Synthesis and evaluation of novel NQO2 inhibitors

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Buthaina Hussein Khalid Hussein

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School of Health Sciences, Division of Pharmacy & Optometry
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List of abbreviations

Å: 10^{-10} m (Angstrom)
Asn: Asparagine
Asp: Aspartic acid
APCI: Atmospheric-pressure chemical ionization spectrometry
Cys: Cysteine
DCPIP: 2,6-Dichlorophenolindophenol
DCM: Dichloromethane
DMAT: 2-dimethylamino-4,5,6,7-tetram bromo-1H-benzimidazole
DMSO: Dimethyl sulfoxide
DMSO-d₆: Deuterated dimethyl sulfoxide
ETC: Electron transport chain
EtOAc: Ethyl acetate
FAD: Flavin adenine dinucleotide
Gly: Glycine
IR: Infra-red spectroscopy
Ile: Isoleucine
Met: Methionine
MT3: Melatonin-binding site 3
NADH: Reduced adenine dinucleotide
NADPH: Reduced adenine phosphate dinucleotide
NQO1: NAD(P)H: quinone oxidoreductase1
NQO2: NRH: quinone oxidoreductase 2

NCI: National Cancer Institute

NF-kB: Nuclear factor-kappa B

NRH: N-Ribosyl dihydronicotinamide

PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase

Phe: Phenylalanine

ppm: Parts per million

ROS: Reactive oxygen species

rt: Room temperature

TBB: 4,5,6,7-tetrabromobenzotriazole

TBBZ: 4,5,6,7-1H-Tetrabromobenzimidazole

THF: Tetrahydrofuran

Thr: Threonine

TNF: Tumour necrosis factor

Trp: Tryptophan

Tyr: Tyrosine
Abstract

(NRH): quinone oxidoreductase 2 (NQO2) is one of the enzymes that belongs to the mammalian quinone reductases (QRs) enzyme family that is responsible for the two-electron reduction of quinone to hydroquinone. There is evidence that NQO2 is associated with cancer initiation via the production of ROS during quinone metabolism. Interest increased in finding novel NQO2 inhibitors, which show good selectivity and potency.

The aim of this project is to design, synthesise and evaluate novel and selective NQO2 inhibitors as potential anticancer candidates, with low toxicity and good stability at physiological pH.

A library of analogues containing the 4-aminoquinoline scaffold has been computationally docked in the crystal structure of the reduced and oxidized NQO2 (PDB code 4U7F and 4FGJ, respectively) using Gold suite (version 5.3). The results of the molecular docking showed an increase in the binding affinity with hydrazine derivatives, compared to the hydrazine, hydrazide, and amine analogues. Introducing an aromatic ring such as phenyl or pyridine at position 2 makes a noticeable increase in the docking score.

Twenty-one compounds from the library were synthesized, which involved condensation of p-anisidine with Meldrum’s acid and trimethyl orthoacetate or trimethyl orthobenzoate to produce the 4-hydrazine-quinoline scaffold.

The potency of the synthesized compounds to inhibit the NQO2 enzyme was determined and measured spectrophotometrically using 2,6-dichlorophenolindophenol as a substrate. These compounds showed potent NQO2 inhibition activity with IC\textsubscript{50} values in the low nano-molar concentrations.

The selectivity of the synthesised compounds toward NQO2 rather than NQO1 has been tested. A few compounds showed activity toward NQO1, however with high micromolar concentrations.

The role of NQO2 inhibitors was also investigated as antimalarial leads where a group of 4-hydrazonequinoline and furan-amidines and their analogues have been synthesised and shown to be active against the malaria parasite \textit{Plasmodium falciparum}.
Declaration

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Last but not the least; I would like to thank my sponsor AL-Zaytoonah University of Jordan for their financial sponsorship.
I dedicate my thesis to my lovely family
1. Introduction

1.1 Introduction to cancer

Cancer is a collection of diseases characterised by abnormal cell growth leading to invasion of surrounding tissues with a potential ability to spread (metastasis) to other tissues to form secondary tumours.\textsuperscript{1,2} It is initiated by one cell with a vital regulatory pathway dysfunction, which then multiplies to billions of cells that constitute a cancer.\textsuperscript{1} Normal cell growth is controlled by internal and external factors, which is lost to a certain degree during carcinogenesis.

Despite the huge amount of cancer research, the incidence of cancer continues to rise.\textsuperscript{3} 1 In 2 people in the UK will develop cancer in their lifetime.\textsuperscript{4} There were 322,197 new cases of cancer recorded in the UK in 2013 and 161,823 deaths attributed to cancer recorded for 2012.\textsuperscript{4}

Risk factors for cancer are tobacco (as primary cause), alcohol, diet, obesity, infectious agents, environmental pollution and radiation.\textsuperscript{1,3}

There are more than 200 different types of cancer,\textsuperscript{5} with breast, colorectal, prostate and lung being responsible for half of all cases.\textsuperscript{5}

1.2 The Eukaryotic Cell cycle

To understand how the cancer cell develops in the body and how some anticancer agents exert their pharmacological effect, it is important to look at cell proliferation and the cell cycle. The cell cycle varies in duration depending on the cell type.\textsuperscript{2} A short cell cycle occurs in early embryo where it takes less than 30 minutes. Other cells divide less frequently like the cells of internal organs (e.g. lung, liver and kidney), however, some cells like the nerve cells do not divide. The typical proliferating human cell cycle is happened within 24 hours (e.g. epithelial cells).\textsuperscript{6} The cells sit in a resting phase called G\textsubscript{0} before they enter the cell cycle, which is divided into two main stages: interphase and mitosis. The cell spends 95\% of its time in the interphase stage (which is around 23 hr). This stage is divided to 3 parts: gap 1(G1), synthesis (S) and gap 2 (G2) (\textbf{Figure 1}).\textsuperscript{7}
During gap1 (G1, 10 hr), the cell is continuously growing but the DNA does not replicate, followed by next phase the synthesis phase (S, 9 hr), where the DNA replication take place then followed by the gap 2 phase (G2, 4 hr) where the cell keep growing and the proteins are synthesised. In the mitosis stage (M, 1 hr) the separation of the daughter chromosomes takes place followed by cell division.\footnote{8}

\section*{1.3 Cancer treatment}

The approaches to treat cancer are surgery treatment, radiotherapy and chemotherapy. Chemotherapy is the main of interest here.

1- Surgical treatment

Surgical treatment is the physical eradication of the tumour mass, which was the main tool used to treat cancerous tumours until 1950s. Surgical treatment is restricted to the solid tumour as it is inefficient in eradication metastatic cancer.\footnote{9}

2- Radiotherapy

Radiation therapy use began in the 1960s to treat local tumours, but, like surgical treatment, could not eradicate metastatic tumours.\footnote{9}

3- Chemotherapy

Chemotherapy is the administration of anticancer cytotoxic chemicals. It began in the 1940s by using antifolate and nitrogen mustards drugs.\footnote{10} It is usually part of the treatment regimen with surgery or radiotherapy. Chemotherapy can be given as a single drug or in combination with two or more drugs to overcome tumour resistance, increase the treatment efficiency and to avoid toxicity to normal cells.\footnote{5,7}
Cytotoxic drugs exhibit their effect on cells by interfering with the cellular functions essential to cell division and growth. These functions are the same in both cancer and the normal cells, which leads to toxicity. However, these drugs tend to exert most of their effect on the rapidly dividing cancerous and normal cell. Therefore, most of their side effects are also affecting these cells, such as, buccal mucosa, bone marrow, gastrointestinal mucosa and hair. Anticancer drugs can be classified based on their mechanism of action; the main groups will now be described:

1.3.1 Alkylating agents

The mode of action of alkylating agents is disrupting the function of DNA by chelating directly to the DNA double strand. Alkylating agents target all the cell cycle phases. Scheme 1 shows the mechanism of bis(chloroethyl)amine which works by forming unstable ethyleneimonium and carbonium ions, which in turn alkylate DNA guanine bases.

Scheme 1. The mechanism of action of the alkylating agents in the cell.
Alkylating agents are categorised into seven sub-classes, as shown in (Table 1) with some examples.

**Table 1. Alkylating agent sub-classes**

<table>
<thead>
<tr>
<th>Alkylating agents sub-classes</th>
<th>Example</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen mustard</td>
<td>Chloroambucil</td>
<td><img src="nitrogen_mustard.png" alt="Structure" /></td>
</tr>
<tr>
<td>Alkyl sulfonates</td>
<td>Busulfan</td>
<td><img src="alkyl_sulfonates.png" alt="Structure" /></td>
</tr>
<tr>
<td>Aziridines</td>
<td>Thiotepa</td>
<td><img src="aziridines.png" alt="Structure" /></td>
</tr>
<tr>
<td>Nitrosoureas</td>
<td>N-Nitroso-N-methylurea</td>
<td><img src="nitrosoureas.png" alt="Structure" /></td>
</tr>
<tr>
<td>Platinum based alkylation agents</td>
<td>Cisplatin</td>
<td><img src="cisplatin.png" alt="Structure" /></td>
</tr>
<tr>
<td>Tetrazines</td>
<td>Dacarbazine</td>
<td><img src="tetrazines.png" alt="Structure" /></td>
</tr>
<tr>
<td>Non-classical alkylation agents</td>
<td>Procarbazine</td>
<td><img src="non_classical.png" alt="Structure" /></td>
</tr>
</tbody>
</table>

1.3.2 Antimetabolites

This class of drugs exert its effect by inhibiting enzymes used in DNA synthesis (metabolic process).\(^5\) Due to chemical structure similarity, the drug may act as an analogues of enzymes precursors involved in the DNA synthetic pathway, or it may behave as an unnatural base in the DNA double helix synthesis, where it resemble the nucleotide or
nucleoside structure, leading to inhibition of DNA duplication and consequently prevents mitosis and tumour growth.\textsuperscript{10} Antimetabolites are cell cycle dependent, where they exert their effect during the synthesis (S) phase. Antimetabolites can be categorised into the following sub-classes:

1.3.2.1 \textbf{Inhibitors of purine and pyrimidine biosynthesis}

These inhibitors interfere with DNA and RNA synthesis \textit{via} inhibiting the biosynthesis of purine and pyrimidine rings.\textsuperscript{13} The drugs resemble the natural nucleoside or nucleobase in structure. The nucleoside is the nucleobase bound to either ribose or deoxyribose sugar through the glyosidic bond.\textsuperscript{14} Nucleobases are classified as purine bases (adenine and guanine) and pyrimidine bases (thymine, cytosine and uracil). Inhibitors may replace the nucleoside or nucleobase in the DNA and RNA biosynthesis or it may inhibit one of the enzymes involved in the cell metabolism.\textsuperscript{15} 6-Mercaptopurine, 6-thioguanine, 5-fluorouracil, capecitabine, cytarabine and gemcitabine are some examples of this class of inhibitors (\textbf{Figure 2}). The majority of these drugs show activity in different types of leukaemia.\textsuperscript{15}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Basic structure of the purine and pyrimidine ring and examples of some inhibitors of purine and pyrimidine biosynthesis.}
\end{figure}
1.3.2.2 Anti-folates

Folic acid plays an important role in DNA synthesis. Folic acid is reduced to dihydrofolate (DHF) then tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR). THF is a necessary cofactor in the synthesis of deoxythymidine monophosphate (DTMP), the DNA building block (Scheme 2).

**Scheme 2.** The mechanism of action of methotrexate in inhibiting DNA synthesis

The folic acid analogues methotrexate, inhibits DHFR, which prevents the conversion of folic acid to tetrahydrofolate THF, a central process in the DNA synthetic pathway. In addition, the low level of THF indirectly inhibits the thymidylate synthase leading to depletion in DTMP. The anticancer drugs, 5-fluorouracil and raltitrexed are known to inhibit thymidylate synthase directly. Figure 3 gives the structure of folic acid and also the antifolates methotrexate, pemetrexed and raltitrexed.

**Figure 3:** Structures of folic acid and anti-folate analogues
1.3.3 Anti-microtubule agents
Microtubules have a key role in mitotic spindle and division, in addition to its role in maintaining the structure of the cell. The polymerization of alpha and beta tubulin protein formed microtubules.\textsuperscript{16} Anti-microtubule agents exert their anticancer effect by blocking mitosis through preventing the formation of the mitotic spindle in G2 and M phases. They are active in both haematopoietic and solid tumours. Antimicrotubule agents are classified into two main sub-types: microtubule destabilizing agents (which binds to tubulin and prevents the assembly of microtubules, examples include vinca alkaloids (vincristine, vinblastine, vinodesine, vinorelbine), cryptophycine, halichondrine and colchicine.\textsuperscript{17}) and microtubule stabilizing agents which prevent microtubule disassembly (for example: paclitaxel, docetaxel and epothilones). Both the stabilising and destabilising agents cause blocking of mitosis.\textsuperscript{16}

1.3.4 Topoisomerase inhibitors
Topoisomerase I and II have a vital role in DNA replication and transcription process, through controlling the breaking down and forming (resealing) the phosphodiester bond of DNA strands. Topoisomerase I cuts one DNA strand, whereas topoisomerase II cuts both of the DNA strands and then reseals the two stands together.\textsuperscript{17} Topoisomerase inhibitors, such as anthracycline bind to the DNA and keep bounding to the end of DNA after the breaking down of the DNA strands has finished. This stable intermediate (DNA-anthracycline) prevents the next step (resealing) to take place.\textsuperscript{5} Blocking this process leads to apoptosis and cell death.\textsuperscript{18} Irinotecan and topotecan are other examples of these inhibitors which used to treat colon and ovarian cancer respectively.\textsuperscript{17}

1.3.5 Cytotoxic antibiotics
The anti-tumour antibiotics (for example, dactinomycine, doxorubicin and bleomycin) exert their anticancer effect through interaction with DNA, leading to stable complexes and consequently disruption of cell division. Therefore, they are considered as cell cycle specific agents. These antibiotics treat a range of cancers such as soft tissue sarcomas, acute leukaemia and squamous cell carcinomas.\textsuperscript{15}

1.4 Chemotherapy resistance
Many cancers such as small cell lung and kidney cancers show resistance to most chemotherapeutics agents from the beginning of the treatment, while others such as breast cancer may respond for a while before developing resistance.\textsuperscript{10} There are several
approaches by which tumour cells can develop resistance as a result of mutation. These approaches include poor uptake of drug by the tumour cell, cells adaptation to stop the activation reactions of some prodrugs, changing the metabolic pathways to avoid the effect of some antimetabolite drugs and efflux of the drugs out of the cell.

Multidrug resistance (MDR) tumours used the last mechanism. P-glycoprotein is the protein which normally responsible to efflux different toxins out of the cell. This protein may become highly expressed in cancer cells and consequently remove the anticancer drugs out of the cell immediately as they enter, leaving the cancer cell resistance to many anticancer drugs. Therefore, combination of drugs with different mechanism of action, could be the solution to overcome drug resistance.

1.5 Introduction to quinones

Quinones are polycyclic organic compounds which are found naturally in animals, plants, fungi, and bacterial (Figure 4).

![Figure 4. Structure of two natural quinones](image)

Every day humans are exposed to quinones through food intake, air-borne pollutants like cigarette smoke or clinically from anticancer, antimalarial or antimicrobial drugs (Figure 5).

![Figure 5. Examples of quinones: anticancer drug- Mitoxantron, antimalarial drug- Atovaquone, and antimicrobial drug- Rhein.](image)
The toxicity of quinones comes from direct exposure, as well as the metabolism of many environmental aromatic hydrocarbons, for example, benzene toxicity arises from its metabolites phenol and hydroquinone.\(^\text{19}\)

Quinone and its derivatives are highly reactive substrates which undergo a catalytic reductive metabolism,\(^\text{20}\) by either one-or two-electron reduction (Scheme 3).\(^\text{21}\) The one-electron reduction catalysed by cytochrome P450 reductase, resulted in unstable semiquinones which react with oxygen molecules to generate highly reactive oxygen species (ROS), which in turn cause cytotoxicity.\(^\text{20}\)

On the other hand, the two-electron reduction catalysed by quinone reductases, produces stable hydroquinones which are conjugated with glutathione or glucuronic acid and then excreted out of the body. The two known quinone reductases are NAD(P)H: quinone oxidoreductase 1 (NQO1) and NRH: quinone oxidoreductase 2 (NQO2).

The two electron reduction competes with one electron reduction for the quinone substrate, but, the formation of hydroquinone is more favourable since it is less reactive than semiquinone. For example, the inhibition of NQO1 will result in an increased production of ROS. On the other hand if the hydroquinone is not removed by conjugation, it will undergo autooxidation resulting in ROS generation.\(^\text{21}\)

**Scheme 3.** Quinone metabolism pathways: one electron reduction (CYP450) and two electron reduction (oxidoreductases).
1.5.1 Reactive oxygen species (ROS)

The reactive oxygen species (ROS) is defined as any molecule that contains unpaired electron. Hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^-$) and superoxide (O$_2^-$) are examples of oxygen radical species. The majority of ROS result from the oxidative phosphorylation taking place in the mitochondrial electron transport chain, where the adenosine triphosphate (ATP) is converted to energy. The other sources are ionization radiation, phagocytic cell and lipid peroxidation (Scheme 4).

![Scheme 4. The initiation and production of different ROS](image)

Under normal conditions the ROS are destroyed before they travel a long distance, otherwise it would be serious effect if ROS reaches the nucleus and interacts with the DNA. If a chain reaction initiated the ROS can show effects over a long distance or generated within the nucleus. In addition, ROS may exhibit their effects on other proteins such as NF-kB by direct oxidation.

(Scheme 5) illustrates the ROS inactivation or termination by the action of some enzymes, such as, superoxide dismutase (SOD), catalase and peroxidase using different reductant molecules, such as, glutathione, vitamin A, C and E where the ROS converted to oxidized glutathione, water and oxygen.

![Scheme 5. The termination of ROS](image)
Altering the reducing environment inside the cell or depletion of antioxidants results in oxidative stress and allows the ROS to mediate its damage effect in the cells leading to cancer induction.

1.6 Introduction to human oxidoreductases

Oxidoreductases are enzymes that catalyse oxidation and reduction reactions, where it transfers electrons from one molecule (electron donor) to another molecule (electron acceptor). These reactions usually require co-factors such as NAD(P)H or NRH. Two human quinone reductases are known; NAD(P)H: quinone oxidoreductase (NQO1) and NRH: quinone oxidoreductase2 (NQO2) where they catalyse the reduction of quinone to hydroquinone.

1.6.1 NAD(P)H: quinone oxidoreductase 1 (NQO1)

NAD(P)H: quinone oxidoreductase 1 or DT-diaphorase was first reported by Ernster and Navazio. It is a flavoprotein that is mainly localized in the cytosol. It has an important role in the quinone detoxification process since it is clearly induced by a variety of chemicals and regulated with other detoxification genes. NQO1 catalyses the two-electron reduction of quinone and its derivatives, utilizing NADH or NADPH as a cofactor (electron donor). This reduction reduces the probability of one-electron reduction catalysed by cytochrome P450, which leads to the formation of free radicals and consequently cytotoxicity. So it is considered as a protective enzyme against quinone toxicity. However, NQO1 has been shown to play a role in quinone activation, to produce unstable hydroquinone which undergoes auto-oxidation to produce ROS or alkylate DNA directly.

The crystal structure of NQO1 shows that it is a homodimer with 274 amino acids (N-terminal 1-220 residues and the C-terminal 221-274 residues) with a molecular weight of 60 kDa (Figure 6).
Chapter 1. Introduction

Figure 6. Crystal structure of human NQO1 (1.7 Å resolution).²⁸

Each monomer contains one molecule of flavin adenine dinucleotide (FAD),²⁴,²⁶ which contains the tricyclic isoalloxazine ring (Figure 7).

NQO1 is expressed in all tissue types.²⁴ The expression of this enzyme is induced in response to xenobiotics, antioxidants, oxidants, heavy metals, UV light and ionizing radiation.²⁰,²⁹

Most human solid tumours (breast, liver, colon and lung) express high levels of NQO1 compared to normal cells.³⁰ NQO1 plays an unknown role in tumour progression. This fact, along with NQO1’s ability to activate quinones and produce ROS has been investigated to develop chemotherapeutic agents.²⁶,³¹ Dicoumarol is a known inhibitor of NQO1, which competes with the NAD(P)H molecule in the binding to FAD.²⁴

Figure 7. The structure of FAD and the isoalloxazine ring
1.6.2 NRH: quinone oxidoreductase 2 (NQO2)

In 1961 NQO2 was described as an unknown flavoprotein which catalyses the oxidation of reduced N-ribosyl- and N-alkyldihydronicotinamides by quinones.\textsuperscript{32} For 36 years it was forgotten until the 1990s when this enzyme was isolated and called NQO2.\textsuperscript{33}

NQO2 is a cytosolic flavoprotein which uses nicotinamide derivatives such as N-ribosyl-, N-methyl- and N-benzyl-dihydronicotinamide (NRH) as reducing agent (co-substrates), (Figure 8). NQO2 is unable to use NAD(P)H as the electron donor in the same efficiency as NQO1.\textsuperscript{27,34} However, NQO2 is still able to use NAD(P)H, but, with a catalytic efficiency three fold less than with NRH.\textsuperscript{25} Ubiquinone, (Figure 9), also known as coenzyme Q\textsubscript{10}, is the only identified natural substrate for NQO2.\textsuperscript{35}

\textbf{Figure 8.} Nicotinamide derivatives, the co-substrates of NQO2.

\textbf{Figure 9.} The structure of ubiquinone, the only identified natural substrate of NQO2

NQO2 is a homodimer of 231 amino acids (MW= 25,821 Da) per monomer.\textsuperscript{33} NQO2 contains the complete N-terminal part found in NQO1 (1-220 residue) but not the complete C-terminal part, since it lacks the 43 amino acid residues found in NQO1 residues (231-274).\textsuperscript{33} The similarity between NQO1 and NQO2 is 54\% for cDNA and 49\% for protein.\textsuperscript{31} Each monomer of NQO2 contains one catalytic binding site, which has a large narrow cavity, 17Å in length and 7 Å wide. This binding site contains amino acid residues from the monomer itself and the FAD isoalloxazine ring which is tightly bound to the enzyme by multiple interactions, forming the floor of this cavity.\textsuperscript{29} In addition, NQO2 has a tetracoordinated metal binding site in each monomer (most likely a copper) which may participate in the enzyme reduction mechanism,\textsuperscript{29,31} however other studies suggest that its
role is just for structural stability and has nothing to do with the enzyme activity because it is far away of the FAD by 13 Å (Figure 10).\textsuperscript{36}

![Figure 10. The structure of human NQO2, (2.1 Å resolution).\textsuperscript{27}}

Human NQO2 is expressed in kidney, liver, blood cells and heart, with minimum expression in the brain and pancreas, but it is absent in the placenta.\textsuperscript{29} The NQO2 expression is induced in response to xenobiotics and antioxidants.\textsuperscript{20}

The current idea about NQO2 is that this enzyme is not a detoxification enzyme like NQO1. It can reduce many ortho-quinones, menadione and some antitumour agents, for example, CB1954 (5-aziridinyl-2,4-dinitrobenzamide), mitomycin C,\textsuperscript{31,37} and Coenzyme Q\textsubscript{0}.\textsuperscript{38} Many recent studies indicate that NQO2 reduces quinones and anticancer drugs to highly reactive species which lead to their cytotoxicity.\textsuperscript{29,39} It is hypothesized that the inhibition of this enzyme may prevent or reduce this toxicity.\textsuperscript{29} therefore, depending on this property it could be an interesting target for chemotherapeutic drugs.\textsuperscript{31,39}

A more recent hypothesis about the cellular function of NQO2 has been proposed.\textsuperscript{25} It is claimed that the main function of NQO2 is a redox singling system, where it is reduced under certain metabolic conditions allowing it to propagate a signal. These conditions should switch on the production of NRH in high amounts hence it is present in very small amounts under normal conditions.\textsuperscript{25}
There are many polymorphic forms of human NQO2, but, two of them are the most common. These two differ in codon 47: the major one has a phenylalanine (NQO2-phe47) and the minor one has a leucine (NQO2-L47). Structurally, codon 47 is close to the boundaries between the two dimers of the enzyme, but, not part of the active site. However this difference leads to alterations in the biochemical properties such as, stability towards proteolytic digestion and thermal denaturation. NQO2-phe47 has been reported to have higher activity in the cell extract than NQO2-L47, This confirmed what has been revealed by different studies that the polymorphism of NQO2 affect the clinical outcome such as, enzyme activity memory recall and cancer susceptibility. On the other hand, the activity differences do not affect the inhibitory activity of resveratrol for both forms.

1.6.3 Enzymatic mechanism
Both NQO1 and NQO2 undergo the same ping-pong mechanism since both of them have relatively similar catalytic binding sites (Scheme 6). The co-substrate and the substrate share the same binding site. The catalytic binding site is first occupied by the co-substrate (electron donor; NADH/NRH; step 1), where it oxidized to NR⁺, then it is replaced by the substrate (electron acceptor; quinone; step 2), where it is reduced to hydroquinone. The overall process is the transfer of two electrons from the co-substrate to the substrate.
1.6.4 Differences between NQO1 and NQO2

There are six main differences between NQO1 and NQO2, each of which is now described:

1-NQO2 is 43 amino acid shorter in its carboxyl-terminus than NQO1.\textsuperscript{20,33} This difference is believed to contribute to the difference in the cofactor requirement since NQO2 uses dihydronicotinamide riboside (NRH), rather than NAD(P)H as electron donor, as well as different inhibitors.\textsuperscript{20,33} This difference maybe because of the deep loop formed by the last 10 amino acids in the C-terminal of NQO2 (221-231), which is structurally different in the corresponding part of NQO1.\textsuperscript{27}

2-NQO2 is resistant to typical inhibitors of NQO1 such as dicoumarol, cibacron blue and phenindone,\textsuperscript{31,36} and vice versa. The well-known NQO2 inhibitors like resveratrol and quercetin only slightly inhibit NQO1.\textsuperscript{36}

3-The catalytic binding site amino acid residue of both enzymes are very similar. The difference is in the three residues Tyr\textsuperscript{126}, Tyr\textsuperscript{128} and Met\textsuperscript{131} in NQO1, which is replaced by...
Phe126, Ile128 and Phe131 in NQO2. This makes the active site of NQO2 much larger and more hydrophobic than NQO1.

4- The crystal structure of NQO2 shows a metal binding site (possibly zinc) which is not found in NQO1.20

5-Gly149, Tyr155, His161 are the three residues which are directly involved in the electron transfer in NQO1, but just His/Asn164 are shown to be involved in NQO2 and the rest is compensated by water molecules.29

6-NQO2 knock down mice show decreased sensitivity to quinone toxicity, while animals with NQO1 knock down mice show increased sensitivity to quinone toxicity.36,44

1.6.5 Hypothesis regarding the physiological role of NQO2
NQO2 has proposed roles in many diseases including malaria, leukaemia, neurodegenerative diseases (e.g. Parkinson's).45 But the exact role of NQO2 is still not clear. Many hypotheses proposed to investigate the physiological role of NQO2.

The role of NQO2 in malaria and cancer has been proposed after the finding that NQO2 is a molecular target for the antimalarial drug chloroquine and the anti-leukemia drug imatinib.45 Some research shows that NQO2 knockout mice are more likely to develop skin cancer,46 as well as the growth inhibition of different tumours linked to up regulation of NQO2.46 Others reported that NQO2 knock down mice showed less sensitivity to menadione induced toxicity compared to control mice.38

Gaikwad and co-workers, suggested that NQO2 may have a significant role in the oestrogen ortho-quinone reduction to their corresponding catechol which may be responsible for cancer initiation.47 At the same time the role of NQO2 in quinone metabolism is still not well known, but the idea that NQO2 generates free radicals during quinone metabolism is proposed. All the above support the idea that the inhibition of NQO2 may protect the cell from free radicals,29 and therefore has potential chemoprotective activity.44,48

Recently, MT3, the third melatonin binding site has been identified to be NQO2 itself. Melatonin binds to the active site of NQO2 leading to enzyme inhibition,38,46 which may lead to the hypothesis that the inhibition of NQO2 may induce other antioxidant mechanisms.29 Along with the observation that coenzyme Q0 an antioxidant, is a good
substrate for this enzyme, this supports a link between NQO2 and antioxidant activity.\textsuperscript{38} Moreover it could be the first binding site for the chemopreventative agent resveratrol at nanomolar concentrations.\textsuperscript{38,45}

1.6.5.1 NQO2 and NF-κB

NF-κB is a transcription factor that regulates the immune and the inflammation response against environmental stress. It regulates cell proliferation and cell survival by protecting cells undergoing apoptosis.\textsuperscript{49} It is believed that activation of the NF-κB pathway may lead to inflammation or cancer.\textsuperscript{50} Deregulated NF-κB activity resulted in high levels of NF-κB in the nucleus of tumour cells like colon, leukaemia, breast, ovarian, and prostate cancer.\textsuperscript{49}

It is also believed that the inhibition of NF-κB induces the apoptosis of different cancer cells or restores the cell’s ability to undergo apoptosis after radiation or chemotherapy.\textsuperscript{51} This makes the development of novel NF-κB inhibitors the major goal for many research groups.\textsuperscript{51} Flavones including quercetin, resveratrol and myricetin can inhibit NF-κB activity and initiate apoptosis by reducing IKK activity (IKK is an enzyme that is part of the upstream NF-κB signal transduction cascade).\textsuperscript{49} NRH, the NQO2 co-substrate can stimulate the NF-κB activity, therefore NQO2 inhibitors may have anticancer effects \textit{via} inhibition of the NF-κB pathway.\textsuperscript{52}

1.6.5.2 NQO2 and P53

P53 is a tumour suppressor protein known as "the guardian of the genome".\textsuperscript{53} P53 is stabilized and activated in response to different carcinogens. This activation leads to cell cycle arrest, senescence, or apoptosis, while the stabilization prevents the cell undergoing apoptosis.\textsuperscript{53} It has been proven that NQO2 stabilizes the tumour suppressor protein P53 that is involved in preventing cancer.\textsuperscript{54} Some studies show that exposure to radiation induces NQO1 and NQO2 levels, which in turn interact with p53, stabilize it and protect it from 20 S proteasome degradation which in turn prevents the cell undergoing apoptosis leading to cancer.\textsuperscript{53} Therefore the inhibition of this enzyme leads to destabilization of P53.\textsuperscript{55} Interestingly chloroquine, a potent NQO2 inhibitor, has been reported to activate the P53 pathway, but it is not clear if this involves NQO2 inhibition or not.\textsuperscript{35}

In another study, Jaiswal and co-worker reported that NQO2 competes with 20 S proteasome in binding with C/EBPα\textsuperscript{2} the transcriptional factor known to regulate
haematopoiesis (formation of blood cell component). Hence, NQO2 protects this transcriptional factor from the degradation by the 20 S proteasome.\textsuperscript{56}

1.6.5.3 Flavin redox switching function

Flavoenzymes utilize flavin mononucleotide (FMN) or flavin dinucleotide (FAD) as cofactors.\textsuperscript{57} These enzymes catalyse one or two electron transfer processes. Hydroxylation, dehydrogenation, DNA repair and electron transfer are examples of reactions catalysed by such enzymes. Recently the interest has increased in the flavin redox switch function.\textsuperscript{57} In flavin redox switches, the flavin regulates the function of some protein, where certain cellular signals or physiological environmental changes alter the redox status of the FMN or FAD combined with conformational changes. This is then translated into a new functional output such as cell signalling, membrane binding or transcriptional regulation. These functional changes could be reversible or irreversible (Scheme 7).\textsuperscript{57}

Leung and co-workers suggested a flavin switch function for NQO2, which requires reduction of FAD by NRH and binding of a suitable ligand.\textsuperscript{35} The NQO2 active site is in the boundaries between the two subunits (protomers) therefore the local structure change in this part affects the whole structure of the enzyme. NQO2 stabilising P53, as explained before, could be an example of a redox switch function.\textsuperscript{35}

![Scheme 7. The oxidized and reduced forms of the isoalloxazine ring in FAD](image)

1.6.6 NQO2 inhibitors

Recently the interest in finding novel potent inhibitors of NQO2 has increased.\textsuperscript{37} Lacking selective and potent NQO2 inhibitors is one of the obstacles regarding research on NQO2.\textsuperscript{29,45} Limited but structurally diverse inhibitors of NQO2 are reported in the literature,\textsuperscript{29,44} for example, resveratrol, casimiroin, melatonin, chloroquine and imatinib. But until now none of these are considered as an ideal NQO2 inhibitor. The first inhibitors identified by
Liao and Williams were atabrine and chlorpromazine with IC$_{50}$ values of 20 µM. The NQO2 inhibitors reported in the literature are discussed below.

1.6.6.1 Flavones

Flavones are a natural compounds that have been found to be competitive inhibitors of NQO2 in the presence of NRH. These include quercetin, morin, galangin, chrysin, (Figure 11). The most potent flavone NQO2 inhibitor is quercetin with a K$_i$ of 21 nM.

![Figure 11. Structures of some flavone inhibitors of NQO2.](image)

1.6.6.2 Melatonin

Melatonin, a known antioxidant, is a hormone synthesized in the pineal gland. It has three binding sites MT1, MT2, and MT3. MT3 has recently identified as an NQO2 binding site. (Table 2) shows the structures of melatonin and its analogues, together with the IC$_{50}$ values (the concentration that produce 50% inhibition) for their binding to NQO2.
Table 2. Structures and IC\textsubscript{50} values of the melatonin-based inhibitors of NQO2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC\textsubscript{50} µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td><img src="image" alt="Melatonin Structure" /></td>
<td>11 µM</td>
</tr>
<tr>
<td>2-Iodomelatonin</td>
<td><img src="image" alt="2-Iodomelatonin Structure" /></td>
<td>1.1 µM</td>
</tr>
<tr>
<td>N-Acetyl-5-hydroxytryptamine</td>
<td><img src="image" alt="N-Acetyl-5-hydroxytryptamine Structure" /></td>
<td>9.9 µM</td>
</tr>
</tbody>
</table>

1.6.6.3 Resveratrol

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenol found in many plants.\textsuperscript{38} The interest of this compound stems from its cancer chemopreventive properties.\textsuperscript{39} Resveratrol is a classical NQO2 inhibitor with an IC\textsubscript{50} value of 5.1µM.\textsuperscript{39} It is rapidly metabolized \textit{in vivo} to its metabolites which are not able to inhibit NQO2, so it is not the ideal NQO2 inhibitor.\textsuperscript{48} Sun and co-workers synthesised resveratrol analogues by changing the substitution on the both resveratrol rings. The new analogues were more potent and show higher affinity toward NQO2 than the parent compound (Figure 12).\textsuperscript{39} These analogues have direct hydrogen bond between the amino group of the compound and the hydroxyl group of residue Thr\textsuperscript{71}. In addition the methoxy group of these analogues interacts with the amide group of residue Asn\textsuperscript{161}. These hydrogen bonds may be responsible for higher potency of these analogues when compared with resveratrol.\textsuperscript{39}
1.6.6.4 Imatinib

Imatinib (Figure 13) is an Abl kinase inhibitor, which is active against acute myeloid leukaemia. Recently NQO2 was identified as an off-target interaction of Imatinib at a concentration of 80nM by competing for binding at the active site with the substrates. This lead to the hypothesis that NQO2 may have a role in leukaemia or Imatinib pharmacodynamics.37

1.6.6.5 Steroidal pyrazolines

Abdalla and co-workers synthesized a series of steroidal pyrazolines and conducted a biological evaluation of these compounds as NQO2 inhibitors. These steroids were potent inhibitors with IC₅₀ values of 20 and 60 nM. These compounds were less toxic than the antitumor cyproterone, the lead compound (Figure 14).59
Figure 14. Structures of steroidal pyrazolines NQO2 inhibitors and their IC$_{50}$ values.

1.6.6.6 Dabigatran and Dabigatran ethyl ester

Michaelis and co-workers have found that the well-known anticoagulant dabigatran and its prodrug dabigatran ethyl ester (under clinical trials) are inhibitors of NQO2 with similar inhibitory potency to Imatinib (0.4 µM). Interestingly, the ethyl prodrug showed more potency than the parent compound (0.8 µM > 10 µM, respectively), (Figure 15).

![Dabigatran and Dabigatran ethyl ester](image)

Figure 15. The structure of dabigatran (R=H) and dabigatran ethyl ester (R=Et) prodrug

1.6.6.7 Indolequinones

Ross and his team have examined the ability of indolequinones to inhibit NQO2 in both cells free and in cellular systems. These compounds were potent inhibitors of NQO2 with IC$_{50}$ values of 1.0 µM with irreversible inhibition. These inhibitors were more potent than quercetin, resveratrol, and melatonin by an order of magnitude (Figure 16).
1.6.6.8 Ammosamides B analogues

Cushman and his team synthesized novel and potent quinoline and pyrroloquinoline NQO2 inhibitors based on ammosamide B as a lead compound. They showed NQO2 inhibition at 61nM (Figure 17).\(^6\) Two series have been synthesised: tricyclic and bicyclic analogues. One of the tricyclic analogues showed an increased potency toward NQO2 by 15 fold, from 61nM to 4.1nM, the structure of which is shown in Figure 17.

![Figure 17](image17.png)

**Figure 17.** a- The general structure of the tricyclic ammosamide B analogues as NQO2 inhibitors, b-the most potent tricyclic analogue (IC\(_{50}\) 4.1 nM).

The bicyclic analogues showed less potency than the tricyclic analogues, with best inhibition at 150 nM. Interestingly both series were nontoxic at a concentration of 10 μM, (Figure 18).\(^6\)

![Figure 18](image18.png)

**Figure 18.** a-The general structure of the bicyclic ammosamide B analogues, b-the most potent bicyclic analogue (IC\(_{50}\) 150 nM).
1.6.6.9 Triazoloacridin-6-one and Imidazoacridin-6-one

In recent studies, the Stratford research group identified triazoloacridin-6-ones as micromolar NQO2 inhibitors from a virtual screening study of the National Cancer Institute (NCI) database.\(^{55}\)

The synthesized compounds of this series showed inhibition activity at nanomolar concentration, the N-oxidation of the tertiary amines of one compound in this series made the compound more potent (~ 100 nM) (Figure 19-b). Then they synthesized a series of imidazoacridine-6-ones derivatives as novel NQO2 inhibitors (Figure 19-c).\(^ {30} \) The inhibitory efficiency of these derivatives was high (14 nM). In both series (triazoloacridin and imidazoacridine) the derivative containing the N- oxide moiety show less toxicity and less DNA protein binding, which make these compounds more potentially acceptable as pharmacological NQO2 inhibitors.\(^ {30, 44} \)

\[ \text{Figure 19. A- The general structure of a triazoloacridine-6-one, B- Triazolo acridine-6-one with N-oxide moiety, C-General structure of imidazoacridine-6-one, D- Imidazoacridine-6-one with N-oxide moiety.} \]

1.6.6.10 Aminoquinolines

The exact mechanism of the well-known antimalarial drugs chloroquine and primaquine (Figure 20) is still not certain, but NQO2 is the only known human target of these drugs. Both compounds have the ability to inhibit NQO2 activity by different mechanisms. Primaquine competes with the co-substrate NRH (binds to NQO2_{ox}), while chloroquine competes with the substrate (bind to NQO2_{red}), and inhibits NQO2 with IC\(_{50}\) values of 7.5 \(\mu\)M and 1.5 \(\mu\)M, respectively.\(^ {35} \) Leung and Shilton proposed a Flavin redox switch
function for NQO2. This proposal stems from the finding that the conformational changes in NQO2\textsubscript{red}-chloroquine complex is different from the NQO2\textsubscript{ox}-chloroquine complex, including movement of the active site loop, crystal packing differences, and changing the environment around the active site.$^{35}$

![Primaquine and Chloroquine](image)

**Figure 20.** Structures of the antimalarial drugs primaquine and chloroquine

### 1.6.6.11 Casimiroin and its analogues

Casimiroin is a natural product found in the seeds of *Zapote blanco*. This compound shows a potential to inhibit carcinogenesis.$^{62}$ Maiti and co-workers were interested in the synthesis of casimiroin and its analogues as aromatase and NQO2 inhibitors. These compounds exhibit activity at low micromolar concentrations. Methylation of the nitrogen atom in the parent compound, in addition to the incorporation of methoxy groups increases the inhibitory activity of the synthesized compound, when compared with the natural compound, (Figure 21).$^{35,62}$

![Casimiroin analogues](image)

**Figure 21.** A-The structure and NQO2 activity of casimiroin, B-The most potent analogue of casimiroin.

### 1.6.6.12 Protein kinase inhibitors (TBBZ, DMAT and TBB)

4,5,6,7-1H-Tetramethylbenzimidazole (TBBZ), 2-dimethylamino-4,5,6,7-tetramethyl-1H-benzimidazole (DMAT) and 4,5,6,7-tetramethylbenzotriazole (TBB) are known to inhibit protein kinase (CK2) at nanomolar concentrations. However, TBBZ and DMAT are still
able to initiate apoptosis in cells exhibiting CK2 inhibitor resistance. This may be attributed to their activity as NQO2 inhibitors.

DMAT has the same affinity to bind to the oxidized and reduced form of NQO2; however the crystal structure revealed different binding mode. In oxidized NQO2, DMAT was buried deeply in the active site, making a direct hydrogen bond with the active site. While in the reduced NQO2 DMAT binds more peripherally and the hydrogen bond is mediated through the water molecule in the active site. Figure 22 shows the structures of the CK2 inhibitors and their IC₅₀ as NQO2 inhibitors.

Figure 22. Structures of CK2 inhibitors and their IC₅₀ for inhibition of NQO2.

1.6.6.13 DNA intercalating agents

Ethidium bromide, acridine orange and doxorubicin are DNA intercalating agent, which were shown by Leung and co-workers to inhibit NQO2 at nanomolar concentrations. Acridine orange and ethidium bromide have a much higher affinity to bind to reduced NQO2 rather than the oxidized form. This could be explained by the positive charge of the pyridine aromatic ring of these agents at neutral pH (Figure 23).
Chapter 1. Introduction

1.6.7 NQO2 inhibitor design

The active site of NQO2 is a large but narrow cavity which is 17 Å in length and 7 Å wide. This makes NQO2 able to receive many different polycyclic and polyaromatic compounds. In contrast to the active site of NQO2, NQO1 is constrained and smaller than the NQO2 active site, therefore designing relatively large inhibitors with more aminoalkyl branching side chains may make these inhibitors more selective toward NQO2.\textsuperscript{37}

Structural planarity is important in the inhibitor design to enhance the $\pi-\pi$ stacking with the FAD molecule in the NQO2 active site\textsuperscript{55,61} (Figure 24).

Figure 23. Structures of some DNA intercalating agents and their IC\textsubscript{50} values for NQO2 inhibition.
Figure 24. The crystal structure of resveratrol bound to the NQO2 active site, FAD in pink and resveratrol in yellow (PDB code 1SG0 resolution at 1.5 Å).

The active site is hydrophobic in nature with some polar residues, which makes it ideal to interact with hydrophobic inhibitors with polar and hydrogen bonding interactions. Pegan and co-workers reported ~100 fold improvement in the binding of tetracyclic compounds and inhibition potency compared to the bicyclic compounds. This maybe a result of the large area of hydrophobic interaction in addition to the tricyclic compounds ability to exclude the water molecules from the active site, which are not excluded by the smaller bicyclic compounds. This is important because as the compounds bind to the residues of the active site or to the FAD directly, rather than binding to the water molecules, the inhibitor becomes more potent. Interestingly, studies have confirmed the importance of water molecules in the active site to improve the compound binding. Therefore for NQO2 inhibitor design we should keep the following essential features: large size, planarity, ability to make hydrophobic interaction (π-π stacking) and a functional group able to make interactions with the polar residue in the active site.

1.6.8 Synthesis of novel NQO2 inhibitors and previous results

None of the above NQO2 inhibitors could be considered as an ideal NQO2 inhibitor, since they are not specific, and the majority of them have other pharmacological effects. Therefore, there is still a need to identify potent and selective NQO2 inhibitors.

Nolan, Stratford and colleagues conducted a virtual screening of the NCI database to identify new NQO2 inhibitors. Structurally diverse inhibitors were identified belonging to the following classes: polyaromatic, ellipticin, acridine, quinoline and furan-amidine. These compounds are considered as lead compounds for the synthesis of novel NQO2
inhibitors. Table 3 shows examples of each scaffold together with their IC$_{50}$ values for inhibition of NQO2.

**Table 3.** Examples of NQO2 inhibitors identified by Nolan and co-workers by virtual screening of the NCI library.$^{52}$

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Structure</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine</td>
<td><img src="image" alt="Acridine Structure" /></td>
<td>420</td>
</tr>
<tr>
<td>Ellipticine</td>
<td><img src="image" alt="Ellipticine Structure" /></td>
<td>50</td>
</tr>
<tr>
<td>Furanamidine</td>
<td><img src="image" alt="Furanamidine Structure" /></td>
<td>140</td>
</tr>
<tr>
<td>Poly fused</td>
<td><img src="image" alt="Poly Fused Structure" /></td>
<td>60</td>
</tr>
<tr>
<td>aromatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinoline</td>
<td><img src="image" alt="Quinoline Structure" /></td>
<td>40</td>
</tr>
</tbody>
</table>

Depending on this virtual screening, derivatives of acridine triazoloacridin-6-one and imidazoacridin-6-one were synthesised and tested as NQO2 inhibitors by the Stratford group (see also, section 1.6.6.9). A series of furan-amidines were also synthesised and tested by Freeman and co-workers.$^{64}$

The previous screening resulted in seven quinoline based compounds (Figure 25) which showed high potency.$^{52}$
Figure 25. The structures of seven aminoquinolines that were found to be NQO2 inhibitors by the virtual screening of the NCI database. These compounds were the starting point for Whitehead and his team in designing and synthesizing NQO2 inhibitors utilizing the aminoquinoline scaffold. It should also be noted that the antimalarial aminoquinolines compounds exhibit NQO2 inhibition activity at nanomolar concentrations. This made the 4-aminoquinolines promising leads for NQO2 inhibitor design. Three series of 4-aminoquinoline analogues and hydrazine quinoline analogues were synthesized and biologically evaluated as potential NQO2 inhibitors. Figure 26 shows the structures of the three different series A – C.
Table 4 shows some of the compounds that were synthesized together with their IC$_{50}$ values.

Table 4. Selected aminoquinoline compounds and their IC$_{50}$ values for the inhibition of NQO2 (Whitehead group, unpublished). Structures for series A-C are shown in Figure 26.

<table>
<thead>
<tr>
<th>$\textbf{R}^1$</th>
<th>$\textbf{R}^2$</th>
<th>$\textbf{R}^3$</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenyl</td>
<td>A</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>Inactive</td>
<td>241</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>H</td>
<td>Phenyl</td>
<td>925</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>2-Fluorophenyl</td>
<td>-</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>H</td>
<td>2-Fluorophenyl</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>2-Fluorophenyl</td>
<td>Inactive</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>H</td>
<td>2-Fluorophenyl</td>
<td>1142</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>2-Pyridyl</td>
<td>-</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>H</td>
<td>2-Pyridyl</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>4-Imidazoyl</td>
<td>-</td>
</tr>
<tr>
<td>OCH$_3$</td>
<td>H</td>
<td>4-Imidazoyl</td>
<td>-</td>
</tr>
</tbody>
</table>

On comparison of the three series, deleting the chlorine atom at position 7 and introducing a methoxy moiety at position 6 converts some derivatives from inactive to active compounds. In addition, increasing the linker length between the ring and side chain also increases the activity. This, may be because it makes the side chain able to freely rotate and take different positions to interact with the amino acid residues in the active site. In general introducing the hydrazone moiety in series C makes the compounds more active. This may be explained by increasing the number of hydrogen bond interactions that the molecule can
Therefore modifying the hydrazone moiety in order to enhance the activity of these compounds is one aim of this project.

**1.6.9 Hydrazone and hydrazide functional groups in pharmaceutical products**

Hydrazones belong to the Schiff bases family.\(^{65}\) The carbon and nitrogen moiety (NHN=\(\text{C}\)) is responsible for the chemical and physical properties of these compounds, and its interaction with electrophiles or nucleophiles.\(^{65}\) Hydrazones and hydrazide scaffolds are emerging as important functional group in drug design. These scaffolds are present in compounds possessing diverse of biological activities,\(^{66,67}\) such as vasodilator, antituberculosis, anti-tumour, antimicrobial, antiplatelet, anti-inflammatory, anticonvulsant and antioxidant\(^{65,67}\) (Table 5).

Hydrazone derivatives emerge again as the moiety of attention in biotechnology, since it aid in targeting drugs by releasing them in the active site.\(^{65}\) The principle of this approach is to attach the monoclonal antibodies (mAbs) of certain cancer cells to the drug through a linker which is stable at neutral pH (such as blood) and undergo hydrolysis under acidic conditions.\(^{68,69}\) Lysosomes offer such conditions, since they is acidic and rich in proteolytic enzymes. Once the mAbs attach to its antigen on the cell surface and enter the cell through endocytosis, the hydrolysis of the hydrazone bond takes place, and the drug is released. The delivery of the anti-tumour drug doxorubicin is an example of this approach\(^ {68}\) (Figure 27).

![mAb-doxorubicin (hydrazone)](Image)

**Figure 27.** Chemical structure of the antitumor doxorubicin linked to a monoclonal antibody.

Hydrazones are relatively stable specially at neutral pH.\(^{65,68}\) Their stability could be attributed to the resonance around the carbon and nitrogen leaving the carbon negatively charged and less electrophilic (Scheme 8).\(^ {70}\)
Scheme 8. Stability of hydrazone by resonance.

Hyrazones undergo acid-catalysed hydrolysis as shown in Scheme 9.

\[
\begin{align*}
R^1\text{N} & \overset{\text{H}^+}{\xrightarrow{\text{H}_2\text{O}}} R^1\text{N}^+ \overset{\text{H}^+}{\xrightarrow{\text{OH}^-}} R^1\text{N}^+ \overset{\text{H}^+}{\xrightarrow{\text{R}^2}} R^1\text{H} + \text{H}_2\text{N}^+ \text{R}^2
\end{align*}
\]

Scheme 9: Mechanism for the hydrolysis of the hydrazone group.

Table 5. Examples of hydrazone and hydrazide derivatives possessing a range of biological activities.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LASSBio-294.71</td>
<td><img src="image1" alt="Structure" /></td>
<td>Vasodilation</td>
</tr>
<tr>
<td>Dihydralazine.72</td>
<td><img src="image2" alt="Structure" /></td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Nifuroxazide.66</td>
<td><img src="image3" alt="Structure" /></td>
<td>Intestinal antibiotic</td>
</tr>
<tr>
<td>Iproniazide.66</td>
<td><img src="image4" alt="Structure" /></td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>4-((2-Phenylhydrazono)methyl)phenol.73</td>
<td><img src="image5" alt="Structure" /></td>
<td>Sepsis</td>
</tr>
<tr>
<td>4-((2-(2-Fluorophenyl)hydrazono)methyl)-2,6-dimethoxyphenol.74</td>
<td><img src="image6" alt="Structure" /></td>
<td>Antioxidant</td>
</tr>
</tbody>
</table>
1.6.10 Aims of the project

According to the previous work (Section 1.6.8), the importance of the hydrazone functional group in medicinal activity, and the criteria mentioned in Section (1.6.7), the hydrazone quinoline derivatives synthesised by the Whitehead group were one of the lead compounds for this project (Figure 28). The effect of introducing a methoxy group at position 6 and the effect of methyl substitution at position 2 will be evaluated, as well as changing the hydrazone moiety to hydrazide and amide moieties.

![Figure 28. The 4-aminoquinoline scaffold](image)

Therefore, the aims of this project were to:

1. Design NQO2 inhibitors with the aid of molecular docking. A library composed of a variety of scaffolds docked in both the reduced and oxidised forms of the enzyme. The affinity of the designed inhibitors toward the enzyme in different redox status has been addressed during this procedure.
2. Design a synthetic pathway to synthesise hydrazone, hydrazide, and amide derivatives of 4-aminoquinolines with high percentage yield.
4. Fully characterise the synthesised compounds by $^1$H NMR, $^{13}$C NMR, melting point, IR and mass spectroscopy to confirm their identity.
5. Evaluate the relationship between NQO2 and malaria and the ability of NQO2 inhibitors to inhibit the malaria parasite growth at nanomolar concentrations.
6. Undertake biological evaluation of the synthesised inhibitors against recombinant NQO2, which may have as crucial role in studying the cellular function of this enzyme, as well as serve as chemoprevention or anticancer candidates.
2. Molecular Modelling

2.1 Introduction

Over the last decades computational drug discovery has emerged as an effective approach to accelerate drug discovery and development. The availability of massive biological macromolecule data facilitates the screening of huge libraries, shortens the whole drug research process and reduces the failure risk.

There are many computational methods serving drug discovery, including virtual screening, molecular modelling, de novo design, pharmacophore modelling and mapping. Computer based methods of molecular docking have become routine in rational drug design.

The molecular docking process is computational prediction of the ligand–protein interaction, including the conformations and orientations (posing) of the ligands in the active site of the target (protein). The docking program measures the affinity of the ligand to bind to the protein and this is called the scoring function. A scoring function works as a fitness function in the optimisation of the ligand binding mode. It should not be expected to be a highly accurate binding affinity prediction, but give a solution with best fitness and compare it to other molecules. In our research, molecular docking has been used to guide drug design of novel inhibitors for NQO2 enzyme, to achieve the best ligand-protein interaction and consequently guide their selectivity and potency as promising anticancer agents.

Scheme 10. The scheme shows the steps to be followed in this project, and illustrates the role of molecular docking in designing new compounds.
2.2. Building the library

Based on previous results from the Stratford and Whitehead groups, the target molecules of this project were designed using the hydrazone quinoline derivatives as the lead compounds. To consider modifications to the hydrazone quinolines or alternative functional groups for the hydrazone to improve the activity and drug likeness of these derivatives, seven related scaffolds were designed to give the docking library (Table 6).

**Table 6.** The chemical structure of the lead compound and scaffolds which are present in the docked library.
The differences between these scaffolds focused mainly on changing the hydrazone moiety at position 4 to different functional groups, such as hydrazine, hydrazide and secondary amine. In addition, change of the bicyclic quinoline to the tricyclic system to mimic known tricyclic NQO2 inhibitors such as ammosamide B analogues and acridine derivatives, was also included. Finally, the steric effect of substituents at position 2 was also explored, by inserting either the methyl or phenyl group (R₁). The (R₂) group on the side chain, which is believed to have some effect on the activity and solubility of the derivatives, was varied on the aromatic rings with a range of substituents, including aliphatic chains (Figure 29).

![Chemical structure of the 4-hydrazone-quinoline scaffold.](image)

**Figure 29.** Chemical structure of the 4-hydrazone-quinoline scaffold.

The library was computationally docked into the NQO2 active site using GOLD (Genetic Optimisation for Ligand Docking) version 5.3. The docking process was carried out using the method described by Stratford and co-workers.³⁶,⁵²

### 2.3. Comparison between oxidized and reduced crystal structures of NQO2

The NQO2 enzyme catalyses oxidation-reduction reactions utilizing the ping-pong mechanism, which requires the redox cofactor FAD to cycle between its reduced and oxidized states. After the NRH co-substrate occupied the active site where it is oxidised and gives two protons to the isoalloxazine ring, it departed leaving behind the FAD in its reduced form, FADH₂. The isoalloxazine ring then recovers the oxidized state by donating these two protons to the substrate (usually quinone) where it is reduced to hydroquinone (as explained in Section 1.6.3 and Scheme 6).²⁵

This gives the NQO2 inhibitor the opportunity to inhibit favourably either the oxidised or reduced enzyme state, either by competing with the co-substrate or the substrate. The inhibitors may also inhibit both enzyme states, but with more affinity to one of them³⁵ (Figure 30).
FAD is a major part of the active site, therefore changing the redox state is likely to affect the shape and conformation of the binding pocket, and consequently the binding affinity and binding mode of the inhibitors.

Many NQO2 crystal structures are available in both the oxidized and reduced forms, binding to different ligands. These crystal structures show the affinity of different ligands to favourably bind either enzyme form. Acridine orange and ethidium have a much higher affinity to bind to reduced NQO2 (NQO2\textsubscript{red}) than the oxidized (NQO2\textsubscript{ox}) state.\textsuperscript{25} Furthermore, primaquine binds to NQO2\textsubscript{ox}, while chloroquine binds to NQO2\textsubscript{red}.\textsuperscript{35} In contrast, the protein kinase inhibitor DMAT has the same affinity to bind to the oxidized and reduced forms, with the crystal structure revealing different binding modes. In oxidized NQO2, DMAT is buried deeply in the active site, making direct hydrogen and halogen bonds with the active site. In the reduced NQO2, DMAT binds more peripherally and the hydrogen bond is mediated through water molecules.\textsuperscript{63}

In this docking study, two different crystal structures of NQO2 have been used: the oxidized form bound to primaquine (PDB code: 4FGJ; resolution 1.35 Å),\textsuperscript{35} and the reduced form bound to CK2 inhibitor DMAT (PDB code: 4U7F; resolution 1.8 Å),\textsuperscript{63} obtained from the Protein Data Bank.
2.3.1 Structural and conformational changes between NQO2\textsubscript{red} and NQO2\textsubscript{ox}.

To address the structural and conformational changes between the reduced and oxidized forms of the NQO2 enzyme, the two crystal structures have been superimposed on each other. Overall, the two crystal structures superimpose very well. However, on close inspection of the FAD and the binding site pocket, there is a tilt of the oxidized form (FAD\textsubscript{ox}) ring down to the bottom of the active site, making a smaller angle between the FAD ring and the carbon of the side chain attached directly to N10 (Figure 31). This makes the FAD\textsubscript{ox} buried more deeply in the active site whereas FAD\textsubscript{red} binds more peripherally in the reduced form (Figure 32).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{fig31.png}
\caption{The structure of the isoalloxazine ring of the FAD.}
\end{figure}

N5 FAD A 434
Figure 32. Two different views of the active site of NQO2 showing the tilt of $\text{FAD}_{\text{ox}}$ to the bottom of the active site in A comparing to more peripheral $\text{FAD}_{\text{red}}$ in B.

Reduction of FAD mainly affects atoms N5 and N1, where hydrogens are added. This tilt could be linked to the hydrogen bond formed between the nitrogen N5 of $\text{FAD}_{\text{ox}}$ and the Trp105 backbone NH group. On the other hand the $\text{FAD}_{\text{red}}$ (with protonated N5) is able only to make a $\pi$-$\pi$ interaction with the Trp105 phenyl ring. (Figure 33)

Figure 33. A: The interaction of $\text{FAD}_{\text{red}}$ with Trp105. B: The interaction of the $\text{FAD}_{\text{ox}}$ with Trp105.
The ability of oxidized FAD molecule to form hydrogen bond with Trp105 pushes the isoalloxazine ring down to the bottom of active site cavity making the angle between isoalloxazine ring and its side chain 110.7°, compared to 112.1° in its reduced state (Figure 34 A and B). As a result, the FAD molecule in the enzyme’s oxidized form (FAD\textsubscript{ox}) is buried more deeply in active site while it has a more peripheral position in the reduced form.

However a different bend called the “butterfly bend” along the N5-N10 axis has been reported by Leung and co-workers.\textsuperscript{35} A slight bend of 1.6° was observed in the FAD\textsubscript{ox} system when bound to primaquine. This concave bend increased to 2.3° with chloroquine binding, which increased to 4.8° with binding to FAD\textsubscript{red}\textsuperscript{35} (Figure 35).

![Figure 34. A: Angle between isoalloxazine ring and its side chain (FAD\textsubscript{red}), B: (FAD\textsubscript{ox}).](image)

![Figure 35. The butterfly bend of NQO2, reported by Leung and co-workers. NQO2\textsubscript{ox} with PQ (black, 1.6°), NQO2\textsubscript{ox} with CQ (red, 2.3°), and NQO2\textsubscript{red} with CQ (blue, 4.8°).](image)
Such a bend was very difficult to observe in this study using the NQO2 crystal structures 4FGJ (NQO2$_{ox}$ with primaquine) and 4U7F (NQO2$_{red}$ with DMAT).

2.3.2 Electronegativity changes between the NQO2$_{red}$ and NQO2$_{ox}$.

Here, a difference in the electronegativity between the FAD$_{ox}$ and FAD$_{red}$ has been observed (Figure 36). Using the same crystal structures, the electronegativity map shows that the FAD$_{ox}$ has a positive electrostatic potential, compared to the negative electrostatic potential of FAD$_{red}$. This could be attributed to the electron delocalization between N1 and the oxygen atom binding to carbon 2, as shown in Figure 30.

![Figure 36](image)

**Figure 36.** The electronegativity maps shows the charge density (A, B for FAD$_{ox}$), and (C, D for FAD$_{red}$), where red represents negative charge, white colour is neutral and blue is positive charge).
Therefore, it is expected that the inhibitors would have different binding affinities to the two enzyme states. However, it should be noticed, that the electronegativity maps were generated using MOE, whereas the docking run was conducted using GOLD, the limitation being that GOLD does not consider the charge density when calculating the score function.

**Figure 36** shows that the isoalloxazine rings of FAD$_{ox}$ and FAD$_{red}$ have different charge distributions, attributed to the differences in their redox state. In addition, FAD$_{red}$ has a slightly more positive electrostatic potential at the ring edges, due to the addition of two hydrogen atoms.

As mentioned before, the active site of NQO2 is lipophilic and the isoalloxazine ring forms the floor of the active site. Therefore the main interaction between the inhibitors and the enzyme via π-π stacking between the aromatic rings of the inhibitors with both the FAD and the lipophilic amino acid residues in the active site. If these aromatic moieties have a positive charge at physiological pH, it tends to bind to the reduced form of the enzyme, where it has a negative electrostatic potential (**Figure 36**).\(^{25}\) In contrast, if the aromatic moiety of the inhibitor was negatively charged, it would bind to the oxidised form of the enzyme. However the electron delocalization/tautomeration in FAD$_{red}$ may reduce the ability of FAD to make π-π stacking with the aromatic rings of the inhibitors, with replacement of these interactions with water molecule.\(^{25}\)

### 2.4 Method validation

The GOLD run approach was validated through extracting and docking of cocrystallized ligands: primaquine and DMAT in NQO2 active site of crystal structures 4FGJ (NQO2$_{ox}$) and 4U7F (NQO2$_{red}$), respectively. The Astex statistical potential (ASP) score was used as a scoring function. Each GOLD run saved the top hundred scoring poses for each ligand. Then, the ligands’ orientation and conformation were compared to their original orientation and conformation in the crystallized structure (**Figure 37**). Finally, the RMSD values for the best scoring poses were calculated and found to be acceptable with a range of (1-4 Å) of the experimental bound ligand poses.
2.5 Results and discussion

Two molecular docking studies have been performed for the proposed library, following the protocol described in the experimental section (2.3), using the reduced and oxidised forms of NQO2. The best docking score reflects the predicted minimum binding energy for the docked ligand in the NQO2 active site. The best scoring poses for each ligand with the lowest energy conformation was then selected.

Table 7 shows the scaffolds structure and the ASP score ranges obtained from the docking run for each scaffold. It is observed that the scores obtained after docking the library compounds using both the oxidized and reduced NQO2 forms were all higher than the NQO2 classical inhibitor, resveratrol (Table 7). In addition, the scores showed high similarity to that of the acridines (scaffold 8), which is also known to inhibit the NQO2 in the nanomolar range. These results led to the hypothesis that the proposed scaffolds 1-7 would have the ability to inhibit NQO2 enzyme through binding to either states of the enzyme. Also, it can be hypothesised that these derivatives would have a higher affinity towards the NQO2 oxidized form since they have higher ASP scores when they were docked in NQO2 active site with an oxidized FAD molecule.

Figure 37. Different poses superimposed with the reference ligand primaquine (in green).
Table 7. The structure of the proposed library scaffolds and the ASP score for each scaffold obtained from docking to the oxidized and reduced NQO2 active site.

<table>
<thead>
<tr>
<th>Scaffold structure</th>
<th>ASPscore (Ox)</th>
<th>ASPscore (Red)</th>
<th>Scaffold structure</th>
<th>ASPscore (Ox)</th>
<th>ASPscore (Red)</th>
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<td>57-66</td>
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<td>57-59</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Aliphatic)R</td>
<td>65-75</td>
<td>69-72</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Scaffold 3</td>
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<td>52-60</td>
<td>(Aromatic)R</td>
<td>60-63</td>
<td>58-62</td>
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<td>Scaffold 5</td>
<td></td>
<td></td>
<td>Scaffold 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Aliphatic)R</td>
<td>60-64</td>
<td>59-63</td>
<td></td>
<td>63-72</td>
<td>61-65</td>
</tr>
<tr>
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<td>51-66</td>
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<td>Scaffold 9</td>
<td></td>
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</tr>
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</table>

From the above results, a correlation between ASP scores for NQO2_{red} and NQO2_{ox} has been calculated. Figure 38 shows this correlation: each point in the curve represents one compound in the library where the NQO2_{red} ASP score is the X coordinate and NQO2_{ox} ASP score is the Y coordinate. The orange line represents the normal linear fit curve where x=y (i.e. if the score of the NQO2_{red} equal the score of the NQO2_{ox}). The blue line represents the actual compounds’ score. By taking the
orange line as a reference, we can conclude that the library compounds have a higher affinity to bind NQO2 enzyme in its oxidized state.

**Figure 38.** The correlation between the NQO2\textsubscript{red} and NQO2\textsubscript{ox} ASP scores. Each point in the curve represents one compound in the library where the NQO2\textsubscript{red} score is the X coordinate and the (NQO2\textsubscript{ox}) score is the Y coordinate.

This higher affinity towards the NQO2\textsubscript{ox} could be attributed to the conformational and electronegativity differences between the oxidised and reduced forms that have been discussed above. On the other hand, most of the library comprises of relatively large molecules which allow them to fit better in the oxidized binding pocket where the FAD is buried deeply in the cavity. Binding deeply in the active site leaves more room to accommodate the whole compound including the side chain of the ligands. This would increase the opportunity of the ligands to bind to the amino acids residues in the cavity. In addition, the negative electrostatic potential of FAD\textsubscript{red} reduces the ability of FAD to make π-π stacking interactions with the aromatic rings of the different ligands.\textsuperscript{25}

### 2.5.1 Comparison between the scaffold scores

Scaffolds 1, 3 and 8 have the highest scores among the docked library scaffolds (Table 7). Scaffold 8, which are the acridine derivatives, will be excluded from this discussion and comparison, since it has already been synthesised by the Stratford group.\textsuperscript{30,55} However, it was included in this docking run as a control together with resveratrol (the classical NQO2 inhibitor). Ammosamide B (Scaffold 9) will not be considered further since it shows the worst scores in comparison with other scaffolds.
Scaffolds 1 and 3 show the best docking scores, which reflect the best binding mode; therefore, they were chosen as the main scaffolds of interest. Particularly the substitution at positions 2 and 4 will be analysed in depth. The substitution at each position will be discussed individually to see how extending the structure modification affects the score.

In order to decide which substitution is the best at position 4, the 6-methoxy-2-methylquinoline nucleus (shown in red) was fixed and the substitution at position 4 was changed to hydrazine, hydrazide and secondary amine with aromatic substitution or hydrazone with an aliphatic chain (Figure 39).

![Figure 39. Structures of 1- hydrazine, 2- hydrazide, 3- hydrazide, 4- secondary amine, and 5- hydrazone with aliphatic chain) derivatives and their ASP scores](image)

It was observed that the hydrazone functionality had a higher score when compared to hydrazine, hydrazide and secondary amine derivatives. At the same time, the hydrazone substituted with an aryl ring was much better than the hydrazone with aliphatic substitution.

The majority of the docked library compounds were planar, having a quinoline ring, which is the main participant in the interaction with the FAD isoalloxazine ring through π-π interaction. Hydrazone derivatives showed better π-π stacking interactions with the FAD than other derivatives because the hydrazone moieties were accommodated deeply inside the binding cavity: the quinoline ring and the hydrazone moieties overlay the FAD isoalloxazine ring and sandwich between the FAD and the ring of phenylalanine Phe B408 (Figure 40).
**Figure 40.** 3D structure of the NQO2 active site showing hydrazone derivatives sandwiched between the coenzyme FAD and the ring of phenylalanine Phe B408 and showing the other amino acids residues in the active site

The deep insertion of the hydrazone derivatives in the active site could be due to the double bond between the nitrogen and carbon atoms of the hydrazone moiety, which helps maintain the planarity of the compound and prevents free rotation of the side chain arm at position-4. In addition, the aryl substituent at the hydrazone moiety is positioned in a way that allows it to participate in the π−π interaction with the FAD and with the phenylalanine Phe B408.

At the same time, the occupancy of the hydrazone derivatives in the active site enables any substitution at the side chain ring to make further interactions with the amino acid residues in the active site, such as with asparagine (Asn A161) (**Figure 42-C, D**). This was the case with both the reduced and oxidized forms of the NQO2 enzyme (**Figure 41**and **Figure 42**).

**Figure 41.** The binding mode of the hydrazone scaffold (yellow) in the active site loop for the oxidised and reduced forms of NQO2: A- NQO2_{ox}; B- NQO2_{red}. The figures were generated using MOE.
Figure 42. Ligand maps showing the binding of the hydrazone scaffold to the oxidised and reduced forms of NQO2: A- hydrazone-NQO2ox; B- hydrazone-NQO2red. D- hydrazone-NQO2ox E- hydrazone-NQO2ox The figures were generated using MOE.

In all the other scaffolds (hydrazine, hydrazide and amine), the quinoline ring was projecting deeply to inside the active site loop, whilst the side chain was projected toward the opening of the active site loop. This pose allows only the quinoline ring to make π-π stacking interactions with the FAD but not with the side chain aromatic ring. At the same time, it reduces the probability of the substitution at the side chain to interact with the active site amino acids residues (Figure 43).
The high scores of the hydrazone scaffold and the proper mode of binding encouraged the study to focus on hydrazone derivatives. The effect of different aromatic substitution on the hydrazone moiety (at position 4) and the effect of substitution at position 2, keeping 6-methoxy-4-hydrazone-2-methylquinoline as the main scaffold (shown in red in Figure 44) have been evaluated.

It was observed that the ASP score increased by having a substitution on the phenyl ring and by replacing the phenyl ring with other heterocyclic rings (Figure 44).
Figure 44. The effect of changing the phenyl substitutions at the hydrazone moiety to other heterocycle rings on the ASP score

In addition, the effect of the substitution at position 2 was also evaluated by comparing the ASP score of different groups attached to the 4-(2-benzylidenehydrazinyl)-6-methoxy-quinoline scaffold (shown in red, Figure 45). Groups with different polarity and size, namely, methyl, phenyl, pyridine, imidazole and oxazole were explored. Pyridine and imidazole gave the highest scores, which could be explained by the presence of a nitrogen atom and its ability to make a hydrogen bond with the amino acid residues in the active site such as asparagine (Asn A161) as shown in Figure 42-C.

Figure 45. The effect of different substituents at position 2 on the ASP score

Comparing scaffold 3 (hydrazone with phenyl substitution at position 2) to scaffold 1 (hydrazone with methyl substitution at position 2), a general pattern was noticed. As the hydrazone moiety was oriented towards the bottom of the active loop in scaffold 1 (as explained above), it was oriented more peripherally toward the opening of the
active site (like the other scaffolds) in scaffold 3, allowing the aromatic substitution at position 2 to make more \( \pi-\pi \) interactions with FAD. Consequently, the scores of scaffold 3 were higher compared to scaffold 1. **Figure 46** shows the differences in the binding mode for related compounds in scaffolds 1 and 3.

**Figure 46.** The ligand interaction maps generated using MOE, showing the differences in the interaction of an example from scaffold 1 and 3.

From the previous **Figure 46** and discussion, an appropriate fitting of hydrazone containing compounds derived from scaffold 1 is clear compared to the corresponding compounds from scaffold 3, where the compounds were more
peripheral, away from the bottom of the active loop. However, the scores for scaffold 3 were higher than scaffold 1. This can be explained by the ligand interaction map, since scaffold 3 is able to make more $\pi-\pi$ stacking interactions with FAD, together with the aid of an extra lipophilic aromatic ring at position-2 compared to scaffold 1. These main differences between the two scaffolds 1 and 3 could be generalized for both oxidized and reduced forms of the enzyme. A slight difference in the orientation of some of the compounds may be observed which may affect the $\pi-\pi$ stacking with the FAD (Figure 47).

However, the electronegativity of the FAD may play a major role in the score differences between the two redox states, as discussed in (section 2.3.2).
Chapter 2. Molecular modelling

**Figure 47.** Examples of the interactions of different ligands (scaffold 3; A, B, scaffold 1; C, D, E, F) with FAD in both the oxidized and reduced forms of NQO2.

Amongst scaffold 3 compounds, imidazole (25) is noteworthy (**Figure 48**) as the ASP score for this compound was the highest in the entire library for both the reduced and oxidised forms of NQO2, with scores of 75.3 for NQO2_{ox} and 71.1 for NQO2_{red}. The binding mode of this compound follows the scaffold 1 pattern where the hydrazone moiety is accommodated deep in the cavity and not projected toward the opening of the active loop, like other compounds derived from scaffold 3.
Accommodation of this pose in the enzyme’s binding site enabled the imidazole ring at the side chain attached to the hydrazone moiety to make a $\pi$-$\pi$ stacking with the phenyl ring of Phe $B\_408$. In addition, the phenyl ring at position 2 made good interactions with glutamine (Gln $B\_352$). At the same time, the methoxy group makes a hydrophobic interaction with FAD as well. These additional interactions of this compound in the active site may be responsible for compounds’ high score function. This different binding mode may also explain the observed activity of this compound against both NQO2 and NQO1, taking into consideration that all the other derivatives are inactive against NQO1.

**Figure 48.** The ligand interaction map of compound (25) in A- NQO$A_{\text{red}}$, B-NQO$A_{\text{ox}}$ active site.

As mentioned before, the NQO2 active site is a deep narrow cavity measuring 17 Å in length and 7 Å in width, which is a relatively large active site able to receive many different structures. Visualising the binding mode of the different scaffolds (which vary in size) and taking into consideration the effect of size or the number of phenyl rings in the compound, lipophilicity can explain the hydrazone (scaffold 1 and 3) and acridine’s (scaffold 8) best fit compared to amines (scaffold 5) and ammosamide (scaffold 9), since they can occupy the cavity of the active site better than the small amines and ammosamide B analogues. **Figure 49** shows the regions occupied by the different scaffolds in the active site of NQO2.
Finally, planarity could be one of the most important characteristics of the compounds, since it enhances the $\pi-\pi$ stacking interaction with the planar isoalloxazine ring of the FAD. This also explains why the hydrazone and acridine scaffolds got the best scores, as well as the poor fitting of the amines and aliphatic hydrazone scaffolds with a freely rotatable side chain. As a conclusion, combining
the substitution at both positions 2 and 4 gave the best scores, as shown in the examples in Figure 50.

![Chemical structures](image)

**Figure 50.** Examples of scaffolds 1 and 3 showing the effect of changing the substitution patterns to improve the scoring function for binding to both the oxidised and reduced forms of NQO2.

**Table 8** shows the structures, ASP scores for both NQO2 redox state and the IC$_{50}$ values for the synthesised compounds belonging to scaffold 1 and 3.
Table 8. Structures, ASP scores for both NQO2 redox state and the IC<sub>50</sub> values for the synthesised compounds

![Chemical structure of compound](image)

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<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
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<td>4-Benzoic acid</td>
<td>60</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>17</td>
<td>-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>4-Nitrophenyl</td>
<td>62</td>
<td>59</td>
<td>83</td>
</tr>
<tr>
<td>18</td>
<td>-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3,5-Dihydroxyphenyl</td>
<td>69</td>
<td>64</td>
<td>46</td>
</tr>
<tr>
<td>19</td>
<td>-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3-Hydroxyphenyl</td>
<td>67</td>
<td>62</td>
<td>137</td>
</tr>
<tr>
<td>20</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>4-N,N-dimethylaniline</td>
<td>66</td>
<td>64</td>
<td>Inactive</td>
</tr>
<tr>
<td>22</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>4-Nitrophenyl</td>
<td>67</td>
<td>63</td>
<td>1420</td>
</tr>
<tr>
<td>23</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Benzyl</td>
<td>52</td>
<td>49</td>
<td>125</td>
</tr>
<tr>
<td>24</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>65</td>
<td>64</td>
<td>595</td>
</tr>
<tr>
<td>25</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>4-Imidazoyl</td>
<td>75</td>
<td>72</td>
<td>90</td>
</tr>
</tbody>
</table>
The results of resveratrol and acridine derivatives (6a1) added to the table as a control and to facilitate the comparison. From the table it is observed that the resveratrol score was around 50-54 and the IC\textsubscript{50} was 900 nM which consider less active than the synthesised inhibitors. This is consistent with the acridine derivative (6a1) result where the ASP score was 67 and the compound was much potent than resveratrol with IC\textsubscript{50} of 120nM. This could be generalized to rest of the synthesised compounds, where the majority of them got higher scores than the resveratrol and they were really much potent. For example the ASP score of compound 25 was the highest among the library and the IC\textsubscript{50} of this compound was just 90 nM.

Overall analysis of the docking results provided the following features to guide the design of potential NQO2 inhibitors with the best docking scores.

- Planarity and hydrophobicity are essential properties for the design of NQO2 inhibitors.
- The hydrazone group gives the best score compared to the secondary amines, hydrazide and hydrazine.
- Introducing an aromatic ring at position-2 makes a noticeable increase in the affinity, which could be explained by enhancing the size or increasing the lipophilikity.
- Substituents on the aromatic rings at positions-2 and -4 that can make hydrogen bonds with the active site is important for both the activity and the solubility of the large and lipophilic compounds.

4-Aminoquinoline derivatives (hydrazone derivatives) were synthesised and tested as inhibitors of NQO2. The effect of introducing a methoxy group at position-6 and the effect of methyl or phenyl substitution at position-2 have been evaluated, as well as changing the hydrazone moiety to hydrazide or a secondary amine moiety.

By using ASP score function, the binding affinities ($\Delta$G\textsubscript{calc}) cannot be calculated, but instead a correlation between the ASP score values and the experimental binding affinities ($\Delta$G\textsubscript{exp}) can be achieved (Figure 51). (Note: $\Delta$G\textsubscript{exp} have been calculated using the IC\textsubscript{50} values obtained from the NQO2 inhibitor studies for the synthesised derivatives).
Figure 51. The correlation coefficient between $\Delta G_{\text{exp}}$ and the ASP score of scaffold 1 bound to NQO2$_{\text{red}}$, scaffold 1 bound to NQO2$_{\text{ox}}$, scaffold 3 bound to NQO2$_{\text{red}}$, and scaffold 3 bound to NQO2$_{\text{ox}}$. 

A. Correlation between the scaffold 1 ASP score of NQO2$_{\text{red}}$ with $\Delta G_{\text{exp}}$.

$$R^2 = 0.1066$$

B. Correlation between the scaffold 1 ASP score of NQO2$_{\text{ox}}$ with $\Delta G_{\text{exp}}$.

$$R^2 = 0.3466$$

C. Correlation between the scaffold 3 ASP score of NQO2$_{\text{red}}$ with $\Delta G_{\text{exp}}$.

$$R^2 = 0.0063$$

D. Correlation between the scaffold 3 ASP score of NQO2$_{\text{ox}}$ with $\Delta G_{\text{exp}}$.

$$R^2 = 0.0005$$
The experimental binding free energies were calculated using the Cheng-Prusoff equation:\textsuperscript{30,44,80}

\[ \Delta G_{\text{exp}} = -RT \ln \left( \frac{1}{IC_{50}} \right) \] \hspace{1cm} \text{eq 1} 

Where R is the gas constant = 8.31 J/K.mol and T is temperature in Kelvin = 298 K.

Only a moderate correlation has been found between the computed and the experimental activity of scaffold 1 derivatives and poor correlation with scaffold 3. This could be explained by the fact that scaffold 1 was more active than scaffold 3. However, this does not match with the score which was higher for scaffold 3 than scaffold 1. However, the correlation with the oxidized NQO2 enzyme results was better than with the reduced NQO2 results.

2.2 Molecular docking procedure

Chem 3D pro 13.0 was used to build the ligand library (~120 ligand). All of the ligands were energy minimized before the docking using MOE software which was also used to view the molecules and the docking poses. GOLD software version 5.3 was used as the docking engine. NQO2 PDB code: 4FGJ and 4U7F X-ray crystal structures were used to dock the library. Each protein was prepared by extracting the original ligand (primaquine or DMAT respectively), all the water molecules were deleted and hydrogen atoms were added. The active site was defined within 5 Å of N5 FAD A 434, an atom of the isoalloxazine ring in chain A of the crystal structure of NQO2 (Figure 52).

The ASP score was used for the scoring function. The Genetic Algorithm (GA) runs hundred times for each ligand. Each run consists of 100 000 genetic operations on a 100 members population. The population was divided into five islands. All NQO2 enzyme figures and docking poses were manipulated using MOE software and the correlation charts were done using the Excel program.

\[ \text{N5 FAD A 434} \]

\[ \text{Figure 52. Isoalloxazine ring of the FAD molecule showing N5 FAD A 434.} \]
3. Synthesis and enzyme assay

3.1 Synthesis

3.1.1 4-Aminoquinolines

4-Aminoquinoline is an important nucleus in medicinal chemistry and in drug design. Malaria, cancer, gastric disorders and some neurodegenerative diseases are examples of diseases treated by 4-aminoquinoline based drugs, for example, Aralen (chloroquine), SKF-97574 and Cognex (tacrine) (Figure 53).81,82

![Tacrine](attachment://tacrine.png)

**Tacrine**
Cholinesterase inhibitor (Alzheimer's disease)

![Aralen](attachment://aralen.png)

**Aralen (chloroquine)**
Antimalarial

![SKF-97574](attachment://skf-97574.png)

**SKF-97574**
Inhibitor of gastric acid secretion

*Figure 53. Examples of 4-aminoquinoline based drugs and their chemical structures.*

Different methods have been developed for the synthesis of the quinoline ring (Scheme 11).
- In the Gould-Jacobs reaction, aniline is condensed with a malonic acid derivative followed by cyclization reaction at high temperature.83
- In the Skraup method, the aniline derivative is heated with glycerol in the presence of an acid to form a stable intermediate that undergoes a cyclization reaction at high temperature.84
In the Doebener reaction, the quinoline ring formed by the interaction between the pyruvic acid, aldehyde and aniline as will describe later in more details.

-A more recent method, described by Price and Roberts, uses a condensation reaction of aniline with ethoxymethylene malonate.

Scheme 11. Different synthetic pathways for the preparation of the quinoline ring.

As mentioned in Section 1.6.10, the aim of this project is to synthesise 4-aminoquinoline derivatives. The synthesis of the quinoline ring is required first, prior to the introduction of the functional group at position 4 (Figure 54).

Figure 54. The structure and synthetic precursor of the target 4-aminoquinoline scaffold.
In this project the 4-aminoquinoline ring has been synthesised using two different pathways, each of which will now be described in detail.

### 3.1.1.1 Quinoline ring synthetic pathway 1

The first synthetic pathway is based up on the Conrad-Limpach reaction (Scheme 12), which involves the acid catalysed condensation of \( p \)-methoxyaniline with \( \beta \)-ketoester (ethyl acetoacetate) to give a 31% yield of intermediate (1), which then undergoes a cyclization reaction under microwave radiation to give quinoline-4-one intermediate (2) in a 51% yield. This is converted to the corresponding 4-chloro-quinoline derivative (3) by reacting with phosphorus oxychloride, which is then converted to the hydrazine derivative (4).\textsuperscript{87,88} Scheme 13 shows the proposed mechanism for this reaction.

![Scheme 12. First synthetic pathway for the synthesis of the quinoline ring.](image-url)
Scheme 13. The mechanism for quinoline synthetic pathway 1.

This synthetic pathway has the advantage of giving intermediate 2 in just two steps, however intermediate 2 could only be isolated in a low percentage yield. This is attributed to the enamine preferring to adopt the geometrical isomer of intermediate 1, whereas cyclization only occurs with intermediate 1. An alternative route to the quinoline ring was therefore developed (Scheme 14).

3.1.1.2 Quinoline ring synthetic pathway 2

Madrid and co-workers modified the Gould-Jacob’s method for the synthesis of the quinoline ring where they used methoxyethylidene Meldrum’s acid instead of diethyl ethoxymethylene-malonate. This modified method has been employed here (Scheme 14).
Scheme 14. The synthetic pathway for 4-hydrazinequinoline (4) using synthetic pathway 2.

The first step of this mechanism is to condense p-anisidine with Meldrum’s acid, which allows a methoxy group to be introduced at position 6, an essential functional group in the desired scaffold. Meldrum’s acid was heated at reflux in trimethyl orthoacetate or trimethyl orthobenzoate to form intermediate (1), this step giving either the methyl or phenyl ring substitution at position 2. The methylene group in Meldrum's acid is acidic, which allows simple alkylation at this position. (Scheme 15), shows the proposed mechanism of this reaction.

Scheme 15. The proposed mechanism of the reaction of Meldrum’s acid with trimethyl orthoacetate (R = Me) or trimethyl orthobenzoate (R = Ph) to form intermediate (6).

Once the p-anisidine is added to intermediate (6) it will initiate an addition-elimination reaction to give enamine intermediate (7). The cyclization reaction of (7) takes place at high temperature to give intermediate (2). This was then treated
with phosphorus oxychloride to introduce the chlorine atom at position 4 to give intermediate (3),\textsuperscript{86} which was then treated with hydrazine monohydrate to give the 4-hydrazonequinoline scaffold (4).\textsuperscript{88,91} This pathway gave a high percent yield, with 83\% for the formation of intermediate (7) and 93\% for intermediate (2) (Scheme 14).

Scheme 17 shows the proposed mechanism of the synthetic pathway 2, the elimination reaction occurring between the nitrogen of the p-anisidine as nucleophile and the electrophile carbon of intermediate (6). The Meldrum’s acid chemistry is responsible for the main features of this reaction, since with heat it undergoes a pericyclic reaction to acetone, ketene, and carbon dioxide (Scheme 16).\textsuperscript{92}

![Scheme 16](image)

Scheme 16. The chemistry of Meldrum’s acid and its pyrolysis at high temperature.

Based on Meldrum’s acid pyrolysis at high temperature, it is often used as a ketene precursor for thermal cyclization. This pyrolysis first give the methyleneketene which then undergoes a 1,3-N-H shift giving imidoylketene, (Scheme 17).\textsuperscript{93}
Scheme 17. Proposed mechanism for the formation of intermediate (7) and its thermal cyclization to precursor (2).

This thermal cyclization reaction was conducted using a microwave synthesiser with diphenyl ether as solvent at 200 °C for 30 min. The highly boiling point of the solvent provides the ability to use a high temperature which can be applied for a short time to minimise side products.\(^93\)

The formation of the stable intermediate (7) was confirmed by \(^1\)H NMR spectroscopy with the peak for the two methyl groups of Meldrum’s acid peak appearing at 1.66 ppm and the protons of the methyl group introduced at position 2 being present at 2.44 ppm. The protons of the methoxy group of the intermediate (6) disappeared, confirmed by just getting a signal for one methoxy group at 3.8 ppm which belongs to the \(p\)-anisidine (Figure 55).
Chapter 3. Synthesis and enzyme assay

Figure 55. The $^1$H NMR spectrum ($d_6$-DMSO) of intermediate (7) with methyl substitution at position 2.

The formation of the intermediate (2) was supported by $^1$H NMR spectroscopy, where the methyl peak of the Meldrum’s acid disappeared and the H-3 proton appeared up-field at 5.8 ppm. In addition, the NH singlet peak appeared at around 11 ppm (Figure 56).
Figure 56. The $^1$H NMR spectrum (d$_6$-DMSO) of intermediate (2)

6-Methoxy-2-methylquinolin-4-ol (2) was then reacted with 3 equivalents of phosphorus oxychloride and heated at reflux for 4 h to give the 4-chloroquinoline derivative (3) (Scheme 18). 84,86

Scheme 18. Proposed mechanism for the formation of 4-chloroquinoline derivative (3).
4-Chloroquinoline (3) was then reacted for 1-3 days with hydrazine monohydrate in a nucleophilic aromatic substitution (addition elimination) reaction to give the key intermediate 4-hydrazine-quinoline (4) (Scheme 19).

Scheme 19. Proposed mechanism for the formation of 4-hydrazine-quinoline (4)

4-Hydrazine-quinoline (4) was then used as a starting material to synthesise the hydrazone, hydrazide and hydrazine quinoline derivatives as explained in detail in the next sections (Scheme 20).

Scheme 20. 4-Hydrazine-quinoline (4) as the starting material for the hydrazone, hydrazide, and hydrazine derivatives.

3.1.1.3 Synthesis of 4-hydrazone quinoline derivatives

4-Hydrazone quinoline derivatives have been synthesised starting from the intermediate 4-hydrazine-quinoline (4) by the reaction with different aldehydes to give the corresponding 4-hydrazone derivatives (Scheme 21). Three equivalents of the appropriate aldehydes were added to the 4-hydrazone quinoline solution in methanol and stirred at room temperature to get the hydrazone derivatives in good yields (30-90%).
Scheme 21. The synthetic pathway of 4-hydrazonequinoline derivatives

This reaction is a nucleophilic addition reaction where the primary amine of the hydrazine moiety attacks the carbonyl group of the aldehydes leading to carbinolamine formation, which then loses a molecule of water to give the imine group. (Scheme 22) shows the mechanism of this reaction.

Scheme 22. The proposed mechanism for the formation of 4-hydrazone quinoline derivatives

Eighteen compounds have been synthesised with methyl or aryl groups at position 2, starting from a variety of aromatic aldehydes (Table 9).
Table 9. Structures of the synthesised 4-hydrazone quinoline derivatives and their % yield of synthesis

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₁</th>
<th>R₂</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>-CH₃</td>
<td>4-Fluorophenyl</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>-CH₃</td>
<td>Phenyl</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>-CH₃</td>
<td>4-Imidazoyl</td>
<td>76</td>
</tr>
<tr>
<td>11</td>
<td>-CH₃</td>
<td>4-Hydroxyphenyl</td>
<td>53</td>
</tr>
<tr>
<td>12</td>
<td>-CH₃</td>
<td>3-Pyridinyl</td>
<td>34</td>
</tr>
<tr>
<td>13</td>
<td>-CH₃</td>
<td>Benzyl</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>-CH₃</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>89</td>
</tr>
<tr>
<td>15</td>
<td>-CH₃</td>
<td>2-Nitrofuranyl</td>
<td>75</td>
</tr>
<tr>
<td>16</td>
<td>-CH₃</td>
<td>4-Benzonic acid</td>
<td>78</td>
</tr>
<tr>
<td>17</td>
<td>-CH₃</td>
<td>4-Nitrophenyl</td>
<td>92</td>
</tr>
<tr>
<td>18</td>
<td>-CH₃</td>
<td>3,5-Dihydroxyphenyl</td>
<td>75</td>
</tr>
<tr>
<td>19</td>
<td>-CH₃</td>
<td>3-Hydroxyphenyl</td>
<td>33</td>
</tr>
<tr>
<td>20</td>
<td>C₆H₅</td>
<td>3-Pyridinyl</td>
<td>89</td>
</tr>
<tr>
<td>21</td>
<td>C₆H₅</td>
<td>4-N,N-dimethylaniline</td>
<td>60</td>
</tr>
<tr>
<td>22</td>
<td>C₆H₅</td>
<td>4-Nitrophenyl</td>
<td>40</td>
</tr>
<tr>
<td>23</td>
<td>C₆H₅</td>
<td>Benzyl</td>
<td>26</td>
</tr>
<tr>
<td>24</td>
<td>C₆H₅</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>91</td>
</tr>
<tr>
<td>25</td>
<td>C₆H₅</td>
<td>4-Imidazoyl</td>
<td>77</td>
</tr>
</tbody>
</table>

The following spectroscopy (Figure 57) shows the ¹H NMR of compound (12) as an example of the formation of the hydrazonequinoline formation. The N=CH peak appeared at 8.98 ppm which confirmed the bond formation between the 4-hydrazinequinoline and the corresponding aldehyde.
3.1.1.4 Synthesis of 4-hydrazide quinoline derivatives

Changing the hydrazone functional group at position 4 to the hydrazide moiety is described here. The synthesis of 4-hydrazide quinoline derivatives take place by reacting the 4-hydrazinequinoline scaffold with the appropriate aryl chloride in the presence of a base to give the corresponding hydrazide derivative (Scheme 23).\(^95\)

**Scheme 23.** The synthetic pathway for the 4-hydrazide quinoline derivatives

In this addition elimination reaction, the lone pair of electrons at the nitrogen atom of the hydrazine moiety attacks the carbonyl of the aryl chloride, and chloride anion departs as the good leaving group.

---

Figure 57. The \(^1\)H NMR spectrum (CD\(_3\)OD) of compound (12)
In the Schotten-Baumann reaction, a base such as pyridine is used to trap the hydrochloric acid by product as the pyridinium hydrochloride salt, to ensure that the unreacted nucleophilic hydrazine remains deprotonated (Scheme 24).

Scheme 24. Mechanism for the formation of 4-hydrazidequinoline derivatives.

Two hydrazide quinolone derivatives have been synthesised as shown in Table 10. No more derivatives were synthesised because of the weak activity of this scaffold, which was much less active than the hydrazine derivatives.

Table 10. Structures of the synthesised 4-hydrazide quinoline derivatives and the % yield of the product.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₁</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Phenyl</td>
<td>28</td>
</tr>
<tr>
<td>27</td>
<td>4-Fluorophenyl</td>
<td>74</td>
</tr>
</tbody>
</table>

3.1.1.5 Synthesis of 4-hydrazine quinoline derivatives

Changing the hydrazone group to the hydrazine moiety was an aim in this project. In theory hydrazine derivatives could be synthesised by reducing the corresponding hydrazone derivatives with a reducing agent, such as sodium borohydride or Raney nickel.96

Unfortunately, several attempts were unsuccessful to reduce the synthesised hydrazone derivatives to the corresponding hydrazine derivatives using different...
reducing agents. All the attempts ended up either by failure to reduce the hydrazone bond or hydrolysis of the hydrazone moiety to give 4-aminequinoline as product (Scheme 25).

![Scheme 25](image)

**Scheme 25.** The results of different reactions to reduce the hydrazone derivative to its corresponding hydrazine derivative.

The NMR spectrum shown in **Figure 58** resulted from the reduction reaction of compound 8 (hydrazone derivative). This spectrum showed that the product was 4-aminoquinoline, since a peak referring to two hydrogen atoms appeared at 6.5 ppm, rather than the desired hydrazine derivative. In addition, the whole side chain containing the aromatic protons of the hydrazone derivatives has been lost, as well as the imine proton.
3. Synthesis and enzyme assay

Figure 58. The $^1$H NMR spectrum (d$_6$-DMSO) of 4-aminoquinoline, the product from the reduction of a hydrazone derivatives with Raney Nickel.

3.1.1.6 Quinoline-4-carboxamide synthetic pathway
Introducing an amide group at position 4 aimed to investigate the effect of the amide group on the activity, reduce the potential toxicity and improve the drug likeness of the designed compound when compared with a hydrazine analogue. The quinoline ring attached directly to a nitrogen atom is more toxic than a quinoline ring attached to a carbon atom. Figure 59 shows the differences between the two scaffolds generated by OSIRIS property explorer, showing that N-methylquinoline-4-amine is predicted to be mutagenic.

Figure 59. Comparison between 4-N-methylamino-quinolin and 4-ethylquinoline in terms of toxicity risk and drug likeness.
It is not possible to synthesise such a scaffold from the hydrazine analogue, therefore it is necessary to re-build the quinoline ring in order to introduce the amide group at position 4. The proposed synthetic pathway started by synthesising quinoline-4-carboxylic acid derivatives using the Doebener reaction. In this multicomponent reaction the quinoline ring was formed by the interaction between pyruvic acid, the appropriate aldehyde and p-anisidine (Scheme 26).

Scheme 26. Synthetic pathway for the quinoline-4-carboxylic acid derivatives based on the Doebener reaction

The reported mechanism for this reaction is shown in Figure 60 where the reaction is initiated by an aldol condensation reaction between the aldehyde and the pyruvic acid to form intermediate (28) which then undergoes a nucleophilic addition reaction with p-anisidine. Acetaldehyde or benzaldehyde could be used to introduce methyl or phenyl groups at position 2, respectively.
**Figure 60.** Mechanism of the Doebner reaction to synthesis 4-carboxylic acid-quinoline derivatives.

Jumade and co-worker\(^8^5\) reported the use of the Doebner reaction to synthesis quinoline-4-carboxylic acid in which \(p\)-anisidine was added dropwise to a refluxing solution of a mixture of the aldehyde and pyruvic acid. Using this procedure, only a low percentage yield of impure product was obtained. Therefore the conditions of the reaction were modified. By changing the order of addition a good yield of pure product was obtained: Benzaldehyde was first reacted with \(p\)-anisidine to give \(N\)-(4-methoxyphenyl)-1-phenylmethanimine (intermediate 31) (Scheme 27) the structure of which was confirmed by \(^1\)H NMR spectroscopy (Figure 61). This intermediate then participated in a nucleophilic addition reaction with pyruvic acid to give the product quinoline-4-carboxylic acid.

**Scheme 27.** The proposed mechanism of the modified Doebner reaction between \(p\)-anisidine, benzaldehyde and pyruvic acid.
Figure 61. The $^1$H NMR spectrum (d$_6$-DMSO) of the imine intermediate 31

The quinoline-4-carboxylic acid scaffold was then used as a starting material to synthesise quinoline-4-carboxamide. Firstly the carboxylic group was converted to acid chloride using thionyl chloride. The formed quinoline 4-acyl chloride derivatives were then reacted with different amines to give the quinoline 4-carboxamide as final products (Scheme 28).

Scheme 28. Pathway for the synthesis of quinoline-4-carboxamide derivatives.

Thionyl chloride is a chlorinating reagent and it is used widely to convert carboxylic acids to acyl chlorides. Scheme 29 shows the mechanism of this reaction.
Chapter 3. Synthesis and enzyme assay

Scheme 29. Mechanism for the conversion of a carboxylic acid to an acyl chloride using thionyl chloride.

The resulting quinoline-acyl chloride derivatives then undergo a nucleophilic substitution reaction with a range of different primary amines,\textsuperscript{99} to give the corresponding quinoline-4-carboxamide derivatives (Scheme 30).

Scheme 30. Mechanism of the nucleophilic substitution reaction of acyl chloride and primary amine.

Only one derivative (Figure 62) was synthesised for this scaffold, due to poor activity: Compound 32 did not show any inhibition of NQO2 at a concentration of 100µM.

Figure 62. Compound 32, the only synthesised quinolone-4-carboxamide derivative
Figure 63. The $^1$H NMR spectrum (CDCl$_3$) of quinoline-4-carboxamide compound 32

The $^1$H NMR spectrum (Figure 63) shows the formation of the amide bond where the two methylene protons on carbon 9 gave a doublet at 4.7 ppm, due to coupling with the NH group. The NH proton is a triplet at 6.5 ppm.

3.1.2 Aminoimidazole derivative

In the presence of the co-factor NRH, NQO2 activates the prodrug CB1954 [5-(1-aziridinyl)-2,4-dinitrobenzamide] by converting it from a monofunctional alkylating agent to a bifunctional agent (Scheme 31), able to interact with two residues of DNA strands and consequently prevents these strands from uncoiling during replication. 100-102

Scheme 31. Activation of CB1954 by NQO2
4-Aminoimidazole-5-carboxamide (AIC) compounds (Figure 64), show their ability to protect CB1954 against this activation,\(^{103}\) which could be through the inhibition of NQO2.

![Image](image_url)

**Figure 64.** The structure of AIC derivatives which protect CB1954 from activation.

In addition, AIC proved to inhibit the pathological activation of the transcription factor NF-\(\kappa\)B, making it potentially useful to treat related diseases (inflammatory disorders, asthma, diabetes, Alzheimer’s and cancer).\(^{104}\)

Taking into consideration that flavones including quercetin, resveratrol and myricetin can inhibit NF-\(\kappa\)B activity and initiate apoptosis by reducing the IKK activity (an enzyme part of the upstream NF-\(\kappa\)B signal transduction cascade).\(^{49}\) NRH, the NQO2 co-substrate can stimulate the NF-\(\kappa\)B activity.\(^{52}\) Therefore AIC may inhibit NF-\(\kappa\)B through the inhibition of NQO2.

These hypotheses make the synthesis of AIC a valuable project. **Figure 65** shows the 5-amino-2-phenyl-1H-imidazole-4-carboxamide (compound 33) which proved to inhibit NF-\(\kappa\)B.\(^{104}\)

![Image](image_url)

**Figure 65.** The NF-\(\kappa\)B inhibitor compound (33)

Although compound (33) is in the literature, there is no available procedure for its synthesis. The approaches attempted here based mainly on condensing \(\alpha\)-amino-
nitriles with imino-ethers. The proposed mechanism for this reaction is shown in Scheme 32.

Scheme 32. The proposed mechanism for the attempted synthesis of compound 33

Scheme 33 shows four pathways for the synthesis of compound 33, however, none of these pathways led to the desired compound. The polarity and consequently the solubility of the α-amino-nitrile starting material was one of the obstacles facing the synthesis of the compound.
Scheme 33. Four synthetic pathways used to attempt the synthesis of 5-amino-2-phenyl-1H-imidazole-4-carboxamide (compound 33)

Pathway D (Scheme 33) has been carried out using ethanol as solvent, which led to the formation of 4-amino-2-phenyloxazole-5-carboxamide (compound 34) as product instead of the desired compound (33) (Figure 66). The resulting compound 34 was confirmed by ES− mass spectrometry where the mass of the product was (202.2) for the formula C\textsubscript{10}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2} (M-H) (instead of (201) for the desired formula C\textsubscript{10}H\textsubscript{9}N\textsubscript{4}O). In addition, the ES+ peaks observed at 204.2 M+H were consistent with the formula C\textsubscript{10}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2}. The accurate mass was then recorded for the absolute confirmation gave an experimental value of 226.0593 which is consistent with the formula C\textsubscript{10}H\textsubscript{9}N\textsubscript{3}O\textsubscript{2} + Na. This could be explained by the Pinner reaction where the Pinner acid salt of an imino ester, reacts with water to form an ester, (Scheme 34).
Scheme 34. Pinner salt reacts with water to form an ester

Analogue (34) has been tested as an NQO2 inhibitor since the oxygen atom is a bioisostere of nitrogen; the valence electron distribution of a di-substituted oxygen atom is the same as a tri-substituted nitrogen atom, but, unfortunately, the compound shows no activity as an NQO2 inhibitor.

![Figure 66](image)

Figure 66. 4-Amino-2-phenyloxazole-5-carboxamide (compound 34)

3.1.3. Reduced N-ribosynicotinamide (NRH)

NRH is the co-factor of NQO2, added to the assay to ensure that the enzyme is able to catalyse its reductive role. The synthesis of NRH was carried out according to the method reported by Long and co-workers. NRH was formed from NADH by its breakdown by two enzymes (Scheme 35). NRH was prepared by dissolving NADH in potassium carbonate/bicarbonate buffer (0.4 M, pH 10.0). Alkaline phosphatase (from bovine mucosal mucosa) and phosphodiesterase 1 type IV (from Crotalus atrox) enzymes were then added and the mixture incubated at 37 °C for 16 h. In this procedure the phosphodiester bond of NADH is cleaved by the phosphodiesterase enzyme, then the alkaline phosphatase, which works optimally at basic pH, removes the phosphate group leading to the formation of NRH. NRH was then purified using HPLC and the sample concentrated by freeze drying.
The product NRH is a heat-labile compound, therefore it is necessary to keep it in an ice bath during the experiments. This sensitivity limits the flexibility of the NRH purification as well. For example, using freeze drying to remove the water is required, since it is difficult to evaporate water using a rotary evaporator without applying heat. This difficulty in synthesising and handling the NRH, encouraged the use of other NRH analogues, such as 1-(2-amino-2-oxoethyl)-1,4-dihydropyridine-3-carboxamide(1-carbamoylmethyl-1,4-dihydronicotinamide) (EP0152R) (Figure 67), instead of NRH.\(^\text{41}\)

![Scheme 35. Synthesis of NRH](image)

**Figure 67.** The structure of NQO2 co-factor EP0152R.
EP0152R is stable at room temperature and structurally very similar to NRH, where both of them are nicotinamide derivatives. Therefore, both of them have the ability to donate two electrons to N5 of FAD\textsuperscript{41} (Scheme 36).

\textbf{Scheme 36}. NRH and EPR are able to reduce FAD by donating two electrons to N5.

3.1.4 Synthesis of 5-(2-(dimethylamino)ethylamino)-8-methoxy-6H-imidazol[4,5,1-de]acridin-6-one N-oxide (compound 71).

Compound 71 (Figure 68) is one of the acridine derivatives that has been synthesised by the Stratford group,\textsuperscript{44} This compound showed good activity as an NQO2 inhibitor at low nanomolar concentration. More compound was required for further biological testing, therefore compound 71 was resynthesized here, as described by Whitehead and co-workers.\textsuperscript{44}

\textbf{Figure 68. Structure of NQO2 inhibitor 71}

2,6-Dichlorobenzoic acid was converted to 2,6-dichloro-3-nitrobenzoic acid using H\textsubscript{2}SO\textsubscript{4} and HNO\textsubscript{3} at 50 °C, which then undergoes an electrophilic aromatic
substitution reaction with \( p \)-anisidine to give intermediate (A), which then cyclized using phosphorus oxychloride to give B. \( N,N \)-Dimethylethlenediamine was used as a source of the amino alkyl side chain to give C, which was then reduced to D using Raney Ni. The two amino group of D formed the imidazole ring of E by condensation with formic acid (derived from trimethyl orthoformate). Compound E was then stirred with m-CPBA to generate the N-oxide moiety to give 71.

**Scheme 37.** Pathway for the synthesis of 71: i) \( \text{H}_2\text{SO}_4, \text{HNO}_3, 70 \, ^\circ\text{C}, 1 \, \text{h} \); ii) \( p \)-anisidine, 24 h; iii) \( \text{POCl}_3, 1,2\)-dichloroethane, 1 h, 90 \( ^\circ\text{C} \); iv) \( N,N \)-dimethylethlenediamine, DMF, reflux, 4 h; v) Raney Ni, THF, 1 h, rt; vi) trimethyl orthoformate, reflux, 24 h; vii) m-CPBA,CHCl\(_3\), 18 h.\(^{44}\)
3.2. Enzyme assay

3.2.1 Inhibition of recombinant human NQO2 enzyme activity

The ability and potency of the synthesized compounds to inhibit the NQO2 enzyme were measured spectrophotometrically using 2,6-dichlorophenolindophenol (DCPIP) as a substrate of NQO2. DCPIP is blue in the oxidized form, which turns colourless once it is reduced (Scheme 38). If the tested compound inhibits NQO2, this will affect the ability of the enzyme to reduce the DCPIP. Since the rate of DCPIP colour change correlates to the rate of NQO2 activity, by measuring the rate of colour change for different inhibitor concentrations, the potency of the inhibitor can be determined.

Scheme 38. Reduction of DCPIP by NQO2

Resveratrol, the classical inhibitor of NQO2, was used as the positive control in this assay. The IC\textsubscript{50} of resveratrol and other inhibitors (compounds 8-27, 32, 34 and 6a1) were determined. The IC\textsubscript{50} value represents the concentration of a drug that is required to inhibit 50\% of the enzyme activity \textit{in vitro}. IC\textsubscript{50} values were determined using non-linear curve fitting in the GraphPad Prism software. The curves were obtained by plotting enzyme activity as a percent of the control versus the log concentration of the inhibitor used (representative curves are shown in appendix I). IC\textsubscript{50} values were determined as 50\% reduction in the enzyme activity compared to control (100\%).

3.2.1.1 4-Hydrazonequinoline derivatives as NQO2 Inhibitors

The IC\textsubscript{50} values of the hydrazone quinoline derivatives from scaffolds 1 and 3, (with methyl or phenyl substitution at position 2, respectively) have been calculated.
Table 11 shows the IC\textsubscript{50} values for resveratrol and the 4-hydrazone quinoline derivatives. The values show that the hydrazone quinolines are good NQO2 inhibitors with IC\textsubscript{50}'s less than 100 nM, compared to resveratrol (IC\textsubscript{50} 900 nM). This means that these inhibitors are nine-fold more potent than resveratrol.

**Table 11.** IC\textsubscript{50} values of different 4-hydrazone quinoline derivatives as NQO2 inhibitors.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R\textsubscript{1}</th>
<th>R\textsubscript{2}</th>
<th>IC\textsubscript{50} (nM) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>-CH\textsubscript{3}</td>
<td>4-Fluorophenyl</td>
<td>103 ± 16.3</td>
</tr>
<tr>
<td>9</td>
<td>-CH\textsubscript{3}</td>
<td>Phenyl</td>
<td>519 ± 14.5</td>
</tr>
<tr>
<td>10</td>
<td>-CH\textsubscript{3}</td>
<td>4-Imidazoyl</td>
<td>71 ± 14.9</td>
</tr>
<tr>
<td>11</td>
<td>-CH\textsubscript{3}</td>
<td>4-Hydroxyphenyl</td>
<td>55 ± 6.6</td>
</tr>
<tr>
<td>12</td>
<td>-CH\textsubscript{3}</td>
<td>3-Pyridinyl</td>
<td>10 ± 0.7</td>
</tr>
<tr>
<td>13</td>
<td>-CH\textsubscript{3}</td>
<td>Benzyl</td>
<td>15 ± 4.4</td>
</tr>
<tr>
<td>14</td>
<td>-CH\textsubscript{3}</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>13 ± 3.1</td>
</tr>
<tr>
<td>15</td>
<td>-CH\textsubscript{3}</td>
<td>2-Nitrofuranyl</td>
<td>18 ± 1.2</td>
</tr>
<tr>
<td>16</td>
<td>-CH\textsubscript{3}</td>
<td>4-Benzoic acid</td>
<td>64 ± 5.9</td>
</tr>
<tr>
<td>17</td>
<td>-CH\textsubscript{3}</td>
<td>4-Nitrophenyl</td>
<td>83 ± 8.0</td>
</tr>
<tr>
<td>18</td>
<td>-CH\textsubscript{3}</td>
<td>3,5-Dihydroxyphenyl</td>
<td>46 ± 8.6</td>
</tr>
<tr>
<td>19</td>
<td>-CH\textsubscript{3}</td>
<td>3-Hydroxyphenyl</td>
<td>137 ± 15.9</td>
</tr>
<tr>
<td>20</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>3-Pyridinyl</td>
<td>372 ± 109.4</td>
</tr>
<tr>
<td>21</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>4-N,N-dimethylaniline</td>
<td>Inactive</td>
</tr>
<tr>
<td>22</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>4-Nitrophenyl</td>
<td>1420 ± 204</td>
</tr>
<tr>
<td>23</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>Benzyl</td>
<td>125 ± 2.8</td>
</tr>
<tr>
<td>24</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>2-Hydroxy-3-methoxyphenyl</td>
<td>595 ± 178</td>
</tr>
<tr>
<td>25</td>
<td>-C\textsubscript{6}H\textsubscript{5}</td>
<td>4-Imidazoyl</td>
<td>90 ± 10.7</td>
</tr>
<tr>
<td><strong>Resveratrol</strong></td>
<td></td>
<td></td>
<td>900</td>
</tr>
</tbody>
</table>
Compounds 8-19, with a methyl group at position 2, showed lower IC₅₀ values (higher activity) than the corresponding compounds 20-25 with a phenyl group at position 2. For example, compound 14 is 45-fold more potent than its corresponding phenyl 24. This variation in potency could be explained by different binding mode of both scaffolds in the NQO2 active site, as explained in Section 2.5.1.

Substituents on the aromatic ring have a noticeable effect on the activity. The para-fluorine substitution in compound 8 makes the compound more potent by five-folds compared to unsubstituted-phenyl analogue 9. In the same manner, compound 11 with a para-hydroxyl substituent is ten-fold more potent than unsubstituted-phenyl analogue 9. However, the fluorine substituent increases the hydrophobic character of the molecule slightly, but this is not consistent with the increasing activity shown by the hydroxyl and other hydrophilic groups as in compounds 11, 14, 16, 18 and 19. This allows for the conclusion that introducing a substitution has the ability to make hydrogen bonds (such as F, OH) with the amino acids residues or with the water molecules in the active site that may enhance the activity of the inhibitors.

In addition, changing the aromatic ring at the side chain also has an effect on the activity. Comparing the activity of the phenyl analogue 9 to the activity of imidazole-analogue 10 and pyridine-analogue 12, leads to the conclusion that changing the phenyl ring to other heterocycle rings may enhance the activity of these inhibitors.

4-Hydrazinequinoline (compound 38) (Figure 69), the nucleus of the hydrazone quinoline derivatives has been tested against the recombinant NQO2 as well. The IC₅₀ of this nucleus was 2047 ± 132 nM. The big difference between the activity of the previous 4-hydrazinequinoline derivatives and the lower activity of the nucleus confirmed that the activity is attributed to the hydrazone functional group in the compounds.

Figure 69. Structure of 4-hydrazinequinoline
3.2.1.2 4-Hydrazidequinoline as NQO2 inhibitors

The two hydrazide derivatives (compounds 26 and 27) were tested and the results are shown in Table (2). The IC\textsubscript{50} values for these two hydrazide derivatives showed either the same or a noticeable decrease in activity compared to the corresponding hydrazonequinoline derivatives 8 and 9. However, compound 26 still has double the activity of resveratrol. These derivatives still have activity at high nanomolar concentrations, compared to other hydrazone derivatives (8, 10-19, 23, 25), which have activity at low nanomolar concentrations. This may give an indication that changing the hydrazine to the hydrazide moiety leads to decrease of the activity. The low % yield of the synthesised derivatives does not encourage the synthesis of further hydrazides to confirm such a conclusion.

Table 12. IC\textsubscript{50} values of different 4-hydrazide quinoline derivatives (26, 27) versus hydrazone derivatives (8, 9) as NQO2 inhibitors

<table>
<thead>
<tr>
<th>Entry</th>
<th>(IC\textsubscript{50} nM)</th>
<th>Entry</th>
<th>(IC\textsubscript{50} nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recombinant NQO2</td>
<td></td>
<td>Recombinant NQO2</td>
</tr>
<tr>
<td>26</td>
<td>472 ± 23.3</td>
<td>9</td>
<td>519 ± 14.5</td>
</tr>
<tr>
<td>27</td>
<td>2906 ± 433</td>
<td>8</td>
<td>103 ± 16.3</td>
</tr>
</tbody>
</table>

3.2.1.3. 4-Carboxamide derivatives as NQO2 inhibitors

4-Carboxamide 32 was inactive against NQO2 at a maximum concentration of 10 \textmu M. Figure 70 shows no dose-response curve with compound 32, with the nanomolar inhibitor concentration on the x-axis versus the percent activity of the NQO2 enzyme on the y-axis. This results support the conclusion that changing the hydrazone or hydrazide group to the amide moiety leads to the loss of activity against NQO2.
3.2.1.4 Summary of the NQO2 Inhibitory Activity

Figure 71) summarizes the synthesised scaffolds and the ranges of the IC\textsubscript{50} values obtained from the enzyme assay. The 4-hydrazonequinoline derivatives with a methyl group at position 2 (scaffold 1) were the most active compounds. Some of the 4-hydrazonequinoline derivatives with a phenyl ring at position 2 (scaffold 3) were also active, however some compounds belonging to this scaffold were poorly active, which may related to poor solubility or the large molecular size. The 4-hydrazide quinoline derivatives (scaffold 7) were less active than the hydrazonequinoline derivatives. Finally, the activity was completely lost with the 4-carboxyamide derivative 32. These differences in the IC\textsubscript{50} values indicate that the nature of the functional group at position 4 is important and essential to give activity.

Figure 70. Dose-response curve generated by GraphPad Prism software for the % activity of compound 32 against NQO2.
Figure 71. Comparisons between the effect of changing the main functional group at position 4 on the activity of the compounds against NQO2.

Scheme 39 shows an analysis of the structure of the synthesised derivatives and the proposed importance and role of each functional group to the activity of the compound and its role in binding to the NQO2 active site.
Scheme 39. Analysis of the structure activity relationship of the synthesised molecules and the importance of each part of the structure to the activity.

3.2.2 Inhibition of recombinant human NQO1 enzyme activity
Selected compounds were assayed for their inhibitory potency against NQO1. The enzyme assay method is described in Section 5.2.2. Dicoumarol, the known inhibitor of NQO1, was used as a control and the IC₅₀ values are tabulated in Table 13.
### Table 13. The inhibitory potency of 4-hydrazine quinoline derivatives (10-16, 20, 24) against NQO1.

<table>
<thead>
<tr>
<th>Entry</th>
<th>(IC$_{50}$ µM) Recombinant NQO1</th>
<th>(IC$_{50}$ µM) Recombinant NQO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicoumarol</td>
<td>0.0044 ± 0.00099</td>
<td>Inactive</td>
</tr>
<tr>
<td>10</td>
<td>38.7 ± 5.2</td>
<td>0.071 ± 0.014</td>
</tr>
<tr>
<td>11</td>
<td>133.9 ± 43.6</td>
<td>0.055 ± 0.006</td>
</tr>
<tr>
<td>13</td>
<td>Inactive</td>
<td>0.015 ± 0.004</td>
</tr>
<tr>
<td>14</td>
<td>73.4 ± 48.7</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>16</td>
<td>Inactive</td>
<td>0.064 ± 0.005</td>
</tr>
<tr>
<td>20</td>
<td>Inactive</td>
<td>0.372 ± 0.109</td>
</tr>
<tr>
<td>24</td>
<td>Inactive</td>
<td>0.595 ± 0.178</td>
</tr>
<tr>
<td>25</td>
<td>0.425 ± 0.09</td>
<td>0.090 ± 0.01</td>
</tr>
<tr>
<td>27</td>
<td>Inactive</td>
<td>2.9 ± 0.4</td>
</tr>
</tbody>
</table>

Compounds 10, 11 and 14 showed very weak activity toward NQO1, whereas they were 500, 2400 and 5500-fold, respectively, more active towards NQO2. This means that the 4-hydrazine quinoline derivatives are NQO2 selective if used in nanomolar to low micromolar concentrations. Compounds 13, 16, 20, 24 and 27 are completely inactive towards NQO1, which means that they are NOQ2 selective. Compound 25 behave in a different manner, as it is showed activity towards both enzymes. Compound 25 is still 5-fold more active towards NQO2, but with a significant activity towards NQO1: This can be rationalised by considering the different binding mode of this compound in the NQO2 active site as explained in Section 2.5.1. The 4-imidazoyl ring of the side chain of compound 25 may also affect the polarity (hydrophilicity) and the compound’s ability to interact with the amino acid residue in the active site. As explained in Section 2.5.1, this difference may help compound 25 bind strongly to the NQO1 as well as the NQO2 active sites.
4. Malaria

4.1 Introduction
Malaria is a mosquito borne disease caused by the malaria parasite, which belongs to the genus *Plasmodium*. It is one of the most spread infectious diseases in the world. 300 to 500 million cases are diagnosed each year and two million patients die due to the lack of proper treatment. Malaria is present mainly in Africa, however the multidrug resistant variant of this disease is spreading to other parts of the world.

There are five known species of malaria parasite which cause disease in humans; *P. falciparum*, *P. malaria*, *P. knowlesi*, *P. vivax*, and *P. ovale*. *P. falciparum* is the most common species and causes the most deaths amongst the malaria patients.

The malaria parasite’s life cycle (*Figure 72*) starts by a bite from infected female *Anopheles* mosquitos, which inoculates its sporozoites containing saliva into the human bloodstream. Sporozoites infect the liver hepatocytes forming schizonts, which are incubated for five to fifteen days before they rupture and release the daughter’s merozoits into blood stream. In the blood the merozoites start invading erythrocytes in which they either mature to trophozoites then schizonts able to release merozoites again or differentiate into female and male gametocytes. where they become infectious to female *Anopheles* mosquitos.
4.2 Malaria treatment

The drug of choice and the treatment regimen for a malaria infection depends on the infection severity and on the parasite sensitivity as explained below:

1-Drug sensitive parasite:

In the areas where the parasite is still sensitive to drugs chloroquine, mefloquine, and a combination of atovaquone and proguanil hydrochloride can be used with good results\(^\text{110}\) (Figure 73).

2-Uncomplicated cases:

Oral treatment is usually used to treat uncomplicated cases; the drug of choice for \(P.falciparum\) is artemisinin in combination with other antimalarial drugs. These combinations are called artemisinin-based combination therapy (ACT) and are
usually effective for 90% uncomplicated cases. Examples of antimalarial drugs that are used in this combination are amodiaquine, lumefantrine, mefloquine, sulfadoxine or pyrimethamine. The second line of treatment is the artesunate or quinone in combination with doxycycline, tetracycline or clindamycin.110

3-Pregnancy:
Clindamycin and quinine are drugs of choice during the 1\textsuperscript{st} trimester of pregnancy, while the ACT could be used in the 2\textsuperscript{nd} and 3\textsuperscript{rd} trimester.110

4-Severe cases of malaria:
For severe cases, intravenous artesunate is used, or intravenous quinone if artesunate is not available\textsuperscript{110} (Figure 73).

![Structures of antimalarial drugs](image_url)

\textbf{Figure 73.} Structures of antimalarial drugs.

Drug resistance to anti-malarial treatment is becoming a growing problem, especially to the cheap and widely used drugs like chloroquine and sulfadoxine-pyrimethamine. Meanwhile, treatment of resistant malaria strains based on artemisinin is considered as an expensive approach. In addition, some of the malaria strains which are resistant even to artemisinin may be untreatable. Therefore, searching for new drug classes...
and/or discovering new targets and mechanisms of action are urgently required to stop the spread of malaria worldwide.\textsuperscript{111-113}

### 4.3 Malaria and NQO2

The role of NQO2 in malaria and cancer has been proposed after the finding that NQO2 is a molecular target for the antimalarial drugs chloroquine and primaquine and the anti-leukaemia drug imatinib.\textsuperscript{45}

The malaria parasite \textit{p. falciparum} has an enzyme called \textit{p. falciparum} NDH2 (\textit{pf}NDH2), which is an alternative enzyme to NADH: ubiquinone oxidoreductases found in other eukaryotes.\textsuperscript{114} The \textit{pf}NDH2 enzyme is functionally very similar to NQO2 as shown in (Table 14).

**Table 14.** Comparison between NQO2 and \textit{pf}NDH2.\textsuperscript{114}

<table>
<thead>
<tr>
<th></th>
<th>NQO2</th>
<th>\textit{pf}NDH2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classification</strong></td>
<td>Flavoprotein</td>
<td>Flavoprotein</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>52 k Da</td>
<td>51.6 kDa</td>
</tr>
<tr>
<td><strong>Quaternary structure</strong></td>
<td>Homodimer</td>
<td>Single subunit</td>
</tr>
<tr>
<td><strong>Cofactor (electron acceptor)</strong></td>
<td>Isoalloxazine ring binds to FAD</td>
<td>Isoalloxazine ring binds to FAD or FMN</td>
</tr>
<tr>
<td><strong>Mechanism of action</strong></td>
<td>Ping-Pong</td>
<td>Ping-Pong</td>
</tr>
<tr>
<td><strong>Substrate</strong></td>
<td>Ubiquinone</td>
<td>Ubiquinone</td>
</tr>
<tr>
<td><strong>Co-substrate</strong></td>
<td>NRH</td>
<td>NADH</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td>Cytoplasm</td>
<td>Mitochondria</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Catalyses reduction of Quinones</td>
<td>Electron donor to electron transport chain (ETC)</td>
</tr>
</tbody>
</table>

The functional similarity between \textit{pf}NDH2 and NQO2, in addition to the fact that NQO2 is a molecular target for the antimalarial drugs chloroquine and primaquine, motivated testing of available NQO2 inhibitors in a malaria model as \textit{pf}NDH2 inhibitors.\textsuperscript{114}
4.4 *P. falciparum* type II NADH: quinone oxidoreductase (*pfNDH2*)

*pfNDH2* belongs to type II NADH: quinone oxidoreductases, which are found in bacteria, plants, and lower eukaryotes. Type II NADH: quinone oxidoreductases do not contain a transmembrane domain, therefore it does not pump protons across the membrane and instead it is considered as a source of NADPH.

*pfNDH2* is a single subunit flavoprotein enzyme with a molecular weight of 52 k. *pfNDH2* utilizes the ping-pong mechanism to catalyse the oxidation of NADH to NAD$^+$ and return back to its oxidized form using ubiquinone (CoQ$_n$) as a substrate. Ubiquinone, also known as coenzyme Q$_{10}$, is the only identified natural substrate for NQO2. It can also use other electron acceptors like menadione and DCPIP. It is considered as an electron donor to the parasite electron transport chain (ETC), which is needed to generate mitochondrial electrochemical membrane potential, an essential function for the parasite life cycle. Therefore, targeting *pfNDH2* enzyme is considered as an attractive approach in malaria chemotherapy.

To date, there is no X-ray crystal structure available for *pfNDH2*. Fisher and co-workers hypothesised a tertiary structure model, which is currently under testing. This model did not identify the natural enzyme cofactor as either FAD or FMN, but it was predicted that the isoalloxazine ring and the nicotinamide of the NADH are parallel to the cofactor (FAD or FMN) with a 3 Å separation, which aids in rapid electron transfer.

4.5 *pfNDH2* inhibitors

A limited number of *pfNDH2* inhibitors have been reported in the literature. Instead, inhibitors of the *p. falciparum* growth as a whole parasite were reported. Diamidine is a good example of these inhibitors, which will be explained in the next section.

Stocks and co-workers reported specific *pfNDH2* inhibitors. Hydroxy-2-dodecyl-4-(1H)-quinolone (HDQ) was identified as a lead compound, and with the aid of more than one cheminformatics method, quinolone was identified to be the core of the HDQ analogues. Some derivatives were developed which showed activity against *pfNDH2* at low nano-molar concentrations (Figure 74).
Figure 74. Structures of some quinolone derivatives as pfNDH2 inhibitors and their IC$_{50}$ values.

4.6 Diamidines as potential antimalarial drugs

Pentamidine (Figure 75) has been used for the clinical treatment of protozoal infections including those caused by the *p. falciparum* parasite. It has been found that pentamidine bind to the toxic ferriprotophyrin IX, which is produced by the malarial parasite during haemoglobin digestion. This binding prevents the polymerisation process of this toxic substance to the nontoxic haemozoin, a vital process in the parasite life cycle. Pentamidine has significant side effects such as renal toxicity and cardiotoxicity, in addition to its poor oral bioavailability.

Figure 75 shows other aromatic diamidines that were inspired by pentamidine, which exerts a broad-spectrum antimicrobial activity. These compounds were highly permeable to *p.falciparum* infected erythrocytes, however they are impermeable to human erythrocytes. These findings demonstrated that the aromatic diamidine scaffold was a promising class of antimalarial drug, acting on a novel target. Diamidine toxicity and poor oral bioavailability can be modified by different substitution.
The exact mechanism of action of the diamidines is still unknown, but several potential mechanisms have been proposed by investigation of their fluorescence properties to study their subcellular localization. Pentamidine was found to be localized in the food vacuole of *P. falciparum* where it inhibits the formation of non-toxic haemozoin by binding to the toxic haem.

Different pentamidine analogues were synthesised (e.g. furan-amidine, pafuran-amidine) with antimicrobial activity, but without the known side effects of pentamidine. DB75 (Figure 76) is a structural analogue of pentamidine which belongs to the furan-amidine class and possesses activity against different parasites including *Trypanosoma rhode-sience*, *Giardia lamblia*, and *p. falciparum*. DB75 has poor oral bioavailability, therefore its orally active prodrug DB289 was developed, which showed 96% activity against *p. falciparum* in a clinical trial.

Figure 75. Structures of aromatic diamidine derivatives which exert antimicrobial activity

Figure 76. The structure of the furan-amidine DB75 and its prodrug DB289.
The nucleus is the cellular target of the furan-amidines, where they interfere with DNA-interacting enzymes such as topoisomerase. This was confirmed by the finding that DB75 accumulates in the DNA containing organelles of *Giardia lamblia* where it can inhibit topoisomerase II. In *Saccharomyces cerevisiae* and trypanosomes, DB75 inhibits the cellular respiration in mitochondria, and accumulates in acidocalcisomes (rounded organelles rich in calcium) of trypanosomes and inhibit calcium homeostasis. These findings can be generalized to *P. falciparum* since it possesses the same processes as other parasites mentioned above. Purfield and co-workers proved that DB75 localizes in the nucleus of the *P. falciparum* parasite and targets nucleic acid synthesis resulting in slowing down parasite maturation at the trophozoite stage (early stage of the parasite’s life). These findings potentially can be applied to other dicationic diamidines compounds.

### 4.7 Previous work

Different non-symmetrical furan-amidine inhibitors of NQO2 recently synthesized by Al-Nabulsi were screened against *P. falciparum* parasite growth, which showed promising results. Figure 77 shows the structures of the non-symmetrical furan-amidine derivatives screened against *P. falciparum*, together with the IC$_{50}$ values.

![IC$_{50}$ values](image)

**Figure 77.** Examples of tested non-symmetrical furan-amidine derivatives and their IC$_{50}$ values.

In cancer cells symmetrical furan-amidine compounds form hydrogen bonds with DNA nucleotides, and at the same time these compounds were found to be localized in the nucleus of *P. falciparum*. A non-symmetrical furan-amidine was
shown not to bind to DNA, which means that non-symmetrical furan-amidine may target other cellular organelles rather than nucleus.

**4.8 Aims of the malaria study**

The aims of this malaria study are to test the hypothesis that NQO2 inhibitors are able to inhibit the malarial parasite growth through the inhibition of the pfNDH2 enzyme. In addition, it is important to establish whether the non-symmetrical furan-amidines do not localize in the nucleus, which would support new mechanism of action not involving interaction with DNA. To test such a hypothesis further non-symmetrical furan-amidine analogues were designed and synthesized. In these analogues a para-fluoro substituent was introduced as the meta-fluoro analogues gave a good biological activity in the previous screening (compound 39), as well as the known importance of the florin atom to the biological activity in general. In addition, changing the furan ring to other bioisosteric heterocycle rings, such as oxazole, pyrrole and imidazole, may enhance activity as well as improving solubility.

In order to further establish the structure-activity relationship of non-symmetrical furan-amidines as potential antimalarial drugs, new simplified furan-amidine analogues have been synthesised and tested. The effect of nitrile, amide, imidate, and amidine moieties on activity, as well as, the presence of one phenyl ring instead of two phenyl rings was investigated.

**4.9 Results and discussion**

**4.9.1 Chemistry**

The new analogues focused on investigating the effect of amidine, imidate, amide and nitrile functional groups on the activity, as well as, simplifying the compounds to one phenyl ring instead of two (Figure 78).
Chapter 4. Malaria

**Figure 78.** Structures of the designed simplified furan derivatives (nitrile 43, amide 44, imidate 45 and amidine 46).

The synthesise of the simplified furan derivatives (43-46) are shown in **Scheme 40**, the key intermediate being 4-(furan-2-yl) benzonitrile (43), the synthesis of which will now be discussed.

**Scheme 40.** Pathways for the synthesis of the nitrile (43), amide (44), imidate (45) and amidine (46) derivatives.

**4.9.1.1 Synthesis of 4-(furan-2-yl)benzonitrile (43)**

Compound 43 has been synthesised using the Suzuki coupling method.\(^{123}\) Recently, this reaction has become one of the most useful methods for the synthesis of C-C bonds.\(^{124}\) Under mild conditions, palladium(0) catalyses the reaction between arylboronic acid and aryl or alkenyl halides, which resulted in carbon-carbon bond formation in high yield (**Scheme 41**).\(^{124}\) The reaction starts by the oxidation of Pd(0)
to Pd(II), with its concomitant insertion into alkyl-halide bond. Potassium bicarbonate acts as a base in this reaction and has a main role, since it activates the boron atom by increasing the polarization of the organic ligand and makes the transmetallation step easier.

Scheme 41. Mechanism of the Suzuki coupling.

Figure 79) shows the $^1$H NMR spectrum for the nitrile (43) that confirms the C-C bond formation between the furan ring of the boronic acid and 4-bromobenzonitrile.
4.9.1.2 Synthesis of 4-(furan-2-yl)benzamide (44)

The synthesis of 4-(furan-2-yl)benzamide (44) takes place using potassium tert-butoxide as nucleophile in a hydration reaction of 4-(furan-2-yl) benzonitrile (43) under mild condition, to give the product in a good yield. Scheme 42 showed the proposed mechanism for the potassium tert-butoxide as oxygen nucleophile source, where it attacks the electron deficient carbon of the nitrile moiety, with the subsequent release of 2-methylpropene.

Figure 79. The $^1$H NMR spectrum (CDCl$_3$) of nitrile (43).
Scheme 42. Mechanism for the synthesis of amide (44).

Figure 80) shows the $^1$H NMR spectrum of amide (44). The amide moiety protons appeared as two broad singlets at 7.33 and 8.04 ppm.

Figure 80. The $^1$H NMR spectrum (d$_6$-DMSO) of amide (44).

4.8.1.3 Synthesis of ethyl 4-(furan-2-yl)benzimidate (45)
Ethyl 4-(furan-2-yl)benzimidate (45) was prepared from the corresponding nitrile derivative (43) using ethanol as solvent and reagent in a nucleophilic addition reaction (Scheme 43).
Scheme 43. Mechanism for the synthesis of compound (45)

Figure 81 shows the $^1$H NMR spectrum of compound 45 where the imidate formation is supported by the appearance of protons for the ethyl group: CH$_3$ appeared as a triplet at 1.64 ppm and CH$_2$ appeared as a quartet at 4.65 ppm.

Figure 81. The $^1$H NMR spectrum (CDCl$_3$) of compound (45)
4.9.1.4 Synthesis of 4-(furan-2-yl)benzimidamide (46)

The simplified amidine derivative (46) was synthesized directly from the imidate (45) (Scheme 40). Ammonium acetate was added to ethyl 4-(furan-2-yl)benzimidate (45) and stirred at room temperature for 1-3 days.\textsuperscript{127}

\begin{equation}
\text{NH}_4\text{OAc} \rightleftharpoons \text{NH}_3 + \text{NH}_4^+ + \text{NH}_2\text{OAc} \rightleftharpoons \text{NH}_3 + \text{NH}_2\text{OAc}
\end{equation}

Scheme 44. Mechanism of the synthesis of 4-(furan-2-yl)benzimidamide (46) using ammonium acetate.

Figure 82 shows the $^1$H NMR spectrum of compound (46) as acetate salt. The methyl group of the acetate moiety appeared at 1.72 ppm and two protons of the amide group appeared at 10.12 ppm.

Figure 82. The $^1$H NMR spectrum (d$_6$-DMSO) of compound (46)
4.9.1.5 Synthesis of non-symmetrical oxazole and furanamidine

As mentioned in section 4.7, many non-symmetrical oxazole and furanamidines have been synthesised by Al-Nabulsi. Here some of these derivatives, in addition to some novel ones, have been resynthesized as they were needed for the biological tests with the malaria parasite. The chemistry of the syntheses of the oxazole and furan-amidine derivatives is presented here in Scheme 45 and Scheme 46 and the details are reported in the experimental section.

Scheme 45. Synthetic pathway for the nitrile, imidate and amidine furan derivatives.

Scheme 46. Synthetic pathway for the nitrile, imidate and amidine oxazole derivatives.
4.9.2 Antimalarial activity

The biological evaluation of the compounds was conducted by Dr Ilaria Russo (FLS, University of Manchester). The first set of non-symmetrical furan-amidines killed the parasite at low nanomolar concentrations 20-60 nM.\textsuperscript{64} These findings encouraged further investigation which showed that these compounds inhibit all the asexual stages and prevented sexual development. These compounds were also tested for cellular accessibility and cellular localization: the results showed a preferential accumulation of the furan-amidines in the infected cells and a selective localization in the mitochondria. For human cell toxicity on four breast cancer cell lines, MDA-MB 468, MDA-MB 231, MCF-7 and T47D, the compounds were only toxic at concentrations 100-1400 times greater than the plasmodium growth inhibition concentration. The simplified furan-amidines (43-46) have been tested in order to understand the importance of the second phenyl ring on anti-malarial activity (Table 15). Comparison of these results to the activity of compounds (39-42) in section (4.7) showed that the simplified furan compounds were 1000 fold more active. The conclusion is that the second phenyl ring has a crucial role in the activity of the inhibitors. This is consistent with the hypothesis that these compounds inhibit pfNDH2, which is similar to NQO2. This active site contains FAD or FMN, which means that the active sites are relatively large and lipophilic, giving increased binding of the furan-amidines with two phenyl ring when compared with the simplified mono-phenyl analogues.

Table 15. The IC\textsubscript{50} of simplified furan analogues for *Plasmodium falciparum* growth inhibition. (Data recorded by Dr Ilaria Russo).

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>IC\textsubscript{50} µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>Nitrile</td>
<td>Inactive</td>
</tr>
<tr>
<td>44</td>
<td>Amide</td>
<td>Inactive</td>
</tr>
<tr>
<td>45</td>
<td>Imidate</td>
<td>62</td>
</tr>
<tr>
<td>46</td>
<td>Amidine</td>
<td>24</td>
</tr>
</tbody>
</table>
Furan and oxazole derivatives (47-56) have also been tested and the IC₅₀ values of these amidines and oxazole derivatives are shown in Table 16.

It is clear that the furanamidine have better activity than the corresponding oxazole derivative. For example furan-amidine 49 is active with IC₅₀ value of 10 nM, whereas the oxazole analogues 56 is over 1000-fold less active with an IC₅₀ value of 16 µM, support the hypothesis that the furan ring is essential to the activity. At the same time, the activity of the amidine containing compound was higher compared to the imidate or nitrile, since amidine 49 is active at 10 nM, whereas the imidate 47 had a higher IC₅₀ value of 31µM and the nitrile was inactive with an IC₅₀ greater than 200 µM.

**Table 16.** The IC₅₀ values of furan and oxazole derivatives for *plasmodium falciparum* growth inhibition. (Data recorded by Dr Ilaria Russo).

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>C</td>
<td>F</td>
<td>Imidate</td>
<td>31.9</td>
</tr>
<tr>
<td>48</td>
<td>C</td>
<td>Ethyl</td>
<td>Imidate</td>
<td>26.5</td>
</tr>
<tr>
<td>49</td>
<td>C</td>
<td>F</td>
<td>Amidine</td>
<td>0.01</td>
</tr>
<tr>
<td>50</td>
<td>C</td>
<td>Ethyl</td>
<td>Amidine</td>
<td>0.024</td>
</tr>
<tr>
<td>51</td>
<td>N</td>
<td>H</td>
<td>Nitrile</td>
<td>&gt;200</td>
</tr>
<tr>
<td>52</td>
<td>N</td>
<td>F</td>
<td>Nitrile</td>
<td>Not tested</td>
</tr>
<tr>
<td>53</td>
<td>N</td>
<td>H</td>
<td>Amidoxime</td>
<td>23.5</td>
</tr>
<tr>
<td>54</td>
<td>N</td>
<td>F</td>
<td>Amidoxime</td>
<td>189</td>
</tr>
<tr>
<td>55</td>
<td>N</td>
<td>H</td>
<td>Amidine</td>
<td>0.313</td>
</tr>
<tr>
<td>56</td>
<td>N</td>
<td>F</td>
<td>Amidine</td>
<td>16</td>
</tr>
</tbody>
</table>

Amidine 49 was chosen for further testing on mice (Figure 83). The % parasitemia (the percentage of infected erythrocyte in blood smears) has been measured over 10 days. Nine mice were intraperitoneally injected with a dose of 5 mg/kg and 25 mg/kg one dose per day, for 4 days. A complete cure was observed at day ten, without any sign of toxicity during the experiment time.
Figure 83: The % parasitemia in mice treated with amidine 49 over 10 days. (Data recorded by Dr Ilaria Russo)

These results confirmed the non-symmetrical furanamidine as a strong antimalarial candidate. Further investigation and evaluation is ongoing to enhance the activity, develop the SAR, and enhance the solubility of these compounds. In addition, more biological evaluation is in progress to establish the exact mechanism for this class of compounds.

4.9.3 Malaria and 4-aminoquinoline derivatives

Primaquine, chloroquine and other aminochinolines are considered to be the main drugs for the treatment of malaria during the last four decades.\textsuperscript{108} Many novel aminochinoline derivatives have been reported in order to overcome the resistance of the current malarial treatments.\textsuperscript{86,108,128}

The high activity against NQO2 of the 4-aminoquinoline derivatives synthesised in this thesis, merits their testing also as antimalarial agents. Table 17 shows the IC\textsubscript{50} values of selected 4-hydrazonequinolines in inhibiting the malaria parasite growth. These compounds showed promising activity in the low nanomolar range, with the best quinoline 24 having an IC\textsubscript{50} of 1 nM. Further investigation and testing is ongoing.
Table 17. The IC<sub>50</sub> values of selected 4-hydrazonequinoline in inhibition malaria parasite growth (for structure see Table 9, section 3.1.1.2). (Data recorded by Dr Ilaria Russo)

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>149</td>
</tr>
<tr>
<td>16</td>
<td>5000</td>
</tr>
<tr>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>24</td>
</tr>
</tbody>
</table>
5. Experimental

5.1. Chemistry

5.1.1 Chemicals and Methods
Chemicals were purchased from Sigma-Aldrich and Fisher Scientific. Solvents and deuterated solvents were purchased from Fisher Scientific. Bruker Avance 300 and 400 spectrometers were used to record $^1$H and $^{13}$C NMR spectra. Chemical shifts are quoted in parts per million (ppm) and referenced to tetramethylsilane (TMS $\delta = 0$). Solvents were evaporated on a Buchi rotavapor R-3 equipped with a Buchi heating bath R-3. Microwave reactions were conducted using a Biotage Initiator synthesiser. Thin layer chromatography (TLC) was performed using silica gel 60 on aluminum sheets with F$_{254}$. The spots were visualized using a UV Mineralight lamp (254/365) UVGL-58. Column chromatography was performed using silica gel with a particle size of 40-63 microns. Infrared spectra were recorded in the solid state using a J.A.S.C.O Fourier transform infrared spectrophotometer. Melting points were measured using a Stuart melting point apparatus SMP10. A BECKMAN DU 7400 spectrophotometer was used to determine enzyme activity. A Grant JB series water bath was used to heat the buffer to 37 °C. Water was evaporated using a Christ alpha1-4 plus freeze dryer equipped with an Edwards vacuum pump. Mass spectrometry was carried out using a Micromass Platform II instrument and Thermo Exactive plus EMR Orbital POS APCI at the School of Chemistry, University of Manchester.

5.1.2 Synthesis

5.1.2.1: 5-(1`-((4`-Methoxyphenyl)amino)ethylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (57).
A solution of Meldrum’s acid (isopropylidene malonate) (1.5 g, 10.40 mmol) in trimethyl orthoacetate (75.0 ml, 589.2 mmol) was heated at reflux for 1 h then cooled to room temperature. P-Methoxyaniline (2.0 g, 16.24 mmol) then was added to the mixture with DMF (2.0 ml) and heated at reflux for another 4 hr. The mixture was then poured into ice and allowed to stir until white crystals formed. The product was collected by suction filtration (2.24 g, 83%). mp: 143-144 °C. IR (cm⁻¹): 1639 (C=C), 1708 (-COO-), 2841 (OMe), 2942 (CH₃), 3069 (C-H); ¹H-NMR (400 MHz, DMSO-d₆) δ ppm: 1.66 (s, 6H, H 7, 8), 2.44 (s, 3H, H 8'), 3.80 (s, 3H, OMe), 7.04 (d, J = 8.0, 2H, H 3',5'), 7.32 (d, J = 8.0, 2H, H 2', 6'), 12.49 (s, 1H, NH). ¹³C-NMR (75 MHz, DMSO-d₆): 26.1 (C 7, 8), 55.4 (OMe), 64.3 (C 2), 84.9 (C 1'), 102.1 (C 5), 114.6 (C 3', 5'), 127.3 (C 2', 6'), 128.6 (C 7'), 158.7 (C 4'), 173.0 (C 4, 6). One Quaternary carbon not observed. MS ES⁻, [M-H, 35%]: 290.1.

5.1.2.2: 5-(((4-Methoxyphenyl)amino)(phenyl)methylene)-2,2-dimethyl-1,3-dioxane-4,6-dione (58).

Compound (58) was prepared by the method described for the synthesis of compound (57), except that trimethyl orthobenzoate was used instead of trimethyl orthoacetate. Compound (58) was isolated as a yellow solid (84%). mp: 185-188 °C. IR (cm⁻¹): 1552 (C=C), 1650 (C=C), 1715(-COO-), 2837 (OMe), 2936 (CH₃), 3059 (C-H); ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 1.75 (s, 6H, H 7, 8), 3.65 (s, 3H, OMe), 6.91 (d, J = 9.0, 2H, H 2', 6'), 6.91 (d, J = 9.0, 2H, H 3', 5'), 7.21-7.32 (m, 5H, Ph), 12.26 (s, 1H, NH); ¹³C-NMR (75 MHz, DMSO-d₆): 26.1 (C 7, 8), 55.2 (OMe), 86.2 (C 5), 102.6 (C 2), 113.8 (C 3',5'), 122.7 (C 9), 126.8 (C 2',6'), 127.9 (C 3'',5''), 128.5 (C 2'',6''), 129.4 (C 4''), 129.6 (C 1',1''), 133.0 (C 4''), 157.5 (C 4, 6), 170.2 (C 9). MS ES⁻, [M-H, 10%]: 354.1.
5.1.2.3: 6-Methoxy-2-methylquinolin-4-ol (59).

![Diagram of 59]

Compound (57) (0.660 g, 2.26 mmol) was dissolved in diphenyl ether (2.0 ml) and heated by microwave radiation at 200 °C for 30 min to give (400 mg, 93%) of compound (59) as a beige powder. mp: 268-270 °C. IR (cm$^{-1}$): 1593 (C=C), 2946 (CH$_3$), 3099 (aromatic C-H); $^1$H-NMR (300 MHz, CDCl$_3$) δ ppm: 2.39 (s, 3H, CH$_3$), 3.88 (s, 3H, OMe), 5.94 (s, 1H, H 3), 7.31 (dd, J = 9.0, J=3.0 1H, H 7), 7.51 (d, J=3.3, 1H, H 5), 7.52 (d, J = 8.4, 1H, H 8), 11.54 (s, 1H, OH). $^{13}$C-NMR (75 MHz, DMSO-d$_6$): 19.3 (Me), 55.2 (OMe), 104.3 (C 5), 107.4 (C 3), 119.4 (C 10), 121.6 (C 7), 125.5 (C 8), 134.7 (C 9), 148.5 (C 6), 155.2 (C 2), 176.0 (C 4). MS ES$^+$, [M+H, 100%]: 190.0.

5.1.2.4: 6-Methoxy-2-phenylquinolin-4-ol (60).

![Diagram of 60]

Compound (60) was prepared by the method described for the synthesis of compound (59) starting from compound (58). Compound (60) was isolated as yellow crystals (84%); mp: >300 °C. IR (cm$^{-1}$): 1581 (C=C), 1591 (C=N), 2833 (OMe), 3070 (C-H); $^1$H-NMR (300 MHz, CDCl$_3$) δ ppm: 3.85 (s, 3H, OMe), 6.31 (s, 1H, H 3), 7.31 (dd, J =9.0, J=3.0 1H, H 7), 7.50 (d, J = 3.0, 1H, H 5), 7.57-7.59 (m, 3H, H 3',4',5'), 7.71 (d, J =9.0, 1H, H 8), 7.81-7.84 (m, 2H, H 2',6'), 11.71 (s, 1H, OH); $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 55.4 (OMe), 103.9 (C 5), 122.2 (C 7), 127.3 (C 2',4',6'), 128.9 (C 3',5'), 130.6 (C 8), 155.7 (C 4). MS ES$^-$, [M-H, 100%]: 250.1.
5.1.2.5: 4-Chloro-6-methoxy-2-methylquinoline (61).

POCl₃ (1 ml, 45 mmol) was added to compound (59) (0.5 g, 2.6 mmol). The reaction mixture was then heated at reflux for 3-4 h at 110 °C. Once the mixture was cooled, it was poured into ice and washed with a saturated solution of NaHCO₃. The resulting solid was collected by vacuum filtration to afford the desired compound (61) as a beige powder (560 mg, 86%), mp 98-101 ºC; IR (cm⁻¹): 1594 (C=C), 1654 (C=N), 2947 (CH₃), 3040 (aromatic C-H); ¹H-NMR (300 MHz, CDCl₃) δ ppm: 2.60 (s, 3H, CH₃), 3.93 (s, 3H, OMe), 7.37 (d, J=3.0, 1H, H 5), 7.45 (dd, J = 9.0, J= 2.8, 1H, H 7), 7.64 (s, 1H, H 3), 7.90 (d, J = 9.0, 1H, H 8); ¹³C-NMR (75 MHz; DMSO-d₆): 23.8 (Me), 55.7 (OMe), 101.6 (C 5), 122.4 (C 3), 123.2 (C 7), 124.9 (C 10), 129.8 (C 8), 156.1 (C 2), 157.9 (C 6). Quaternary C 4 and C 9 not observed. MS ES⁺, [M+H, 100%]: 208.0.

5.1.2.6: 4-Chloro-6-methoxy-2-phenylquinoline (62).

Compound (62) was prepared by the method described for the synthesis of compound (61) starting from compound (60). Compound (62) was isolated as a yellow powder (89%); mp: 120-123 °C. IR (cm⁻¹): 1574 (C=C), 1620 (C=N), 2827 (OMe), 3055(C-H); ¹H-NMR (300 MHz, CDCl₃) δ ppm: 3.92 (s, 3H, OMe), 7.32-7.47 (m, 5H, H 3,7,3’,4’,5’), 7.86 (s, 1H, H 5), 7.99-8.04 (m, 3H, H 8, 2’,6’); ¹³C-NMR (75 MHz; DMSO-d₆): 55.7 (OMe), 101.7 (C 5), 119.3 (C 3), 123.4 (C 10),
126.4 (C 7), 127.3 (C 2,6′), 128.9 (C 3′,5′), 129.5 (C 4′), 131.6 (C 8), 138.6 (C 1′), 141.6 (C 4), 145.0 (C 9), 154.8 (C 2), 158.7 (C 6). MS ES⁺, [M+H, 100%]: 270.0.

5.1.2.7: 4-Hydrazinyl-6-methoxy-2-methylquinoline (38).

Hydrazine monohydrate (1.16 ml, 2.4 mmol) was added to a solution of compound 61 (0.5 mg, 2.4 mmol) in ethanol (25 ml). This mixture was heated at reflux 1-3 days and monitored by TLC until the reaction went to completion. The resulting solid was collected by suction filtration (80%). mp: 243-245 °C. IR (cm⁻¹): 1247 (C-N 2° amine), 1599 (C-C), 2939 (CH₃), 3026 (aromatic C-H), 3302 (NH₂ 1° amine); ¹H-NMR (400 MHz, DMSO-d₆) ppm: 2.58 (s, 3H, CH₃), 3.89 (s, 3H, OMe), 6.92 (s, 1H, H 3), 7.44 (dd, ³J = 8.0, ⁴J = 4.0, 1H, H 7), 7.80 (d, J = 4.0, 1H, H 5), 7.84 (d, J = 8.0, 1H, H 8); ¹³C-NMR (75 MHz; DMSO-d₆): 22.4 (Me), 56.3 (OMe), 98.2 (C 5), 102.2 (C 3), 116.0 (C 8), 122.7 (C 7), 125.1 (C 6), 153.6 (q-C), 154.3 (q-C), 156.6 (C 2), one carbon not observed. MS ES⁻: 238.1 [M+35Cl, 100%], 240.1 [M+37Cl, 30%].

5.1.2.8: 4-Hydrazinyl-6-methoxy-2-phenylquinoline (63).

Compound (63) was prepared by the method described for the synthesis of compound (38) starting from compound (62). Compound (63) was isolated as a yellow powder (50%). mp: 158-160 °C. IR (cm⁻¹): 1585 (C=C), 1624 (C=N), 2830 (OMe), 3172 (C-H), 3298 (2° amine), 3349 (1° amine); ¹H-NMR (300 MHz, DMSO-d₆) ppm: 3.87 (s, 3H, OMe), 4.45 (s, 2H, NH₂), 7.24 (dd, ³J = 9.3, ⁴J = 2.4, 1H, H 7), 7.42-7.52 (m, 5H, H 3’,4’,5’,3, 5), 7.75 (d, J = 9.3, 1H, H 8), 8.14 (d, J = 8.1, 2H, H
2',6'), 8.30 (s, 1H, NH); 13C-NMR (75 MHz; DMSO-d6): 55.6 (OMe), 95.4 (C 5), 100.8 (C 3), 117.1 (C 8), 120.6 (C 7), 126.7 (C 2',4',6'), 128.4 (C 3',5'), 130.7 (C 10), 140.4 (C 9), 143.6 (C1'), 152.9 (C 4), 154.3 (C 6), 155.8 (C2). MS ES⁺, [M+H, 100%]: 266.5.

5.1.2.9: 6-Methoxy-2-methylquinolin-4-amine (64).

Compound (38) (25.0 mmol) was dissolved in methanol. Raney Ni (11.0 mmol) and sodium borohydride (79.0 mmol) were added and stirred at 40 °C for 2 h. On completion of the reaction, Raney Ni was filtered off using Celite. The filtrate was washed with water and the product extracted with ethyl acetate, dried using magnesium sulfate. The solvent was removed using a rotary evaporator to give the product as a yellow solid (90%). mp: 194-196 °C. IR (cm⁻¹): 1589 (C-C), 1646 (C=C), 2921 (CH₃), 3102 (arenes C-H), 3316 (arenes C-H) 3371 (NH₂ 1° amine); ¹H-NMR (400 MHz, DMSO-d6) ppm: 2.37 (s, 3H, CH₃), 3.85 (s, 3H, OMe), 6.39 (s, 1H, H3), 6.50 (br s, 2H, NH₂) 7.19 (dd, ³J =9.0, ⁴J = 2.7, 1H, H7), 7.45 (d, J = 2.7, 1H, H5), 7.57 (d, J = 9.0, 1H, H8); ¹²C-NMR (75 MHz; DMSO-d6): 24.5 (Me), 55.5 (OMe), 101.2 (C 5), 102.1 (C 3), 117.5 (C 10), 120.4 (C 7), 129.4 (C 8), 143.9 (C 9), 150.6 (C 4), 155.1 (C 6), 155.6 (C 2). MS ES⁺, [M+H, 100%]: 189.2.

5.1.2.10: General procedure for the synthesis of hydrazone derivatives (8-25)

Compound 38 or compound 63 (1.0 mmol) and the appropriate aldehyde (3.0 mmol) were dissolved in methanol (3.0 ml) and stirred for 3 h. The solid formed was then collected by filtration, washed with cold methanol and dried by suction filtration to get the hydrazone derivative.
5.1.2.11: 4-(2-(4-Fluorobenzylidene) hydrazinyl)-6-methoxy-2-methylquinoline (8).

Yellow solid (90mg, 56%). mp: 227-230 °C. IR (cm⁻¹): 1505 (C=C), 1591(C=N), 2921 (CH₃), 3001 (arenes C-H), 3425 (-NH- ²amine). ¹H-NMR (400 MHz, DMSO-d₆) δ ppm: 2.74 (s, 3H, CH₃), 4.00 (s, 3H, OMe), 7.38 (dd-t, J₈₇ = J₈F = 8.0, 2H, H 3°, 5°), 7.55 (s, 1H, H 3), 7.64 (dd, ³J = 8.0, ⁴J = 4.0, 1H, H 7), 7.93 (d, J = 8.0, 1H, H 8), 7.99 (dd, ³J = 8.0, ⁴J = 4.0, 2H, H 2°, 6°), 8.15 (d, J = 4.0, 1H, H 5), 8.86 (s, 1H, N=CH), 12.6 (s, 1H, NH). ¹³C-NMR (75 MHz; DMSO-d₆): 56.3 (OMe), 101.7 (C 5), 107.1 (C 3), 116.4 (d, ³J_CF = 12.7, C 3°, 5°), 116.5 (C 7), 129.3 (d, ³J_CF = 8.8, C ²°, 6°), 131.9 (C 1°), 133.4 (C=N), 156.5 (C 2), 162.3 (d, J_CF =147.0, C 4°). Quaternary carbons 4, 9, 10 not observed. MS ES⁺ [M+H, 100%]: 310.5. Accurate mass calculated for C₁₈H₁₆FN₃O + H: 310.1350. Found: 310.1333, error -5.482 ppm.

5.1.2.12: 4-(2-Benzylidenehydrazinyl)-6-methoxy-2-methylquinoline (9).

Yellow solid (75mg, 52%). mp: 282-285 °C. IR (cm⁻¹): 1501 (C=C), 1600 (C=N), 2845 (CH₃), 3121 (aromatic C-H stretch), 3353 (-NH- ²amine). ¹H-NMR (400 MHz, DMSO-d₆) δ ppm: 2.60 (s, 3H, CH₃), 3.99 (s, 3H, OMe), 7.33 (s, 1H, H 3), 7.36 (dd, ³J = 8.0, ⁴J = 4.0, 1H, H 7), 7.47 (t, J₈₇ = J₈H = 8.0, 1H, H 4°), 7.53 (t, J = 8.0, 2H, H 3°, 5°), 7.67 (d, J = 4.0, 1H, H 5), 7.78 (d, J=8.0, 1H, H 8), 7.86 (d, J = 8.0, 2H, H 2°, 6°), 8.45 (s, 1H, C=N), 10.92 (s, 1H, NH). ¹³C-NMR (75 MHz; DMSO-d₆): 19.7 (Me), 56.3 (OMe), 101.7 (C 5), 107.1 (C 3), 116.4 (d, ³J_CF = 12.7, C 3°, 5°), 116.5 (C 7), 129.3 (d, ³J_CF = 8.8, C ²°, 6°), 131.9 (C 1°), 133.4 (C=N), 156.5 (C 2), 162.3 (d, J_CF =147.0, C 4°). Quaternary carbons 4, 9, 10 not observed. MS ES⁺ [M+H, 100%]: 310.5. Accurate mass calculated for C₁₈H₁₆FN₃O + H: 310.1350. Found: 310.1333, error -5.482 ppm.
56.7 (OMe), 100.1 (C5), 103.2 (C3), 115.3 (C3’,5’), 121.2 (C 6), 124.8 (C 7), 127.5 (C 1’), 128.9 (C 8), 130.7 (C 6’), 133.5 (C 2’), 133.7 (C=N), 149.4 (C 9), 151.0 (C 4), 152.1 (C 6), 157.5 (C 2). MS ES $^+$, [M+H, 100%]: 292.5. Accurate mass calculated for C$_{18}$H$_{17}$N$_3$O + H: 292.1444. Found: 292.1438, error: -2.046 ppm.

5.1.2.13: 4-(2-((1H-Imidazol-5-yl)methylene)hydrazinyl)-6-methoxy-2-methylquinoline (10).

Bright yellow solid (105 mg, 76%). mp: 226-229 °C. IR (cm$^{-1}$): 1570 (C=C), 1599 (C=N), 2833 (OMe), 2928 (CH$_3$), 3121 (C-H), 3368 (2° amine); $^1$H-NMR (300 MHz, DMSO-d$_6$) δ ppm: 2.69 (s, 3H, CH$_3$), 3.98 (s, 3H, OMe), 7.42 (s, 1H, H 3), 7.57 (d, J = 9.3, 1H, H 7), 7.68 (s, 1H, H 5’), 7.88 (s, 1H, H 5), 7.91(d, J = 9.3, 1H, H 8), 8.25 (s, 1H, H 3’), 8.89 (s, 1H, HCN), 12.66 (s, 2H, NH). $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 19.8 (Me), 56.7 (OMe), 99.7 (C 5), 103.39 (C 3), 115.2 (C 5’), 121.3 (C 7), 124.4 (C 8), 133.7 (C 3’), 137.8 (C=N), 150.6 (C 4), 151.6 (C 6), 157.3 (C 2). Quaternary carbons (1’, 9 and 10) not observed. MS ES’, [M-H, 100%]: 280.1. Accurate mass calculated for C$_{15}$H$_{15}$N$_3$O+H: 282.1349. Found: 282.1329, error-7.0888 ppm.

5.1.2.14: 4-(2-((1H-Imidazol-4-yl)methylene)hydrazinyl)-6-methoxy-2-phenylquinoline (25).
Compound (25) collected as brown solid (100 mg, 77%). mp: 184-186 °C. IR (cm\(^{-1}\)): 1585 (C=C), 1624 (C=N), 2830 (OMe), 3063 (C-H), 3295 (2\(^\text{nd} \) amine). ¹H-NMR (300 MHz, DMSO-d\(_6\)) δ ppm: 3.96 (s, 3H, OMe), 7.38 (dd, \(^3\)J = 9.0, \(^4\)J = 2.4, 1H, H 7), 7.47-7.57 (m, 4H, H 3, 3\(^\text{rd} \), 4\(^\text{th} \), 5\(^\text{th} \)), 7.67 (d, J = 2.4, 1H, H 5), 7.84 (d, J=6.9, 2H, H 6\(^\text{th} \), 8), 7.89 (s, 1H, H 4\(^\text{th} \)), 8.20 (d, J = 7.5, 2H, H 2\(^\text{nd} \), 6\(^\text{th} \)), 8.40 (s, 1H, C=N), 10.90 (s, 1H, NH), 12.73 (s, 1H, NH); ¹³C-NMR (75 MHz; DMSO-d\(_6\)): 55.8 (OMe), 97.6 (C 5), 100.8 (C 3), 116.6 (C 6\(^\text{th} \)), 121.3 (C 7, 8), 126.9 (C 3\(^\text{rd} \), 5\(^\text{th} \)), 128.5 (C 2\(^\text{nd} \), 6\(^\text{th} \)), 128.9 (C 4\(^\text{th} \)), 130.3 (C 9), 137.3 (C 4\(^\text{th} \)), 139.4 (C 1\(^\text{st} \)), 147.2 (C 1\(^\text{st} \)), 153.9 (C 4), 156.3 (C 2, 6). MS ES\(^+\), [M+H, 100%]: 344.3. Accurate mass calculated for C\(_{20}\)H\(_{17}\)N\(_3\)O\(_2\) + H: 344.151. Found: 344.1487, error -5.5208 ppm.

5.1.2.15: 4-((2-(6-Methoxy-2-methylquinolin-4-yl) hydrazono)methyl)phenol (11).

Yellow solid (80 mg, 53%). mp: 208-210 °C. IR (cm\(^{-1}\)): 1570 (C=C), 1599 (C=N), 2833 (OMe), 2928 (CH\(_3\)), 3121(C-H), 3368 (NH), 3462 (OH). ¹H-NMR (300 MHz, DMSO-d\(_6\)) δ ppm: 2.71(s, 1H, Me), 3.98 (s, 3H, OMe), 6.89 (d, J = 9.0, 2H, H 3\(^\text{rd} \),5\(^\text{th} \)), 7.44 (s, 1H, H 3), 7.58 (dd, \(^4\)J = 6.0, \(^3\)J= 3.0, 1H, H 7), 7.72 (d, J = 9.0, 2H, H 2\(^\text{nd} \),6\(^\text{th} \)), 7.89 (d, J= 9.0, 1H, H 8), 8.15 (s, 1H, H 5), 8.77 (s, 1H, C=N), 10.15 (s, 1H, OH), 12.39 (s, 1H, NH). ¹³C-NMR (75 MHz; DMSO-d\(_6\)): 19.7 (Me), 56.6 (OMe), 99.6 (C 5), 103.1 (C 3), 115.1 (C 5\(^\text{th} \)), 115.9 (C 3\(^\text{rd} \)), 121.1 (C 10), 124.5 (C 7), 124.6 (C 8), 129.4 (C 2\(^\text{nd} \),6\(^\text{th} \)), 133.4 (C=N), 149.8 (C 9), 150.7 (C 4), 151.7 (C 6), 157.3 (C 2), 160.1 (C1\(^\text{st} \)). MS ES\(^+\), [M+H, 100%]: 308.1. Accurate mass calculated for C\(_{18}\)H\(_{17}\)N\(_2\)O\(_2\) + H: 308.1394. Found: 308.1389, error -1.6226 ppm.
5.1.2.16: 3-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)phenol (19).

Beige solid (0.5 g, 33%). mp: >300 °C. IR (cm\(^{-1}\)): 1570 (C=C), 1606 (C=N), 2895 (OMe), 3157 (C-H), 3284 (OH). \(^1\)H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 2.73 (s, 3H, CH\(_3\)), 3.99 (s, 3H, OMe), 6.91 (d, \(J = 6.0\), 1H, H 6\(^{\prime}\)), 7.30-7.34 (m, 3H, H 2\(^{\prime}\), 3\(^{\prime}\), 5\(^{\prime}\)), 7.45 (s, 1H, H 3), 7.62 (d, \(J = 9.3\), 1H, H 7), 7.92 (d, \(J = 9.0\), 1H, H 8), 8.14 (s, 1H, H 5), 8.78 (s, 1H, N=CH), 9.75 (s, 1H, OH). \(^{13}\)C-NMR (75 MHz; DMSO-\(d_6\)): 19.9 (Me), 56.6 (OMe), 100.0 (C 5), 103.0 (C 3), 113.5 (C 2\(^{\prime}\)), 115.3 (C 6\(^{\prime}\)), 117.9 (C 10), 118.7 (C 7), 121.4 (C 4\(^{\prime}\)), 124.7 (C 8), 129.9 (C 5\(^{\prime}\)), 133.8 (C 3\(^{\prime}\)), 134.9 (C=N), 149.5 (C 9), 150.8 (C 4), 152.2 (C 6), 157.5 (C 1\(^{\prime}\)), 157.8 (C 2). MS ES\(^+\), [M+H, 100%]: 308.6. Accurate mass calculated for C\(_{18}\)H\(_{17}\)N\(_3\)O\(_2\)+H: 308.1394. Found: 308.1388, error -1.947 ppm.

5.1.2.17: 5-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)benzene-1,3-diol (18).

Bright yellow (0.12 g, 75%). mp: >300 °C. IR (cm\(^{-1}\)): 1599 (C=C), 1643 (C=N), 2961 (OMe), 2997 (CH\(_3\)), 3081 (C-H), 3295 (NH). \(^1\)H-NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) ppm: 2.72 (s, 3H, CH\(_3\)), 3.99 (s, 3H, OMe), 6.38 (s, 1H, H 6\(^{\prime}\)), 6.73 (s, 2H, H 2\(^{\prime}\), 4\(^{\prime}\)), 7.39 (s, 1H, H 3), 7.62 (d, \(J = 5.4\), 1H, H 7), 7.93 (d, \(J = 5.7\), 1H, H 8), 8.16 (s, 1H, H 5).
5), 8.70 (s, 1H, HC=N), 9.58 (s, 2H, 2 x OH), 12.47 (s, 1H, NH). $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 19.9 (Me), 56.5 (OMe), 99.9 (C 5), 102.9 (C 3), 105.2 (C 6'), 105.6 (C 2', 4'), 115.3 (C10), 124.7 (C 7), 135.2 (C 4, 3'), 149.9 (C=N), 150.8 (C 9), 152.2 (C 6), 157.5 (C 2), 158.8 (C 1', 5'). MS $ES^{-}$, [M+H, 100%]: 324.6. Accurate mass calculated for C$_{18}$H$_{17}$N$_3$O$_3$ + H: 324.1343. Found: 324.1339, error -1.234 ppm.

5.1.2.18: 6-Methoxy-2-methyl-4-(2-(pyridin-3-ylmethylene)hydrazinyl)quinolone (12).

![Structure of 12]

Beige powder (50 mg, 34%), mp: >290°C (Dec). IR (cm$^{-1}$): 1570 (C=C), 1591 (C=N), 2830 (OMe), 2917 (CH$_3$). $^1$H-NMR (300 MHz, CD$_3$OD) δ ppm: 2.79 (s, 3H, CH$_3$), 4.06 (s, 3H, OMe), 7.57-7.66 (m, 3H, H 3, 7, 5'), 7.82 (d, J = 9.3, 1H, H 8), 7.91 (s, 1H, H 5), 8.39 (d, J = 6.3, 1H, H 6'), 8.65 (s, 2H, H 2', 4'), 8.98 (s, 1H, HC=N). $^{13}$C-NMR (75 MHz, CD$_3$OD): 20.2 (C Me), 56.9 (OMe), 102.1 (C 5), 102.8 (C 3), 117.1 (C 10), 122.4 (C 7), 125.7 (C 5'), 126.7 (C 8), 131.9 (C 1'), 135.4 (C 6'), 136.2 (C=N), 134.4 (C 9), 149.7 (C 4), 151.8 (C 2'), 153.2 (C 4'), 153.8 (C 6), 160.1 (C 2). MS $ES^{-}$, [M+H, 100%]:293.3. Accurate mass calculated for C$_{17}$H$_{16}$N$_4$O + H: 293.140. Found: 293.1397, error: -6.4816 ppm.

5.1.2.19: 6-Methoxy-2-phenyl-4-(2-(pyridin-3-ylmethylene)hydrazinyl)quinoline (20).

![Structure of 20]
Yellow solid (120 mg, 89%). mp: 215-218 °C. IR (cm⁻¹): 1585 (C=C), 1624 (C=N), 2798 (OMe), 3063 (C-H), 3219 (NH). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 3.97 (s, 3H, OMe), 7.37 (dd, ⁴ J = 9.0, ³ J = 1.8, 1H, H 7), 7.43-7.56 (m, 4H, H 3, 3`, 4`, 5`). 7.69 (s, 1H, H 5), 7.90 (d, J = 9.3, 1H, H 8), 7.93 (t, J = 6.3, 1H, H 5`). 8.23 (d, J = 7.8, 2H, H 2`, 6`). 8.29 (d, J = 7.8, 1H, C 6`) 8.46 (s, 1H, C=N), 8.59 (d, J = 4.8, 1H, H 4`) 8.97 (s, 1H, H 2`). 11.17 (s, 1H, NH). ¹³C-NMR (75 MHz; DMSO-d₆): 55.8 (OMe), 98.1 (C 5), 100.5 (C 3), 116.7 (C 10), 121.3 (C 8), 123.9 (C 7), 126.8 (C 2`, 6`), 128.6 (C 3`, 5`), 128.8 (C 4`), 130.8 (C 1`), 131.0 (C 5`), 132.9 (C 6`), 139.5 (C 9), 139.7 (C=N), 144.4 (C 4), 146.5 (C 1`), 148.2 (C 2`), 149.8 (C 4`), 154.3 (C 6), 156.4 (C 2). MS ES⁺, [M+H, 100%]: 355.2. Accurate mass calculated for C₂₂H₁₈N₄O + H: 355.1553. Found 355.1551, error -0.5631 ppm.

5.1.2.20: 6-Methoxy-2-methyl-4-(2-((5-nitrofuran-2-yl) methylene) hydrazinyl) quinolone (15).

Dark yellow powder (80 mg, 75%). mp: 264-266 °C. IR (cm⁻¹): 1247 (C=O-C), 1511(C=C), 1577 (NO), 1603 (C=N), 2722 (OMe), 3110 (C-H), 3425 (NH). ¹H-NMR (300 MHz, CD₃OD) δ ppm: 2.76 (s, 3H, Me), 4.00 (s, 3H, OMe), 7.45 (s, 1H, H 3), 7.48 (d, J = 6.0, 1H, H 3`), 7.65 (dd, ⁴ J = 9.0, ³ J= 3.0, 1H, H 7), 7.88 (d, J = 3.0, 1H, H 4`), 7.92 (d, J = 9.0, 1H, H 8), 8.17 (s, 1H, H 5), 8.82 (s, 1H, C=N). ¹³C-NMR (75 MHz; CD₃OD): 19.7 (Me), 55.6 (OMe), 100.6 (C 5), 103.0 (C 3), 115.3 (C 4`, 3`), 121.3 (C 7), 124.0 (C 10), 124.8 (C=N), 133.6 (C 8), 146.4 (C 9), 148.9 (C 4), 151.0 (C 5`), 151.1 (C 6), 152.3 (C 2`), 157.5 (C 2). MS ES⁺, [M+H, 100%]: 327.1. Accurate mass calculated for C₁₆H₁₄N₄O₄ + H: 327.1088. Found: 327.1085, error -0.9171 ppm.
5.1.2.21: 2-Methoxy-6-((2-(6-methoxy-2-methylquinolin-4-yl) hydrazono) methyl) phenol (14).

Yellow powder (120 mg, 89%). mp: 242-245 °C. IR (cm⁻¹): 1574 (C=C), 1603 (C=N), 2794 (OMe), 2841 (OMe), 2925 (CH₃), 3016 (C-H), 3226 (NH), 3411 (OH). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 2.54 (s, 3H, CH₃), 3.84 (s, 3H, OMe), 3.92 (s, 3H, OMe), 6.85 (t, J = 7.8, 1H, H 4'), 6.99 (d, J = 7.8, 2H, H 3', 3'), 7.35 (d, J = 8.5, 2H, H 7, 5'), 7.67 (d, J = 9.3, 1H, H 8), 7.80 (d, J = 2.1, 1H, H 5), 8.86 (s, 1H, C=N), 10.12 (s, 1H, NH), 11.90 (s, 1H, OH). ¹³C-NMR (75 MHz; DMSO-d₆): 21.9 (Me), 55.7 (OMe), 55.9 (OMe), 102.1 (C 5), 113.0 (C 3), 113.0 (C 3', 4'), 120.4 (C 7), 121.8 (C 5'), 146.6 (C=N), 147.9 (C 1', 2'), 156.1 (C 2). Quaternary carbons not observed. MS ES⁺, [M+H, 100%]: 338.3. Accurate mass calculated for C₁₉H₁₉N₃O₃ + H: 338.1499. Found: 338.1480, error -5.6188 ppm.

5.1.2.22: 2-Methoxy-6-((2-(6-methoxy-2-phenylquinolin-4-yl) hydrazono) methyl) phenol (24).

Bright yellow powder (132 mg, 91%). mp: 120-123 °C. IR (cm⁻¹): 1566 (C=C), 1620 (C=N), 2830 (OMe?), 2946 (OMe?), 3059 (C-H), 3230 (NH), 3509 (OH). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 3.85(s, 3H, OMe), 3.96 (s, 3H, OMe), 6.87 (t, J=7.8, 1H, H 4'), 7.00 (d, J = 7.8, 1H, H 3'), 7.35 (d, J = 9.0, 1H, H 7), 7.46-7.56 (m, 4H, H
5', 3\'', 4\'', 5\''\), 7.70 (s, 1H, C 3), 7.76 (s, 1H, H 5), 7.86 (d, J = 9.3, 1H, H 8), 8.16 (d, J = 7.2, 2H, H 2\'', 6\''), 8.80 (s, 1H, HC=N), 9.71 (s, 1H, NH), 11.00 (s, 1H, OH).

\(^{13}\)C-NMR (75 MHz; DMSO-d\(_6\)): 55.9 (2C, OMe), 97.3 (C 5), 100.5 (C 3), 112.6 (C 3\''), 118.2 (C 4\'), 118.3 (C 6'), 119.3 (C 10), 120.9 (C 8), 121.3 (C 7), 122.0 (C 4\''), 126.7 (C 2\'', 6\''), 128.6 (C 5, 5\''), 130.9 (C 4\''), 139.8 (C 9), 140.9 (C 1\''), 145.6 (C=N), 147.9 (C 4, 1'), 148.5 (C 4'), 154.3 (C 6), 156.3 (C 2). MS ES\(^+\), [M+H, 100%]: 400.2. Accurate mass calculated for C\(_{24}\)H\(_{21}\)N\(_3\)O\(_3\) + H: 400.1689. Found: 400.1649, error -1.7493 ppm.

5.1.2.23: 6-Methoxy-2-methyl-4-(2-(2-phenylethylidene)hydrazinyl)quinolone (13).

![Diagram of 13]

Cream solid (43mg, 30%). mp: 235-237 °C. IR (cm\(^{-1}\)): 1566 (C=C), 1610 (C=N), 2786 (OMe), 3004 (CH\(_2\)), 3154 (C-H), 3423 (NH). \(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) ppm: 2.68 (s, 3H, CH\(_3\)), 3.75 (d, J = 5.7, 2H, H2\'''), 3.93 (s, 3H, OMe), 7.25 (s, 1H, H 3), 7.28-7.40 (m, 5H, phenyl), 7.56 (d, J = 8.7, 1H, H 7), 7.89 (d, J = 9.0, 1H, H 8), 8.12 (s, 1H, H 5), 8.37 (t, J= 5.4, 1H, HC=N), 12.39 (s, 1H, NH). \(^{13}\)C-NMR (75 MHz; DMSO-d\(_6\)): 19.9 (Me), 35.0 (C 2\', visible in dept), 56.5 (OMe), 99.5 (C 5), 102.9 (C 3), 114.9 (C 10\''), 124.6 (C 7), 126.8 (C 6'), 128.8 (C 5\', 7\'), 128.91 (C 4\', 8\'), 136.3 (C 3\''), 152.2 (C=N), 153.1 (C 6), 157.3 (C 2). Other quaternary carbons not observed. MS ES\(^+\): 306.1 [M+H, 30%]. Accurate mass calculated for C\(_{19}\)H\(_{19}\)N\(_3\)O + H: 306.1601. Found: 306.1581, error:-6.5325 ppm.

5.1.2.24: 6-Methoxy-2-methyl-4-(2-(2-phenylethylidene) hydrazinyl) quinolone (23).

![Diagram of 23]
Beige powder (56mg, 26%). mp: 180-182 °C. IR (cm\(^{-1}\)): 1585 (C=C), 1617 (C=N), 2910 (OMe), 2968 (CH\(_2\)), 3026 (C-H). \(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) ppm: 3.75 (d, J=5.7, 2H, H 2`), 3.90 (s, 1H, OMe), 7.25 -7.6 (m, 10H, H 3, 7, 3`,4`,5`,4``, 5``, 6`,7``,8`) , 7.71 (s, 1H, H 5), 7.81-7.87 (m, J = 9.0, 2H, H 8, N=CH), 8.11 (d, J = 7.5, 2H, H 2`,6`), 10.59 (s, 1H, NH). \(^1\)C-NMR (75 MHz; DMSO-d\(_6\)): 38.4 (C 2``), 55.7 (OMe), 97.3 (C 5), 100.5 (C 3), 116.4 (C 10), 121.1 (C 8), 126.5 (C 7), 126.7 (2C, 4`, 6``), 128.5 (C 5``), 128.6 (C 2`,6`), 128.7 (C 8``), 128.9 (C 3`, 5`), 130.9 (C 4``), 137.4 (C 3``), 139.9 (C 9), 144.3 (C 1`), 145.5 (C=N), 147.1 (C 4), 154.2 (C 6), 156.1 (C 2). The quaternary and CH\(_2\) carbons confirmed by carbon dept. MS ES\(^+\), [M+H, 100%]: 368.2. Accurate mass calculated for C\(_{24}\)H\(_{21}\)N\(_3\)O +H: 368.1757. Found: 368.1763, error 1.629 ppm.

5.1.2.25: 4-((2-(6-Methoxy-2-phenylquinolin-4-yl)hydrazono)methyl)-N,N-dimethylaniline (21).

Brown powder (90mg, 60%). mp: 115-118 °C. IR (cm\(^{-1}\)): 1589 (C=C), 2802 (OMe), 2892 (OMe), 2997 (CH\(_3\)), 3234 (C-H), 3513 (NH). \(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) ppm: 2.98 (s, 6H, N(CH\(_3\))\(_2\)), 3.95 (s, 3H, OMe), 6.74-6.82 (m, 3H, H 3, 5, 3``), 7.33 (d, J = 9.3, 1H, H 7``), 7.42-7.55 (m, 3H, H 3`, 4`, 5`), 7.61-7.67 (m, 2H, H 2, 6), 7.82 (s, 1H, H 5``), 7.84 (d, J = 9.3, 1H, H 8``), 8.16 (d, J = 7.5, 2H, H 2`, 6`), 8.33 (s, 1H, HC=N), 10.71 (s, 1H, NH). \(^1\)C-NMR (75 MHz; DMSO-d\(_6\)): 55.7 (OMe), 97.2 (C 5``), 100.6 (C 3``), 111.7 (C7``), 112.0 (C 3, 5), 121.0 (C 8``), 121.5 (C 10``), 122.4 (C 1), 126.7 (C 2`, 6`), 127.8 (C 2, 6), 128.6 (C 4``), 128.7 (C 3`, 5`), 129.4 (C 1`), 130.9 (C 9``), 143.6 (C=N), 146.9 (C 4``), 151.08 (C 4), 156.1 (C 2``), 160 (C 6``)(NMe\(_2\) carbons not observed, as under the d6-DMSO). MS ES\(^+\), [M+H, 100%]: 297.3. Accurate mass calculated for C\(_{25}\)H\(_{24}\)N\(_3\)O +H: 297.2023. Found: 297.2016, error -1.76 ppm.
5.1.2.26: 4-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)benzoic acid (16).

Yellow solid (130mg, 78%), mp: 272-275 °C. IR (cm⁻¹): 1577 (C=C), 1603 (C=N), 1675 (C=O), 2648 (OMe), 2928 (CH₃), 3004(CH-H), 3314 (2° amine). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 2.74 (s, 3H, CH₃), 3.99 (s, 3H, OMe), 7.56 (s, 1H, H 3'), 7.62 (d, J = 9.3, 1H, H 7'), 7.93 (d, J = 9.3, 1H, H 8'), 7.99-8.07 (m, 5H, H 2, 3, 5, 6, 5'), 8.14 (s, 1H, N=C), 8.89 (s, 1H, NH), 12.65 (s, 1H, OH). ¹³C-NMR (75 MHz; DMSO-d₆): 20.0 (Me), 56.6 (OMe), 100.6 (C 5'), 102.9 (C 3'), 115.5 (C10'), 121.9 (C 7'), 124.5 (C 8'), 127.4 (C 3, 5), 129.8 (C 2, 6), 132.1 (C 1), 134.3 (C 4), 137.8 (C=N), 147.5 (C 9'), 150.7 (C 4'), 152.5 (C 6'), 157.4 (C 2'), 166.8 (C=O). MS ES⁺, [M+H, 100%]: 336.1. Accurate mass calculated for C₁₉H₁₇N₃O₃+H: 336.1343. Found: 336.1339, error -1.19 ppm.

5.1.2.27: 6-Methoxy-2-methyl-4-(2-(4-nitrobenzylidene) hydrazinyl) quinolone (17).

Bright yellow solid (133mg, 92%), mp: 220-223 °C. ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: IR (cm⁻¹): 1341 (N=O), 1574 (C=C), 1510 (C=N), 2856 (OMe), 2917 (CH₃), 3366 (2° amine). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 2.64 (s, 3H, CH₃), 3.96 (s, 3H, OMe), 7.42 (s, 1H, H 3), 7.47(d, J = 9.0, 1H, H 7), 7.82 (d, J = 9.0, 1H, H 8), 7.95(d, 2H, H 2', 6'), 7.98(s, 1H, H 5), 8.03 (d, 2H, J = 8.7, H 3', 5'), 8.70 (br s, 1H, N=CH), 12.73 (s, 1H, NH). ¹³C-NMR (75 MHz; DMSO-d₆): 21.9 (Me), 56.1 (C,
OMe), 100.8 (C 5), 101.9 (C 3), 115.9 (C10), 126.9 (C 7, 3`, 5`), 128.5 (C 1`), 129.8 (C 2`, 6`), 131.5 (C 8), 138.3 (C=N), 156.7 (C 2), 166.9 (C 6). Three quaternary carbons (4, 9, 4`) not observed. MS ES+, [M+H, 100%]: 337.1. Accurate mass calculated for C$_{18}$H$_{16}$N$_{4}$O$_{3}$ + H: 337.1295. Found: 337.1293, error: -0.593 ppm.

5.1.2.28: 6-Methoxy-4-(2-(4-nitrobenzylidene)hydrazinyl)-2-phenylquinoline (22).

Red brown powder (53mg, 40%). mp: 230-234 °C. IR (cm$^{-1}$): 1556 (NO), 1595 (C=C), 2848 (OMe), 2921 (C-H), 3357 (NH). $^{1}$H-NMR (300 MHz, DMSO-d$_{6}$) δ ppm: 4.03 (s, 3H, OMe), 7.47 (d, J = 9.0, 1H, H 7), 7.51-7.63 (m, 3H, H 3`, 4`, 5`), 7.77 (s, 1H, H 3), 7.96-8.01 (m, 2H, H 8, 5), 8.15 (d, 2H, J = 8.1, H 2`, 6`), 8.28 (d, 2H, J = 7.2, H 2`, 6`), 8.37 (d, 2H, J = 8.1, H 3`, 5`), 8.58 (s, 1H, N=CH), 11.43 (br s, 1H, NH). $^{13}$C-NMR (75 MHz; DMSO-d$_{6}$): 55.9 (OMe), 99.5 (C 5), 100.5 (C 3), 116.8 (C 10), 121.4 (C 7, 8), 124.1 (C 3`, 5`), 126.8 (C 2`, 6`), 127.3 (C 2`, 4`, 6`), 128.6 (C 3`, 5`), 128.9 (C 9), 131.1 (C 1`), 139.8 (C 1`), 141.3 (C=N), 146.2 (C 4), 147.1 (C 4`), 154.3 (C 6), 156.5 (C 2). MS ES+, [M+H, 100%]: 399.1. Accurate mass calculated for C$_{23}$H$_{18}$N$_{4}$O$_{3}$+H: 399.1452, Found: 399.1451, error -0.250 ppm.

5.1.2.29: General procedure for the hydrazide derivatives (26-27)
Compound 38 (1 mmol) was dissolved in dichloromethane (10 ml), cooled (ice bath) and stirred for 15 minutes. Pyridine (1.5 mmol) was added slowly followed by the dropwise addition of a solution of the appropriate acyl chloride (1 mmol) in dichloromethane (5 ml) over a period of 2 h. Once the addition had finished, the ice bath was removed and the mixture was left to stir for another 3 hr at room temperature. The formed solid was collected by filtration, washed with water and recrystallized from ethanol to give the final product.
5.1.2.31: N-(6-Methoxy-2-methylquinolin-4-yl)benzohydrazide (26).

Beige powder (45 mg, 28%). mp: >285°C (Dec). IR (cm⁻¹): 1599 (C=C), 1620 (C=O amide), 2946 (CH₃), 3020 (C-H arene), 3205 (NH-amide), 3302 (NH-aryl).

^1H-NMR (300 MHz, DMSO-d₆) δ ppm: 2.65 (s, 3H, CH₃), 3.94 (s, 3H, OMe), 6.86 (s, 1H, H 3'), 7.56-7.65 (m, 4H, H 4', 5', 7', 8'), 7.94 (m, 2H, H 2, 6), 8.02 (d, J = 7.2, 2H, H 3, 5), 8.13 (d, J = 8.1, 1H, NH), 11.20 (s, 1H, NHCO).

^13C-NMR (75 MHz; DMSO-d₆): 19.6 (Me), 56.1 (OMe), 102.5 (C 5’), 114.8 (C 10’), 120.9 (C 3’), 123.9 (C 7’), 127.8 (C 2, 6), 128.6 (C 3, 5), 131.8 (C 1), 132.4 (C 8’), 132.6 (C 4), 151.2 (C 9’), 152.8 (C 4’), 155.2 (C 6’), 157.0 (C 2’), 166.0 (C=O). MS ES⁻, [M+Cl, 20%]: 309.0. Molecular ion not observed. Cleavage to hydrazine starting material has been detected.

5.1.2.30: 4-Fluoro-N’-(6-methoxy-2-methylquinolin-4-yl)benzohydrazide (27)

Yellow solid (238 mg, 74%). Mp: 284-286°C. IR (cm⁻¹) 1501 (C-C), 1599 (C=C), 1636 (amide), 2594 (C-H), 2939 (CH₃), 3030 (arenes C-H), 3248 (NH₂ 1° amine).

^1H-NMR (400 MHz, DMSO-d₆) δ ppm: 2.43 (s, 3H, CH₃), 3.90 (s, 3H, OMe), 6.49 (s, 1H, H 3’), 7.29 (dd, 3 J = 9.2, 4 J = 3.2 1H, H 7’), 7.41 (dd-t, 3 J_HH = 3 J_HF = 8.0, 2H, H 3, 5), 7.62 (d, J=2.8 1H, H 5’), 7.70 (d, J= 9.0, 1H, H 8’), 8.05 (dd, 3 J_HH = 8.0, 4 J_HF
= 4.0, 2H, H 2, 6), 9.10 (s, 1H, NH), 10.63 (s, 1H, NH). $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 24.9 (Me), 55.9 (OMe), 106.2 (C 5'), 107.9 (C 3'), 116.0 (C 5), 116.2 (d C 3, 5??), 116.3 (C 10'), 116.4 (C 7'), 127.8 (C 1), 132.5 (C 2, 6), 132.6 (C 8'), 133.0 (C 9'), 156.4 (C 4'), 164.4 (C 6'), 164.7 (C 2'), 166.4 (C=O), 166.9 (C 4). Molecular ion not observed in the mass spectrum: Cleavage to hydrazine starting material was detected.

5.1.2.32: 6-Methoxy-2-phenylquinoline-4-carboxylic acid (30).

A solution of $p$-anisidine (0.35g, 2.8mmol) and benzaldehyde (0.3 ml, 2.8mmol) in (10.0 ml) ethanol was heated to 80 °C for 1h. The mixture was stirred overnight at room temperature after which the solution was heated to 80°C and a pyruvic acid (0.4 ml, 5.7mmol) added dropwise over 1hr. The mixture was heated at reflux for 3hr. The resulting precipitate was filtered off and washed with hexane to give the product as a brown powder. mp: 238-240 °C. IR cm$^{-1}$: 1505 (C=C), 1614 (C=N), 1726 (C=O ), 2645 (OH), 2936 (OMe), 3077 (arene C-H). $^1$H-NMR (300 MHz, DMSO-d$_6$) ppm: 3.92 (s, 3H, OMe), 7.49-7.58 (m, 4H, H 7,3',4',5'), 8.08 (d, J = 9.0, 1H, H 8), 8.14 (d, J = 2.4, 1H, H 5), 8.24 (d, J = 7.2, 2H, H 2', 6'), 8.46 (s, 1H, H 3). $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 56.6 (OMe), 103.7 (C 5), 119.9 (C 7), 122.8 (C10), 125.0 (C 3), 127.0 (C 4'), 128.9 (C 2'), 129.7 (C 7'), 130.8 (C 6'), 135.9 (C 3'), 137.5 (C 4), 144.0 (C 1'), 153.0 (C 9), 158.4 (C 2'), 167.5 (C=O). MS ES$^+$, [M+H]: 280.2.
5.1.2.33: 6-Methoxy-2-phenylquinoline-4-carbonyl chloride (65).

A solution of 6-methoxy-2-phenylquinoline-4-carboxylic acid (0.7g, 2.5mmol) in thionyl chloride (12 ml) and DMF (0.2ml) was heated at reflux for 3hr. The excess thionyl chloride was removed by washing with toluene (2 x 3ml) and evaporated under vacuum to give the product as a yellow brown powder. mp: 140-142 °C. IR cm⁻¹: 1541 (C=C), 1614 (C=N), 1788 (C=O), 2841 (OMe), 3073 (arene C-H). ¹H-NMR (300 MHz, CDCl₃) ppm: 4.12 (s, 3H, OMe), 7.54-7.62 (m, 3H, H 3`, 4`, 5`), 7.67 (d, J = 9.3, 1H, H 7), 7.92 (s, 1H, C 3), 8.18 (dd, J = 7.5, J = 1.2, 2H, H 2`, 6`), 8.43 (d, J = 9.3, 1H, H 8). ¹³C-NMR (75 MHz; CDCl₃): 57.3 (OMe), 114.8 (C 5), 118.6 (C 7), 119.2 (C 10), 120.44(C 4`)127.5 (C 8), 128.8 (C 2`, 6`), 129.5 (C 3`, 5`), 132.1 (C 3), 133.3 (C 4), 140.7 (C 1`), 144.8 (C 9), 154.1 (C 6), 155.6 (C 2), 167.6 (C=O). Late note: H 5 was not observed in the ¹H NMR spectrum. It is proposed that chlorination occurred at position 5, as explained below for compound 32. The acid chloride was not sufficiently stable to be detected by mass spectrometry.

5.1.2.34: N-Benzyl-6-methoxy-2-phenylquinoline-4-carboxamide (32).

6-Methoxy-2-phenylquinoline-4-carbonyl chloride (0.1g, 0.33mmol) and benzylamine (0.73ml) was dissolved in ethanol (5 ml) and heated using a Biotage
microwave synthesiser for 10 min at 120 °C. The precipitate was filtered off and washed with hexane to give the product as a brown powder. mp: 239-241 °C. IR (cm⁻¹): 1639 (C=O of amide), 2917 (OMe), 3048 (C-H), 3248 (NH of amide). ¹H-NMR (300 MHz, CDCl₃) δ ppm: 4.04 (s, 3H, OMe), 4.73 (d, J=5.4, 3H, CH₂), 6.51 (t, J=5.4, 1H, NH), 7.33-7.49 (m, 9H aromatic), 7.69 (s, 1H, H 3), 7.98-8.03 (m, 3H aromatic). ¹³C-NMR (75 MHz; CDCl₃): 44.9 (CH₂ confirmed by DEPT), 56.9 (OMe), 114.9 (C 5), 116.8 (C 10), 119.3 (C 7), 121.9 (C 3), 127.2 (C 2″, 6″), 127.9 (C 4″), 128.6 (C 2′, 6′), 128.87 (C 3″,5″), 128.92 (C 3′, 5′), 129.8 (C 4′), 130.3 (C 8), 137.2 (C 1″), 137.9 (C 1′), 141.8 (C 9), 144.6 (C 4), 153.7 (C 2), 154.6 (C 6), 168.9 (C=O). Late note: One aromatic proton was not observed, proposed to be C5. The mass spectrum confirmed the presence of one chlorine atom in the molecule: MS ES⁺ 403.2 [M+H ³⁵Cl, 100%], 405.2 [M+H ³⁷Cl. 30%]; MS ES⁻ 437.2 [M+Cl, 100%, ³⁵Cl, ³⁷Cl], 439.2 [M+Cl, 60%, ³⁵Cl³⁷Cl]; 441 [M+Cl, 10%, ³⁵Cl³⁷Cl]. The proposed structure is 32b.

5.1.2.35: 4-Amino-2-phenyloxazole-5-carboxamide (34).

2-Amino-2-cyanoacetamide (0.20g, 2.01 mmol) was dissolved in ethanol (50 ml) and heated up to 80°C until it completely dissolved. Ethyl benzimidate hydrochloride (0.32g, 1.72 mmol) was then added to the solution and left to stir at 80°C for 2-3 days (more ethyl benzimidate may need to be added if consumed before reaction completion). The product was extracted with ethyl acetate–water, the organic layer was dried (MgSO₄) and purified by column chromatography (40% ethyl acetate : 55% hexane : 5% methanol) to give the product as a white solid (50mg, 12%). mp:
168-172 °C. IR cm⁻¹: 1120 (C=O), 1620 (C=C), 1650 (C=O amide), 3059 (arene C-H), 3164 (N-H amide), 3364 (NH₂ 1° amine). ¹H-NMR (300 MHz, CDCl₃) δ ppm: 2.40 (s, 3H, NH), 5.96 (s, 2H, NH₂), 7.45-7.59 (m, 3H, H 3’, 4’, 5’), 7.83-7.85 (m, 2H, H 2’, 6’). ¹³C-NMR (75 MHz; CDCl₃): 127.4 (C2’, 6’), 128.7 (C3’, 5’), 132.1 (C4’), 169.4 (C=O), other quaternary carbons not observed. MS ES⁻, [M-H, 100%]: 202.2. MS ES⁺, [M+Na, 30%]: 226.2. Accurate mass calculated for C₁₀H₉N₃O₂+Na: 226.0593, Found: 226.0592, error -0.44 ppm.

5.1.2.36: 4-(Furan-2-yl)benzonitrile (43).

![Diagram of 4-(Furan-2-yl)benzonitrile (43)](image)

4-Bromobenzonitrile (1.0 mol) was added to a solution of water (10 ml), EtOH (7.5 ml), and DMF (8 ml). The mixture was heated to 80 °C until it completely dissolved. Pd-C (0.8% mol) was then added, along with KHCO₃ (2.0 mol) to adjust the pH to 10. The 2-furanyl boronic acid (1.5 mol) then added portionwise and the mixture left to stir 3-4 hr at 80 °C. After cooling, the product was extracted with EtOAc (3-4 times), dried (MgSO₄) and the organic layer was purified by column chromatography eluting with 10% EtOAc: 90% hexane to get the product as a white solid (400mg, 64%). mp: 73-75 °C. IR (cm⁻¹): 1283 (C=O-C), 1505 (C=C), 2227 (C≡N); ¹H-NMR (300 MHz, CDCl₃) δ ppm: 6.54 (dd, J₃.3, J = 1.8, 1H, H 4’), 6.83 (d, J = 3.3, 1H, H 5’), 7.55 (bs, 1H, H 3’), 7.67 (d, J = 8.7, 2H, H 2, 6), 7.76 (d, J = 8.4, 2H, C 3, 5). ¹³C-NMR (75 MHz; CDCl₃): 108.1 (C 3’), 110.4 (C 4’), 112.2 (C1), 118.9 (C≡N), 124.0 (C 3, 5), 132.6 (C 2, 6), 134.7 (C 4), 143.7 (C 5’), 152.0 (C 2’). MS ES⁺, [M+H, 100%]: 169.9. Accurate mass calculated for C₁₁H₇NO+H: 170.0600. Found: 170.0601, error: 0.588 ppm.
5.1.2.37: 4-(Furan-2-yl)benzamide (44).

![Structural formula of 4-(Furan-2-yl)benzamide (44)](image)

Compound (43) (1.0 mol) and potassium tert-butoxide (3.0 mol) were dissolved in dry toluene (volume) with stirring under dry conditions. The mixture was heated at reflux for 3-5 hr until the reaction was complete. The solution quenched with water, the organic layer separated and evaporated under vacuum to give the product as a white solid (31 mg, 56%). mp: 290 °C (Dec). IR cm⁻¹: 1283 (C=O-C), 1559 (C=C), 1614 (C=N), 1646 (C=O amide), 3186 (arene C-H), 3393 (N-H amide). ¹H-NMR (300 MHz, DMSO) δ ppm: 6.63 (dd, J= 3.3, J = 1.8, 1H, H 4’), 7.09 (d, J = 3.3, 1H, H 3’), 7.37 (br s, 1H, amide), 7.77 (d, J = 8.4, 2H, H 2, 6), 7.81 (bs, 1H, H 5’), 7.92 (d, J = 8.4, 2H, H 3, 5), 8.00 (br s, 1H, amide). ¹³C-NMR (75 MHz; CD₃OD): 104.9 (C 3’), 111.9 (C 4’), 121.8 (C 3, 5), 128.8 (C 2, 6), 128.8 (C 4), 142.3 (C 1), 143.0 (C 5’), 153.8 (C 2’), 170.6 (C=O). GCMS (Rt 14 min) [M, 100%]: 187.1.

5.1.2.38: Ethyl 4-(furan-2-yl)benzimidinium chloride (45).

![Structural formula of Ethyl 4-(furan-2-yl)benzimidinium chloride (45)](image)

A solution of compound (43) (0.6 g, 0.29 mmol) in a mixture of ethanol (3.0 ml) and water (0.05 ml, 0.23mmol) was cooled in an ice bath. Thionyl chloride (2.4 ml, 2.7 mmol) was then added dropwise. The resulting mixture was stirred at room temperature for 36h. The precipitate was collected by filtration, washed twice with Et₂O and dried using vacuum filtration to give the product as a yellow powder (330mg, 43%). Mp: 200 °C (Dec). IR (cm⁻¹): 1291(C=O-C), 1606 (C=C), 2863(CH₃-O-); ¹H-NMR (300 MHz, CD₃OH) δ ppm: 1.64 (t, J = 6.8, 3H, CH₃), 4.66 (q, J = 6.8, 2H, CH₂), 6.64 (dd, J = 3.3, J= 1.8, 1H, H 4’), 7.14 ( dd, J = 0.4, J = 3.6, 1H, H 3’),
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7.73 (dd, J = 0.4, J = 2.0, 1H, H 5’), 7.97 (d, J = 9.2, 2H, H 2, 6), 8.10 (d, J = 8.8, 2H, H 5, 3). \(^{13}\)C-NMR (75 MHz; CD\(_3\)OH): 12.5 (CH\(_3\)), 69.6 (CH\(_2\)), 109.2 (C 3’), 112.2 (C 4’), 123.6 (C 3, 5), 129.4 (C 2, 6), 129.6(C 1), 137.3 (C 4), 144.4(C 5’), 151.8(C 2’), 172.4(C=NH).

MS ES+, [M+H, 100%]: 216.4.

5.1.2.39: Amino(4-(furan-2-yl)phenyl)methaniminium acetate (46).

Ammonium acetate (4.0 mmol) was added to a stirred suspension of compound (45) (1.0 mmol) in anhydrous (10 ml) ethanol. The mixture was stirred at room temperature for 1-3 days. The light yellow solid was filtered off under vacuum and rinsed with ethanol to give the product as white solid (200mg, 64%). mp: 260 °C (Dec). IR (cm\(^{-1}\)): 1291 (C-O-C), 1556 (C=C), 1617 (C=N), 2961 (CH\(_3\)), 3160 (C-H).

\(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) δ ppm: 1.72(s, 3H, acetate), 6.66 (dd, J = 3.3, J= 1.8, 1H, H 4’), 7.19 (d, J = 3.3, 1H, H 3’), 7.89-7.83 (m, 5H, H 5’, 2, 3, 5, 6), 10.12 (br s, 2H, NH\(_2\)). \(^{13}\)C-NMR (75 MHz; DMSO-d\(_6\)): 23.9 (CH\(_3\) of acetate), 108.5 (C 3’), 112.5 (C 4’), 123.3 (C 3, 7), 128.3 (C 2, 6), 134.1 (C 1), 144.2 (C 4, 5’), 151.7 (C 2’), 165.2 (C=NH\(_2\)), 175.4 (C=O). For the cation MS ES+ [M+H, 100%]: 187.0. Accurate mass calculated for C\(_{11}\)H\(_{10}\)N\(_2\)O + H: 187.0866. Found: 187.0855, error -5.8796 ppm.

5.1.2.40: General procedure for the synthesis of the asymmetric aryl 1,4-diketones (66, 67).

Zinc chloride (9.0 mmol) was added to dry toluene (5.0 ml), dry ethanol (6.8 mmol) and triethylamine (6.8 mmol). The mixture was stirred at room temperature for 1-2 h. Methyl aryl ketone (4-fluoroacetophenone (for compound 66) or 4-ethylacetophenone (for compound 67) (6.8 mmol) and 2-bromo-4’-cyanobenzonitrile
(4.5 mmol) were added. The mixture was stirred at room temperature for 5-7 days and monitored by TLC (20% EtOAc / 80% hexane).

5.1.2.41: 4-(4-(4-Fluorophenyl)-4-oxobutanoyl)benzonitrile (66).

The reaction mixture was diluted with ethyl acetate (10 ml). Ethyl acetate and toluene were decanted and the solvents removed by evaporated to give a yellow solid. Re-crystallization from methanol gave the required compound (0.16 g, 26.0 %) as bright yellow crystals: mp 162-165 °C; IR (cm\(^{-1}\)) : 1591 (C=C), 1679 (C=O), 2227 (C≡N), 2913 (CH\(_2\)), 3070 (C-H).

\(^1\)H-NMR (300 MHz, CDCl\(_3\)) \(\delta\) ppm: 3.47 (s, 4H, H2`, 3`), 7.18 (dd ~ t, \(J_{HH} \sim J_{HF} \sim 8.4\), 2H, H 3```, 5````), 7.82 (d, \(J = 8.1, 2H, H 3, 5\)), 8.07 (dd, 2H, J = 5.4, J = 8.4, H 2```, 6````), 8.14 (d, \(J = 8.1, 2H, H 2, 6\)). \(^{13}\)C NMR (75 MHz; CDCl\(_3\)) : 32.4 (CH\(_2\)), 32.8 (CH\(_2\)), 115.8 (d, \(\text{CF} = 22.0\), C 3```, 5````), 116.5 (C 4), 118.0 (CN), 128.6 (2 × CH), 130.8 (d, \(\text{CF} = 9.0\), C 2```, 6````), 132.6 (2 × CH), 132.9 (d, \(\text{CF} = 3.0\), C 1````), 139.7 (C), 165.9 (d, \(\text{CF} = 253.0\), C4````), 196.6 (C=O), 197.4 (C=O). MS ES\(^+\) [M+H, 100%] : 282.

5.1.2.42: 4-(4-(4-Ethylphenyl)-4-oxobutanoyl)benzonitrile (67).

The reaction was quenched by the addition of 10.0 % (v/v) aqueous sulfuric acid and the product was extracted with ethyl acetate (20 ml). The organic layer was washed with brine (10.0 ml), dried (MgSO\(_4\)) and concentrated. The compound was crystallized from methanol to give 0.38 (15.0 %) of the product as white powder: mp 126-128 °C. IR (cm\(^{-1}\)) : 1606 (C=C), 1708 (C=O), 2227 (C≡N), 2928 (CH\(_2\),CH\(_3\)), 143
3048(C-H). $^1$H-NMR (300 MHz, CDCl$_3$) δ ppm: 1.19 (M, 3H, CH$_3$), 2.65 (q, J = 7.8, 2H, CH$_2$), 3.32-3.34 (m, 4H, H 2`, 3`), 7.24 (d, J = 8.1, 2H, H 3`, 5`), 7.72 (d, J = 8.4, 2H, H 3, 5), 7.87 (d, J = 8.1, 2H, H 2`, 6`) 8.05 (d, 2H, J = 8.4, H 2, 6). $^{13}$C NMR (75 MHz; CDCl$_3$): 15.2 (CH$_3$), 29.0 (CH$_3$CH$_2$), 32.5 (CH$_2$), 32.8 (CH$_2$), 116.4 (C 4), 118.0 (CN), 128.2 (C 3``), 128.4 (2 × CH), 128.6 (2 × CH), 132.5 (2 × CH), 134.2 (C), 139.9 (C), 150.4 (C), 197.7 (C=O), 197.9 (C=O); MS, MS ES+ [M+H, 100%]: 292.1.

5.1.2.43: General procedure for the synthesis of ethyl furan-imidate hydrochloride (47, 48).

Acetyl chloride (28.4 mmol) was added dropwise to a suspension of 4-(4-(4-fluorophenyl)-4-oxobutanoyl)benzonitrile (66) or 4-(4-(4-ethylphenyl)-4-oxobutanoyl)benzonitrile (67) (1.0 mmol) in absolute ethanol (24 mmol) and dry chloroform (10 ml) at 0 °C. The reaction mixture was allowed to warm up to room temperature and stirred for 2-3 days.

5.1.2.44: Ethyl 4-(5-(4-fluorophenyl)furan-2-yl)benzoate (47).

![Diagram of the molecule](image)

The yellow precipitate was filtered off under vacuum and rinsed with hexane to give the required product 0.22 g (89.0%): mp 190-192 °C. $^1$H-NMR (300 MHz, CDCl$_3$) δ ppm: 1.66 (s, 3H, CH$_3$), 4.96 (d, J = 6.3, 2H, CH$_2$), 6.74 (d, J = 3.3, 1H, H 3`), 6.96 (d, J = 3.6, 1H, H 4`), 7.14 (t, J = 8.4, 2H, H 3`, 5`), 7.73 (dd, 2H, J = 5.4, J = 8.4, H 2`, 6`), 7.87 (d, J = 8.1, 2H, H 3, 5), 8.43 (d, J = 8.1, 2H, H 2, 6), 11.58 (s, 1H, NH). MS ES+ [M+H, 100%]: 310.2.

5.1.2.45: Ethyl 4-(5-(4-ethylphenyl)furan-2-yl)benzoate (48).
The reaction was cooled to 0 °C and a saturated solution of sodium bicarbonate was added dropwise until gas evolution ceased. The organic layer was extracted with ethyl acetate, washed with water, dried (MgSO₄) and evaporated to give the product (400mg, 95.0 %) as a yellow solid: mp 198-204 °C. IR (cm⁻¹): 1287 (C-O-C), 1505 (C=C), 1600 (C=N), 2903 (CH₃, CH₂), 3396 (C=N-H). MS E5⁺[M+H, 100%]: 320.3.

5.1.2.46: General procedure for the synthesis of furan-amidines (49, 50).
Ammonium acetate (4.0 mmol) was added to a stirred suspension of furan-imidates (47, 48) (1.0 mmol) in anhydrous ethanol. The mixture was stirred at room temperature for 1-3 days.

5.1.2.47: 4-(5-(4-Fluorophenyl)furan-2-yl)benzamidine (49).

The white precipitate was filtered off under vacuum and rinsed with hexane. The product was re-crystallized from ethanol to give the product (400mg, 84 %): mp 180-183 °C. IR (cm⁻¹): 1297 (C-O-C), 1490 (CH benzene), 1556 (C=C), 1614 (C=N), 2965 (C-H), 3164 (N-H). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 7.16 (d, J = 3.6, 1H, H 3′), 7.33 (dd ~ t, J_HH~J_HF = 8.7, 2H, H 3″, 5″), 7.37 (d, J = 3.6, 1H, H 4′), 7.87 (d, J = 8.4, 2H, H 3, 5) 7.93 (dd, 2H, J = 5.4, J = 8.7, H 2″, 6″), 7.99 (d, J = 8.4, 2H, H 2, 6), 9.51 (br s, 3H, NH, NH₂). ¹³C-NMR (75 MHz, DMSO-d₆): 108.5 (C 3′), 111.3 (C 4′), 115.95 (d, ²J_CF = 21.75, C 3″, C 5″), 123.4 (C 3, 5), 126.0 (d, ³J_CF = 8.1, C 2″, C 6″), 126.4 (d, ⁴J_CF = 3.0, C 1″), 126.5 (C 1), 128.7 (C 2, 6), 134.4 (C 4), 151.0 (C 5′), 153.2 (C 2″), 161.8.0 (d, ¹J_CF = 244.1, C 4″), 164.8 (C=NH₂). MS E5⁺[M+H, 100%]: 281.5.
5.1.2.48: 4-(5-(4-Ethylphenyl)furan-2-yl)benzamidine (50).

The light yellow precipitate was filtered off under vacuum and rinsed with hexane to give the product (190mg, 55.0 %): mp 249-252 °C. IR (cm⁻¹): 1287 (C=O-C), 1410 (C-H bending), 1552 (C=C), 1610 (C=N), 2921(C-H), 3125 (N-H).¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 1.18 (t, J_HH 7.2, 3H, CH₃), 2.64 (q, J_HH 7.2, 2H, CH₂), 7.06 (s, 1H, H 3′), 7.29 (m, 3H, H 4′, 3″, 5″′), 7.76 (d, J = 7.5, 2H, H 2″, 6″′), 7.95 (m, 4H, H 2, 3, 5, 6), 9.64 (br s, 3H, NH, NH₂).¹³C-NMR (75 MHz; DMSO): 15.4 (CH₃), 27.9 (CH₂), 107.9 (C 3′), 111.2 (C 4′), 123.3 (C 3, 5), 123.9 (C 2″, 6″), 126.4 (C 1″), 127.3 (C 1), 128.3 (C 3″, 5″′), 128.6 (C 2, 6), 134.4 (C 4), 143.9 (C 4″), 150.7 (C 2′), 154.3 (C 5′), 164.2 (C=NH₂). MS ES⁺ [M+H, 100%]: 291.6.

5.1.2.49: General procedure for the synthesis of N-(2-oxo-2-phenylethyl) benzamide derivatives (68 and 69).
Saturated sodium bicarbonate solution (12.0 ml) and dichloromethane (10.5 ml) were stirred vigorously and cooled in an ice bath. 4-Cyanobenzoyl chloride (6.0 mmol) and 2-amino-1-phenylethanone hydrochloride (4.0 mmol) or 2-amino-4-fluoroacetophenone (compound 70) (4.0 mmol) was added and the reaction mixture was stirred at room temperature for 2 h.¹²⁹ The organic layer was extracted, dried (MgSO₄) and evaporated to give the product.

5.1.2.50: 4-Cyano-N-(2-oxo-2-phenylethyl)benzamide (68).
White solid (980mg, 62.0%): mp 178-180°C. IR (cm\(^{-1}\)): 1595 (C=C), 1639 (C=O), 1686 (C=O), 2227 (C≡N), 3361 (N-H amide). \(^1\)H-NMR (300 MHz, DMSO-d\(_6\)) \(\delta\) ppm: 4.82 (d, J = 5.4, 2H, H 1), 7.57 (t, J = 7.5, 2H, H 3\(^`\),5\(^`\)), 7.69 (t, J = 7.6, 1H, H 4\(^`\) ) 7.98-8.08 (m, 6H, H 2\(^`\),6\(^`\), 2\(^``\), 6\(^``\), 3\(^``\), 5\(^``\)), 9.21 (t, J = 6.3, 1H, NH). \(^13\)C-NMR (75 MHz; DMSO-d\(_6\)): 46.5 (C1), 113.8 (C 4\(^``\)), 118.3 (C≡N), 127.9 (C 2\(^`\), 6\(^``\)), 128.1 (C 3\(^`\), 5\(^`\)), 128.8 (C 2\(^`\), 6\(^`\)), 132.5 (C 3\(^``\), 5\(^``\)), 133.6 (C 4\(^`\)), 134.9 (C 1\(^`\)), 137.9 (C1\(^``\)), 165.2 (C=O of amide), 194.9 (C=O of ketone). MS ES\(^+\) [M+Na, 80%]: 287.1.

5.1.2.51: 4-Cyano-N-(2-(4-fluorophenyl)-2-oxoethyl)benzamide (69).

White powder (200mg, 83%), mp 194-198 °C. IR (cm\(^{-1}\)): 1595 (C=C), 1665 (C=O of amide), 1690 (C=O of ketone), 2227 (C≡N), 3390 (N-H). \(^1\)H-NMR (300 MHz, CDCl\(_3\)) \(\delta\) ppm: 4.87 (d, J = 5.7, 2H, H 1), 7.47 (dd ~ t, J = 8.7, 2H, H 3\(^`\),5\(^`\)), 8.06 (d, J = 8.7, 2H, H 3\(^``\), 5\(^``\)), 8.11 (d, J = 8.4, 2H, H 2\(^``\),6\(^``\)), 8.19 (dd, 2H, J = 2.6, J = 5.7, H 2\(^`\),6\(^`\)), 9.22 (t, J = 5.7, 1H, NH). \(^13\)C-NMR (75 MHz; DMSO-d\(_6\)): 46.9 (C 1 confirmed by DEPT), 116.0 (C 4\(^``\)), 116.5 (C 3\(^`\), 5\(^`\)), 118.6 (C≡N), 128.61 (C 2\(^`\), 6\(^`\)), 131.4 (C 6\(^`\)), 131.5 (C 2\(^`\)), 132.2 (C 1\(^`\)), 133.0 (C 3\(^``\), 5\(^``\)), 138.3 (C 1\(^``\)), 164.7 (C4\(^`\)), 165.7 (C=O of the amide), 194.1 (C=O of ketone). MS ES\(^+\)[M-H, 80%]: 281.1.

5.1.2.52: General procedure for the synthesis of benzonitrile (51 and 52).

To a solution of 4-cyano-N-(2-oxo-2-phenylethyl)benzamide (68) or 4-cyano-N-(2-(4-fluorophenyl)-2-oxoethyl)benzamide (69) (3.8 mmol) in acetic anhydride (18.0
ml) was added concentrated sulfuric acid (6 drops). The reaction mixture was stirred at room temperature for 20 min. The resulting mixture was neutralized by addition of saturated NaHCO₃ and the product extracted with ethyl acetate.

5.1.2.53: 4-(5-Phenyl-oxazol-2-yl)benzonitrile (51).

![Chemical structure of 51]

White precipitate (800mg, 79 %): mp 181-183 °C. IR (cm⁻¹): 1276 (C-O-C), 1591(=C-C), 2224 (C≡N), 3059 (C-H). 1H-NMR (300 MHz, DMSO-d₆) δ ppm: 7.42 (t, J= 7.5, 1H, H 4″), 7.52 (t, J= 7.5, 2H, H 3″, 5″), 7.88 (d, J = 7.5, 2H, H 2″, 6″), 7.95 (s, 1H, H 4′), 8.02 (d, J = 8.4, 2H, H 2, 6), 8.24 (d, J = 8.4, 2H, H 3, 5). ¹³C-NMR (75 MHz, DMSO-d₆): 112.6 (C 4), 118.4 (C= N), 124.4 (C 2″, 6″), 124.8 (C 4″), 126.5 (C 2, 6), 126.9 (C 4″), 129.0 (C 1″), 129.1 (C 3″, 5″), 130.5 (C 1), 133.1 (C 3, 5), 151.8 (C 5′), 158.6 (C 2′), MS ES⁺ [M-H, 72%]: 246.1.

5.1.2.54: 4-(5-(4-Fluorophenyl)oxazol-2-yl)benzonitrile (52).

![Chemical structure of 52]

Brown powder (500mg, 87%), IR (cm⁻¹): 1221 (C-O-C), 1595 (C=C), 1643 (C=N), 2227 (C≡N), 2925 (C-H). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 1.91 (s, CH₃ of acetate), 7.38 (dd-t, J = 9.0, 2H, H 3″, 5″), 7.94-7.98 (m, 3H, H 4′, 2″,6″), 8.03 (d, J = 8.1, 2H, H 3, 5), 8.25 (d, J = 8.1, 2H, H 2, 6), 12.01 (br s, NH). ¹³C-NMR (75 MHz; DMSO-d₆): 20.9 (CH₃), 112.6 (C 4), 116.1 (d, JCF = 22.1, C 3″, 5″), 118.4
(C≡N), 123.7 (C 4’), 124.6 (C 1’’), 126.5 (C 2, 6), 126.7 (d, J\textsubscript{CF} = 8.4, C 2’’), 130.4 (C 1), 133.1 (C 3, 5), 151.0 (C 5’), 158.6 (C 2’), 162.5 (d, J\textsubscript{CF} = 182.5, C 4’’), 171.9 (C=O of acetate).

5.1.2.55: General procedure for the synthesis of N-hydroxy amidines (amidoximes) (53 and 54).

Hydroxylamine hydrochloride (10.0 mmol) was suspended in dry DMSO and the mixture was cooled to 0 °C. Potassium tert-butoxide (10.0 mmol) was added portionwise to the reaction mixture under an argon atmosphere and the reaction mixture was stirred at room temperature for 1 h. Compound (51) or compound (52) (1.0 mmol) was added and the reaction mixture was stirred at room temperature for 1-3 days and monitored by TLC (10.0 % MeOH/CHCl\textsubscript{3}).

5.1.2.56: N-Hydroxy-4-(2-phenyloxazol-5-yl) benzimidamide (53).

The reaction mixture was poured into iced water and the precipitate was filtered off under vacuum, rinsed with hexane to give a white solid (421 mg 88.0 %): mp 215-217 °C. IR (cm\textsuperscript{-1}): 1544 (C=C), 1675 (C=O), 3051 (C-H), 3343 (OH), 3419 (NH). \textsuperscript{1}H-NMR (300 MHz, DMSO-d\textsubscript{6}) δ ppm: 5.94 (br s, 2H, NH\textsubscript{2}), 7.40 (t, J = 7.5, 1H, H 4’’), 7.52 (t, J = 7.5, 2H, H 3’’, 5’’), 7.85-7.88 (m, 5H, H 3, 4’, 5, 2’’, 6’’), 8.09 (d, J = 8.4, 2H, H 2, 6), 9.85 ( s, 1H, OH). \textsuperscript{13}C-NMR (75 MHz; DMSO-d\textsubscript{6}): 124.1 (C 2’’), 124.3 (C 4’), 125.7 (C 3, 5), 125.9 (C 3’’, 5’’), 126.9 (C 1’’), 127.3 (C 1), 128.6 (C 4’’), 129.1 (C 2, 6), 135.2 (C4), 150.1 (C 5’), 150.9 (C 2’), 159.9 (C=NOH). MS ES\textsuperscript{+} [M+H, 100%]: 280.1.

5.1.2.57: 4-(5-(4-Fluorophenyl)oxazol-2-yl)-N-hydroxybenzimidamide (54).
The reaction mixture was poured into iced water; the precipitate was filtered under vacuum and rinsed with hexane to give the product as a yellow solid (225 mg, 44.0 %): mp 194-197 °C. IR (cm⁻¹): 1229 (C=O-C), 1505 (C=N), 3190 (C-H), 3343 (OH), 3425 (NH). ¹H-NMR (300 MHz, DMSO-d₆) δ ppm: 5.93 (s, NH₂), 7.37 (dd-t, J = 9.0, 2H, H 3```, 5```), 7.85 (d, J = 8.1, 3H, H 2, 6, 4''), 7.93 (dd, J = 8.4, 4''H₂ = 5.4, 2H, H 2```, 6````), 8.08 (d, J = 8.4, 2H, H 3, 5), 9.85 (s, OH). ¹³C-NMR (75 MHz; DMSO-d₆): 116.2 (d, ²JₐCF = 21.9, C 3```, 5````), 124.0 (C 4''), 124.1 (C 4), 125.7 (C 2, 6), 125.9 (C 3, 5), 126.4 (d, ³JₐCF = 8.3, C 2``, 6````), 126.8 (C 5'''), 135.2 (C 1), 150.1 (d, ⁴JₐCF = 4.1, C 1````'), 159.9 (C=N), 162.1 (d, ¹JₐCF = 245.2, C 4''''). MS ES⁺ [M+H, 100%]: 298.1.

5.1.2.58: General procedure for the synthesis of amidines (55 and 56).
Ammonium formate (5.0 mmol) and Pd/C (10.0 % g/ml) were added slowly to a solution of the amidoxime (53 or 54) (1.0 mmol) in glacial acetic acid. The reaction mixture was heated at reflux and monitored by TLC (1% MeOH / 99% CHCl₃). Upon consumption of the starting amidoxime, the reaction mixture was cooled to room temperature, filtered through Celite 521 and rinsed with glacial acetic acid. The reaction mixture was quenched with 1.0 M NaOH, adjusting the pH to ~7.0 and stirred at room temperature for 1 h.

5.1.2.59: 4-(5-Phenyloxazol-2-yl)benzamidine (55).

The grey-coloured precipitate was filtered off under vacuum, rinsed with H₂O and re-crystallized from ethanol to give the compound as a pale red solid (60 mg, 23.6 %): mp 193-195 °C. IR (cm⁻¹): 1113 (C-O-C), 1432 (C-H bending), 1574 (C=C), 1639
(C=N), 3037 (C-H), 3353 (C=N-H), 3458 (NH2). 1H-NMR (300 MHz, DMSO-d6) δ ppm: 5.94 (br s, 2H, NH2), 7.41 (t, J = 5.5, 1H, H 4``), 7.52 (t, J = 5.7, 2H, H 3``, 5``), 7.88 (d, J = 5.4, 2H, H 2`` , 6``), 7.91 (s, 1H, H 4`), 7.96 (d, J = 6.0, 2H, H 2, 6), 8.12 (d, J = 6.6, 2H, H 3, 5). 13C-NMR (75 MHz; DMSO-d6): 124.1 (C 2``, 6``), 124.4 (C 4`), 125.6 (C 2, 6), 127.3 (C 4``), 127.4 (C 3``, 5``), 127.8 (C 4), 128.7 (C 1), 129.1 (C 3, 5), 151.0 (C 5`), 152.7 (C 2'), 159.8 (C=NH). One quaternary carbon (C1``) not observed. MS ES+ [M+H, 100%]: 264.1.

5.1.2.60: 4-(5-(4-Fluorophenyl) oxazol-2-yl)benzamidine (56).

The beige-coloured precipitate was filtered off under vacuum, rinsed with H2O and re-crystallized from ethanol to give the product (75 mg, 79 %). mp 230-233 ºC. IR (cm⁻¹): 1603 (C=N), 3125 (N-H amide), 3353 (NH2). 1H-NMR (300 MHz, DMSO-d6) δ ppm: 7.38 (dd ~ t, 3JHF = 8.4, 2H, H 3`` , 5``), 7.84-7.94 (m, 4H), 8.06 (dd, 4JHF = 13.8, 4JHF = 8.4, 2H, H 2``, 6``), 8.18(t, J = 8.4, NH), 8.26 (d, J = 6.6, 2H, H 2, 6). 13C-NMR (75 MHz; DMSO-d6): 97.2 (C 4`), 116.2 (d, 2JCF = 21.9, C 3``, 5``), 126.7 (d, 3JCF = 11.2, C 2``, 6``), 127.68 (C 2, 6), 133.2(C 3, 5), 152.7 (C). MS ES+ [M+H, 70%]: 282.1.

5.1.2.61: 2-Amino-1-(4-fluorophenyl)ethan-1-one (70).

Hexamethylenetetramine (0.164g, 1.17mmol) added to a solution of 2-bromo-4-fluoroacetophenone (0.25g, 1.15mmol) in (15.0 ml) chloroform. The mixture was stirred at room temperature for 16hr, after which the white precipitate was collected by suction filtration and suspended in methanol (10 ml). The mixture was heated at
reflux for 4 h, after which the solvent was removed by evaporation. The resulting solid was purified by column chromatography (5% methanol / 95% DCM) to give the product as a reddish brown powder (500mg, 70%). mp 198-200 °C. IR (cm\(^{-1}\)): 1595 (C=C), 1670 (C=O), 3004 (CH\(_2\)), 3113 (C-H), 3400 (NH\(_2\)). \(^1\)H-NMR (300 MHz, CD\(_3\)OD) δ ppm: 4.62 (s, 2H, H\(_2\)), 7.33 (dd~ t, J\(_{HH} \sim J_{HF} = 8.7\), 2H, H 3’, 5’), 8.12-8.17 (m, 2H, H 2’, 6’). \(^13\)C-NMR (75 MHz; CD\(_3\)OD): 89.7 (C 2), 115.2 (d, \(^4\)J\(_{CF} = 22.5\), C1’), 115.8 (d, \(^2\)J\(_{CF} = 22.3\), C 3’, 5’), 130.9 (d, \(^3\)J\(_{CF} = 9.7\), C 2’, 6’), 166.6 (d, \(^1\)J\(_{FC} = 253.0\), C 4’), 190.5(C=O). MS ES+ [M+H, 100%]: 154.0.

5.1.2.62. Reduced N-ribosylnicotinamide (NRH)

NADH (0.5 g, 0.69 mmol) was dissolved in 20 ml of 0.4 M sodium carbonate/bicarbonate buffer, pH 10.0, and incubated at 37°C for 16 hr with 0.1 unit of phosphodiesterase 1 type IV (phosphodiesterase I from Crotalus atrox western diamondback rattlesnake) and 500 units of alkaline phosphatase type VII-S. After complete digestion of NADH the mixture was freeze dried. The dried powder was extracted with methanol (5×5 ml), and this methanol extract was dried by rotary evaporation and dissolved in 5 ml of water. The NRH was then purified by preparative HPLC performed on a microsorb C18 column (21.2 X 250 mm), eluted with 10 % methanol in water over 15 minutes at a flow rate of 15 ml/min, using a 100 µl injection size. The NRH peak was detected at a wavelength of 350 nm. This peak from each injection was collected, freeze dried and stored at 4°C. \(^1\)H-NMR (400 MHz; D\(_2\)O) δ ppm: 2.92 (dd \(^3\)J = 5.4, \(^4\)J = 1.8, 2H, H6), 3.54 (dd, \(^2\)J = 12.4, \(^3\)J = 4.8, 1H17), 3.60 (dd, \(^2\)J = 13.4, \(^3\)J = 4.0, 1H, H17), 3.80-3.85 (m, H12), 3.82 (dd, J=8.0, J=4.0, H13), 4.05 (dd, J = 8.0, J= 4.0, H14), 4.73 (d, J = 8, H10), 4.85 (dt, J\(_{2,6} = 8.0\), J\(_5 =4.0\), H1), 5.95 (dq, J = 8.0, J=2.0, H2), 7.00 (d, J=4.0, H4).
5.2 Enzyme assay

5.2.1 Inhibition of recombinant human NQO2 and NQO1 enzyme activity

5.2.1.1 Materials and methods
The enzyme activity measured spectrophotometrically using a BECKMAN DU 7400 spectrophotometer. The buffer heated up to 37 °C using Grant JB series water bath. Recombinant human NQO2 and NQO1 enzymes were purchased from Sigma-Aldrich. NRH used was synthesized according to the procedure described by Long and coworkers, in section (4.1.2). NADH and Dichlorophenolindophenol (DCPIP) were purchased from Sigma-Aldrich.

5.2.1.2 General procedure for the NQO2 and NQO1 enzyme activity
The rates of the NQO2 and NQO1 enzyme activity were determined spectrophotometrically by recording the rates of DCPIP colour change at 600 nm, over 1 minute at 37 °C. The cuvette is 1.0 cm in width and containing a final volume of 1.0 ml: 940 µl phosphate buffer (50 mM, pH 7.4), 10 µl NQO2 (5 x 10^(-2) mg/ml), 20 µl NRH (10 mM) in case of NQO2 and 20 µl NADH (10 mM) in case of NQO1, 20 µl DCPIP (2 mM), 10 µl DMSO or NaOH (in the control sample), and 10 µl inhibitor (5 samples of different concentration).

All the experiments were performed in triplicates, three independent times. In each independent experiment fresh solutions of NRH (10 mM), DCPIP (2 mM) and NQO2 and NQO1 (5 x 10^(-2) mg/ml) were prepared. NRH, NADH, NQO1 and NQO2 were dissolved in phosphate buffer (50 mM, pH 7.4) and kept at 0 °C during the experiment time. DCPIP was dissolved in deionized water. The inhibitors stock solutions were prepared by dissolving the inhibitors in DMSO or 0.13M NaOH to give 10Mm solutions. A 10-fold serial dilution was completed using DMSO or 0.13M NaOH to end up with five different concentrations of the inhibitors which is in between 1nM -10000nM for NQO2, and 0.01-100µM for NQO1.

Both NQO1 and NQO2 were prepared by dissolving them in phosphate buffer. 5µM of FAD and 250 mM of sucrose added to end up by 5mg/ml stock solution.

IC_{50} values were determined using non-linear curve fitting in the Graph pad prism software. The curves were obtained from plotting enzyme activity as a percent of the
control versus the log concentration of the inhibitor used. IC\textsubscript{50} values were determined as 50% reduction in the enzyme activity compared to control (100%).
B027

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100

B028

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100

B030

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100

B031

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100

B032

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100

B034

% Activity

Concentration (nm)

0 10 20 30 40 50 60 70 80 90 100
Appendix II

Paracetemia % for compounds 43, 44, 45, 46
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