Discovery of potent 4-aminoquinoline hydrazone inhibitors of NRH:quinoneoxidoreductase-2 (NQO2)

Buthaina Hussein[a,§], Balqees Ikhmais[a,§], Manikandan Kadirvel[a], Rachael N. Magwaza[a], Gavin Halbert[b], Richard A. Bryce[a], Ian J. Stratford[a,*], Sally Freeman[a,*]

[a]Division of Pharmacy & Optometry, School of Health Sciences, Faculty of Biology, Medicine & Health, University of Manchester, Manchester, M13 9PT, UK

[b]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0NR, UK

*Corresponding authors

Email: Sally.Freeman@manchester.ac.uk & Ian.Stratford@manchester.ac.uk

§ Authors have made an equal contribution

ABSTRACT

(NRH):quinone oxidoreductase 2 (NQO2) is associated with various processes involved in cancer initiation and progression probably *via* the production of ROS during quinone metabolism. Thus, there is a need to develop inhibitors of NQO2 that are active *in vitro* and *in vivo*. As part of a strategy to achieve this we have used the 4-aminoquinoline backbone as a starting point and synthesized 21 novel analogues. The syntheses utilised *p*-anisidine with Meldrum's acid and trimethyl orthoacetate or trimethyl orthobenzoate to give the 4-hydrazin-quinoline scaffold, which was derivatised with aldehydes or acid chlorides to give hydrazone or hydrazide analogues, respectively. The hydrazones were the most potent inhibitors of NQO2 in cell free systems, some with low nano-molar IC_{50} values. Structure-activity analysis highlighted the importance of a small substituent at the 2-position of the 4-aminoquinoline ring, to reduce steric hindrance and improve engagement of the scaffold within the NQO2 active site.

Cytotoxicity and NQO2–inhibitory activity *in vitro* was evaluated using ovarian cancer SKOV-3 and TOV-112 cells (expressing high and low levels of NQO2, respectively). Generally, the hydrazones were more toxic than hydrazide analogues and further, toxicity is unrelated to cellular NQO2 activity. Pharmacological inhibition of NQO2 in cells was measured using the toxicity of CB1954 as a surrogate end-point. Both the hydrazone and hydrazide derivatives are functionally active as inhibitors of NQO2 in the cells, but at different inhibitory potency levels. In particular, 4-((2-(6-methoxy-2-methylquinolin-4-yl)hydrazono)methyl)phenol has the greatest potency of any compound yet evaluated (53nM), which is 50-fold lower than its toxicity IC_{50}. This compound and some of its analogues could serve as useful pharmacological probes to determine the functional role of NQO2 in cancer development and response to therapy.

Keywords

NQO2 inhibitors; anticancer; 4-aminoquinoline; hydrazine; hydrazine; ovarian cancer; SKOV-3 cells; TOV-112D cells; CB1954.

1. Introduction

There is continued interest in dissecting the role of the flavoprotein NQO2 (Quinone oxidoreductase 2, QR2, EC.1.10.99.2) in cancer biology and in the development of various neurological conditions.[1-9] NQO2 is a FAD-containing protein capable of oxidizing a variety of analogues of dihydronicotinamide. It is a structural analogue of NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC.1.6.99.2) and while the two enzymes have similar properties there are major functional differences.[10-12] In particular, the two enzymes catalyse the reduction of various quinones into hydroquinones.[12-15] The reduction process proceeds *via* a ping-pong mechanism in which two electrons are transferred from the co-substrate to FAD molecule and subsequently to the substrate.[16, 17] However, a major difference between the enzymes is that whereas NQO1 can use NADPH or NADH as reducing cofactors, the enzymatic function of NQO2 can only be supported by nicotinamide derivatives such as N^1-ribosyl-, N^1-methyl-, N^1-benzyl-dihydronicotinamide (NRH) and 1-carbamoylmethyl-3-carbamoylpiridinium iodide (EPR) as reducing cofactors.[12, 17-21]

There is evidence linking NQO2 activity to p53 function and degradation, to NFkB activity and to the induction of metastases in prostate cancers.[22-24] Also, NQO2 is reported to be highly expressed in a number of cancer cell lines and hence needs to be evaluated as a potential therapeutic target in the treatment of cancer.

Structurally diverse inhibitors of NQO2 are known, which include resveratrol, casimiroin, melatonin, chloroquine and imatinib. However, none are considered as ideal NQO2 inhibitors for therapeutic intervention as they have other pharmacological effects. Nolan *et al.* (2012) conducted a virtual screening of the NCI database to identify potential new NQO2 inhibitors.[25] Lead inhibitors were identified that belonged to the polyaromatic, ellipticine, acridine, quinoline and furan-amidine classes of compound. This screen further identified seven quinoline compounds (Table 1) that showed potent inhibition of NQO2.[25] We have thus used 4-aminoquinolines (Figure 1) as the basis for the design of novel inhibitors of NQO2 and here we report the synthesis and evaluation of three different series of compounds. These are based on hydrazine, hydrazide and hydrazone-quinolines and were chosen because these scaffolds are emerging as important functional groups in drug design as they are present in compounds possessing diverse biological activities, [26, 27] such as vasodilator, anti-tuberculosis, anti-tumour, anti-microbial, anti-platelet, anti-inflammatory, anti-convulsant and anti-oxidant activities.[27, 28]

Table 1. NSC numbers of seven aminoquinolines and their IC_{50} values for the inhibition of recombinant NQO2.[25] Compound structures are given in the SI.

NSC number	IC_{50} (μM) ± SD
13484	0.55 ± 0.05
76750	0.50 ± 0.06
101984	0.64 ± 0.05
270904	0.17 ± 0.09
273829	0.25 ± 0.05
617933	0.04 ± 0.01
617939	0.07 ± 0.01

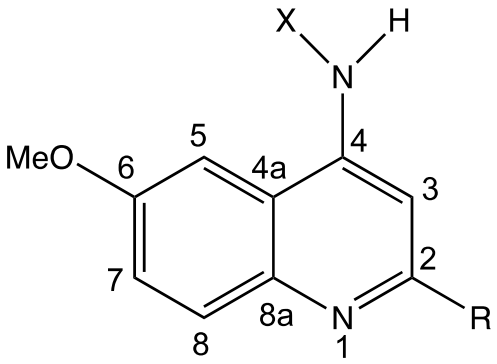
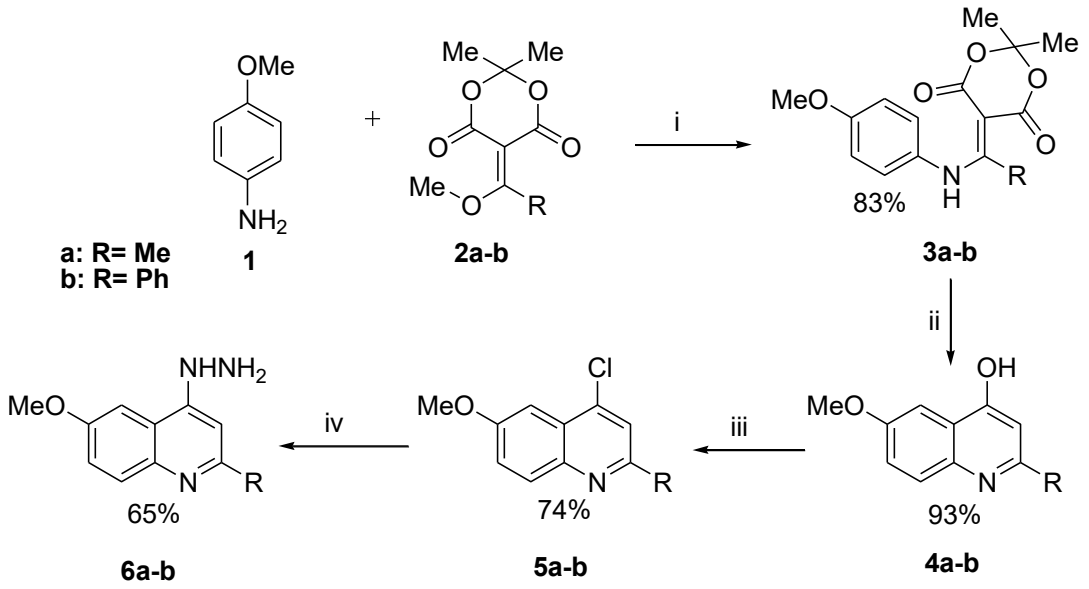


Figure 1. The 4-aminoquinoline scaffold.

2. Results and discussion

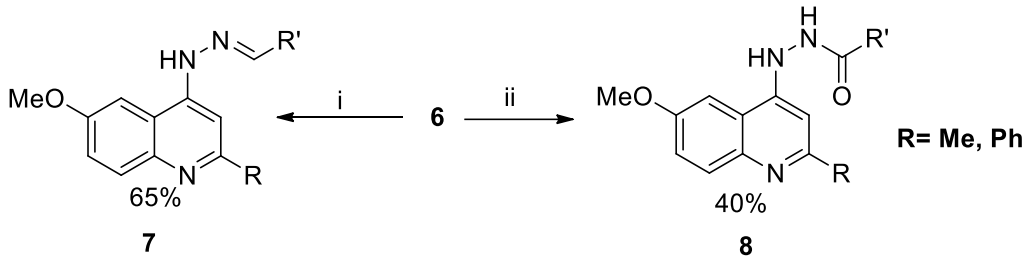
2.1 Synthesis

The syntheses of 4-hydrazine-quinolines **6a-b** are shown in **Scheme 1**. This first requires the synthesis of the quinoline ring (**4a-b**) by reacting p-anisidine with methoxyethylidene Meldrum's acid [29] **2a** or phenoxyethylidene Meldrum's acid **2b**,[29, 30] to give enamine intermediates **3a-b** [31]. The thermal pericyclic reaction of **3a-b** takes place at high temperature (microwave, diphenyl ether, 200 ˚C, 30 min) to give intermediates **4a-b**[29, 32] with the release of acetone and carbon dioxide.[33] 6-Methoxy-quinolin-4-ols **4a-b** were then treated with phosphorus oxychloride to give 4-chloroquinolines **5a-b**,[29] which were then reacted with hydrazine monohydrate to give the 4-hydrazine-quinolines **6a-b**[34, 35] in very good yields (Scheme 1).



Scheme 1. Synthesis of 4-hydrazine-quinolines **6a (R = Me)** and **6b (R = Ph)**. Reagents and conditions: (i) reflux, 3h; (ii) 200 °C, 30 min, MW; (iii) $POCl_3$; (iv) NH_2NH_2.

Eighteen 4-hydrazone quinoline derivatives **7a-r** were synthesized in good yields (30-90 %) by the reaction of the 4-hydrazine-quinoline **6a-b** with a range of aldehydes (Scheme 2) (Table 2).[35, 36] The syntheses of the 4-hydrazide quinoline derivatives **8** were achieved by reacting the 4-hydrazinequinoline scaffold **6b** with the appropriate aryl chloride in the presence of pyridine (Scheme 2).[37] Two hydrazide quinoline derivatives **8a-b** were prepared, however they were much less active than the hydrazone derivatives (Table 2), therefore further hydrazide analogues were not synthesised.



Scheme 2. Synthetic pathways for 4-hydrazonequinoline **7** and 4-hydrazidequinoline **8** derivatives: Reagents: (i) R'C(O)H, (ii) R'COCl.

Table 2. Sides chains (R and R') of the parent hydrazine- (**6a**), 4-hydrazone- (**7a-r**) and 4-hydrazide- (**8a-b**) quinoline derivatives, % yields for the syntheses, IC_{50} values for inhibition of recombinant NQO2 and NQO1, and toxicity towards SKOV-3 cells (following 24 h and 96 h exposure).

Entry	R	R`	Yield %	NQO2 IC_{50} (nM±SE)	NQO1 IC_{50} (nM±SE)	Toxicity IC_{50} (μM±SE)	
						24 h	96 h
6a	-	-	80	2047±132	-	84±16	41±8.9
7a	Me	4-Fluorophenyl	56	103±16.3	-	2.3±0.18	2.3±0.06
7b	Me	Phenyl	52	519±14.5	-	1.77±0.09	1.73±0.09
7c	Me	4-Imidazoyl	76	71±14.9	38700 ±5200	32.0±2.08	4.2±0.56
7d	Me	4-Hydroxyphenyl	53	55±6.6	133900 ±43600	2.75±0.26	2.3±0.26
7e	Me	3-Pyridinyl	34	10±0.7	-	-	-
7f	Me	Benzyl	30	15±4.4	Inactive	50±1	40.67±2.3
7g	Me	2-Hydroxy-3-methoxyphenyl	89	13±3.1	73400 ±48700	2.1±0.25	2.0±0.06
7h	Me	2-Nitrofuranyl	75	18±1.2	-	-	-
7i	Me	4-Benzoic acid	78	64±5.9	Inactive	>100	70.0±4.93
7j	Me	4-Nitrophenyl	92	83±8.0	-	-	-
7k	Me	3,5-Dihydroxylphenyl	75	46±8.6	-	-	-
7l	Me	3-Hydroxyphenyl	33	137±15.9	-	-	-
7m	Ph	3-Pyridinyl	89	372±109.4	Inactive	2.8±0.36	2.2±0.12
7n	Ph	4-*N,N*-Dimethylaniline	60	>10000	-	1.57±0.42	1.53±0.44
7o	Ph	4-Nitrophenyl	40	1420±204	-	-	-
7p	Ph	Benzyl	26	125±2.8	-	-	-
7q	Ph	2-Hydroxy-3-methoxyphenyl	91	595±178	Inactive	2.88±0.22	2.73±0.22
7r	Ph	4-Imidazoyl	77	90±10.7	425±90	2.47±0.30	2.17±0.22
8a	Me	4-Fluorophenyl	74	2906±433	Inactive	>100	>100
8b	Me	Phenyl	28	472±23.3	-	68.3±4.06	43.3±6.49
Resveratrol				900±7.8	-	91.7±4.4	36.3±1.3
9-Aminoacridine (9AA)				440±90	-	5.43±0.47	2.78±0.17

2.2 Inhibition of recombinant human NQO2 and NQO1 enzyme activity

The ability of the synthesized compounds to inhibit the NQO2 enzyme was determined by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) using EPR as a co-substrate.[18] The IC_{50} values for the hydrazonequinoline derivatives **7a-r** are given in Table 2. Results are also given for the standard NQO2 inhibitor resveratrol and for 9-aminoacridine (9-AA), a compound we showed previously to be a potent inhibitor of NQO2.[38] Many of the hydrazonequinolines are excellent NQO2 inhibitors with IC_{50} values less than 100 nM, being more potent than both resveratrol and 9-AA.

The nucleus of the hydrazonequinoline derivatives, 4-hydrazine-6-methoxy-2-methyl-quinoline (**6a**), was also tested against recombinant human NQO2, giving an IC_{50} of 2047 nM. The greater activity of the 4-hydrazonequinoline derivatives when compared with the scaffold (**6a**), confirmed that the inhibitory activity can be attributed to the hydrazone functionality in the compounds.

We then went on to evaluate the NQO2-inhibitory potency of representative hydrazidequinolines **8a-b (Table 2)** which showed modest inhibitory activity. The lack of improvement in inhibitory activity of the hydrazide- versus the hydrazonequinolines precluded any further development of this series.

To test the enzyme selectivity of the hydrazones and hydrazides, some of the compounds were assayed for their activity against NQO1 (Table 2). Dicoumarol, the standard inhibitor of NQO1 has an IC_{50} value of around 4nM whereas only compounds **7c**, **7d, 7g** and **7r** showed any (μM) activity. Compounds **7f, 7i, 7m, 7q** and **8b** were completely inactive towards NQO1, therefore are NQO2 specific. Compound **7r** had an inhibitory activity towards NQO1 of 425 nM, but is still much more selective towards NQO2.

2.3 Structure-activity analysis

Probing NQO2 enzyme inhibition using the range of synthesized compounds indicate some key structural features. Compounds **7a-7l** (2-Me group), showed lower IC_{50} values than the corresponding compounds **7m-7r** (2-Ph group). For example, 2-methyl analogue **7e** is 37-fold more potent than its corresponding 2-phenyl analogue **7m** (Table 1). This variation in potency may be explained by different binding modes of **7e** and **7m** in the NQO2 active site: for the 2-methyl substituted compound **7e** (Figure 2A), we find that computational docking predicts that the compound binds well in two distinct orientations in the NQO2 site. In the first binding pose, the aryl-hydrazone moiety of **7e** fully enters into the NQO2 active site, allowing π-stacking of the 3-pyridinyl group, as well as the quinoline scaffold, against the FAD isoalloxazine ring (yellow, Figure 2A). In the second pose, the ligand is in a 180° flipped orientation, such that the 3-pyridinyl group can occupy a pocket formed by residues Val160, Asn161 and Phe178' (cyan, Figure 2A). By contrast, docking of the 2-phenyl analogue **7m** leads to a markedly different predicted orientation in the NQO2 cavity (Figure 2B): although the quinoline scaffold can π-stack with the FAD cofactor, the additional steric bulk of the 2-phenyl group of **7m** precludes the aryl-hydrazone from entering the active site; instead the aryl-hydrazone and 3-pyridinyl moieties project out of the active site without forming specific amino acid contacts except a weak nonpolar contact with the sidechain of Ile128 (Figure 2B).

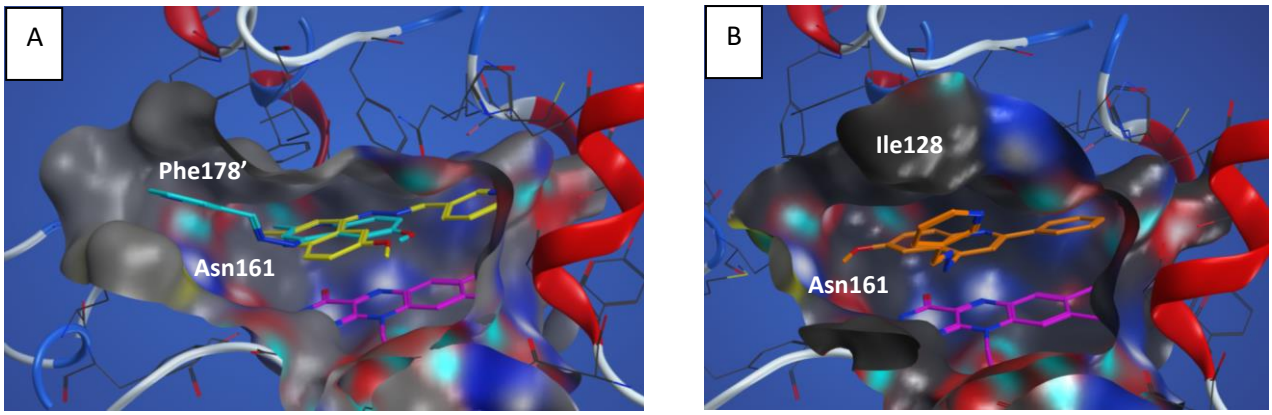


Figure 2. (A) Docked poses of 2-methyl analogue **7e** in the active site of NQO2. (B) Docked pose of 2-phenyl analogue **7m** in the active site of NQO2.

We further note that substituents on the aromatic ring have a noticeable effect on the inhibitory activity of the synthesized compounds. The *para*-fluorine substitution in compound **7a** makes the compound five-fold more potent compared to unsubstituted-phenyl analogue **7b** (Table 2). In the same manner, compound **7d** with a *para*-hydroxyl substituent is ten-fold more potent than unsubstituted-phenyl analogue **7b**. 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 **7d, 7g, 7i, 7k** and **7l**. This may suggest that introducing a group (such as F, OH) that has the ability to make hydrogen bonds with the amino acid residues, such as the proximal polar residue Asn161 (Figure 2A), is favourable. Other potent inhibitor compounds have been shown to form interactions with this residue, such as resveratrol analogues.[39]

2.4 Evaluation of cytotoxicity

SKOV-3 ovarian cancer cells were used to first assess the toxicity of the hydrazone- and hydrazide-quinolines. This cell line was chosen as these cells showed relatively high levels and activity of NQO2 (unpublished data). Cells were exposed to the different drugs for 24 h or 96 h prior to an assessment of toxicity using the MTT assay. Typical survival data is illustrated in Figure 3 for compounds **7d** and **7f** given to cells for either 24 h or 96 h. It is clearly that **7d** is the more toxic of the two drugs. Survival curves were obtained for each drug and from these curves, the concentration of drug required to reduce cell survival to 50% (IC_{50}) was determined (Table 2). For comparison, the toxicity of the NQO2 inhibitors resveratrol and 9-AA were also recorded. Since it was considered a possibility that the N=C double bond of the hydrazone moiety might be labile in cellular milieu resulting in hydrolysis to give the parent compound **6a**, this was also assessed for toxicity (Table 2).

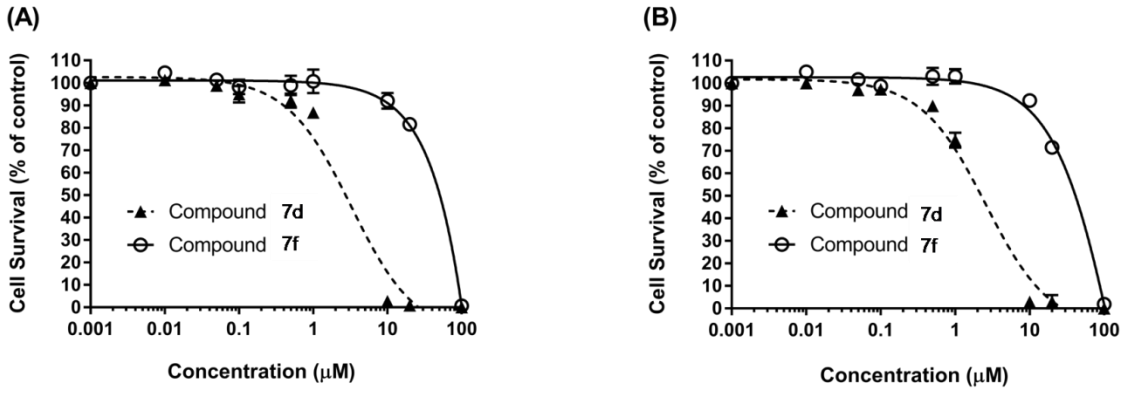


Figure 3. Toxicity of selected 4-hydrazonequinolines **7d** and **7f** towards SKOV-3 ovarian cancer cells as measured by the MTT assay. Panels A and B show values of cell survival following 24 h and 96 h exposure to the different compounds, respectively. Survival values (± SE) were derived from four independent experiments.

For all the hydrazone-and hydrazide-quinolines evaluated, with exception of **7c**, the levels of toxicity were not dependent on exposure time. This may suggest that the drugs are degraded in the culture medium within the first 24 h so that none is available to elicit toxicity at longer times. By comparison of hydrazone **7b** (R' = Ph) with hydrazide **8b** (R = Ph), it shows that the hydrazone shows greater toxicity than the hydrazide. The similarity in the toxicity of structurally diverse hydrazones suggests that this moiety might be the main contributor to the compounds' toxicity**.** It is noteworthy that the parent hydrazine **6a** is less toxic than most of the hydrazones, which further suggests that the hydrazone moiety is primarily responsible for the toxicity.

Further inspection of the toxicity data in Table 2 suggests there is no obvious correlation between the toxicity of the compounds and their ability to inhibit the activity of recombinant NQO2. To interrogate this further, we evaluated the toxicity of some of the hydrazones and hydrazides along with resveratrol and 9-AA in isogenic pairs of cell lines where the expression of NQO2 was genetically altered (unpublished data). To do this, we first used SKOV-3 cells which were stably transfected with doxycycline-inducible shRNA directed to a portion of the NQO2 gene (SKOV-3 sh27 cells). This resulted in a reduction of cellular activity of NQO2 from 140.5±4.0 to 93.8±2.2 nmol/min/mg of protein in cells treated with and without doxycycline (Dox) (unpublished data). Secondly, and in contrast, we also used TOV-112D ovarian cancer cells, which normally have barely detectable levels of NQO2 and we constitutively over-expressed NQO2 in these cells (TOV-112D NQO2-OE cells). This resulted in a change in activity from 5.2±0.5 nmol/min/mg of protein in the wild type cells to 128.3±4.9 nmol/min/mg of protein in the transfected cells (unpublished data). The values of IC_{50} for the control and transfected cell lines exposed to the different drugs for 96 h are given in Table 3. An important feature of this data is that results for "control" cells are based on the following. Firstly, for the SKOV-3 cells, we used cells that were transfected with a non-targeted vector (NTC), both NTC cells and the sh27 cells were then treated

with doxycycline (which only gives down-regulation of NQO2 in the sh27 cells). Secondly, the TOV-112D cells were transfected with an empty vector (EV). The results show that none of the compounds have any dependence on NQO2 activity for their toxicity and this includes those compounds that are standard inhibitors of NQO2 (resveratrol and 9-AA).

Table 3. Toxicity of the various compounds towards isogenic cell lines where the activity of NQO2 has been either down regulated or over-expressed (see text). Cells were exposed to compounds for 96 h prior to measuring toxicity using the MTT assay. IC_{50} values (± SE) were determined from dose-response curves generated from three independent experiments.

Compound Number/ Name	96 h Toxicity (IC_{50} (μM) ± SE)			
	SKOV-3		TOV-112D	
	NTC	Sh27	EV	NQO2-OE
	+Dox	+Dox		
7c	6.9 ± 0.30	6.73 ± 0.41	3.65 ± 0.29	3.72 ± 0.24
7d	2.87 ± 0.15	2.58 ± 0.10	3.17 ± 0.22	2.98 ± 0.38
7f	44.67 ± 6.9	49.67 ± 0.88	42.5 ± 2.57	41.0 ± 5.0
7g	2.7 ± 0.1	2.78 ± 0.17	3.4 ± 0.10	3.77 ± 0.19
7i	65.0 ± 2.89	61.3 ± 3.48	>100	>100
8a	>100	>100	61.3 ± 3.3	57.3 ± 2.9
Resveratrol	29.0 ± 5.51	39.67 ± 2.91	10.67 ± 0.6	11.0 ± 0.58
9AA	2.53 ± 0.32	2.57 ± 0.20	3.6 ± 0.21	3.15 ± 0.22

2.5 Functional inhibition of NQO2 in cancer cells

One of the purposes for seeking novel inhibitors of NQO2 is to identify potential pharmacological probes for the activity of NQO2, as well as its function, in living cells. The fact that none of the hydrazone- and hydrazide- quinolines show any dependence for toxicity on NQO2 inhibitory potency (as measured by activity against recombinant protein) means that they could be potentially useful probes. To test this we established whether these compounds were functionally active as NQO2 inhibitors in cells. To do this, we took advantage of a unique property of NQO2, which is to reductively activate the drug CB1954 to a cytotoxic species.[25] Thus, cells with relatively high levels of NQO2 will be sensitive to CB1954 (as a consequence of NQO2-mediated reductive activation), whereas in the presence of inhibitors the toxicity of CB1954 will be much reduced.[25]

Experiments were carried out with SKOV-3 cells treated with varying concentrations of CB1954 together with the activating co-factor EPR in the presence and absence of varying concentrations of a selection of

the putative NQO2 inhibitors. Typical survival curves are given in Figure 4. The closed circles in the Figure show the survival of the SKOV-3 cells given CB1954 and EPR, whereas in the presence of the inhibitors (the other symbols) it is clear that the toxicity is much reduced. Indeed, the toxicity curve obtained in the presence of 0.5μM **7d** is identical to that obtained when the exposure to CB1954 is carried out in the absence of EPR (data not shown), which strongly suggests that the inhibitors are acting in cells by competing with the activating co-factor for access to the enzyme active site. Importantly, these data confirm that these compounds are functionally active as NQO2 inhibitors in cells.

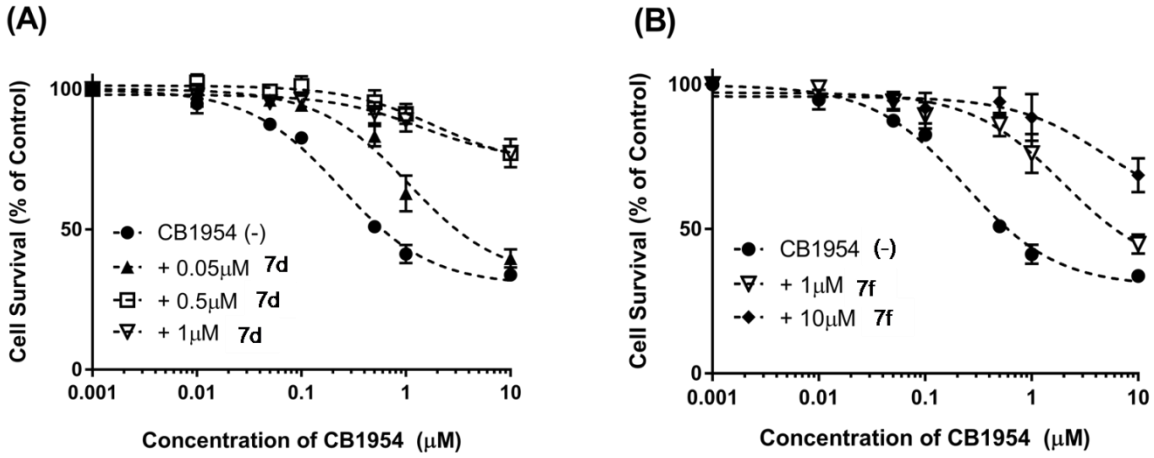


Figure 4. Data showing that the 4-hydrazonequinolines can be functionally active as NQO2 inhibitors in SKOV-3 ovarian cancer cells. Cells were exposed to the CB1954/EPR ± the inhibitors for 24 h prior to measuring toxicity with the MTT assay 72 h later. Panels A and B show the ability of different concentrations of the NQO2 inhibitors **7d** and **7f**, respectively, to reduce the toxicity of CB1954. Data points (± SE) were derived from four independent experiments.

In order to evaluate the efficiency of the different compounds to act as inhibitors of NQO2 in cells, we exposed SKOV-3 cells to a single concentration of CB1954 (1μM) and EPR, together with a wide range of concentrations of the different putative inhibitors. From these experiments we were able to calculate the concentration of inhibitor that could cause half-maximal inhibition of CB1954 toxicity. These results are recorded in Table 4. We have already identified 9-AA as an extremely potent agent for inhibiting NQO2 in cells.[25] This previous work was carried out with MDA-MB-468 cells and here, using SKOV-3 cells, it can be seen that 9-AA retains its potent inhibitory activity. However, it is quite apparent that some of the hydrazonequinolines show comparable efficiency to 9-AA in the SKOV-3 cells. Indeed, compound **7d** is some ten-fold more efficient, making it one of the most potent inhibitors of NQO2 in cells yet identified. An important feature of this set of results, which complements our previous findings [25] is that the ability of compounds to inhibit recombinant enzyme is not necessarily a guide to the most efficient inhibitor in cells. For example, compounds **7f** and **7g** show approximately four-fold greater potency as inhibitors than **7d** against recombinant NQO2 enzyme; in contrast, in cells, they are 10 to 20 fold less efficient than **7d**.

Table 4. Efficiency of compounds to act as inhibitors of NQO2 in wild type SKOV-3 cells. Data are derived from experiments similar to that given in Figure 4 with the exception that the cells were exposed to a fixed concentration of CB1954 (1μM) plus its activating co-factor EPR in the presence of a range of non-toxic concentrations of the different putative inhibitors of NQO2. Cells were exposed to the agents for 24 h prior to measuring toxicity with the MTT assay 72 h later.

Compound Number/ Name	Concentration of inhibitor required to reduce maximal toxicity of CB1954/ EPR by 50% (μM ± SE)
7c	2.07 ± 0.5
7d	0.053 ± 0.007
7f	0.98 ± 0.22
7g	0.44 ± 0.22
7i	22.7 ± 8.9
8a	74 ± 11.4
9AA	0.45 ± 0.14

2.6 Conclusion

4-Hydrazone- and 4-hydrazide-quinoline derivatives have been prepared using multistep syntheses in good yields. Several of the 4-hydrazone analogues showed low nanomolar inhibition against recombinant NQO2, whereas the 4-hydrazides were less active. This activity profile was rationalized by qualitative docking to the active site of NQO2. Further the hydrazine inhibitors are selective for NQO2, showing little if any activity against NQO1. The hydrazones and hydrazides were toxic at micro-molar concentrations which are, however, much higher than the concentrations required to inhibit cellular NQO2 activity in cells. The study provides two important conclusions. Firstly, inhibitory potency against recombinant enzyme is not necessarily the best guide to identify the inhibitors likely to be most active in cells. Secondly, compound **7d** is shown to be one of the most active NQO2 inhibitors in cells yet reported. This compound could prove to be useful as a pharmacological probe for interrogating the role of NQO2 in cancer and a variety of other biological conditions.

3. Experimental

3.1 Synthesis

Chemicals, solvents and deuterated solvents were purchased from Sigma-Aldrich and 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 solvent peak or tetramethylsilane (TMS δ = 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 F254. The spots were visualized using a UV GL-58 Mineral-Light lamp. 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 spectrometer. 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. LC-MS spectrometry was carried out using the ACQUITY UPLC H-class system. The mass spectrometry data was acquired in the positive (ES+) and negative (ES-) modes, scanned from 100- 1000 m/z. The LC data was obtained for Waters ACQUITY UPLC PDA detector scanning from 210 – 400nm Mass spectrometry was carried out using a Micromass Platform II instrument at the School of Chemistry, University of Manchester. The ^{1}H NMR and LC-MS spectra for most compounds are provided in Appendix A Supplementary data.

*3.1.1 General procedure for the synthesis of intermediates **3a-b***

A solution of Meldrum's acid (isopropylidine malonate) (10.40 mmol) in trimethylorthoacetate for **3a** or trimethylorthobenzoate for **3b** (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 a further 4 h. The mixture was then poured into ice and allowed to stir until white crystals formed. The product was collected by suction filtration.

*3.1.1.1 5-(1`-((4`-Methoxyphenyl)amino)ethylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (**3a**).* The product was collected as white solid (2.24 g, 83%). Mp: 143-144 ºC; IR (cm^{-1}): 1708 (-COO-), 1889 (C=C), 2942 (CH_3), 3019 (2° -amine); ^{1}H-NMR (400 MHz; DMSO-d_6): 1.66 (s, 6H, H 7, 8), 2.44 (s, 3H, H 8`), 3.80 (s, 3H, OMe), 7.04 (d, J = 8.0 Hz, 2H, H 3`,5`), 7.32 (d, J= 8.0 Hz, 2H, H 2`, 6`), 12.49 (s, 1H, NH); $^{13}$C-NMR (75 MHz, DMSO-$d_6$, 1 quat. C not observed): 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); MS *ES*$^{-}$, [M-H, 35%]: 290.1.

*3.1.1.1 5-(((4-Methoxyphenyl)amino)(phenyl)methylene)-2,2-dimethyl-1,3-dioxane-4,6-dione (**3b**).* The product was collected as a yellow solid (84%). Mp: 185-188 ºC; IR (cm^{-1}): 1552 (C=C), 1650 (C=C), 1715 (-COO-), 2837 (OMe), 2936 (Me), 2990 (Me), 3059 (C-H); ^{1}H-NMR (300 MHz; DMSO-d_6): 1.75 (s, 6H, H 7, 8), 3.65 (s, 3H, OMe), 6.91 (d, J = 9.0 Hz, 2H, H 2`, 6`), 6.91 (d, J= 9.0 Hz, 2H, H 3`, 5`), 7.21-7.32 (m, 5H, Ph), 12.26 (s, 1H, NH); ^{13}C-NMR (75 MHz, DMSO-d_6): 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.

*3.1.2 General procedure for the synthesis of intermediates **4a-b***

Intermediate **3a** or **3b** (2.26 mmol) was dissolved in diphenyl ether (2.0 ml) and heated by microwave radiation at 200 ˚C for 30 min to give **4a** or**4b**, respectively.

*3.1.2.1 6-Methoxy-2-methylquinolin-4-ol (**4a**).* The product was collected as a beige powder (400 mg, 93%). Mp: 268-270 ºC; IR (cm^{-1}): 1593 (C=C), 2946 (CH_3), 3099 (phenol); ^{1}H-NMR (300 MHz; $CDCl_3$): 2.39

(s, 3H, Me), 3.88 (s, 3H, OMe), 5.94 (s, 1H, H 3), 7.31 (dd, J = 9.0 Hz, J = 3.0 Hz, 1H, H 7), 7.51 (d, J = 3.3 Hz, 1H, H 5), 7.52 (d, J = 8.4 Hz, 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.

*3.1.2.2 6-Methoxy-2-phenylquinolin-4-ol (**4b**).* The product 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; $CDCl_3$): 3.85 (s, 3H, OMe), 6.31 (s, 1H, H 3), 7.31 (dd, J = 9.0 Hz, J = 3.0 Hz, 1H, H 7), 7.50 (d, J = 3.0 Hz, 1H, H 5), 7.57-7.59 (m, 3H, H 3`,4`,5`), 7.71 (d, J = 9.0 Hz, 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 2). MS *ES*$^-$, [M-H, 100%]: 250.1.

*3.1.3 General procedure for the synthesis of intermediates **5a-b***

$POCl_3$ (1 ml, 45mmol) was added to intermediate **4a** or **4b** (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 $NaHCO_3$. The resulting solid was collected by vacuum filtration to afford the intermediate **5a**or **5b**.

*3.1.3.1 4-Chloro-6-methoxy-2-methylquinoline (**5a**).* The product was collected as a beige powder (560 mg, 86%). Mp: 98-101˚; IR (cm^{-1}): 1594 (C=C), 1654 (C=N), 2947 (Me), 3040 (arene C-H); ^{1}H-NMR (300 MHz; $CDCl_3$): 2.60 (s, 3H, Me), 3.93 (s, 3H, OMe), 7.37 (d, J = 3.0 Hz, 1H, H 5), 7.45 (dd, J = 9.0 Hz, J = 2.8 Hz, 1H, H 7), 7.64 (s, 1H, H 3), 7.90 (d, J = 9.0 Hz, 1H, H 8); ^{13}C-NMR (75 MHz; DMSO-d_6, quat. C 4 and C 9 not observed): 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); MS *ES*$^+$, [M+H, 100%]: 208.0.

*3.1.3.24-Chloro-6-methoxy-2-phenylquinoline (**5b**).* The product 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); ^{1}H-NMR (300 MHz; $CDCl_3$): 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`); $^{13}$C-NMR (75 MHz; DMSO-$d_6$): 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.

*3.1.4 General procedure for the synthesis of intermediates **6a-b***

Hydrazine monohydrate (1.16 ml, 2.4 mmol) was added to a solution of compound **5a** or **5b** (2.4 mmol) in ethanol (25ml). This mixture was heated at reflux for 1-3 days and monitored by TLC until the reaction went to completion. The resulting solid **6a** or **6b** was collected by suction filtration.

*3.1.4.1 4-Hydrazinyl-6-methoxy-2-methylquinoline (**6a**).* The product was collected as beige powder 80%. Mp: 243-245 °C. IR (cm^{-1}): 1247 (C-N 2° amine), 1599 (C-C), 2939 (CH_3), 3026 (arene C-H), 3302 (NH_2, 1° amine); ^{1}H-NMR (400 MHz; DMSO-d_6, 1 quat C not observed): 2.58 (s, 3H, CH_3), 3.89 (s, 3H, OMe), 6.92 (s, 1H, H 3), 7.44 (dd, ^{3}J = 8.0 Hz, ^{4}J = 4.0 Hz, 1H, H 7), 7.80 (d, J = 4.0 Hz, 1H, H 5), 7.84 (d, J = 8.0 Hz, 1H, H 8); ^{13}C-NMR (75 MHz; DMSO-d_6): 22.4 (Me), 56.3 (OMe), 98.2 (C 5), 102.2 (C 3), 116.0 (C8),

122.7 (C 7), 125.1 (C 6), 153.6 (q C), 154.3 (q C), 156.6 (C 2); MS *ES*$^-$: 238.1 [M+^{35}Cl, 100%], 240.1 [M+^{37}Cl, 30%].

*3.1.4.2 4-Hydrazinyl-6-methoxy-2-phenylquinoline (**6b**).* The product 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); ^{1}H-NMR (300 MHz; DMSO-d$_6$): 3.87 (s, 3H, OMe), 4.45 (s, 2H, NH$_2$), 7.24 (dd, ^{3}J = 9.3 Hz, ^{4}J = 2.4 Hz, 1H, H 7), 7.42-7.52 (m, 5H, H 3`,4`,5`,3, 5), 7.75 (d, J = 9.3 Hz, 1H, H 8), 8.14 (d, J = 8.1 Hz, 2H, H 2`,6`), 8.30 (s, 1H, NH); $^{13}$C-NMR (75 MHz; DMSO-d$_6$): 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.

*3.1.5 General procedure for the synthesis of hydrazone derivatives (**7a-r**)*

Intermediate **6a** or **6b** (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.

*3.1.5.1 4-(2-(4-Fluorobenzylidene)hydrazinyl)-6-methoxy-2-methylquinoline (**7a**).* Product isolated as a yellow solid (90mg, 56%). Mp: 227-230 °C; IR (cm^{-1}): 1505 (C=C), 1591 (C=N), 2921 (Me), 3001 (arene C-H), 3425 (-NH- 2° amine); ^{1}H-NMR (400 MHz; DMSO-d$_6$): 2.74 (s, 3H, Me), 4.00 (s, 3H, OMe), 7.33 (dd~t, $J_{HH} = J_{HF}$ = 8.0 Hz, 2 H, H 3`, 5`), 7.46 (s, 1H, H 3), 7.55 (dd, ^{3}J = 8.0 Hz, ^{4}J = 4.0 Hz, 1H, H 7), 7.88 (d, J = 8.0 Hz, 1H, H 8), 7.94 (dd, ^{3}J = 8.0 Hz, ^{4}J = 4.0 Hz, 2H, H 2`, 6`), 8.07 (d, J= 4.0 Hz, 1H, H 5), 8.86 (s, 1H, N=CH), 12.4 (s, 1H, NH). ^{13}C-NMR (75 MHz; DMSO-d$_6$; Quat. C 4, 4a, 8a not observed): 56.3 (OMe), 101.7 (C 5), 107.1 (C 3), 116.4 (d, $^2J_{CF}$ = 12.7 Hz, C 3`, 5`), 116.5 (C 7), 129.3 (d, $^3J_{CF}$ = 8.8 Hz, C 2`, 6`), 131.9 (C 1`), 133.4 (C=N), 156.5 (C 2), 162.3 (d, $J_{CF}$ = 147.0 Hz, C 4`); MS *ES*$^+$, [M+H, 100%]: 310.5. Accurate mass calculated for $C_{18}H_{16}FN_3O$ + H: 310.136. Found: 310.1333, error: -5.482 ppm.

*3.1.5.2 4-(2-Benzylidenehydrazinyl)-6-methoxy-2-methylquinoline (**7b**).* Product isolated as a yellow solid (75mg, 52%). Mp: 282-285 °C. IR (cm^{-1}): 1501 (C=C), 1600 (C=N), 2845 (Me), 3121 (aromatic C-H stretch), 3353 (-NH- 2° amine); ^{1}H-NMR (400 MHz, DMSO-d$_6$): 2.60 (s, 3H, CH$_3$), 3.99 (s, 3H, OMe), 7.33 (s, 1H, H 3), 7.36 (dd, ^{3}J = 8.0 Hz, ^{4}J = 4.0 Hz, 1H, H 7), 7.47 (t, J = 8.0 Hz, 1H, H 4`), 7.53 (t, J = 8.0 Hz, 2H, H 3`, 5`), 7.67 (d, J = 4.0 Hz, 1H, H 5), 7.78 (d, J = 8.0 Hz, 1H, H 8), 7.86 (d, J = 8.0 Hz, 2H, H 2`,6`), 8.45 (s, 1H, C=N), 10.92 (s, 1H, NH). $^{13}$C-NMR (75 MHz; DMSO-d$_6$; Quat. C 4a not observed): 19.7 (Me), 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 8a), 151.0 (C 4), 152.1 (C 6), 157.5 (C 2); MS *ES*$^+$, [M+H, 100%]: 292.5. Accurate mass calculated for $C_{18}H_{17}N_3O$ + H: 292.145. Found: 292.1438, error: -2.046 ppm.

*3.1.5.3 4-(2-((1H-Imidazol-5-yl)methylene)hydrazinyl)-6-methoxy-2-methylquinoline (**7c**).* Product isolated as a bright yellow solid (105 mg, 76%). Mp: 226-229 °C; IR (cm^{-1}): 1570 (C=C), 1599 (C=N), 2833 (OMe), 2928 (Me), 3121 (C-H), 3368 (2° amine); ^{1}H-NMR (300 MHz, DMSO-d$_6$): 2.69 (s, 3H, Me), 3.98 (s, 3H, OMe), 7.42 (s, 1H, H 3), 7.57 (d, J = 9.3 Hz, 1H, H 7), 7.68 (s, 1H, H 5`), 7.88 (s, 1H, H 5), 7.91 (d, J = 9.3 Hz, 1H, H 8), 8.25 (s, 1H, H 3`), 8.89 (s, 1H, HC=N), 12.66 (s, 2H, NH); ^{13}C-NMR (75 MHz; DMSO-d$_6$; Quat. C 1`, 4a, 8a not observed): 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); MS *ES*$^{-}$, [M-H, 100%]: 280.1. Accurate mass calculated for $C_{15}H_{15}N_5O$+H: 282.135. Found: 282.1329, error:-7.0888 ppm.

*3.1.5.4 4-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)phenol (**7d**).* Product isolated as a 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); ^{1}H-NMR (300 MHz; DMSO-d_6): 2.71 (s, 1H, Me), 3.98 (s, 3H, OMe), 6.91 (d, J = 9.0 Hz, 2H, H 3`,5`), 7.46 (s, 1H, H 3), 7.62 (dd, ^{4}J = 6.0 Hz, ^{3}J = 3.0 Hz, 1H, H 7), 7.75 (d, J = 9.0 Hz, 2H, H 2`,6`), 7.89 (d, J = 9.0 Hz, 1H, H 8), 8.08 (s, 1H, H 5), 8.71 (s, 1H, C=N), 10.15 (s, 1H, OH), 12.3 (s, 1H, NH); ^{13}C-NMR (75 MHz; DMSO-d_6): 19.7 (Me), 56.6 (OMe), 99.6 (C 5), 103.1 (C 3), 115.1 (C 5`), 115.9 (C 3`), 121.1 (C 4a), 124.5 (C 7), 124.6 (C 8), 129.4 (C 2`,6`), 133.4 (C=N), 149.8 (C 8a), 150.7 (C 4), 151.7 (C 6), 157.3 (C 2), 160.1 (C1`); MS *ES*$^{+}$, [M+H, 100%]: 308.1. Accurate mass calculated for $C_{18}H_{17}N_3O_2$ + H: 308.140. Found: 308.1389, error, -1.6226 ppm.

*3.1.5.5 6-Methoxy-2-methyl-4-(2-(pyridin-3-ylmethylene)hydrazinyl)quinoline (**7e**).* Product isolated as a beige powder (50 mg, 34%). Mp: >290ºC (dec); IR (cm^{-1}): 1570 (C=C), 1591 (C=N), 2830 (OMe), 2917 (CH_3); ^{1}H-NMR (300 MHz; CD_3OD): 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 Hz, 1H, H 8), 7.91 (s, 1H, H 5), 8.39 (d, J = 6.3 Hz, 1H, H 6`), 8.65 (s, 2H, H 2`, 4`), 8.98 (s, 1H, HC=N); ^{13}C-NMR (75 MHz; CD_3OD): 20.2 (C Me), 56.9 (OMe), 102.1 (C 5), 102.8 (C 3), 117.1 (C 4a), 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 8a), 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 $C_{17}H_{16}N_4O$ + H: 293.140. Found: 293.1378, error: -6.4816 ppm.

*3.1.5.6 6-Methoxy-2-methyl-4-(2-(2-phenylethylidene)hydrazinyl)quinoline (**7f**).* Product isolated as a cream solid (43mg, 30%). Mp: 235-237 ºC; 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) δ ppm: 2.68 (s, 3H, CH_3), 3.75 (d, J = 5.7 Hz, 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 Hz, 1H, H 7), 7.89 (d, J = 9.0 Hz, 1H, H 8), 8.12 (s, 1H, H 5), 8.37 (t, J = 5.4 Hz, 1H, HC=N), 12.39 (s, 1H, NH). $^{13}$C-NMR (75 MHz; DMSO-$d_6$, Quat. C not observed): 19.9 (Me), 35.0 (C 2`, observed in DEPT), 56.5 (OMe), 99.5 (C 5), 102.9 (C 3), 114.9 (C10`), 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); MS ES-, [M+Na, 100%]: 326.1. Accurate mass calculated for $C_{19}H_{19}N_3O$ + Na: 328.143. Found: 306.1581, error:-6.5325 ppm.

*3.1.5.7 2-Methoxy-6-((2-(6-methoxy-2-methylquinolin-4-yl)hydrazono)methyl)phenol (**7g**).* Product isolated as a yellow powder (120 mg, 89%). Mp: 242-245 ºC. IR (cm^{-1}): 1574 (C=C), 1603 (C=N), 2794 (OMe), 2841 (OMe), 2925 (CH_3), 3016 (C-H), 3226 (NH), 3411 (OH). ^{1}H-NMR (300 MHz; DMSO-d_6) δ: 2.54 (s, 3H, CH3), 3.84 (s, 3H, OMe), 3.92 (s, 3H, OMe), 6.85 (t, J = 7.8 Hz, 1H, H 4`), 6.99 (d, J = 7.8 Hz, 2H, H 3, 3`), 7.35 (d, J = 8.5 Hz, 2H, H 7, 5`), 7.67 (d, J = 9.3 Hz, 1H, H 8), 7.80 (d, J = 2.1 Hz, 1H, H 5), 8.86 (s, 1H, C=N), 10.12 (s, 1H, NH), 11.90 (s, 1H, OH). $^{13}$C-NMR (75 MHz; DMSO-$d_6$, Quat. C not observed): 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); MS *ES*$^{+}$, [M+H, 100%]: 338.3. Accurate mass calculated for $C_{19}H_{19}N_3O_3$ + H: 338.150. Found: 338.1480, error: -5.6188 ppm.

*3.1.5.8 6-Methoxy-2-methyl-4-(2-((5-nitrofuran-2-yl)methylene)hydrazinyl)quinoline (**7h**).* Product isolated as a 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). ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 2.76 (s, 3H, Me), 4.00 (s, 3H, OMe), 7.45 (s, 1H, H 3), 7.49 (d, J=6.0 Hz, 1H, H 3`), 7.67 (dd, $^{4}$J= 9.0 Hz, $^{3}$J= 3.0 Hz, 1H, H 7), 7.89 (d, J= 3.0 Hz, 1H, H 4`), 7.95 (d, J = 9.0 Hz, 1H, H 8), 8.21 (s, 1H, H 5), 8.86 (s, 1H, C=N), 13.0 (s, 1H, NH); ^{13}C-NMR (75 MHz; CD_3OD): 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 4a), 124.8 (C=N), 133.6 (C 8), 146.4 (C 8a), 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 $C_{16}H_{14}N_4O_4$ + H: 327.109. Found: 327.1085, error: -0.9171 ppm.

*3.1.5.9 4-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)benzoic acid (**7i**).* Product isolated as a yellow solid (130 mg, 78%). Mp: 272-275 °C; IR (cm^{-1}): 1577 (C=C), 1603 (C=N), 1675 (C=O), 2648 (OMe), 2928 (CH_3), 3004(C-H), 3314 (2° amine); ^{1}H-NMR (300 MHz, DMSO-d_6) δ ppm: 2.71 (s, 3H, CH_3), 3.98 (s, 3H, OMe), 7.51 (s, 1H, H 3`), 7.57 (d, J = 9.3 Hz, 1H, H 7`), 7.90 (d, J= 9.3 Hz, 1H, H 8`), 7.97-8.05 (m, 5H, H 2, 3, 5, 6, 5`), 8.11 (s, 1H, N=C), 8.87 (s, 1H, NH), 13.0 (br s, 1H, OH); ^{13}C-NMR (75 MHz; DMSO-d_6): 20.0 (Me), 56.6 (OMe), 100.6 (C 5`), 102.9 (C 3`), 115.5 (C 4`a), 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 8`a), 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 $C_{19}H_{17}N_3O_3$ + H: 336.135. Found: 336.1339, error: -1.19 ppm.

*3.1.5.10 6-Methoxy-2-methyl-4-(2-(4-nitrobenzylidene)hydrazinyl)quinoline (**7j**).* Product isolated as a bright yellow solid (133 mg, 92%). Mp: 220-223 °C; IR (cm^{-1}): 1341 (N=O), 1574 (C=C), 1510 (C=N), 2856 (OMe), 2917 (CH_3), 3366 (2° amine); ^{1}H-NMR (300 MHz, DMSO-d_6): 2.64 (s, 3H, CH_3), 3.96 (s, 3H, OMe), 7.42 (s, 1H, H 3), 7.47 (d, J = 9.0 Hz, 1H, H 7), 7.82 (d, J = 9.0 Hz, 1H, H 8), 7.95 (d, 2H, J = 8.7 Hz, H 2`, 6`), 7.98 (s, 1H, H 5), 8.03 (d, 2H, J = 8.7 Hz, H 3`, 5`), 8.70 (br s, 1H, N=CH), 12.73 (s, 1H, NH); ^{13}C-NMR (75 MHz; DMSO-d_6; Quat. C4, 8a, 4` not observed): 21.9 (Me), 56.1 (C, OMe), 100.8 (C 5), 101.9 (C 3), 115.9 (C 4a), 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); MS *ES*$^{-}$, [M+H, 100%]: 337.1. Accurate mass calculated for $C_{18}H_{16}N_4O_3$ + H: 337.130. Found: 337.1293, error: -0.593 ppm.

*3.1.5.11 5-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)benzene-1,3-diol (**7k**).* Product isolated as a bright yellow solid (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): 2.72 (s, 3H, CH_3), 3.99 (s, 3H, OMe), 6.38 (s, 1H, H 6`), 6.73 (s, 2H, H 2`, 4`), 7.40 (s, 1H, H 3), 7.62 (d, J = 5.4 Hz, 1H, H 7), 7.64 (d, J = 5.7 Hz, 1H, H 8), 8.09 (s, 1H, H 5), 8.63 (s, 1H, HC=N), 9.57 (s, 2H, 2 x OH), 12.3 (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 (C 4a), 124.7 (C 7), 135.2 (C 4, 3`), 149.9 (C=N), 150.8 (C 8a), 152.2 (C 6), 157.5 (C 2), 158.8 (C 1`, 5`); MS *ES*$^{+}$, [M+H, 100%]: 324.6. Accurate mass calculated for $C_{18}H_{17}N_3O_3$ + H: 324.135. Found: 324.1339, error:-1.234 ppm.

*3.1.5.12 3'-((2-(6-Methoxy-2-methylquinolin-4-yl)hydrazono)methyl)phenol (**7l**).* Product isolated as a beige solid (0.5 g, 33%). Mp: >300 °C; IR (cm^{-1}): 1570 (C=C), 1606 (C=N), 2895 (OMe), 2965 (CH_3), 3157 (C-H), 3284 (OH); ^{1}H-NMR (400 MHz, DMSO-d_6): 2.73 (s, 3H, CH_3), 3.99 (s, 3H, OMe), 6.91 (d, J = 6.0 Hz, 1H, H 6`), 7.30-7.34 (m, 3H, H 2`, 3`, 5`), 7.47 (s, 1H, H 3), 7.63 (d, J = 9.3 Hz, 1H, H 7), 7.91 (d, J=9.0 Hz, 1H, H 8), 8.07 (s, 1H, H 5), 8.7 (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`), 115.3 (C 6`), 117.9 (C 4a), 118.7 (C 7), 121.4 (C 4`), 124.7 (C 8), 129.9 (C 5`), 133.8 (C 3`), 134.9 (C=N), 149.5 ( C 8a), 150.8 (C 4), 152.2 ( C 6), 157.5 (C 1`),

157.8 (C 2); MS *ES*$^+$, [M+H, 100%]: 308.6. Accurate mass calculated for $C_{18}H_{17}N_3O_2$+H: 308.140. Found: 308.1388, error -1.947 ppm.

*3.1.5.13 6-Methoxy-2-phenyl-4-(2-(pyridin-3-ylmethylene)hydrazinyl)quinoline (**7m**).* Product isolated as a yellow solid (120 mg, 89%). Mp: 215-218 ºC. IR (cm^{-1}): 1585 (C=C), 1624 (C=N), 2798 (OMe), 3063 (C-H), 3219 (NH); ^{1}H-NMR (300 MHz, DMSO-d_6): 3.97 (s, 3H, OMe), 7.37 (dd, ^{4}J= 9.0 Hz, ^{3}J= 1.8 Hz, 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 Hz, 1H, H 8), 7.93 (t, J = 6.3 Hz, 1H, H 5`), 8.23 (d, J = 7.8 Hz, 2H, H 2``, 6``), 8.29 (d, J = 7.8 Hz, 1H, C 6`), 8.46 (s, 1H, C=N), 8.59 (d, J = 4.8 Hz, 1H, H 4`), 8.97 (s, 1H, H 2`), 11.17 (s, 1H, NH); $^{13}$C-NMR (75 MHz; DMSO-$d_6$): 55.8 (OMe), 98.1 (C 5), 100.5 (C 3), 116.7 (C 4a), 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 8a), 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 $C_{22}H_{18}N_4O$ + H: 355.156. Found 355.1551, error: -0.5631ppm.

*3.1.5.14 4-((2-(6-Methoxy-2-phenylquinolin-4-yl)hydrazono)methyl)-N,N-dimethylaniline (**7n**).* Product isolated as a brown powder (90 mg, 60%). Mp: 115-118 ºC. 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) δ 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 Hz, 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 Hz, 1H, H 8``), 8.16 (d, J= 7.5 Hz, 2H, H 2`, 6`), 8.33 (s, 1H, HC=N), 10.71 (s, 1H, NH); ^{13}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 4``a), 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 8``a), 143.6 (C=N), 146.9 (C 4``), 151.08 (C 4), 156.1 (C 2``), 160 (C 6``); MS *ES*$^+$, [M+H, 100%]: 397.3. Accurate mass calculated for $C_{25}H_{24}N_4O$+H: 397.203. Found: 397.2016, error: -1.7623 ppm.

*3.1.5.15 6-Methoxy-4-(2-(4-nitrobenzylidene)hydrazinyl)-2-phenylquinoline (**7o**).* Product isolated as a red-brown powder (53 mg, 40%). Mp: 230-234 ºC; IR (cm^{-1}): 1556 (NO), 1595 (C=C), 2848 (OMe), 2921 (C-H), 3357 (NH). ^{1}H-NMR (300 MHz, DMSO-d_6): 3.97 (s, 3H, OMe), 7.41 (d, J = 8.0 Hz, 1H, H 7), 7.48-7.56 (m, 3H, H 3``, 4``, 5``), 7.72 (s, 1H, H 3), 7.92 (d, J = 8.0 Hz, 1H, H 8), 7.91-7.96 (s, 1H, H 5), 8.11 (d, 2H, J = 8.1 Hz, H 2`, 6`), 8.22 (d, 2H, J = 7.2 Hz, H 2``, 6``), 8.32 (d, 2H, J = 8.1 Hz, H 3`, 5`), 8.52 (s, 1H, N=CH), 11.37 (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 4a), 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 8a), 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 $C_{23}H_{18}N_4O_3$+H: 399.146. Found: 399.1451, error: -0.250 ppm.

*3.1.5.16 6-Methoxy-2-methyl-4-(2-(2-phenylethylidene)hydrazinyl)quinoline (**7p**).* Product isolated as a beige powder (56 mg, 26%). Mp: 180-182 ºC; 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): 3.75 (d, J = 5.7 Hz, 2H, H 2``), 3.90 (s, 1H, OMe), 7.25 -7.56 (m, 10H, H 3, 7, 3`,4`,5`,4``, 5``, 6``,7``,8``), 7.71 (s, 1H, H 5), 7.82-7.86 (m, 2H, H 8, N=CH), 8.11 (d, J = 7.5 Hz, 2H, H 2`,6`), 10.59 (s, 1H, NH); ^{13}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 4a), 121.1 (C 8), 126.5 (C 7), 126.7 (2C, 4`, 6``), 128.5 (C 5``,7``), 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 8a), 144.3 (C 1`), 145.5 (C=N), 147.1 (C 4), 154.2 (C 6), 156.1 (C 2); MS *ES*$^+$, [M+H, 100%]: 368.2. Accurate mass calculated for $C_{24}H_{21}N_3O$+H: 368.176. Found: 368.1763, error: 1.629 ppm.

*3.1.5.17 2-Methoxy-6-((2-(6-methoxy-2-phenylquinolin-4-yl)hydrazono)methyl)phenol (**7q**).* Product isolated as a 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); ^{1}H-NMR (300 MHz, DMSO-d_6): 3.85 (s, 3H, OMe), 3.96 (s, 3H, OMe), 6.87 (t, J=7.8 Hz, 1H, H 4`), 7.00 (d, J = 7.8 Hz, 1H, H 3`), 7.37 (d, J= 9.0 Hz, 1H, H 7), 7.46-7.56 (m, 4H, H 5`, 3``, 4``, 5``), 7.70 (s, 1H, H 3), 7.76 (s, 1H, H 5), 7.88 (d, J= 9.3 Hz, 1H, H 8), 8.16 (d, J=7.2 Hz, 2H, H 2``, 6``), 8.81 (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 (C6`), 119.3 (C 4a), 120.9 (C 8), 121.3 (C 7), 122.0 (C 4`), 126.7 (C2``, 6``), 128.6 (C 5, 5``), 130.9 (C 4``), 139.8 (C 8a), 140.9 (C1``), 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 $C_{24}H_{21}N_3O_3$+H: 400.166. Found: 400.1649, error: -1.7493 ppm.

*3.1.5.18 4-(2-((1H-Imidazol-4-yl)methylene)hydrazinyl)-6-methoxy-2-phenylquinoline (**7r**).* Product isolated 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° amine); ^{1}H-NMR (300 MHz, DMSO-d_6): 3.96 (s, 3H, OMe), 7.38 (dd, ^{3}J = 9.0 Hz, ^{4}J = 2.4 Hz, 1H, H 7), 7.47-7.57 (m, 4H, H 3, 3``, 4``, 5``), 7.67 (d, J = 2.4 Hz, 1H, H 5), 7.84 (d, J = 6.9 Hz, 2H, H 6`, 8), 7.89 (s, 1H, H 4`), 8.20 (d, J = 7.5 Hz, 2H, H 2``, 6``), 8.40 (s, 1H, C=N), 10.90 (s, 1H, NH), 12.73 (s, 1H, NH); $^{13}$C-NMR (75 MHz; DMSO-$d_6$, C4a not observed): 55.8 (OMe), 97.6 (C 5), 100.8 (C 3), 116.6 (C 6`), 121.3 (C 7, 8), 126.9 (C 3``, 5``), 128.5 (C 2``, 6``), 128.9 (C 4``), 130.3 (C 8a), 137.3 (C 4`), 139.4 (C 1`), 147.2 (C 1``), 153.9 (C 4), 156.3 (C 2, 6); MS *ES*$^{+}$, [M+H, 100%]: 344.3. Accurate mass calculated for $C_{20}H_{17}N_5O$+H: 344.151. Found: 344.1487, error: -5.5208 ppm.

*3.1.6 General procedure for the hydrazide derivatives **8a-b***

Compound **6a** (1 mmol) was dissolved in dichloromethane (10 ml), cooled in an ice bath and stirred for 15 min. 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 h at room temperature. The formed solid was collected by filtration, washed with water and recrystallized from ethanol to give the final product.

3.1.6.1 4-Fluoro-N'-(6-methoxy-2-methylquinolin-4-yl)benzohydrazide (**8a**). Product was isolated as a 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): 2.43 (s, 3H, CH3), 3.90 (s, 3H, OMe), 6.49 (s, 1H, H 3`), 7.29 (dd, $^{3}$J = 9.2 Hz, $^{4}$J = 3.2 Hz, 1H, H 7`), 7.41 (dd~t,3 JHH = 3JHF = 8.0 Hz, 2H, H 3, 5), 7.62 (d, J = 2.8 Hz, 1H, H 5`), 7.70 (d, J = 9.0 Hz, 1H, H 8`), 8.05 (dd, 3JHH = 8.0 Hz, 4JHF = 4.0 Hz, 2H, H 2, 6), 9.10 (s, 1H, NH), 10.63 (s, 1H, NH); 13C-NMR (75 MHz; DMSO-d6): 24.9 (Me), 55.9 (OMe), 106.2 (C 5`), 107.9 (C 3`), 116.1 (d JCF = 12.7 Hz, 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); MS ES+ [M+H, 10%]: 326.1.

3.1.6.2 N'-(6-Methoxy-2-methylquinolin-4-yl)benzohydrazide (**8b**). Product was isolated as a beige powder (45 mg, 28%). Mp: >285°C (Dec); IR (cm^{-1}): 1599 (C=C), 1620 (C=O amide), 2946 (CH_3), 3020 (C-H arene), 3205 (NH-amide), 3302 (NH-aryl); 1H-NMR (300 MHz; DMSO-d6): 2.65 (s, 3H, CH_3), 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 Hz, 2H, H

3,5), 8.13 (d, J = 8.1 Hz, 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, 100%]: 238.1 (cleavage to hydrazine 6a observed).

3.2 Molecular modelling

Compounds were built using Chem 3D pro 13.0 and energy minimized using the MOE software package [39] prior to docking. The compounds were docked into the NQO2 X-ray structure PDB code 4FGJ.[40] Waters of crystallization were deleted from the protein and hydrogens were added, checking protonation and tautomeric states. Docking was performed using GOLD version 5.3[41] with the ASP scoring function.

3.3 Biology

3.3.1 Inhibition of recombinant human NQO2 and NQO1 enzyme activity

Recombinant human NQO2 and NQO1, NADH, and dichlorophenolindophenol (DCPIP) were purchased from (Sigma-Aldrich, UK) and EPR was synthesized at the Strathclyde Institute of Pharmacy and Biomedical Sciences.

The rates of the NQO2 and NQO1 enzyme activity were determined spectrophotometrically by recording the rates of DCPIP colour change at 600nm, over 1 min at 37 ˚C using Beckman DU 7400 spectrophotometer. Reaction mixtures were made up to a 1 ml final volume by sequential mixing of 940 µl phosphate buffer (50 mM, pH 7.4) with 20 µl DCPIP (2 mM), 20 µl enzyme cofactor (10 mM) (either EPR for NQO2 or NADH for NQO1) and 10 µl inhibitor (concentration ranging from 0.001 to 1000 µM) or an appropriate concentration of DMSO or NaOH (in the control sample). The reaction was initiated by the addition of 10 µl rhNQO2 or rhNQO1 (5 x 10^{-3} mg/ml).

All experiments were performed in triplicate on three independent occassions. In each independent experiment solutions of NRH (10 mM), DCPIP (2 mM) and NQO2 and NQO1 (5 x 10^{-3} mg/ml) were freshly prepared. NRH, NADH, NQO1 and NQO2 were dissolved in phosphate buffer (50 mM, pH 7.4) and kept at 0 ˚C. DCPIP was dissolved in deionized water. The inhibitors stock solutions were prepared by dissolving the inhibitors in DMSO or 0.13 M NaOH to give 10mM solutions. A 10-fold serial dilution was completed using DMSO or 0.13 M NaOH to give five different concentrations of the inhibitors: 1-10000 nM for NQO2, and 0.01-100 µM for NQO1. Both NQO1 and NQO2 were reconstituted in phosphate buffer containing 5µM of FAD and 250mM of sucrose at a stock concentration of 5 mg/ml.

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_{50} values were determined as 50% reduction in the enzyme activity compared to control (100%).

3.3.2 Cell lines and cell culture

Two human ovarian cancer (OVC) cell lines, SKOV-3 and TOV-112D, were provided by the Viral Oncology Labs, St Mary's Hospital, and authenticated using the DNA sequencing facility at the University of Manchester. All cells were cultured in RPMI-1640 medium (Sigma, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS; GIBCO, UK) and (2 mmol/L) L-glutamine (Sigma, UK). To maintain pure cultures of NQO2 genetically modified TOV-112D and SKOV-3 cell sub-lines, the cultures were held in RPMI medium containing 1μg/ml of puromycin (Sigma, UK) before sub-culture in fresh medium free of antibiotics immediately prior to experimentation. For induction of NQO2 silencing in SKOV-3 cells, Doxycycline Hyclate (Sigma, UK) was added to the culture medium at a concentration of 1μg/ml on alternate days for five days before seeding for the experiment. This ensured that the silencing levels of NQO2 were stable throughout the experiment; DOX was also added directly to the culture medium on alternate days throughout the experiment.

3.3.3 Toxicity Assays

SKOV-3 and TOV-112D cells (either WT or genetically derived sublines) were seeded at 1500 and 1000 cells, respectively, per well into 96-well plates and left overnight to allow cell attachment. Cells were then treated with different compounds at various concentrations ranging between 0.001 and 100 μM for 24 h or 96h. For the shorter exposure period, the drug-containing medium was removed after 24 h and replenished with fresh growth medium for the remainder of the 96 h period. The number of surviving cells was then determined using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay.[42] MTT solution (2.5 mg/ml) was prepared in sterile PBS. Following 96h incubation, 50μl MTT solution was added to the cells and incubated for 4 h at 37°C. The medium was then removed and the remaining formazan crystals were dissolved in 200 μl dimethyl sulfoxide (DMSO) (Fisher Scientific, UK). Optical densities were then measured on a multi-well scanning spectrophotometer at 540 nm and then analyzed using the Gen 5 Software package (BioTek). The results were then used to calculate the surviving fraction of cells relative to solvent only controls. Dose-response curves were generated using the GraphPad Prism **5.01** Software and nonlinear regression analysis was used to fit the data. IC_{50} values (defined as the concentration of drug required to decrease cell survival by 50% relative to controls) were determined from these curves.

To determine the ability of the novel quinoline compounds to act as pharmacological inhibitors of NQO2 in cells, we use inhibition of CB1954 toxicity as a surrogate measure of effects on NQO2. SKOV-3 cells were treated for 24 h, with either a fixed concentration of inhibitors and varying concentrations of CB1954 (0.001-10μM) combined with 100 μM EPR or a fixed concentration of CB1954 (1 μM) plus 100 μM EPR and varying non-toxic concentrations of the putative inhibitors. Following 24 h incubation with the combination therapy, the medium containing treatment was replaced with fresh growth medium for the remainder of 96 h period. Thereafter, the MTT assay was used to determine cell viability. Percentages of surviving cells were determined and used to generate dose-response curves.

3.3.4 Statistical analysis

The primary analysis of data was performed on Microsoft Excel software (2007) and then transferred to GraphPad Prism (version **5.01** for Windows, La Jolla California, USA, www.graphpad.com).

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Appendix A. Supplementary Data

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