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Quinone Reductase 2 is a Flavin Redox Switch

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Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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QUINONE REDUCTASE 2 IS A FLAVIN REDOX SWITCH

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by

Kevin Ka Ki Leung

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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Abstract and Keywords

Quinone reductase 2 (NQO2) is a mammalian enzyme that catalyzes the reduction of quinone using an unusual co-substrate, dihydronicotinamide riboside (NRH). In addition, NQO2 has a secondary function, which is to modulate the 20S proteasomal degradation of p53 in a redox-dependent manner. This alternate function has been characterized in its sister enzyme quinone reductase 1 (NQO1) and yeast quinone reductase Lot6p, but relatively few studies have investigated the role of NQO2 beyond quinone catalysis. From studies of Lot6p, it was proposed that quinone reductases could be categorized as flavin redox switches. In this thesis, the how NQO2 functions as a flavin redox switch and how inhibitors can modulate NQO2 was investigated. Initially, a method was developed to reconstitute recombinant NQO2 with FAD. Having purified functional NQO2, reduced structures of NQO2 were solved in complex with four different inhibitors: the antimalarial drug chloroquine, CK2 inhibitor DMAT, DNA intercalating agent ethidium bromide, and acridine orange. In all four reduced structures, the hydrogen bond network and the orientation of inhibitors in the active site of NQO2 were changed by the presence of water molecules compared to the oxidized structures. For the structure of reduced NQO2 in complex with chloroquine, there was also a global conformational change. These reduced structures together showed that NQO2 has two functional states, and indicated mechanistic features of a flavin redox switch in NQO2. To understand how these inhibitors affect NQO2 signalling in cells, the CK2 inhibitors and DNA intercalating agents were further studied in tissue culture. Even though the cytotoxicity of these compounds was not dependent on NQO2, p53 levels were modulated in an NQO2-dependent manner. In conclusion, this thesis has shown that in addition to quinone catalysis, NQO2 functions as a flavin redox switch, and NQO2 inhibitors have the ability to modulate this process.

Keywords

Quinone reductase 2, flavoprotein, antimalarial drugs, primaquine, chloroquine, CK2 inhibitors, TBB, TBBz, DMAT, DNA intercalating agents, ethidium bromide, acridine orange, doxorubicin, X-ray crystallography, enzyme kinetics, ping-pong mechanism, flavin redox switch, 20S proteasomal degradation, p53 degradation.
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List of Abbreviations

9AA – 9 aminoacridine

Abl – Abelson kinase

ADP – adenosine diphosphate

AMP – adenosine monophosphate

AMP – adenosine monophosphate

AO – acridine orange

ARNT – aryl hydrocarbon receptor nuclear translocator protein

ATP – adenosine triphosphate

BCR-Abl – BCR-Abelson kinase

BNAH – 1-benzyl-1,4-dihydronicotinamide

CA – Carbon α

CK2 – protein kinase CK2

CML – Chronic myelogenous leukemia

COSMIC – catalogue of somatic mutations in cancer

CQ – chloroquine

CSD – Cambridge Structural Database

DHODH – dihydroorotate dehydrogenase

DMAT – 2-dimethylamino-4,5,6,7-tetabromo-1H-benzimidazole

EDTA – ethylenediaminetetraacetic acid
EtBr – ethidium bromide
FAD – flavin adenine dinucleotide
FMN – flavin mononucleotide
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
HLADH – horse liver alcohol dehydrogenase
HPLC – high-performance liquid chromatography
IC\textsubscript{50} – inhibition constant
ITC – Isothermal Titration Calorimetry
LOV – light oxygen voltage
Md – menadione
MDR1 – multidrug resistance protein 1, or P-glycoprotein
MMN\textsuperscript{+} – nicotinamide mononucleotide
MRP – multidrug resistance-associated protein
MRP1 – multidrug resistance-associated protein 1
NAD\textsuperscript{+} / NADH – nicotinamide adenine dinucleotide / dihydronicotinamide adenine dinucleotide
nADP – normalized atomic displacement parameter
NAMPT – nicotinamide phosphoribosyltransferase
NF\textkappa B – nuclear factor kappa-light-chain-enhancer of activated B cells
NMNH – dihydronicotinamide mononucleotide
NQO1 – quinone reductase 1
NQO2 – quinone reductase 2

NR\(^+\) / NRH – nicotinamide riboside / dihydronicotinamide riboside

PARP – poly-ADP ribose polymerase

PAS – PER-ARNT-SIM

PER – period protein

PIG3 – p53 inducible gene 3

PKC – protein kinase C

PQ – primaquine

QC – quinacrine

Rb – retinoblastoma protein

ROS – reactive oxygen species

SAXS – small angle X-ray scattering

SCDP – 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine

SILAC – stable isotope labeling by amino acids in cell culture

SIM – single-minded protein

SRB – sulforhodamine B

TBB – 4,5,6,7-tetramethylbenzotriazole

TBBz – 4,5,6,7-tetramethylbenzimidazole
Chapter 1

1 Introduction to Quinone Reductase 2

1.1 General Introduction

Cancer is the uncontrolled growth of cells caused by the induction of oncogenes, and inactivation of tumor suppressors. On this basis, the development of molecular targeted therapy operates on the principle that if these drivers of cancer can be controlled individually, then a regimen of therapeutic agents can ultimately reverse the process of cancer progression. This principle was clearly demonstrated by the success of the drug imatinib to induce complete cytogenetic remission in patients with chronic myelogenous leukemia (CML) by targeting the BCR-Abelson kinase (BCR-Abl) (1). The hallmark of CML is a translocation of chromosome 9 to chromosome 22 in which the normally inactive Abl kinase is fused to BCR, forming the onco-gene BCR-Abl (2). By inhibiting the onco-protein that drives the proliferation of cells, imatinib successfully prevented cells with BCR-Abl from multiplying. While this illustrated the success of molecular targeted therapy, cancer is rarely driven by a single onco-protein.

Validation studies revealed that imatinib also bound to other targets. Using a chemical proteomics approach, two groups independently showed that quinone reductase 2 (NQO2) was a prominent non-kinase target of imatinib (3, 4). NQO2 is a detoxification enzyme, which alternatively functions to stabilize the tumor suppressor p53 from degradation (5, 6) \(^1\). The antileukemic effects of imatinib are dependent on p53 activation in addition to BCR-Abl inhibition, so the interaction between imatinib and NQO2 may contribute to the therapeutic effects of the drug (7). These studies together illustrated the

\(^{1}\) Reference 6 by Gong et al. has been retracted as of February 1\(^{st}\), 2015. Retraction: NRH:Quinone Oxidoreductase 2 and NAD(P)H:Quinone Oxidoreductase 1 Protect Tumor Suppressor p53 against 20S Proteasomal Degradation Leading to Stabilization and Activation of p53. (2015). Cancer Research, 75(3), 615–615.
point that although compounds are designed to inhibit one target, they can act in combination with other targets to elicit a favorable therapeutic effect.

Protein kinase CK2 (CK2) is another dysregulated kinase in cancer. The overexpression of CK2 in cancer attenuates apoptosis by the phosphorylation of caspases and caspase substrates, and inhibition of CK2 can induce apoptosis in cancer cells (8). In the past two decades, numerous CK2 inhibitors have been developed, yet how the inhibitors affect the phosphorylation pattern in cells is still poorly understood. In an attempt to identify CK2 substrates and to validate the inhibitors’ mechanism of action, an inhibitor-resistant CK2 construct was made to restore CK2 activity in the presence of inhibitors (9). Despite the restoration of CK2 activity in the presence of two inhibitors, TBBz and DMAT, both inhibitors still induced apoptosis in cells. Thus, CK2 inhibition was not entirely responsible for cell death and the inhibition of other proteins contributed to apoptosis. Using a proteomics approach, NQO2 was again identified to be an unintended off-target binding protein of TBBz and DMAT (9). Together, this raises the possibility that the efficacy of CK2 inhibitors is also partially due to the off-target inhibition of NQO2.

Initially discovered in the 1960’s by William-Ashman and coworkers, NQO2 was purified as a mammalian cytosolic flavo-protein. It catalyzed the reduction of quinone using dihydronicotinamide riboside (NRH), but not NAD(P)H or dihydronicotinamide mononucleotide (NMNH) (10). NQO2 was left unstudied for almost three decades until it was discovered to be genetically and structurally related to the widely-known enzyme quinone reductase 1 (NQO1) (11). The identification of NQO2 as a homologue of NQO1 suggested that the two enzymes shared the same catalytic machinery and function, but their substrate and inhibitor specificity was strikingly different (11). For the next ten years, several groups identified NQO2 to be the target of different bioactive compounds. These compounds included targeted kinase inhibitors (imatinib and nilotinib (3, 4), CK2 inhibitors (9), PKC inhibitors (12)), antimalarial compounds (primaquine, chloroquine, and quinacrine (13)), natural flavonoid (quercetin (5)), stilbenoid (resveratrol (14)), and hormone (melatonin (15)). While these inhibitors can prevent NQO2 catalysis at micromolar to low nanomolar concentrations, how they disrupt normal cell functions in an NQO2-dependent manner is uncertain. During this period, NQO1 was shown to
stabilize p53 from 20S proteasomal degradation (16). This led to the discovery that NQO2, like NQO1, also modulated p53 protein levels (6, 17). Recently, NQO2 was also shown to indirectly regulate the AKT/cyclin-D1/Rb and the NFκB signalling pathways (18, 19). Together, it would appear that NQO2 has cellular signalling functions in addition to quinone reduction. Similarly, studies of yeast quinone reductase Lot6p also showed that Lot6p has a cell signalling function (20). Lot6p was a poor catalyst of quinones but was able to stabilize transcription factor Yap4 from 20S proteasomal degradation in an NADH dependent manner (20, 21). In this regard, it was postulated that quinone reductase could be categorized in a larger protein family called the flavin redox switch. From these studies, it would appear that NQO2 has physiological roles as a flavin redox switch.

In this thesis, I hypothesize that NQO2 functions as a flavin redox switch that senses intracellular NRH and recognizes potential cytotoxic compounds to modulate p53 levels in cells. To this end, several oxidized and reduced structures of NQO2 in complex with inhibitors were solved. The structural changes in NQO2 upon reduction revealed mechanistic features in NQO2 that resembled flavin redox switches. Extending the investigation of inhibitors to cell culture, it was also found that inhibitors modulate p53 differently compared to NQO1. In this chapter, major concepts pertaining to the role of NQO2 as a flavin switch will be presented. First, NQO2 catalysis will be introduced because it is fundamental to NQO2’s function as an enzyme and as a flavin redox switch. Next, the biological process of p53 stabilization from 20S proteasomal degradation, and how quinone reductase could function as a flavin redox switch will be explained in greater detail. Finally, several NQO2 inhibitors will be introduced to show the diversity of compounds that bind NQO2 and affect p53 modulation.

1.2 Structure and Kinetics of Quinone Reductase 2

Historically, NQO2 was thought to function as a detoxification enzyme because it was a homologue of quinone reductase 1 (NQO1) with 48% sequence identity (11). NQO1 catalyzes an obligate two-electron reduction of quinone to hydroquinone, and prevents the catalysis of single electron reductions of quinone by phase I enzymes such as
cytochrome P450, which generates semi-quinone species. Since semi-quinones can undergo redox-cycling that generates harmful reactive oxygen species, NQO1 functions to detoxify quinone compounds. In this regard, NQO2 was anticipated to perform the same function.

NQO2 is a 26 kDa metallo-flavo-protein that is catalytically active as a dimer. Structurally, each monomer is composed of a Rossmann fold that coordinates one copy of flavin adenine dinucleotide (FAD). NQO2 is catalytically dead when depleted of its flavin co-enzyme, but its activity can be restored using FAD or a high concentration of flavin mononucleotide (FMN) (10). The active site of the enzyme is located at the interface between the two protomers where the isoalloxazine ring of FAD makes up the base of the active site, and residues from the second protomer make up the top (Figure 1.1). Each NQO2 protomer coordinates one FAD co-enzyme, and there are two active sites in the NQO2 dimer. Since most crystal structures of NQO2 are composed of a dimer

![Figure 1.1 Surface Representation of Quinone Reductase 2.](image)

Each protomer of NQO2 (coloured as red or blue) coordinates an FAD co-enzyme and the active site is made up of the isoalloxazine ring from one protomer and residues from the other protomer (at the center of the figure).
in the asymmetric unit, the two protomers are not identical. It is currently not known whether the two subunits act independently or cooperatively, since structural studies show that inhibitors can occupy the two active sites in different orientations. Another feature of NQO2 is the presence of a metal ion located roughly 10 Å away from the active site (22). Between the metal ion and the active site are four residues that make up an electron transport bridge. The identity of the metal ion is copper when isolated from a mammalian source, and zinc when purified from *Escherichia coli* using a recombinant construct. However, the role of the metal ion in the catalysis of quinone is currently unknown since 100 mM EDTA did not decrease its catalytic activity (23). In the following sections, a detailed introduction on each component of NQO2 catalysis (FAD co-enzyme, nicotinamide co-substrate, quinone substrate, and inhibitors) and how they influence the role of NQO2 as a flavin redox switch will be presented.

1.2.1 The Ping-pong Mechanism of NQO2 Catalysis

Flavin redox switches are a class of proteins that can adopt different conformations depending on the redox state of its co-enzyme FAD. For NQO2, the redox state of FAD is dependent on the amounts of substrates and co-substrate available in the environment. In this regard, a comprehensive understanding of NQO2 catalysis is required to appreciate the complexity of NQO2 as a flavin redox switch. Similar to NQO1, NQO2 is able to catalyze the reduction of quinone using a ping-pong mechanism (Figure 1.2)(11). This catalytic mechanism can be described as two half-reactions: an oxidation reaction of NRH, and a reduction reaction of quinone. In the oxidation half-reaction, a nicotinamide co-substrate enters the active site and transfers two electrons to the isoalloxazine rings of FAD, reducing FAD to FADH$_2$. The oxidized nicotinamide would then exit the active site, leaving NQO2 in its reduced state. In the reduction half-reaction, a quinone substrate enters the active site to pick up the two electrons, producing hydroquinone. After the hydroquinone leaves the active site, NQO2 returns to its original oxidized state. In summary, the dihydronicotinamide co-substrate and the quinone substrate alternately occupy the active site, and NQO2 cycles through two redox states for each catalytic turn over.
Figure 1.2 Ping-pong Mechanism of NQO2 Catalysis and Inhibition.

Due to the nature of the ping-pong mechanism where each catalytic turn over requires the entry and exit of two substrates, NQO2 catalysis is not considered to be an enzyme with high catalytic rates. In a review of enzyme catalysis documented in the BRENDA database, it was found that the median catalytic turnover ($K_{cat}$) was roughly $10 \text{ s}^{-1}$ (24). Compared to carbonic anhydrase, one of the fastest enzymes known, with $K_{cat}$ value of over $1 \times 10^5 \text{ s}^{-1}$, the catalysis of menadione reduction using NRH by NQO2 is relatively slow ($K_{cat} = 43 \text{ s}^{-1}$)(5). To further understand the efficiency of NQO2 catalysis, the steady-state kinetics for a ping-pong mechanism described by equation 1.1 will be examined.

$$\frac{V_{obs}}{V_{max}} = \frac{[A][B]}{K_{M(A)}[B] + K_{M(B)}[A] + [A][B]}$$

Equation 1.1 Ping-pong Mechanism.

The rate of quinone reduction is dependent on the two Michaelis constants ($K_m$) for the quinone substrate (A) and the nicotinamide co-substrate (B), the substrate concentrations, and the maximum velocity ($V_{max}$). A detailed examination of this equation indicates that the catalysis of NQO2 in practice could be much slower than theoretical estimates. To illustrate this point, consider the catalytic rate when both substrate concentrations are present at their respective $K_m$ values (ie. substitute all the variables with 1). The $V_{obs}/V_{max}$ will be at 1/3, meaning that the rate of catalysis is only one third of maximum velocity. At five times the concentration of their respective $K_m$ values (ie. Substitute [A], [B] with 5, and $K_{m(A)}$ and $K_{m(B)}$ with 1), $V_{obs}/V_{max}$ will be at 25/35, or 71%. Therefore, the theoretical $k_{cat}$ or $V_{max}$ is only attainable at very high concentrations of both substrate and co-substrate. From these calculations, it appears that NQO2 catalysis is largely dependent
on the availability of both the quinone substrate and dihydronicotinamide co-substrate. Without dihydronicotinamide, NQO2 will likely be in an oxidized state even in the presence of quinone, and vice versa. In this regard, the catalytic efficiency of NQO2 as a detoxification enzyme may be much lower in a cellular context. As a flavin redox switch, the redox state of NQO2 is intimately tied to the presence of substrates and co-substrates in cells.

1.2.2 The FAD Co-enzyme

FAD is crucial to NQO2’s enzymatic and signalling function. As an enzyme, the obligate two-electron transfer to quinone is only possible with the FAD co-enzyme. During a catalytic turnover, a hydride ion (a proton and two electrons) is transferred to the isoalloxazine ring of FAD. At this stage, FAD would be reduced to FADH\(^{-}\) where one proton and one electron reside at the N5 atom of FAD, while the other electron is shared between the N1, O2, and O4 atom in a tautomeric state (Figure 1.3). In NQO1, theoretical calculations showed that the second electron preferentially resides at the O2 atom and could be protonated by a nearby residue (25). Upon reduction, the electron transfer at the N5 atom breaks the planarity of the three ringed members of isoalloxazine. In protein-free FADH\(_2\), the isoalloxazine ring is characterized by a “butterfly bend” of 10 - 28\(^{\circ}\) along the N5-N10 axis (26). In the protein bound form, the butterfly bend varies. For example, the FAD ring bent up to 34\(^{\circ}\) in reduced thioredoxin reductase, while the bending of FAD in NQO2 was roughly 5\(^{\circ}\) (27, 28). Maintaining FADH\(^{-}\) in a planar formation requires relatively little energy, around 4 kcal/mol according to Dixon and coworker’s estimation (26), which could be stabilized by a single hydrogen bond (~4.3 kcal/mol). As an enzyme, the relatively planar geometry of FAD could contribute to its catalytic efficiency and substrate specificity. As a signalling molecule, the additional hydrogen bond could alter the conformation of the protein (29).
1.2.3 The Nicotinamide Co-substrate

The discovery of NQO2 in 1962 by William-Ashman and coworkers showed that NQO2 was unable to use the common co-substrates NAD(P)H and NMNH, but used NRH instead (Figure 1.4) (10). This was followed up in 1997 by Jaiswal and co-workers who showed that while NQO1 efficiently catalyzed the reduction of quinone using NAD(P)H ($k_{cat}/K_m = 440 \text{ min}^{-1} \mu\text{M}^{-1}$), NQO2 used NAD(P)H poorly with a catalytic efficiency roughly 700-fold lower than NQO1 ($k_{cat}/K_m = 0.62 \text{ min}^{-1} \mu\text{M}^{-1}$) (5). Instead, NQO2 used NRH nearly as efficiently as NQO1 used NAD(P)H ($k_{cat}/K_m = 95 \text{ min}^{-1} \mu\text{M}^{-1}$). To further illustrate that NQO1 and NQO2’s co-substrates are exclusive to each other, a study also showed that NQO1 used NRH 500-fold less efficiently than NAD(P)H, as NRH inhibited NQO1 catalysis with a $K_i$ value of 2.39 mM (30). Since then, several other N-alkyl-dihydronicotinamide derivatives have been identified to be co-substrates of NQO2 with an appreciable kinetic efficiency (31). Though virtually all *in vitro* studies of NQO2 utilized these dihydronicotinamide derivatives, it is not known why NQO1 and NQO2 use the dihydronicotinamide co-substrates exclusively.
Figure 1.4 Chemical Structure of Nicotinamide Derivatives and Dihydronicotinamide.

Quinone reductase is part of the flavodoxin superfamily that can catalyze a diverse range of quinones. They are widely found in organisms ranging from bacteria to mammals. Based on sequence similarity, quinone reductases can be organized into three groups: those with C-terminal domains (NQO1-like), those lacking C-terminal domains (NQO2-like), and those lacking C-terminal domains with an internal deletion of 18 residues (flavodoxin-like) (32). In a review of quinone reductase from 14 species of plants, fungi, and bacteria, it was surprising that NQO2 was the only enzyme that preferentially used NRH (33). A structural study of NQO1 in complex with NADP⁺ showed that the C-terminal domain of NQO1 coordinated the negatively-charged phosphate and AMP moiety of NADH during catalysis (34). But neither truncation of the C-terminal domain of NQO1, nor insertion of the C-terminal domain from NQO1 to NQO2, changed their co-substrate specificity (35). This indicated that the C-terminal domain alone was inadequate in conferring substrate specificity towards NADH. Mutating residues in the NQO2 active sites to resemble NQO1 was also unable to alter the co-substrate specificity to use NADH (36). Instead, it increased the overall catalytic activity of NQO2 using NRH and synthetic BNAH. These studies together indicated that NQO2 had evolved to avoid using NADH, yet it is unclear whether NRH is readily available in cells.

Knox and coworkers have a long-standing interest in using the prodrug CB1954 in treating cancer. They have found that reduction of CB1954 by NQO2 can increase the cytotoxicity of CB1954 up to three orders of magnitude (31). V79 cells are Chinese
hamster lung fibroblast cells that have minimal NQO2 expression and activity. In these cells, the toxicity of CB1954 was only increased 1.4 times in the presence of NRH. But when NQO2 was transfected into these cells, the IC50 of CB1954 was increased up to 3000-fold (31). Strikingly, the presence of NQO2 did not change the IC50 of CB1954 in cells without NRH co-treatment. This indicated that NRH and other NQO2 co-substrates were absent in normal proliferating cells, and suggested that NQO2 by itself was not catalytically active. In contrast, NQO1 substrates mitomycin C and EO9 were readily reduced without the addition of NADH (37, 38). Furthermore, how NRH is produced in cell is currently unknown. Given that NQO2 requires a high concentration of co-substrate to efficiently catalyze quinone, the lack of NRH in cells challenges the perspective that NQO2 functions primarily as a catalyst.

1.2.4 The Quinone Substrates

The notion that NQO2 is a detoxification enzyme is based on its similarity to NQO1 in catalyzing a two-electron transfer to quinone. For NQO1, menadione (vitamin K3) and several benzo- and napthoquinones are classic examples of the quinone substrate (39). NQO2 was also able to reduce menadione, but it did so 15-fold less efficiently than NQO1 when its preferred co-substrate was used (5). Furthermore, while napthoquinones are common substrates of NQO1, they were not recognized by NQO2 (40). In a screen of commercially available quinones, only one substrate, coenzyme Q0 was identified for NQO2 (40). Since coenzyme Q0 is not a naturally occurring compound, it is unclear what natural quinone compounds NQO2 recognizes. From these observations, it appears that NQO1 and NQO2 catalyze different quinone substrates.

At high concentrations, menadione exhibits substrate inhibition for both NQO1 and NQO2. This indicates that it could act as both a substrate and an inhibitor of the system depending on its concentration (23, 41). How menadione behaves in an enzymatic assay is well characterized, but whether menadione acts as a substrate or inhibitor in cells is not clear. On one hand, mice deficient of NQO1 were more susceptible to menadione toxicity compared to wild-type mice (42). On the other hand, mice deficient of NQO2 were less sensitive to menadione toxicity when challenged with the same compound compared to
wild-type mice (43). Therefore, NQO1 and NQO2 deletion had opposite effects in mice treated with menadione. In the scenario where NQO1 is actively metabolizing menadione in the presence of NAD(P)H, the deletion of NQO1 could sensitize cells to menadione toxicity. In contrast, if NQO2 is being inhibited by menadione by the lack of NRH in healthy cells, this interaction could elicit cellular signals that increase menadione’s cytotoxicity. As such, the deletion of NQO2 would abrogate this signalling event, rendering menadione less cytotoxic. Drawing from these speculations, it demonstrates that the physiological function of NQO2 is very complex. NQO2 function could be vastly different from NQO1, and the role of NQO2 as a detoxifying enzyme or a signalling protein is intimately linked to the availability of a reducing co-substrate.

In addition to menadione, several synthetic substrates of NQO2 have been identified. They include prodrug CB1954, several quinone methides, acetaminophen and potentially paraquat (Figure 1.5). The prodrug CB1954 is a dinitrobenzamide aziridine that functions as a DNA alkylating agent. Upon a four electron transfer to the nitrogen groups by quinone reductase, CB1954 is able to form DNA crosslinks and is up to 3000-fold more cytotoxic than its parent compound (31). Initially, it was found that rat NQO1 can activate CB1954 and selectively kill Walker 256 (rat breast carcinoma) cells while sparing HEK295 (human embryonic kidney) cells (44). However, human NQO1 was not able to catalyze this reaction, and CB1954 was unable to selectively kill human cancer cells. Instead, human NQO2 reduced the CB1954 1000-fold more efficiently than NQO1 when cells were supplemented with NRH or other NQO2 co-substrates (5). This illustrates that the substrates are unique to NQO1 and NQO2.

Both NQO1 and NQO2 were able to reduce ortho- and para-quinone methides in vitro (45). In these molecules, one of the two carbonyl groups was replaced with a methylene group. Biologically, these compounds were more reactive than quinone and could undergo redox cycling when reduced (46). Finally, paraquat and acetaminophen toxicity were shown to be mediated by NQO2 and their toxicity diminished in the presence of NQO2 inhibitors (47, 48). Paraquat is an herbicide that is lethal when ingested and could be an indirect substrate of NQO2. Acetaminophen is a common medication and was shown to be a weak substrate of NQO2. From these observations, it appears NQO2-
mediated reduction of compounds is responsible for activating toxic compounds rather than detoxifying them.

Figure 1.5 Chemical Structure of NQO2 Substrates.

1.2.5 ROS Generation and Auto-oxidation

It was recently shown that NQO2 can produce reactive oxygen species (ROS) in the presence of substrates and co-substrates (49). When the first structural analysis of NQO2 was published (PDB ID: 1QR2), Foster and co-workers noted a bound metal ion in NQO2. It was connected to the O(2) atom of FAD through the following four residues, forming an electron transfer bridge: Y155, N161, Y132, and H173 (22). The metal ion was not present in NQO1 and it was proposed that it could be important in the reduction of quinone in NQO2. However, mutating the residues that coordinated the metal ion did not significantly change the rate of quinone reduction, and EDTA did not noticeably affect NQO2 activity either (23, 36). Thus, the role of the bound metal ion is currently unknown. When NQO2 was isolated from a mammalian source, it was found to contain copper. Since copper is a reactive metal, it is possible that this metal participates in ROS generation (50). As an enzyme, the generation of ROS indicates that it could mediate oxidative stress in cells. As a flavin redox switch, auto-oxidation represents a mechanism
of switching from a reduced state to an oxidized state that could be important in temporally regulating NQO2’s signalling function.

1.2.6 The Inhibition Mechanism

In 1962, William-Ashman and coworkers showed that a small concentration of anthracene derivatives (10 nM) was able to inhibit the catalytic activity of NQO2 (10). They further showed that much like the dihydronicotinamide co-substrates, inhibitors of NQO1 and NQO2 were exclusive to one another. The active site of NQO2 is a large hydrophobic pocket and numerous molecules with a restricted planar moiety could inhibit NQO2 catalysis. In the past two decades, numerous bioactive compounds were found to inhibit NQO2 using two methods: pull-down of NQO2 from a compound-conjugated matrix, and direct screening. The former identified types of molecules such as kinase inhibitors, antimalarial compounds, natural stilbenoids, and hormones. The latter identified compounds like flavonoids (40) and DNA intercalating agents (51). All of these compounds also disrupt cellular proliferation, but it is currently unknown whether this results from NQO2 inhibition.

The interactions between a compound and an enzyme can be described by enzyme inhibition or by direct binding experiments. For NQO2, which catalyzes using a ping-pong mechanism, inhibitors can either compete against dihydronicotinamide, quinone, or both substrates (Figure 1.2). Described in terms of direct binding, inhibitors can directly bind to oxidized, reduced, or both redox states of NQO2. To investigate the mode of inhibition, Kwiek and co-workers presented a comprehensive analysis of three NQO2 inhibitors: the antimalarial drugs primaquine (PQ), chloroquine (CQ), and quinacrine (QC) (23). In their study, PQ competed with the dihydronicotinamide co-substrate for the oxidized FAD site. In contrast, CQ and QC competed with the quinone substrate for the reduced FAD site. A direct binding assay further confirmed that PQ bound oxidized NQO2 while CQ did not. These observations showed that not only could these compounds inhibit catalysis, but they also have a binding preference towards a specific redox-state of NQO2. By directly binding to either redox-states, NQO2 inhibitors could stabilize the protein in an oxidized or reduced state. For a flavin redox switch, these
interactions could propagate distinct signals in a cellular environment. From this perspective, NQO2 inhibitors could induce signalling events even when concentrations of a substrate or co-substrate are low in a cell.

### 1.2.7 Role of NQO2 Catalysis

In this section, each component of NQO2 catalysis was expounded. The substrates, co-substrates, and inhibitors of NQO1 and NQO2 are almost exclusive to one another, and the inferred detoxification role of NQO2 was challenged. To catalyze quinones efficiently, NQO2 would require a high concentration of dihydronicotinamide co-substrate. However, it appears that little, if any, endogenous levels of NQO2 dihydronicotinamide co-substrate are present in cells. Furthermore, inhibitors of NQO2 also have the potential to stabilize NQO2 in a redox-specific state. From these observations, the ability of NQO2 to reduce quinone is questioned. In the following section, how NQO2 could function as a flavin redox switch will be explored.

### 1.3 NQO2 as a Flavin Redox Switch

The wild-type p53 gene is a tumor suppressor that protects a cell from genomic instability by inducing cell cycle arrest or apoptosis in response to genomic stress. In a study of p53-mediated apoptosis, p53 activated redox-related genes to generate ROS that damaged the mitochondria (52). Among the p53-induced genes identified was one that shared sequence homology with mammalian NADPH-quinone oxidoreductase and ζ crystallin, and was named p53-inducible gene 3 (PIG3). Based on this finding, Asher and coworkers speculated that oxidoreductase could regulate p53 in a feedback loop, and experimentally showed that p53 degradation was modulated by NQO1 (16). However, PIG3 and NQO1 were very different proteins in terms of sequence, structure, and function. Instead of detoxifying quinone to prevent ROS generation, PIG3 produced ROS (53, 54). Regardless, since the discovery that NQO1 can modulate p53 degradation, NQO2 was also shown to modulate p53 (6, 23). In this section, an introduction of the flavin redox switch will be presented and the notion that quinone reductase can function as a flavin redox switch will be illustrated. Next, the specific mechanism of how NQO1 and NQO2 stabilize p53 from 20S proteasomal degradation will be presented.
1.3.1 Flavin Redox Switch

Flavin redox switches are a class of proteins that senses specific physiological conditions and propagate a signal for the cell to respond to (29). In the presence of a specific substrate, electron donor, or light, the reduction of its flavin co-enzyme FAD or FMN activates the flavin redox switch. The reduced isoalloxazine ring of flavin then alters the hydrogen bond network within the protein, leading to a change in protein conformation. In turn, the activated reduced state is responsible for regulating transcription factors, controlling membrane binding, and modulating post-translational modifications. The flavin redox switch can be turned off by the oxidation of flavin in most cases, but it is irreversible in some. An example of a flavin redox switch is phototropin, which regulates photosynthetic proteins in plants. They are light sensing proteins that contain two light-oxygen-voltage (LOV) domains and a kinase domain. The FMN co-enzyme in the LOV domain is reduced by light and makes a covalent bond with a nearby cysteine residue. This causes a conformational change in the LOV domain, resulting in the activation of the kinase domain. The phosphorylation of phototropin substrates then induces the production of photosynthetic proteins in cells.

Most flavin redox switches are characterized by having a PER-ARNT-SIM\(^2\) (PAS) domain; this encompasses the LOV domain (55). In contrast, quinone reductase is composed of a flavodoxin-like domain that is structurally and sequentially distinct from the PAS domain (33). As such, the function of quinone reductase as a flavin redox switch cannot be directly inferred. In yeast, Lot6p is the sole orthologue of human NQO1 and NQO2, and it was found to physically associate with the 20S proteasome in a redox-dependent manner (20). Specifically, Lot6p modulated the degradation of Yap4, a yeast transcription factor involved in oxidative stress response (20, 56). Further investigation showed that oxidative stress induced apoptosis-like cell death in a Lot6p-dependent manner (57). As an enzyme, Lot6p was shown to be a poor catalyst because it reduced quinone slower than non-enzymatic reactions (21). From this perspective, it was

\(^2\) PER – Period protein; ARNT – aryl hydrocarbon receptor nuclear translocator protein; and SIM – single-minded protein.
speculated that Lot6p functioned primarily as a regulator of 20S proteasome rather than as a catalyst. Furthermore, inhibitors of NQO1 and NQO2 (curcumin and resveratrol respectively) inhibited Lot6p catalysis and also stabilized the protein from thermal denaturation (58). This indicated a possible role for inhibitors to affect how Lot6p regulates the 20S proteasomal degradation of Yap4. While there is no p53 homologue in yeast, these studies demonstrated that the quinone reductase family could have a functional role in modulating protein degradation as a flavin redox switch in addition to quinone catalysis.

1.3.2 The p53 Tumor Suppressor

The tumor suppressor p53 is the “master guardian and executioner” of the genome (59). It is a transcription factor that induces cell cycle arrest or apoptosis in response to a diverse range of genotoxic and oncogenic stress. Structurally, p53 is composed of an N-terminal unstructured region, a stable DNA binding domain region, and a trans-activation C-terminal region. A number of post-translational modifications in p53 drives the translocation of p53 into the nucleus where it forms a homo-tetramer to induce various pro-apoptotic genes (60). In the COSMIC database that curates all available cancer genomes, the p53 gene was found to be mutated in roughly 30% of all cancer types (http://cancer.sanger.ac.uk/, accessed July 11, 2015)(61). Since cells with defective p53 are impeded from activating apoptosis in the presence of genomic stress, cancers lacking p53 function could tolerate a higher level of genomic instability and become more aggressive. Recently, it was shown that restoration of wildtype-p53 led to cancer remission in a mouse model (62). Therefore, being able to manipulate NQO2 as a flavin redox switch using NQO2 specific inhibitors could be an avenue of inducing wildtype-p53 in cells.

1.3.3 Mechanism of p53 Degradation by 20S proteasome

The degradation of p53 has been an area of extensive research because it is rapidly degraded and only has a half-life of 20 minutes (63). In the conventional pathway of p53 degradation, the protein is first poly-ubiquitinatated by hdm2 E3 ligase. It is then recognized by the 26S proteasome and degraded in an ATP-dependent manner (Figure
1.6) NQO1 was initially identified to protect p53 from proteasomal degradation because it was thought that oxidoreductases could regulate p53 (16). It was shown that dicoumarol, an inhibitor of NQO1, enhanced the degradation p53. Furthermore, the inhibitor-induced degradation was prevented by the overexpression of NQO1, the treatment with proteasome inhibitor MG132, and the presence of SV40 large T antigen. The degradation of p53 was independent on the hdmi2 regulation pathway, and wild-type NQO1 was necessary to mediate the effects of dicoumarol (64). Surprisingly, mutant p53 with the most frequently mutated arginine residues (R174H, R273H, and R248H) were resistant to NQO1 inhibitor-induced degradation (65). Mechanistically, p53 was degraded by the 20S core proteasome particle (proteasome 26S without the 19S regulatory subunit) alone, without ubiquitination or the addition of ATP (66). This alternate degradation pathway of p53 was regulated by NQO1 in a NADH dependent manner, and was thought to be the default pathway of p53 degradation in cells (67, 68). Since the 20S core proteasome could degrade intrinsically disordered proteins, it was speculated that the proteasome recognized the unstructured regions of p53 (68, 69). In addition to p53, NQO1 also prevented 20S proteasomal degradation of p73 and ornithine decarboxylase.

![Diagram of p53 Degradation Pathway by 26S and 20S Proteasome.](image)

Figure 1.6 p53 Degradation Pathway by 26S and 20S Proteasome.
which indicated that NQO1 could modulate other proteins with intrinsically disordered regions (66, 70). These studies together showed that NQO1 can stabilize the basal levels of proteins including p53 by regulating the 20S proteasome, and NQO1 inhibitors can prevent this to some degree (71).

Similar to NQO1, NQO2 was also shown to stabilize p53 in the presence of NRH. Mice deficient of NQO2 were highly sensitive to tumorigenesis in a chemically induced skin cancer model and had a lowered basal level of p53 (72). Moreover, mice deficient of both NQO1 and NQO2 were more susceptible to tumorigenesis, and its p53 regulation was drastically impaired compared to the single knockout mice (73). At the molecular level, the degradation of p53 by 20S proteasome was verified using an in vitro degradation assay of $[^{35}\text{S}]$-methionine-labeled p53, and NQO2 was able to stabilize p53 in an NRH dependent manner (6). From these studies, it is clear that NQO1 and NQO2 can modulate basal expression of p53 by controlling its 20S proteasomal degradation. To date, the only known physiological condition known to activate p53 in an NQO1/NQO2 dependent manner is the inhibition of pyrimidine nucleotide synthesis (17). However, the mechanism behind how NQO1 or NQO2 modulates this process is currently unknown.

The function and regulation of p53 rely on numerous signals and cellular conditions in addition to 20S proteasomal degradation. In light of the complex modulation of p53, how NQO2 behaves as a flavin redox switch and how NQO2 inhibitors affect this switch remains poorly understood. In the following section, several inhibitors of NQO2 will be introduced to illustrate the potential link between NQO2 inhibition and cell signalling.

1.4 Inhibitors of NQO2

1.4.1 Discovery of NQO2 Inhibitors

NQO2 inhibitors normally characterized to prevent catalysis of quinone reduction could stabilize NQO2 in a specific redox-state to modulate p53 stabilization in cells. Since the discovery of NQO2, a number of proteomic studies have identified NQO2 to be the target of biologically active compounds such as antimalarial drugs, kinase inhibitors, resveratrol, and melatonin. These unrelated compounds bound to NQO2 with a sub-
micromolar affinity and they represent the majority NQO2 inhibitors studied in depth to date. While these compounds perturb biological processes, most of these inhibitors also bind to other targets. Accordingly, how NQO2 mediates their biological effects remains ambiguous. Another approach to discovering NQO2 inhibitors was by screening known or newly synthesized compounds that inhibited NQO2 catalysis. Motivated by making a more selective NQO2 inhibitor, NQO2 inhibitors with nanomolar affinity have been developed. In this section, a brief overview of the inhibitors used in this thesis will be presented, with an emphasis on how they were discovered to bind NQO2. The following well-characterized compounds will also be introduced because they affect cellular p53 levels in some way: 9-aminoacridine, imatinib and nilotinib, melatonin, and resveratrol. The list of all the compounds discussed here is presented in figure 1.7.

1.4.2 Primaquine and Chloroquine

Primaquine (PQ) and chloroquine (CQ) are antimalarial drugs routinely used to treat parasitic infections of Plasmodium sporozoites transferred by mosquitoes. The two inhibitors target different stages of malarial infection: PQ clears infections from the liver while CQ clears infections from blood (74, 75). To investigate how these drugs function, Graves and co-workers employed a proteomics strategy to search for a protein target of PQ and CQ (13). Cell lysate from malaria-infected blood cells was immobilized on an ATP-sepharose column and proteins that eluted in the presence of PQ and CQ were identified. Surprisingly, no parasitic protein was found. Instead, NQO2 was identified and was potently inhibited by the concentration of drugs used in clinical treatment. This discovery demonstrated the possibility that inhibition of NQO2 could contribute to antimalarial effects.

The interaction between the two drugs and NQO2 also indicated that NQO2 could be involved in other diseases for which PQ and CQ are used as therapeutics. Specifically, PQ is used to treat pneumocystis pneumonia, and is active against leishmaniasis and trypanosomiasis in vivo (76–78). CQ has been used to treat rheumatoid arthritis, systemic lupus erythematosus, amoebic hepatitis, and is under clinical trials for use in HIV-1/AIDS (79, 80). Furthermore, CQ is cytotoxic to several human cancer cell lines and is
Figure 1.7 Chemical Structures of NQO2 Inhibitors.
being investigated for use as a cancer therapeutic agent (81–83). Mechanistically, CQ was shown to stabilize wild-type p53 from degradation and induced apoptosis in glioblastoma cells (84). However, it is unclear how NQO2 mediate the pharmacological effects of these two inhibitors. To understand how the two inhibitors affect NQO2 as a flavin redox switch, a structural analysis of NQO2 and the two inhibitors will be presented in chapter 3.

1.4.3 CK2 Inhibitors TBB, TBBz, and DMAT

Protein kinase CK2 (CK2) is a serine/threonine protein kinase, involved in cell cycle control, cell proliferation signalling, tumorigenesis, and other cellular processes (8, 85, 86). Since it is overexpressed in many tumor types, it is a potential cancer therapeutic target and numerous CK2 inhibitors have been developed (87). In particular, 4,5,6,7-tetrabromobenzotriazole (TBB), 4,5,6,7-tetrabromobenzimidazole (TBBz), and 2-dimethylamino-4,5,6,7-tetramethyl-1H-benzimidazole (DMAT), are three widely-used CK2 inhibitors that can induce apoptosis in cells (88, 89). Despite being optimized for CK2 specific inhibition, both TBBz and DMAT inhibit at least three other subfamilies of kinases unrelated to CK2 (DYRKs, PIM, and HIPK (90)). Therefore, the exact pathways through which CK2 inhibitors induce apoptosis in cells remain debatable. Using a similar approach to find targets for CQ and PQ, NQO2 was identified as a target of TBBz and DMAT (9). Specifically, NQO2 was eluted from an ATP-Sepharose column in the presence of TBBz and DMAT, but not TBB. This correlated with the observation that in cells TBBz and DMAT, but not TBB, induced apoptosis response even when CK2 activity was restored. Together, this shows that inhibition of NQO2 may contribute to apoptosis.

In prostate cancer cells, TBB induced apoptosis in cell lines that had wildtype p53 but not in cell lines with a mutant or no p53 expression (91). TBB and DMAT also induced p53 expression and apoptosis in biliary tract cancer cells (92). However, the direct involvement of NQO2 in inhibitor-induced apoptosis has not been demonstrated. In Chapter 4, the inhibition of NQO2 by TBB, TBBz, and DMAT will be characterized, and cellular studies with these compounds will be presented in Chapter 6.
1.4.4 DNA Intercalating Agents

In a virtual screen for NQO2 inhibitors, a number of planar molecules were identified (93, 94). Among these were 9-aminoacridine (9AA) and imidazoacridine-6-one C1311 (C1311), which were also known to intercalate DNA. While DNA intercalating agents can cause DNA damage leading to p53 activation, 9AA induced p53 expression independent of genotoxic stress (82). C1311 was also shown to be cytotoxic to a number of solid tumors and leukemia, but p53 status was not a determinant of the inhibitor’s efficacy (95). When C1311 was modified so that the DNA intercalating properties were diminished without changing its ability to inhibit NQO2, it became less cytotoxic (96). From these findings, even though NQO2 inhibition was not responsible for the cytotoxicity of the compounds, p53 could still be modulated in an NQO2 dependent manner. In chapter 5, an investigation into whether other DNA intercalating agents can also inhibit NQO2 or not will be presented.

1.4.5 Imatinib and Nilotinib

Imatinib and nilotinib are inhibitors of BCR-Abl. In normal cells, wild-type Abl is under strict regulation and remains inactive. In CML, a chromosomal translocation of the Abl gene to the BCR gene makes the fusion gene BCR-Abl. This renders the gene product BCR-Abl kinase constitutively active. By binding to the ATP site of BCR-Abl, imatinib and nilotinib inhibited the unregulated activity of BCR-Abl and reversed the oncogenicity of CML (1). Using an inhibitor-conjugated affinity matrix, two groups independently showed that NQO2 was a prominent unintended non-kinase target of imatinib and nilotinib (3, 4). This interaction was further characterized by X-ray crystallography, but how NQO2 inhibition contributes to the efficacy of these inhibitors is currently unknown (97). Using a mouse CML model, leukemic cells deficient of p53 were less sensitive to the cytotoxic effects of imatinib, even though BCR-Abl was inhibited (7). This showed that p53 was partially responsible for the antiproliferative effects of imatinib, and points to the possibility that NQO2 was involved.
1.4.6  Melatonin

Melatonin is a hormone pivotal to the regulation of circadian rhythm, which was shown to have anticancer effects (98). Using radioactive-labeled melatonin, two high affinity targets (MT1 and MT2) and a low-affinity target (MT3) were discovered in hamsters (99). MT1 and MT2 were identified as membrane bound G-protein coupled receptors, and MT3 was identified as NQO2 (15). At one nanomolar concentration (1 nM), melatonin induced p53 expression (100). However, the induction of p53 was likely caused by MT1 and MT2 interactions because NQO2 inhibition would require a greater concentration of melatonin ($K_i = 130 \mu M$; (101)). Based on the melatonin moiety, a number of high affinity NQO2 inhibitors were developed (101). The compound with the strongest affinity was S29434\(^3\) with an IC\(_{50}\) of 14 nM, and it was recently used to study the NQO2-mediated toxicity of paraquat and the generation of ROS by NQO2 (47, 49). As high affinity inhibitors of NQO2 such as S29434 continue to be used, it would offer insight into how targeted NQO2 inhibition affects cell signalling pathways.

1.4.7  Resveratrol

Resveratrol is an antioxidant commonly found in grape skins and red wine. This compound has been studied intensively due to its potential role in cardioprotection and cancer prevention. Using a resveratrol-immobilized affinity column, NQO2 was identified to be a prominent target of resveratrol (14). To date, twenty other targets have been identified, but resveratrol binds to NQO2 with the highest affinity ($K_a = 35nM$) (102). Several studies have shown that resveratrol induced p53 expression, but the concentrations used in these experiments would also bind to targets other than NQO2 (103, 104). As such, it is currently unclear how the inhibition of NQO2 by resveratrol contributes to p53 regulation.

\(^3\) S29434 was initially named as NMDPEF: N-[2-[(2-methoxy-6H-dipyrido[2,3-a:3,2-e]pyrrolizin-11-y1)ethyl]-2-furamide]
1.4.8 Effects of NQO2 Inhibition

In this section, several inhibitors of NQO2 that are cytotoxic to cells were highlighted. Some of these compounds affected apoptosis and p53 induction independent of NQO2 catalysis. Many of the inhibitors also bound to a wide range of proteins while others intercalated DNA. In addition, inhibitors of NQO2 can suppress NF-κB and affect AKT/Rb/Cyclin D1 signalling, which can modulate p53 (18, 19, 105). In light of the complex regulation of p53, it is difficult to specify how NQO2 inhibitors affect p53 protein levels through direct binding to NQO2. Since inhibitors have the potential to alter NQO2’s function as a flavin redox switch by stabilizing NQO2 in either oxidized or reduced states, these inhibitors could directly modulate p53 levels though NQO2 interactions.

1.5 Scope of Thesis Project

NQO2 is an enzyme inhibited by a myriad of compounds and has cellular signalling functions. My hypothesis is that NQO2 functions as a flavin redox switch that senses intracellular NRH and recognizes many potential cytotoxic compounds to modulate p53 levels in cells. In this thesis, the structural understanding of how NQO2 functions as a flavin redox switch was advanced and how inhibitors interact with NQO2 were investigated. The studies of inhibitors were also extended into cellular systems to show that some inhibitors do modulate p53, but they do so in an unexpected manner.

In preparing recombinant NQO2 expressed in Escherichia coli, it was found that NQO2 lacked the co-enzyme FAD, which prevented accurate analysis of kinetic and structure studies. This led to the development of a method to fully reconstitute NQO2 with FAD (Chapter 2). Overcoming this hurdle, the oxidized NQO2 structure with the inhibitors PQ and CQ were solved. Next, the first crystal structure of reduced NQO2-inhibitor complex was obtained by reducing NQO2-CQ crystals in an anoxic environment. A global conformational change of NQO2 was seen in the reduced state, which showed that NQO2 shared common features with other flavin redox switches (chapter 3).
The identification of NQO2 as a target of CK2 inhibitors prompted a kinetics and structural investigation with these inhibitors. Using a modified inhibited-kinetic model, the degree to which TBB, TBBz, and DMAT competed with the dihydronicotinamide co-substrate or the quinone substrate was determined. Specifically, TBBz was found to bind oxidized NQO2 exclusively and DMAT bound both oxidized and reduced NQO2 with similar affinities, while TBB bound to NQO2 with a low affinity. Compared to the ATP binding site of CK2, the electrostatic potential of the NQO2 active site was different in charge. The difference in how the compounds bound NQO2 and CK2 indicated that the binding of NQO2 by kinase inhibitors was fortuitous rather than by design (Chapter 4).

Next, three DNA intercalating agents were identified as novel inhibitors of NQO2. EtBr, AO, and doxorubicin were found to inhibit NQO2 with nanomolar affinities. Specifically, EtBr and AO were identified to be the highest affinity reduced-state inhibitors of NQO2. Structural analysis of these compounds with reduced NQO2 confirmed that hydrogen bond network in the NQO2 active site was different compared to its oxidized active site. It was further shown that a non-toxic concentration of AO could inhibit NQO2 catalysis in cells, thus AO is a suitable mechanistic probe of NQO2 function in cells (Chapter 5).

To further understand how inhibitors affect the signalling role of NQO2, NQO2 inhibitors were studied in tissue cultures. Endogenous NQO2 was knocked out in human HCT116 cancer cells using CRISPR/Cas9 technology to compare the role of NQO2 in cells treated with substrate, co-substrate, and inhibitors. It was found that while NQO2 status did not affect the cytotoxicity of NQO2 inhibitors, the presence of NQO2 affected how inhibitors modulated p53 levels (Chapter 6).

Using both structural and cellular techniques, the work presented in this thesis has advanced the molecular understanding of how NQO2 behaves as a flavin redox switch. Reduced NQO2 in complex with four different inhibitors were characterized in detail and together illustrated that NQO2 could be differentially modulated by state-specific inhibitors. In this regard, several anoxic techniques were also developed in crystallography and ITC to study reduced NQO2. Cellular investigations revealed that NQO2 modulation of signalling events was a very complex process. Altogether, this
thesis supports the role of NQO2 as a flavin redox switch and shows that inhibitors could modulate NQO2 in ways in addition to inactivating NQO2 catalysis.
1.6 References


Chapter 2

2 Flavin Adenine Dinucleotide Content of Quinone Reductase 2: Analysis and Optimization for Structure-Function Studies

Quinone reductase 2 (NQO2) is a broadly expressed enzyme implicated in responses to a number of compounds, including protein kinase inhibitors, resveratrol, and antimalarial drugs. NQO2 includes a flavin adenine dinucleotide (FAD) co-enzyme, but X-ray crystallographic analysis of human NQO2 expressed in *Escherichia coli* showed that electron density for the isoalloxazine ring of FAD was weak and there was no electron density for the adenine mononucleotide moiety. Reversed-phase high-performance liquid chromatography (HPLC) of the NQO2 preparation indicated that FAD was not present and only 38% of the protomers contained flavin mononucleotide (FMN), explaining the weak electron density for FAD in the crystallographic analysis. A method for purifying NQO2 and reconstituting with FAD such that the final content approaches 100% occupancy with FAD is presented here. The enzyme prepared in this manner has a high specific activity, and there is strong electron density for the FAD co-enzyme in the crystal structure. Analysis of NQO2 crystal structures present in the Protein Data Bank indicates that many may have sub-stoichiometric co-enzyme content and/or contain FMN rather than FAD. This method of purification and reconstitution will help to optimize structural and functional studies of NQO2 and possibly other flavoproteins.

2.1 Introduction

Quinone reductase 2 (NQO2) is an unusual enzyme with an interesting history. During the early 1960s, Williams-Ashman and coworkers observed that rat tissue extracts, especially from kidney, liver, and heart, have the ability to couple the oxidation of dihydronicotinamide riboside (NRH) to the reduction of menadione. This novel enzyme

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activity could be distinguished from NAD(P)H-linked reducing activities because the NRH-dependent activity was inhibited by estradiol but not by dicoumarol (1). The enzyme in question was purified from bovine kidney and shown to include tightly bound flavin adenine dinucleotide (FAD) as a co-enzyme. At the time, this was the only enzyme known to couple oxidation of NRH to reduction of compounds such as menadione, and its physiological function was a mystery. Because a degradative pathway for NRH was not known, Williams-Ashman and coworkers speculated that the enzyme may function to oxidize cellular NRH so that the resulting nicotinamide riboside (NR) could be degraded.

These early observations did not receive further attention until 1990 when a human cDNA (complementary DNA) corresponding to the enzyme was discovered (2). The sequence had very strong similarity (49% identity) to quinone reductase 1 (NQO1), and on this basis the protein product was defined as “quinone reductase 2” (NQO2). The connection between NQO2 and the enzyme purified by the Williams-Ashman group was finally made in 1997 when Talalay and coworkers published a detailed enzymatic characterization of NQO2 and showed that it had properties similar to those described by Williams-Ashman and coworkers (3).

NQO2 remains a fascinating and enigmatic enzyme. The substrates and physiological function of NQO2 are not well defined. NQO2 is the only enzyme listed in the BRENDA database (http://www.brenda-enzymes.org) that preferentially uses NRH as a reductant, but the biological “rationale” for this preference is not obvious and it is not known what nicotinamide derivative is the dominant co-substrate under physiological conditions. Its “sister” enzyme, NQO1, uses NADH or NADPH as a co-substrate and is thought to provide a cytoprotective function, acting as a detoxification protein that catalyzes an obligatory two-electron transfer to quinone substrates, thereby preventing a single electron reduction and the generation of reactive radical species (4). Despite the structural similarities between the enzymes, an analogous cytoprotective function for NQO2 is not consistently supported by the available data; however, NQO2 is receiving increasing attention because of its interaction with, and/or modification of, a number of drugs and biologically active molecules, including kinase inhibitors, resveratrol, and antimalarial drugs (5).
NQO2 catalysis proceeds via a ping-pong kinetic mechanism, where the bound FAD co-enzyme is first reduced by NRH or a related dihydronicotinamide derivative and then the substrate replaces the nicotinamide at the active site. NQO2 is inhibited by compounds with planar aromatic ring systems, and in all cases examined so far the inhibitors bind adjacent to the isoalloxazine ring of FAD and are coordinated by tryptophan residues in the catalytic pocket. Our interest in NQO2 arose because it was identified as a target for two inhibitors of the protein kinase CK2 (6). To understand the specificity determinants for this interaction, we co-crystallized a recombinant form of NQO2 with the inhibitors; although the crystals diffracted to high resolution and yielded excellent electron density maps for protein atoms, the electron density for the FAD co-enzyme was weak and there was no electron density for the inhibitors. We traced the origin of this problem to a sub-stoichiometric amount of FAD in the enzyme preparation, resulting in a low occupancy for bound FAD in the crystals. The relatively low occupancy of FAD in the crystals means that there is a smaller contribution from the co-enzyme to the diffraction intensities, which is accounted for in the crystallographic model by elevated atomic displacement parameters (ADPs) for the FAD. An analysis of the ADPs for the FAD co-enzyme in available NQO2 structures indicates that this may be a fairly common occurrence for recombinant NQO2 expressed in *Escherichia coli*. To obtain stronger electron density for FAD and bound substrate or inhibitor, a simple step was added to the purification procedure that yields enzyme with a stoichiometric amount of FAD. Crystals of this NQO2 preparation yield greatly improved electron density maps for the entire FAD co-enzyme, and this modification will help us to obtain detailed information on inhibitor binding.

### 2.2 Materials and Methods

#### 2.2.1 Reagents

FAD, flavin mononucleotide (FMN), phenylmethanesulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were purchased from Sigma–Aldrich. Tris base, (NH₄)₂SO₄, Hepes, NaH₂PO₄, and NaCl were purchased from EMD Chemicals. Lysozyme, ampicillin, dithiothreitol (DTT), and guanidine–HCl were purchased from Bioshop Canada. Routine protein determinations were made using the Lowry assay with
a BSA standard. Enzymatic substrates 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium (MTT), 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCDP), and menadione were purchased from Sigma–Aldrich.

2.2.2 Cloning and Protein Expression

Full-length human NQO2 was cloned into the vector pPro-Ex HTa (Invitrogen) to a
His6–NQO2 fusion construct with a tobacco etch virus (TEV) protease cleavage site
between the hexahistidine tag and NQO2. The construct was verified by sequencing and
transformed into an E. coli BL21(DE3) background for protein expression. Cultures were
grown at 37°C in 2×YT medium with ampicillin (3 mM) to an OD$_{600}$ of 0.6 to 0.9, and
induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 4.2 mM), cells
were grown for an additional 4 h. Cells were harvested by centrifugation and resuspended
in 50 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole (pH 7.0) (buffer A),
treated with lysozyme (0.1 mg/ml) and PMSF (1 mM), and lysed using a French press at
10 kPa.

2.2.3 Protein Purification

All steps were carried out at 4 °C. The cell lysate was applied to a Chelating Sepharose
Fast Flow column (5 ml, GE Healthcare) with bound Ni$^{2+}$ and equilibrated with buffer A.
The column was washed exhaustively with buffer A, and protein was eluted by raising
the imidazole concentration to 250 mM. NQO2-containing fractions were treated with
TEV protease (7) at a 1:100 protease/ protein ratio, including DTT (5 mM), at 4 °C
overnight. Three further additions of TEV protease and DTT were made at 6-h intervals;
complete cleavage of the affinity tag was confirmed by sodium dodecyl sulfate–
polyacrylamide gel electrophoresis (SDS–PAGE), and untagged NQO2 was dialyzed into
50 mM Tris–HCl (pH 8.0) in preparation for anion-exchange chromatography. The
partially purified NQO2 was applied to a 1.6 × 15-cm column of Q-Sepharose HP (GE
Healthcare) equilibrated with 50 mM Tris–HCl and eluted with a linear gradient of NaCl
from 0 to 500 mM over 200 ml. Fractions containing NQO2 were pooled, concentrated to
5 ml, and applied to a 2.6 × 65-cm column of Superdex 200 (GE Healthcare) equilibrated
with 50 mM Tris–HCl and 150 mM NaCl (pH 8.0) and running at 2 ml/min. Fractions
containing NQO2 were pooled and concentrated to 15 to 35 mg/ml by ultrafiltration. Purified NQO2 was aliquoted, flash-frozen, and stored at -80 °C for analysis and crystallization.

2.2.4 Optimization of FAD Content in NQO2

To remove bound FMN and achieve stoichiometric amounts of bound FAD, the purification method outlined above was used with the following changes. Pooled fractions from the anion exchange column were concentrated to 5ml (protein concentration ~10 mg/ml or 390 µM) and supplemented with guanidine–HCl and FAD to final concentrations of 3 M and 10 mM, respectively. The solution was incubated on ice for 5 min and applied to the gel filtration column equilibrated with 50mM Tris–HCl, 150 mM NaCl, and 10 µM FAD (pH 8.0).

2.2.5 Crystallization, X-ray Diffraction, and Refinement

NQO2 was crystallized by hanging drop vapor diffusion against reservoirs containing 0.1 M Hepes and 1.3 to 2.0 M (NH₄)₂SO₄ (pH 7.0). Data were collected at beamline CMCF-1 of the Canadian Light Source or a laboratory source. Data were processed using MOSFLM (8) and Scala (9); structure solution and refinement was carried out using PHENIX (10) with 1QR2 (11) as the starting model.

2.2.6 Determination of Flavin Nucleotide Content in NQO2 Preparations

The amounts of NQO2 in the final preparations (Table 1) were determined by amino acid analysis (Advanced Protein Technology Centre, Hospital for Sick Children, Toronto, Canada, http://www.sickkids.ca/Research/APTC/Amino-Acid-Analysis/index.html). The FAD and FMN contents of NQO2 were quantified by reversed-phase HPLC based on a published method (12). To release bound nucleotide, samples of NQO2 were heated to 100 °C for 10 min and passed through a syringe filter (0.45 µm nominal pore diameter) to remove denatured protein. To resolve FAD and FMN, a Symmetry C300 C18 column (Waters) was developed isocratically with 10% (by volume) methanol and 10 mM ammonium acetate (pH 5.0). The eluent was monitored at 450 nm, and integrated peak
areas were calibrated to the amount of flavin nucleotide using a series of 50-µl injections of mixtures containing 86 to 860 pmol of FAD and 156 to 1560 pmol of FMN. NQO2 samples were diluted to a concentration of less than 40µM so that less than 2000 pmol FAD/FMN would be sampled in each 50-µl injection.

2.2.7 Steady-state Kinetic Assay

Enzyme activity was measured using a coupled assay, where menadione reduced by NQO2 subsequently reduces MTT. The assay buffer contained 240 µM (0.1 mg/ml) MTT, 500 µM SCDP as co-substrate (13), and 50 µM menadione as substrate. The assay was carried out in a 1-ml volume under stirring at 30 °C. The reaction was initiated by injection of 2 to 3 ng (1 µl of 2–3 mg/ml) of NQO2-containing solution. The change in absorbance was monitored at 610 nm using a Cary 100 spectrophotometer with associated kinetics software (Varian Instruments). The change in absorbance was converted to moles of menadione reduced per minute using an extinction coefficient for MTT of 11.3 × 10^3 M⁻¹ cm⁻¹.

2.2.8 B-factor Analysis

Average atomic displacement factors (ADPs), or B-factors, were calculated using the ‘‘What If’’ web interface (http://swift.cmbi.ru.nl/servers/html/index.html) and Moleman (http://xray.bmc.uu.se/usf/). The average ADPs and normalized ADPs (nADPs) for FAD, and the separate FMN and adenosine monophosphate (AMP) moieties, were calculated in Excel. Hydrogen atoms were not included in these calculations.
2.3 Results

2.3.1 Expression, Purification, and Crystal Structure Analysis of NQO2

NQO2 was expressed as a hexahistidine-tagged construct with a TEV protease cleavable linker. After affinity purification and removal of the affinity tag, NQO2 was purified by ion exchange and gel filtration chromatography. Analysis of the final preparations by SDS–PAGE indicated that the affinity tag had been removed and the protein was greater than 90% pure (Figure 2.1). This method of purification yielded NQO2 that readily formed crystals diffracting to high resolution. For preparation 1, the refined structure yielded excellent agreement with crystallographic data and good stereochemical parameters (Table 2.1). However, the average ADP for the FAD co-enzyme was 40.4 Å², nearly double the average ADP for protein atoms (22.3 Å²) and higher than the average solvent ADP (30.5 Å²). Closer analysis showed that the FAD could be divided into FMN and AMP on the basis of average ADP values of 28.2 and 57.2 Å², respectively (Table 2.1). Consistent with these high ADPs, electron density for the isoalloxazine ring and associated ribityl was relatively weak and discontinuous and was almost absent for the AMP moiety (Figure 2.2A).

![Figure 2.1 SDS-PAGE Analysis of Purified NQO2.](image)
The final preparation 1 (left lane) and preparation 2 (right lane) were analyzed, and the positions of molecular weight markers (kDa) are shown.
<table>
<thead>
<tr>
<th></th>
<th>NQO2 Preparation 1</th>
<th>NQO2 Preparation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.9791</td>
<td>1.5418</td>
</tr>
<tr>
<td>Space Group</td>
<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Unit Cell Dimensions (Å)</td>
<td>55.95, 83.15, 106.57</td>
<td>56.45, 82.99, 106.49</td>
</tr>
<tr>
<td>Resolution</td>
<td>35.5 - 1.84 (1.94 - 1.84)ᵃ</td>
<td>15.0 - 1.96 (2.07 - 1.96)ᵃ</td>
</tr>
<tr>
<td>R_sym</td>
<td>0.050 (0.168)ᵃ</td>
<td>0.066 (0.273)ᵃ</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>24.8 (9.8)</td>
<td>14.8 (4.8)</td>
</tr>
<tr>
<td>Completeness</td>
<td>99.1 (94.6)</td>
<td>97.7 (95.7)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.3 (6.6)</td>
<td>4.0 (3.9)</td>
</tr>
<tr>
<td>Unique Reflections</td>
<td>55611</td>
<td>34670</td>
</tr>
<tr>
<td>R_work/R_free</td>
<td>0.184/0.218</td>
<td>0.158/0.211</td>
</tr>
<tr>
<td><strong>Ramachandran Plot</strong> (b) (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most Favoured</td>
<td>89.2</td>
<td>89.7</td>
</tr>
<tr>
<td>Additionally Allowed</td>
<td>10.3</td>
<td>9.8</td>
</tr>
<tr>
<td>Generously Allowed</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Disallowedᵇ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>RMS Deviations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond Lengths (Å)</td>
<td>0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>Bond Angles (degrees)</td>
<td>1.795ᵃ</td>
<td>1.665ᵃ</td>
</tr>
<tr>
<td>Dihedral Angles (degrees)</td>
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<td>19.1°</td>
</tr>
<tr>
<td><strong>Mean B values (Å²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein All Atoms</td>
<td>22.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Main Chain Atoms</td>
<td>20.8</td>
<td>17.7</td>
</tr>
<tr>
<td>Side Chain Atoms</td>
<td>23.5</td>
<td>20.6</td>
</tr>
<tr>
<td>Solvent</td>
<td>30.5</td>
<td>27.5</td>
</tr>
<tr>
<td>FAD</td>
<td>40.4</td>
<td>20.4</td>
</tr>
<tr>
<td>FMN</td>
<td>28.2</td>
<td>13.9</td>
</tr>
<tr>
<td>AMP</td>
<td>57.2</td>
<td>29.3</td>
</tr>
</tbody>
</table>

ᵃValues in parentheses represent the highest resolution shell
ᵇAs defined in Procheck (14).
Figure 2.2 Electron Density Maps for FAD in NQO2 Preparations.

Electron density maps ($2F_o-F_c$, contoured at 1.2 $\sigma$) are shown for the FAD molecule in refined NQO2 crystal structures from preparation 1 (A), which contained 0.32 equivalents of FMN per protomer, and preparation 2 (B), which was saturated with FAD. Crystallographic data and refinement statistics for the two structures are provided in Table 2.1.

2.3.2 Analysis and Optimization of FAD Content of NQO2

The relatively high ADPs and weak electron density for the bound co-enzyme prompted us to measure the flavin content of the NQO2 preparation. Using HPLC, FAD was not detectable in preparation 1; instead, 0.38 equivalents of FMN were present per NQO2 protomer (Table 2). We attempted to increase the FAD content of the enzyme by simply adding 100 $\mu$M FAD to the protein solution obtained after Ni$^{2+}$-NTA (nitritotriacetic acid) affinity chromatography, which increased the amount of bound FAD but failed to exchange FAD for FMN (data not shown). We also attempted, unsuccessfully, to exchange FMN for FAD by gel filtration chromatography in the presence of 10 $\mu$M FAD (data not shown). The expected $K_D$ for flavin co-enzymes is in the mid- to low-nanomolar range (15) and a very slow rate of exchange is consistent with our inability to displace bound FMN. To facilitate rapid exchange of bound FMN with FAD, guanidine was included in the buffer to partially denature the protein (15); NQO2 was incubated briefly
on ice in a solution of 3 M guanidine–HCl along with a high concentration (10 mM) of FAD and then gel-filtered in a nondenaturing buffer (preparation 2) (Figure 2.1 and Table 2.2). NQO2 prepared in this manner is fully saturated with FAD, and crystals formed from this preparation yield structures with relatively low ADPs for the bound FAD (Table 2.1). Electron density maps for the refined structure are strong and continuous for the entire FAD molecule (Figure 2.2B).

2.3.3 Spectroscopic Properties of NQO2

The absorption spectrum of NQO2 that is fully saturated with FAD (preparation 2) is provided in Figure 2.3 along with the absorption spectrum for preparation 1, which had 0.38 equivalents of FMN per protomer and no FAD. The two spectra have been adjusted to represent a solution of NQO2 at a concentration of 1 mg/ml. At wavelengths above 320nm, the spectra appear to be qualitatively similar and are characteristic of the oxidized flavin (16). The peak at 450 nm has absorbance of 0.20 and 0.52 for preparations 1 and 2, respectively, roughly in line with the relative flavin contents of the two preparations determined by HPLC (Table 2.2).

![Figure 2.3 Absorbance Spectra of NQO2 Preparations 1 and 2.](image)

Shown are the absorbance spectra for NQO2 preparation 1, with 0.38 equivalents of FMN (gray curve), and preparation 2, which is fully saturated with FAD (black curve). The curves have been scaled to represent the proteins at a concentration of 1 mg/ml.
Table 2.2 Flavin Co-enzyme Content of QR2 Preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Fractional Flavin Saturationa</th>
<th>kcatb (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FMN</td>
<td>FAD</td>
</tr>
<tr>
<td>1c</td>
<td>0.38 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>2d</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

aAverage ± S.D. for three determinations.
bAverage ± S.D. for four determinations with 500 µM SCDP as co-substrate and 50 µM menadione as substrate.
cprotein purification was unmodified
dprotein purification was modified by incubation with 3M guanidine and 10mM FAD prior to gel filtration

At wavelengths in the UV, the presence of the adenine ring in the bound FAD of preparation 2 increases the absorption of the complex and blue-shifts its maximum from 280 to 273 nm compared with preparation 1, which contains only FMN. NQO2 that is fully saturated with FAD has an absorption maximum of 273 nm with an absorbance (1 mg/ml solution) of 3.73, yielding an extinction coefficient at 273 nm of $1.06 \times 10^5$ M⁻¹ cm⁻¹ with an $A_{273}/A_{450}$ ratio of 7.2. The extinction coefficient at 280 nm is slightly lower at $1.02 \times 10^5$ M⁻¹ cm⁻¹ with an $A_{280}/A_{450}$ ratio of 6.9. Together, the absorption maximum at 273 nm with an $A_{273}/A_{450}$ ratio of 7.2 should be a reasonably good indicator that an NQO2 preparation is fully saturated with FAD. Note that if one is not concerned with the nature of the bound flavin—whether FMN or FAD—the flavin content of NQO2 can be determined spectrophotometrically by denaturation of a known amount of NQO2 with 0.2% SDS and then measure the liberated total flavin (as FMN and/or FAD) at 473 nm, where the two have the same extinction coefficient of 9200 M⁻¹ cm⁻¹ (12).

2.3.4 FAD B-Factors and Occupancy in NQO2 Crystal Structures

In the NQO2 crystals from preparation 2, which is saturated with FAD, the FMN moiety exhibits average ADPs that are significantly lower than the average ADPs for the protein (13.9 Å² for FMN moiety vs. 19.3 Å² for all protein atoms), whereas the AMP portion has average ADPs that are close to those of the bound solvent (29.3 Å² for the AMP moiety vs. 27.5 Å² for bound solvent). Because there is no difference in the occupancy in this case (i.e., there is no free FMN associated with the protein according to the HPLC analysis), the difference in ADPs must be due to increased motion or disorder in the AMP
portion of FAD. This is consistent with a surface accessibility calculation showing that less than 14% of the FMN portion is accessible to solvent compared with just over 27% for the AMP portion.

The FAD ADP analysis was extended to NQO2 structures deposited in the Protein Data Bank (as of March 2011). To make comparisons of ADP between different structures, the ADPs were normalized to the protein ADP (nADP) (17); an nADP is equal to the difference between the ADP of the atom in question and the average ADP for the protein divided by the standard deviation of the protein ADPs. Thus, nADPs have a mean of 0 and a standard deviation of 1, facilitating comparisons between structural models refined against different crystallographic datasets. The average nADP for the entire FAD molecule ranges from 0 to 3 for the available NQO2 structures (Figure 2.4, bottom panel). Each structural model incorporates FAD at full occupancy (i.e., an occupancy of 1 in the coordinate file). Given that bacterial cultures can become depleted in FAD during protein overexpression, the most likely explanation for the range of nADPs for the FAD moiety is that the structures have sub-stoichiometric amounts of FAD and/or are populated by FMN in place of FAD. For example, in the case of the NQO2 preparations we have examined, the nADP for FAD in crystals from preparation 1, with only 0.38 equivalents of FMN per subunit, was roughly 1.4 compared with an nADP of 0 for preparation 2, which was fully saturated with FAD.

The FAD in these structures was further analyzed by dividing it into FMN and AMP portions (Figure 2.4, top and middle panels, respectively). Most of the structures, including that derived from NQO2 preparation 2 (this study), had negative values for the normalized ADP of FMN, indicating that its overall ADP is lower than the average for the protein. Based on these observations and the known FAD content of preparation 2, it is possible that structures with normalized ADPs above approximately -0.5 for the FMN portion have less than a full equivalent of bound co-enzyme. Similarly, for the AMP portion, a normalized ADP above approximately 1 may be due to the presence of FMN rather than FAD and/or the presence of apo protomers.
The average nADPs for the FAD molecules in available NQO2 crystal structures are shown in the bottom panel. Also shown are the average nADPs for the FMN and AMP portions (top and middle panels, respectively). All of the crystal structures except 3FW1 were in space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with an NQO2 dimer in the asymmetric unit; the gray and black bars represent the A and B chains of the dimer, respectively. 3FW1 was crystallized in space group I422 with one protomer in the asymmetric unit. The structures are described in the following references: 1QR2 and 2QR2, Foster and coworkers (11); 2QWX, 2QX4, 2QX6, 2QX8, and 2QX9, Calamini and coworkers (18); 1XI2, Fu and coworkers (19); 3GAM and 3G5M, Maiti and coworkers (20); 2BZS, Abu Khader and coworkers (21); 2QMY and 2QMZ, Fu and coworkers (22); 1SG0, Buryanovskyy and coworkers (23); and 3FW1, Winger and coworkers (24).
2.4 Discussion

It can be difficult to achieve stoichiometric ligand occupancy in protein crystals because the crystallization conditions often include high concentrations of salts and other molecules that can compete with and/or weaken the binding of the ligand. The result is that ligand will be present at partial occupancy, which manifests itself during refinement as elevated ADPs and relatively poor electron density for the ligand. This problem is compounded in NQO2 because the FAD co-enzyme itself is noncovalently bound. Ligands that bind tightly to NQO2 generally contain a planar polyaromatic moiety that stacks on the isoalloxazine ring of FAD; therefore, if the FAD co-enzyme itself is present at less than 1:1 stoichiometry, obtaining crystals with a full ligand occupancy will be impossible and the electron density for the bound ligand will be relatively poor.

Flavin synthesis in *E. coli* is sufficient to support exponential growth and continues for up to 2 h after reaching stationary phase (25). However, overexpression in *E. coli* of FAD-containing proteins such as NQO2 can result in depletion of the flavin pool and/or insufficient production of FAD from FMN, leading to production of proteins with sub-stoichiometric amounts of bound FAD. *E. coli* does not have the genes required for flavin import (26, 27); therefore, the addition of riboflavin to the culture medium will not solve this problem. Attenuating the expression of the recombinant gene by using a weak promoter or a lower concentration of IPTG improved the production of holo-glycerophosphate oxidase (28), another FAD-linked enzyme. On this basis, differences in the expression vector and/or culture conditions could result in varying proportions of the FAD-bound, FMN-bound, and apo NQO2 enzymes that cannot be distinguished by conventional chromatographic techniques. This might explain some of the variation seen in NQO2 crystal structures available in the Protein Data Bank.

We wanted large quantities of NQO2 for structural studies, and because the *apo* form of NQO2 appears to be quite stable, our approach was to saturate NQO2 with FAD after overexpression and partial purification. To effect full exchange of FMN with FAD, NQO2 was incubated for a short time with a high concentration of FAD under partially
denaturing conditions (3 M guanidine at 4 °C) and then gel-filtered to rapidly remove the denaturant and unbound FMN. This simple procedure yielded NQO2 that was fully saturated with FAD and easily crystallized. We are currently using enzyme prepared in this manner to obtain structures of NQO2 in complex with new ligands, and it is clear that fully saturating NQO2 with FAD has markedly improved the quality of the electron density maps.
2.5 References


Chapter 3

3 Chloroquine Binding Reveals Flavin Redox Switch Function of Quinone Reductase 2

Quinone reductase 2 (NQO2) is an FAD-linked enzyme and the only known human target of two antimalarial drugs, primaquine (PQ) and chloroquine (CQ). The structural differences between oxidized and reduced NQO2 and the structural basis for inhibition by PQ and CQ were investigated by x-ray crystallography. Structures of oxidized NQO2 in complex with PQ and CQ were solved at 1.4 Å resolution. CQ binds preferentially to reduced NQO2, and upon reduction of NQO2-CQ crystals, the space group changed from P2₁2₁2₁ to P2₁, with 1-Å decreases in all three unit cell dimensions. The change in crystal packing originated in the negative charge and 4–5° bend in the reduced isoalloxazine ring of FAD, which resulted in a new mode of CQ binding and closure of a flexible loop (F126–L136) over the active site. This first structure of a reduced quinone reductase shows that reduction of the FAD cofactor and binding of a specific inhibitor lead to global changes in NQO2 structure and is consistent with a functional role for NQO2 as a flavin redox switch.

3.1 Introduction

Despite 6 decades of routine use in treating malaria, the pharmacologic mechanisms of primaquine (PQ) and chloroquine (CQ) are not completely understood, although it is known that the two antimalarial drugs work at different stages of the Plasmodium life cycle. Malaria begins when Plasmodium sporozoites gain entry to the bloodstream via a bite from an infected mosquito, after which the parasite infects liver cells. During this liver stage infection, the 8-aminoquinoline, PQ (Figure 3.1), acts as hepatic schizontocide to clear infection (1). If left untreated, the parasites mature into merozoites and are

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released into the bloodstream to infect erythrocytes. In erythrocytes, the parasites either spawn into more merozoites or mature into gametocytes capable of infecting mosquitoes through additional bites. During the erythrocytic stage of infection, the 4-aminoquinoline, CQ (Figure 3.1), acts as a blood schizontocide to clear infection (2). As such, PQ and CQ are routine medications for treating malaria. In recent years, however, the emergence of chloroquine-resistant Plasmodium falciparum has rendered CQ ineffective in most endemic areas. PQ, on the other hand, is still widely used as a prophylaxis of malaria because parasites rarely exhibit resistance against this drug.

Figure 3.1 Structures of Primaquine and Chloroquine
Primaquine (left) and chloroquine (right) are two antimalarial drugs that act at different stages of the Plasmodium life cycle.

In terms of therapeutic mechanisms, PQ is thought to work by generating toxic oxygen species via its reactive metabolites (3). CQ, on the other hand, is thought to prevent a detoxification of excess heme in the parasite, which is generated as a result of its metabolism of hemoglobin (3). Despite these findings, no protein target has been identified for either drug in the parasite, causing some controversy about the mode of action of the two drugs (4, 5). This led Graves and coworkers (6) to search for target proteins of CQ and PQ using a proteomics approach. Surprisingly, no parasitic protein targets were identified; instead, two human proteins present in infected erythrocytes, quinone reductase 2 (NQO2) and aldehyde dehydrogenase 1, were identified. Of the two proteins, NQO2 was potently inhibited by concentrations of the quinoline drugs used in clinical treatment, whereas aldehyde dehydrogenase 1 was not. It is not known whether
the inhibition of NQO2 by PQ and/or CQ plays a role in the antimalarial effects of the drugs.

The identification of NQO2 as a human target of PQ and CQ is intriguing because both drugs are used to treat non-malarial diseases. For instance, PQ is used to treat pneumocystis pneumonia and is active against leishmaniasis and trypanosomiasis in vivo (7–9). Similarly, CQ is used to treat rheumatoid arthritis, systemic lupus erythematosus, and amoebic hepatitis; is under clinical trials for use in HIV-1/AIDS; and is being investigated for use in cancer chemotherapy (10–12). Thus, the inhibition of NQO2 may contribute to the therapeutic effects of PQ and CQ against diseases other than malaria.

NQO2 is a cytosolic and ubiquitously expressed metalloflavoprotein that catalyzes the two-electron reduction of quinone substrates. It has been the subject of extensive study over the last 15 years and binds a number of bioactive compounds, including imatinib (13, 14), inhibitors against CKII and PKC (15, 16), resveratrol (17), and melatonin (18, 19); however, the only natural substrate identified is ubiquinone, and the physiological function of NQO2 is not understood (20). NQO2 is a member of the thioredoxin family of enzymes but is unique in that it uses dihydronicotinamide riboside (NRH) as a reducing co-substrate rather than NADH or NADPH. The oxidized form of the co-substrate, nicotinamide riboside, is involved in NAD metabolism (21), but the cellular source of the reduced form, NRH, is not known, and it is not clear why NQO2 has evolved to use NRH. In addition to an enzymatic role in quinone reduction, NQO2 stabilizes the p53 tumor suppressor against 20S proteasomal degradation in the presence of NRH (22, 23). Furthermore, NQO2 is capable of generating reactive oxygen species (24). Yet the current understanding of NQO2 and its inhibition does not adequately explain the effects of PQ and CQ on malaria and non-malarial diseases.

PQ and CQ inhibit NQO2 with different mechanisms. Kinetic studies show that PQ and CQ both inhibit NQO2 in the micromolar range, with $K_I$ values of 1.0 and 0.6 µM, respectively (25), but PQ exhibits competitive inhibition against the reducing co-substrate (NRH), whereas CQ competes with the quinone substrate. This pattern of inhibition can be explained by the ping-pong kinetic mechanism that NQO2 uses. The tightly bound
FAD co-enzyme is first reduced by NRH to FADH$_2$; nicotinamide riboside is replaced by the quinone substrate, which is then reduced by FADH2. As such, NQO2 can exist as either the oxidized (NQO2$_{ox}$) or reduced (NQO2$_{red}$) form, and inhibitors may have a higher affinity for one or the other.

To provide a structural basis for the difference in specificity between PQ and CQ, we solved high resolution crystal structures of NOQ2$_{ox}$ in complex with either PQ or CQ as well as NQO2$_{red}$ in complex with CQ. The NQO2$_{red}$-CQ complex is particularly interesting because CQ is present in a completely different orientation compared with the NQO2$_{ox}$-CQ complex and this difference in binding is accompanied by movement of an active site loop, a change in crystal packing, and a change in space group symmetry. The structure of the NQO2$_{red}$-CQ complex provides support for the idea that, in common with some other flavin-containing proteins (26), NQO2 acts as a redox-sensitive “switch” that is responsive to the presence of cellular NRH in conjunction with binding of certain bioactive compounds.
3.2 Methods

3.2.1 Protein expression and Purification

Recombinant NQO2 was expressed in Escherichia coli and purified as described previously (27). A critical step in the purification was full reconstitution of the enzyme with the FAD cofactor. When expressed in *E. coli*, recombinant NQO2 typically contains substoichiometric levels of flavin mononucleotide (FMN) and no FAD. A partial denaturation of NQO2 and reconstitution with FAD was incorporated into the purification procedure, leading to full saturation of the enzyme with FAD, as reported previously (27).

3.2.2 Crystallization of NQO2

NQO2<oxid> was co-crystallized with 1 mM PQ or CQ by hanging drop vapor diffusion against reservoirs containing 0.1 M HEPES, pH 7.5, and 1.3–2.0 M (NH₄)₂SO₄. To prepare NQO2<reduced>-CQ crystals, crystals of NQO2-CQ were soaked in 1 µl of reducing-soak solution consisting of 0.1 M HEPES, pH 7.5, 2.0 M (NH₄)₂SO₄, 10 mM 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCDP; an NRH analog), and 1 mM chloroquine, for 2 min; the soak was repeated until crystals turned pale yellow. Whereas larger rod shaped crystals (0.5 mm × 0.2 mm) cracked when introduced into the reducing soak, medium size crystals (0.3 mm × 0.1 mm) remained intact. The reduced crystals were transferred to a soak of 1 mM chloroquine, 2.0 M (NH₄)₂SO₄, 0.1 M HEPES, pH 7.5, to decrease the of the amount of SCDP, which may be competitive with CQ at high concentrations. Crystals were then briefly passed through a cryoprotectant solution (20% glycerol, 2.0 M (NH₄)₂SO₄, 0.1 M HEPES, pH 7.5) and plunged into liquid nitrogen. To prevent autoxidation of NQO2, the entire crystal mounting process from harvesting to cryocooling was performed under an anoxic atmosphere in a glove bag purged with N₂. To prepare for the reduction protocol, the glove bag was initially evacuated and flushed with nitrogen for 5 min. All soaking solutions were then deoxygenated by sparging with nitrogen gas inside the glove bag. In the dry nitrogen atmosphere, crystals and crystal soak solutions quickly evaporated; therefore, to maintain an appropriate level of humidity, the nitrogen used to flush the glovebag was bubbled.
through a flask of crystallization reservoir solution (0.1 M HEPES, pH 7.5, 2.0 M (NH₄)₂SO₄).

3.2.3 X-ray Data Collection, Refinement, and Analysis

Data were collected at the CMCF-1 beamline of the Canadian Light Source, processed using XDS (28) or MOSFLM (29), and merged using Scala (30). The structures were solved by molecular replacement with Protein Data Bank entry 1QR2 as a starting model (31); refinement was carried out using PHENIX (32). Given the high resolution of the crystallographic data, NCS restraints were not used for any of the refinements.

Topology files for PQ and CQ were generated using PHENIX.ELBOW in conjunction with small molecule crystal structures of the two compounds (33, 34). For refinement of the NQO₂_red structure, the FAD topology file was modified to allow bending along the N5–N10 axis of FAD: the planarity restraints incorporating N5 and N10 of the isoalloxazine ring were removed so that it was separated into two planes, one incorporating the pyrimidine ring and the second incorporating the benzyl ring. Also, the bond length, bond angle, and dihedral angle restraints involving N5 and N10 were removed. The degree of bending in the isoalloxazine ring was calculated by finding equations for the two planes using principal component analysis incorporating all atoms in each of the two planes. The final structures for NQO₂_ox-PQ, NQO₂_ox-CQ, and NQO₂_red-CQ were deposited in the Protein Data Bank as 4FGJ, 4FGK, and 4FGL respectively.

To validate the observed changes in FAD structure and, in particular, to assess the contribution of the FAD and FADH₂ stereochemical restraint files to the structure of the isoalloxazine ring, each model was refined using an identical simulated annealing strategy with the FADH₂ stereochemical restraint file to assess the degree of bending along the N5–N10 axis. In each case, the refinement was performed three times using the same starting model with a different random seed to calculate a final average bending angle. To ensure an “active” convergence to an optimal conformation, FAD in the starting model of NQO₂ was bent to an angle of around 30°.
3.3 Results

3.3.1 Oxidized NQO2-Primaquine Complex

NQO2 was co-crystallized with PQ in space group P2_12_121. Crystals of NQO2-PQ diffracted to 1.35 Å and contained a homodimer in the asymmetric unit; the structure was solved by molecular replacement and refined to an $R_{free}$ value of 0.167 (Table 3.1). Primaquine was initially modeled in a single orientation in both active sites of the homodimer (Figure 3.2A), and after several rounds of refinement, a second orientation was incorporated to fully account for the electron density (Figure 3.2B). The occupancies for bound PQ molecules refined to values of around 0.5 for each of the conformations, indicating that PQ binds with roughly equal affinity in each mode. In both orientations, the quinoline ring of PQ is buried in the active site, making planar stacking interactions with the FAD isalloxazine ring and the phenyl ring of F178. PQ also makes contact with additional aromatic residues (W105, F106, and F126), which form the interior wall of the active site. As well, both orientations of PQ make a water-mediated hydrogen bond with the side chain of Asn161. In the first orientation of PQ (Figure 3.2A), a water molecule (position 557 in A chain and 450 in B chain) mediates hydrogen bonding between the aromatic nitrogen in the quinoline ring and the side chain of N161. In the alternate orientation (Figure 3.2B), the quinoline ring is flipped 180°, and the water molecule mediates a hydrogen bond between the 6-methoxy group of PQ and the side chain of N161.

3.3.2 Oxidized NQO2-Chloroquine Complex

NQO2 catalysis proceeds by a ping-pong mechanism. The reducing co-substrate, NRH, binds in the active site, reduces the FAD, and then dissociates; the quinone substrate then enters the active site and undergoes a two-electron reduction. Thus, NQO2 can exist in either an oxidized (NQO2$_{ox}$) or reduced (NQO2$_{red}$) state, and inhibitors of NOQ2 will exhibit a preference for one or the other. Kinetic studies indicate that CQ exhibits competitive inhibition against the quinone substrate and therefore binds preferentially to NQO2$_{red}$ (25). However, high concentrations of CQ will also compete against the reducing co-substrate, NRH, indicating that CQ also has affinity with NQO2$_{ox}$. To fully
characterize CQ binding to NQO2, we co-crystallized NQO2$_{\text{ox}}$ in complex with CQ. NQO2$_{\text{ox}}$-CQ crystals diffracted to 1.4 Å, and the structure was refined to an $R_{\text{free}}$ of 0.175 (Table 3.1).

### Table 3.1 Crystallographic Data Collection and Refinement Statistics.

<table>
<thead>
<tr>
<th>Crystal Ligand</th>
<th>Oxidized NQO2</th>
<th>Reduced NQO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primaquine</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.033217</td>
<td>1.033217</td>
</tr>
<tr>
<td>Space Group</td>
<td>P2$_1$2$_1$2$_1$</td>
<td>P2$_1$2$_1$2$_1$</td>
</tr>
<tr>
<td></td>
<td>56.5, 83.0, 106.5,</td>
<td>56.37, 83.11, 106.60,</td>
</tr>
<tr>
<td>β = 90.17°</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution</td>
<td>30.1 - 1.35</td>
<td>32.7 - 1.40</td>
</tr>
<tr>
<td>$I/\sigma(I)$</td>
<td>0.066 (0.63)</td>
<td>0.065 (0.50)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.8</td>
<td>7.3</td>
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<td>Unique Reflections</td>
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<td>98537</td>
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<tr>
<td>$R_{\text{work}}/R_{\text{free}}$</td>
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<td>0.1325/0.1745</td>
</tr>
<tr>
<td>Ramachandran Plot$^b$ (%)</td>
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<td></td>
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<tr>
<td>Most Favoured</td>
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<td>Additionally Allowed</td>
<td>8.8</td>
<td>9.5</td>
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<td>0.5</td>
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<tr>
<td>Disallowed</td>
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<td>0</td>
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<tr>
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</tr>
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<td>Dihedral Angles (deg)</td>
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<td>Mean A.D.P. values (Å$^2$)</td>
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</tr>
<tr>
<td>Protein All Atoms</td>
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<tr>
<td>Main Chain</td>
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</tr>
<tr>
<td>Side Chain</td>
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</tr>
<tr>
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</tr>
<tr>
<td>FAD</td>
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<td>17.3</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>17.5</td>
<td>26.3</td>
</tr>
</tbody>
</table>

$^a$ Values in parentheses refer to the highest resolution shell.

$^b$ Ramachandran plot statistics were calculated using PROCHECK (35).
Figure 3.2 Binding of Primaquine and Chloroquine to Oxidized NQO2

(A - B) PQ is bound to the A subunit in two alternate conformations. The B subunit of the NQO2_{ox}-PQ homodimer contained similar electron density and was modeled in the same way as the A subunit. (C - D) The active sites for the NQO2_{ox}-CQ structure. Electron density in the NQO2_{ox}-CQ subunit A (C) was different from that observed in the B subunit (D), and CQ was modeled in two alternate conformations in the B subunit. The electron density represents the final 2F_o - F_c maps contoured at 1σ around the inhibitors. (E) The two positions of CQ in the B subunit correspond to two different conformations of the active site loop, comprising residues 126–136. The active site loop in the “open” conformation (light shading) accommodates CQ when it is deeply buried in the active site, whereas the “closed” conformation (dark shading) is adopted when CQ is bound in a more peripheral location. One-letter amino acid codes are used.
Electron density for the bound CQ was initially quite poor, but a large peak (greater than 10 $\sigma$) in the difference ($F_o - F_c$) electron density map was used to position the CQ chlorine atom, after which the rest of the CQ molecule was placed in the remaining electron density. For the “A” subunit of the homodimer, CQ is present in one position, with the quinoline ring less deeply buried than primaquine and the aminopentane arm projecting toward the solvent (Figure 3.2C). In the active site of the “B” subunit, additional difference density was observed after several rounds of manual adjustment and refinement, and an alternate orientation of CQ was modeled into the structure (Figure 3.2D). The occupancies for the two modes of CQ binding to the B subunit refined to 0.52 and 0.48. In conjunction with these two positions for CQ, an active site loop consisting of residues 126–136 was modeled in two different conformations (Figure 3.2E). In the position of CQ unique to the B subunit, in which the quinoline ring is more deeply buried in the active site, the bulky aminopentane arm of CQ interferes with the ability of the 126–136 loop to close over the active site, and therefore the loop adopts an “open” conformation (Figure 3.2E). There is no evidence of cooperative interactions or nonequivalence between the two subunits of NQO2, and therefore this minor difference in CQ binding between the A and B subunits probably originates from different crystal packing environments.

### 3.3.3 Reduced NQO2-Chloroquine Complex

The reduced form of NQO2 (NQO2$_{\text{red}}$) was not sufficiently stable to allow for co-crystallization with CQ. To obtain crystals of NQO2$_{\text{red}}$ in complex with CQ, crystals of NQO2$_{\text{ox}}$-CQ were treated with SCDP (an NRH analog) in an anoxic environment until the crystal was bleached, indicating reduction of FAD to FADH$_2$, and then frozen and stored in liquid nitrogen for data collection. Data from three such crystals of NQO2$_{\text{red}}$-CQ were collected and processed (Table 3.2). In all three cases, the crystallographic data for NQO2$_{\text{red}}$ could be processed using the P2$_1$2$_1$2$_1$ space group, with unit cell dimensions ~1 Å shorter in all three directions compared with those for NQO2$_{\text{ox}}$-CQ. However, data processed in P2$_1$2$_1$2$_1$ yielded unacceptably high $R_{\text{sym}}$ values, making it clear that the crystals had undergone a structural transition to a lower symmetry space group with four rather than two protomers in the asymmetric unit. Data processed and merged in P2$_1$
yielded acceptable $R_{\text{sym}}$ values (Table 3.2), and the highest resolution data set was used for refinement and analysis (Table 3.1). The NQO2 structure underwent several rounds of manual rebuilding and refinement to yield an $R_{\text{free}}$ of 0.17. Analysis of the crystallographic data indicated the presence of pseudomerohedral twinning, and further refinement in Phenix using the twin operator ($h$, - $k$, -$l$) yielded an estimated twin fraction of 0.15, with a final $R_{\text{free}}$ value of 0.136 and improved electron density maps. This is the first structure of a reduced quinone reductase, and the combined effects of FAD reduction and chloroquine binding are described below.

### 3.3.4 Chloroquine Binding to Reduced NQO2

The reduced isoalloxazine ring is expected to be deprotonated and carry a negative charge when it is bound to NQO2. In solution, N1 of FADH$_2$ exhibits a $pK_a$ of 6.8 (36); when FAD is bound to NQO2, N1 accepts a hydrogen bond from the amide nitrogen of G149, making it highly unlikely that N1 is protonated in NQO2$_{\text{red}}$. Instead, the negative charge, which would be delocalized between N1 and O2, is neutralized by two hydrogen bonds donated to O2, one from the ring hydroxyl of Y155 and a second from the amide of G150.

**Table 3.2 Space Group Change upon Reduction of NQO2-chloroquine Crystals**

<table>
<thead>
<tr>
<th></th>
<th>Crystal 1$^a$ (1.24 Å resolution)</th>
<th>Crystal 2$^a$ (1.35 Å resolution)</th>
<th>Crystal 3$^{a,b}$ (1.15 Å resolution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit Cell</td>
<td>P$_2_1$2$_1$2$_1$&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P$_2_1$&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P$_2_1$&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>a</td>
<td>53.75</td>
<td>53.90</td>
<td>54.24</td>
</tr>
<tr>
<td>b</td>
<td>81.68</td>
<td>81.84</td>
<td>82.02</td>
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<tr>
<td>c</td>
<td>105.67</td>
<td>105.66</td>
<td>105.71</td>
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<tr>
<td>$\beta$</td>
<td>90</td>
<td>90.35</td>
<td>90</td>
</tr>
<tr>
<td>$R_{\text{sym}}$</td>
<td>0.171</td>
<td>0.327</td>
<td>0.347</td>
</tr>
</tbody>
</table>

$^a$Crystals of NQO2$_{\text{ox}}$ in complex with chloroquine were soaked with SCDP, an NRH analogue, to effect reduction of the FAD cofactor.

$^b$Crystal 3 was used for structure solution, refinement, and detailed analysis.

$^c$Data were indexed, processed and merged in either P$_2_1$2$_1$ or P$_2_1$ using Mosflm and Scala.
Reduction of FAD drastically changes the nature of its interaction with CQ. In NQO2\(_{ox}\), CQ tends to be rather deeply buried and able to make direct contact with aromatic residues that line the back wall of the active site (Figure 3.2C), with the chlorine atom positioned above C5A of the isoalloxazine ring. In the NQO2\(_{red}\) structure, the quinoline ring of CQ has flipped so that the opposite face interacts with the surface of reduced FAD, and the chlorine atom is positioned roughly above O2 on the periphery of the isoalloxazine ring (Figure 3.3A-B). The mode of CQ binding in NQO2\(_{red}\) allows CQ to participate in a water mediated hydrogen-bonding network that is centered on the proton on N5, a characteristic feature of reduced FAD (Figure 3.3C). The keystone for this network is a water molecule (HOH 32, 312, 204, and 92 in chains A, B, C, and D, respectively) that accepts hydrogen bonds from the N5 proton of FAD and also from the proton on N4 of the CQ quinoline ring. The keystone water donates a hydrogen bond to a second water molecule (HOH 149, 154, 157, and 63 in chains A, B, C, and D, respectively) that in turn donates hydrogen bonds to O4 of the FAD and to the carbonyl oxygen of G174. The relative positions of these water molecules are conserved in all four subunits of the asymmetric unit. The geometry of the interaction between the keystone water and the FAD N5 atom deviates from what would be expected for a good hydrogen bond; the angle between CQ-N4, the keystone water, and FAD-N5 is 79 ± 1° (mean ± S.D. for four subunits) rather than a more typical angle of 110°. In addition to these hydrogen bonds, the aromatic rings of W105, F106, F126, and F178 enclose the two water molecules; indeed, the keystone water appears to donate a hydrogen bond to the indole ring of W105, whereas the second water may donate a hydrogen bond to the benzene ring of F178 (37).

The \(2F_o - F_c\) electron density for bound CQ is extremely well defined in one of the NQO2 dimers (C and D chains) of the asymmetric unit, and there is clear density for a third water molecule in the active sites of the CD dimer (Figure 3.3B). This third water molecule donates a hydrogen bond to the second water and to the chlorine atom on CQ, and it accepts a hydrogen bond from the side-chain amide nitrogen of N161. In the second dimer (A and B chains) of the asymmetric unit, there was additional electron density for the bound CQ, and therefore a second CQ molecule was included in a slightly
Figure 3.3 Binding of Chloroquine to reduced NQO2.

(A-B) CQ binds NQO2_red active sites in the two dimers of the asymmetric unit; the electron density corresponds to 2Fo - Fc maps contoured at 1σ around CQ. (A) In the active site of the A subunit, CQ bound in two alternative positions, one of which corresponds to the position observed in the C and D subunits, whereas the second is shifted slightly. The electron density and positions of bound CQ were similar for the B subunit. (B) In the active site of the C subunit, CQ was modeled in the same single position. The electron density in the D subunit was similar. (C) The hydrogen bonding network, with the “keystone water” bridging N4 of CQ and N5 of the FAD isoalloxazine ring. (D) The active site loop (residues 126–136) is shown in the fully closed conformation (green) observed in NQO2_red-CQ; in this state, residue I128 makes van der Waals contact with CQ. For reference, the conformations of the loop observed in crystals of oxidized NQO2 are shown in gray.
different orientation from the first (Figure 3.3A). For the A and B subunits, the third water molecule was not present. This minor difference in CQ binding between the two reduced NQO2 dimers in the asymmetric unit may be a product of their interaction with each other or may be due to differences in the surrounding crystal packing environment.

Regarding the stereochemistry of the bound inhibitors, it is noteworthy that both CQ and PQ have a single stereocenter and exist as racemic mixtures. For the structures of NQO2\textsubscript{ox} with bound PQ or CQ, the electron density for the aminopentane arm was generally weak, and it was not possible to discern whether the protein selectively bound a single enantiomer. In the case of NQO2\textsubscript{red}, however, the (R)-CQ enantiomer was built into the structure, and there was enough electron density for the aminopentane arm to exclude binding of the (S)-CQ enantiomer. In this regard, binding of CQ to reduced NQO2 may be stereoselective for the (R)-enantiomer.

3.3.5 Structural Changes in FAD

Crystals of NQO2\textsubscript{ox} in complex with CQ were consistently isomorphous with other oxidized forms, indicating that the CQ itself is not sufficient for the observed structural change in crystals of the NQO2\textsubscript{red}-CQ complex. Instead, the origin of the change in structure is the reduction of the FAD cofactor. The FAD isoalloxazine ring is planar, but reduction should lead to a “butterfly bend” along the N5–N10 axis (38) (Figure 3.4). The electron density of the isoalloxazine ring in NQO2\textsubscript{red}-CQ indicated that the bending upon reduction is relatively minor. Refinement using a modified stereochemical restraint set for FAD, in which planar restraints and associated bond and angle restraints around N5 and N10 were removed, indicated an angle of $\sim$4-5$^\circ$ between the two planes of the “butterfly wings” (Figure 3.4).

To validate that the small change in the FAD isoalloxazine structure was due to reduction of the FAD, and not simply removal of stereochemical restraints, the refined NQO2 structures were subjected to simulated annealing refinement against data from both oxidized and reduced crystals. In the absence of stereochemical restraints around N5 and N10, the isoalloxazine ring of NQO2\textsubscript{ox} in complex with PQ refined to an average bend of $\sim$1.6$^\circ$; for NQO2\textsubscript{ox} in complex with CQ, the average bend was 2.3$^\circ$; whereas for NQO2\textsubscript{red}
Figure 3.4 Structural Changes in the Isoalloxazine ring.

(A) Chemical structure and atom designations in the FAD isoalloxazine ring, and the axis for the “butterfly bend” that is brought about by reduction of FAD to FADH$_2$. (B) An edge-on view of the structure of the isoalloxazine ring for the following: NQO$_{2\text{ox}}$ with PQ (black), NQO$_{2\text{ox}}$ with CQ (red), and NQO$_{2\text{red}}$ with CQ (blue). (C) Binding of PQ induces a slight (∼1.6°) concave bend in the isoalloxazine system, whereas binding of CQ induces a convex bend of ∼2.3°. Reduction of FAD further increases this bend to 4.8°. To determine the bend in the isoalloxazine rings, the crystal structures were subjected to simulated annealing without stereochemical restraints at the N5 and N10 positions. FAD bending along the N5-N10 axis (butterfly bend) was calculated using the atomic positions of the dimethylbenzene and pyrimidine “wings” and principal component analysis to find the angle between the two best fit planes. Error bars, S.D.
in complex with CQ, the bend was 4.8° (Figure 3.4C). It is noteworthy that for NQO2<sub>ox</sub>, PQ brings about a small “upward” bend in the ring, forming a very slight concave binding surface, whereas with CQ, the isoalloxazine system bends in the opposite direction, with CQ binding to a slightly convex surface. Reduction of the FAD further enhances this bend by 2–3°. Other notable changes occur around N5, which moves toward a tetrahedral configuration in NQO2<sub>red</sub>; the C4A-N5-C5A bond angle was decreased from 116–117° in NQO2<sub>ox</sub>-CQ and NQO2<sub>ox</sub>-PQ to 112° in NQO2<sub>red</sub>-CQ (Table 3.3). In contrast, there was no significant difference in the C10-N10-C9A angle between the oxidized and reduced forms. Thus, binding of CQ to NQO2<sub>ox</sub> appears to push the isoalloxazine ring toward a slightly bent conformation, and reduction of FAD to FADH<sub>2</sub> results in a further increase in the bend as well as the transition of N5 toward a tetrahedral configuration.

Table 3.3 Conformational Changes in the Isoalloxazine Ring upon Reduction

<table>
<thead>
<tr>
<th>NQO2</th>
<th>Inhibitor</th>
<th>Subunit</th>
<th>N5-N10 bend&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>C4X-N5-C5X angle&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C10-N10-C9A angle&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized Primaquine</td>
<td>A</td>
<td>-1.56 ± 0.01</td>
<td>117.1 ± 0.2</td>
<td>120.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-1.64 ± 0.00</td>
<td>116.9 ± 0.1</td>
<td>118.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>-1.60</td>
<td>117.0</td>
<td>119.3</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>A</td>
<td>1.69 ± 0.00</td>
<td>118.2 ± 0.2</td>
<td>118.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2.94 ± 0.11</td>
<td>113.8 ± 0.2</td>
<td>116.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>2.32</td>
<td>116.0</td>
<td>117.7</td>
<td></td>
</tr>
<tr>
<td>Reduced Chloroquine</td>
<td>A</td>
<td>4.61 ± 0.28</td>
<td>112.2 ± 0.5</td>
<td>117.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.24 ± 0.05</td>
<td>112.1 ± 0.4</td>
<td>118.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5.36 ± 0.19</td>
<td>112.3 ± 1.2</td>
<td>116.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>3.94 ± 0.30</td>
<td>113.1 ± 1.1</td>
<td>117.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>4.79</td>
<td>112.4</td>
<td>117.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Both oxidized and reduced structures were subjected to three separate rounds of simulated annealing refinement in the absence of planar restraints for N5-N10 axis and angle restraints for the C4X-N5-C5X/C10-N10-C9A angles.

<sup>b</sup>The “butterfly” bend along the N5-N10 axis was calculated using the atomic positions of the dimethylbenzene and pyrimidine “wings” and principal component analysis to find the angle between the two best-fit planes.

3.3.6 Conformational Changes in Reduced NQO2-Chloroquine

The monoclinic crystals of NQO2<sub>red</sub>-CQ contained four NQO2 protomers, two functional dimers, in the asymmetric unit, rather than the single functional dimer observed in the
asymmetric unit of orthorhombic crystals of NQO2\textsubscript{ox}. Thus, reduction and CQ binding caused a reorientation of the two dimers, removing the crystallographic symmetry that related them in orthorhombic crystals of NQO2\textsubscript{ox} (Figure 3.5A).

The active sites in the NQO2 dimer are at the interface of the two protomers. One of the protomers is largely responsible for binding FAD, whereas the second protomer contributes elements that cover the surface of the isoalloxazine ring. One such element is an irregular loop formed by residues 126–136 (Figure 3.3D). In NQO2\textsubscript{red}-CQ, the loop closes over the active site, with I128 coming into direct contact with the CQ quinoline ring, which becomes sandwiched between the side chain of I128 and the isoalloxazine ring of FAD. This conformation is seen in the B, C, and D chains of the NQO2\textsubscript{red}-CQ structure, with the loop in a slightly more open conformation in the A chain.

The loop movement outlined above, as well as other changes in the structure that could explain the repacking of the crystal upon reduction of FAD and binding of CQ, are relatively small and difficult to distinguish from crystal packing artifacts. To assess the structural changes that take place upon reduction and binding of CQ, we made use of the four NQO2 protomers in the asymmetric unit of NQO2\textsubscript{red}-CQ to eliminate random changes in atomic positions and find regions that show a concerted difference from protomers in crystals of oxidized NQO2. All of the individual subunits from reduced and oxidized NQO2-CQ were superimposed, and average carbon α (CA) positions were calculated for each of the four oxidized protomers (two protomers from each of the NQO2\textsubscript{ox}-PQ and NQO2\textsubscript{ox}-CQ structures) and the four reduced protomers (contained in the NQO2\textsubscript{red}-CQ asymmetric unit). Differences in the average CA positions for the oxidized and reduced protomers were calculated, plotted, and mapped to the structure (Figure 3.5B,C). There were seven regions where consistent differences between the oxidized and reduced NQO2 subunits were observed. Two of these regions (regions 4 and 5 in Figure 3.5C) make direct contact with the bound chloroquine and comprise the 126–136 active site loop and an oddly structured connecting loop that includes N161; three other regions (regions 1, 2, and 3) form a surface patch that is not in direct contact with either the FAD cofactor or bound chloroquine; the last two regions (regions 6 and 7)
Figure 3.5 Conformational Changes in NQO2 upon Reduction and Binding of CQ. (A) Stereodiagram illustrating the relationship between NQO2 in the oxidized (red) and reduced (blue) states. CA traces of two dimers are shown, with the origin of the unit cells indicated by a yellow sphere; the FAD molecules are drawn as stick representations. To show the changes that take place in the crystal, the dimer from the orthorhombic (P2₁2₁2₁) NQO2\textsubscript{ox}-CQ crystal has been superimposed on one of the dimers from the monoclinic (P2₁) crystal of NQO2\textsubscript{red}-CQ. In orthorhombic crystals of NQO2\textsubscript{ox}, there is a single dimer in the asymmetric unit, and the second dimer shown is related by crystallographic symmetry. When NQO2-CQ is reduced (blue), the relationship between the two dimers changes, and the crystallographic symmetry is broken, resulting in a monoclinic space group with two dimers in the asymmetric unit. (B) A plot of differences in average CA positions between protomers of oxidized NQO2 (NQO2\textsubscript{ox}-PQ and NQO2\textsubscript{ox}-CQ) and protomers of NQO2\textsubscript{red}-CQ. (C) Two views of the NQO2 dimer (CA trace) colored from blue to red according to the magnitude of the difference in average CA position between reduced and oxidized NQO2-CQ. The views are related by 90° rotation around a vertical axis; the FAD co-enzyme and bound CQ are represented as yellow and magenta CPK models. Regions of NQO2 (numbered 1–7) that demonstrate a significant shift upon reduction and binding of CQ (B) are indicated.
are at the N- and C-terminal ends, respectively, of a surface-exposed helix comprising residues 196–212. Region 7 is in direct contact with the adenosine moiety of FAD but is still far removed from the active site. In summary, only two of seven regions that exhibit structural changes between NQO2\textsubscript{ox} and NQO2\textsubscript{red}-CQ are in direct contact with CQ and the isoalloxazine ring. On this basis, the combination of reduction and CQ binding appears to exert a global change in structure, consistent with the observed reorientation of NQO2 dimers within the crystal lattice.

3.4 Discussion

NQO2 was identified as a human target of both CQ and PQ; kinetic studies showed that PQ binds preferentially to NQO2\textsubscript{ox}, whereas chloroquine binds to NQO2\textsubscript{red} (6, 25). Binding of inhibitors to NQO2\textsubscript{red} has not been studied, and the goal of the current work was to understand the structural basis for the difference in binding specificity. The NQO2\textsubscript{red}-CQ complex is the first structure of reduced NQO2, and it shows that the mode of CQ binding to NQO2\textsubscript{red} is completely different from what is observed for CQ binding to NQO2\textsubscript{ox}. The ring nitrogen of 4-aminoquinoline has a pK\textsubscript{a} of 9.2 (39), and therefore CQ will be protonated and positively charged at physiological pH; in contrast, the reduced form of the NQO2-bound isoalloxazine ring will have a negative charge, whereas the oxidized form will be neutral. The negative charge on the reduced isoalloxazine ring explains the preference of CQ for NQO2\textsubscript{red} and will contribute to the striking difference in the mode of CQ binding to NQO2\textsubscript{red} and NQO2\textsubscript{ox}.

The difference in CQ binding to NQO2\textsubscript{ox} and NQO2\textsubscript{red} is noteworthy but not as surprising and interesting as the effect of reduction and CQ binding on the overall structure of NQO2. There are 40 NQO2\textsubscript{ox} structures in the Protein Data Bank, most of which were crystallized as complexes with different inhibitors. Of the 40 structures, 38 were crystallized in the same orthorhombic space group observed for the NQO2\textsubscript{ox}-PQ and NQO2\textsubscript{ox}-CQ complexes; one structure was crystallized in I\textsubscript{4}22 with a single protomer in the asymmetric unit; and one structure was crystallized in P1 with two dimers in the asymmetric unit. The monoclinic space group and close packing of the two dimers observed for NQO2\textsubscript{red}-CQ are therefore unique and arise due to the reduction of FAD and chloroquine binding rather than random crystal packing effects. The unexpected
conformational change in NQO2 provides a structural basis for the proposed role of quinone reductases as flavin redox switches (40, 41). Flavin redox switches are FAD- or FMN-containing proteins in which the oxidation state of the cofactor regulates interactions with other proteins, nucleic acids, or membranes (26). The oxidation state of the cofactor is coupled to the conformation of the switch protein through a hydrogen-bonding network that incorporates the N5 atom of the isoalloxazine ring. For example, in the LOV family of light-sensing domains, light-induced reduction of the flavin involves reaction of a cysteine thiolate with C4A of the isoalloxazine ring; the ensuing protonation of N5 causes the side chain amide of a conserved glutamine residue to flip, and this change in hydrogen bonding in the vicinity of N5 is communicated to other parts of the protein (42, 43). In the case of NQO2, reduction of FAD leads to a unique mode of CQ binding; protonation of the isoalloxazine N5 allows it to donate a hydrogen bond to the “keystone” water that is held in place by a second hydrogen bond from CQ. Binding of CQ to NQO2_red in this fashion stabilizes an active site loop in a “closed” conformation. Reduction and CQ binding also cause the isoalloxazine ring of FAD to bend by 4.8°. Because the active site of NQO2 is at the interface between the two protomers, the “local” changes in the active site affect the overall structure of the dimer. The reorientation of the dimers in the crystal lattice indicates global changes in structure and/or dynamics. Thus, the flavin switch function of NQO2 requires both reduction of FAD and binding of an appropriate ligand to the reduced isoalloxazine ring to stabilize the protein in an alternate conformation.

Although the cellular function of NQO2 is not understood, it binds many drugs and bioactive compounds and has been implicated in apoptosis and cancer (22, 44–46). A role as a flavin redox switch, the function of which is dependent on cellular redox conditions and the presence of an appropriate ligand, could explain the elusive nature of NQO2 cellular function. CQ is a good example of an NQO2-interacting compound that is used to treat a variety of diseases, but the mechanisms underlying the cellular effects of CQ are poorly understood. As an antimalarial, CQ is thought to interact with undimerized ferriprotoporphyrin and prevent its detoxification and storage as β-hematin (4). On the other hand, the process of hemoglobin digestion and storage of ferriprotoporphyrin by the parasite involves endocytosis and a number of steps in which endosomes must fuse with
digestive vacuoles, and CQ and related quinolone drugs may interfere with these processes (4). Along these lines, the accumulation of CQ in lysosomes is thought to be responsible for its inhibition of autophagy (47). Furthermore, CQ has effects on glucocorticoid signalling and has been linked to p53 function (48, 49). It is conceivable that some of the cellular effects of CQ are linked to its binding of NQO2 and the ensuing conformational switch, which would only occur under an appropriate metabolic state where NQO2 is reduced.

Both NQO1 and NQO2 have been shown to protect p53 against 20S proteosomal degradation in the presence of their reduced nicotinamide co-substrates (22, 23, 50, 51). In fact, the stabilization and localization of a transcription factor is a common function of flavin redox switches. For example, the oxidized form of the flavin switch NifL forms an inactive complex with the transcription factor NifA; reduction of NifL leads to dissociation of the complex, allowing NifA to activate genes involved in nitrogen fixation. Lot6p, a yeast homologue of human NQO1 and NQO2 (52), provides an example of a quinone reductase that functions as a flavin redox switch; in this case, reduction of Lot6p helps it to stabilize the Yap4p transcription factor against 20S proteosomal degradation, similar to the way in which NQO1 and NQO2 appear to stabilize p53 (40, 53). Lot6p is actually a poor catalyst (the rate of the oxidative half-reaction is slower than that of non-enzymatic model reactions), and this led Sollner and coworkers (41) to propose that Lot6p may have evolved primarily as a flavin redox switch to regulate 20S proteosomal degradation. The conformational switch that we have observed with NQO2 provides a structural basis for the proposed role of NQO2 and possibly other quinone reductases in the redox-dependent regulation of p53.

As a flavin redox switch, it is not clear why NQO2 uses NRH as an electron donor instead of NAD(P)H, which is used by NQO1 and all other members of the flavodoxin family. NQO2 is the only enzyme known to use NRH, and although oxidized nicotinamide riboside exists as a metabolite of NAD, the origin of cellular NRH is not known. On this basis, the metabolic signal that facilitates conformational switching of NQO2 remains elusive. CQ has been reported to activate the p53 pathway, but whether this process involves NQO2 was not determined (49). In any case, there may be a number
of small molecule “inhibitors” of NQO2, as well as proteins or peptides, that could interact specifically with the reduced form of NQO2 and function in a manner similar to CQ to stabilize NQO2 in an alternate conformation. Thus, although there are many unanswered questions regarding the cellular function of NQO2, a flavin switch function that is dependent on the cellular redox state and the presence of an appropriate ligand provides an intriguing, metabolically regulated, link between NQO2, p53, and the poorly defined cellular effects of the many drugs and bioactive compounds that interact with NQO2.
3.5 References


Chapter 4

4 Quinone Reductase 2 Is an Adventitious Target of Protein Kinase CK2 Inhibitors TBBz (TBI) and DMAT

Quinone reductase 2 (NQO2) exhibits off-target interactions with two protein kinase CK2 inhibitors, 4,5,6,7-1H-tetribromobenzimidazole (TBBz) and 2-dimethylamino-4,5,6,7-tetribromo-1H-benzimidazole (DMAT). TBBz and DMAT induce apoptosis in cells expressing an inhibitor-resistant CK2, suggesting that the interaction with NQO2 may mediate some of their pharmacological effects. In this study, we have fully characterized the binding of TBBz and DMAT to NQO2. Fluorescence titrations showed that TBBz and DMAT bind oxidized NQO2 in the low nanomolar range; in the case of TBBz, the affinity for NQO2 was 40-fold greater than its affinity for CK2. A related CK2 inhibitor, 4,5,6,7-tetribromobenzotriazole (TBB), which failed to cause apoptosis in cells expressing inhibitor-resistant CK2, binds NQO2 with an affinity 1000-fold lower than those of TBBz and DMAT. Kinetic analysis indicated that DMAT inhibits NQO2 by binding with similar affinities to the oxidized and reduced forms. Crystal structure analysis showed that DMAT binds reduced NQO2 in a manner different from that in the oxidized state. In oxidized NQO2, TBBz and DMAT are deeply buried in the active site and make direct hydrogen and halogen bonds to the enzyme. In reduced NQO2, DMAT occupies a more peripheral region and hydrogen and halogen bonds with the enzyme are mediated through three water molecules. Therefore, although TBB, TBBz, and DMAT are all potent inhibitors of CK2, they exhibit different activity profiles toward NQO2. We conclude that the active site of NQO2 is fundamentally different from the ATP binding site of CK2 and the inhibition of NQO2 by CK2 inhibitors is adventitious.

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4.1 Introduction

Protein kinases are often deregulated in cancer, and the development of kinase inhibitors for cancer chemotherapy has received much attention in the past 30 years (1). Protein kinase CK2 (CK2) is a constitutively active kinase that phosphorylates more than 200 protein targets and regulates a myriad of cell signalling events in cell cycle, apoptosis, and transcriptional control (2, 3). With regard to apoptosis, CK2 attenuates cell death by phosphorylation of caspases and caspase targets (4). Therefore, the overexpression of CK2 in many tumor types suggests that CK2 is involved in tumorigenesis by preventing cell death (5). As such, CK2 is an attractive cancer therapy target and CK2 inhibitors that cause apoptosis in cancer cells have been extensively developed over the past two decades (6). However, despite tremendous efforts to increase the specificity of inhibitors for CK2, many of these compounds exhibit off-target interactions.

The tetrabromobenzene compounds 4,5,6,7-tetrabromobenzotriazole (TBB), 4,5,6,7-tetrabromobenzimidazole (TBBz), and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) are some of the most widely used inhibitors of CK2 (Figure 4.1). TBB inhibits CK2 activity in the submicromolar range with relatively high specificity over other kinases (7–9), and treatment of cells in culture with TBB leads to apoptosis (10). Further development of TBB led to the discovery of another potent inhibitor of CK2, TBBz, that is more selective for yeast CK2 (11, 12). Based on the tetrabromobenzene moiety in TBBz, another CK2 inhibitor, DMAT, was developed that was more selective for CK2 than TBB and bound with an affinity 10-fold greater than that of either TBB or TBBz (13, 14). Despite being optimized for CK2 inhibition, both TBBz and DMAT inhibit at least three other subfamilies of kinases: DYRKs, PIM, and HIPK2 (15). Furthermore, they also bind several non-kinase targets with ATP binding sites (16). Such nontargeted interactions may contribute to the cellular effects of the inhibitors.

In a series of unbiased validation studies of the specificity of these inhibitors, Duncan and co-workers identified quinone reductase 2 (NQO2) as a particularly interesting non-kinase target of TBBz and DMAT (17). Briefly, an inhibitor-resistant mutant of CK2 was transfected into cells to rescue them from CK2 inhibitor-induced apoptosis; however,
Figure 4.1 Chemical Structure and Electrostatic Properties of CK2 Inhibitors. Chemical structure and electrostatic properties of CK2 inhibitors 4,5,6,7-tetrabromobenzotriazole (TBB), 4,5,6,7-tetrabromo-1H-benzimidazole (TBBz), and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). Partial charges were calculated and displayed on the molecular surfaces using Marvin 6.3.1 by ChemAxon (http://www.chemaxon.com).

only TBB-treated cells were rescued, while TBBz- and DMAT-treated cells underwent apoptosis. Therefore, inhibition of CK2 alone did not fully account for the cell death caused by TBBz and DMAT treatment. This challenges the notion that CK2 is the bona fide target of the two inhibitors and indicates that TBBz and DMAT act through alternate mechanisms to cause cellular apoptosis. Further investigation using a proteomics approach identified a non-kinase target, NQO2, that eluted from an ATP-Sepharose affinity column when it was washed with TBBz and DMAT but not with a similar concentration of TBB. This corresponded to the rescue experiment in which TBBz and DMAT but not TBB caused cell death and suggests that NQO2 inhibition may contribute to the apoptotic effects of the drugs. Thus, it prompted our investigation of NQO2 inhibition by the three inhibitors.

Historically, NQO2 and related quinone reductases have been characterized as enzymes involved in quinone detoxification. They do so by catalyzing an obligate two-electron
transfer to a quinone to produce the relatively stable quinonol, thus preventing non-
enzyme-catalyzed single-electron transfer that produces redox active semiquinones (18).
NQO2 is unusual, however, because it uses dihydronicotinamide riboside (NRH) as a
reducing co-substrate, rather than NAD(P)H (19); moreover, in the presence of NRH,
NQO2 was found to protect p53 from degradation by the 20S proteasome (20, 21). At the
molecular level, reduction of NQO2 and binding of chloroquine (an antimalarial) were
shown to induce a conformational change in NQO2, suggesting that NQO2 may function
as a flavin redox switch (22). Therefore, in addition to its role in quinone detoxification,
NQO2 appears to have a signalling function, possibly connecting the metabolic status of
the cell to p53 levels. While its cellular functions remain to be determined, mice deficient
for NQO2 develop skin tumors more frequently than wild-type mice in a chemical
carcinogenesis model (23), indicating that NQO2 plays a role in cell proliferation and/or
apoptosis.

In addition to CK2 inhibitors, several chemotherapeutic inhibitors of the Abelson kinase
and protein kinase C (PKC) were found to inhibit NQO2 with nanomolar and micromolar
affinity, respectively (24–27). The fact that multiple kinase-directed drugs also inhibit
NQO2 raised the question of whether the drug binding site of NQO2 somehow mimics
the ATP binding site of kinases. In this study, we performed kinetic and structural
analyses to characterize the inhibition of NQO2 by TBB, TBBz, and DMAT. The three
inhibitors exhibit different binding profiles with respect to NQO2 that do not correspond
to their interactions with CK2.
4.2 Methods

4.2.1 Protein Expression and Purification

Recombinant NQO2 was expressed in Escherichia coli and purified as previously described (28). A critical step in the purification was full reconstitution of the enzyme with the FAD co-enzyme. When expressed in *E. coli*, recombinant NQO2 typically contains substoichiometric levels of flavin mononucleotide (FMN) and no FAD. A partial denaturation of NQO2 and reconstitution with FAD was incorporated into the purification procedure, leading to full saturation of the enzyme with FAD (28).

4.2.2 Affinity of Inhibitors by Fluorescence Quenching.

To assess direct binding of inhibitors to oxidized NQO2 (NQO2<sub>ox</sub>), fluorescence quenching of FAD was monitored with an excitation wavelength of 350 nm and an emission wavelength of 430 nm. NQO2 (775 or 38.75 nM) was titrated with the indicated concentrations of TBB, TBBz, and DMAT. The binding data were fit to equation 4.1 to obtain the dissociation constant (*K*<sub>D</sub>) for binding of the inhibitors to NQO2<sub>ox</sub>.

\[
\frac{F - F_o}{F_{max} - F_o} = \frac{K_D + I + NQO2 - \sqrt{(K_D + I + NQO2)^2 - 4(I)(NQO2)}}{2(NQO2)}
\]

**Equation 4.1 Fluorescence Quenching by Inhibitors**

where NQO2 and *I* are the final concentrations in the cuvette of the enzyme and inhibitor, respectively, *F*<sub>o</sub> is the initial fluorescence reading in counts per second without an inhibitor, and *F* is the fluorescence after the addition(s) of TBBz or DMAT. *K*<sub>D</sub> and *F*<sub>max</sub> were determined by nonlinear regression using Prism 4.0b (GraphPad Software Inc., San Diego, CA). The data are presented as fractional saturation of NQO2 with an inhibitor (*F* - *F*<sub>o</sub>)/(*F*<sub>max</sub> - *F*<sub>o</sub>).

4.2.3 Enzymatic Assays.

Enzyme activity was measured by monitoring the consumption of 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCDP) (Sigma) co-substrate (a dihydronicotinamide riboside analogue) at an absorbance peak of 360 nm using a Cary 100-Bio spectrophotometer (Varian). The reaction was initiated by addition of NQO2 at a
final concentration of 154 pM to a stirring cuvette containing SCDP (10-365 µM) as co-substrate and menadione (0.5-50 µM) (Sigma) as the substrate at 30 °C. The linear portion of the decrease in absorbance was determined using Cary Kinetics Software (Varian) and converted to turnover number (inverse seconds) using the extinction coefficient of SCDP (ε_{360} = 4480 M\(^{-1}\) cm\(^{-1}\)). Because menadione inhibits the reaction at high concentrations by competing with SCDP for binding to the oxidized enzyme, kinetic data were fit to Equation 4.2 that describes a ping-pong kinetic mechanism with substrate inhibition (29).

\[
V_{\text{obs}} = \frac{V_{\text{max}} [\text{SCDP}][\text{Md}]}{K_{\text{M(SCDP)}}[\text{Md}]+K_{\text{M(Md)}}[\text{SCDP}]+[\text{SCDP}][\text{Md}]}
\]

\[\text{where } K_{\text{M(SCDP)}}^{\text{app}} = K_{\text{M(SCDP)}}(1+\frac{[\text{Md}]}{K_{I(Md)}})\]

**Equation 4.2 Ping-pong Mechanism of NQO2 Catalysis**

where \(V_{\text{obs}}\) is the observed rate of reaction, \(V_{\text{max}}\) is the maximal rate, [SCDP] and [Md] are the concentrations of SCDP and menadione, respectively, \(K_{\text{M(SCDP)}}\) and \(K_{\text{M(Md)}}\) are the Michaelis constants for substrates SCDP and menadione, respectively, and \(K_{I(Md)}\) is the competitive binding constant of menadione. Initial values were obtained from the previous study of NQO2 kinetics using N-methyldihydronicotinamide (NeMH) as the co-substrate (29). Kinetic parameters were fit to the data by nonlinear regression using Igor Pro (Wavemetrics, Portland, OR); the \(\chi^2\) values were calculated as

\[\chi^2 = \sum_i \left(\frac{y-y_i}{\sigma_i}\right)^2\]

**Equation 4.3 Determination of Chi-squared Values**

where \(y\) and \(y_i\) are the fitted and measured values, respectively, and \(\sigma_i\) is the estimated standard deviation of the measured value.
4.2.4 Enzyme Inhibition by TBBz and DMAT.

To determine the constants for inhibition ($K_I$ values) of NQO2 by TBBz and DMAT, inhibition kinetics were performed using the same techniques that were used for the uninhibited reaction described above. For each inhibitor, kinetic assays were performed either with a constant SCDP concentration of 150 µM and a varying menadione concentration or with a constant menadione concentration of 5 µM and a varying SCDP concentration. Because inhibitors of NQO2 can be competitive against SCDP, menadione, or both, we adapted the kinetic equation to account for both types of inhibition:

$$V_{\text{obs}} = \frac{V_{\text{max}} [\text{SCDP}] [\text{Md}]}{K_{M(\text{SCDP})} [\text{Md}] + K_{M(\text{MD})} [\text{SCDP}] + [\text{SCDP}] [\text{Md}]}$$

where

$$K_{M(\text{SCDP})}^{\text{app}} = K_{M(\text{SCDP})} (1 + \frac{[\text{Md}]}{K_{I(\alpha)}} + \frac{[I]}{K_{I(\alpha)}})$$

and

$$K_{M(\text{MD})}^{\text{app}} = K_{M(\text{MD})} (1 + \frac{[I]}{K_{I(\beta)}})$$

Equation 4.4 Inhibition of NQO2 Catalysis

On the basis of equation 4.2, $[I]$, $K_{I(\alpha)}$, and $K_{I(\beta)}$ were introduced into equation 4.3, where $[I]$ is the concentration of inhibitor and $K_{I(\alpha)}$ and $K_{I(\beta)}$ are the inhibition constants that modify the “apparent” Michaelis constants $K_{M(\text{SCDP})}^{\text{app}}$ and $K_{M(\text{MD})}^{\text{app}}$ respectively. That is, $K_{I(\alpha)}$ is the constant that accounts for the change in $K_{M(\text{SCDP})}$ in the presence of inhibitor; hence, it describes the component of inhibition that is competitive against SCDP. $K_{I(\beta)}$ is the constant that accounts for the change in $K_{M(\text{MD})}$ in the presence of inhibitor; hence, it describes the component of inhibition that is competitive against menadione. The kinetic parameters $K_{M(\text{SCDP})}$, $K_{M(\text{MD})}$, and $K_{I(\text{MD})}$ were fixed using values determined for the uninhibited reaction (Table 4.2), while $V_{\text{max}}$, $K_{I(\alpha)}$, and $K_{I(\beta)}$ were determined by globally fitting data to equation 4.4 using nonlinear regression (Igor Pro version 6.34A) with the IC$_{50}$ values as initial values.
4.2.5  IC$_{50}$ Measurements.

To determine the IC$_{50}$ value of each inhibitor, reactions were initiated by addition of 154 pM NQO2 to a reaction buffer containing 150 µM SCDP and 5 µM menadione with TBB (0.625-640 µM), TBBz (2.5-640 nM), or DMAT (2.5-640 nM). IC$_{50}$ values were then calculated using a dose-response model and represented as relative inhibition.

4.2.6  Crystallization of NQO2.

Oxidized NQO2 (NQO2$_{ox}$) was co-crystallized with TBBz or DMAT by hanging drop vapor diffusion against reservoirs containing 0.1 M Hepes (pH 7.5) and 1.3-2.0 M (NH$_4$)$_2$SO$_4$. Because TBBz and DMAT are not soluble in mother liquor, TBBz or DMAT (100 µM) was added to a final concentration of 2 mg/mL (77 µM) of NQO2. The NQO2-inhibitor complex was then concentrated 10-fold to 20 mg/mL and used for crystallization.

To obtain a structure of reduced NQO2 in complex with DMAT, reduction of NQO2$_{ox}$-DMAT crystals was performed as previously described (22). Briefly, N NQO2$_{ox}$-DMAT crystals were repeatedly soaked into 1 µL of a reducing soak solution with 0.1 M Hepes (pH 7.5), 2.0 M (NH$_4$)$_2$SO$_4$, 10 mM SCDP, and 1 mM DMAT, for 2 min intervals until the crystals were bleached. They were then transferred to a soak without SCDP before briefly being passed through a cryoprotectant solution [2.0 M (NH$_4$)$_2$SO$_4$, 0.1 Hepes (pH 7.5), and 20% glycerol] and plunged into liquid nitrogen. To prevent oxidation of NQO2, the entire crystal mounting process from harvesting to cryocooling was performed under an anoxic atmosphere in a glovebag purged with N$_2$.

4.2.7  X-ray Data Collection, Refinement, and Analysis.

Crystallographic data were collected at Canadian Light Source beamline 08ID-1 or from a rotating anode source, processed using MOSFLM (30), and merged using Scala (31). The structures were determined by molecular replacement with Protein Data Bank (PDB) entry 1QR2 as a starting model (32); refinement was conducted using PHENIX (33). Given the high resolution of the crystallographic data, NCS restraints were not used for any of the refinements. For structures with detectable anomalous scattering (NQO2$_{ox}$-
TBBz and NQO$_{\text{red}}$-DMAT), zinc and bromine were refined as anomalous groups. For the oxidized NQO2-DMAT structure, the high resolution of the data allowed for anisotropic refinement of the zinc and bromine atoms. These refinement strategies lowered both $R_{\text{work}}$ and $R_{\text{free}}$.

Topology files for TBBz and DMAT were generated using PHENIX.ELBOW (33). For refinement of the NQO$_{\text{red}}$-DMAT structure, the FAD topology file was modified to allow bending along the N5-N10 axis of FAD: the planarity restraints incorporating N5 and N10 of the isoalloxazine ring were removed so that it was separated into two planes, one incorporating the pyrimidine ring and the second the benzyl ring. Also, the estimated standard deviations of the bond lengths attached to N5 and N10 were increased 10-fold, and the bond angle and dihedral angle restraints involving N5 and N10 were removed.

To compare FAD bending among the three structures, each structure was refined three times using simulated annealing with the relaxed FAD topology parameters that allow for bending of the isoalloxazine ring. The degree of bending in the isoalloxazine ring was then calculated using principal component analysis incorporating all atoms in each of the two planes, and the angles in each of the three refined structures were averaged. The NQO$_{\text{ox}}$-TBBz, NQO$_{\text{ox}}$-DMAT, and NQO$_{\text{red}}$-DMAT final structures were deposited as PDB entries 4U7G, 4U7H, and 4U7F, respectively.
4.3 Results

4.3.1 NQO2 Is a Target of TBB, TBBz, and DMAT.

Halogenated benzotriazoles were identified as inhibitors of CK1 and CK2, with 4,5,6,7-tetrabromobenzotriazole (TBB) strongly inhibiting CK2 (7). From this starting point, brominated benzimidazoles, in particular, 4,5,6,7-1H-tetrabromobenzimidazole (TBBz) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) (Figure 4.1), that increased selectivity for CK2 were developed (11, 14). DMAT is the most potent of these compounds, exhibiting a 10-fold lower dissociation constant and a 3-fold lower IC\(_{50}\) compared to those of TBB and TBBz (Table 4.1). All three compounds target the ATP binding site of CK2, and on this basis, it is not surprising that they are also active against other kinases (15). On the other hand, it was surprising that both TBBz and DMAT were identified as interactors with NQO2, which neither is a kinase nor has a known ATP binding site (17). However, NQO2 does bind other kinase inhibitors (24, 27), raising the possibility that its active site mimics a kinase binding site. Our goal in this study was to characterize the binding and inhibition of NQO2 by these three compounds and compare their activities against NQO2 with those against CK2.

Table 4.1 Binding Affinity and Inhibition of TBB, TBBz, and DMAT towards CK2 and NQO2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(K_D)</th>
<th>(IC_{50})</th>
</tr>
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<tbody>
<tr>
<td><strong>CK2</strong></td>
<td><strong>NQO2</strong></td>
<td><strong>CK2</strong></td>
</tr>
<tr>
<td>TBB</td>
<td>400 nM</td>
<td>7.11 ± 0.04 µM</td>
</tr>
<tr>
<td>TBBz</td>
<td>700 nM</td>
<td>18.1 ± 0.4 nM</td>
</tr>
<tr>
<td>DMAT</td>
<td>40 nM</td>
<td>36.4 ± 1.7 nM</td>
</tr>
</tbody>
</table>

\(a\)\(K_D\) and \(IC_{50}\) values for CK2 were reported previously (14, 34).

\(b\)The dissociation constants for oxidized NQO2 were determined by fluorescence titrations; values are mean ± standard deviation for three independent titrations.

\(c\)\(IC_{50}\) values for NQO2 were determined by an *in vitro* assay with 150 µM SCDP and 5 µM menadione and varying concentrations of inhibitors. Kinetic data were fit to a sigmoidal response curve (Figure 4.2B); values are the estimated \(IC_{50}\) ± the range of the 95% confidence interval.

TBB, TBBz, and DMAT are planar aromatic compounds and as such are expected to bind to NQO2 by stacking onto the isoalloxazine ring of FAD. On this basis, fluorescence titrations were used to directly measure binding affinities for oxidized NQO2. Both TBBz
and DMAT bound tightly to NQO2, with \( K_D \) values of 18 and 36 nM, respectively, while TBB bound much more weakly with a \( K_D \) of 7.1 \( \mu \)M (Table 4.1 and Figure 4.2A).

To characterize the ability of these compounds to inhibit NQO2’s enzymatic activity, \( IC_{50} \) values were determined using an in vitro assay (Figure 4.2B). NQO2 operates by a ping-pong catalytic mechanism: the first step is reduction of the FAD isoalloxazine ring by dihydronicotinamide riboside (NRH), followed by dissociation of the oxidized nicotinamide and binding of a quinone substrate. For the assay, a commercially available NRH analogue, 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCDP), which exhibits a change in absorbance at 360 nm upon oxidation by NQO2, was used along with menadione as the quinone substrate. All three compounds inhibited the enzymatic activity of NQO2. The \( IC_{50} \) values were all higher than the dissociation constants, but the difference depended on the compound (Table 4.1). That is, the \( IC_{50} \) value for DMAT was > 12-fold higher than its \( K_D \), while the \( IC_{50} \) value for TBBz was 4-fold higher; for the relatively weak binding inhibitor, TBB, the \( IC_{50} \) was only slightly greater than the \( K_D \) (Table 1). This indicates that the compounds are inhibiting NQO2 through somewhat different mechanisms.

The activity profiles of TBB, TBBz, and DMAT against NQO2 are different from those against CK2. First, both TBB and TBBz bind to CK2 with similar affinity (\( K_D \) values of 400 and 700 nM, respectively), whereas with NQO2, the binding of TBB is almost 3 orders of magnitude weaker than that of TBBz. Second, a comparison of DMAT and TBBz shows that DMAT binds to CK2 with an affinity more than 1 order of magnitude greater than that of TBBz, whereas binding of DMAT to NQO2 is slightly weaker than that of TBBz (Table 1). Finally, the large differences between \( K_D \) and \( IC_{50} \) values observed for NQO2 (particularly in the case of DMAT) were not observed for CK2; instead, for CK2, the \( IC_{50} \) and \( K_D \) values for TBB and TBBz were almost equal, and the \( IC_{50} \) for DMAT was only 3-fold greater than the \( K_D \). On the basis of these binding and inhibition data, the properties of the NQO2 binding site do not fully mimic the properties of the CK2 ATP binding site. To improve our understanding of the interactions between NQO2 and the nanomolar inhibitors TBBz and DMAT, we performed a more detailed kinetic analysis to determine the mode of enzymatic inhibition.
(A) Representative data for binding of TBBz (▲), DMAT (▼), and TBB (●) to oxidized NQO2. Binding was characterized by fluorescence titrations of the inhibitors against oxidized NQO2 at a concentration of 38.75 nM (TBBz and DMAT) or 775 nM (TBB). The stirred sample was monitored at an excitation wavelength of 350 nm and an emission wavelength of 430 nm. The solid curves represent the binding isotherms, which were determined by nonlinear regression as outlined in Methods; the dissociation constants for the inhibitors are listed in Table 1. (B) Inhibition of steady-state catalysis by TBBz (▲), DMAT (▼), and TBB (●). Reactions were initiated with the addition of 154 pM NQO2 to 150 μM SCDP and 5 μM menadione and varying concentrations of the inhibitors; \( IC_{50} \) values were calculated from these data and are listed in Table 4.1.

4.3.2 Kinetic Analysis of Inhibition of NQO2 by TBBz and DMAT.

The ping-pong catalytic mechanism of NQO2 means that the active site cycles through an oxidized state and a reduced state for each catalytic turnover. In this study, the steady-state kinetic parameters of the uninhibited reaction were determined using the NRH analogue SCDP as the co-substrate and menadione as the substrate. Because previous studies have shown that menadione acts as a competitive inhibitor toward SCDP (29), substrate inhibition by menadione was incorporated into the kinetic model (equation 4.2, Methods). Consistent with previous results, our kinetic data (Figure 4.3) confirm that the binding of menadione to oxidized NQO2 \( [K_{i(Md)}] \) is similar to the Michaelis constant of menadione \( [K_{M(Md)}] \) (Table 4.2). Thus, menadione exhibits limited discrimination between the two redox states of NQO2.
Figure 4.3 Steady-state Ping-pong Kinetics of NQO2 with Substrate Inhibition. Steady-state kinetic analysis was performed to obtain the kinetic parameters listed in Table 2. Initial concentrations of SCDP were 10 (■), 25 (▲), 50 (▼), 100 (◆), and 365 (●) μM, and concentrations of menadione varied as indicated. Data points are measured rates, and solid curves represent the nonlinear regression fits derived from globally fitting all data to equation 4.1 (Methods) that describes a ping-pong kinetic mechanism with substrate inhibition by menadione. The $\chi^2$ value for the global fit was $7.4 \times 10^4$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$</td>
<td>$981 \pm 90.4$ s$^{-1}$</td>
</tr>
<tr>
<td>$K_{M(SCDP)}$</td>
<td>$142 \pm 23.2$ μM</td>
</tr>
<tr>
<td>$K_{M(menadione)}$</td>
<td>$1.62 \pm 0.31$ μM</td>
</tr>
<tr>
<td>$K_{I(menadione)}$</td>
<td>$8.07 \pm 1.48$ μM</td>
</tr>
</tbody>
</table>

*Calculated by globally fitting rate data to equation 4.2 as described in Materials and Methods.

An inhibitor of NQO2 can be competitive toward the SCDP co-substrate and/or the quinone substrate depending on whether the inhibitor binds preferentially to the oxidized or reduced form of NQO2. We used equation 4.4 (Methods) to account for the competitive inhibition model. In this model, each inhibitor is expected to compete with SCDP, menadione, or both, and the inhibition constants $K_{I(a)}$ and $K_{I(β)}$ describe the degree to which the presence of the inhibitor alters the apparent Michaelis constants for the reaction. Another way of viewing the $K_{I(a)}$ or $K_{I(β)}$ constants is that their relative values should indicate the preference of the inhibitor for either the oxidized or reduced form of NQO2, respectively. Global fitting of the rate data (Figure 4.4) to equation 4.4 yielded values of $K_{I(a)}$ and $K_{I(β)}$ for TBBz and DMAT as well as $k_{cat}$ values (Table 4.3). Thus, the inhibition constant of TBBz toward SCDP ($K_{I(a)} = 19.6 \pm 2.9$ nM) is very close to the binding affinity of TBBz for oxidized NQO2 ($K_D = 18.1 \pm 0.4$ nM). The high value and very high standard error for the inhibition constant of TBBz toward the quinone substrate,
Figure 4.4 Kinetic Analysis of NQO2 Inhibition by TBBz and DMAT.
Steady-state rate measurements of NQO2-catalyzed reduction of menadione were taken in the presence of TBBz (A and B) or DMAT (C and D) to obtain the values for $K_{I(\alpha)}$, $K_{I(\beta)}$, and $k_{cat}$ listed in Table 3. In panels A and C, assays included a constant menadione concentration of 5 µM, while in panels B and D, assays included a constant SCDP concentration of 150 µM. The concentrations of TBBz were 0 ( ■ ), 50 ( ▲ ), 100 ( ▼ ), and 500 ( ◆ ) nM (A) and 0 ( ■ ), 25 ( ▲ ), 50 ( ▼ ), and 100 ( ◆ ) nM (B). The concentrations of DMAT were 0 ( ■ ), 100 ( ▲ ), 500 ( ▼ ), and 1000 ( ◆ ) nM (C) and 0 ( ■ ), 25 ( ▲ ), 50 ( ▼ ), 100 ( ◆ ), and 500 ( ● ) nM (D). The solid curves in the panels represent global fits to all of the data in panels A and B [to obtain $K_{I(\alpha)}$, $K_{I(\beta)}$, and $k_{cat}$ for inhibition by TBBz] or panels C and D [to obtain $K_{I(\alpha)}$, $K_{I(\beta)}$, and $k_{cat}$ for inhibition by DMAT]. The $\chi^2$ value for the global fit to TBBz-inhibited NQO2 (A and B) was $1.4 \times 10^5$, while that for the DMAT-inhibited NQO2 (C and D) was $4.6 \times 10^4$.

Table 4.3 Inhibition of NQO2 by TBBz and DMAT

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{I(\alpha)}$ (nM)</th>
<th>$K_{I(\beta)}$ (nM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBz$^c$</td>
<td>19.3 ± 2.5</td>
<td>801 ± 3280</td>
<td>948.0 ± 34.4</td>
</tr>
<tr>
<td>DMAT$^d$</td>
<td>131.2 ± 27.5</td>
<td>252.0 ± 124.0</td>
<td>929.9 ± 24.7</td>
</tr>
</tbody>
</table>

$^a$ $K_{I(\alpha)}$ is the component of the inhibition that is competitive against SCDP.

$^b$ $K_{I(\beta)}$ is the component of the inhibition that is competitive against menadione.

$^c$ The parameters (with standard errors) were calculated by globally fitting the rate data illustrated in Figure 4.4, Panels A and B.

$^d$ The parameters (with standard errors) were calculated by globally fitting the rate data illustrated in Figure 4.4, Panels C and D.
menadione ($K_{i(β)} = 865 ± 3790 \text{nM}$), indicate that the interaction of TBBz with the oxidized form of NQO2 is sufficient to completely account for TBBz-mediated inhibition, and therefore, binding to the reduced form cannot be assessed using steady-state kinetics. The situation for DMAT is more complicated: the inhibition constant of DMAT toward SCDP ($K_{i(α)} = 121 ± 20 \text{nM}$) is 3-fold higher than its affinity for oxidized NQO2 ($K_D = 36.4 ± 1.7 \text{nM}$). Furthermore, we find that the inhibition constant of DMAT toward menadione ($K_{i(β)} = 302 ± 131 \text{nM}$) is similar to its inhibition constant against SCDP. This indicates that DMAT is binding to both oxidized and reduced NQO2; it exhibits relatively poor discrimination between the two. This was surprising but not unprecedented as the substrate menadione also shows poor discrimination between the two redox states of NQO2; however, DMAT is the first inhibitor shown to exhibit similar affinity for both redox states of NQO2.

**4.3.3 Structures of Oxidized NQO2 with TBBz and DMAT.**

Crystal structures of NQO2–inhibitor complexes were used to determine the structural basis for binding of TBBz and DMAT to oxidized NQO2. Cocrystallization of NQO2 and TBBz yielded crystals that diffracted to 1.86 Å; the structure was determined by molecular replacement and refined to an $R_{free}$ value of 0.2288 (Table 4.4). Crystals of NQO2 contain a dimer in the asymmetric unit, and TBBz was modeled in two orientations in both active sites of the NQO2 dimer. Because TBBz has four bromine atoms that contribute to anomalous scattering, the first orientation of TBBz was modeled using an anomalous difference map that indicated four prominent peaks (two peaks at 5–6 $σ$ and two peaks at 4 $σ$) in the active site of NQO2 (Figure 4.5A). In the first orientation, the tetrabromobenzene makes $π$-stacking interactions with the FAD isoalloxazine ring and the phenyl ring of F178. Bromine atoms 4 and 5 of TBBz make halogen bonds (35) to the backbone carbonyl of G174 and to the carboxyamide of N161. The benzimidazole nitrogen makes a $π$-hydrogen bond (36) with the benzene ring of the W105 indole side chain. During the refinement, inconsistencies in the relative values for the atomic displacement parameters (ADPs) within the imidazole ring of TBBz suggested that TBBz was binding in an alternative orientation (Figure 4.5B). The second orientation of TBBz is related by a 60° rotation around the benzene ring that places a bromine atom
Figure 4.5 Binding of TBBz and DMAT to Oxidized (A-D) and Reduced (E-F) NQO2

Complexes between oxidized NQO2 and TBBz or DMAT (A–D) show the inhibitors are sandwiched between the isoalloxazine ring of FAD (yellow) and F178 (above the plane of the inhibitor). (A) In one orientation, TBBz makes two halogen bonds and a \(\pi\)-hydrogen bond to N161, G174, and W105, respectively. (B) In its second orientation, TBBz is rotated 60° to make three halogen bonds with N161, G174, and W105. (C) DMAT makes two halogen bonds to N161 and G174 in one orientation, but the presence of the dimethylamine group hinders the imidazole nitrogen from \(\pi\)-hydrogen bonding with W105. (D) In the second orientation, DMAT makes three halogen bonds with N161, G174, and W105, similar to what was observed for TBBz. In the structure of reduced NQO2 (E and F), DMAT occupies a more peripheral position, makes fewer \(\pi\)-stacking interaction with FAD, no longer contacts F178, and instead makes somewhat stronger hydrophobic contact with F126. (E) In one orientation of DMAT, bromine 6 makes a halogen bond to a water molecule, which is in turn bonded to N161, while bromine 5 makes a halogen bond to W105. (F) In the second orientation with reduced NQO2, DMAT is rotated 60° and the same halogen bonds are mediated by bromines 4 and 5. The electron density maps represent the \(F_o - F_c\) (green mesh) and anomalous (magenta mesh) differences calculated after simulated annealing with the inhibitors omitted from the structure; all electron density maps were contoured at 3\(\sigma\) around the inhibitors. In all three structures, the inhibitor binding orientations are the same for both subunits (the A and B chains) of the NQO2 dimer that constitutes the asymmetric unit of the crystal.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Oxidized NQO2</th>
<th>Reduced NQO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBz</td>
<td>1.54</td>
<td>1.54</td>
</tr>
<tr>
<td>DMAT</td>
<td>1.03</td>
<td>1.03</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Crystal Structure Data Collection and Refinement Statistics</th>
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<tbody>
<tr>
<td><strong>Crystal</strong> Oxidized NQO2 Reduced NQO2</td>
</tr>
<tr>
<td><strong>Wavelength (Å)</strong> 1.54 1.03 1.54</td>
</tr>
<tr>
<td><strong>Space Group</strong> P2₁2₁ P2₁2₁ P2₁2₁</td>
</tr>
<tr>
<td><strong>Unit Cell Dimensions (Å)</strong> 56.24, 83.17, 106.65 56.25, 83.01, 106.48 56.12, 83.14, 106.46</td>
</tr>
<tr>
<td><strong>Resolution</strong> 18.18-1.91 30.02-1.45 17.99 - 1.73</td>
</tr>
<tr>
<td><strong>aRₜₚₚ</strong> 0.098 (0.57) 0.06 (0.43) 0.073 (0.66)</td>
</tr>
<tr>
<td><strong>aI/σ(I)</strong> 7.5 (2.0) 9.7 (1.7) 14.8 (2.6)</td>
</tr>
<tr>
<td><strong>aCompleteness</strong> 92.8 (57.3) 96.0 (74.5) 89.9 (74.5)</td>
</tr>
<tr>
<td><strong>Redundancy</strong> 4.4 4.6 6.2</td>
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<tr>
<td><strong>Unique Reflections</strong> 36697 85781 47314</td>
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<tr>
<td><strong>Rₜₒₜ/Rₚₒₑ</strong> 0.1745/0.2288 0.1843/0.1958 0.1840/0.2254</td>
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**Ramachandran Plot**

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<th>Most Favoured</th>
<th>90.2%</th>
<th>91.7%</th>
<th>90.7%</th>
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</thead>
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<td>Additionally Allowed</td>
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<td>7.8%</td>
<td>8.8%</td>
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<tr>
<td>Generously Allowed</td>
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<td>0.5%</td>
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</tr>
<tr>
<td>Disallowed</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

**RMS Deviations**

| Bond Lengths (Å) | 0.009 | 0.010 | 0.009 |
| Bond Angles (deg) | 1.150 | 1.353 | 1.128 |

**Mean A.D.P. values (Å²)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>All Atoms</th>
<th>20.990</th>
<th>21.913</th>
<th>23.698</th>
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<td>Side Chain</td>
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<td>24.809</td>
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<td>Solvent</td>
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</tr>
<tr>
<td>Water</td>
<td>27.553</td>
<td>31.850</td>
<td>26.960</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>22.305</td>
<td>22.682</td>
<td>25.113</td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td>26.988</td>
<td>46.165</td>
<td>37.759</td>
<td></td>
</tr>
</tbody>
</table>

* a Values in parentheses refer to the highest resolution shell.

* b Ramachandran plot statistics were calculated using PROCHECK (Laskowski et al. 1993).
in the position of one of the benzimidazole nitrogens. Refinement of the structure with the two TBBz orientations yielded consistent values for the ADPs. As a result of the rotation, the halogen bonds to G174 and N161 are maintained but are made with bromines 5 and 6 rather than bromines 4 and 5 as in the first orientation. In addition, the π-hydrogen bond to the W105 indole is replaced with an interaction between bromine 4 and the W105 side chain. Thus, both orientations of TBBz involve similar interactions with NQO2.

Oxidized NQO2 was also co-crystallized with DMAT. Data from an NQO2_{ox}–DMAT crystal were collected to 1.5 Å, and the structure was refined to an $R_{\text{free}}$ value of 0.2023 (Table 4.4). Because these data were collected from a synchrotron source at a wavelength farther from the bromine edge, the anomalous signal for bromine was weaker. Nonetheless, DMAT was modeled in two orientations by anchoring the bromines to anomalous scattering peaks (Figure 4.5C,D). Like the NQO2_{ox}–TBBz complex, DMAT makes π-stacking interactions with the FAD isoalloxazine ring and the phenyl ring of F178. The first orientation of DMAT is similar to that of TBBz, in which the inhibitor makes two halogen bonds to the backbone carbonyl of G174 and the carboxamide of N161; however, the dimethylamine substituent of DMAT prevents the benzimidazole nitrogen from making a π-hydrogen bond with W105. The alternate orientation of DMAT (Figure 4.5D) is rotated 60° clockwise and binds in an orientation almost identical to that of TBBz (Figure 4.5B). As one might anticipate from their structures, TBBz and DMAT show very similar modes of binding to the NQO2 active site. The fact that the dimethylamine moiety of DMAT prevents it from binding optimally in the preferred mode explains its slightly lower affinity for NQO2 (a $K_D$ of 36 nM compared to a $K_D$ of 18 nM for TBBz).

4.3.4 Binding of DMAT to Reduced NQO2.

Our kinetic analysis indicated that DMAT binds with a similar affinity to the reduced and oxidized forms of NQO2. To elucidate the mode of binding of DMAT to reduced NQO2, crystals of oxidized NQO2 in complex with DMAT (NQO2_{ox}–DMAT) were reduced in a soaking solution containing SCDP. A data set from a crystal of the NQO2_{red}–DMAT complex was collected to 1.90 Å and the structure refined to an $R_{\text{free}}$ value of 0.2268
(Table 4.4). It was clear that reduction of the FAD co-substrate had a major effect on binding of DMAT: it was less deeply buried in NQO2\text{red} than in NQO2\text{ox}, and the space made available was filled with water molecules that mediate interactions between DMAT and reduced NQO2. As with the NQO2\text{ox}-DMAT complex, DMAT bound to the reduced enzyme in two orientations: this was clear from the presence of three strong anomalous scattering peaks representing the common positions of the bromine atoms in the two orientations (Figure 4.5 E,F). Two of the bromines interact with three water molecules (numbers 32, 16, and 85) that in turn are hydrogen-bonded to the backbone carbonyl of G174 and the carboxyamide of N161. The third common bromine atom makes a π-halogen bond to the W105 indole group. The less deeply buried position of DMAT in the reduced NQO2 structure means that it no longer makes π-stacking interactions with F178, and the area in contact with the isoalloxazine ring is also reduced.

4.3.5 Structural Changes in FAD in the NQO2\text{red}-DMAT Complex.

The reduction of the planar isoalloxazine ring of FAD leads to a “butterfly bend” along the N5-N10 axis (37). On this basis, refinement of the NQO2\text{red}-DMAT structure was conducted using a modified stereochemical restraint set for FAD, in which planar restraints and associated bond and angle restraints around N5 and N10 were removed. The final refined structure indicated butterfly bend angles of 3.0° and 5.4° for the isoalloxazine ring systems in chains A and B, respectively. To validate that the small change in the FAD isoalloxazine structure was due to reduction of the FAD, and not simply removal of stereochemical restraints, the refined NQO2 structures were further subjected to simulated annealing refinement against data from both oxidized and reduced crystals (Table 4.5). In the absence of stereochemical restraints around N5 and N10, the isoalloxazine ring of NQO2\text{ox} in complex with TBBz refined to 1.8° and 2.4° for each FAD co-substrate; for NQO2\text{ox} in complex with DMAT, the bend was 2.7° and 2.4° for each FAD co-substrate. Thus, FAD is bent approximately 2° upon reduction and binding of DMAT.
Table 4.5 Conformational Changes in the Isoalloxazine Ring upon Reduction

<table>
<thead>
<tr>
<th>NQO2</th>
<th>Inhibitor</th>
<th>Subunit</th>
<th>N5-N10 bend a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>TBBz</td>
<td>A</td>
<td>1.81 ± 0.06</td>
</tr>
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<td></td>
<td></td>
<td>B</td>
<td>2.44 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>2.13</td>
</tr>
<tr>
<td>DMAT</td>
<td>A</td>
<td>2.74 ± 0.01</td>
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</tr>
<tr>
<td></td>
<td>B</td>
<td>2.39 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>DMAT</td>
<td>A</td>
<td>2.95 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.37 ± 0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>4.16</td>
<td></td>
</tr>
</tbody>
</table>

a Both oxidized and reduced structures were subjected to three separate rounds of simulated annealing refinement in the absence of planar restraints for N5-N10 axis and angle restraints for the C4X-N5-C5X/C10-N10-C9A angles.

b The “butterfly” bend along the N5-N10 axis was calculated using the atomic positions of the dimethylbenzene and pyrimidine “wings” and principle component analysis to find the angle between the two best-fit planes.
4.4 Discussion

TBB, TBBz, and DMAT are ATP-competitive inhibitors of CK2 that reduce the level of autophosphorylation of CK2β by CK2α and CK2α′. The three inhibitors induce apoptosis in HeLa, Jurkat, and HL-60 cells (10, 14, 17, 34) ostensibly by preventing CK2 function to promote cell survival over apoptosis (3, 38, 39). However, both TBBz and DMAT, but not TBB, were potent inducers of apoptosis in cells expressing an inhibitor-resistant CK2. Furthermore, in cells expressing wild-type CK2, both TBBz and DMAT are more potent inducers of apoptosis than TBB (10, 14, 17, 34). On this basis, interactions of TBBz and DMAT with molecule(s) other than CK2 appear to contribute to their induction of apoptosis. Through a proteomics screen, NQO2 was identified as a target of TBBz and DMAT, but not TBB. In the study presented here, we have shown that both TBBz and DMAT bind to and inhibit NQO2 at nanomolar concentrations, whereas the interaction between NQO2 and TBB is much weaker. The interactions of TBB, TBBz, and DMAT with NQO2 therefore correlate with their apoptotic effects in cells transformed with inhibitor-resistant CK2 (17). Because several other kinase inhibitors also bind NQO2 (24, 25, 27, 40), we sought to understand the structural basis for the interaction between NQO2 and TBBz or DMAT. In particular, we wanted to determine how similar the active site of NQO2 was to the ATP binding site of CK2 and other kinases.

While TBB, TBBz, and DMAT all bind CK2 with nanomolar affinity (11, 14, 41) (Table 4.1), the affinity of TBB for oxidized NQO2 is 3 orders of magnitude lower than that of TBBz and DMAT (Table 4.1). Similarly, the IC₅₀ values of TBB, TBBz, and DMAT for inhibition of NQO2 are dramatically different (Table 4.1), with TBB exhibiting much weaker inhibition of NQO2, consistent with its relatively low binding affinity. The difference in binding affinities between TBB and TBBz/DMAT can be explained by the charge of the inhibitors (Figure 4.1). The triazole function of TBB has an estimated pKₐ of 5 and is expected to be anionic at physiological pH, whereas the imidazole functions of TBBz and DMAT have pKₐ values of approximately 9 and will therefore be neutral at physiological pH (34). In CK2, the negatively charged triazole portion of TBB interacts with a region of positive electrostatic potential (Figure 4.6A,B) (6, 42). In the case of oxidized NQO2, the surface of the active site has a negative electrostatic potential that
precludes high-affinity interactions with anionic TBB (Figure 4.6C). Thus, differences in the electrostatic potential of the active sites of NQO2 and CK2 explain why TBB binds with a relatively low affinity to the NQO2 active site.

DMAT and TBBz bind with a similar high affinity to oxidized NQO2, and our structural analysis shows that they bind in a similar manner, stacking on top of the isoalloxazine ring of FAD with the bromine atoms making halogen bonds to nearby carbonyl groups as well as the indole side chain of W105 (Figure 4.5). Both TBBz and DMAT bind in two orientations. For TBBz, the preferred orientation is one in which two bromine atoms and one of the imidazole nitrogens make bonds with two carbonyl groups and W105, respectively (Figure 4.5A). The small difference in affinity between TBBz and DMAT is likely due to the inability of DMAT to fully adopt the preferred conformation (Figure 4.5A) because of a steric clash between its dimethylamine function and the indole side chain of W105 (Figure 4.5C). Structures of CK2 in complex with TBBz and DMAT show that the two inhibitors bind to the CK2 active site in an identical manner (PDB entries 1ZOE and 2OXY, respectively) (42, 43). In CK2, the two drugs are sandwiched between hydrophobic residues and two of the bromine atoms make halogen bonds with backbone carbonyl groups of E114 and V116 (Figure 4.6B). In the case of CK2, DMAT binds with 10-fold greater affinity than TBBz because of additional favorable interactions mediated by the dimethylamine group.

In summary, the NQO2 active site appears to mimic the adenine binding site of CK2 in the sense that it provides a hydrophobic crevice that can accommodate planar aromatic molecules, and there are two carbonyl groups that are well placed to mediate charge interactions with the bromine atoms of these inhibitors in a manner similar to that observed in CK2. On the other hand, NQO2 provides an environment that allows both TBBz and DMAT to bind in alternate orientations, whereas binding to CK2 is limited to a single orientation. Furthermore, the presence of the dimethylamine group on DMAT favors binding to CK2 but disfavors binding to NQO2. These observations, in addition to the striking differences in the electrostatic potential in the two active sites, indicate that the binding of TBBz and DMAT to NQO2 is adventitious and on the whole the NQO2 active site is not a good mimic of the CK2 active site.
Figure 4.6 Binding of DMAT to CK2, Oxidized NQO2, and Reduced NQO2.

(A) The molecular surface of the CK2 active site is shown with bound DMAT (PDB entry 1ZOE, (42)) and colored according to electrostatic potential. (B) Details of interactions between DMAT (orange carbons) and the CK2 active site. Note that TBBz (not displayed) binds in exactly the same manner as DMAT(42). The position of CK2-bound TBB (green carbons, PDB entry 1J91,(44)) is also shown: the negative charge of the triazole moiety (Figure 4.1) causes a shift toward a more positively charged region in the CK2 active site (42). (C) Molecular surface of the active site in oxidized NQO2 with bound DMAT in two orientations. (D) Details of the binding of DMAT to oxidized NQO2. (E) Molecular surface of reduced NQO2 with bound DMAT in two orientations, including three water molecules (cyan) that are present in the reduced, but not the oxidized, structure. (F) Details of the binding of DMAT to reduced NQO2. Electrostatic potentials were calculated using APBS (45), and partial charges were calculated using PDB entry 2PQR (46). In the case of oxidized FAD, the sum of the partial charges in the isoalloxazine ring yielded an overall charge of -0.2; for reduced FAD, an additional full charge was added and distributed roughly evenly between the N1 and O2 atoms. The figures were made in PyMol (version 1.7.0.5, Schrödinger, LLC), and the color limits for the electrostatic surfaces were set to -12 kT (red) to 12 kT (blue) for all three structures.
Another major difference between the active sites of NQO2 and CK2 is that the NQO2 active site can exist in either an oxidized or reduced form. The origin of the change in oxidation state is the isoalloxazine ring of FAD: upon interaction with a reduced nicotinamide co-substrate (NRH or SCDP), two electrons are transferred onto the isoalloxazine ring of FAD (47). Upon reduction, the N5 atom is protonated and its orbital changes from planar sp$^2$ to trigonal pyramidal sp$^3$ hybridization. These changes allow it to donate a hydrogen bond, and the change in orbital hybridization also disrupts the planarity of the isoalloxazine ring, leading to a small butterfly bend. Thus, reduction of FAD leads to a relatively minor structural change in the isoalloxazine ring but more importantly imparts a negative charge that will be largely centered around N1 and O2 of the isoalloxazine ring. Our crystallographic study indicates that reduction of the isoalloxazine ring completely changes the mode of binding of DMAT: it binds in a more peripheral location, and the interactions of the bromine atoms with the carbonyl groups are mediated through intervening water molecules.

Compounds that bind with high affinity to only one form of NQO2, either oxidized or reduced, exhibit predictable effects on the steady-state kinetics of the system. Thus, TBBz binds with high affinity to oxidized NQO2 and competes with SCDP, resulting in significant changes in the apparent Michaelis constant for SCDP; furthermore, the inhibition constant against SCDP is close to its dissociation constant, as expected. This does not mean that TBBz completely fails to bind to reduced NQO2; in fact, given the similarity between TBBz and DMAT, it is likely that TBBz binds to the reduced form of NQO2 in a manner similar to that observed for DMAT. However, because the affinity of the interaction between TBBz and reduced NQO2 is relatively weak compared to binding of TBBz to oxidized NQO2, the inhibition observed in the steady-state kinetic analysis is due almost entirely to its binding to oxidized NQO2, and therefore, TBBz has little impact on the Michaelis constant for menadione.

The situation appears to be more complicated for an inhibitor such as DMAT that binds with similar affinities to both oxidized and reduced NQO2. The lower affinity of DMAT for oxidized NQO2 made the interaction between DMAT and reduced NQO2 apparent in our steady-state kinetic analysis, where the presence of DMAT had a significant effect on
the Michaelis constants for both SCDP and menadione. In this case, changes in both Michaelis constants were used to calculate inhibition constants ($K_I$ values) of 121 nM against SCDP and 302 nM against menadione, both of which are significantly higher than the dissociation constant (36 nM) for binding of DMAT to oxidized NQO2. This is a confusing result because the inhibition constant is expected to represent a dissociation constant for the inhibitor. The relatively high $K_I$ values for DMAT are in line with the high IC$_{50}$ for DMAT, which is 6-fold higher than the IC$_{50}$ for TBBz, despite the fact that DMAT binds to oxidized NQO2 with a $K_D$ that is only twice as high as that of TBBz. It seems as though the ability of DMAT to bind with similar affinities to both the oxidized and reduced forms of NQO2 actually weakens its ability to inhibit NQO2-mediated catalysis. Although we can only speculate about the molecular origins of this effect, it highlights the complicated nature of NQO2 and the fact that binding interactions may have unpredictable effects on catalysis and, by extension, in vivo function.

### 4.4.1 Inhibition of NQO2 by Other Kinase Inhibitors

Interestingly, NQO2 was also found to be the only non-kinase target of two Abl kinase inhibitors as well as protein kinase C bisindolylmaleimide inhibitors (24, 25, 27). Specifically, the cancer wonder drug imatinib and its second-generation derivative, nilotinib, were found to bind and inhibit NQO2. Structural investigation showed that rings A and B of imatinib that occupy the ATP binding site of the Abelson kinase were also found to occupy the active site of NQO2 (26). While imatinib does not inhibit CK2 (48) and CK2 inhibitors do not inhibit the BCR-ABL kinase (15), both were found to interact with the non-kinase target NQO2 (17, 25). Therefore, there are several classes of kinase inhibitors that bind NQO2 fortuitously, and the specific cellular effects of these kinase inhibitors may be due in part to their interaction with NQO2.

One of the cellular functions of NQO2 may be the detoxification of quinones by two-electron reduction; however, the natural substrates have not been identified, and it is not obvious why NQO2 would have evolved a preference for NRH over NAD(P)H to fulfill a detoxification function. A second possible function for NQO2 may involve cellular signalling in response to changes in metabolic and/or redox status. In particular, NQO2 was shown to stabilize p53 from 20S proteasomal degradation in the presence of NRH.
While the canonical degradation pathway of p53 is ubiquitin-dependent and uses the 26S proteasome, degradation by the 20S proteasome is ubiquitin-independent and is modulated by quinone reductase 1 (NQO1) and NQO2 (20, 21, 49). Interestingly, NQO1, the “sister enzyme” of NQO2, uses NAD(P)H as a co-substrate and is specific for a different set of inhibitors. Given their difference in co-substrate specificity, NQO1 and NQO2 may be regulating p53 stability in response to changes in the cellular redox state, as reflected in the levels of NAD(P)H and NRH, but this remains a matter of speculation. Strikingly, numerous NQO2 inhibitors such as melatonin, resveratrol, chloroquine, imatinib, 9-aminoacridine, and quinacrine have been shown to induce p53-dependent apoptosis (50–54). Melatonin and resveratrol both induce p53 phosphorylation at serine 15, which is essential to the transactivation of p53-induced apoptotic genes (50, 53); chloroquine, 9-aminoacridine, and quinacrine, on the other hand, increase p53 levels without phosphorylation at serine 15, which is thought to lead to transcription-independent apoptosis modulated by p53 (51, 54, 55). The fact that NQO2 binds all of these bioactive compounds is remarkable, but a contribution from NQO2 to the cellular effects of these compounds has yet to be demonstrated.

The cellular roles of NQO2 are still very much a matter of debate. In this regard, the cellular effects of two high-affinity (nanomolar) inhibitors of NQO2 have been investigated. S29434 was developed by the French pharmaceutical company Servier as a compound that inhibits the third melatonin binding site (MT3), which is NQO2, without inhibition of the two other melatonin binding sites, both of which are G-protein coupled receptors (56, 57). S29434 prevents generation of reactive oxygen species in vitro and in vivo, and it prevents paraquat induced toxicity to cell lines in a ROS-dependent manner (58, 59). Similarly, NQO2 knockout mice were resistant to menadione induced toxicity (60), which likely occurs through oxidative stress resulting from NQO2-mediated metabolism of menadione. Therefore, inhibitors of NQO2 can prevent cell death caused by compounds that are toxic when they are metabolized by NQO2; however, inhibition of NQO2 appears to have little effect on cells in the absence of these toxic compounds.

A second series of inhibitors, based on an imidazoacridin-6-one core, was identified through a computational screen of the National Cancer Institute chemical database (61).
The compound that was initially identified (C1311 or NSC645809) is a known DNA intercalating agent and an inhibitor of FLT3 kinase (62). This compound, as well as related imidazoacridin-6-ones, inhibits NQO2 in the nanomolar range and is cytotoxic to cells. N-Oxide derivatives of the imidazoacridin-6-one compounds that retained nanomolar affinity for NQO2 but had reduced DNA intercalating properties were developed (61). Surprisingly, the presence of the N-oxide modification also decreased cytotoxicity by 10 to 20-fold in a variety of cell lines. As such, it appears that the apoptotic effects of this class of inhibitors are due largely to their ability to intercalate DNA. It was also noteworthy that the seven breast cancer cell lines tested in the study exhibited up to 50-fold differences in NQO2 activity but virtually no difference in their sensitivity to the imidazoacridin-6-one inhibitors (61). The N-oxide derivatives of the imidazoacridin-6-one compounds were much less toxic than the parent compounds but still had toxicity IC$\text{}_{50}$ values in the low micromolar range; this toxicity was probably not due to inhibition of NQO2, because the IC$\text{}_{50}$ values for NQO2 inhibition were typically 2–3 orders of magnitude lower than the toxicity IC$\text{}_{50}$ values. That is, for N-oxide derivatives of the imidazoacridin-6-one compounds, concentrations that would completely inhibit NQO2 do not appear to be cytotoxic.

From the studies with S29434 and the N-oxide derivatives of imidazoacridin-6-one compounds, it appears that inhibition of NQO2 is not cytotoxic; similarly, NQO2 knockout mice were almost identical to wild-type littermates, although there were some differences, including myeloid hyperplasia due to a decreased level of apoptosis of myeloid cells (60). The NQO2 knockout mice were also more susceptible to benzopyrene induced carcinogenesis, and the absence of NQO2 prevented TNFα-induced apoptosis in an NFκB-dependent manner (63, 64). In summary, it appears that, on its own, inhibition of NQO2 or genetic knockout is not toxic to cells. Instead, it may be that inhibition or removal of NQO2 in conjunction with other “inputs” – metabolic or oxidative stress, drug mediated changes in the activity of kinases, DNA damage, and so on – leads to more pronounced toxicity and/or other cellular effects. Because kinase inhibitors are designed to be kinase specific, the fact that a number of efficacious inhibitors bind NQO2 with high affinity challenges the “single-target” approach in drug discovery. It may be that binding of both NQO2 and kinases is important for the cellular effects of the inhibitors,
including enhanced apoptosis of transformed cells. As CK2 and other kinase inhibitors continue to be developed and used as therapeutics, it will be crucial to characterize off-target interactions with NQO2 and possibly other non-kinase targets to fully understand the cellular effects of these compounds and the roles of the individual targets in these effects.
4.5 References


64. Iskander, K., Paquet, M., Brayton, C., and Jaiswal, A. K. (2004) Deficiency of NRH:quinone oxidoreductase 2 increases susceptibility to 7,12-dimethylbenz(a)anthracene and benzo(a)pyrene-induced skin carcinogenesis. Cancer Res. 64, 5925–8
Chapter 5

The Binding of DNA Intercalating Agents to Oxidized and Reduced Quinone Reductase 2

Quinone reductase 2 (NQO2) is an enzyme that may also have intracellular signalling functions. A wide range of planar aromatic compounds bind NQO2, and we have identified three DNA intercalating agents – ethidium bromide (EtBr), acridine orange (AO), and doxorubicin – as novel nanomolar inhibitors of NQO2. The cationic EtBr and AO bound reduced NQO2 with 50-fold higher affinity than oxidized NQO2, while doxorubicin only bound oxidized NQO2. Crystallographic analysis of oxidized NQO2 in complex with the inhibitors indicated that inhibitors bound oxidized NQO2 deeply in the active site. The aromatic faces were sandwiched between the isoalloxazine ring of FAD and the phenyl ring of F178, and their edges making direct contacts with active site residues. In reduced NQO2, EtBr and AO occupied a more peripheral position in the active site, allowing several water molecules to interact with the polar end of the negatively charged isoalloxazine ring. We also showed that AO inhibited NQO2 at a nontoxic concentration in cells while EtBr was less effective at inhibiting NQO2 in cells. Together, this study suggested a role of NQO2 as a sensor for potential DNA intercalating agents.

5.1 Introduction

Quinone Reductase 2 (NQO2) was historically classified as a detoxification enzyme responsible for the reduction of potentially cytotoxic quinones(1). Similar to its sister enzyme Quinone Reductase 1 (NQO1), NQO2 catalyzes an obligate 2-electron transfer to generate the hydroquinone, thereby preventing production of reactive semi-quinones. While NQO1 efficiently catalyzed quinone reduction using NAD(P)H as a reducing coenzyme \( k_{\text{cat}}/K_M = 440 \text{ min}^{-1} \cdot \mu\text{M}^{-1} \), NQO2 used NAD(P)H inefficiently, with a catalytic efficiency 700-fold lower than NQO1 \( k_{\text{cat}}/K_M = 0.62 \text{ min}^{-1} \cdot \mu\text{M}^{-1} \) (2). On this
basis, it appears that NQO2 has evolved to avoid using the canonical reducing coenzymes NADH and/or NADPH. Instead, NQO2 exhibits a preference for unusual, and possibly non-physiological, reducing coenzymes, such as dihydro-nicotinamide riboside (NRH) (2). Although nicotinamide riboside (NR\(^+\)) participates in NAD metabolism, it is currently unknown how the reduced form, NRH, is generated in cells. On this basis, the availability of the reducing coenzyme for NQO2 in cells is currently an open question. Furthermore, the only cellular quinone substrate of NQO2 is ubiquinone, which resides in the mitochondria and is inaccessible to NQO2 (3). Given its inefficient utilization of NAD(P)H and lack of physiologically relevant quinone substrates, NQO2 may have important cellular functions beyond reduction of quinones.

NQO2 has been implicated in cell signalling as well as the formation of reactive oxygen species (4–6). Specifically, NQO2 modulated the 20S proteosomal degradation of p53 in a NRH-dependent manner (4, 7). Furthermore, NQO2 also indirectly regulates the AKT/cyclin-D1/Rb and NFκB signalling pathways (5, 8). Numerous bioactive compounds interact with NQO2, raising the possibility that some of the cellular effects of the compounds may be attributed to NQO2. These compounds ranged from targeted kinase inhibitors (imatinib and nilotinib (9, 10), CK2 inhibitors (11), PKC inhibitors (12)), antimalarial compounds (primaquine, chloroquine, and quinacrine (13)), natural flavonoids (quercetin (2)), stilbenoid (resveratrol (14)), and hormones (melatonin (15)). All of these compounds perturb cellular proliferation and/or apoptosis, but the role of NQO2 in these processes is not known.

The active site of NQO2 is composed of a large hydrophobic pocket with a planar aromatic surface provided by the isoalloxazine ring of the FAD cofactor (16). As such, the planar aromatic portions of NQO2 inhibitors bind to the active site through pi-pi stacking interactions with the isoalloxazine ring and hydrophobic residues. Given the planar nature of NQO2 inhibitors, it is perhaps not surprising that a number of them, such as imatinib, quinacrine, and 9-aminoacridine can also intercalate DNA (17–19). In fact, several compounds known to intercalate into DNA (9-aminoacridine and C1311) were identified from an in silico screening of NQO2 inhibitors (8, 20). Given the potential
roles of NQO2 in cell signalling and the nature of compounds that bind NQO2, we investigated whether NQO2 is a target for additional DNA intercalating agents.

DNA intercalating compounds are polycyclic aromatic molecules that act as potent cytotoxic and mutagenic agents to cells. In 1961, Leonard Lerman proposed and eventually showed that these compounds can unwind and extend DNA by inserting themselves between two nucleotide bases (21). In highly proliferating cells, these compounds are cytostatic by inhibiting DNA replication or transcription (22, 23). For cells that overcome replicative stress, the intercalating agents elongate DNA and cause DNA slippage leading to indel mutations during DNA replication (24, 25). When present in the environment, cells can deal with these harmful compounds by active excretion through efflux pumps such as P-glycoprotein (multidrug resistant-associated protein) and/or metabolism by cytochrome P450 (26); however, cells unable to clear DNA intercalators are at risk of cell growth retardation and genomic instability.

Since DNA intercalators can be detrimental to cells, we hypothesize that NQO2 can recognize potential DNA intercalating agents and function, at least in part, as a sensor for these compounds. In this study, we have tested three well-characterized DNA intercalating agents – ethidium bromide (EtBr), acridine orange (AO), and doxorubicin – and found that they are all nanomolar inhibitors of NQO2. We further characterized these three novel inhibitors of NQO2 by means of enzyme kinetics, isothermal titration calorimetry, and crystallographic analysis. We found that the cationic EtBr and AO bound reduced NQO2 with a 50-fold higher affinity than oxidized NQO2; this can be explained by the increased negative electrostatic potential in the active site of reduced NQO2. In this regard, we have identified AO to be the highest affinity inhibitor for reduced NQO2, with a dissociation constant of 0.36 nM. Structurally, the inhibitors bind to the NQO2 active site in a manner that resembles their interaction with DNA. Lastly, we demonstrate that a non-toxic concentration of AO can inhibit NQO2 activity in cells.
5.2 Materials and Methods

5.2.1 Reagents

Recombinant NQO2 was expressed in *E. coli* and purified as previously described (27). 1-(3-sulfonatopropyl)-3-carbamoyl-1,4-dihydropyrimidine (SCDP) is an analogue of NRH obtained from Sigma-Aldrich. Nicotinamide riboside (NR) was purchased from High Performance Nutrition (Irving, CA, USA) and dihydronicotinamide riboside (NRH) was prepared according to previously described protocol with NR (28). Ethidium bromide was obtained from Bioshop, AO and doxorubicin were obtained from Caymen Chemical.

5.2.2 Enzymatic Activity Measurements and Analysis

Enzyme activity was measured by monitoring the consumption of SCDP at an absorbance peak of 360 nm using a Cary 100-Bio spectrophotometer (Varian). The reaction was initiated by addition of NQO2 to a final concentration of 154 pM in a stirred cuvette containing 150 µM SCDP and 5 µM menadione (Sigma) at 30°C with EtBr (1.9 nM to 2.6 µM), AO (7.8 nM to 1 µM) or doxorubicin (80 nM to 50 µM) in a buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). IC\textsubscript{50} values were calculated using a dose-response model and represented as relative inhibition.

The constants for inhibition ($K_I$ values) of NQO2 by EtBr were determined using the same kinetics assay described for determination of IC\textsubscript{50} values. For each inhibitor, kinetic assays were performed either with a constant SCDP concentration of 150 µM and a varying menadione concentration or with a constant menadione concentration of 5 µM and a varying SCDP concentration. NQO2 is subject to substrate inhibition by menadione, and inhibitors of NQO2 can be competitive against SCDP, menadione, or both. On this basis, we used an equation for steady-state ping-pong catalysis that includes a term for substrate inhibition by menadione ($K_{I(Md)}$), as well as constants for inhibition against both the oxidized and reduced forms of the enzyme (29):
In Equation 5.1, \([\text{SCDP}]\) and \([\text{Md}]\) are the concentrations of SCDP and menadione respectively. \(K_{I(\text{MD})}\) is the inhibition constant that accounts for substrate inhibition. \([I]\) is the concentration of inhibitor and \(K_{I(\alpha)}\) and \(K_{I(\beta)}\) are the inhibition constants that modify the apparent Michaelis constants \(K_{M(\text{SCDP})}\) and \(K_{M(\text{Md})}\) respectively. That is, \(K_{I(\alpha)}\) is the constant that accounts for the change in \(K_{M(\text{SCDP})}\) in the presence of inhibitor; hence, it describes the component of inhibition that is competitive against SCDP. \(K_{I(\beta)}\) is the constant that accounts for the change in \(K_{M(\text{Md})}\) in the presence of inhibitor, and it describes the component of inhibition that is competitive against menadione. Fixing the kinetic parameters of the uninhibited steady-state kinetics \(K_{M(\text{SCDP})}\) (142 µM), \(K_{M(\text{Md})}\) (8.07 µM), and \(K_{I(\text{Md})}\) (1.62 µM) with values previously determined (29), \(V_{\text{max}}\), \(K_{I(\alpha)}\), and \(K_{I(\beta)}\) were determined by globally fitting data to Equation 5.1 using nonlinear regression (Igor Pro version 6.34A) with the IC\(_{50}\) values as initial estimates.

5.2.3 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was used to assess the direct binding of the inhibitors to NQO2. Titrations were performed using a MicroCal VP-ITC microcalorimeter and all data were analyzed using Origin 7.0 (MicroCal). Samples of concentrated NQO2 (700 µM in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and concentrated inhibitors (20 to 60 mM in H\(_2\)O or DMSO) were diluted into ITC buffer (50 mM sodium phosphate, 150 mM NaCl pH 7.55) to final concentrations of 8 µM and 80 to 260 µM respectively. In cases where inhibitor stocks were suspended in DMSO, an equal amount of DMSO was also added to the protein sample to match the buffer composition.
between the sample and titrant. All solutions were thoroughly degassed by stirring under vacuum. All titrations were performed at 25°C or 35°C with 3-10 µl injections spaced by 3 to 5 minute intervals. The integrated binding isotherms were corrected for the heat of dilution using data from titrations of the individual inhibitors into the respective matched buffers in the absence of NQO2. For the binding of inhibitors to oxidized NQO2, the 1.35 mL sample cell was filled with an 8 µM solution of NQO2 and the 250 µL syringe was filled with 80 µM EtBr, AO, or doxorubicin. All ITC titration data towards oxidized NQO2 were fitted to a single-site binding model with MicroCal Origin7 software.

Reduced NQO2 is subject to rapid oxidation (30); however, the calorimeter provided a closed environment that limited diffusion of oxygen into the solutions. To titrate compounds against reduced NQO2, SCDP was added to degassed solutions of both NQO2 and the inhibitors to a final concentration of 500 µM. To measure the very high affinities of EtBr and AO towards reduced NQO2, competition titrations were performed by adding 80 µM of EtBr or AO into 8 µM of NQO2 pre-mixed with 80 µM chloroquine (CQ) and 500 µM SCDP. Additionally, the binding of CQ alone to reduced NQO2 was determined by titrating 160 µM CQ into 8 µM of NQO2 and 500 µM SCDP. This second binding isotherm was fitted to a single-site binding model. Using the thermodynamic parameters of CQ to reduced NQO2, the binding isotherms of the displacement titrations were fitted to a competitive binding model using MicroCal Origin7 software to determine the thermodynamic parameters EtBr and AO.

5.2.4 Crystal Structure Analysis

Oxidized NQO2 (NQO2ox) was co-crystallized with EtBr, AO, or doxorubicin by hanging drop vapour diffusion against reservoirs containing 0.1 M HEPES pH 7.5, and 1.3 – 2.0 M (NH₄)₂SO₄. To obtain crystals of reduced NQO2 in complex with EtBr or AO, reduction of NQO2ox-inhibitor crystals was performed as previously described (30). Briefly, crystals of NQO2ox-EtBr or AO were repeatedly soaked into 1 µL of reducing-soak solution with 0.1 M HEPES pH 7.5, 2.0 M (NH₄)₂SO₄, 10 mM SCDP, and 1 mM of inhibitor, for 2 minute intervals until the crystals bleached. They were then transferred to a soak without SCDP before briefly passing through a cryoprotectant solution (2.0 M (NH₄)₂SO₄, 0.1 Hepes pH 7.5, 20% glycerol) and plunged into liquid nitrogen. To
prevent auto-oxidation of NQO2, the entire crystal mounting process from harvesting to cryo-cooling was performed under an anoxic atmosphere in a glove bag purged with N2.

Crystallographic data were collected from a rotating anode source, processed using MOSFLM (31), and merged using Scala (32). The structures were solved by molecular replacement with PDB-ID 1QR2 as a starting model (16); refinement was carried out using PHENIX (33). All crystals had the same primitive orthorhombic space group and contained a dimer in the asymmetric unit; given the high resolution of the crystallographic data, NCS restraints were not used for any of the refinements. Topology files for EtBr and AO were generated using PHENIX.ELBOW (33). The “butterfly bend” of the isoalloxazine ring was determined by simulated annealing with planar restraints removed, as previously described (30).

5.2.5 DNA Binding Pocket Comparison

The solvent accessible area in DNA or NQO2 binding pocket was calculated using POVMF 2.0 (34, 35). Briefly, a contiguous area was generated from an arbitrary point in the binding site of the ethidium unwound DNA structure (36) or reduced NQO2-EtBr structure for a 10 Å spherical radius. Dimensions of the binding pockets were then measured at arbitrary points along the edge of the binding pocket.

5.2.6 Effect of AO on HCT116 Colon Carcinoma Cells

To determine the cytotoxicity of AO, HCT116 cells were seeded at 1000 cells per well in 96-well plates and allowed to attach overnight. Cells were then treated with AO (0.014 to 230 µM) and grown for an additional 48 hrs before assaying using Sulforhodamine B (SRB; Sigma) assay according to established protocol(37). Briefly, cells are fixed with trichloroacetic acid for 1 hr at 4°C and washed with water 3 times. Cells were then stained with 0.3% SRB in 1% acetic acid for 20 mins at room temperature and washed with 1% acetic acid 4 times. The dye was re-solubilized in 10 mM Tris base and absorbance was measured using at 560 nm (Victor multi-plate reader, Perkin Elmer). Cell growth in treated samples was normalized against controls that were not treated. Data were analyzed and plotted using Prism 6.0f (GraphPad Software Inc).
To assay the ability of AO to inhibit NQO2, HCT116 cells were seeded at 1000 cells per well in 96-well plates and allowed to attach overnight. Cells were then treated with 7.8 µM to 1000 µM CB1954 with 50 µM NRH, with 50 µM NRH and 78 nM AO, or without NRH. Cells grew for an additional 48 hrs before being assayed using MTT dye. Briefly, cells were treated with 1 mM of MTT dye and were incubated at 37°C for 2-4 hrs. Media was completely removed and the dye was solubilized in DMSO to be measured at 560 nm using a plate reader. Cell growth in treatment wells was normalized against cells with no treatment and data were analyzed using Prism 6.0f (GraphPad Software Inc).
5.3 Results

5.3.1 DNA Intercalating Agents Inhibit NQO2

Ashman and co-workers first characterized NQO2 in 1962 and showed that a small concentration (10 nM) of a number of anthracene derivatives inhibited its activity (38). Since then, many other planar polycyclic aromatic compounds were identified as inhibitors of NQO2 including compounds that also intercalate DNA (8, 20, 39). To characterize the interaction between NQO2 and DNA intercalating agents in greater detail, we investigated the ability of 5 different DNA intercalating molecules to inhibit NQO2 (Figure 5.1). Three of the intercalating agents – ethidium bromide (EtBr), acridine orange (AO), and doxorubicin – exhibited IC\textsubscript{50} values in the nanomolar range; the other two, mitoxantrone and methylene blue, showed relatively weak inhibition of NQO2 catalysis with IC\textsubscript{50} values greater than 10 µM (Figure 5.2A and Table 5.1).

![Chemical Structures of DNA Intercalating Agents.](image)

NQO2 catalyzes quinone reduction by a ping-pong mechanism: the dihydronicotinamide co-enzyme is first oxidized, transferring a hydride ion to the NQO2-bound FAD co-factor; the quinone substrate then binds to reduced NQO2, and electrons are transferred to produce the corresponding hydroquinone. Thus, NQO2 can exist in either an oxidized or...
reduced state, and inhibitors can be competitive towards the dihydronicotinamide coenzyme or the quinone substrate. In a kinetic assay, this means that an inhibitor can raise the apparent $K_M$ for the dihydronicotinamide coenzyme or the quinone substrate. Using our previously established kinetic model (Equation 5.1), the terms $K_{I(\alpha)}$ and $K_{I(\beta)}$ account for changes in the apparent $K_M$ of SCDP (an NRH analogue) and menadione (the quinone substrate) respectively (29). Our kinetic characterization of EtBr inhibition towards NQO2 showed that competition with menadione was the dominant mode of NQO2 inhibition ($K_{I(\beta)} = 6.7 \text{ nM}$ versus $K_{I(\alpha)} > 500 \text{ nM}$; Figure 5.2B, Table 5.2). In other words, EtBr binds reduced NQO2 with much greater affinity compared to oxidized NQO2, and this interaction is responsible for inhibition of NQO2 catalysis.

**Figure 5.2 Ethidium Bromide, Acridine Orange, and Doxorubicin are Inhibitors of NQO2.**

(A) Determination of IC$_{50}$ values for EtBr (●), AO (■), and doxorubicin (▲) against NQO2-catalyzed reduction of menadione. The IC$_{50}$ values are listed in Table 1. (B) Determination of kinetic inhibition constants for EtBr. The inhibition of NQO2 by EtBr was characterized using a constant menadione concentration (5 µM) and varying SCDP (left), and a constant SCDP concentration (150 µM) with varying menadione (right). The enzymatic activity was measured in the absence of EtBr (●) and at EtBr concentrations of 2 nM (■), 10 nM (▲), 50 nM (▼), and 250 nM (◆). The data were globally fitted to Equation 5.1 as described in Materials and Methods. The kinetic constants are summarized in Table 5.2.

**Table 5.1 Inhibition of NQO2 by DNA Intercalating Agents.**

<table>
<thead>
<tr>
<th>Intercalating Agent</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium Bromide</td>
<td>11.8 ± 0.4</td>
</tr>
<tr>
<td>Acridine Orange</td>
<td>24.6 ± 4.1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>254 ± 41.7</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>&gt; 10 000</td>
</tr>
<tr>
<td>Methylene Blue</td>
<td>&gt; 10 000</td>
</tr>
</tbody>
</table>
Table 5.2 Steady-state Kinetic Parameters for Inhibition of NQO2 by EtBr.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}^{a}$</td>
<td>1184 ± 30 s$^{-1}$</td>
</tr>
<tr>
<td>$K_{I(\alpha)}^{a,c}$</td>
<td>555 ± 2270 nM</td>
</tr>
<tr>
<td>$K_{I(\beta)}^{a,c}$</td>
<td>6.7 ± 1.24 nM</td>
</tr>
<tr>
<td>$K_m$ (SCDP)$^b$</td>
<td>142 µM</td>
</tr>
<tr>
<td>$K_m$ (menadione)$^b$</td>
<td>1.62 µM</td>
</tr>
<tr>
<td>$K_i$ (menadione)$^b$</td>
<td>8.07 µM</td>
</tr>
</tbody>
</table>

$^a$ Calculated by globally fitting data to equation 1 as described in Methods. 
$^b$ Steady state kinetic parameters for the uninhibited reaction were determined previously (29). 
$^c$ $K_{I(\alpha)}$ and $K_{I(\beta)}$ are the constants for inhibition against SCDP and menadione, respectively.

5.3.2 Binding of DNA Intercalating Agents to Oxidized and Reduced NQO2

The preferential binding of EtBr to reduced NQO2 is of interest because the reduced form of NQO2 appears to play an important role in cells. That is NQO2 was shown to inhibit p53 degradation in the presence of NRH, and on this basis it appears that reduced NQO2 is the “active” state of the enzyme that is capable of regulating p53 (4). To further characterize interactions between DNA intercalating agents and NQO2, the binding of EtBr, AO, and doxorubicin to both oxidized and reduced NQO2 was measured using Isothermal Titration Calorimetry (ITC). Of the three compounds, AO bound oxidized NQO2 with the highest affinity, almost 10-fold greater than both EtBr and doxorubicin (Table 5.3, Figure S5.1A-C). Preliminary titrations of AO and EtBr against reduced NQO2 indicated that their binding affinities were much higher compared to oxidized NQO2. To accurately characterize the very high affinity binding of both EtBr and AO to reduced NQO2, competition ITC was used (40). In competition ITC, a compound with high binding affinity is titrated against the target protein in complex with a relatively low affinity ligand. In the case of NQO2, chloroquine (CQ) was used as the low-affinity ligand. A direct titration of reduced NQO2 with CQ yielded a $K_D$ value of 0.57 µM (Table 5.3, Figure S5.1G) similar to its inhibition constant (0.6 µM) determined previously using enzyme kinetics (13). Consistent with the kinetics assays, EtBr bound most tightly to reduced NQO2, with a $K_D$ over 60 times lower compared to the oxidized form (Table 5.3, Figure S5.1D). The situation was similar for AO, which bound
preferentially to reduced NQO2 with sub-nanomolar affinity (Table 5.3, Figure S5.1E). Doxorubicin bound to oxidized NQO2 with sub-micromolar affinity but there was no observable binding to the reduced form (Table 5.3, Figure S5.1F).

### Table 5.3 Binding of NQO2 Inhibitors to Oxidized and Reduced NQO2

<table>
<thead>
<tr>
<th>NQO2</th>
<th>Inhibitor</th>
<th>$K_D$ (nM)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$-T\Delta S$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>EtBr</td>
<td>215 ± 33</td>
<td>-9.4</td>
<td>-7.8 ± 0.1</td>
<td>-1.6</td>
</tr>
<tr>
<td></td>
<td>AO</td>
<td>29.4 ± 28.7</td>
<td>-10.3</td>
<td>-7.6 ± 0.2</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>274 ± 49</td>
<td>-8.9</td>
<td>-8.1 ± 0.2</td>
<td>-0.8</td>
</tr>
<tr>
<td>Reduced</td>
<td>EtBr$^a$</td>
<td>3.47 ± 0.33</td>
<td>-11.5</td>
<td>-6.7 ± 0.1</td>
<td>-4.8</td>
</tr>
<tr>
<td></td>
<td>AO$^a$</td>
<td>0.36 ± 0.12</td>
<td>-12.9</td>
<td>-6.5 ± 0.1</td>
<td>-6.3</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin$^b$</td>
<td>NB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CQ</td>
<td>578 ± 116</td>
<td>-8.5</td>
<td>-17.7 ± 0.5</td>
<td>9.2</td>
</tr>
</tbody>
</table>

$^a$ Thermodynamic parameters were determined by competition titrations against NQO2 in complex with CQ as described in Methods.

$^b$ There was no observable binding of doxorubicin to reduced NQO2 (Figure S5.1F).

#### 5.3.3 Crystal Structures of NQO2 with DNA Intercalating Agents

There are numerous structures of oxidized NQO2 in complex with a variety of inhibitors, but only two in which inhibitor binding to reduced NQO2 has been structurally characterized (29, 30). In both cases, reduction of the FAD cofactor led to a striking change in the binding mode of the inhibitors. We have extended this comparative analysis by solving the high resolution crystal structures of oxidized and reduced NQO2 in complex with EtBr and AO, as well as doxorubicin in complex oxidized NQO2 (Table S5.1). In all of the oxidized NQO2 inhibitor complexes, the inhibitors are deeply buried in the active site and sandwiched between the isoalloxazine ring of FAD and the phenyl ring of F178 (Figure 5.3A, C, and E). As such, binding of the inhibitors to oxidized NQO2 includes common aromatic stacking interactions. In the structure of reduced NQO2 with EtBr (Figure 5.3B) the overall orientation of ethidium has not changed, but it is positioned less deeply in the active site. The space vacated by the ethidium is filled with three water molecules that mediate hydrogen bonds between the amino group on the ethidium and N161 and G174 of NQO2. The situation is similar for AO: when bound to oxidized NQO2, AO is positioned deep in the active site, stacking over the oxygens of the isoalloxazine ring and making direct contact with N161 and G174 (Figure 5.3C).
However, AO has rotated and occupies a more peripheral location when bound to reduced NQO2 (Figure 5.3D), and again the space next to N161 and G174 is filled with water molecules.

The difference in binding mode for EtBr and AO resembles what was observed for chloroquine (CQ) (30) and the protein kinase CK2 inhibitor DMAT (29), which both bound to oxidized NQO2 such that they were positioned deep in the active site and made direct contact with N161 and G174. When bound to reduced NQO2, however, both CQ and DMAT were positioned in a more peripheral location, and there were water molecules filling the space between N161, G174, and the inhibitors. It appears that the properties of the reduced isoalloxazine ring, which include a “butterfly bend” of approximately 5° (Table S5.1 and Figure S5.2) and a negative charge that will be delocalized between N1 and O2, make the region above the isoalloxazine oxygens less suitable for the aromatic stacking interactions observed in the oxidized structures. Instead, the inhibitors move away from this region which is then occupied by polar, non-aromatic water molecules.

The active site of NQO2 has a negative electrostatic potential that becomes much stronger when the isoalloxazine ring is reduced and carries a formal negative charge (29). Both EtBr and AO are positively charged at neutral pH, which explains their preference towards the negatively charged FADH over neutral FAD. Doxorubicin also has a positive net charge, but the charge resides on non-aromatic portions of the molecule that are excluded from the active site (Figure 5.3E). Therefore, a positive charge in the planar aromatic portions of NQO2 inhibitors appears to determine their preference for reduced NQO2. This is consistent with the two other NQO2 inhibitors, chloroquine and quinacrine, which both carry a positive charge in their aromatic portions and exhibit a marked preference for binding to reduced NQO2 (13).

NQO2 crystallizes with a dimer in the asymmetric unit, and inhibitors can sometimes adopt different orientations in the two crystallographically distinct binding sites. This was the case for both the NQO2ox-EtBr and NQO2red-EtBr structures where electron density
for a second ethidium molecule in the B-chain active site became evident during refinement (Figure S5.3). The second ethidium interacts with the first by means of pi-pi

**Figure 5.3** Binding of Ethidium, Acridine Orange, and Doxorubicin to NQO2
The inhibitors are sandwiched between the FAD co-factor (below the plane of inhibitor) and F178 (above the plane of the inhibitor) in oxidized NQO2 (A, C, E), and the inhibitors are excluded from this region by water molecules in reduced NQO2 (B, D). (A) Ethidium is deeply buried in the active site of oxidized NQO2 making hydrogen bonds with N161 directly and to D117, T71, and G68 via two water molecules. (B) Ethidium is less buried in reduced NQO2 and makes hydrogen bonds with N161 and G174 via three water molecules on the left side of the binding site, and with G68 and T71 directly on the right side of the binding site. (C) Acridine orange binds oxidized NQO2 by making hydrogen bonds to N161 via two water molecules. (D) In the reduced structure of NQO2 in complex with AO, AO makes one hydrogen bond to E193 via a water molecule. (E) The planar moiety of doxorubicin is inserted into the left side of the binding cleft where it
is anchored by a hydrogen bond to N161. All electron density maps surrounding the inhibitors are $F_c-F_o$ omit maps generated after three rounds of simulated annealing and contoured at $3\sigma$.

planar stacking of their benzyl substituents, and the rest of the ethidium is loosely sandwiched between surface residues surrounding the B-chain active site and residues from a symmetry-related molecule. We believe that this mode of binding is induced primarily by crystal packing and likely irrelevant for NQO2 in solution. In contrast to EtBr, both AO and doxorubicin bound in exactly the same manner to both subunits of the NQO2 dimer.

5.3.4 Comparison between Intercalators Binding to DNA and NQO2

The high affinity binding of DNA intercalating agents prompted us to compare the binding pocket in unwound (intercalated) DNA with the active site of NQO2. Molecules that intercalate DNA create a cavity with pi-pi stacking interactions mediated by the bases and the potential for hydrogen bonds to the DNA backbone, as shown in the structure of ethidium bound to DNA (36) (Figure 5.4A). The binding pocket in the DNA could accommodate molecules up to 13.5 Å in length at the major groove side and 7.9 Å in length at the minor groove side. Since the DNA is unwound by EtBr, the adjacent base pairs are not parallel with the space between them varying between 2.5 Å to 3.7 Å. In NQO2, the isoalloxazine ring in the bottom of the pocket provides pi-pi stacking interactions, and additional interactions with the other planar face of the inhibitor are mediated by hydrophobic and aromatic residues at the top of the pocket (Figure 5.4B). Similar to the binding pocket in DNA, the NQO2 active site can accommodate molecules up to 13.9 Å in length and 2.7 Å in height. When the reduced NQO2-EtBr structure was superimposed to the known DNA-EtBr structure by aligning the coordinates of EtBr, the many residues of the NQO2 binding pocket also superimposed with the unwound DNA as shown by the overlap of the molecular surfaces of the two molecules (Figure 5.4C). In the reduced NQO2-AO to DNA-AO (41) comparison, the positively charged nitrogenous base of AO makes two hydrogen bonds to the hydroxyl group of the two adjacent guanosine nucleotides via water molecules, in addition to pi-pi stacking interactions (Figure 5.4D). Similarly in NQO2, the nitrogenous base of AO is anchored to E193 by
hydrogen bonds via a water molecule. Therefore, the active site of NQO2 bears a surprising resemblance to the binding pocket in DNA.

Figure 5.4 Comparison of the Inhibitor Binding Sites in Reduced NQO2 and DNA

(A-B) Solvent accessible area (green blob) of the unwound DNA in the DNA-ethidium structure (A; Cambridge Structure Database ID ETHUAD10) and the NQO2 active site (B) viewed from the front (left panel) showing the hydrophobic and polar regions of the binding sites. Views from the top (middle), and from the side (right panel) show the estimated dimensions in the binding sites. (C) Molecular surface of reduced NQO2 bound to EtBr (left), DNA intercalated with EtBr (middle), and superimposition of EtBr (right) to compare the molecular surfaces of the NQO2 and DNA ethidium binding sites. (D) Molecular surface of reduced NQO2 bound to AO (left) showing that the nitrogenous base of AO makes a hydrogen bond to E193 via a water molecule; DNA intercalated with AO (middle, CSD ID ACCYGA10) showing that the nitrogenous base of AO makes a
hydrogen bond with the hydroxyl group of two adjacent guanosine bases via water molecules; and superimposition of AO (right) showing the overlap of molecular surfaces between NQO2 and DNA.

Even though the active site of NQO2 bears some resemblance to the binding pocket in unwound (intercalated) DNA, the affinities of DNA intercalators for NQO2 do not correlate well with their affinities for DNA. For example, of the agents studied in this manuscript, doxorubicin binds DNA most tightly ($K_D = 384 \text{ nM}$) (42), while binding of EtBr and AO is much weaker ($K_D$ values of 15 and 36 $\mu\text{M}$, respectively) (43). In the case of NQO2, EtBr and AO bind reduced NQO2 with very high affinity, with dissociation constants that are 4 to 5 orders of magnitude lower (i.e. 3.47 and 0.57 nM; Table 5.1) than those for their binding to DNA; doxorubicin, on the other hand, displays a relatively modest affinity for NQO2, roughly the same as its affinity for DNA. Furthermore, the other DNA intercalating agents, methylene blue and mitoxantrone, that did not display observable inhibition of NQO2 exhibit bind DNA with similar affinity as EtBr and AO (43, 44). In summary, even though there are similarities between the NQO2 active site and the space in DNA occupied by intercalators, it does not seem that NQO2 is a completely faithful mimic of DNA.

5.3.5 Inhibition of NQO2 in Cells

Beyond an in vitro characterization of these novel inhibitors of NQO2, we were also interested in whether AO, to our knowledge the highest affinity inhibitor of reduced NQO2 characterized to date, could inhibit NQO2 in cells. First, we established the cytotoxicity of AO in HCT116 cells. HCT116 cells are colorectal carcinoma cells with a high level of NQO2 expression and activity (39); in addition, these cells do not express P-glycoprotein (45), preventing active efflux of AO and making them a good model to assay in-cell inhibition of NQO2 by AO. We found that AO inhibited HCT116 cell proliferation with an IC$_{50}$ value of $3.9 \pm 0.5 \mu\text{M}$ and on this basis concentrations of AO below approximately 500 nM do not affect cell proliferation (Figure 5.5A).

To determine whether NQO2 is inhibited by AO in cells, we used the cancer prodrug CB1954 that is specifically activated by NQO2 in the presence of NRH (28). CB1954 is a DNA alkylating agent that can be reduced to a much more toxic DNA cross-linking agent
by NQO2 in the presence of NRH. Without NRH, the cytotoxicity of CB1954 was 348 ± 41 µM, and in the presence of 50 µM NRH, the cytotoxicity of CB1954 increased 20-fold to 17.8 ± 1.4 µM (figure 5.5B). Addition of a non-toxic dose of AO (78nM) was able to partially reverse the NRH-dependent activation of CB1954 and lower its cytotoxicity in the presence of NRH to 114 ± 15 µM (Figure 5.5C). We also tested whether EtBr was able to inhibit NQO2 with a non-toxic concentration and found that EtBr had an IC$_{50}$ value of 9.1 ± 0.9 µM. The highest concentration of EtBr tested (312nM) was only able to slightly reverse the activation of CB1954 by NRH (data not shown). Therefore, a non-toxic concentration of AO can effectively inhibit NQO2 in cells while EtBr is less effective at inhibiting NQO2.

Figure 5.5 Validation of NQO2 Inhibition by AO in HCT116 cells

NQO2 is inhibited by AO in HCT116 cells. (A) IC$_{50}$ of AO in cells. HCT116 cells were treated with AO at the indicated concentration for 48 hours before being assayed by SRB. Cell growth of the treated cells was normalized to the cell growth of untreated cells and the data were fitted to a dose-response curve to calculate an IC$_{50}$ for AO of 3.9 ± 0.5 µM. (B) Induction of CB1954 cytotoxicity by NRH. Cells were treated with the indicated concentration of CB1954 with (■) and without (●) 50 µM NRH for 48 hours before being tested for viability using an MTT assay. (C) Cellular inhibition of NQO2 by AO. Induction of CB1954 cytotoxicity with 50 µM NRH was partially reversed by co-treatment with 78 nM AO (▲). Cytotoxicity of CB1954 with (■) and without (●) 50 µM NRH are shown for reference in grey.
5.4 Discussion

We have identified and characterized the binding of three well-known DNA intercalating agents to NQO2. Positively charged polycyclic compounds are thought to be better DNA intercalating agents because they are recruited to the negatively charged phosphate backbone prior to intercalating DNA. Here, we have shown that when the positive charge resides in the planar moiety of compounds such as EtBr and AO, they exhibit a marked preference for binding to reduced NQO2, with affinities towards reduced NQO2 that are over 50-fold greater compared to those for oxidized NQO2. In contrast, a positive charge outside the planar aromatic portions of the inhibitor, as in the case of doxorubicin, does little to enhance binding affinity for reduced NQO2. The results with EtBr and AO are in line with those of two other NQO2 inhibitors, chloroquine and quinacrine, that bind preferentially to reduced NQO2 and carry a positive charge in their aromatic ring systems (13). In summary, the available data indicate that a positive charge in the aromatic ring system of an NQO2 inhibitor will enhance preferential binding to the reduced form of the protein.

As an alternative function to the enzymatic reduction of quinones, NQO2 has been implicated in the regulation of p53, where it was shown that NQO2 in the presence of NRH protects p53 from 20S proteosomal degradation (4, 7). Thus, the proposed role for NQO2 in the regulation of p53 requires the presence of NRH, suggesting that it is the reduced form of NQO2 that is the relevant species. Along these lines, however, is the question of how NQO2 is reduced in vivo. NQO2 is inefficiently reduced by NAD(P)H, and it is not clear that NRH is actually present in cells. On this basis, NQO2 in cells may exist primarily in an oxidized state. Molecules that bind with very high affinity to reduced NQO2 would be expected to stabilize it and extend its lifetime. Thus, molecules such as AO and EtBr, with nanomolar affinities to NQO2, could “prime” the cell to respond to impinging DNA damage and/or apoptotic signals.

NQO2 has been implicated in neurodegenerative disease and cancer tumorigenesis (46, 47), although the cellular functions of NQO2 remain a matter of debate. NQO2 knockout
mice appear to have lower levels of p53, a weaker induction of p53 by cell stressors, and decreased apoptosis (47, 48). In addition, NQO2 has been repeatedly identified as a target for a variety of bioactive compounds, such as resveratrol (14), melatonin (15), antimalarials (13, 30), and kinase-targeted therapeutics (11, 29). Despite these provocative observations, the knockout or inhibition of NQO2 does not bring about acute cellular effects. For example, Karen Nolan and coworkers found that imidazoacridine-6-one compounds are potent NQO2 inhibitors but also intercalate DNA at similar concentrations (20). An N-oxide modification of the parent compound reduced its ability to intercalate DNA and lowered its cytotoxicity 20-fold while retaining its affinity towards NQO2 (49). Similarly, we have shown concentrations of AO that are sufficient to inhibit NQO2 have no obvious effect on cell proliferation. It appears that the function of NQO2 becomes manifest only when cells are challenged by some other insult.
5.5 References


5.6 Supporting tables and figures

Figure S 5.1 Inhibitor Binding to NQO2 Assayed by Isothermal Titration Calorimetry

Titration of EtBr (A), AO (B), and doxorubicin (C) to oxidized NQO2. A syringe filled with 80 µM of the inhibitor was titrated to a solution of 8 µM NQO2. Competition ITC of EtBr (D) and AO (E) to NQO2 in the presence of 500 µM SCDP and 80 µM of chloroquine (CQ). Direct titration of doxorubicin (F) and chloroquine (G) to NQO2 in the presence of 500 µM SCDP. Titration data were fit to a single substrate model for all direct titrations, or to a competitive model using fixed values from binding parameters determined by a titration of CQ to reduced NQO2 (Table 5.3). The thermodynamics parameters are summarized in Table 5.3.
Table S 5.1 Crystallographic Statistics

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<tr>
<td>Subunit B</td>
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<td>-3.79</td>
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<sup>a</sup> Values in parentheses refer to the highest resolution shell.
<sup>b</sup> The equations of the two planes of FAD were first calculated from the coordinates of FAD using principal component analysis in MATLAB (MathWorks R2015a). The angle between the planes was then calculated and each structure was visually inspected for direction of bending. Positive values indicates downward bending.
Figure S 5.2 Superimposition of FAD Co-enzyme

Seven atoms from ring A of the isoalloxazine ring were aligned to reveal the downward butterfly bend along the N5-N10 axis in ring B. FAD of the oxidized structures in complex with EtBr, AO, and doxorubicin were colored in blue, and FAD of the reduced structures in complex with EtBr and AO were colored in red.

Figure S 5.3 Second Binding Site of Ethidium in NQO2

Two copies of ethidium were modeled in the active site of subunit B of oxidized NQO2 (A) and reduced NQO2 (B) to account for prominent non-solvent electron density near the active site. The electron density map surrounding ethidium is an $F_c-F_o$ omit map generated after three rounds of simulated annealing contoured at 3 $\sigma$. 
Chapter 6

6 Inhibitors of Quinone Reductase 2 Modulate p53 in HCT116 cells

6.1 Introduction

NQO2 is a cytosolic flavoprotein constitutively expressed in most tissues (1). Historically, it was characterized as a detoxifying enzyme that prevents quinone from acquiring a single electron and forming cytotoxic quinone radical by catalyzing an obligate two-electron transfer from dihydronicotinamide riboside (NRH) to quinone (2). By virtue of this detoxification role, inhibition of NQO2 could sensitize cells to oxidative stress generated by quinone radicals. In the past two decades, a wide range of bioactive compounds was found to inhibit NQO2 catalytic activity. These compounds included kinase targeted inhibitors (3–6), antimalarial drugs (7), natural flavonoids (8, 9), resveratrol (10), melatonin (11), and DNA intercalating agents (12). All of these compounds perturb cellular functions, but it remains unclear whether inhibition of NQO2 contributes to their effects.

In addition to catalysis of quinone reduction, NQO2 was shown to modulate cellular proteins involved in cell cycle and apoptotic pathways. These proteins included AKT, NF-κB, CEBPα, and most notably p53 (13–18). The role of p53 regulation was initially discovered in quinone reductase 1 (NQO1). When NQO1 was knocked down, mutated, or inhibited in HCT116 human colorectal carcinoma (HCT116) cells, p53 levels decreased (19, 20). Further investigation showed that NQO1 mediated the 20S proteasomal degradation of p53 in a NAD(P)H-dependent manner (21). From these observations, NQO1 was thought to regulate the basal level of p53 in cells (22). Subsequent studies revealed that NQO2 stabilized p53 from 20S proteasomal degradation in a NRH-dependent manner (17, 18)\(^8\). Mice deficient for NQO2 had lower p53 levels, and developed skin tumors more readily when challenged with a chemical carcinogen

\(^8\) Reference 17 was retracted as of February 1\(^{st}\), 2015.
compared to control mice (23). This suggested that NQO2 functioned as a tumor suppressor by maintaining basal p53 levels. From these studies, NQO2 appears to have additional roles beyond catalyzing quinone reduction that correlated to its redox state.

This alternate function of NQO2 in stabilizing p53 in a redox-dependent manner suggested that NQO2 could function as a flavin redox switch, which is a class of proteins that participate in cell signalling by responding to light and/or changes in redox state (24). The first indication of this relationship originated from studies of Lot6p, the sole orthologue of NQO1 and NQO2 in yeast (25). Lot6p is a poor catalyst of quinone reduction, but it prevented the 20S proteasomal degradation of the transcription factor Yap4 in response to elevated NADH levels (26, 27). Similarly, human NQO1 and NQO2 could be functioning as flavin redox switches by sensing cellular dihydronicotinamide levels and modulating 20S proteasomal degradation of p53. Along these lines, structural studies of oxidized and reduced NQO2 revealed mechanistic features of a flavin redox switch in NQO2 (28). Together, this prompted the present investigation into the signalling role of NQO2 and how NQO2 inhibitors modulate this switch function. Specifically, two classes of NQO2 inhibitors were investigated: CK2 inhibitors and DNA intercalating agents.

Protein kinase CK2 (CK2) is elevated in many cancer types, and the development of CK2 inhibitors that induce apoptosis in cancer has received much attention in the past decade (29, 30). However, CK2 inhibitors are not very specific and how CK2 inhibitors work remains largely unknown (31). For example, the inhibitors 4,5,6,7-tetrabromobenzimidazole (TBBz) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), induced apoptosis even when CK2 activity was restored (5). Coincidentally, TBBz and DMAT were identified to be targets of NQO2, and further analysis showed that they bound NQO2 with nanomolar affinity (32). In contrast, another CK2 inhibitor, 4,5,6,7-tetramethylbenzotriazole (TBB), did not induce apoptosis in cells when CK2 activity was restored. It also bound NQO2, but with much lower affinity when compared to TBBz and DMAT. Therefore, whether CK2 inhibitors induce apoptosis by interaction with NQO2 or not remains a topic for further research.
Recently, three additional nanomolar affinity inhibitors of NQO2 were identified: ethidium bromide (EtBr), acridine orange (AO), and doxorubicin (33). EtBr and AO bound to reduced NQO2 with a dissociation constant of 4 nM and 0.4 nM respectively. To date, these two compounds have the highest affinity for reduced NQO2. As DNA intercalators, these compounds inhibit DNA replication and RNA transcription; for cells that overcome this cytostatic stress, they are also mutagenic. Based on these findings, interest arose firstly, in how inhibition of NQO2 by DNA intercalators affects cellular proliferation, and secondly, if reduced-state NQO2 inhibitors differ from oxidized-state NQO2 inhibitors in cells.

In this present study, endogenous NQO2 was knocked out using CRISPR/Cas9 technology in HCT116 cells, and the NQO2-specific effects of substrates and inhibitors were characterized. Initial findings revealed that the isolated HCT116\textsuperscript{ΔNQO2} clones had a slower growth rate compared to the parental HCT116 cells. The IC\textsubscript{50} of CK2 inhibitors (TBB, TBBz, and DMAT) and DNA intercalators (EtBr, AO, and doxorubicin) were determined to be the same in both HCT116 and HCT116\textsuperscript{ΔNQO2} cells. Moreover, attempts to activate NQO2 by co-treating cells with NRH and the aforementioned inhibitors did not alter the inhibitors’ cytotoxicity. The basal level of p53 was also equal in both the HCT116 and HCT116\textsuperscript{ΔNQO2} cells, but the parental cells were found to modulate p53 more dynamically in response to inhibitor treatment. Taken together, our results indicated that NQO2’s role in p53 regulation was different than that of NQO1’s.
6.2 Methods

6.2.1 Reagents

TBB, TBBz, and DMAT (Sigma) were a gift from Dr. David Litchfield. Ethidium bromide was obtained from Bioshop (Burlington, ON, CAD). AO and doxorubicin were obtained from Caymen Chemical (Ann Arbor, MI, USA).

6.2.2 NRH Synthesis

Nicotinamide riboside (NR$^+$) was synthesized and purchased from High Performance Nutrients (Irving, CA, USA) as a dietary supplement capsule (34). Analysis of the commercial preparation by NMR spectroscopy indicated that it was 92% pure, and it matched the spectrum of the synthesized compound (Biomolecular NMR Facility, Western University, London, ON). Both synthetic and commercial NR$^+$ contained traces of nicotinamide.

Commercial NR$^+$ was then reduced to dihydronicotinamide riboside (NRH) according to the established protocol (35). Powder NR$^+$ from the supplement capsule was dissolved in water and insoluble material was removed using a 0.45 µm syringe filter (Millipore). Roughly 15 mM of NR$^+$ was reacted with 57 mM of sodium dithionite in a 200 mM sodium carbonate-sodium bicarbonate buffer (pH 9.9) in ddH$_2$O. The reaction mixture was incubated at 37 °C for 30 mins, then cooled on ice. NRH was then separated by preparative HPLC using a Waters µ-Bondapak C18 column (19mm x 150mm). The reaction mixture was injected 5 mls at a time into the reverse phase column and eluted at 10 mL/min by an isocratic mixture of 20% acetonitrile in water. The absorbance of the eluent was monitored at 280 nm and 340 nm. For each injection, four species with absorbance at 340nm were resolved by the C18 column and eluted at distinct time points. Each species was collected, pooled, and tested for their ability to reduce MTT (an NQO2 substrate) in an NQO2-dependent manner. One fraction showed NQO2-dependent activity and was lyophilized into a yellow amorphous powder. Absorbance scan of this species was identical to NR$^+$ with an additional absorbance peak at 360 nm.
6.2.3 CRISPR/Cas9 Knockout of NQO2

To knockout endogenous NQO2, the NQO2 was disrupted using CRISPR guided Cas9 nickase to generate single stranded breaks at two neighboring sites in the fourth exonic region. The plasmid pSpCas9n(BB)-2A-Puro (PX462) encoding both the CRISPR guided RNA cassette and Cas9 nickase was a gift from Feng Zhang (Addgene plasmid # 48141). Using an online CRISPR Design Tool (http://crispr.mit.edu/), CRISPR motifs targeting nucleotide 46 and 108 of exon 4 were identified as the top sequences with minimal off-targets (Figure S6.1). Oligonucleotides corresponding to the pair of guide RNAs (sgRNA) were cloned into PX462 according to previous protocol (36) to produce the two vectors NQO24_46 and NQO24_108 (Table 6.1). HCT116 cells at 70% confluence were then co-transfected with the sequence verified vectors NQO24_46 and NQO24_108 using Lipofectamine 2000 (Life technologies) according to the manufacturer’s manual. 24 hrs after transfection, the media was supplemented with 0.7 µg/mL of puromycin (Alfa Aesar, Ward Hill, MA, USA) for 72 hrs. Surviving cells were trypsinized and serially diluted into 96-well plates supplemented with puromycin to select for single colonies carrying the transfected plasmid(s). After two weeks, five clones were isolated and expanded.

6.2.4 Validation of NQO2 Knockout

Cellular NQO2 was detected by Western blotting using rabbit anti-NQO2 polyclonal antibodies (a gift from Dr. Tim Haystead at Duke University). Cell lysates from each clone were resolved using SDS-PAGE, and then transferred onto a PVDF membrane. After blocking with 5% non-fat milk, the blot was probed for NQO2 was using a 1:1000 dilution of the rabbit-anti-NQO2 antibody and visualized with 1:5000 fluorescence labeled secondary goat anti-rabbit antibody (Licor). To validate the excision of the targeted genome on exon 4, genomic DNA was isolated from each HCT116ΔNQO2 clones and parental HCT116 cells according to instructions from DNA extraction kit (Froggabio). Primers exon4_F1 and exon4_R1 were designed using Primer-Blast to amplify exon 4 of NQO2 (Table S6.2; www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR was performed using taq polymerase according to the manufacturer’s instructions (Biobasic). PCR products were resolved on a 3% polyacrylamide gel to detect the
excision of a DNA fragment in exon 4. Since no PCR products were detected in clones 2 and 4, two additional primers (exon4_F2 and exon4_R2) were designed to amplify DNA regions that flanked the targeted cut sites (Figure S6.1, Table S6.2). PCR amplification of these flanking regions would determine whether the exon 4 was present but disjointed or was completely absent. Furthermore, primers were also made to validate that the Cas9 nickase did not introduce unwanted mutations in off-target sites for the paired guide RNA used (Table S6.2).

6.2.5 Functional Validation of HCT116<sup>ΔNQO2</sup> cells

To validate that functional NQO2 was absent in the cells, activation of CB1954 by NRH was used as a reporter of NQO2 activity (15). HCT116 or HCT116<sup>ΔNQO2</sup> clone 3 cells were seeded at 1000 cells per well 96-well plates, and were allowed to attach overnight. Cells were then treated with: 1) 7.8 – 1000 μM of CB1954 and 6 – 200 μM of NRH, 2) 7.8 – 1000 μM of CB1954 alone, 3) 4.8 – 625 μM of NRH alone or 4) nothing (n=3). Cells were allowed to grow for an additional 48 hrs before they were counted using sulforhodamine B (SRB) assay.

6.2.6 SRB Assay

The SRB assay was a technique that stained total protein content of fixed cells (37). Cells were first fixed with trichloroacetic acid (TCA) for 1 hr at 4°C, then washed with water 3 times. They were then stained with SRB dye (Sigma) for 20 mins at room temperature, then washed with 1% acetic acid. The dye was re-solubilized in 10 mM Tris base and its absorbance was measured using a multi-reader at 560 nm (Victor multi-plate reader, Perkin Elmer). Absorbance readings of treated cells were normalized against untreated cells, and the data was fitted to a dose-response curve and graphed using Prism 6.0f (GraphPad Software Inc).

6.2.7 Growth Curve of HCT116<sup>ΔNQO2</sup> Cell Lines

To characterize cell growth, HCT116 and HCT116<sup>ΔNQO2</sup> cells from clone 3 and clone 5 were seeded at 1000 cells in 96-well plates. For 8 consecutive days, cells were trypsinized and counted using a haemocytometer each day. In a parallel experiment, cell
growth of HCT116 and HCT116\textsuperscript{Δ\text{NQO2}} clones 2, 3, and 4 were determined using the SRB assay. In the SRB assay, cells were fixed each day with TCA for 20 mins at room temperature, washed four times with water, dried, and then returned to the incubator. On day 8, all fixed cells were stained with SRB and protein content was measured as described above.

6.2.8 Cell Cycle Analysis by Flow Cytometry

Cell cycle analysis using flow cytometry was performed as previously described (38). Briefly, HCT116 and HCT116\textsuperscript{Δ\text{NQO2}} cells were seeded at 500,000 cells in a 10 cm culture dish to grow for 40 hrs until reaching 50-70% confluence. The cells were then labeled with bromodeoxyuridine (BrdU) for 1 hr, harvested, fixed in 95% ethanol, and stored at 4ºC until they were used. Prior to flow cytometry analysis, the cells were stained with mouse-anti-BrdU (BD Bioscience), FITC conjugated goat-anti-mouse IgG antibodies, and propidium iodide (PI).

6.2.9 Toxicity of Inhibitors of NQO2

To determine the toxicity of NQO2 inhibitors, HCT116 and HCT116\textsuperscript{Δ\text{NQO2}} cells were seeded in 96-well plates, 1000 per well, and allowed to attach overnight. The two cell lines were then treated with one of the following compounds: 7 nM – 115 \(\mu\)M of TBB, TBBz, or DMAT, or 14 nM – 230 \(\mu\)M of EtBr, AO, or doxorubicin. The experiment was then replicated with the addition of 61 \(\mu\)M of NRH co-treatment to determine whether activation of NQO2 sensitize cells to inhibitor-induced cytotoxicity. Cells were harvested after 48 hrs and were counted using the SRB assay.

6.2.10 Western Blot Analysis of Inhibitor-treated Cells

HCT116 and HCT116\textsuperscript{Δ\text{NQO2}} cells were seeded at 250,000 cells per well in 6-well plates and allowed to attach overnight. They were then treated with the concentrations of CK2 inhibitors and DNA intercalating agents indicated in individual experiments. The cells were harvested at the indicated time points by the addition of Tris lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% Deoxycholic acid) with a cocktail of protease inhibitors (1 mM PMSF, 7 \(\mu\)g/ml Pepstatin A, 20 \(\mu\)g/ml Leupeptin, and 2.9
µg/ml Aprotinin). Since cells treated with a high concentration of TBB detached from their plates, all non-adherent cells were collected and pooled with the adherent cells. The concentration of cell lysates was determined using a BCA assay (Bio-RAD) and normalized to the least concentrated sample. For each sample, 10 – 15 µg of lysate was separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was then blocked with 3% milk and probed with one of the following primary antibodies: monoclonal mouse-anti-p53 (DO-1, Santa Cruz), monoclonal mouse-anti-GAPDH (Millipore), polyclonal rabbit-anti-PARP (Cell Signal), or monoclonal rabbit-anti-cleaved PARP (Asp214, Cell Signal). The blot was then probed with fluorescence labeled goat-anti-mouse/rabbit secondary antibodies and visualized on a LiCor scanner (LiCor). All the antibodies used were a generous gift from Dr. David Litchfield (University of Western Ontario).
6.3 Results

6.3.1 CRISPR/Cas9 Knockout of NQO2

Disrupting the expression of endogenous NQO2 using RNA interference was effective in some cell lines but was challenging in others (39, 40). Previous attempts to knock-down NQO2 in Hela cells using shRNA resulted in roughly 60 percent decrease in NQO2 protein levels (data not shown). Therefore, the decision was made to completely abolish endogenous NQO2 expression using CRISPR/Cas9 technology (14). This way, unpredictable cellular changes caused by transfection reagents and residual quantities of NQO2 were avoided. To minimize off-target DNA double-strand cleavage events, the Cas9 nickase was targeted to two neighboring sequences in the fourth exon of NQO2 by a pair of guide RNA to generate two single stranded breaks (Figure 6.1A). HCT116 colorectal carcinoma cells were chosen to model NQO2-mediated p53 regulation for the following reasons: they have a normal p53 status, they have high levels of NQO2 expression and activity, and there is no basal expression of the P-glycoprotein efflux pump known to excrete toxic compounds (41–43). Five clonal populations of HCT116 cells were isolated after transfection of the CRISPR/Cas9 nickase constructs. In four of these clones, there was no detectable level of NQO2 expression (Figure 6.1B). To verify the success of the NQO2 gene disruption, PCR amplification of the targeted exon was performed. Clones 2 and 4 had no PCR amplicon, while clones 3 and 5 had a shorter PCR amplicon compared to the parental HCT116 cells (Figure 6.1C). Sequence analysis of the PCR amplicon of clones 3 and 5 showed that 18 DNA basepairs were deleted near the targeted exonic region. Since the two clones had the same genotype, it is possible they originated from the same colony.

The sequence analysis of the PCR amplicon from parental HCT116 showed a single nucleotide deletion in the intronic region upstream of exon 4 in one allele. However, this heterozygosity was not present in the PCR amplicons of HCT116ΔNQO2 clone 3 and 5, indicating that only one of the NQO2 exon 4 alleles was amplified. CRISPR/Cas9 mediated genome editing can cause excessive gene deletion and/or chromosomal translocations (44). Furthermore, HCT116 cells are known to be defective in their mismatch repair machinery, and their ability to repair DNA damage using non-
Figure 6.1 NQO2 Gene Knock Out in HCT116 using CRISPR/Cas9 Nickase.
(A) Illustration of the knockout strategy. The paired guide RNA was designed to generate a single strand nick at positions 46 and 108 of the fourth exon of NQO2. (B) Western blot of NQO2 in HCT116 (WT) and five knock out clones (numbered 1-5) transfected with the paired guide RNA. Four of the clones (2 to 5) had no detectable amount of NQO2. A non-specific band (*) was detected by the polyclonal anti-NQO2 antibody. (C) PCR amplification of exon 4 of NQO2 in HCT116 and HCT116 ΔNQO2 clones. Clone 1 showed a product size of 425 bp as expected, clones 2 and 4 did not have a PCR product, and clones 3 and 5 had a shorter PCR amplicon (top). PCR products were separated on a 3% polyacrylamide gel. Regions upstream and downstream of exon 4 were also individually amplified (bottom). A PCR product was observed for all clones in the 5’ flank and 3’ flank regions of exon 4. Thus, the two flanking regions of exon 4 exist in the genome but are disjointed in clone 2 and 4. PCR products of the flanking regions were separated on a 1% agarose gel.
homologous end-joining (NHEJ) is also impaired (45). Therefore, it is also possible that HCT116 cells are prone to genomic instability when manipulated by CRISPR/Cas9 genome editing tools. Accordingly, the regions that flanked the targeted cut sites were amplified to determine whether the genomic content was lost or disjointed. To our surprise, the 5’ and 3’ regions flanking the targeted cut sites were present in all clones and the genomic content was not lost (Figure 6.1C). Therefore, it is likely that a translocation event had disjointed the 4th exon of NQO2 in both alleles of clone 2 and 4, and in one allele of clone 3 and clone 5. Since one allele could be accounted for in clone 3 and clone 5, all subsequent experiments were performed using clone 3 unless otherwise specified.

6.3.2 Characterization of HCT116ΔNQO2 Cell Lines

HCT116 cells are colorectal carcinoma cells that have epithelial-like cell morphology. An initial comparison in morphology between the parent HCT116 cell and HCT116ΔNQO2 showed no observable differences. However, a slower overall growth rate was observed in the HCT116ΔNQO2 clones during normal cell passage. Consistent with these observations, it was previously reported that knockdown of NQO2 in prostate cancer cells slowed cell growth (16). To further investigate changes in proliferation, growth curves were generated for the HCT116 parental and HCT116ΔNQO2 cell lines. The growth rates of HCT116ΔNQO2 clones 3 and 5 were markedly slower than the parental cell line (Figure 6.2A). The greatest difference in cell counts occurred at the end of an exponential growth phase on day 4. At this point, there were 4- and 6-fold fewer cells in clones 3 and 5, respectively. The growth curves for HCT116ΔNQO2 clones 2-4 were also generated using SRB assay and similar growth suppression was observed (Figure S6.2).

To understand the cause of growth suppression, the cell cycle distribution was analyzed by flow cytometry using BrdU and PI staining (38). Asynchronous HCT116ΔNQO2 cells from clones 2-4 had up to a 2-fold higher distribution in the S phase compared to parental HCT116 (Figure 6.2B). HCT116ΔNQO2 also had an overall lower distribution in the G1 and G2/M phases. In combination with the slow growth phenotype observed in HCT116ΔNQO2 cells, a higher distribution of cells in the S phase is indicative of a defect in the intra-S phase checkpoint. Since NQO2 was shown to modulate AKT, NF-κB,
Figure 6.2 Characterization of HCT116\textsuperscript{ΔNQO2} clones
(A) Growth curve of HCT116 (○), HCT116\textsuperscript{ΔNQO2} clone 3 (■) and clone 5 (▲) assayed by cell count (left). Cells were initially seeded at 1000 cells/well in a 96-well plate. Each day, cells were trypsinized for cell counting using haemocytometer immediately (n=4). On day 4, cell counts were plotted on a linear scale to illustrate the difference in cellular proliferation between the three cell lines (right). (B) Increased S phase distribution in HCT116\textsuperscript{ΔNQO2} cells. Asynchronously proliferating cells of HCT116 (○), HCT116\textsuperscript{ΔNQO2} clone 2 (■), clone 3 (▲), and clone 4 (▼) were stained with propidium iodide and BrdU, followed by flow cytometry analysis. As a reference, a line was drawn for the cell cycle distribution of parental HCT116.

CEBP\textalpha, and p53 (13–16), cell cycle perturbation could be the result of dysregulation of these proteins.

6.3.3 Cytotoxicity of Substrate CB1954
To determine the catalytic activity of NQO2 in cells, Knox and coworkers established a protocol in which the cytotoxic compound CB1954 can be activated up to 1000-fold in the presence of NRH when transfected NQO2 was over-expressed (35). Here, it was found that as little as 12.5 µM of NRH could maximally induce the cytotoxicity of CB1954 in parental HCT116 cells. The IC\textsubscript{50} value of CB1954 was decreased over 40-fold from 203 ± 19.1 µM to 4.5 ± 0.4 µM (Figure 6.3A,C). In contrast, activation of CB1954 by NRH in HCT116\textsuperscript{ΔNQO2} cells was completely abolished even when co-treated with 200 µM NRH (Figure 6.3B-C). As such, cellular activity of NQO2 was completely abolished. In the absence of NRH co-treatment, the toxicity (IC\textsubscript{50}) of CB1954 was the same between the HCT116 and HCT116\textsuperscript{ΔNQO2} cell lines. This indirectly indicated that NRH (or any other NQO2 reducing co-substrate) was not abundant in HCT116 cells.
6.3.4 Toxicity of NR and NRH

The bioactivation of CB1954 in cells required the addition of exogenous NQO2 co-substrate (35). To this end, the cytotoxicity of NR\(^+\) and NRH was initially determined as an experimental control to find a non-toxic concentration of NRH suitable to activate CB1954. Unexpectedly, treating cells with NR\(^+\) or NRH alone at concentrations above 100 \(\mu\)M or 200 \(\mu\)M respectively were cytotoxic to both HCT116 and HCT116\(^{ΔNQO2}\) cells (Figure 6.3C). This was contrary to previous findings by Knox and coworkers, who indicated that treating cells with up to 10 mM of NRH or other NQO2 co-substrates were not cytotoxic in four human cancer cell lines (PC-3 prostate carcinoma, U373-MG and U87-MG glioblastoma multiforme, and T98G glioblastoma) (35). Furthermore, up to 1 mM of the same commercial NR\(^+\) used in this experiment was not cytotoxic to human aortic endothelial cells, HITC6 smooth muscle cells, and primary mouse smooth muscle cells (Krista Hawrylyshyn, personal communications). From these findings, it appears that HCT116 cell proliferation was sensitive to high concentrations of NR\(^+\) or NRH. In a recent study, mice deficient of a gene implicated in NAD\(^+\) synthesis more readily developed liver tumors when challenged with a carcinogen (46). Restoration of cellular NAD\(^+\) levels with a high NR\(^+\) diet prevented tumor formation and progression. In the same regard, an increase of NAD\(^+\) levels in HCT116 by NR\(^+\) and NRH treatment may be the cause of cytotoxicity for these cells, while it was non-toxic to other cell lines.

6.3.5 Toxicity of NQO2 Inhibitors

It is well known that CK2 inhibitors (TBB, TBBz, and DMAT) and DNA intercalators (EtBr, AO, and doxorubicin) are cytotoxic. These compounds were all previously identified to be NQO2 inhibitors (5, 33), but it is unclear how NQO2 inhibition contributes to the cellular effects of these chemicals. To explore this question, the IC\(_{50}\) of each inhibitor was determined in HCT116 and HCT116\(^{ΔNQO2}\) cells. Surprisingly, the status of NQO2 did not affect the cytotoxicity of these inhibitors (Figure 6.3D, Table 6.1). Since NRH was required by NQO2 to stabilize p53 \textit{in vitro} (17), NRH co-treatment could sensitize cells towards the apoptotic effects of the inhibitors. However, the addition of 61 \(\mu\)M NRH did not change the IC\(_{50}\) values of the inhibitors tested. Comparing the
Figure 6.3 Cytotoxicity of Substrate, Co-substrate, and Inhibitors in HCT116 and HCT116$^{\Delta}$NQO2 cells.

(A) Cytotoxicity of substrate CB1954. HCT116 (left) and HCT116$^{\Delta}$NQO2 (right) cells were treated with CB1954 at the indicated concentration with 6 µM (●), 12.5 µM (■) 25 µM (▲), 50 µM (▼), 100 µM (▲), 200 µM (◆), or without NRH (○) for 48 hrs and assayed for cell proliferation using SRB dye (n=3). All cellular growth measured by SRB assay was normalized to cell growth without treatment, and data was fitted to a dose-response curve. Fitted curve was only shown for 100 µM NRH co-treatment cells (◆) for clarity.

(B) IC$_{50}$ values CB1954 in HCT116 (●) and HCT116$^{\Delta}$NQO2 (○) cells when co-treated with NRH. The cytotoxicity of CB1954 increased 50-fold in the presence of NRH in parental HCT116 but the induction of CB1954 toxicity was completely abolished in HCT116$^{\Delta}$NQO2.

(C) Cytotoxicity of co-substrate NR$^+$ and NRH. HCT116 (●) and HCT116$^{\Delta}$NQO2 (○) cells were treated with NR$^+$ (left) and NRH (right) at the indicated concentration for 48 hrs and assayed for cell proliferation using SRB dye (n=3). Cellular proliferation was attenuated at NR$^+$ concentrations above 100 µM and NRH concentrations above 200 µM as indicated by the dotted line. (D) Representative curve of cytotoxicity of inhibitor treatment. HCT116 (●) and HCT116$^{\Delta}$NQO2 (○) were treated with TBBz at the indicated concentrations for 48 hrs and assayed for cellular proliferation using SRB dye (n=2). Further co-treatment with 61 µM NRH in HCT116 (■) and HCT116$^{\Delta}$NQO2 (□) cells did not affect cell growth (n=2). All cellular growth measured by SRB assay was normalized to cell growth without treatment. Data was fitted to a dose-response curve and IC$_{50}$ values are summarized in Table 1. Fitted curve was only shown for TBBz treatment of HCT116 WT cells (●) for clarity.
Table 6.1 IC$_{50}$ (µM) of Inhibitors in HCT116 and HCT116$^{\Delta NQO2}$ Cells$^a$

<table>
<thead>
<tr>
<th></th>
<th>NRH$^b$</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
<th>$K_D$ (nM)</th>
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<tbody>
<tr>
<td>TBB</td>
<td>43 ± 8.1</td>
<td>68 ± 23</td>
<td>57 ± 15</td>
<td>74 ± 23</td>
<td>7110 ± 40$^c$</td>
<td></td>
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<tr>
<td>TBBz</td>
<td>12 ± 2.4</td>
<td>16 ± 3.9</td>
<td>12 ± 2.5</td>
<td>18 ± 4.1</td>
<td>18 ± 0.4$^c$</td>
<td></td>
</tr>
<tr>
<td>DMAT</td>
<td>182 ± 31</td>
<td>134 ± 18</td>
<td>139 ± 25</td>
<td>147 ± 27</td>
<td>36 ± 1.7$^c$</td>
<td></td>
</tr>
<tr>
<td>EtBr</td>
<td>9.1 ± 0.9</td>
<td>9.5 ± 1.3</td>
<td>12 ± 1.7</td>
<td>8.1 ± 0.9</td>
<td>3.5 ± 0.3$^{d,e}$</td>
<td></td>
</tr>
<tr>
<td>AO</td>
<td>3.9 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>4.5 ± 0.5</td>
<td>3.7 ± 0.4</td>
<td>0.4 ± 0.1$^{d,e}$</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.61 ± 0.14</td>
<td>0.37 ± 0.07</td>
<td>-</td>
<td>-</td>
<td>274 ± 49$^d$</td>
<td></td>
</tr>
</tbody>
</table>

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$^a$ IC$_{50}$ values were determined by treatment of inhibitors at various concentrations for 48 hrs. Fitted curves are shown in Figure S6.2.

$^b$ Cells were co-treated with and without 61 µM NRH.

$^c$ $K_D$ values of TBB, TBBz, and DMAT towards NQO2 were previously determined using fluorescence titration (32).

$^d$ $K_D$ values of EtBr, AO, and doxorubicin towards NQO2 were previously determined by ITC (To be published).

$^e$ Dissociation constants of EtBr and AO towards reduced NQO2.

IC$_{50}$ values of each inhibitor to their respective in vitro $K_D$ values, the concentrations needed to inhibit cell growth were roughly 2 to 3 orders of magnitude higher than their dissociation constants for NQO2 (Table 6.1). TBB and doxorubicin were the only exception where their cellular IC$_{50}$ values were similar to their in vitro $K_D$ values.

Altogether, it appears that NQO2 inhibition was not directly responsible for the cytotoxic effects of the inhibitors even though the concentrations used adequately inhibited NQO2 in vitro.

### 6.3.6 Modulation of p53 Expression Levels by NQO2 Inhibitors

NQO2 protected p53 from degradation in vitro. Accordingly, mice deficient in NQO2 also had a lower expression of p53 (17, 23). According to these findings, a down-regulation of p53 expression in HCT116$^{\Delta NQO2}$ cells was expected. However, the basal level of p53 expression remained the same even when endogenous NQO2 was knocked out in HCT116 cells (Figure 6.4A). This was contrary to previous findings and raised the question of whether NQO2 is active in stabilizing p53 in tumorigenic cells.

NQO2 status did not change how CK2 inhibitors and DNA intercalators attenuated cell growth, but the six inhibitors modulated p53 levels in an NQO2-dependent manner (Figure 6.4B-C). To compare the effects of these inhibitors, HCT116 and HCT116$^{\Delta NQO2}$
cells were treated with inhibitors at concentrations of either 1/5<sup>th</sup> of the IC<sub>50</sub> or IC<sub>50</sub> for 18 or 24 hrs (Table 6.1). When cells were treated with inhibitors at 1/5<sup>th</sup> concentrations of IC<sub>50</sub>, p53 levels did not vary greatly. Specifically, p53 was slightly down-regulated in TBB and AO treated cells, while p53 levels increased in doxorubicin treated cells. At the IC<sub>50</sub> concentration where all the inhibitors attenuated cell growth to the same degree, the modulated levels of p53 expression were drastically different. TBBz, EtBr, and doxorubicin highly induced p53 expression, while TBB and DMAT down-regulated p53. In comparison, AO had the least effect on p53 regulation despite binding most tightly to NQO2 <em>in vitro</em>. Therefore, NQO2 inhibitors did not uniformly modulate p53 expression. Furthermore, inhibitor-induced changes in p53 levels did not differ much between HCT116 and HCT116<sup>ΔNQO2</sup> cells. The most drastic difference was observed in TBBz treatment at the IC<sub>50</sub> concentration where p53 levels was roughly two times greater in HCT116 cells compared to HCT116<sup>ΔNQO2</sup> cells. NQO1 inhibitors dicoumarol and curcumin induced p53 degradation in HCT116 cells (19, 47), yet our data showed that NQO2 inhibitors did not degrade p53 in a consistent manner. Drawing from these observations, NQO2 could function differently compared to NQO1 in HCT116 cells.

6.3.7 Apoptotic Response and p53 Modulation by TBB and TBBz

Since mutation or down-regulation of endogenous NQO1 prevented inhibitor-induced p53 degradation (19, 20, 47), NQO2 could modulate inhibitor-induced p53 degradation. As such, apoptotic signalling and p53 modulation by TBB and TBBz were more carefully examined. HCT116 and HCT116<sup>ΔNQO2</sup> cells were treated with three concentrations of TBB and TBBz (corresponding to 1/5<sup>th</sup> of IC<sub>50</sub>, IC<sub>50</sub>, and 5 times the IC<sub>50</sub>) for 6, 12, 18, and 24 hrs. At 5 times the IC<sub>50</sub> concentration of TBB, cells detached from the plates after 12 hrs. Despite detaching, they were still viable as determined by a trypan blue exclusion assay. In light of this finding, all cells were pooled for western blot analysis. Initial blotting using a polyclonal PARP antibody was unable to reliably detect cleaved PARP products (Figure S6.4). Using a monoclonal antibody against cleaved PARP, this apoptotic signal was clearly detected and appeared to be no different between HCT116 and HCT116<sup>ΔNQO2</sup> cells (Figure 6.5A). Following TBB treatment, apoptosis was
Figure 6.4 Western Blot Analysis of p53 in HCT116 and HCT116ΔNQO2 Cells Treated with Six NQO2 Inhibitors

(A) Western blot of p53, NQO2, and GAPDH in HCT116 cells and HCT116ΔNQO2 clone 2-5. Fold difference was calculated by integrated intensity of p53 normalized to GAPDH compared to levels of p53 of WT cell line. (B) HCT116 and HCT116ΔNQO2 cells were treated with six NQO2 inhibitors at 1/5th IC50 and IC50 concentrations for 18 and 24hrs (refer to table 6.1). Cell lysates were analyzed by western blot for p53 and GAPDH. (C) Quantification of p53 was performed by normalizing the intensity of each p53 band against GAPDH band and shown as a bar graph.
only apparent in the detached cells treated with 5 times the IC<sub>50</sub> concentration at 6 and 12 hrs. In contrast, TBBz induced apoptosis most strongly at the IC<sub>50</sub> concentration at 18 and 24 hrs, but was almost absent at 1/5<sup>th</sup> and 5 times the IC<sub>50</sub> concentrations. Therefore, inhibitor-induced apoptosis was dependent on time and concentration of inhibitors used, but not dependent on NQO2 status.

The inhibitor-induced modulation of p53 also appeared to be time and dose dependent (Figure 6.5A-B). Overall, TBB treatment down regulated p53 levels while TBBz induced its expression. For the low and medium concentrations of TBB, p53 levels were not drastically perturbed. At 5 times the IC<sub>50</sub> concentration of TBB, however, p53 was down regulated in HCT116<sup>ΔNQO2</sup> cells and almost undetectable in parental HCT116 cells. In contrast, all three concentrations of TBBz treatment induced p53 expression. At the IC<sub>50</sub> concentration, TBBz induced p53 almost twice as much in HCT116<sup>ΔNQO2</sup> cells compared to the parental HCT116 cells at 12, 18, and 24 hrs. In terms of temporal differences, changes in p53 levels were more gradual in cells deficient of NQO2 compared to HCT116 cells for all concentrations of TBB and TBBz treatment. Specifically, inhibitor-mediated p53 levels rose and fell at an earlier time point in parental HCT116 cells compared to the same inhibitor treatment in HCT116<sup>ΔNQO2</sup> cells. Finally, HCT116<sup>ΔNQO2</sup> cells had an overall lower p53 levels compared to parental HCT116 cells. The only exception was for TBB treatment at the IC<sub>50</sub> concentration. On the whole, the data showed that NQO2 played a role in modulating p53 in very complex manner.
Figure 6.5 Analysis of PARP Cleavage and p53 Modulation in HCT116 and HCT116ΔNQO2 Cells Treated with TBB and TBBz

(A) Cells were treated with 11, 55, or 275 µM TBB and or 2.8, 14, or 69 µM TBBz (corresponding to 1/5th IC_{50}, IC_{50}, and 5 x IC_{50} of the individual inhibitors). At 6, 12, 18, and 24 hrs, cells lysed with Tris lysis buffer. At 12, 18, and 24 hrs, cells treated with 275 µM TBB all lifted from plate. As such, all cells were pooled for analysis. The concentration of cell lysates was normalized and 15 µg of each sample were resolved on SDS-PAGE. After being transferred onto a PVDF membrane, it was probed with anti-cleaved PARP, anti-p53, and anti-GAPDH antibodies. The membrane was then probed with fluorescence labeled secondary antibodies and visualized using a LiCor imaging system. (B) Quantification of p53 was performed by normalizing the intensity of each p53 band against GAPDH band and was shown as a bar graph.
6.4 Discussion

NQO2 was traditionally characterized as an enzyme, but recent findings suggest it also functions as a flavin redox switch to modulate p53 degradation (17, 28). As such, NQO2 inhibitors that inactivate catalysis may be modulating NQO2 in an unexpected manner by modifying the switch function of NQO2. Furthermore, numerous bioactive compounds were found to interact with NQO2 as an “off-target” but how these interactions contribute to their effects is poorly understood. To this end, we knocked out endogenous NQO2 in HCT116 cells using CRISPR/Cas9 technology to study the effects of NQO2 inhibitors. However, the six NQO2 inhibitors studied did not affect cell viability in an NQO2-dependent manner. Thus, the cytotoxic effects of NQO2 inhibitors were not directly caused by NQO2 inhibition. Instead, inhibitors modulated p53 in a time-, dosage-, and NQO2-dependent manner.

In 2001, the modulation of p53 by NQO1 was shown for the first time (19). In that study, an NQO1 specific inhibitor, dicourmarol, drastically decrease p53 levels in HCT116 cells. Since NQO2 was thought to function similarly to NQO1, NQO2 inhibitors were expected to affect p53 levels in the same way. However, this was not the case in this current study. For the six inhibitors studied, each inhibitor modulated p53 levels differently. Some inhibitors induced p53 (TBBz, EtBr, and doxorubicin), others downregulated p53 (TBB, DMAT), and one inhibitor didn’t change p53 levels (AO). Furthermore, the two inhibitors specific to reduced NQO2 (EtBr and AO) modulate p53 in an unpredictable way compared to inhibitors specific to oxidized NQO2. From these results, the role of NQO2 in modulating p53 appears to be different compared to NQO1.

The stabilization of p53 by NQO2 and NQO1 was dependent on NRH and NADH respectively (17). Since the co-substrate specificity of NQO2 and NQO1 are exclusive to one other (8), cellular concentrations of NRH and NADH would be crucial in determining how NQO2 and NQO1 modulate p53. However, the availability of cytosolic NRH is questionable. On one hand, activation of the NQO2 substrate CB1954 in cells required the addition of either NRH or another NQO2 co-substrate (35); as confirmed in this study. On the other hand, activation of the NQO1 substrate EO9 in cells did not require the addition of NADH (48, 49). Therefore, cellular concentrations of NQO2 co-
substrates are normally too low for reduction of substrates, while concentrations of NQO1 co-substrates are sufficient for catalysis. On this basis, it is possible that NQO2 remains in an oxidized state in the absence of NRH, unable to stabilize p53 from degradation; hence inhibition of NQO2 has no effect on p53. In contrast, NQO1 could stay in a reduced state in the presence of NADH, which allows it to maintain basal p53 levels. The inhibition of NQO1 then prevents NQO1 reduction and induces p53 degradation. Along these lines, NQO2 and NQO1 may be constantly sensing the balance between NR\(^+\)/NRH and NAD\(^+\)/NADH levels in cells in regulating p53. Together, NQO2 as a flavin redox switch appears to be regulated differently compared to NQO1.

To further complicate the interpretation of our observations, the NQO2 inhibitors used in this study were not very specific to NQO2. The six NQO2 inhibitors were studied for their NQO2-dependent effects at concentrations that attenuated cell growth by 50% (IC\(_{50}\). With the exception of TBB and doxorubicin, the inhibitor’s IC\(_{50}\) were 2 – 3 orders of magnitude higher than their dissociation constants (K\(_D\)) towards NQO2. As such, the inhibitors could also be binding to other targets and affecting multiple cellular processes: TBB, TBBz, and DMAT were originally designed to inhibit CK2, while EtBr, AO, and doxorubicin were well-characterized DNA intercalating agents. Furthermore, some of these compounds also inhibited additional targets in cells. For example, TBBz and DMAT potently inhibit kinase from three other families, and doxorubicin inhibits topoisomerase II (31, 50). Consequently, the cytotoxicity and p53 expression induced by inhibitors may not be direct effects of NQO2 interactions.

Differences in how HCT116 and HCT116\(^{ΔNQO2}\) modulated inhibitor-induced p53 changes were initially not apparent in the six inhibitors studied. Further investigation into cells treated with TBB and TBBz showed that p53 was modulated in a time-, dose-, and NQO2-dependent manner. As such, NQO2-mediated modulation of p53 was not easily detected. Owing to the lack of NRH in normal proliferating cells, three possible scenarios regarding how inhibitors could affects p53 modulation in an NQO2-dependent manner were postulated. Firstly, the direct binding of inhibitors to oxidized NQO2 may generate a novel state of NQO2 whose functions are directly responsible for p53 regulation. Secondly, since inhibitors also target other cellular processes, oxidized NQO2 may play
an indirect role in modulating p53. Lastly, the inhibitors of NQO2 could induce NRH production through an unknown mechanism. In turn, elevated NRH concentrations could reduce NQO2 to stabilize p53 levels. In all three scenarios, the mechanism of p53 modulation by NQO2 is complicated and deciphering it will require further investigation using specific inhibitors, different cell lines, and robust p53 detection techniques.

In summary, endogenous NQO2 was successfully knocked out in HCT116 cells using the CRISPR/Cas9 genome editing tool. Even though NQO2 was historically characterized to function similarly to NQO1, NQO2 inhibitors did not consistently degrade p53 as NQO1 inhibitors were shown to do. Furthermore, the lack of NQO2 slowed cell growth, but had no effect on cell viability when treated with inhibitors. Drawing from these observations, the role of NQO2 as a flavin redox switch is complex and is different compared to its homologue NQO1.
6.5 References


32. Leung, K. K. K., and Shilton, B. H. (2015) Quinone Reductase 2 Is an Adventitious Target of Protein Kinase CK2 Inhibitors TBBz (TBI) and DMAT. *Biochemistry*. 54, 47–59
33. Leung, K. K. K., and Shilton, B. H. *The Binding of DNA Intercalating Agents to Oxidized and Reduced Quinone Reductase 2*


6.6 Supporting information

Table S 6.1 Oligonucleotides Used for CRISPR Cassette Construct.

<table>
<thead>
<tr>
<th>Guide RNA oligos</th>
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<tr>
<td>Exon4_46_top</td>
<td>CACCGACCCTTTGCTTGTAGGCTTCG</td>
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<tr>
<td>Exon4_46_bottom</td>
<td>AAACCCAAGCTACAAGCAAAGGT</td>
</tr>
<tr>
<td>Exon4_108_top</td>
<td>CACCGTGAGCAGAAAAAGGTTCGGG</td>
</tr>
<tr>
<td>Exon4_108_bottom</td>
<td>AAACCCGAACCTTTTTCTGCTCA</td>
</tr>
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Table S 6.2 Oligonucleotides Used for PCR Validation of HCT116^{ΔNQO2} cells

<table>
<thead>
<tr>
<th>Primers</th>
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</tr>
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<tr>
<td>Exon4_F1</td>
<td>TGCTAGGTAGCAAGTGCTCAATC</td>
</tr>
<tr>
<td>Exon4_R1</td>
<td>CTTCCAGAAAGCAGCACAATACTC</td>
</tr>
<tr>
<td>Exon4_F2</td>
<td>CTGCCCTAGTATATGGTTTTGTC0</td>
</tr>
<tr>
<td>Exon4_R2</td>
<td>GTCCTGCCTCATAATTGAAACCTCAG</td>
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<tr>
<td>46oF1</td>
<td>ATGGAAAGCAGGAACTCAGAAAACTCA</td>
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<tr>
<td>46oR1</td>
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</tr>
<tr>
<td>108oF1</td>
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</tr>
<tr>
<td>108oR1</td>
<td>TTCTGCTCAAAGGTTCGCC</td>
</tr>
</tbody>
</table>

Figure S 6.1 Cartoon Illustration of Primers Used for PCR Validation in of HCT116^{ΔNQO2} Cells.

PCR amplification of wild-type NQO2 gene using primers Exon4_F1 and Exon4_R1 will generate a PCR product of 425bp, while the PCR product of the knockout gene is estimated to be 363bp if exonuclease excise single stranded nucleotides up to the targeted cut sites. PCR amplification of the 5’ flank and 3’ flank regions in genomes where full-length PCR products are absent will show that the two ends of exon4 still exist in the genomic DNA. This suggests that a chromosomal translocation event has occurred.
Figure S 6.2 Growth Curve of HCT116 and HCT116ΔNQO2 Cells Assayed by SRB. HCT116 (●), HCT116ΔNQO2 clone 2 (■), clone 3 (▲), and clone 4 (▼) cells were seeded at 1000 cells/well in a 96-well plate (n=3). Each day, cells were fixed with 10% TCA for 20 mins at room temperature and returned to the incubator. On day 8, the fixed cells were stained with 0.3% SRB dye and absorbance at 560nm was recorded using a plate reader (left). On day 4, HCT116 cell proliferation was markedly higher than any of the clones of HCT116ΔNQO2 (right).
Figure S 6.3 Cytotoxicity of NQO2 Inhibitors in HCT116 and HCT116ΔNQO2 cells. HCT116 (●) and HCT116ΔNQO2 (■) cells were treated with TBB (A), TBBz (B), DMAT (C), EtBr (D), AO (E), and doxorubicin (F) at the indicated concentrations for 48 hrs and assayed for cellular proliferation using SRB dye (n=2). HCT116 (▲) and HCT116ΔNQO2 (▼) cells co-treated with inhibitors and 61 µM NRH did not show marked differences in proliferation (n=2). All cellular growth measured by SRB assay was normalized to cell growth without treatment. Data was fitted to a dose-response curve and IC₅₀ values are summarized in Table 1. Fitted curves are only shown for inhibitor treatment of HCT116 WT cells (●) for clarity.
Figure S 6.4 Analysis of PARP Cleavage and p53 Modulation in HCT116 and HCT116ΔNQO2 Cells Treated with TBB and TBBz

(A) Cells were treated with 11, 55, or 275 µM TBB and or 2.8, 14, or 69 µM TBBz (corresponding to 1/5th IC₅₀, IC₅₀, and 5 x IC₅₀ of the individual inhibitors). At 6, 12, 18, and 24 hrs, cells lysed with Tris lysis buffer. At 12, 18, and 24 hrs, cells treated with 275 µM TBB all lifted from plate. As such, all cells were pooled for analysis. The concentration of cell lysates was normalized and 15 µg of each sample were resolved on SDS-PAGE. After being transferred onto a PVDF membrane, it was probed with polyclonal anti-PARP, anti-p53, and anti-GAPDH antibodies. The membrane was then probed with fluorescence labeled secondary antibodies and visualized using a LiCor imaging system. (B) Quantification of p53 was performed by normalizing the intensity of each p53 band against GAPDH band and shown as a bar graph.
7 Summary and Perspectives

NQO2 was historically characterized as an enzyme that catalyzed the reduction of quinone. However, recent evidence had revealed that NQO2 also had a role in regulating the 20S proteasomal degradation of p53. On this basis, my hypothesis is that NQO2 functions as a flavin redox switch that senses intracellular NRH levels and recognizes inhibitors. The work presented in this thesis addressed the molecular aspects of how NQO2 behaved as a flavin redox switch, and showed that inhibitors could perturb cellular signalling by modulating p53 stabilization. In this chapter, the structural features that allow NQO2 to function as a flavin redox switch will be summarized. Next, the fundamental differences between how NQO2 and NQO1 function as a flavin redox switch will be discussed. Finally, a hypothesis of how NQO2 could function as a multi-drug sensor will be explored. In each section, some of the unpublished work initiated for NQO2 will also be highlighted and the direction of research will be proposed.

7.1 Structural Indication of NQO2 as a Flavin Redox Switch

The ability of NQO2 to stabilize p53 is redox-dependent (1). However, little is known about reduced NQO2. Using one of the two known reduced-state inhibitors of NQO2 at that time, the structure of reduced NQO2 in complex with chloroquine (CQ) was solved (Chapter 3). This was a challenging endeavor because NQO2 spontaneously oxidizes in the presence of oxygen. By reducing the oxidized protein-ligand crystal complex in an anoxic environment, NQO2 was kept reduced. Solving this structure, reduced NQO2 in complex with an inhibitor was characterized for the first time. Compared to oxidized NQO2, three observations were made in reduced NQO2-CQ: an FAD bend, an altered hydrogen bond network at the active site mediated by several water molecules, and a global conformation change.

Subsequently, the structures of oxidized and reduced NQO2 were obtained in complex with the following four inhibitors: DMAT (chapter 4), ethidium, acridine orange (chapter 5), and quinacrine (unpublished). Compared to their respective oxidized NQO2-inhibitor structures, all the reduced NQO2-inhibitor complexes had approximate a 5° “butterfly”
bend at the isoalloxazine ring of the reduced FAD that was repeatedly detected. Additionally, all the reduced structures showed a consistent rearrangement of the hydrogen bond network mediated by several water molecules at the active site. The water molecules prevented the inhibitors from binding in a deeply buried manner, which indicated that they were coordinated more strongly in reduced NQO2 compared to oxidized NQO2.

In the reduced NQO2-CQ structure, a global conformation change indicated by a loss in crystallographic spacegroup symmetry was also observed. However, this change was absent in the other four reduced NQO2 structures. From a technical perspective, the change in spacegroup may be only apparent at higher-resolutions. The data of reduced NQO2-CQ crystals was collected to 1.25Å, whereas the dataset for the other reduced NQO2-inhibitor crystals were collected to 1.9Å. Another explanation for this unique conformational change could be attributed to the chemical structure of CQ. Compared to the other inhibitors, CQ has a tail moiety in addition to a planar moiety. In the reduced NQO2-CQ structure, this tail moiety of CQ was in close contact with I128. This particular isoleucine residue was part of a loop region in NQO2 that was in contact with symmetry-related NQO2 molecules. Therefore, the “closing” of the loop region mediated by CQ (Figure 3.3) could be responsible for the change in spacegroup.

Since reduced NQO2 underwent a global conformation change in the crystal structure, reduction of NQO2 in solution was also investigated using small angle X-ray scattering (SAXS). SAXS is a low-resolution technique used to probe conformational changes of proteins in solution. To determine whether NQO2 was drastically different in its two redox states, oxidized NQO2, reduced NQO2, and reduced NQO2 in the presence of CQ were analyzed using SAXS and the data was summarized in Table 7.1. It was found that the radiuses of gyration ($R_g$) between the three samples were no different from each other. In essence, conformational changes seen in the reduced crystal structure were too small to be observed using SAXS.
Table 7.1 SAXS Analysis of NQO2 in Solution

<table>
<thead>
<tr>
<th></th>
<th>( I_{(0)} )</th>
<th>( R_g )</th>
<th>( D_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO2 alone</td>
<td>55.10</td>
<td>23.31</td>
<td>71</td>
</tr>
<tr>
<td>NQO2 with SCDP</td>
<td>55.80</td>
<td>23.28</td>
<td>72</td>
</tr>
<tr>
<td>NQO2 with SCDP and CQ</td>
<td>55.10</td>
<td>23.12</td>
<td>72</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>27.90</td>
<td>13.75</td>
<td>40</td>
</tr>
</tbody>
</table>

*Data was collected at the Advance Photon Source BioCAT 18ID Beamline
Data was analyzed using Scatter (Robert Rambo, Diamond Light Source (Didcot, UK))

To investigate minute changes in reduced NQO2, one approach would be to compare protein dynamics at the molecular level. In a crystal structure, the atomic displacement parameter of each atom describes how “mobile” a certain residue is. Using this data, the normalized atomic displacement factors (nADP, as described in chapter 2) between the main chain atoms of oxidized and reduced NQO2-CQ were compared (Figure 7.1). In the reduced NQO2-CQ structure, an increase in nADP was localized on one side of the protein, while a decrease was localized on the opposite side. These concerted changes in nADP indicated that the reduction of NQO2 in complex with CQ facilitated the mobility of residues globally. Therefore, these minute changes in protein dynamics may be crucial in mediating its interaction with the 20S proteasome and p53.

**Figure 7.1 Protein Dynamic Changes in Reduced NQO2.**
The NQO2 dimer was coloured according to the difference in the normalized atomic displacement parameter (nADP) between oxidized and reduced NQO2-CQ complex. Increases in nADP (indicated in red) was localized to the bottom half of NQO2, and decreases in nADP (indicated in blue) was localized to the top half of NQO2 shown in this figure. The two views of the NQO2 dimer are related by a 90° turn about the vertical axis.
Having solved the reduced structures of NQO2 with four other inhibitors, a comprehensive comparison of nADP between oxidized and reduced NQO2 could reveal trends in protein dynamics. Pairing such analysis with molecular dynamic simulations of oxidized and reduced NQO2 could reveal the nature of how NQO2 behaves in a redox dependent manner. In conclusion, further molecular analysis will shed more light on the protein dynamics of NQO2 as a flavin redox switch.

7.2 Cellular Inhibition of NQO2

Historically, NQO2 was thought to function in a similar fashion to NQO1. While the mechanism of quinone catalysis was the same, their co-substrate, substrate, and inhibitor specificity were exclusive to one another. In this regard, it was unclear whether NQO2 had the same role in modulating p53 degradation as NQO1. For NQO1, p53 levels decreased in response to NQO1 knockdown, mutation, or inhibition, in HCT116 cells (2–5). Furthermore, p53 was undetectable in mice deficient of NQO1 (6). For NQO2, p53 degradation was dependent on NRH, and mice deficient of NQO2 had a lowered expression of p53 (1, 7). However, it remained unknown how NQO2 inhibitors would affect p53 degradation. In chapter 6, six NQO2 inhibitors were shown to modulate p53 levels, albeit inconsistently, in HCT116 cells. Knocking out endogenous NQO2 had the effect of partially dampening the inhibitor-mediated induction/degradation of p53. Therefore, NQO2 inhibitors modulated p53 in a different manner compared to NQO1, but exactly how NQO2 participates in p53 degradation remains poorly understood (2).

As a flavin redox switch, the one fundamental difference between NQO1 and NQO2 is their co-substrate specificity. NQO1- and NQO2-mediated degradation of p53 in vitro is dependent on NADH and NRH respectively (1). On this basis, the ability of NQO1 and NQO2 to stabilize p53 would depend on the availability of these dihydronicotinamide concentrations in cells. However, endogenous concentrations of NRH or another NQO2 co-substrate was insufficient to reduce NQO2 specific substrate CB1954 (Chapter 6, (8)). In contrast, NQO1 specific substrates EO9 and mitomycin C were readily activated in cells without the addition of NADH (9–11). As a result of these findings, the belief that NRH is a natural co-substrate of NQO2 is questionable. Inferring from the different cellular concentrations of NRH and NADH, it appears that NQO2 is normally in the
oxidized state, while NQO1 is in the reduced state in proliferating HCT116 cells. In the next section, the potential sources of NRH will be further discussed.

7.2.1 Source of NRH

There are three potential mechanisms of NRH production: 1) transfer of a hydride ion from NADPH to NR$^+$ by transhydrogenase, 2) truncation of NAD(P)H to NRH by NAD-consuming enzymes, and 3) reduction of free NR$^+$ to NRH by dehydrogenase. Transhydrogenases are proteins found on the inner membrane of mitochondria that transfer the hydride ion from NADPH to NAD$^+$ to generate NADP$^+$ and NADH. However, no transhydrogenases have been identified to catalyze this function on the cytosolic side of a mammalian cell, and it is unclear whether this enzyme can utilize NR$^+$ (12). The second potential source of NRH could originate from enzymes that consume NAD$^+$. These include poly-ADP ribose polymerase (PARP), mono-ADP-ribosyltransferase, and deacetylase (ie. Sirtuins). These proteins transfer the ADP-ribose moiety of NAD$^+$ to lysine or arginine side chains, leaving nicotinamide as a by-product. This way, NR$^+$ may be generated by nicotinamide phosphoribosyltransferase (NAMPT) through the NAD$^+$ salvage pathway. However, these enzymes are strictly dependent on NAD$^+$ and do not utilize reduced NADH. Therefore, the first two potential sources of intracellular NRH seem unlikely.

In a study investigating NAD$^+$ derivatives in alcohol metabolism, Sicsic and co-workers showed that NR$^+$ can be used in place of NAD$^+$ to oxidize ethanol by horse liver alcohol dehydrogenase (HLADH) in the presence of AMP (13). Consequently, it is not inconceivable that other mammalian dehydrogenases have the ability to reduce NR$^+$ to NRH. When cells are insulted by oxidative stress or DNA damage, NAD$^+$ depletion by PARP activation is often accompanied by ATP depletion via the inhibition of glycolysis (14). In this low energy state, adenylate kinase is known to catalyze the reaction $2$ ADP $\rightarrow$ AMP + ATP to maintain high ATP levels for cell survival. Therefore, NR$^+$ and AMP could be abundant when cells are insulted by genotoxic and oxidative stress. Along these lines, NRH could be produced by an abundant dehydrogenase such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Together, NRH could be produced when NR$^+$, AMP, and the reducing substrates are present at the right concentrations within a cell.
Identifying enzymes capable of generating NRH will be the first step in understanding the mechanism of NQO2 reduction in cells.

To date, the only physiological condition known to stimulate NQO2-mediated stabilization of p53 is the inhibition of dihydroorotate dehydrogenase (DHODH) (15). DHODH is part of the de novo pyrimidine biosynthesis pathway, and the inhibition of this enzyme depletes pyrimidine nucleotide cells. Even though inhibition of DHODH induced p53 stabilization in an NQO2 dependent manner, it was unclear how this type of metabolic stress affects NQO2. One possibility is that NRH, or another NQO2 substrate, is generated under these circumstances. To determine the cellular concentration of NRH, CB1954 could be used as a surrogate reporter because it is strictly dependent on human NQO2 and NRH. Using this assay, it would be valuable to determine whether common signals of p53 induction such as oxidative stress, UV rays, gamma irradiation, and hypoxia, could induce NRH production. Further metabolomics studies using mass spectrometry can validate the identity of the NQO2 co-substrate. Altogether, there is a need to determine how NRH is produced in cells and how NQO2 is reduced in a cellular context.

7.3 NQO2 as a Sensor of Toxic Compounds

The function and regulation of p53 is very complex. As a transcription factor, it can activate genes responsible for cell cycle arrest and apoptosis. Independent of transcriptional activation, p53 can induce apoptosis by directly interacting with pro-apoptotic signalling proteins (16). Furthermore, it can modulate proteins and transcriptional factors involved in other cellular processes. An investigation into six NQO2 inhibitors showed that NQO2 was not responsible for the cytotoxicity or apoptotic signalling mediated by the inhibitors (Chapter 6). However, TBB and TBBz modulated p53 to some degree in an NQO2 dependent manner. This indicated that NQO2’s role in cells as a flavin redox switch could involve cellular processes other than p53-induced apoptosis. In this section, the subject of identifying CK2 inhibitors and DNA intercalating agents as NQO2 inhibitors will be revisited. Given the diverse nature of the compounds that bind NQO2, the potential role of NQO2 as a sensor for cytotoxic compounds will be
proposed. Finally, a perspective on how to study NQO2’s role as a flavin redox switch using a proteomics approach is presented.

7.3.1 NQO2 inhibition by Kinase Inhibitors

CK2 is a constitutively active kinase essential for cell survival. It is capable of phosphorylating a diverse range of substrates. The long-standing interest in understanding the cellular response to CK2 inhibition at David Litchfield’s lab identified NQO2 as the unintended target of TBBz and DMAT (17). This prompted a full investigation into the kinetic and structural interactions between CK2 inhibitors and NQO2 (Chapter 4). The initial rationale for obtaining the structure of NQO2 in complex with TBBz and DMAT was to gain insight towards designing an inhibitor-resistant NQO2 construct. Such mutant constructs could restore NQO2 activity in cells to study NQO2-specific cellular effects. It was previously shown that mutations in the active site of NQO2 decreased the affinity of the melatonin derivative $[^{125}\text{I}]$iodo-MCA-NAT$^9$ from binding to NQO2 without decreasing its catalytic activity (18). Therefore, it appears feasible to design an inhibitor-resistant NQO2 mutant to validate inhibitor-induced cellular effects caused by NQO2 inhibition alone.

Since TBB and TBBz modulated p53 degradation in an NQO2-dependent manner (Chapter 6), NQO2’s interactions with other kinase inhibitors may also affect this cellular process. To determine whether other CK2 inhibitors bind to NQO2, a more specific CK2 inhibitor, CX-4945, was found. NQO2 catalytic activity was reduced to 75% in the presence of 5 µM CX-4945, thus it was able to inhibit NQO2 weakly (Figure 7.2).

NQO2 was also known to be an unintended target of BCR-Abl kinase inhibitors imatinib and nilotinib. While the BCR-Abl inhibitors do not inhibit CK2 and CK2 inhibitors (TBBz and DNAT) do not inhibit Abl-kinase, both inhibited NQO2 (17, 19–22). Even though these kinase inhibitors were initially designed to inhibit kinase activity, they are further selected based on favorable clinical outcomes. Therefore, there is a possibility that...

$^9$ $[^{125}\text{I}]$iodo-MCA-NAT: 2-$[^{125}\text{I}]$iodo-5-methoxycarbonylamino-N-acetyltryptamine
a compound able to inhibit both the kinase and NQO2 outperformed compounds that only inhibited the kinase. In this regard, a comprehensive screen of kinase inhibitors towards NQO2 catalysis could show whether or not NQO2 inhibition contributes to the efficacy of kinase inhibitors in general.

**Figure 7.2 Inhibition of NQO2 Catalysis by CX4945.** Data was fitted to a dose-response curve.

### 7.3.2 Inhibition of NQO2 by DNA Intercalating Agents

Studies demonstrating that some NQO2 inhibitors could intercalate DNA prompted an investigation into whether other DNA intercalators could perform likewise (23, 24). In Chapter 5, three DNA intercalators were identified to be novel inhibitors of NQO2. Most NQO2 inhibitors identified to date are oxidized-state inhibitors, so it was unexpected to find ethidium (EtBr) and acridine orange (AO) to be reduced-state inhibitors. The common feature between EtBr, AO, and the two previously identified reduced-state inhibitors CQ and quinacrine was a positive charge in the planar moiety. It appears that positively charged compounds interacted favorably with the reduced FAD. Further structural comparisons between extended (unwound) DNA and the NQO2 active site showed numerous similarities between the two binding cavities. Drawing from these observations, NQO2 has the potential to recognize a diverse range of DNA intercalating agents in cell.

The identification of DNA intercalating agents as NQO2 inhibitors also raised the question of whether other NQO2 inhibitors also intercalate DNA. Some NQO2 inhibitors such as imatinib, 9-aminoacridine, and resveratrol have been independently shown to interact with DNA (25–27). Along these lines, an important question regarding NQO2
inhibitors is whether their cytotoxic effects are primarily due to DNA intercalation or inhibition of protein targets. A comprehensive analysis of known NQO2 inhibitors’ ability to intercalate DNA could clarify why NQO2 inhibitors are cytotoxic to cells.

7.3.3 Potential Role of NQO2 as a Sensor of Toxins

Given the ability of NQO2 to recognize low concentrations of CK2 inhibitors and DNA intercalating agents, it is unclear if these interactions serve a function. One possible downstream effect of inhibitor binding is to induce multidrug efflux pumps mediated by p53. Bacterium gain drug resistances by expressing multidrug efflux pumps that excrete non-specific compounds (28). Many of these transporters are under transcriptional regulation by multidrug binding proteins. For example, BmrR in Bacillus subtilis (29), QacR in Staphylococcus aureus (30), and EmrR in Escherichia coli (31) are transcriptional regulators of multidrug efflux pumps. BmrR acted as a repressor of the efflux pump Bmr by directly binding to the promoter region. In the presence of a toxin, BmrR induced Bmr expression by remodeling the DNA structure to be accessed by RNA polymerase (32). Additionally, an elaborate study on QacR showed that a range of toxic compounds, both hydrophobic and cationic, can activate the QacA efflux pump (33).

From these studies, it appeared that bacterial multidrug proteins could sense a variety of compounds, and act as transcriptional activators and repressors. While these proteins do not share sequential or structural homology with mammalian quinone reductase, this regulatory pathway could represent a primitive model of how NQO2 functions as a mammalian multidrug efflux pump regulator.

In mammals, P-glycoproteins (MDR1) and the family of multidrug resistance-associated proteins (MRPs) are ATP-binding cassette transmembrane transporters (ABC transportors) that pumps xenobiotic compounds out of the cell (34). MRPs’ expression can be induced by the presence of toxins and drugs, but their mechanism of induction is unclear (35). Specifically, no multidrug binding protein that directly recognizes a toxic compound has been reported in mammalian cells. As such, the fact that NQO2 is the target of a plethora of cytotoxic compounds, and is able to modulate p53, makes it a compelling candidate towards its identification as a mammalian multidrug binding protein.
While the regulation of MDR1 and MRPs is complex, a pathway through which NQO2 can modulate these proteins is by p53 and the transcription factor Sp1 (Sp1). Sp1 was normally down-regulated by direct binding to p53, but it can induce a member of the MRP family, MRP1, in response to a range of cytotoxic stresses (36, 37). This regulation was further shown by an induction of MRP1 expression when p53 was inactivated or mutated (38). Therefore, a basal p53 expression prevented the expression of MRP1, while inactivation of p53 induced MRP1 expression. Through this transcriptional regulation mechanism, NQO2 inhibitors could induce the degradation of p53 and release Sp1 to initiate MRP1 expression. In this regard, NQO2 may be functioning as a multi-drug binding protein that signals the activation of a multidrug efflux pump. Furthermore, there are also four Sp1 recognition sites upstream of the NQO2 gene (39), which could act as positive regulator of MRP1 until the cell clears all cytotoxic compounds. Following these suppositions, an investigation of whether NQO2 plays a role in the regulation of MRP1 is warranted.

A myriad of compounds inhibit NQO2 with affinities ranging from high micromolar to nanomolar. Given that NQO2-mediated modulation of p53 may have functions other than inducing cell cycle arrest and apoptosis, a role for regulating MRP1 efflux pump is proposed: toxic DNA intercalating agents recognized by NQO2 could induce MPR1 and be excreted in normal proliferating cells. In this regard, the success of chemotherapeutics such as kinase inhibitors may not be due solely to their ability to effectively kill cancer cells. Instead, it may be due to the ability of normal cells to excrete these compounds such that side effects are minimized. Extending this hypothesis, kinase inhibitors recognized by NQO2 could also induce multidrug resistance in cancer cells if the concentrations used are not high enough to kill the cell. Taken together, NQO2 may act as a direct sensor for toxic compounds to modulate signalling events in cells.

In the next section, the modulation of p53 by NQO2 will be revisited once more to point out that NQO2 has the potential to regulate a number of proteins. To investigate the role of NQO2 as a sensor for toxic compounds, an analysis of MRP1 and other proteins using a proteomics approach will help decipher the complex function of NQO2.
7.3.4 Protein Stabilization by NQO2

Both NQO1 and NQO2 were shown to stabilize p53 from 20S proteasomal degradation (1). To understand this biological process, recombinant p53 from E. coli and 20S proteasome from lamb liver were purified. However, in vitro degradation of p53 was not detected (data not shown). This was contrary to a previous demonstration that in vitro translation of p53 by rabbit reticulocyte lysates were degraded by rabbit 20S proteasomes (1). This illustrated that p53 may require certain post-translational modifications to be degraded, or that the degradation of p53 was mediated by additional components from the rabbit reticulocyte lysate. In addition to p53 modulation, NQO2 may also stabilize other proteins. For example, NQO1 also directly modulated the degradation of p73α and ornithine decarboxylase (5, 40). All three of these proteins have an intrinsic disordered region that could be recognized by the 20S proteasome, and were degraded in an ubiquitin-independent manner (41–43). This led Yosef Shaul and coworkers to hypothesized that quinone reductase could be modulating 20S proteasomal degradation in general (44). As for NQO2, it also modulated Rb/cyclin D1 (45), NFκB (46), CEBPα (47), and AKT (48) pathways, though it is not clear if these proteins were directly modulated. These studies demonstrated that NQO2 could be directly regulating the degradation of an array of intrinsically disordered proteins by the 20S proteasome. To gain a comprehensive perspective on the function of NQO2, a global proteomics approach is needed to decipher how NQO2 regulates protein stabilization.

Initially, the overall proteome of HCT116 and HCT116ΔNQO2 cells could be compared using a 2D gel. Given sufficient changes in protein levels, SILAC-based (stable isotope labeling by amino acids in cell culture) mass spectrometry techniques could be used to quantify the differences in protein levels on a global scale (49). Having established the basal protein difference between HCT116 and HCT116ΔNQO2, the effects of NRH on the proteome will demonstrate how the redox state of NQO2 changes the protein stability. Super-SILAC can also be used to monitor several NRH concentrations to detect changes in protein levels that are most directly and immediately affected by NQO2 (50). To further characterize specific proteins that NQO2 associates with in cells, proximity labeling of proteins near NQO2 could be identified using cross-linking reagents or a BIO-
ID mass-spectrometry based approach (51). Having established these mass-spectrometry techniques, a comprehensive analysis of the proteins that NQO2 modulates directly will illuminate the function of NQO2 as a flavin redox switch. This general approach can then be extended to decipher how high affinity NQO2 inhibitors such as S29434 and acridine orange affect the proteome.

7.4 Conclusion

The central question in this thesis was how NQO2 functions as a flavin redox switch. To this end, structural analyses of NQO2 and NQO2 inhibitors revealed the mechanistic features of a flavin redox switch. Cellular studies further identified that NQO2 exists in an oxidized state, contrary to NQO1, which exist in a reduced state. Though the role of NQO2 as a flavin redox switch was delineated, many questions remain. Answers to the following questions will illuminate how NQO2 modulates cellular processes when challenged by the diverse range of NQO2 inhibitors: How does the redox state of NQO2 affect protein degradation? What is the mechanism of NRH production, and is NRH really the natural co-substrate? What other compounds do NQO2 recognize? What are the consequences of NQO2 activation? What proteins do NQO2 stabilize? By using advance techniques such as protein molecular dynamic simulation, high throughput screening of NQO2 inhibitors, and biological mass spectrometry, the knowledge of how NQO2 functions as a flavin redox switch will be further advanced.
7.5 References


8  Curriculum Vitae

Kevin Ka Ki Leung

Education
Doctoral candidate in Biochemistry, University of Western Ontario
Honors B.M.Sc. (Microbiology and Immunology), University of Western Ontario, 2010

Scholarship and Awards
2014-2015  CIHR doctoral Award - Frederick Banting and Charles Best Canada Graduate Scholarships (CGS-D) for the top ranking applicants in Canada
2013-2014  Western University Ontario Graduate Scholarship/ Queen Elizabeth II Graduate Scholarship for the top ranking applicants in the university
2013  Graduate Thesis Research Award, University of Western Ontario,
2012  Louis Delbaere Pauling Poster Prize for the best Canadian poster, American Crystallographic Association annual meeting
2012-2013  Ontario Graduate Scholarship Program for the top ranking applicants in Ontario
2010-2014  Western Graduate Research Scholarship, University of Western Ontario
2009  Millennium Bursary, Canada Millennium Scholarship Foundation
2008  Millennium Bursary, Canada Millennium Scholarship Foundation
2008  Laurene Paterson Scholars, University of Western Ontario
2006  UWO Admission Scholarship, University of Western Ontario

Referred Publications
Leung, K.K., and Shilton, B. H. (2015) Quinone Reductase 2 Is an Adventitious Target of Protein Kinase CK2 Inhibitors TBBz (TBI) and DMAT. Biochemistry 54, 47–59


Non-referred Publications
Leung, K.K. (April, 2010). Structure of Quinone Reductase II with Casein Kinase II Inhibitors. Thesis submitted and accepted as fulfillment of Honors Bachelor of Science (Microbiology and Immunology) at The University of Western Ontario, class of 2010
Lin, Y., Tam, A., Leung, K.K., Callum, J.L. (October, 2006). Assessment of fetomaternal hemorrhage (FMH) using hemoglobin F quantitation by flow cytometry (HFQ) and the Kleihauer-Betke (KB) test in a large obstetrical population. Presented at the 59th AABB Annual Meeting and TXPO, Miami Beach, FL. Transfusion 46(9s):143A

**Conference Presentations**

Leung, K.K. and Shilton, B. (June 1st, 2014). Quinone reductase 2 is an adventitious target of CK2 inhibitors. Poster presentation at the 47th International School of Crystallography: Structural Basis of Pharmacology.

Leung, K.K. and Shilton, B. (July 22nd, 2013). Off-Target Interactions of Quinone Reductase 2 with Kinase-Targeted Inhibitors. Poster presentation at the American Crystallographic Association annual meeting, Honolulu, HI, USA. (June 21st, 2013) Poster presentation at the 10th Annual Department of Oncology Research and Education Day, London, Ontario, Canada.

Leung, K.K. and Shilton, B. (November 2nd, 2012) Conformational Switching in quinone reductase 2 by reduction of FAD and chloroquine binding. Oral presentation at the 21st Annual Buffalo-Hamilton-Toronto Symposium, Hamilton, ON, Canada; (July 30th, 2012). Poster presentation at the American Crystallographic Association annual meeting, Boston, MA, USA.


**Teaching Assistantships**

2012 - 2014  Biochemistry Laboratory 3380G
Demonstrated cloning, protein purification, kinetics and other biochemistry techniques to a small group of 8 students. Also assisted them to write scientifically and graded lab reports.

2012 - 2014  Biochemistry and Molecular Biology 2280A
Managed course forum, held office hours, conducted tutorial sessions, and acted as a substituted lecturer to a class of 800 student.

**Other Research Assistantship**

2010  Summer Research Student at University of Western Ontario (Biochemistry)
Supervised by Dr. Brian Shilton and Dr. David Litchfield for work with NQO2

2008  Summer Research Student at Sunnybrook Health Science Center (Haematology)
Supervised by Dr. Jeannie Callum and Dr. Matthew Oliver for clinical studies with nephology patients
2008 Summer Research Student at Sunnybrook Research Institute (Odette cancer research)
Supervised by Dr. Richard Wells for work with orphan EAR2 nuclear receptor

2008 Work-study Student University of Western Ontario (Immunology)
Supervised by Dr. Mansour Haeryfar for routine laboratorial chores

2007 Summer Research Student Sunnybrook Health Science Center (Haematology)
Supervised by Dr. Jeannie Callum, Dr. Yulia Lin, and Dr. Sandro Rizoli for multiple clinical studies in hematology, trauma, and thrombosis unit.

2005-2006 Sanofi-Aventis Biotechnology Challenge participant at York University (Seneca College)
Supervised by Dr. Stephanie Ditta and Dr. Michael Gadsden for work in purifying DUTPase from Staphylococcus Epidermidis

Other Awards
2014 Course grant for the 47th International School of Crystallography
2013 American Crystallographic Association travel award
2011 Publication Incentive, University of Western Ontario (Biochemistry)
2011 Travel award, University of Saskatchewan (1st Annual Canadian Light Source Macromolecular crystallography Data Collection School)
2007-2009 Dean’s Honor Roll at University of Western Ontario
2007 Certificate of Merit in the Case Study Competition of the “Ethical Leadership for the New Generation”
2003-2006 Ranked top twenty-five percent of contestants in Pascal, Fermat, and Canadian Open Math Contest
2006 Award of Merit for Sanofi-Aventis Biotechnology Challenge 2006
2006 Coop Award, University of Toronto (Sunnybrook Health Science Center)
2005 Award of Merit for Aventis Biotechnology Challenge 2005

Other activities
2014 Visiting Speaker Representative for the Biochemistry Department
Acted as host for lunch with visiting speaker with graduate students around twice a month. Also invited speaker for our Annual Stewart Lecture and acted as host for the event.