concentrations (50µM and 100µM) results in a dose-dependent increase in the clonogenicity of NQO2-OE cells, compared to EV/EPR, Figure (5.2, C), with a 1.3- and 3-fold increase in the colony numbers respectively. As the only difference between these cells (NQO2-OE and EV) lies in the NQO2 levels, this increase in their clonogenicity could be due to the induction of NQO2 cellular activity via EPR. The dose-dependent reduction in the survival fraction associated with the addition of EPR is found to be more pronounced in EV cells in comparison to NQO2-OE, indicating the non-involvement of NQO2 in EPR toxicity, Figure (5.2, C).
Figure 5.2 Clonogenicity of TOV-112D cells upon upregulating NQO2 protein and activity levels

A. TOV-112D cell sub-lines (EV and NQO2-OE) were plated in 6 cm dishes at an ultra-low seeding density. Following overnight incubation, they were treated with 50µM and 100µM concentrations of EPR and incubated along with non-treated cells for 14 days. Colonies were fixed and stained with methylene blue at the end of the incubation period. B. The plating efficiency (PE) of NQO2-OE was estimated and compared to that of control EV cells. The PE was measured relative to the initial number of plated cells. C. Survival fractions (SF) were calculated based on the PE of treated cells relative to the PE of control untreated cells for each cell sub-line under each EPR concentration. The PE and SF values were represented on bar graphs as means ± SEM calculated from four independent experiments.
5.2.1.3 Effects of upregulating the NQO2 levels on the cell cycle progression of TOV-112D cells

The cell-division cycle is a tightly controlled process which is activated under certain circumstances and ends with the multiplication of cell populations. In cancer, damaged cells persistently divide by passing through the cell cycle unlimited times, developing large masses of cancer cells [1]. Several pathways have been found to mediate the persistent induction of the cell cycle progression in cancer cells, which ultimately causes deregulated cell proliferation [3]. To further explore the molecular events behind the differences in proliferation rates between TOV-112D cell sub-lines, a flow cytometric analysis of the cell cycle was conducted for determining and comparing the distribution patterns of these cells through the cell cycle phases. The DNA content of PI-stained cells was measured as described in section (2.13.1). The intensity of PI fluorescence is directly proportional to the DNA content of cells, and is used to build up the cells’ DNA histogram profiles, as represented in Figure (5.3). The histograms were afterwards analysed with the assistance of Mr Michael Jackson to determine the relative percentages of cells in each cell cycle phase at all time points tested. The percentages from three independent experiments are plotted on linear graphs against time, Figure (5.4). Two-way repeated-measures ANOVA followed by Sidak’s multiple comparisons test were used to determine the level of significance in cell percentages between EV and NQO2-OE cells at each time point.
Figure 5.3 Histographic representation of cell cycle profiles of TOV-112D cells upon upregulating NQO2 protein and activity levels

The distribution pattern of cells through the cell cycle phases was determined by measuring the fluorescence intensity of PI stained cells using an LSR Fortessa FACS machine. The intensity of PI stain is directly proportional to the DNA content of cells. Cell proportions containing a particular quantity of DNA were then graphically represented on histograms. **A.** Representative histograms refer to the overlay cell cycle profiles of TOV-112D cell sub-lines, including EV control cells (■) and NQO2-OE cells (□), which were generated at a 24hr time interval over a period of 96hr. **B.** Representative histograms refer to the overlay cell cycle profiles of TOV-112D cell sub-lines treated with 100µM EPR, which were denoted as EV/EPR (■) and NQO2-OE/EPR cells (□). These histograms were also generated at 24hr time intervals over a period of 96hr.

It is worth mentioning that a preliminary experiment was performed to determine the optimal cell seeding numbers required for each time point to produce 70% confluence cultures of unstressed cells. Minimising variations in the culture conditions which the cells were exposed to at different time intervals reduced the probability of misinterpreting the results, and allowed to refer any alterations in the findings to cell characteristics rather than culture conditions. Cells were plated in 6 cm dishes at different seeding densities. Following a four-hour incubation, the cells were treated with 100µM EPR and incubated along with untreated control cells until harvesting time, which was on a 24hr time interval basis during the 96hr test. The cells were then fixed and stored until the
samples from all time points were ready for staining and analysis on the same day, thereby minimising technical-related variations in the results.

Figure 5.4 Cell cycle phases distribution of TOV-112D cell sub-lines under different cellular conditions

A. The effects of overexpressing NQO2 on the progression of NQO2-OE cells (▲) through the cell cycle phases (G1, S and G2/M phase) were analysed and compared to that of EV control cells (●). B. These figures illustrate the effects of inducing NQO2 activity via EPR on the progression of EV/EPR (●) and NQO2-OE/EPR cells (▲) through the cell cycle phases on a 24hr time interval basis over a period of 96hr. All samples from different conditions were prepared and handled in the same manner at each 24hr time interval, up to 96hr. They were then all stained with PI to measure DNA content in the population and to subsequently establish cell cycle profiles. These profiles underwent further analysis using the FlowJo Software V10.2 to mathematically estimate the percentage of cells in all phases. The represented figures were generated by plotting cell percentages in each phase against a duration of 96hr. Points represent the mean values of three independent experiments with SEM. The asterisk refers to the significant level of difference at p value<0.05, which was determined by two-way repeated-measures ANOVA and a post hoc test called Sidak’s multiple comparisons test.

Figure (5.4, A) compares the percentages of cells within and between TOV-112D cell sub-lines in each phase of the cell cycle and at all of the tested time points. It is apparent that both EV and NQO2-OE cells had similar patterns of distribution through the cell cycle phases; the highest
Chapter 5

percentage of cells (approximately 50%) was found to be in the G1 phase, while the rest of the cells were distributed between the S and G2/M phase, at 30% and 20% respectively. Despite this similarity, there were slight but significant differences in the percentages of G1 and S cells between EV and NQO2-OE cell sub-lines. It was demonstrated that overexpressing NQO2 in TOV-112D cells allowed a higher percentage of NQO2-OE cells to progress through the G1/S phase, resulting in a reduction in G1-cells and a concomitant increase in S-cells, as compared to EV. These differences appeared from the early time points, and became more pronounced at the latter points (72hr and 96hr).

Interestingly, the proportions of NQO2-OE cells in each cell cycle phase remained comparatively similar along the tested duration of 24hr time intervals, (i.e. maintaining the cells in the cultures for a shorter or longer amount of time had no impact on their progression through the cell cycle phases). In contrast, EV cells showed different progression patterns through the phases during the tested period. It was shown that incubating EV cells for the extended periods of 72hr and 96hr was associated with cell accumulation in the G1 phase, causing a significant increase in G1-cells and a concomitant reduction in S-cells, compared to the early time points (24hr and 48hr). These alterations in the cell cycle progression of EV cells were responsible for the significant differences in the G1-cells between EV and NQO2-OE, particularly at the 96hr time period, with a six percent difference.

With regards to the effects of EPR on the cell cycle, it was revealed that the progression of TOV-112D cell sub-lines through the cell cycle phases in the presence of EPR was similar to that of in its absence. As can be seen from Figure (5.4, B), the differences in the percentages of G1- and S-cells between EV/EPR and NQO2-OE/EPR were also found to be very comparable with those observed in the absence of EPR. After comparing the 96hr cultures of EV/EPR and NQO2-OE/EPR cells, there appeared to also be a significant difference in the percentages of cells in G1 and S phase. This difference resulted from the accumulation of a higher percentage of EV cells in the G1 phase, and the concomitant reduction in its percentage in the S phase when compared to NQO2-OE. The high similarity in the NQO2-OE cell cycle profiles at different treatment conditions (-/+EPR) led to the conclusion that high levels of NQO2 in TOV-112D were sufficient to develop the
aforementioned changes in the TOV-112D cell cycle profile, whilst no additional effects can be obtained by inducing its activity via EPR.

5.2.1.4 Assessment of intracellular ROS levels in NQO2-overexpressing TOV-112D cells

There is a growing body of evidence that supports the central roles of the cellular redox status in regulating critical physiological processes such as cell proliferation and death [249-251]. As NQO2 is involved in regulating cellular redox, it was of utmost interest to investigate whether the changes observed in the growth characteristics of TOV-112D cells overexpressing NQO2 occurred as a result of an alteration in the redox status of cells mediated by the elevated NQO2 levels. To investigate this, a flow cytometric analysis of ROS levels was conducted for NQO2-OE and EV cells using DCFDA cell permeable dye which upon oxidative metabolism it generated an ultimate fluorescent DCF metabolite which can be detected at 530nm by a Fortessa machine. The intensity of the DCF fluorescence is directly proportional to the intracellular ROS levels [15]. ROS levels were measured in cultures maintained for both short and long time periods. To do this, the cells were seeded on different days and incubated for the intended duration of 24hr time intervals, up to 96hr when all samples were collected for analysing the ROS levels. It is worth mentioning that the seeding densities used in this assay were similar to those previously used for the cell cycle analysis, so as to ensure that the cells in all cultures grew in similar nutrient-replete and unstressed conditions. Therefore, any alterations in ROS levels were ascribed to changes in the cellular processes rather than the culture conditions. As the effects of catalysing NQO2 activity on intracellular ROS levels also needed to be investigated, a parallel set of EPR-treated cultures was incubated along with untreated samples, and collected at 24hr time intervals. Further details about the methods used for sample preparation and data analysis can be found in section (2.13.2). The DCF fluorescence of stained samples was first normalised to the background fluorescence of their respective unstained samples. The fold changes in the ROS levels of EV (48-96hr) and NQO2-OE (24-96hr) samples were calculated relative to the control EV at the 24hr mark. The data was represented on bar graphs as mean values of four independent experiments ± SEM.
It can be seen from the data in Figure (5.5, A) that EV and NQO2-OE cultures maintained for 48hr show a substantial increase in ROS levels when compared to the 24hr culture. However, maintaining the cultures for the prolonged durations of 72hr or 96hr was associated with a significant reduction in ROS levels compared to that of the 48hr culture.

The same trend in ROS level changes was also observed within and between EV/EPR and NQO2-OE/EPR samples treated with EPR as that of those recorded in its absence over time. ROS levels were also found to be lower in NQO2-OE/EPR cultures maintained for more than 24hr when compared to EV/EPR. The lack of significant difference in the ROS levels between cultures treated with and without EPR allowed to conclude that these changes in ROS levels were due to overexpressing NQO2; no additional changes could be obtained by the addition of EPR. Two-way repeated-measures ANOVA followed by Sidak’s multiple comparisons test were used to determine the levels of significance in the ROS levels within EV and NQO2-OE cells across different time points.
Figure 5.5 Determination of ROS-mediated fluorescent levels in NQO2-overexpressing TOV-112D cells over a long duration of 96hr

A flow cytometric analysis of ROS levels was conducted in TOV-112D cell sub-lines using DCFDA dye. The levels of ROS were measured in cultures maintained for both a short and long time period. The intensity of fluorescence emitted from the stained samples was first normalised to that emitted from their respective unstained samples. The fold changes in ROS levels were then calculated relative to the untreated control EV at the 24hr mark, and plotted as bars representing the mean values of four independent experiments ± SEM. (A) The graph shows the effect of overexpressing NQO2 on ROS levels compared to EV during a 96hr testing period. (B) The graph shows the effect of inducing NQO2 catalytic activity via EPR on ROS levels in NQO2-OE/EPR compared to EV/EPR during the 96hr testing period. The ROS levels were compared in between and among different time points for both cell sub-lines using two-way repeated-measures ANOVA followed by Sidak’s multiple comparisons test. The asterisks appearing as (*) and (**) indicate the significant levels of difference between ROS levels which were at p value<0.05 and <0.01 respectively.

5.2.1.5 Assessment of expression levels of G1 phase regulatory markers in stably NQO2-overexpressing TOV-112D cells

It was demonstrated earlier that there is a trend for TOV-112D cells overexpressing NQO2 to show enhanced proliferative ability, probably by facilitating the progression of cells through cell cycle phases, particularly through the G1/S phase. To gain more information about the effect of upregulating NQO2 in TOV-112D cells on the cell cycle, a Western blot analysis for G1 phase regulators was performed, as the most significant differences in cell proportions between EV and
NQO2-OE cells were found to be at the G1 phase. Furthermore, NQO2 has been shown to play a critical role in regulating the activity of kinases enzymes involved in cyclin D1 turnover in prostate cancer cells [162, 166]. Thus, it was necessary to address the changes in the expression of G1-regulators within and between EV and NQO2-OE, in cultures maintained for short and long time periods. The cells were plated at the same seeding densities used earlier for the cell cycle analysis experiment. The effects of inducing the catalytic activity of overexpressed NQO2 on the expression levels of G1-regulators were also examined in these cultures. For this purpose, the cells were exposed to 100µM EPR following the cell seeding by four hours. At each 24hr time interval, the samples were collected in a RIPA buffer. The protein concentrations were then determined and volumes containing 30µg protein were loaded for Western blot analysis. Multiple blots were prepared for immunoblotting and subsequent densitometric analysis of the bands’ intensities. The results were normalised to β-actin prior to calculating the ratios of changes relative to the EV (24hr) sample. To facilitate the tracking of the changes in the protein levels among samples, the ratios were represented under the blot as mean values ± SEM. All blots in Figure (5.6) are representative of six independent experiments. Two-way repeated-measures ANOVA followed by Tukey’s and Sidak’s multiple comparisons tests were used to determine the levels of significance difference in the ratios between EV and NQO2-OE cells at the tested time points.

The distinct expression levels of NQO2 in EV and NQO2-OE cells were first measured. Figure (5.6, A) illustrates that NQO2-OE cells stably overexpress NQO2 to a similar extent among tested time points compared to EV cells.

As the phosphorylation of Rb is important in inducing the transition of cells from the G1 to S phase, it was imperative to first evaluate the changes in p-Rb/Rb expression within and between EV and NQO2-OE cells. The blots in Figure (5.6, B) demonstrate that at the 48hr mark, hyperphosphorylated Rb (p-Rb) expression levels at Ser-807/811 in EV substantially increased, and reached their highest level at the 96hr mark which shows 2.6-fold increase compared to EV at the 24hr mark. The expression levels of p-Rb in NQO2-OE cells were found to be similar among the early time points (24-72hr), but were slightly reduced at 96hr compared to the 24hr mark. A similar pattern of expression for p-Rb was also found in EV and NQO2-OE cells treated with EPR.
Comparing the p-Rb expression levels between EV and NQO2-OE cells throughout the duration of 96hr shows that EV cells expressed relatively higher levels of p-Rb than NQO2-OE, with statistically significant differences of 2.6- and 2-folds at the 96hr mark, in the absence and presence of EPR respectively. The level of significance for this set of data was at p<0.05. These findings also demonstrate the predominant role of elevated NQO2 expression levels in inducing these changes in p-Rb/Rb; no significant changes were found upon the addition of EPR. Notably, there were high levels of consistency in expression patterns between hypophosphorylated Rb (Rb) and p-Rb within and between EV and NQO2-OE cells.

In an attempt to understand the reason behind the differences in the expression of p-Rb/Rb proteins between TOV-112D cell sub-lines, the expression levels of the upstream regulatory complex consisting of cyclin D1 and CDK4 were also measured. As shown in Figure (5.6, C), cyclin D1 is expressed in EV cells at comparatively similar levels among the tested time points. On the contrary, there were non-significant reductions in cyclin D1 expression in NQO2-OE cells over time. They reached their highest level at the 96hr mark, with a 30% reduction compared to the 24hr mark.

Upon comparing cyclin D1 levels between TOV-112D cell sub-lines, it was noticed that the expression levels of cyclin D1 in NQO2-OE cells were significantly less than that in EV cells, particularly at 72hr and 96hr, with a 30% and 50% reduction respectively. Figure (5.6, C) also illustrates that the cyclin D1 expression patterns in TOV-112D cell sub-lines are not altered in the presence of EPR, compared to in its absence. Therefore, similar levels of significant difference were also detected between EV/EPR and NQO2-OE/EPR cells at the 72hr and 96hr mark, with p<0.005.

As phosphorylation-induced proteasomal degradation is one of the mechanisms involved in regulating cyclin D1 levels in the cells, cyclin D1 phosphorylation at Thr286 were followed. It was found that NQO2-OE cells expressed slightly higher levels of p-cyclin D1 (Thr286) compared to that of EV cells, and this occurred both in the absence and presence of EPR. This provided strong
evidence for the differences noticed in cyclin D1 levels between the cells sub-lines expressing different levels of NQO2.

In accord with the expression pattern of cyclin D1, overexpressing NQO2 in TOV-112D cells leads to significant reductions of approximately 30% in CDK4, particularly at 72hr and 96hr time periods, compared to EV, with p<0.05 and <0.005 respectively, Figure (5.6, D). Interestingly, there appeared to be only small, non-significant reductions in CDK4 levels in NQO2-OE/EPR cells exposed to EPR when compared to EV/EPR at the latter time points (72hr and 96hr).

Based on these results, it can be concluded that the alterations in the expression pattern of G1 factors were consistent and strongly correlated with one another. The reduction in the levels of cyclin D1 and its partner CDK4 in NQO2-OE cells was accompanied by a reduction in p-Rb and, consequently, in the total Rb, whereas stabilising cyclin D1 and CDK4 in EV was accompanied by an elevation in p-Rb levels and, consequently, in the total Rb. Moreover, these results also provided explanations for the differences noticed in the cell cycle progression and ultimately in the proliferation rates between TOV-112D cell sub-lines. In NQO2-OE cells overexpressing NQO2, cyclin D1 degradation was promoted to facilitate the transition of cells through the G1/S phase for fast proliferation, whereas in EV cells expressing low basal levels of NQO2, cyclin D1 formed active complexes with CDK4 to induce the phosphorylation activation of Rb, in turn inducing cell progression through the G1 phase, thereby promoting the proliferation of slow-growing EV cells.
### Chapter 5

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<tr>
<td>P-cyclin D1 (Thr286)</td>
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<td>Ratio (mean)</td>
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A western blot analysis was conducted to assess and compare the protein levels of G1 phase regulatory factors within and between TOV-112D cell sub-lines (EV and NQO2-OE) during the 96hr testing period. The effects of inducing NQO2 catalytic activity on the G1 factors levels were also examined following catalysing NQO2 activity via EPR. Cells were seeded in 10cm dishes and, four hours later, a parallel set of dishes was treated with 100µM EPR. Samples were collected in a RIPA buffer on a 24hr time interval basis. 30µg protein was used for the analysis. These blots are representative of six independent blots which afterward underwent densitometric analysis to measure bands’ intensities. The results were first normalised to β-actin, then the ratios of changes were calculated relative to either the control EV or EV/EPR at the 24hr mark, and represented under the blots as mean values ± SEM. Several markers were analysed in the present study; each one is represented in an individual blot such as blots (A) for NQO2, (B) for hyperphosphorylated Rb at Ser(807/811) and total Rb, (C) for cyclin D1 and phosphorylated cyclin D1 (Thr286) and (D) for CDK4. The left side columns include the blots representing untreated cells while the right side columns include the blots representing EPR-treated cells. The asterisks appearing as (*), (**) and (***) were used to describe the levels of statistical significance in ratios of changes between EV and NQO2-OE at the tested time points, which were at p<0.05, p<0.01 and p<0.001 respectively. They were determined using two-way repeated-measures ANOVA and a post hoc test called Sidak’s multiple comparisons test.

5.2.2 Investigating the influence of silencing NQO2 on SKOV-3 cells growth and proliferation

5.2.2.1 Proliferation rates of SKOV-3 cells

Since overexpressing NQO2 in TOV-112D cells caused an increase in the growth rate of exponentially growing cells, it was of interest to validate the correlation between NQO2 levels and the proliferative abilities of OVC cells by using another OVC cell line, such as SKOV-3 cells possessing high NQO2 basal levels. Proliferation rates and other cellular growth characteristics
were also determined and compared following the modulation of NQO2 levels via either EPR or dox.

5.2.2.1.1 Proliferation rates of EPR-treated SKOV-3 cells

The effects of EPR-catalysed basal NQO2 activity on the proliferation rates of SKOV-3 cells were first investigated using a trypan blue exclusion assay. To do this, growth curves of EPR-treated and -untreated cells were established using the protocol earlier described in section (5.2.1.1). Cells were treated with a 100µM concentration of EPR. This concentration is highly efficient at inducing intracellular NQO2 activity, as demonstrated in chapter 4 Figure (4.4). EPR was left in the culture for the duration of the experiment (192hr). Cells were harvested at a 24hr time interval for counting. Semi-log graphs were also used to plot the cell counts of living cells against the collection time points, thus generating the cells' growth curves.

Figure (5.7) demonstrates that the proliferative patterns and growth rates of SKOV-3 sub-lines and their respective EPR-treated cells are similar, resulting in comparable doubling times of approximately 28hr. The cells spent approximately 96hr in the exponential phase (24-120hr) before entering the plateau phase. Despite this similarity, there appeared to be a slight reduction in the growth rates of EPR-treated cells compared to that of untreated cells, particularly when exposed to EPR for more than 96hr. These results were in agreement with the MTT assay findings for EPR co-factor toxicity Figure (9.2) in the appendix chapter, which also demonstrates that EPR generates slight cytotoxic effects after a 96hr incubation.
SKOV-3 (NTC and sh27) cell sub-lines expressing high basal levels of NQO2 were seeded and then treated with 100µM EPR to investigate the effects of inducing NQO2 activity on the growth characteristics of cells. Cell suspensions were prepared every 24hr over a period of 192hr. Representative growth curves of untreated cells (e.g. NTC (●) and sh27 (▲)) and EPR-treated cells (e.g. NTC/+EPR (■) and sh27/+EPR (▼)) were generated by plotting average number counts of living cells calculated from three independent experiments against 24hr time interval collection. These curves were used to determine the Tc of cells.

5.2.2.1.2  Proliferation rates of SKOV-3 cells following induction of NQO2 silencing

The effects of NQO2 silencing on the proliferative pattern and growth rates of SKOV-3 cells were also investigated. To do this, the growth curves of SKOV-3 cell sub-lines treated with dox were generated and compared with their respective untreated cells expressing high basal levels of NQO2, Figure (5.8). To address the impact of NQO2 silencing from the first day counting, sh27 cells were treated with dox for five days prior to being seeded for the experiment. Cells remained under the dox effect throughout the experiment by continuous addition of dox on an alternate day basis, in an attempt to maintain the silencing levels of NQO2 constant throughout the assay duration.
SKOV-3 cell sub-lines (NTC and sh27) were first exposed to dox for five days. They were then seeded for counting every 24hr during the 192hr test. Growth curves of untreated cells including (NTC (●) and sh27 (▲)) and dox-treated including (NTC/+dox ( ■) and sh27/+dox ( ▼)) were established by plotting the living cell counts against the time points of collection on a semi-log graph. Points represent the average of three independent experiments, while bars indicate the SEM. These curves were used to determine the Tc.

As can be seen from Figure (5.8), the growth rates of dox-treated cells were observed to be slightly slower than that of untreated cells, resulting in an approximate three hours difference in the Tc value between them; see Table (5.2). It is critical to note that sh27/+dox cells had similar growth characteristics to that of NTC/+dox and therefore, the changes occurring in the presence of dox were due to the off-target cellular activities of dox rather than from the NQO2 silencing.
### Table 5.2 Doubling times of SKOV-3 cell sub-lines expressing varying levels of NQO2

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<th>Conditions</th>
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<td>NTC</td>
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<td>sh27</td>
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<td>28.1 ± 0.5</td>
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<td>sh27/ +dox</td>
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Tc was calculated from exponential phases of the growth curves of SKOV-3 cell sub-lines shown in Figure (5.8). Tc results were tabulated as mean values ± SEM calculated from three independent experiments.

#### 5.2.2.2 Clonogenic capacity of SKOV-3 cells following induction of NQO2 silencing

The effects of silencing NQO2 on the reproductive viability of SKOV-3 cell sub-lines for the eventual formation of colonies were assessed using a clonogenic survival assay. To do this, the cell sub-lines (NTC and sh27) were initially treated with dox for five days prior to being seeded for the experiment. Cells remained under the effect of dox for the duration of the experiment (14 days) by continuous addition of dox on an alternate day basis. The PE of NTC and sh27 cells in the absence and presence of dox were first determined, in order to calculate SF as described in section (2.10.3).

From the data in Figure (5.9), it is apparent that the treatment of NTC and sh27 with dox (+dox) is associated with a significant reduction in the cell survival fractions, at p<0.0001 level, compared to untreated control cells. However, this reduction was comparable between NTC/+dox (expressing high basal levels of NQO2) and sh27/+dox (possessing modified low levels of NQO2), allowing to conclude that downregulating NQO2 in SKOV-3 cells did not alter their capacity to produce colonies.
NTC and sh27 cells were exposed to dox for five days prior to being seeded, and throughout the experiment to keep the silencing levels of NQO2 constant. Cells were seeded at an ultra-low seeding density and incubated for 14 days before the experiment was terminated by fixing the colonies and staining them with methylene blue. Survival fractions of dox-treated cells were calculated relative to untreated cells. Results were analysed based on a one-way ANOVA followed by Tukey’s multiple comparisons test. The asterisks indicate the significance level of difference between the untreated control and dox-treated cells, which was at \( p<0.0001 \) level.

5.2.2.3 Effects of modulating NQO2 levels via either EPR or dox on the cell cycle progression of SKOV-3 cells

SKOV-3 cells were used as another representative model for ovarian cancer to investigate the cell cycle effects of NQO2 following the modulation of its basal levels under three different treatment conditions. Firstly, under the effect of EPR-induced cellular activity of NQO2, where the cells were treated with a high concentration of 100\( \mu \)M EPR for the maximum induction of NQO2 activity. Secondly, under the effect of dox-induced NQO2 silencing in sh27 cells, where the cells were exposed to dox (1\( \mu \)g/ml) for five days prior to seeding as well as throughout the assay and finally, under the effect of a combination treatment of dox plus EPR. Investigating the effects of EPR in the absence of NQO2 allowed to determine whether the changes in the cell cycle progression...
observed in the presence of EPR were due to EPR-mediated NQO2 activity or EPR off-target effects. To examine this, the cells were exposed to dox prior to being seeded with EPR (100µM). Cell cycles from all treatment conditions were investigated on a 24hr time interval up to 96hr. Further details regarding the protocol are described in section (2.13.1). The seeding density required for each time point of the assay was optimised in a preliminary experiment. The cell cycle profiles of cells from different conditions were generated and represented in Figure (9.5) as shown in the appendix chapter. They then underwent further analysis to determine the relative percentages of cells distributed through cell cycle phases at the tested time points. The cell percentages in each phase were then plotted on linear graphs against time, as shown in Figure (5.10). Untreated NTC and sh27 were used here as controls for comparison.

From the data in Figure (5.10, A), it can be seen that inducing NQO2 activity in SKOV-3 cells via EPR promotes a higher percentage of NTC/EPR and sh27/EPR cells to progress from the G1 to S phase compared to untreated control cells, with a slight reduction in the G1-cell population, accompanied by a concomitant increase in the S-cell population. For example, incubating the cells with EPR for 72hr and 96hr allowed higher percentages of NTC/EPR and sh27/EPR cells to progress through the G1/S phase compared to the control, with a difference of approximately 12%.

To determine whether these changes associated with EPR treatment were mediated by functionally active NQO2, the effects of EPR on cell cycle were addressed again, but in the presence of attenuated NQO2 levels, for comparison purposes. To do this, it was necessary to first investigate the effects of dox-induced NQO2 silencing on cell cycle. Exposing sh27 cells to dox is apparent to induce NQO2 silencing, as illustrated in Figure (5.11, A).

As shown in Figure (5.10, B), silencing NQO2 in sh27/+dox has no noticeable impact on the progression of the cells through the entire cycle phases when compared to NTC/+dox control cells; there are no significant differences in the cell percentages progressing through cycle phases between NTC/+dox and sh27/+dox at all time points. The putative role of active NQO2 in controlling the cell cycle progression of SKOV-3 cells was then investigated. It is apparent that the distribution patterns and proportions of sh27/+dox/+EPR cells progressing through the cell cycle phases were the same as that of NTC/+dox/+EPR and that of NTC/EPR treated with EPR alone,
Figure (5.10, C). This allowed to conclude that the changes in the cell cycle progression associated with the addition of EPR were not mediated by activating NQO2, but were rather related to the other cellular activities of EPR.

Figure 5.10 Cell cycle phases distribution of SKOV-3 cell sub-lines under different treatment conditions

A. The effects of inducing NQO2 activity via EPR on the cell cycle progression of NTC/+EPR (■) and sh27/+EPR (▼) cells were examined and compared with that of untreated NTC (○) and sh27 (▲) cells. Following seeding, cells were treated with 100µM EPR and collected along with the control cells on a 24hr time interval for the duration of the 96hr test. B. The effects of inducing NQO2 silencing via dox on the cell cycle progression of NTC/+dox (■) and sh27/+dox (▼) were also examined and compared with NTC (○) and sh27 (▲) cells every 24hr for 96hr. C. To investigate whether the changes in the cell cycle observed in the presence of EPR in NTC/+EPR (■) and sh27/+EPR (▼) were mediated by active NQO2, the cell cycle effects of EPR were reinvestigated in the presence of dox (1µg/ml) in NTC/+dox/+EPR (★) and sh27/+dox/+EPR (◆) cells. Fixed cells were stained with PI to determine DNA content in the cell population, so as to generate DNA histogram profiles of cells (please refer to the appendix, Figure (9.5)). These profiles underwent further analysis using the FlowJo Software V10.2 to mathematically estimate the percentage of cells in all phases. The represented figures were generated by plotting cell percentages in each phase over a period of time. The points represent the mean values of three independent experiments ± SEM.
5.2.2.4 Assessment of expression levels of G1 phase regulators in SKOV-3 cells following induction of NQO2 silencing

To further validate the contributory roles of NQO2 in controlling the expression of G1 phase regulators in OVC, SKOV-3-derived cell sub-lines expressing high basal levels and silencing NQO2 were selected to analyse G1 regulators for comparison purposes. The western blot technique was used to measure the levels of these regulators, including p-Rb (Ser807/811), total Rb, cyclin D1, p-cyclin D1 (Thr286) and CDK4, in samples prepared on a 24hr interval basis over a period of 96hr. NQO2 silencing was first necessary to be induced via dox (1µg/ml) prior to seeding and throughout the experiment. The seeding densities used in this experiment were similar to those which were optimised for cell cycle analysis, so that NTC and sh27 cells were seeded at the same density for each tested time point but differently among time points, generating 70% confluence cultures of unstressed cells. Cells were lysed in a RIPA buffer. The expression levels of the regulators were quantified in samples containing 30µg protein through densitometric analysis. The blots shown in Figure (5.11) are representative of four independent experiments. It is worth noting that any differences in the expression levels of G1 regulators observed between samples treated with and without dox were due to the dox-associated activities of inducing NQO2 silencing and/or other off-target activities in the cells. This is because those cells underwent similar culture conditions, with the exception of the dox presence. To address the G1 expression level changes in either NTC or sh27, the ratios of changes in all samples (including the untreated ones at time points [48-96hr] and the dox-treated ones at time points [24-96hr]) were calculated relative to the untreated control (either NTC or sh27) at the 24hr mark. These ratios are represented under the blots strips as mean values of three independent experiments ± SEM, as shown in Figure (5.11). The results were analysed based on two-way repeated-measures ANOVA followed by Tukey’s multiple comparisons test to determine the levels of significance in the ratios between NTC and sh27 cells.

Firstly, it was necessary to confirm that NQO2 levels in sh27/+dox samples were downregulated maximally and stably throughout the duration of the test, and that NQO2 levels in NTC samples remained unaffected by dox exposure. This was successfully confirmed and is demonstrated in Figure (5.11, A).
Thereafter, the expression of p-Rb (Ser807/811) involved in controlling the G1/S phase transition was measured in NTC and sh27. In Figure (5.11, B.a), untreated NTC cells showed a clear trend of reduction in the expression of p-Rb over time, to reach the maximum reduction in the cultures maintained for longer durations of 72hr and 96hr. This trend was also revealed in NTC samples treated with dox, (i.e. dox did not alter the natural trend of p-Rb expression in NTC cells). Sh27 cells also showed a similar trend of reduction in p-Rb in long-term cultures, not only in the presence of NQO2 but also in its absence, when the samples were exposed to dox, as illustrated in Figure (5.11, B.c). When all is considered, it can be concluded that NQO2 may not play a potential role in regulating the phosphorylation of Rb in SKOV-3 cells, as p-Rb levels were similar in both the presence and absence of NQO2.

By comparing the expression trends of Rb with p-Rb, it is obvious that there is a high consistency in their expressions throughout the tested duration, Figure (5.11, B, a-d), thereby supporting the changes observed in p-Rb levels. The lack of significant difference in Rb levels between NTC/+dox and sh27/+dox confirmed the previous finding of the non-involvement of NQO2 in the phosphorylation of Rb. These results collectively provide explanations for the earlier findings regarding the lack of difference in proliferative ability and cell cycle progression between the control NTC/+dox and sh27/+dox cells.

As was elucidated earlier in section (5.2.1.4), overexpressing NQO2 in TOV-112D cells resulted in the destabilisation of cyclin D1 via inducing its phosphorylation at Thr286. Following this it was imperative to investigate the correlation between NQO2 levels and cyclin D1 turnover using another model from ovarian cancer cells. SKOV-3 cell sub-lines were selected to investigate this correlation by comparing cyclin D1 and p-cyclin D1 (Th286) levels between NTC/+dox cells (possessing high basal levels of NQO2) and sh27/+dox (possessing modified low levels of NQO2). From the data in Figure (5.11, C, a), it is apparent that exposing NTC cells to dox causes significant reductions in cyclin D1 at these time points (72hr and 96hr), pointing to the participatory roles of dox in modulating cyclin D1 expression. To determine the changes in cyclin D1 accompanied solely by NQO2 silencing, the effect of dox in sh27 cells was first examined. As Figure (5.11, C, c) shows, there are slight, non-significant reductions in cyclin D1 levels in
sh27/+dox at all tested time points when compared with untreated sh27. As there appear to be a slight difference in cyclin D1 levels between untreated NTC and untreated sh27 cells, fold changes in cyclin D1 induced by dox need to be calculated for each cell sub-line, relative to their respective untreated cells; these are represented under the cyclin D1 blot strip in highlighted orange boxes, as shown in Figure (5.11, C, a and c). As the fold changes in cyclin D1 levels between these cell sub-lines were similar, the reduction which appeared in the presence of dox is due to the off-target effects of dox rather than to the NQO2 silencing. The role of NQO2 in stabilising and degrading cyclin D1 is further supported by the fact that silencing NQO2 in sh27 results in a significant reduction in p-cyclin D, particularly at the 96hr mark, compared to NTC/+dox (time 96hr), as illustrated in Figure (5.11, C, b and d). This indicates that the stability in cyclin D1 levels observed upon NQO2 silencing might have resulted from the attenuation of the cyclin D1 phosphorylation, and subsequent degradation. These results were in agreement with the earlier findings for TOV-112D cells.

As cyclin D1 is a central partner of CDK4 and essential for its activity, the CDK4 levels were also measured. No significant differences were found between dox-treated and untreated samples of either NTC or sh27 cells, indicating that silencing NQO2 did not alter the basal levels of CDK4 in SKOV-3 cells, as illustrated in Figure (5.11, D, a and b).
Figure 5.11 Assessment of expression levels of G1 phase factors in stably expressing shNQO2 SKOV-3 cells

A western blot analysis was conducted to assess the protein levels of the G1 phase cell cycle regulatory factors in SKOV-3 cell sub-lines including NTC control (possessing high basal levels of NQO2) and sh27 (possessing modified low levels of NQO2 induced by dox) for comparison purposes. The samples prepared from cultures exposed to dox were denoted as (+dox) whereas the samples prepared from parallel cultures without dox were denoted (-dox). It is worth mentioning that NTC and sh27 cells were exposed to dox (1µg/ml) five days prior to seeding for the experiment for the maximum induction of sequences expression. The cells were then seeded in 10cm dishes and collected in a RIPA buffer in 24hr intervals up to 96hr. The G1 factors were analysed in samples containing 30µg protein. Uppercase letters (A-D) refer to the blots that represent the levels of (A) NQO2, (B) p-Rb and Rb, (C) cyclin D1 and p-cyclin D1 and (D) CDK4 respectively, while lowercase letters refer to NTC (a and b) and sh27 cells (c and d). These blots were representative of four independent experiments, which underwent densitometric analysis to measure the bands’ intensity. The results were first normalised to β-actin, then the ratios of changes in G1 regulator levels at all time points were calculated, relative to their respective untreated sample at the 24hr mark. This allowed to address and compare the G1 level changes in each cell sub-line over time, in both the presence and absence of dox. The ratios were represented as mean values ± SEM under the blots strips. The orange box present in Figure C demonstrates the fold changes in cyclin D1 expression in dox-treated samples relative to their respective untreated samples over the tested time points. Asterisks appearing as (*) were used to denote the levels of statistical significance in the ratio of the changes between NTC (+dox) and sh27 (+dox) at p<0.05, which were determined using two-way repeated-measures ANOVA and a post hoc test called Tukey’s multiple comparisons test.
5.3 Discussion

Cancer is frequently recognised as “a disease of the cell cycle” [152]; deregulated expression and aberrant activation of cyclin/CDK complexes occur in many types of cancers, including prostate [166], pancreatic [151] and ovarian cancers [152]. Ovarian serous carcinoma is the most lethal subtype of ovarian cancer, which has a high proliferative ability [152]. In this study, it was shown that NQO2 expression and activity levels varied widely in a panel of OVC cell lines, being the highest in SKOV-3 cells from the serous histological subtype. Thus, it was of great importance to investigate whether modulating the NQO2 levels in ovarian cancer cells led to an alteration in their proliferative ability, cell cycle progression and expression of G1 phase regulators. To do this, two models of ovarian cancer cell lines, TOV-112D and SKOV-3 cells expressing low and high basal levels of NQO2 respectively, were analysed with their derived sub-lines possessing genetically modified levels of NQO2.

5.3.1 TOV-112D

This study’s results show that upregulating NQO2 expression and activity levels in TOV-112D cells causes slight increases in the proliferative ability and colony-forming capacity of these cells, compared to control EV cells. The present findings seem to be consistent with other research, which found that the knock-down of NQO2 in K562 erythroleukemia [130] and CWR22Rv1 prostate cancer cells [142, 166] results in the suppression of cells’ proliferation rates. Furthermore, it has been shown that treating those cells with resveratrol is also accompanied by significant reductions in cell proliferation [130, 166]. As NQO2 is considered to be the cellular target protein of resveratrol, it has been suggested that the anti-proliferative activity of resveratrol in prostate cancer cells might be mediated through the inhibition of NQO2 activity [126].

To understand the underlying mechanism behind the elevation in the proliferation rates of TOV-112D cells overexpressing NQO2, a flow cytometric analysis of the cell cycle was performed. The results showed an increase in the progression of NQO2-OE cells through the G1/S phase, with a reduction in the G1 phase cell proportion and a concomitant increase in S phase cell proportion,
compared to EV cells. These differences in cell proportions between NQO2-OE and EV cells became more pronounced at the latter time points of 72hr and 96hr, post-seeding. Therefore, the increase observed in the proliferation rate of NQO2-OE cells might be attributed to their ability to progress at higher percentages through cell cycle phases, thus duplicating the cell numbers in a shorter amount of time compared to EV. This interpretation is in accordance with the previous study which attributes the suppression in the proliferation rate of CWR22Rv1 cells to the NQO2 knock-down, leading to the arrest of cells in the G1 phase [166].

It is interesting to note that the proportions of NQO2-OE cells distributed through the cell-cycle phases remained relatively constant with each cycle of cell division over the duration of 96hr. However, this was not the case with EV cells, which showed alterations in the proportions of G1 and S phase cells over time, with significant accumulations in the G1 phase and concomitant reductions in the S phase cell proportion at the latter time points of 72hr and 96hr post-seeding. A possible explanation for this is that EV cells in the long-term cultures underwent more stress, which extensively affected their proliferative ability compared to NQO2-OE cells. As the seeding densities used for the short and long-term cultures were already optimised to produce 70% confluence cultures upon collection time, and the cell culture conditions were optimal, the probability of the stress stemming from the culture conditions themselves is very low. It can therefore be assumed that the stress was most likely due to natural cellular processes that blocked cell cycle progression and consequently impaired the proliferation of EV cells. This assumption is supported by several studies that demonstrated the contributory roles of ROS and accompanied stress in regulating critical cellular processes, including cell cycle progression [19, 252]. The results of this study show that the ROS levels in EV and NQO2-OE cells were unstable over time. In the 48hr cultures, the ROS levels substantially increased, compared to the 24hr culture, and then significantly reduced in 72hr and 96hr cultures. Notably, this reduction was more pronounced in NQO2-OE cells than EV cells. As NQO2-OE cells express much higher levels of NQO2 than EV cells, the observed reduction in ROS levels can be explained by taking the antioxidant activity of NQO2 into account [122]. This fluctuation in the ROS levels led to changes in the cellular redox status, which may consequently affect the cell cycle progression and cell proliferation rate. In accordance with the
present results, previous studies have demonstrated that the changes in ROS levels in cancer cells are mediated by natural compensatory mechanisms that the cells rely on to promote their proliferation and maintain their survival. In the initial stage of cancer development, the production of ROS is substantially increased to stimulate the intracellular signalling cascade and subsequently promote the proliferation of cancer cells. Thereafter, the cells respond to the high toxicity of ROS by increasing the antioxidant levels, thereby reducing cell stress [253]. The overexpression of NQO2 in NQO2-OE may allow the cells to manage the high stress levels much better than EV cells, by declining ROS levels to an extent higher than that of EV cells, thereby reducing the stress-associated toxicity. Moreover, the inexorable demands of cancer cells for energy and biosynthetic precursors to support their high proliferation rate are associated with massive increases in oxidative stress [254]. It was hypothesised that maintaining TOV-112D cells for longer periods of time in 72 and 96hr cultures allowed them to proliferate more and therefore generate more stress. Given the fact that NQO2 is an antioxidant enzyme [122], its overexpression in NQO2-OE cells is expected to confer resistance to these cells against oxidative stress-associated toxicity by reducing higher levels of ROS than in EV cells, as demonstrated in Figure (5.5). Also, the low ROS levels in NQO2-OE cells may support their survival and promote their proliferation. Low ROS levels have been shown to have a positive regulatory role in supporting cancer cell proliferation; however, high ROS levels block cell cycle progression, resulting in growth inhibition [252]. A reduction in the cell cycle progression has been also noticed in Chinese Hamster ovary cells when they are exposed to $\text{H}_2\text{O}_2$ [252].

To probe further into the effects of upregulating NQO2 in TOV-112D cells on cell cycle, the expression profile of G1 phase regulators was established. Deregulation in the G1/S phase transition has been found to be involved in ovarian carcinogenesis [152]. The aberrant expression of cyclin D1 is commonly observed in many types of cancers, and is suggested to have a positive impact on cancer cell proliferation; the aberrant increase in cyclin D1 levels enhances the phosphorylation of Rb, consequently promoting cell progression through the G1 phase, resulting in uncontrolled cell proliferation [166]. Although the earlier findings elucidated that NQO2 may play a positive participatory role in supporting ovarian cancer cell proliferation, the expression levels of
cyclin D1 with its respective partner CDK4 and their downstream target (Rb) were all found to be contrastingly much lower in NQO2-OE cells compared to EV. These results seem to contradict the NQO2-OE cells’ ultimate response. However, by looking at the results from a different angle, it can be concluded that the NQO2-OE cells might have been in another phase when they were collected along with EV cells for the Western blot analysis. There might have been high proportions of NQO2-OE cells in the G1/S-phase boundary and in the S phase, where cyclin D1 levels ought to be low. This also indicated that overexpressing NQO2 results in destabilisation of cyclin D1. It has been found that the reduction of cyclin D1 levels is imperative to inducing the G1/S phase transition, as the presence of low levels of cyclin D1 allows the cells to synthesise DNA, facilitating entry into the S phase [160, 255]. Cyclin D1 plays a repressive role in the DNA synthesis process, through its binding to PCNA and CDK2 [155]. Therefore, the degradation of cyclin D1 is necessary to allow DNA replication. This process has been found to be regulated by the proteasomal degradation of phosphorylated cyclin D1 (Thr286). This indicates that the turnover of cyclin D1 is primarily dependent on the induction of its phosphorylation at Thr286, which has been found to be mediated by GSK-3β [155, 159, 256]. In this study, overexpressing NQO2 was found to cause slight increases in the expression of p-cyclin D1 (Thr286), compared to EV cells expressing low levels of NQO2. These findings therefore supported the assumption that the reduction of cyclin D1 levels was due to the induction of its phosphorylation at Thr286, which was probably mediated by overexpressed levels of NQO2 allowing the DNA synthesis.

The underlying molecular mechanism through which the overexpression of NQO2 causes the destabilisation of cyclin D1 in NQO2-OE cells was not investigated in the present study. However, Hsieh et al. delineates the role of NQO2 in controlling AKT activity, which acts as a negative regulator of GSK-3β activity [162]. It has been shown that the knock-down of NQO2 in CWR22Rv1 cells allowed the activation of AKT and subsequent blockage of GSK-3β activity, consequently preventing the phosphorylation of cyclin D1 and maintaining its levels stable in the cells. The activation of this cascade has been associated with an accumulation of cells at the G1 phase, and a consequent reduction in their proliferation rate [166]. The findings of previous studies allowed me to conclude that the reduction observed herein in cyclin D1 levels might be promoted by the NQO2-
mediated inhibition of AKT and subsequent activation of GSK-3β, thereby allowing the proteolytic phosphorylation of cyclin D1. Therefore, the presence of high NQO2 levels might be critical in inducing this cascade and ultimately reinforce the cells to enter the S phase, thereby accelerating cell proliferation. However, this was not the case with EV cells that expressed relatively high stable levels of cyclin D1 and CDK4, which promote the progression of cells through the G1 phase in an attempt to overcome the negative impacts of high stress levels on cell proliferation.

On the other hand, the expression of low levels of G1 phase regulators in NQO2-OE cells allowed to conclude that the cells may partially rely on the cyclin D1/CDK4/Rb pathway to progress through the cell cycle, meaning they may have alternative pathways that are activated under certain circumstances and supported by high NQO2 levels. This suggestion was extrapolated from previous studies that demonstrate the ability of p-Rb deficient cells to progress through the G1/S phase by relying on cyclin E/CDK2 [150]. As NQO2 has been shown to have pivotal roles in the stabilisation of cyclin D1 and in the turnover of central proteins involved in regulating the DNA damage repair, cell apoptosis and cell cycle-phases arrest [166], it was speculated that the presence of high NQO2 levels in NQO2-OE cells might be crucial in mediating the stability of cyclin E/CDK2, which are also considered as S phase initiating factors [151], thus allowing high proportions of cells to progress through the G1/S phase. In other words, the contributory functions of NQO2 might allow these cells to switch to the secondary pathways under certain circumstances, to support the continuity of the cell cycle progression and cancer cell proliferation at the same rate. It is worth mentioning that there were high similarities in all cellular responses studied herein between NQO2-OE and NQO2-OE/EPR cells. This indicates that even in the absence of endogenous co-factors, NQO2 was still functional in supporting the cancer cell proliferation.

5.3.2 SKOV-3

The current study on SKOV-3 cells set out to assess the influences of silencing NQO2 on the proliferative ability of ovarian cancer, and investigate whether the possible changes were mediated by NQO2’s putative roles involved in regulating cell cycle progression and stabilising G1 phase key regulators. In this study, the genetic silencing of NQO2 was found to maintain the proliferation rate
of sh27/+dox cells similar to that of sh27 cells where NQO2 levels are high. Furthermore, there were no differences in the colony-forming capacity of cells between NTC/+dox (expressing NQO2) and sh27/+dox (possessing silencing NQO2). The significant reduction in the survival fractions observed in the presence of dox was ascribed to off-target actions of dox in the cells. These results are in agreement with a recent study that demonstrates the efficiency of dox in attenuating the proliferative properties of breast cancer, causing a significant repression in the colony formation of MDA-MB-468 and MCF-7 cells [257].

The lack of differences in the proliferative abilities between NTC and sh27 treated with dox was further supported by the findings of the cell cycle analysis, which demonstrated high similarities in the cell cycle progression, and therefore cell cycle phases distribution between these cell sub-lines. This allowed to conclude that the effects of modulating NQO2 levels on the growth behaviour of cancer cells seem to vary among ovarian cancer cell lines. Silencing NQO2 in SKOV-3 cells maintains their proliferative ability similar to that of the sh27 culture, whereas overexpression in TOV-112D make the cells highly proliferative, due to the positive regulatory functions of NQO2 supporting their growth. It might be that the NQO2 silencing levels in SKOV-3 might not have been sufficient to clearly show the nature of growth response. Although these cells stemmed from the same type of cancer, they responded to the modulated levels of NQO2 differently; this is not a unique occurrence. It has been found that treating MCF-7 breast cancer cells with gammatoctotrienol was accompanied by an upregulation in NQO2 expression and activity levels; this might be responsible for the observed repression in cell proliferation [213]. Interestingly, the genetic silencing of NQO2 in MDA-MB-231 breast cancer cells has also led to a repression in cell proliferation, accompanied by concomitant alterations in the expression of cell cycle regulators [99].

Regarding the nature of NQO2's regulatory roles in the G1 phase factors expression in SKOV-3 cells, this study found that silencing NQO2 did not significantly alter the basal expression levels of Rb and p-Rb in comparison to the control cells. The same observation was also found with cyclin D1, which was expressed in sh27/+dox at levels similar to that of the basal levels expressed in control NTC/+dox cells. This stability was suggested to be mediated by a significant reduction in p-cyclin D1, as observed in sh27/+dox compared to NTC/+dox cells. These findings were supportive
of the notion that NQO2 is involved in cyclin D1 turnover by controlling the phosphorylation of cyclin D1 at Thr286. The present findings are consistent with Hsieh et al.’s research, which found that an NQO2 knock-down in CWR22Rv1 cancer cells enhances the stability of cyclin D1 levels in the cells via the suppression of cyclin D1’s phosphorylation. The changes observed in p-cyclin D1 upon silencing NQO2 allowed to conclude that NQO2 might contribute to the control of cell cycle regulators’ expression in SKOV-3 cells and, consequently, cell proliferation. The level of NQO2 silencing however was not sufficient to cause appreciable alterations in the growth behaviour of cancer cells.

In summary, the results in this chapter show that upregulating NQO2 levels provided some survival and proliferative advantages to TOV-112D cancer cells. NQO2 played crucial roles in controlling the stability and degradation of cyclin D1 in the tested ovarian cancer cell line, thereby affecting their proliferative ability. However, it did so to different extents. Furthermore, the antioxidant effects of NQO2 were also suggested to support the cancer cell survival and growth. The differences in cellular responses to the modulations of NQO2 levels seemed strongly affected by the cells subtype and/or degree of NQO2 levels modulation. In conclusion, as NQO2 may induce cancer cell proliferation upon its overexpression, inhibition of NQO2 might be a promising therapeutic strategy for ovarian cancer.
Chapter 6

Investigating the impact of silencing NQO2 genetically and/or pharmacologically on the susceptibility of ovarian cancer cells to adriamycin
Chapter 6

6 Investigating the impact of silencing NQO2 genetically and/or pharmacologically on the susceptibility of ovarian cancer cells to adriamycin

6.1 Introduction

The limited outcomes associated with the primary standard therapy of OVC are one of the main reasons that lead to a high mortality rate among OVC patients [6]. Platinum-resistant ovarian cancer is frequently recognised as an incurable disease. Thus, there is a strong need to develop new therapeutic agents that target pathways uniquely present in OVC patients [258]. Adriamycin (ADR) has been approved for use as a single agent in the second-line treatment for OVC [258] as well as for the treatment of platinum-refractory relapsed OVC patients [6]. The usage of ADR in combination with topotecan has been shown to have high therapeutic efficacy for treatment of OVC, reducing the need for high therapeutic doses; consequently patients are protected against ADR-associated side effects [258]. Currently, the combination of ADR and carboplatin is considered an optimal therapeutic approach recommended for treatment of platinum-sensitive relapsed OVC [259]. ADR is an anthracyclin quinone [1] that exerts its antitumour activity through several mechanisms; the production of free radicals-associated with oxidative damage is considered a critical factor mediating its antitumour activity [95].

Resistance to chemotherapy is recognised as a main concern for the failure of cancer therapy. Such resistance is due to multiple factors including the increased drug efflux and enhanced antioxidant capacity of cancer cells [260]. Overexpression of P-gp is a common characteristic of cancer cells that show high resistance against chemotherapeutic drugs [1]. Ovarian cancer cells particularly the ones that have resistance characteristics have shown high expression levels of P-gp. P-gp is a membrane transporter that contributes to pumping lipophilic drugs out of the cancer cells, thereby reducing their intracellular therapeutic concentrations and efficacy [66]. Furthermore, the presence of high levels of antioxidants agents and detoxifying enzymes in cancer cells allows the cells to adapt to the exogenous oxidative stress generated by anticancer drug such as ADR attenuating the cells’ responsiveness to ADR treatment [260]. Therefore, identifying the potential factors that contribute to the resistance of cancer cells to ADR is important to restore its efficacy
thereby protecting non-cancerous cells from unwanted side effects associated with the high refractory doses [261]. Evaluating the impact of modulating NQO2 levels on the efficacy of ADR is the focus of the work described in this chapter.

NQO1 is an antioxidant enzyme that has been found to confer resistance to ADR through its free radical scavenging activity which protects cancer cells from the oxidative damage associated with ADR [97]. NQO2, which is functionally related to NQO1, has also demonstrated a similar detoxifying activity in cancer cells [122]. Accordingly, we hypothesised that the presence of high expression levels of NQO2 in cancer cells may impair the anticancer efficacy of ADR. Thus, NQO2 might be a novel therapeutic molecule to be targeted by chemosensitising agents (such as potent inhibitors) in an attempt to enhance the responsiveness of cancer cells to oxidative stress inducers (e.g. ADR). Previous work carried out in Prof Stratford’s laboratory suggests that NQO2 plays a contributory role in determining the cytotoxic effects of ADR in MDA-MB-231 breast cancer cells; this occurs via regulating the expression of P-gp and changing drug uptake [98, 99]. Thus, this chapter aims to:

1. Investigate impact of silencing NQO2 levels genetically and/or pharmacologically on the toxicity of ADR in OVC cells.
2. Investigate the effect of downregulating NQO2 on the P-gp expression and intracellular basal ROS levels.
3. Identify the nature of correlation between aforementioned factors (P-gp and ROS) and resistance of OVC to ADR, and whether NQO2 levels mediate this correlation.
6.2 Results

6.2.1 Investigating the correlation between NQO2 levels and the susceptibility of OVC cells to ADR treatment

6.2.1.1 Comparing ADR toxicity profiles of SKOV-3 and TOV-112D (WT) cells expressing varying levels of NQO2

It was demonstrated earlier in this study that NQO2 levels varied widely in the panel of OVC cells being the highest in SKOV-3 and lowest in TOV-112D. To investigate whether there is a correlation between NQO2 levels and the sensitivity of OVC cells to ADR, cytotoxicity of ADR was evaluated and compared in SKOV-3 and TOV-112D (WT) cells using MTT assay. The plated cells were treated with a range of ADR (0.001-10µM) for five hours. Thereafter, the medium was replaced with a fresh one for 96hr. The experiment was terminated by the addition of MTT solution to the cells. The percentages of cells that remained unaffected by ADR treatment were calculated relative to the untreated control. The percentages of survival cells were plotted against drug doses generating dose-response curves, as shown in Figure (6.1).

From the data in Figure (6.1), it is apparent that the sensitivity of SKOV-3 cells to ADR is significantly lower than that of TOV-112D cells; with 3-fold difference in the IC\textsubscript{50} values being 0.31 ± 0.04µM and 0.1 ± 0.009µM respectively. These findings support the possible correlation between cellular NQO2 levels and responsiveness of cancer cells to ADR treatment.
Figure 6.1 Sensitivity of OVC cell lines expressing varying levels of NQO2 to ADR

The 5hr cytotoxicity of ADR was evaluated in SKOV-3 (WT) cells (possessing high basal levels of NQO2) and TOV-112D (WT) cells (possessing low basal levels of NQO2) over a range of concentrations of (0.001-10µM). The proportions of survival cells at these concentrations were estimated using MTT assay following 96hr incubation with fresh medium and calculated as percentages relative to the solvent control. Points on the graphs are representative of mean values of cell survival percentages calculated from three independent experiments, which are connected with the best fit lines. Representative curves are dose-response curves for SKOV-3 (●) and TOV-112D (▲). Perpendicular lines are used to indicate the concentrations of ADR which developed 50% toxicity in SKOV-3 and TOV-112D cells. Significance difference between mean values of IC50s was determined by unpaired t-test. Asterisks refer to the level of significance which was at p value =0.01.

6.2.1.2 The impact of silencing of NQO2 genetically and/or pharmacologically on the cytotoxicity of ADR in SKOV-3 cells

The effects of downregulating NQO2 on the cytotoxicity of ADR were investigated in SKOV-3 (sh27) cells under different treatment conditions whereby NQO2 levels were modulated genetically and/or pharmacologically. To address changes in ADR toxicity associated with genetic silencing of NQO2, sh27 cells were treated with and without dox at a concentration of (1µg/ml) for five days prior to seeding for the experiment. The cells were then seeded in a 96-well plate and treated with ADR at the range of concentration (0.001-10µM) for five hours prior to replenishing them with a fresh medium for the remainder of 96hr period. MTT assay was also used to evaluate cell survival. The resultant dose-response curve is represented in Figure (6.2). To address changes in ADR toxicity associated with pharmacological inhibition of NQO2, a selection of potent novel and typical
inhibitors of NQO2 (e.g. compound 11, compound 13, 9AA and resveratrol) were used at fixed concentrations to treat sh27 cells expressing high basal levels of NQO2 for 19hr. This was followed by adding varying concentrations of ADR treatment ranging from 0.001 to 10µM for further five hours. The medium containing inhibitor plus ADR was then substituted by a fresh medium for 96hr. Percentages of survival cells that remained unaffected by ADR toxicity were calculated relative to inhibitor-treated control and plotted against tested range of concentrations of ADR to generate dose-response curves, as shown in Figure (6.3, A-D). Lastly, to address the effects of maximising the NQO2 inhibition to a high extent on ADR toxicity, NQO2 was inhibited sequentially by dox followed by NQO2 inhibitors. The cells were first treated with dox (1µg/ml) for five days. Thereafter, they were seeded in 96-well plates and treated with fixed concentrations of those inhibitors for 19hr prior to adding varying concentrations of ADR (ranging from 0.001µM to 10µM) for a period of five hours. The medium containing treatments were then replaced with a fresh medium for 96hr. Percentages of survival cells were determined and plotted against ADR concentration to generate dose-response curves, as shown in Figure (6.3, A*-D*). The highlighted curves refer to the dose-response curves of ADR generated following the exposure of sh27 cells to a combination therapy of dox and inhibitors whereas the plain ones for those generated following exposure to inhibitors only. The IC₅₀ values of ADR in the presence of inhibitors alone and in combination of dox were determined from the dose-response curves and represented in Table (6.1).
SKOV-3 (sh27) cells were treated with and without dox (1µg/ml) for five days prior to starting the experiment. The 5hr cytotoxicity of ADR was evaluated in sh27 and sh27/+dox over a range of concentrations of (0.001-10µM). This was conducted using MTT assay following 96hr incubation with fresh medium. The percentages of cell survival were calculated relative to solvent control. Points on the graphs are representative for the mean values of the percentages of survival cells calculated from three independent experiments, which are connected with the best fit lines. Representative curves are dose-response curves for sh27 (●) and sh27/+dox (▲).

The data, as shown in Figure (6.2), demonstrates the null effect of genetic silencing of NQO2 on ADR toxicity; with no difference in the IC50 values (approximately 0.3 ± 0.04µM) of sh27 cells treated with and without dox. On the other hand, pre-exposing the cells to compound 11, compound 13 and 9AA resulted in slight non-significant reductions in the toxicity of ADR, (i.e. these inhibitors potentiated the resistance of cells to ADR in a dose-dependent manner as reflected by the slight upward shifting of the curves). In contrast, resveratrol showed a completely opposite trend of significant enhancement in the ADR toxicity as marked by the substantial downward shifting of the curves. To examine whether these changes in ADR toxicity were mediated by pharmacological inhibition of NQO2 activity, the responsiveness of sh27 cells to ADR following exposure to the inhibitors was evaluated again but this time it was in the presence of modified low levels of NQO2. The same trends of alterations in ADR toxicity were also found in both sh27 and sh27/+dox cells, as demonstrated in Table (6.1), indicating the non-involvement of NQO2 in mediating inhibitors’ activity-induced these alterations in ADR toxicity.
It is worth mentioning that dox itself had no impact on the cytotoxicity of ADR as demonstrated in Figure (9.6) in the appendix chapter where NTC control cells respond to ADR similarly in the presence and absence of dox; with IC$_{50}$ values of 0.34 ± 0.02µM and 0.31 ± 0.04µM respectively.
Figure 6.3 Sensitivity of SKOV-3 cells to ADR following pharmacological inhibition and/or gene silencing of NQO2 (continue on next page)
The 5hr toxicity of ADR was evaluated in SKOV-3 (sh27) cells following the pharmacological inhibition of NQO2 using MTT assay. To do this, the cells were first treated with fixed concentrations of inhibitors for 19hr and then varying concentrations of ADR (0.001-10µM) were added for a further five hours. The medium was replaced after this time with a fresh one for 96hr. Percentages of survival cells were calculated relative to solvent control and plotted against ADR concentrations range to generate dose-response curves. Figures (A-D) are representative of the dose-response curves of ADR in sh27 following inhibition of cellular NQO2 activity pharmacologically using compound 11, compound 13, 9AA and resveratrol (RV) respectively at fixed concentrations of 0.05µM (○), 0.5µM (■), 1µM (●), 2µM (◇), 10µM (▲), 25µM (◆) and 50µM (★). Dose-response curve of ADR alone (●) was also included for comparison. The effects of downregulating NQO2 activity sequentially by dox followed by NQO2 inhibitors on ADR cytotoxicity were also addressed following the same protocol described earlier with the exception of using sh27/+dox cells instead, which were pre-treated with dox (1µg/ml) for five days prior to being seeded for toxicity assay. The same inhibitors and concentrations were also used in this regard. The highlighted Figures (A*-D*) are representative of the dose-response curves of ADR in sh27/+dox following downregulating NQO2 genetically and pharmacologically. Dose-response curve of ADR in sh27 treated with dox alone (▲) was also included for comparison purposes.

Table 6.1 Toxicity of ADR following downregulation of NQO2 activity in SKOV-3 cells

<table>
<thead>
<tr>
<th>NQO2 inhibitors</th>
<th>Concentration (µM)</th>
<th>5hr-Toxicity of ADR (IC50 µM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>sh27</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>0.67 ± 0.12</td>
</tr>
<tr>
<td>Compound 11</td>
<td>0.05</td>
<td>0.85 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.1 ± 0.46</td>
</tr>
<tr>
<td>Compound 13</td>
<td>1</td>
<td>0.84 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.08 ± 0.30</td>
</tr>
<tr>
<td>9AA</td>
<td>0.5</td>
<td>0.64 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.3 ± 0.40</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>25</td>
<td><strong>0.22 ± 0.048</strong></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td><em>0.2975 ± 0.043</em></td>
</tr>
</tbody>
</table>

IC50 values of ADR were determined from dose-response curves shown in Figures (6.2 and 6.3), and used as measures of its toxicity in SKOV-3 (sh27) cells under different conditions where NQO2 was either completely present or downregulated by genetic and/or pharmacologic approaches. They were represented as mean values of three independent experiments ± SEM. Asterisks refer to the significance levels of difference in the ADR toxicity between controls (either sh27 or sh27/+dox) and their corresponding treated cells which were statistically determined using paired t-test at *p<0.05 and **p<0.001.
6.2.1.3 The effect of downregulating NQO2 genetically and/or pharmacologically on the P-gp expression levels in SKOV-3 cells

The contributory role of NQO2 in regulating the expression of P-gp was examined in SKOV-3 (P-gp-positive cells [186]) upon modulating NQO2 levels genetically and/or pharmacologically using Western blot analysis. Samples used for analysis were prepared as follows:

1. For dox-treated samples, sh27 cells were seeded in a 6cm dish and treated with and without dox (1µg/ml) for five days on an alternate day basis for maximum genetic silencing of NQO2.

2. For inhibitor-treated samples, sh27 cells were seeded in 6cm dishes and incubated for 24hr prior to being treated with NQO2 inhibitors including novel hydrazone-quinoline derivatives such as compound 11 (at a concentration of 1µM) and compound 13 (at a concentration of 10µM), and typical inhibitors such as resveratrol (at a concentration of 50µM) and 9AA (at a concentration of 2µM). These sub-IC$_{50}$ concentrations have previously been demonstrated to be highly efficient at silencing NQO2 in vitro. The cells were exposed to the treatments for 24hr prior to collecting the samples in RIPA buffer.

3. For the samples treated with a combination treatment of dox and inhibitors, sh27 cells were cultured in a big flask with dox (1µg/ml) for five days and then seeded in a 6cm dishes. Following a 24hr incubation, the cells were treated with those inhibitors at the same concentrations and collected after 24hr in RIPA buffer.

Samples containing 20µg protein were loaded in 7% SDS-gels for electrophoresis. Wet protein blotting was used to transfer the proteins. The blots in Figures (6.4 and 6.5) are representative of three independent experiments. Figure (6.4, A) shows the effects of silencing NQO2 gene on P-gp levels while Figure (6.5, A and B) shows the effects of pharmacological inhibition or pharmacological plus genetic silencing of NQO2 in sh27 cells on P-gp expression levels respectively. Densitometric analysis was performed on the whole Western blots. The P-gp bands were normalised first to their respective β-actin and then the ratio of changes in P-gp levels were
calculated relative to untreated sh27. The mean of ratios calculated from three independent experiments were represented graphically, as illustrated in Figure (6.4, B) and Figure (6.5, C). The results were analysed based on one-way ANOVA followed by Dunnett’s multiple comparisons tests.

**Figure 6.4 P-gp expression levels in SKOV-3 cells following genetic silencing of NQO2**

A. Western blot analysis was performed to investigate the effects of silencing the NQO2 gene on P-gp levels. SKOV-3 (sh27) cells were first exposed to dox (1µg/ml) for five days before collection for analysis. Sh27 untreated with dox was also seeded and exposed to the same culture condition. 20µg was the amount of protein loaded for electrophoresis. This blot is representative of three independent blots that underwent densitometric analysis to measure the bands intensity. B. Bands referring to the P-gp protein levels were normalised first to their respective β-actin and then the mean of fold changes in P-gp expression in sh27/+dox was calculated relative to untreated sh27 control cells and represented in bar graph.

The data, as shown in Figure (6.4), demonstrates that genetic silencing of NQO2 results in a significant reduction in P-gp expression levels compared to untreated sh27 control; with 1.5–fold difference. The level of significance was p=0.006. It is noteworthy to mention that dox itself caused no significant alterations in the basal levels of P-gp in SKOV-3 cells, as illustrated in Figure (9.7) in the appendix chapter; there is no difference in the P-gp expression levels between untreated NTC
and dox-treated NTC cells. This means that the reduction observed in the P-gp levels in sh27/+dox cells is mainly attributable to the silencing of NQO2.

The ability of the most potent NQO2 inhibitors to alter the expression levels of P-gp in sh27 cells was also examined. No significant reduction in P-gp levels was found between untreated control and samples treated with compound 11 and 13, as illustrated in Figure (6.4, A). In contrast, treating the samples with resveratrol or 9AA caused non-significant increase in the P-gp levels when compared to untreated samples. Based on these findings, it can be suggested that the pharmacological inhibition of NQO2 induced by quinolines might not be sufficient to generate appreciable changes in the expression of P-gp and therefore amplifying the NQO2 silencing levels might cause substantial alterations in the P-gp levels. Furthermore, by doing this, it would be possible to investigate whether the increases observed in P-gp expression in resveratrol- and 9AA-treated samples were actually attributed to inhibiting NQO2 or other off-target effects of these inhibitors.

From the data in Figure (6.5, B and C), it is apparent that inhibition of NQO2 by dox plus compounds 11 and 13 results in significant reductions in P-gp levels as compared to untreated control, but at the same time in non-significant differences comparing with a dox-treated control sample, (i.e. no change in P-gp levels greater than that induced by dox was detected). In addition, no significant differences in P-gp levels were found between resveratrol- and 9AA-treated samples and the corresponding ones treated with a combination of either resveratrol/+dox or 9AA/+dox, thereby demonstrating the alterations in P-gp expression to the off-target, NQO2-independent activities of these classical inhibitors.
Figure 6.5 P-gp expression levels in SKOV-3 cells following genetic and/or pharmacological inhibition of NQO2

A. Western blot analysis was performed to investigate the effects of pharmacological inhibition of NQO2 on P-gp levels. SKOV-3 (sh27) cells possessing basal high-levels of NQO2 were treated with the most potent NQO2 inhibitors from hydrazone-quinoline class, including compound 11 and 13 at concentrations of 1µM and 10µM respectively, and with typical inhibitors such as RV and 9AA at concentrations of 50µM and 2µM respectively. The cells were exposed to the treatments for 24hr prior to collecting them for further analysis. B. The same protocol was followed to address the changes in P-gp expression levels accompanied by a concomitant inhibition of NQO2 genetically and pharmacologically. Basically, the cells were first exposed to dox (1µg/ml) for five day prior to being treated with these inhibitors for 24hr using the same concentrations. 20µg was the amount of protein loaded for electrophoresis. C. Blots (A and B) are representative of three independent blots that underwent densitometric analysis to measure the bands intensity. Bands referring to P-gp were first normalised to their respective β-actin and then fold changes in P-gp expression in the treated samples were determined relative to untreated control (ctrl). Vertical dashed line was used to address the fold differences in P-gp expression levels between sh27 cells treated with inhibitors alone (left-hand side) and those treated with a combination of dox and inhibitors (right-hand side). Asterisks refer to the significance level which was determined using one-way ANOVA and post hoc Dunnett’s tests at p<0.01.
6.2.1.4 The effects of silencing NQO2 genetically and/or pharmacologically on ROS levels in SKOV-3 cells

Various studies have suggested that ROS play an important participatory role in modulating the cells' response to chemotherapy [70, 262]. It has been found that the cells possessing high basal levels of oxidative stress are much more sensitive to oxidative damage inducers (such as anticancer drugs) than that possessing the low basal levels of stress [260]. Thus, modulating the intrinsic levels of oxidative stress in SKOV-3 cells through downregulating NQO2 might alter the susceptibility of the cells to ADR. To investigate this hypothesis, cells were seeded and treated with the same compounds and at the same concentrations that mentioned earlier in section (6.2.1.3). However, the subsequent steps for ROS levels analysis were applied following the protocol described in section (2.13.2). The fluorescence intensity of DCF emitted from the stained samples was directly proportional with ROS levels and measured at 530nm using Fortessa machine. The mean fluorescence of DCF-stained samples were first normalised to their respective unstained samples and then the fold changes in ROS levels were calculated relative to untreated control. It is worth mentioning that the possible alterations in the basal ROS levels generated by off-target cellular effects of dox were also investigated. As such, NTC cells were exposed to the same culture and treatment conditions as sh27 cells. The results were analysed based on one-way ANOVA and post hoc Dunnette’s tests.

From the data in Figure (6.6, A), it is evident that treating NTC cells with dox caused significant elevation of ROS levels in the cells. This elevation in ROS was thought to be due to off-target effects of dox in SKOV-3 cells. Thus, subsequent comparisons of dox-treated sh27 samples were made with NTC/+dox to exclude undesirable effects of dox from sh27 findings and therefore relate any change in ROS levels to the silencing of NQO2. Interestingly, a slight non-significant reduction in ROS level was observed in sh27/+dox when compared to NTC/+dox. Thus, it was of interest to investigate the implications of the pharmacological inhibition of NQO2 on ROS levels. No significant differences in ROS levels were found between untreated NTC control and all samples-treated with inhibitors with the exception of resveratrol, as illustrated in Figure (6.6, B). Resveratrol interestingly caused substantial increase in ROS after a 24hr exposure. To investigate whether this
increase associated with resveratrol was basically mediated by resveratrol targeting NQO2 activity, sh27/+dox cells possessing low-levels of NQO2 were treated with resveratrol and analysed for ROS levels along with NTC/+dox/+resveratrol cells. In addition, the consequences of amplifying NQO2 silencing using a combination treatment of dox plus inhibitors on the ROS levels were addressed in the same manner. Figure (6.6, C) demonstrates that the elevation in ROS levels observed in resveratrol-treated samples might be mediated by NQO2 as there is a slight reduction in ROS levels in sh27/+dox/+resveratrol cells possessing silenced NQO2 when compared to NTC/+dox/+resveratrol. This figure also shows that sh27 samples treated with combination therapy (dox+inhibitors) have slightly less levels of ROS than those of NTC control samples exposed to the same treatment conditions. However, this reduction was primarily mediated by the genetic silencing of NQO2 as comparable levels of reduction in ROS levels were observed between sh27/+dox and sh27/+dox/+inhibitors.
Figure 6.6 Assessing ROS levels in SKOV-3 cells sub-lines following genetic and/or pharmacological silencing of NQO2 (continue on next page)
A. SKOV-3 (NTC and sh27) cells were treated with and without dox (1µg/ml) for five days prior to the start of the experiment. Cells were re-seeded in medium containing dox until harvesting for ROS analysis. B. SKOV-3 cells were seeded and incubated for 24hr prior to being treated with NQO2 inhibitors including compound 11, compound 13, 9AA and resveratrol (RV) at the following concentrations: 1µM, 10µM, 2µM and 50µM respectively. Control cells were also included and termed (ctrl). Following a 24hr incubation with the treatments, the cells were harvested for ROS analysis. C. Cultures of NTC and sh27 cells were treated with and without dox (1µg/ml) for five days prior to seeding for the experiment. The cells were re-seeded and then treated with the same concentrations of the aforementioned inhibitors. Following a 24hr incubation with inhibitors, the cells were harvested for ROS analysis. The collected samples were stained in parallel with 10µM DCFDA for 30min at 37ºC. DCFDA is metabolised in the cells to DCF as a final fluorescent product. The fluorescence intensity of DCF was measured at 530nm using flow cytometer. The resultant DCF fluorescence of stained samples were first normalised to background fluorescence of their respective unstained samples. The fold changes in ROS levels were then calculated relative to untreated control and plotted as bars representing the mean of three independent experiments ± SEM. Asterisks refer to the significance levels of difference between untreated control and treated samples, which was determined based on one-way ANOVA and post hoc Dunnett's tests at *p<0.05 and **p<0.01.

6.3 Discussion

The problem of chemotherapy resistance constitutes a major obstacle for the successful treatment of the cancer patients [70]. Therefore, understanding the underlying mechanisms behind chemoresistance allows us to identify contributory factors that need to be targeted to restore the susceptibility of cancer patients to chemotherapeutic agents [260]. Enhancing the susceptibility of cancer cells to chemotherapeutic agents allows to use low therapeutic doses thereby reducing their unwanted side effects [263-265]. Thus, this study aimed to investigate whether the response of OVC cells to ADR can be potentiated through downregulating NQO2 levels genetically and/or pharmacologically in the cells.

The current study found that the responsiveness of OVC WT cell lines (e.g. SKOV-3 and TOV-112D) to ADR is significantly different. This is not unexpected as there is growing evidence that demonstrates that the histological subtypes of OVC cells respond differently to chemotherapeutic agents [9]. The difference noticed in this study may in part be due to a wide variation in the expression levels of NQO2; SKOV-3 cells with high basal levels of NQO2 were less responsive to ADR than TOV-112D cells with low basal levels of NQO2. A similar study was conducted on a panel of breast cancer cells to evaluate the correlation between NQO2 levels and sensitivity of the
cells to ADR, and showed the presence of a strong correlation between these variables (ADR toxicity and NQO2 levels). MDA-MB-231, MDA-MB-468 and SKBr3 cells expressing high levels of NQO2 were much less sensitive to ADR than MCF-7 and Cal-51 cells expressing low levels of NQO2 [98]. In order to validate this correlation, NQO2 was genetically silenced in MDA-MB-231 cells. Silencing NQO2 consequently caused a substantial increase in the cells’ sensitivity to ADR with significant difference in the IC50 values in comparison with control [99]. Similarly, the pharmacological inhibition of NQO2 in HCT116 cells using triazoloacridine-6-one (A6B1), a potent inhibitor of NQO2, resulted in an increase in the susceptibility of the cells to ADR [98]. Taken together, the findings of the previous studies have validated the contributory role of NQO2 in the response of breast cancer cells to ADR treatment. Thus, it was of great interest to investigate whether the responsiveness of SKOV-3 to ADR can be improved by modulating NQO2 levels.

The toxicity findings of ADR in SKOV-3 demonstrated that neither genetic silencing nor pharmacologic inhibition of NQO2 result in an enhancement in the cell’s response to ADR with the exception of resveratrol which caused a significant increase in the sensitivity of the cells to ADR in a NQO2-independent manner. The lack of difference in the responsiveness of the cells to ADR upon silencing NQO2 genetically raised the question of whether the reduction in NQO2 levels was not sufficient to alter the toxicity of ADR. To answer this question, the most potent classical and novel inhibitors of NQO2 including resveratrol, 9AA, compound 10 and compound 11 were used both alone and in combination with dox for maximal inhibition of NQO2 prior to treating the cells with ADR. These inhibitors showed higher efficacy in inhibiting NQO2 activity in SKOV-3 cells when they were used in combination with dox as compared with individual treatment, as shown in Figure (4.13). The lack of alterations in ADR toxicity under both treatment conditions allow us to conclude that the differences in the genetic profiles and metabolic processes of breast and ovarian cancer cells might be the reason behind these variations in the cells’ responses to ADR following NQO2 modulations.

The screening study evaluating the responsiveness of the breast cancer cells to ADR ascribes the variations in the cells’ response to the presence of varying expression levels of P-gp which, interestingly, are demonstrated to be in direct correlations with the NQO2 levels; the most resistant
cells are found to express the highest levels of NQO2 and P-gp, and vice versa [98]. This has been further proved in MDA-MB-231 cells which show a significant reduction in P-gp expression following genetic silencing of NQO2. This reduction has been elucidated to be accompanied by a substantial increase in the intracellular concentration of ADR as illustrated by the increase in the fluorescence intensity emitted from cells treated with ADR [99]. As it has been suggested that NQO2 plays a participatory role in regulating P-gp expression in breast cancer cells, it was of great importance to investigate this again in OVC cells. Measuring the expression levels of P-gp under different treatment conditions also allow us to understand the reason behind the variations in the cells’ response to ADR and whether they are mediated by modulated levels of NQO2. The current study found that genetic silencing of NQO2 resulted in a significant reduction in the expression levels of P-gp and this was in agreement with what has been shown in breast cancer [99]. The lack of change in SKOV-3 cells’ response to ADR despite the reduction in P-gp indicates that there might be other pathways that predominantly affect the cells’ response to ADR, in which NQO2 is not involved in them. Nevertheless, NQO2 may impact the sensitivity of OVC cells to other types of anticancer drugs which are compromised by the high expression levels of P-gp. On the other hand, there appear to be substantial increase in the cells’ response to ADR following exposure to resveratrol despite the increased P-gp levels; this supports the idea that P-gp might not be the mediator of ADR resistance in OVC. Contradictory, it has been found that chemosensitising effects of resveratrol to ADR in breast cancer can be the result of modulating P-gp levels [266]. The differences in the pathways-mediated chemosensitising effect of resveratrol to ADR of different types of cancer reinforce the idea that the variations observed in the cells’ response to the modulated levels of NQO2 may primarily be affected by the cell types and their distinct genetic and proteomic compositions.

Chemotherapy resistance is a multifactorial problem and the capacity of antioxidant defence systems in the cells is a critical factor [260]. The elevated antioxidant capacity of the cells makes them more tolerable and resistant to the elevated oxidative stress levels induced by exogenous sources such as anticancer drugs as demonstrated in hepatoma cells against ADR [260, 267]. Oxidative damage associated with one-electron redox cycling of ADR is considered the main factor
involved in anticancer activity in the cells [95]. It has been demonstrated that susceptibility of the cells to ADR is correlated with the basal levels of oxidative stress; the cells that experience high levels of oxidative stress are more sensitive to the anticancer drugs with oxidative stress inducing properties (e.g. ADR) than that with low basal levels of oxidative stress [260]. Manipulation of ROS levels in the cancer cells is a new strategy that has recently been used to enhance the toxicity of chemotherapies in particular the oxidative stress inducers drugs. Modulating the intracellular oxidative stress levels pharmacologically plays a critical role in altering the response of the cells to the chemotherapy [260]. This strategy was used in this study in that NQO2 activity was inhibited genetically and/or pharmacologically prior treating SKOV-3 cells with ADR in an attempt to inhibit its antioxidant activity and therefore increase the basal oxidative stress levels in the cells, thereby enhancing the susceptibility of the cells to ADR treatment. The basal levels of ROS in SKOV-3 cells were measured following the treatment with inhibitors and the results showed slight non-significant reduction in the ROS levels. These findings provide explanations for the lack of difference in the cell response to ADR following the silencing of NQO2 as evident by the no significant increase in the internal ROS levels associated with inhibiting antioxidant activity of NQO2. The contributory role of basal ROS levels in potentiating the sensitivity of SKOV-3 cells to ADR was further supported when manipulating ROS levels in the cells by resveratrol resulted in enhancing the response of the cells to ADR. In other words, the increase in sensitivity of the cells to ADR was directly correlated with the basal levels of ROS which however was not significantly enhanced upon silencing NQO2. The role of resveratrol in sensitising cancer cells to ADR through enhancing ROS levels has recently been confirmed by a number of studies that demonstrated that the dose, exposure time and cell types are all factors that affect the nature of function of resveratrol whether as an antioxidant or a suppressor of antioxidant defence system [268]. The reason of slight changes in ROS levels associated with NQO2 silencing can be due to the high endogenous activity levels of NQO1 in SKOV-3 cells as demonstrated in Table (3.1). The antioxidant ability of NQO1 is suggested to be involved in maintaining the baseline of oxidative stress levels stable in the cells even in the presence of modified low levels of NQO2. This means that NQO1 might also contribute to the resistance of the cells to ADR and that inhibition of NQO1 along with NQO2 might be crucial to potentiate the susceptibility of cancer cells to ADR. Several studies have delineated the
implications of antioxidant activity of NQO1 in the resistance of cells to oxidative stress inducer like anticancer drugs (e.g. ADR) [97]. They have also demonstrated the chemosensitising effects of dicoumarol in enhancing toxicity of ADR in urothelial and bladder cancer cells [97, 269]. This evidence indicates the necessity of downregulating NQO1 along with NQO2 in SKOV-3 cells in order to potentiate their susceptibility to ADR.

In summary, the initial findings in the present study demonstrate that there is the strong correlation between NQO2 levels and sensitivity of OVC cells to ADR. They also demonstrate the contributory role of NQO2 in regulating the expression of P-gp in SKOV-3 cells. The lack of difference in the responsiveness of cells to ADR upon silencing NQO2 emphasise the importance of conducting an initial analysis for the expression levels of antioxidants enzymes in the cells to select adequate chemosensitising agents to compromise their activity and consequently to enhance sensitivity of the cells to ADR maximally. Furthermore, this study re-emphasises the importance of manipulating the baseline of oxidative stress prior to treating the cells with ADR as a strategy used to enhance their sensitivity to ADR.
Conclusion and future directions
7 Conclusions and future directions

7.1 Conclusions

NQO2 is the second member of NAD(P)H: quinone oxidoreductase family, which was discovered 56 years ago. The biological roles of NQO2 are still ambiguous; there exist several controversial assumptions regarding the involvement of NQO2 in different pathological conditions. NQO2 has been proposed to be implicated in the development of cancer [139-141, 270], malaria [172] and neurodegenerative diseases [121, 179] and this has been linked through its putative roles in regulating; firstly, cellular redox status [167]; secondly, the activity of TNF-mediated signalling pathways and NF-κB activation [167] and lastly, cyclin D1 turnover [166]. NQO2 expression has been found to be high in different types of cancer including breast, prostate, hepatocellular tumour and leukaemia. The potential roles of NQO2 in cancer are yet to be discovered. To the best of our knowledge, the biological roles of NQO2 in ovarian cancer progression have not been studied yet. Identification of the nature of alterations in ovarian cancer cells behaviour associated with modulating NQO2 levels allows us to determine whether NQO2 plays contributory roles in the studied pathways or not and consequently whether it has a repressive, progressive or null impact on cancer. NQO2 levels were modulated using two different approaches: first, using NQO2 genetically modified cells, and second, using NQO2 inhibitors.

Screening study on a panel of OVC cell lines for NQO2 expression and activity levels demonstrated that NQO2 levels were varied widely with 35-fold difference among cell lines. A strong correlation was found between NQO2 protein and activity levels in OVC cells. The highest and lowest levels of NQO2 were detected in SKOV-3 and TOV-112D cell lines which were subsequently used to generate NQO2 genetically modified cells. The lentiviral strategy was highly efficient at modulating NQO2 levels in OVC cells. The stably NQO2-overexpressing TOV-112D cells were successfully prepared and the substantial increases in NQO2 protein and activity levels were demonstrated. SKOV-3 (sh27) cells were amongst the cells selected to represent the NQO2 silencing model as the sh27 sequence showed high specificity at targeting NQO2 as well as the highest efficiency at inducing NQO2 silencing compared to the other tested sequences (such as
sh22 and sh29). Therefore, they were preferably used along with TOV-112D (NQO2-OE) cells to investigate the potential roles of NQO2 in OVC.

The availability of less effective inhibitors to study the biological roles of NQO2 in cancer required the synthesis of novel inhibitors with better properties such as high potency and low toxicity. Quinoline scaffold has led Dr Buthaina Hussein attention from Dr Freeman's lab to synthesise novel derivatives with diverse structural modifications with the purpose of improving the compounds’ properties relative to that of typical NQO2 inhibitors. The compounds’ characteristics were then investigated in a cell-free system and in vitro using CB1954 cytotoxicity approach.

Evaluation of CB1954 cytotoxicity is a reliable approach to be used for the investigation of the functional activity and inhibitory potency of the novel compounds against cellular NQO2. The strong correlation between cellular NQO2 activity levels and CB1954 cytotoxicity enhancement ratios allowed us to conclude that NQO2 can be the primary determinant of the CB1954 bioactivation extent and subsequent toxicity in ovarian cancer cells and therefore the modulation of NQO2 activity levels pharmacologically would consequently affect the toxicity extent of CB1954. Thus, cytotoxicity levels of CB1954 can be used as an indirect measure of the compounds’ intracellular inhibitory potency against NQO2.

This strong correlation also confirmed the notion that NQO2 is a nitroreductive enzyme that is responsible for CB1954 bioactivation in human cells. The significant enhancement in the cytotoxicity of CB1954 upon the addition of EPR co-factor supported the theory that reductive activity of NQO2 requires further support from an exogenous co-factor to catalyse CB1954 bioactivation, thereby strengthening the general theory in the literature about reductive characteristics of NQO2.

The unique ability of NQO2 to activate CB1954 anticancer drug as well as the high expression levels of NQO2 in some OVC cell lines NQO2 re-emphasise the potential of using NQO2 in the enzyme-directed tumour approach for CB1954 therapy of human malignant diseases. Another important conclusion that could be drawn from this is that using CB1954 as an alternative therapy
to carboplatin in platinum-resistant tumours; there appeared to be more than 100-fold and 248-fold
difference in the IC$_{50}$ values for cytotoxicity between CB1954/EPR and carboplatin in SKOV-3 and
TOV-112D (NQO2-OE) cells respectively, indicating that these cells respond much better to
CB1954/EPR compared to carboplatin.

Evaluation of the extracellular inhibitory potency of the novel compounds allowed us to conclude
that hydrazone-quinoline derivatives are the most potent inhibitors of NQO2 comparing with other
novel quinoline derivatives including hydrazide and carboxamide derivatives as well as typical
NQO2 inhibitors such as resveratrol and 9AA. Amongst hydrazone derivatives, compounds 10, 11,
13, 14 and 16 have the highest inhibitory potency against recombinant human NQO2. CB1954
findings also demonstrated that the intracellular inhibitory potency of the hydrazone-quinoline
derivatives particularly compounds 10, 11, 13, 14 and 16 is in an excellent correlation with their
extracellular activity and they can, therefore, be considered the most potent NQO2 inhibitors when
compared to other novel inhibitors and to typical ones. Compound 27 from hydrazide class also
showed comparatively high potency against NQO2. The weak activity of the aforementioned
compounds toward NQO1 highlights their high specificity to target NQO2 instead. Despite the high
potency of the hydrazone-quinolines against NQO2, they showed higher toxicity than typical
inhibitors in SKOV-3 cells. However, the toxicity of these compounds is irrelevant to their inhibition
potency to the NQO2.

The inhibitory potency findings of the novel compounds in both systems support the molecular
docking results which suggest that hydrazone derivatives particularly compounds 10, 11, 13, 14
and 16 are the most potent inhibitors. The substitutions on these inhibitors allow them to form
multiple interactions with the binding sites of NQO2 thereby enhancing their binding affinity and
subsequent potency. The substitutions that can be suggested to be introduced to the future design
of highly potent inhibitors are methyl group at position 2 of quinoline ring and/or heterocycles,
hydrophilic substituents or aromatic rings at position 4 of quinoline ring. In addition of being potent,
the high availability of compounds 11 and 13 enables their usage as valuable pharmacological
tools to investigate the contributory roles of NQO2 in the responsiveness of OVC cells to
adriamycin therapy and whether pharmacological inhibition of NQO2 activity can potentiate the cytotoxicity of ADR.

The participatory role of NQO2 in controlling the growth of ovarian cancer cells was also investigated using genetic approach. Overexpression of NQO2 was associated with slight increases in the cell proliferation and clonogenicity of TOV-112D cells. The cell cycle analysis study demonstrated that the upregulation of NQO2 in the cells allowed them to progress at higher percentages through the cell cycle phases particularly from G1 to S phase and consequently to duplicate the cell number in a shorter time comparing with that of the control cells. Furthermore, the presence of overexpressed NQO2 was important to maintain the proportion of cells progressing through the cell cycle phases constant while the cells divide. This however was not the case with EV cells expressing low basal levels of NQO2 which were arrested at G1 phase in the long-term culture highlighting the potential role of NQO2 in supporting the continuity of cancer growth. These findings were further supported by the expression analysis study for G1-phase regulators which demonstrated the involvement of NQO2 in regulating cyclin D1 turnover through modulating cyclin D1 phosphorylation at Thr286. Overexpressing NQO2 enhanced phosphorylation degradation of cyclin D1 thereby allowing DNA synthesis and facilitating the progression of the cells from G1 to S phase.

The potential role of NQO2 in regulating intracellular basal levels of ROS in TOV-112D cells was suggested to be responsible for the elevated cell proliferation. Upregulation of NQO2’s functional activity in TOV-112D allowed NQO2-OE cells to manage the increase in the ROS levels associated with the high cellular proliferation, reducing ROS levels to higher extent comparing with that of the control cells having low basal levels of NQO2. This, therefore, highlights the role of NQO2 in conferring resistance to the cells against oxidative stress-associated toxicity. In addition, reduction in the ROS levels mediated by overexpressed NQO2 may have a positive impact on cell proliferation as the low levels of ROS are found to act as a positive regulator of cell growth. In other words, the presence of high levels of NQO2 may confer growth advantageous properties to the cells through altering cyclin D1 levels and intracellular ROS levels. It is worth noting that NQO2 can generate most of these changes in the cells’ growth behaviour even in the presence of low
endogenous levels of co-factor; the findings of most studies were similar in the presence and absence of exogenous EPR. Furthermore, these findings strengthen the theory that NQO2 is involved in regulating cyclin D1 levels and cellular redox status.

On the other hand, silencing NQO2 in SKOV-3 maintains the proliferation rate of the cells similar to that of the control expressing high basal levels of NQO2 and this was further supported by the cell cycle analysis study. In spite of this, the expression analysis study demonstrates a significant reduction in the phosphorylated form of cyclin D1 and subsequent stabilisation in the cyclin D1 levels in the cells. This allows us to conclude that NQO2 may actually participate in regulating growth of SKOV-3 cells, but this was not noticeable as silencing levels of NQO2 were not sufficient to cause appreciable alteration in their proliferation rates. Furthermore, differences in the genetic and proteomic profiles between TOV-112D and SKOV-3 subtype of ovarian cancer cells may also be responsible for the variations observed in their responses to these modulations in NQO2 levels.

In conclusion, NQO2 is a potential therapeutic target for pharmacological intervention which upon inhibition may attenuate cancer cell growth.

As it has been demonstrated that NQO2 has a contributory role in determining the cytotoxicity of ADR in breast cancer, it was of interest to investigate this again in ovarian cancers using genetic and pharmacological approaches. Downregulating NQO2 even to the maximal level using combination therapies does not alter the response of SKOV-3 cells to ADR. In ovarian cancer, alterations in the ADR toxicity are found to be affected by basal ROS levels rather than P-gp expression. Therefore, the presence of highly active NQO1 along with NQO2 in SKOV-3 might compromise the chemosensitising effect of silenced NQO2 to ADR in SKOV-3 cells. Moreover, the reduction in the P-gp expression levels upon silencing NQO2 allows us to conclude that NQO2 may participate in enhancing the susceptibility of the cells to other types of anticancer drugs where P-gp levels interfere with their response. It has been demonstrated that attenuating P-gp expression in ovarian cancer enhances the cells' response to docetaxel through increasing its intracellular therapeutic concentration [271].
In summary, NQO2 contributes to regulating ovarian cancer cells growth but to different extent depending on the cell subtype; this occurs through regulating cyclin D1 turnover and ROS levels. NQO2’s participatory roles in mediating cells’ response to ADR are dependent on the type of cells and their antioxidant capacity as there appear to be a strong correlation between ROS levels and ADR toxicity. Therefore, these findings collectively support the notion that NQO2 is a therapeutic target for pharmacological intervention which aims to improve the available therapeutic options for ovarian cancer treatment.

7.2 Future directions

To enhance the work presented in this project a number of lines of enquiry could be pursued. First, exploring the underlying mechanisms through which the NQO2 could regulate cyclin D1 turnover in ovarian cancer cells requires further investigation. Assessing the effects of modulating NQO2 on activity of proteasome-mediated stability of cyclin D1 upstream regulators including GSK-3β and AKT would be very useful to understand the reason behind the differential expression in cyclin D1 and p-cyclin D1 between each pair of NQO2 genetically modified cells lines. This can be conducted by performing Western blot analysis of AKT and GSK-3β expression levels in these cells following treatment with proteasome inhibitor (e.g. MG132). In addition, correlation between NQO2 levels and activity of AKT-involved in GSK-3β control is also suggested to be evaluated in these cells possessing varying levels of NQO2. This, therefore, enables us to decide whether NQO2 can also act as non-kinase inhibitor of AKT in ovarian cancer cells via generating physical interactions with it.

Second, it is suggested that the hypothesis regarding the involvement of NQO2 in regulating the stability of other cell cycle regulatory factors in cancer cells including cyclin E and CDK2 should be investigated. This would enable the discovery of novel pathways through which NQO2 can maintain the continuity of cancer cell growth.

Third, it would be useful to investigate the effect of downregulating NQO1 levels on ovarian cancer cells’ response to ADR in cells expressing different levels of NQO2. NQO1 cellular activity can be
inhibited either genetically using siRNA approach or pharmacologically using dicoumarol. This will allow for understanding whether the presence of NQO1 is responsible for the lack of difference in the cells’ response to ADR upon silencing NQO2 comparing with control. In addition, it may also emphasise the necessity of downregulating both enzymes in parallel in cancer cells to avoid their interference effect on the activity of each other or to get better response to ADR treatment.
8 References


124. Scott, K.A., Analogues of dicoumarol as inhibitors of NQO1, in School of Pharmacy and Pharmaceutical sciences. 2010, University of Manchester. p. 2-284.


9 Appendices

9.1 Evaluation of specificity of secondary antibody for IF assay

Figure 9.1 Specificity of anti-NQO2 secondary antibody conjugated with green fluorescence staining

To investigate the specificity of the secondary antibody, the fixed cells were incubated with only the blocking buffer instead of primary antibody.
9.2 Evaluation of EPR toxicity in SKOV-3

Figure 9.2 Toxicity of EPR in SKOV-3 cell at different time points

MTT assay was used as a read out of cell survival following treatment with 1, 10, 50, 100 and 1000µM concentrations of EPR for 24 (○), 48 (◊), 72 (◆) and 96hr (□).
9.3 Investigation of functional activity of putative inhibitors of NQO2 using CB1954 approach

(F)

(F')

(G)

(G')

(H)

(H')

(I)

(I')

Figure 9.3 Effect of putative inhibitors of NQO2 on CB1954 toxicity in SKOV-3 cells (continue on next page)
The protective effect of NQO2 inhibitor from enzymatic bioactivation and subsequent cytotoxicity of CB1954 was measured using MTT assay. SKOV-3 cells were treated with a range of concentration of CB1954, 0.01-10µM plus EPR (●), and also in combination with compound 14 (F/F*), 16 (G/G*), 25 (H/H*), and resveratrol (RV) (I/I*) for (24 hr and 96 hr*). Each inhibitor was tested at varying concentrations selected depending on its cytotoxicity: at 0.05µM (●), 0.1µM (●), 0.5µM (●), 1µM (●), 5µM (●), 10µM (●), 25µM (●), 50µM (●) and 80µM (●). It is evident that these inhibitors protect the cells from CB1954 cytotoxicity in a dose-dependent manner via NQO2 inhibition.
9.4 Investigation of inhibitory potency of NQO2 inhibitors

The inhibitory potency of the compounds against NQO2 was evaluated by measuring their protection levels against cytotoxicity of CB1954 using MTT assay. SKOV-3 cells were concomitantly treated with a fixed concentration of 1µM of CB1954 plus EPR and varying concentrations of NQO2 inhibitor for 24hr (●) and 96hr (○). Figures (F-I) illustrate concomitant treatments with compound 16, 25, resveratrol and quercetin, respectively. Flat curve indicated that the compound was not effective at protective the cells against CB1954 toxicity over this tested range of concentration.
9.5 Evaluation of effect of different treatment conditions on the cell cycle profile of SKOV-3 cells

(A) Representative histograms refer to the overlay cell cycle profiles of untreated control cells including NTC (□) and sh27 cells (■) and EPR-treated cells including NTC/+EPR (■) and sh27/+EPR (■), which give an overview about the effect of EPR on the cell cycle at a 24hr time point interval over a period of 96hr.

(B) Representative histograms refer to the overlay cell cycle profiles of SKOV-3 cells sub-lines including NTC/+dox (□) and sh27/+dox (■), which also illustrated the effects of silencing NQO2 via dox on the cell cycle at a 24hr time intervals over a period of 96hr.

(C) Representative histograms refer to the overlay cell cycle profiles of EPR-treated SKOV-3 cell sub-lines and dox plus EPR treated cells including NTC/+dox/+EPR (■) and sh27/+dox/+EPR (■), which were used to compare the effect of EPR on cell cycle phases distribution and cell proportions the presence and absence of NQO2. Arrows were used to address the difference in the proportions of untreated control cells and EPR-treated cells in G1 phase.
9.6 Investigation of effects of doxycycline on sensitivity of SKOV-3/ NTC control cells to ADR

![Graph showing dose-response curves for NTC and NTC/ +dox](image)

**Figure 9.6 Effect of dox on sensitivity of SKOV-3/ NTC cells to ADR**

To investigate whether pre-treating SKOV-3 cells with dox interfere with sensitivity of the cells to ADR, 5hr cytotoxicity of ADR was measured in NTC cells which were exposed to dox (1µg/ml) five days prior to seeding for the experiment. MTT assay was also used as a read out of cell survival. Representative curves are dose-response curves for NTC (●) and NTC/+dox (▲).
9.7 Investigation of effects of doxycycline on the P-gp expression levels

Western blot analysis was performed to investigate the effects of dox itself on P-gp levels. Thus, SKOV-3/NTC control cells were first exposed to dox (1µg/ml) for five days before undergoing for further analysis. Bands referring to P-gp were first normalised to their respective β-actin and then the fold change in P-gp expression of NTC/+dox cells was determined relative to the untreated control and represented under the blot as mean ± dox.
9.8 Chemical structures of 4-hydrazone-quinoline derivatives
9.9 Chemical structures of 4-hydrazide-quinoline derivatives
9.10 Chemical structures of 4-carboxamide-quinoline derivative

![Chemical structure of 4-carboxamide-quinoline derivative](image)