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Indolequinone Inhibitors of NRH:Quinone Oxidoreductase 2 (NQO2). Characterization of Mechanism of Inhibition in both Cell-free and Cellular Systems. †

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Abstract

We describe a series of indolequinones as efficient mechanism-based inhibitors of NRH:quinone oxidoreductase 2 (NQO2) for use either in cellular or cell-free systems. Compounds were designed to be reduced in the active site of the enzyme leading to loss of a substituted phenol leaving group and generation of a reactive iminium electrophile. Inhibition of NQO2 activity was assessed in both cell-free systems and in the human leukemia K562 cell line. Inhibition of recombinant human NQO2 by the indolequinones was NRH-dependent with kinetic parameters characteristic of mechanism-based inhibition and partition ratios as low as 2.0. Indolequinones inhibited NQO2 activity in K562 cells at nanomolar concentrations which did not inhibit NQO1 and were non-toxic to cells. Computational-based molecular modeling simulations demonstrated favorable conformations of indolequinones positioned directly above and in parallel to the isoalloxazine ring of FAD and mass spectrometry extended our previous finding of adduction of the FAD in the active site of NQO2 by an indolequinone-derived iminium electrophile to the wider series of indolequinone inhibitors. Modeling combined with biochemical testing identified key structural parameters for effective inhibition including a 5-aminoalkylamino side chain. Hydrogen bonding of the terminal amine nitrogen in the aminoalkylamino side chain was found to be critical for correct orientation of the inhibitors in the active site. These indolequinones were irreversible inhibitors and were found to be at least an order of magnitude more potent than any previously documented competitive inhibitors of NQO2 and represent the first mechanism-based inhibitors of NQO2 to be characterized in cellular systems.

There are two quinone reductases that occur in mammalian systems NAD(P)H:quinone oxidoreductase 1 (NQO1, EC 1.6.99.2) and NRH:quinone oxidoreductase 2 (NQO2, EC 1.10.99.2). NQO1 was originally characterized by Ernster and Navazio (1, 2) and was probably identical to an enzyme isolated by Martius a few years earlier (3, 4). Interestingly,

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Conflict of Interest Statement CJM, DR and DS are co-founders and stockholders in QGenta Inc. which holds an option to license molecules described in this article.

Supporting Information Available.

Structural frames from the molecular docking simulations and mass spectrometry data for some of the indolequinones, as well as the methods for the preparation of indolequinone **3** and **6** are shown in supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

NQO2 was cloned and fully characterized by Jaiswal et al. (5) but as highlighted by Zhao et al. (6) was also found to be identical to a flavoprotein isolated 30 years earlier (7). Both NQO1 and NQO2 are homodimeric flavoproteins, containing one FAD site per monomer that utilize pyridine nucleotide cofactors to catalyze the direct two-electron reduction of a broad range of quinone substrates (6, 8, 9). However, NQO2 differs from NQO1 in that it utilizes dihydronicotinamide riboside (NRH) instead of NAD(P)H as the cofactor. In addition, in comparison to NQO1 which is usually highly expressed in solid tumors (10), higher levels of NQO2 expression are found in red blood cells (11) and in leukemias (12). With respect to quinone substrates, NQO2 has been suggested to preferentially reduce *ortho*-quinones derived from catecholamines and estrogen which has led to its proposed involvement in neurodegenerative diseases and breast cancer (13, 14). NQO2 has been shown to reduce numerous antitumor quinones *in vitro* including mitomycin C (15), RH1 (16) and the HSP90 inhibitor 17AAG (17) while the antitumor activity of CB1954, a non-quinone dinitrobenzamide-containing compound currently in clinical trials, relies on targeted activation by NQO2 via nitroreduction (18).

The identification of inhibitors for NQO2 has generated considerable interest. Despite structural similarities between NQO2 and NQO1, commonly used NQO1 inhibitors such as dicoumarol (19) and ES936 (20) are extremely poor inhibitors of NQO2 while conversely; inhibitors of NQO2 such as resveratrol and quercetin have been shown to selectively inhibit NQO2 but not NQO1 (21–23). Previous studies have shown that resveratrol (21, 22), quercetin (23), chloroquine (11, 24), and melatonin (9, 25) can inhibit the catalytic activity of NQO2 but do so reversibly. In addition to inhibiting NQO2 these compounds have also been shown to inhibit other enzymes and have direct anti-oxidant activities. Most recently, NQO2 has been found to be the major non-kinase target of imatinib in leukemia cells (12, 26) suggesting it may play an as yet uncharacterized role in leukemia and/or imatinib pharmacodynamics. All of these studies point to an emerging role for NQO2 in diverse physiological and disease process but one major obstacle in defining the role of NQO2 in complex cellular systems has been the absence of potent and specific inhibitors of the enzyme. We have recently examined the structural requirements for selective inhibition of NQO2 relative to NQO1 (27) and proposed a novel mechanism of inhibition involving flavin adduction. In this study, we have characterized a series of indolequinones as mechanism-based inhibitors of NQO2 that can be utilized in both cell-free and cellular systems. In addition we have utilized molecular modeling in combination with biochemical studies and mass spectrometry to define the structural parameters of this indolequinone series that are necessary for effective inhibition of NQO2.

Materials and Methods

Materials

NADH, FAD, 2,6-dichlorophenolindophenol (DCPIP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), menadione, resveratrol, chloroquine, quercetin, and melatonin were obtained from Sigma (St. Louis, MO). Imatinib mesylate was purchased from LC laboratories (Woburn, MA). The indolequinones 5-(4-aminobutyl)amino-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (**1**), 5-(4-aminobutyl)amino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**2**), 6-(4-aminobutyl)amino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**3**), 5-(3-aminopropyl)amino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**4**), 5-(3-dimethylamino)propylamino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**5**), 6-(3-dimethylamino)propylamino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**6**), 5-(3-dimethylamino)propylmethylamino-1,2-dimethyl-3-[(2,4,6-trifluorophenoxy)methyl]indole-4,7-dione (**7**), 5-(3-dimethylamino)propylamino-1,2-

dimethyl-3-(phenoxyethyl)indole-4,7-dione (**8**), and 5-(3-dimethylamino)propylamino-1,2-dimethyl-3-(hydroxymethyl)indole-4,7-dione (**9**) were synthesized using published methods (27) except that indolequinones **3** and **6** were prepared as described in the supporting information. Recombinant human NQO1 (rhNQO1) was purified from *Escherichia coli* using Cibacron blue affinity chromatography as previously described (28). Recombinant human NQO2 (rhNQO2) was purchased from Sigma (St. Louis, MO) and dissolved in 250mM sucrose and stored at -80°C . Dihydronicotinamide riboside (NRH) was prepared from NADH using previously reported methods (16, 29).

Cell Lines

The human leukemia cell line K562 was obtained from ATCC (Manassas, VA) and grown in complete RPMI1640 medium supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. Cells were maintained in a humidified incubator containing 5% carbon dioxide at 37°C .

Inhibitor Screening for NQO2

Mechanism-based inactivation of NQO2 by this indolequinone series was assayed using purified human recombinant NQO2 (rhNQO2). In these reactions (0.5 mL final volume) rhNQO2 (4 $\mu\text{g/mL}$) was incubated with 0.1–10.0 μM indolequinone in the absence and presence of 0.2 mM NRH in 50 mM potassium phosphate buffer, pH 7.4, containing 5 μM FAD, 125 mM NaCl, and 1 mg/mL bovine serum albumin at room temperature. After 5 min a 50 μL aliquot was removed and diluted 50-fold in stop buffer [50 mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 5 μM FAD, 0.1% (v/v) Tween-20, and 0.25mg/ml MTT]. An aliquot (960 μL) of the mixture was then transferred to a cuvette and mixed with 200 μM NRH and 10 μM menadione (final volume 1 mL) and the linear increase in absorbance was monitored spectrophotometrically at 550 nm for 2 min at room temperature. NQO2 activity was measured as NRH-dependent menadione reductase activity using MTT as the final electron acceptor (23).

Inhibitor Characterization

Partition ratios for inactivation of rhNQO2 by the indolequinones that demonstrated mechanism-based inhibition were determined by using the methods described above except that the indolequinones and rhNQO2 were incubated in the presence of 0.2 mM NRH for 15 min, with defined molar ratios of indolequinone to rhNQO2 monomer (active sites)(range 0.25:1 to 120:1). The percent of activity remaining for each concentration was then plotted against the molar ratios; a straight line was generated using linear regression and extrapolated and the x-intercept of the line represents the partition ratio (number of molecules of inhibitor metabolized to inactivate one molecule of enzyme) (30).

The concentration- and time-dependent inactivation of NQO2 by the selected indolequinone **1** and **5** was examined to confirm mechanism-based inhibition. For these studies inactivation of NQO2 by indolequinone **1** or **5** was performed at 4°C in a reaction system (final volume 1ml) containing 50 mM potassium phosphate buffer, pH 7.4, 0.1% Tween-20, 125mM NaCl, 200 μM NRH, 5 μM FAD and 4 $\mu\text{g/mL}$ rhNQO2. Indolequinones **1** and **5** were then added independently (final concentration from 0 to 2 μM) and at the indicated time points an aliquot (40 μL) was removed and diluted 100-fold in stop buffer [50 mM potassium phosphate buffer, pH 7.4, containing 250 mM sucrose, 5 μM FAD, 200 μM NRH and 0.1% (v/v) Tween-20] and frozen immediately in liquid nitrogen. NQO2 activity was then measured at room temperature in samples (960 μL) supplemented with 10 μM menadione and 0.1 mg/ml MTT (final volume 1ml) and the linear increase in absorbance at 550nm was monitored for 3 min. The data generated were analyzed using the Kitz and Wilson plot (31) and the K_i and k_{inact} values were calculated accordingly.

Comparison of indolequinones with known competitive inhibitors of NQO2

The ability of various compounds to inhibit NQO2 was compared in an assay adapted for a 96-well format using resazurin as NQO2 substrate. A 100 μ l of reaction was setup for each drug concentration containing 50 mM potassium phosphate buffer (pH 7.4), 1 mg/ml BSA, 125 mM NaCl, 5 μ M FAD, 200 μ M NRH, and 20 ng NQO2. Drugs were then introduced at the final concentration from 0.0025 μ M to 250 μ M and incubated at room temperature for 5 min; 10 μ l of resazurin stock solution was then added into each well to achieve a final concentration of 15 μ M. The fluorescence of reduced resazurin was then monitored at 530 nm excitation/590 nm emission for 5 min using a molecular device SpectraMax fluorescence plate reader. The rate of change in fluorescence per minute was calculated and the NQO2 activity remaining expressed as percentage of DMSO-treated control.

Inactivation of NQO2 and NQO1 in K562 cells

The inhibition of NQO2 activity in K562 cells by these indolequinones was determined using the following procedure. K562 cells (3×10^6) were treated with various concentrations of inhibitors for 15 min in 1 ml of complete medium, after which the medium was then removed by centrifugation (1500 rpm $\times$ 3 min), and cells were washed twice with cold PBS. Cells were then resuspended in 25 mM Tris buffer, pH 7.4, containing 250 mM sucrose and 5 μ M FAD and sonicated on ice. The protein concentrations of sonicates were determined by the method of Lowry (32). NQO2 activity was determined using NRH-dependent CB1954 metabolism (23). For these studies, 25 μ l of cell sonicates were mixed with 25 μ l of 25 mM Tris-HCl buffer, pH 7.4, containing 400 μ M NRH and 100 μ M CB1954 and allowed to react at room temperature for 15 min. The reaction was then stopped by the addition of 50 μ l cold acetonitrile, centrifuged at 13k rpm (4°C) and 25 μ l of supernatant was removed and analyzed for CB1954 metabolism by HPLC as previously described (23). The amount of CB1954 consumed (proportional to NQO2 activity) was then calculated for each sample. NQO2 activity was normalized to protein content and the results were expressed as percent of DMSO-treated control. NQO1 activity of cell sonicates were determined by measuring NADH-dependent reduction of DCPIP in 1 ml reactions at room temperature. Briefly, an aliquot of sonicate was added to 50 mM potassium phosphate buffer containing 0.1% Tween-20, 200 μ M NADH and 40 μ M DCPIP and the linear decrease in absorbance was measured at 600 nm over 1 min.

Growth Inhibition Assay

Growth inhibition of K562 cells was measured using the MTT colorimetric assay (33). For these studies K562 cells were seeded at 5×10^4 cells per well in 96-well plates, in triplicate for each compound and then allowed to recover for 1 h. Cells were then treated with the appropriate indolequinone (0–5000 nM) in complete medium. After 72 h, 10 μ l of complete medium containing MTT (5 mg/ml) was added to each well and incubated for an additional 4 h. Plates were then centrifuged at 1000 rpm for 5 min and the medium removed and DMSO (100 μ L) was then added to each well to dissolve the crystalline formazan product derived from cellular metabolism of MTT. Optical density was determined at 550 nm using a Molecular Devices Thermomax microplate reader. The IC₅₀ values were defined as the concentration of indolequinone that resulted in 50% reduction in cell number compared to the DMSO-treated control, determined from semilogarithmic plots of percentage of control versus indolequinone concentration.

Molecular Docking of the Indolequinone Ligands into NQO2

All simulations were performed within the Discovery Studio software package (Version 2.5.5; Accelrys Inc., San Diego, CA). The 1.8 Å structure of human NQO2 co-crystallized with indolequinone **1** (PDB: 3O73), has been resolved by our group and deposited in the

RCSB protein databank (<http://www.pdb.org>). The CHARMM force field (34) was applied to NQO2 structure to represent the protonation state of the protein at physiological pH. The binding site was defined as whole residues within an interface 12Å radius subset encompassing the FAD cofactor. LigandFit (35) was used for the molecular docking of indolequinones **1–9** into the binding site of NQO2. Fifty structural outputs were specified and the identification of a docked conformation was followed by a minimization (1000 iterations) using the conjugate gradient method to a convergence of 0.001 kcal/mol. The Poisson-Boltzmann method was used to calculate binding energy, with a non-bonded list radius of 14Å (36). The conformations for each docked complex were ranked on the basis of binding energy and interactions examined. The top-ranked poses were subsequently chosen based on binding energies and interactions known to be essential for efficient mechanism-based inhibition including hydrogen bonding and π -stacking between the indolequinone and FAD cofactor (20, 27, 37, 38).

HPLC and Mass Spectrometry Analysis of FAD Adducts

Indolequinone-inactivated human recombinant NQO2 protein for HPLC and MS analysis was prepared by incubating 25 µg of rhNQO2 with a 10-fold molar excess of indolequinones in 25 mM potassium phosphate buffer, pH 7.4 (40µl final volume) in the presence or absence of 0.5 mM NRH. Reactions were stopped after 10 minutes by the addition of 60 µl ice-cold methanol to denature NQO2 and release FAD. Precipitated protein was removed by centrifugation (13,000 rpm × 5 min at 4°C) and 50 µl of the supernatant was then loaded onto a C₁₈ reverse-phase HPLC column (Luna C18(2), 5 µ, 250 × 4.6 mm, Phenomenex, Torrance, CA) and eluted at 1 ml/min with a mobile phase of 10 mM ammonium acetate (A) and 100% acetonitrile (B) with a linear gradient of 5–50% B over 15 min using a detection wavelength of 260 nm. For LC-MS analysis, rhNQO2 was inactivated and FAD extracted as describe above and then concentrated under vacuum (SpeedVac, Savant AES-1000) and 8 µl of sample was introduced into an Agilent 1100 capillary HPLC system utilizing a 1.0 × 150 mm Grace Vydac C₁₈ MS column (218MS5115) and a mobile phase of 10 mM ammonium acetate (A) and 10 mM ammonium acetate in acetonitrile (B) with a linear gradient of 5–60% B over 10 min at a flow rate of 50 µl/min. Spectra were recorded on a Waters QTOFII mass spectrometer in positive mode, scanning from m/z 200–1500 using a 1 sec scan time.

Results

Indolequinones

A series of indolequinones were selected for this study varying in both the 5- and 6-substituents and in the leaving group at the 3-position. The 5- and 6-substituents were chosen to examine the effect of structural variations in the side chain on inhibition of NQO2 and the impact of 5 versus 6 substitutions. Compounds were designed to have either very good leaving groups at the indole 3-position, such as 4-nitrophenoxy and 2,4,6-trifluorophenoxy, or very poor leaving groups under physiological conditions (3-phenoxy and 3-hydroxy). Chemical structures of the indolequinones used in these studies are shown in Figure 1.

NRH-dependent NQO2 Inhibition by Indolequinones

The ability of indolequinones to inhibit recombinant human NQO2 protein was examined in the presence and absence of the co-factor NRH. For these studies rhNQO2 was incubated with indolequinones in the presence and absence of NRH for 5 min after which a sample of the reaction was removed, diluted and NQO2 activity was measured spectrophotometrically (550nm) using NRH-dependent menadione reduction coupled to MTT reduction (23). The incubation of **1**, **2**, **4**, and **5** (1 µM) with NRH and rhNQO2 resulted in greater than 95% inhibition of NQO2 catalytic activity (Fig. 2, Table 1), and this inactivation was dependent

upon NRH, suggestive of mechanism-based inhibition. These data demonstrated that variations in the 5-position amino alkyl chain length (CH₂)_n where n = 3 (compounds **4** and **5**) or n = 4 (compounds **1** and **2**) as well as substitutions on the terminal amine (compound **5**) were tolerated and resulted in marked NQO2 inhibition. However, aminoalkylamino substitutions at the 6-position (compounds **3** and **6**) resulted in compounds that were much poorer inhibitors when compared to indolequinones substituted at the 5-position. No inhibition of rhNQO2 was observed with the indolequinones **7**, **8**, and **9** in the presence or absence of NRH. Data using compound **7** illustrates that branching of the proximal nitrogen in the aminoalkylamino chain is not optimal for inhibition while the studies with compounds **8** and **9** demonstrated that a leaving group is required at the 3-position for inhibition. Inhibition of NQO2 by the indolequinones appeared to be irreversible since desalting of the indolequinone-inhibited protein using the Millipore Microcon Molecular Cutoff device did not result in recovery of enzyme activity (data not shown). To determine if these compounds could also inhibit the related enzyme NQO1 we incubated rhNQO1 with indolequinones (1 μM) in the presence and absence of NADH and in these studies no inhibition of NQO1 was detected (27).

Kinetic Analysis of NQO2 Inhibition by Indolequinones

Indolequinone **1** and **5** were selected for kinetic analysis since they were the most efficient NQO2 inhibitors. The inactivation of NQO2 by both indolequinones was very rapid, and consequently, the time and concentration dependence of the inhibition kinetics were determined at 4°C. As shown in Figure 3, the inhibition of NQO2 by compound **1** (A) and **5** (C) was both time- and concentration-dependent. The K_i and k_{inact} were determined from Kitz and Wilson plots (Fig. 3B, D) as described (31).

Partition Ratio Determination

The partition ratio for a mechanism-based inhibitor is the number of catalytic cycles required to inactivate one molecule of enzyme (30); therefore, the lower the partition ratio, the more efficient the inhibitor. To compare the efficiency of NQO2 inhibition the partition ratio for each indolequinone that demonstrated NRH-dependent NQO2 inhibition was determined (Table 1). Partition ratios of approximately 2 were determined for inactivation of NQO2 by indolequinones **1**, **2**, **4**, and **5** indicating that these indolequinones were extremely efficient mechanism-based inhibitors of NQO2. The 6-aminoalkylamino compounds, **3** and **6**, had partition ratios of 14.0 and 3.7, respectively, displaying less efficient mechanism-based inhibition of NQO2 when compared to their 5-aminoalkylamino counterparts. Partition ratios for the indolequinones **8** and **9** were not determined since they did not inhibit NQO2.

Potency of indolequinone inhibitors relative to previously characterized competitive inhibitors of NQO2

The ability of indolequinones to inactivate NQO2 was compared with previously reported competitive NQO2 inhibitors using a 96-well based assay as described in Materials and Methods. Indolequinones **1**, **2** and **5** were considerably more potent as inhibitors of NQO2 than either quercetin, imatinib, resveratrol, chloroquine or melatonin (Fig. 4). A molar comparison showed at least an order of magnitude more potent inhibition with the indolequinone inhibitors than other compounds used.

Inhibition of NQO2 Activity by Indolequinones in K562 Cells

The ability of the indolequinones **1**, **2**, **4**, and **5** to inhibit NQO2 activity in cells was examined in the human leukemia cell line K562 (Fig. 5). K562 cells were chosen because they contain very high levels of NQO2 activity and protein and only trace levels of NQO1

activity and protein (Fig. 5, inset). For these studies K562 cells were treated with increasing concentrations of indolequinones for 15 min, after which the cells were collected, and NQO2 (Fig. 5A) and NQO1 (Fig. 5B) activities were measured. Results from these studies demonstrated that these indolequinones could enter cells and efficiently inhibit NQO2. Greater than 95% of NQO2 activity could be inhibited following treatment with indolequinones (Fig. 5A). Importantly, NQO1 activity was not affected under the same conditions (Fig 5B). These data demonstrated that aminoalkyl-substituted indolequinones have very good selectivity for NQO2 over NQO1 in cells.

Growth Inhibition by Indolequinones in K562 cells

Growth inhibition studies were performed in K562 cells. Cells were treated for 72 h with various concentrations of indolequinones. The IC₅₀ values were calculated from the survival curve and reported in Table 1. All of the indolequinones tested have an IC₅₀ greater than 10 μ M (the highest concentration tested). These results indicate that these indolequinones are non-toxic to K562 cells at concentrations that result in greater than 95% inhibition of NQO2 activity.

Molecular Modeling of Indolequinones into NQO2

Molecular docking simulations were performed for indolequinones **1–9** in the active site of NQO2 with the anionic form of the reduced FAD cofactor which represents the physiological situation where quinone encounters reduced FAD (8, 39). High-ranking binding conformations of the indolequinones in the active site of NQO2 were identified and shown in Figure 6 (**1–3, 5** and **7**) and Figure S1 (**4, 6, 8, 9**). The binding energies corresponding to the selected conformation for each complex were calculated and listed in Table 1. The conformations of the inhibitors (**1–7**) with the indolequinone system stacked in parallel above the isoalloxazine ring of FAD resulted in more favorable binding energies, which could be attributed to the optimal aromatic π - π interactions between the indolequinone ring and the reduced isoalloxazine ring system of FAD (40, 41). Indolequinones **8, 9** have high binding energies (Table 1) but are not inhibitors due to the absence of an efficient leaving group. The overall conformations of the potent inhibitors **1** (Fig. 6A), **2** (Fig. 6B), **4** (Fig. S1A), **5** (Fig. 6D) in the active site of NQO2 were very similar. As observed in the co-crystallized structure (27) the indolequinone ring was aligned parallel to the isoalloxazine ring of FAD and the C7 carbonyl of the quinone moiety was positioned directly above the N-5 of the FAD, enabling efficient hydride transfer from the FAD during reduction (8, 39). The hydrophobic interaction between the indole 2-methyl and residues Phe178 and Phe106 also contributes favorably to the binding energy. These studies also revealed the importance of the interaction between the terminal amine of the aminoalkylamino side chain and the NQO2 active site which further contributed to the binding energy. The hydrogen bond formed between the terminal amine of the aminoalkylamino side chain and either Gln122, Glu193 or FAD (Fig. 6 and Fig. S1, black dotted line) at the edge of the binding pocket, acts as an anchor which appears to stabilize the binding of the indolequinone in the active site after reduction. The aminoalkylamino substitution at the 6-position (**3** and **6**) results in steric hindrance between the indolequinone and the active site (Fig. 6C and Fig. S1B), making them less efficient inhibitors compared with the 5-substituted analogs (Fig. 6A and Fig. 6D). Incorporating a proximal N-methyl branch to the aminoalkylamino side chain (**7**) hinders the accessibility of the indolequinone into the active site and affected alignment with the FAD (Fig. 6E). Consequently, **7** was predicted to be ineffective as an inhibitor of NQO2 which was confirmed by biochemical testing (Fig. 2).

Modeling also provided an explanation of the inability of the indolequinone NQO2 inhibitors to inhibit NQO1. The active site of NQO1 is more constrained than NQO2 and the

bulky aminoalkylamino at the 5 position blocked entrance of the inhibitors into the NQO1 active site (Fig. 6F). The impaired accessibility of the indolequinone inhibitors into the active site of NQO1 relative to NQO2 is shown in Figure 6 using indolequinone **1** as an example.

MS analysis

Our previous data using compound **1** suggested a novel mechanism of inhibition of NQO2 by the indolequinones involving FAD adduction (27). To confirm and extend these observations we examined three additional compounds using ESI-LC/MS. To determine whether the indolequinones were adducted to the non-covalently bound FAD cofactor, rhNQO2 protein was incubated with indolequinones in the presence and absence of NRH and the liberated FAD was then analyzed using HPLC and ESI-LC/MS for potential indolequinone-derived adducts. HPLC analysis of FAD extracted from NQO2 following incubation with indolequinone **5** and NRH clearly showed a decrease in the parent FAD peak with the appearance of a new, slightly more hydrophobic peak when compared to incubations performed in the absence of NRH (Fig. 7A). LC/MS analysis (positive mode) of the new peak gave the spectrum shown in Fig. 7B (MH^+ $m/z = 1075.35$, detailed fragmentation analysis is reported in Supporting Information, Fig. S2). The mass spectrum confirms the presence of a **5** iminium-FAD adduct (FAD, $m/z = 785$ plus the reactive iminium intermediate of **5**, $m/z = 288$) and suggest the formation of the hydroquinone form of the iminium-FAD adduct (Scheme 1). To confirm adduction of FAD by the series of indolequinones, these experiments were extended to indolequinones **4** (Fig. 7C and Fig. S3) and **2** (Fig. 7D and Fig. S4). In these studies iminium-FAD adducts could be detected that differ in the molecular weight corresponding to the 5-position aminoalkylamino side chain with all indolequinone NQO2 inhibitors tested demonstrating the generality of this mechanism. Detailed fragmentation analysis of iminium-FAD adducts generated from indolequinones **5**, **4**, **2** are shown in Supporting Information (Figures S2–4).

Discussion

Several structurally diverse compounds have been shown to be competitive inhibitors of NQO2, including the natural products resveratrol (21, 22), quercetin (23), casimiroin (42), and melatonin (9, 25) and the drugs chloroquine (11, 24) and imatinib (12, 26). The indolequinones we have developed were designed to undergo NQO2-mediated reduction to the hydroquinone form and subsequent elimination of a leaving group from the (indol-3-yl) methyl position. This results in the generation of a reactive iminium ion that directly alkylates within the NQO2 active site, resulting in the irreversible inhibition of NQO2 enzymatic activity. An important measure of mechanism-based inhibition is the requirement for the catalytic step to occur prior to inactivation of the enzyme. The inhibition of rhNQO2 activity by this series of indolequinones was therefore monitored in the presence and absence of NRH to determine if the inhibition of rhNQO2 was mechanism-based. Indolequinones **1–6** demonstrated NRH-dependent NQO2 inhibition, which supports mechanism-based inactivation. In addition, inhibition of NQO2 by indolequinones was time- and concentration-dependent, which is characteristic of mechanism-based inhibitors. Moreover, compounds **8** and **9**, which are NQO2 substrates but have a poor leaving group at the 3-position and therefore could not generate an iminium intermediate, were not inhibitors of NQO2, suggesting that reduction and expulsion of the leaving group is a prerequisite for the mechanism-based inhibition of NQO2. The indolequinone inhibitors of NQO2 were at least an order of magnitude more potent than previously characterized competitive inhibitors; quercetin, imatinib, chloroquine, resveratrol and melatonin.

Structural requirements for effective inhibition of NQO2 were examined and found to include an aminoalkylamino side chain at the 5-position. Compounds where the aminoalkyl

chain terminated in either an unsubstituted amino group (**1**, **2**, **4**) or a dimethyl substituted amino group (**5**) were efficient inhibitors of NQO2. Compounds with aminoalkylamino substitutions at the 6-position (**3**, **6**) were not efficient mechanism-based inhibitors of NQO2. As discussed above, a good leaving group at the 3-position (either 4-nitrophenoxy or 4-trifluorophenoxy) which enables generation of a reactive iminium electrophile following enzymatic reduction is essential for efficient NQO2 inhibition by these compounds and since indolequinones **8** and **9**, do not meet this requirement, they were not inhibitors of NQO2. Our molecular modeling simulations suggested tight binding between the inhibitors and the NQO2 active site. The terminal amine nitrogen in the aminoalkylamino side chain was shown to be critical for the binding of the indolequinones by forming hydrogen bonds with Gln122 or Glu193 or FAD and providing an anchor point for the indolequinones in the active site. The molecular modeling simulations also provided a possible explanation for the inefficiency of **3** and **6** in inhibiting NQO2 comparing to **2** and **5**. As shown in Figure 6 and Figure S1 indolequinones with a side chain located at the 5-position are positioned in parallel above the isoalloxazine rings and orientated such that the quinone is directly above the N5 nitrogen in FAD. In contrast, indolequinones with a side chain located at the 6-position do not orient themselves with the quinone ring positioned directly above the N5 nitrogen of FAD resulting in less efficient inhibition of NQO2. If the side chain is branched at the nitrogen proximal to the quinone ring (see indolequinone **7**), then docking of the compound into the active site of NQO2 is blocked. This hypothesis was confirmed as indolequinone **7** was a very poor inhibitor of NQO2 in biochemical studies.

Although based on an identical indolequinone backbone as the previously reported series of NQO1 inhibitors (37, 43), the compounds reported in these studies exhibited selectivity for inhibition of NQO2. In a cell free system, rhNQO1 was only slightly inhibited by indolequinones using concentrations one order of magnitude greater than those required for complete inhibition of NQO2 (27). Studies in K562 cells demonstrated that NQO1 activity was not affected despite greater than 95% inhibition of NQO2 activity after 15 min of treatment with indolequinones (Figure 5). The selective inhibition of NQO2 over NQO1 could be attributed to the bulky aminoalkylamino substituent at the 5-position, which blocks entrance of the indolequinones into the NQO1 active site (Figure 6F). The NQO2 active site is wider and more hydrophobic than that of NQO1 and could adequately accommodate these larger indolequinones. We cannot exclude the possibility that other flavoproteins could also be inhibited by the indolequinones. However, this seems unlikely given the requirement for two-electron reduction to release the reactive iminium, the need for an active site which will accommodate the indolequinone in an optimal binding orientation and the specificity of these indolequinones for NQO2 relative to the structurally-related NQO1.

Analysis of the active site of NQO2 using molecular modeling simulations demonstrated a lack of amino acids in the active site suitable for covalent adduction by electrophiles and we were unable to detect the adduction of amino acids following incubation of either of the indolequinones **1** or **5** with NQO2 and NRH using LC/MS analysis. In contrast, adducts could readily be detected using the indolequinone ES936 and the related enzyme NQO1 as we have previously reported (20). Our previous data (27) indicated that nucleophilic sites in FAD were potential adduction sites and we confirmed this data and extended it to three other additional indolequinone inhibitors indicating the generality of the FAD adduction mechanism for this series of inhibitors. ESI-LC/MS confirmed the presence of a FAD adduct generated from the iminium electrophile obtained after reduction of the indolequinone and loss of the leaving group consistent with the proposed scheme for activation (Scheme 1). These FAD adducts are relatively unstable once FAD is extracted from NQO2 (data not shown) and unequivocal assignment of the structure of the FAD adducts formed will require further studies most probably using biological NMR.

A unique feature of NQO2 is that it is usually expressed at very low levels in human solid tumors but is expressed at higher levels in leukemias (6) and could be a potential therapeutic target. NQO2 has been shown to be the major non-kinase target of imatinib in leukemias raising the intriguing possibility that NQO2 may contribute to leukemia cell growth or alternatively may act as a sink for imatinib altering its pharmacodynamics. Our data indicates that indolequinones were non-toxic to K562 cells at the concentrations where complete NQO2 inhibition was achieved and although this was not the major goal of the present study it does suggest that inhibition of NQO2 alone is not sufficient to cause leukemia cell death. However, it is possible that NQO2 inhibition may have a synergistic effect with Bcr-Abl inhibition in the presence of imatinib, or alternatively that NQO2 inhibition by indolequinone could potentiate imatinib toxicity by increasing the concentration of free drug. Both genetic and pharmacologic approaches are currently underway in our laboratory to fully understand the role of NQO2 in leukemias and in imatinib pharmacology.

In summary, we have generated and characterized a series of indolequinone compounds as mechanism-based inhibitors of NQO2 in cell-free and cellular systems. The indolequinones are the most effective inhibitors of NQO2 to be described to date and utilizing modeling, biochemical studies and mass spectrometry we have characterized both their mechanism of inhibition and structural parameters required for effective inhibition of NQO2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

NQO1	NADH:quinone oxidoreductase 1
NQO2	NRH:quinone oxidoreductase 2
NRH	nicotinamide riboside
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
ESI-LC/MS	Electrospray ionization-liquid chromatography/mass spectrometry

References

1. Ernster L, Navazio F. Soluble diaphorase in animal tissues. *Acta Chem Scand*. 1958; 12:595–602.
2. Ernster L, Ljunggren M, Danielson L. Purification and some properties of a highly dicumarol-sensitive liver diaphorase. *Biochem Biophys Res Commun*. 1960; 2:88–92.
3. Martius C. [The place of vitamin K1 in respiratory chain; preliminary report.]. *Biochem Z*. 1954; 326:26–27. [PubMed: 13230055]
4. Ernster L. DT-diaphorase: a historical review. *Chemica Scripta*. 1987; 27A:1–13.
5. Jaiswal AK, Burnett P, Adesnik M, McBride OW. Nucleotide and deduced amino acid sequence of a human cDNA (NQO2) corresponding to a second member of the NAD(P)H:quinone oxidoreductase gene family. Extensive polymorphism at the NQO2 gene locus on chromosome 6. *Biochemistry*. 1990; 29:1899–1906. [PubMed: 1691923]
6. Zhao Q, Yang XL, Holtzclaw WD, Talalay P. Unexpected genetic and structural relationships of a long-forgotten flavoenzyme to NAD(P)H:quinone reductase (DT-diaphorase). *Proc Natl Acad Sci U S A*. 1997; 94:1669–1674. [PubMed: 9050836]
7. Liao S, Dulaney JT, Williams-Ashman HG. Purification and properties of a flavoprotein catalyzing the oxidation of reduced ribosyl nicotinamide. *J Biol Chem*. 1962; 237:2981–2987. [PubMed: 14465018]

8. Bianchet MA, Faig M, Amzel LM. Structure and mechanism of NAD(P)H:quinone acceptor oxidoreductases (NQO). *Methods Enzymol.* 2004; 382:144–174. [PubMed: 15047101]
9. Vella F, Ferry G, Delagrangé P, Boutin JA. NRH:quinone reductase 2: an enzyme of surprises and mysteries. *Biochem Pharmacol.* 2005; 71:1–12. [PubMed: 16253210]
10. Siegel D, Ross D. Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. *Free Radic Biol Med.* 2000; 29:246–253. [PubMed: 11035253]
11. Graves PR, Kwiek JJ, Fadden P, Ray R, Hardeman K, Coley AM, Foley M, Haystead TA. Discovery of novel targets of quinoline drugs in the human purine binding proteome. *Mol Pharmacol.* 2002; 62:1364–1372. [PubMed: 12435804]
12. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol.* 2007; 25:1035–1044. [PubMed: 17721511]
13. Fu Y, Buryanovskyy L, Zhang Z. Quinone reductase 2 is a catechol quinone reductase. *J Biol Chem.* 2008; 283:23829–23835. [PubMed: 18579530]
14. Gaikwad NW, Yang L, Rogan EG, Cavalieri EL. Evidence for NQO2-mediated reduction of the carcinogenic estrogen ortho-quinones. *Free Radic Biol Med.* 2009; 46:253–262. [PubMed: 18996184]
15. Jamieson D, Tung AT, Knox RJ, Boddy AV. Reduction of mitomycin C is catalysed by human recombinant NRH:quinone oxidoreductase 2 using reduced nicotinamide adenine dinucleotide as an electron donating co-factor. *Br J Cancer.* 2006; 95:1229–1233. [PubMed: 17031400]
16. Yan C, Kepa JK, Siegel D, Stratford IJ, Ross D. Dissecting the role of multiple reductases in bioactivation and cytotoxicity of the antitumor agent 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1). *Mol Pharmacol.* 2008; 74:1657–1665. [PubMed: 18794327]
17. Siegel D.; Reigan, P.; Guo, W.; Ross, D. Proceedings of the American Association for Cancer Research. Los Angeles: 2007. The metabolism of antitumor quinones by NRH:quinone oxidoreductase 2 (NQO2).
18. Knox RJ, Jenkins TC, Hobbs SM, Chen S, Melton RG, Burke PJ. Bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by human NAD(P)H quinone oxidoreductase 2: a novel co-substrate-mediated antitumor prodrug therapy. *Cancer Res.* 2000; 60:4179–4186. [PubMed: 10945627]
19. Hollander PM, Ernster L. Studies on the reaction mechanism of DT diaphorase. Action of dead-end inhibitors and effects of phospholipids. *Arch Biochem Biophys.* 1975; 169:560–567. [PubMed: 52345]
20. Winski SL, Faig M, Bianchet MA, Siegel D, Swann E, Fung K, Duncan MW, Moody CJ, Amzel LM, Ross D. Characterization of a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1 by biochemical, X-ray crystallographic, and mass spectrometric approaches. *Biochemistry.* 2001; 40:15135–15142. [PubMed: 11735396]
21. Buryanovskyy L, Fu Y, Boyd M, Ma Y, Hsieh TC, Wu JM, Zhang Z. Crystal structure of quinone reductase 2 in complex with resveratrol. *Biochemistry.* 2004; 43:11417–11426. [PubMed: 15350128]
22. Wang Z, Hsieh TC, Zhang Z, Ma Y, Wu JM. Identification and purification of resveratrol targeting proteins using immobilized resveratrol affinity chromatography. *Biochem Biophys Res Commun.* 2004; 323:743–749. [PubMed: 15381063]
23. Wu K, Knox R, Sun XZ, Joseph P, Jaiswal AK, Zhang D, Deng PS, Chen S. Catalytic properties of NAD(P)H:quinone oxidoreductase-2 (NQO2), a dihydronicotinamide riboside dependent oxidoreductase. *Arch Biochem Biophys.* 1997; 347:221–228. [PubMed: 9367528]
24. Kwiek JJ, Haystead TA, Rudolph J. Kinetic mechanism of quinone oxidoreductase 2 and its inhibition by the antimalarial quinolines. *Biochemistry.* 2004; 43:4538–4547. [PubMed: 15078100]
25. Mailliet F, Ferry G, Vella F, Berger S, Coge F, Chomarat P, Mallet C, Guenin SP, Guillaumet G, Viaud-Massuard MC, Yous S, Delagrangé P, Boutin JA. Characterization of the melatoninergic

- MT3 binding site on the NRH:quinone oxidoreductase 2 enzyme. *Biochem Pharmacol.* 2005; 71:74–88. [PubMed: 16293234]
26. Winger JA, Hantschel O, Superti-Furga G, Kuriyan J. The structure of the leukemia drug imatinib bound to human quinone reductase 2 (NQO2). *BMC Struct Biol.* 2009; 9:7. [PubMed: 19236722]
 27. Dufour M, Yan C, Siegel D, Colucci MA, Jenner M, Oldham NJ, Gomez J, Reigan P, Li Y, Matteis CID, Ross D, Moody CJ. Mechanism-based inhibition of quinone reductase 2 (NQO2). Selectivity for NQO2 over NQO1 and structural basis for flavoprotein inhibition. *ChemBiolChem.* 2011 In press.
 28. Beall HD, Mulcahy RT, Siegel D, Traver RD, Gibson NW, Ross D. Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. *Cancer Res.* 1994; 54:3196–3201. [PubMed: 8205540]
 29. Friedlos F, Jarman M, Davies LC, Boland MP, Knox RJ. Identification of novel reduced pyridinium derivatives as synthetic co-factors for the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2). *Biochem Pharmacol.* 1992; 44:25–31. [PubMed: 1385952]
 30. Silverman, RB. *Chemistry and Enzymology.* Boca Raton, FL: CRC Press; 1988. Mechanism based enzyme inhibition; p. 3-30.
 31. Kitz R, Wilson IB. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J Biol Chem.* 1962; 237:3245–3249. [PubMed: 14033211]
 32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193:265–275. [PubMed: 14907713]
 33. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65:55–63. [PubMed: 6606682]
 34. Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S. CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. *J Comput Chem.* 1983; 4:187–217.
 35. Luty BA, Wasserman ZR, Stouten PFW, Hodge CN, Zacharias M, McCammon JA. A molecular mechanics/grid method for evaluation of ligand-receptor interactions. *J Comput Chem.* 1995; 16:454–464.
 36. Gilson MK, Honig B. Calculation of the total electrostatic energy of a macromolecular system: solvation energies, binding energies, and conformational analysis. *Proteins.* 1988; 4:7–18. [PubMed: 3186692]
 37. Reigan P, Colucci MA, Siegel D, Chilloux A, Moody CJ, Ross D. Development of indolequinone mechanism-based inhibitors of NAD(P)H:quinone oxidoreductase 1 (NQO1): NQO1 inhibition and growth inhibitory activity in human pancreatic MIA PaCa-2 cancer cells. *Biochemistry.* 2007; 46:5941–5950. [PubMed: 17455910]
 38. Nolan KA, Zhao H, Faulder PF, Frenkel AD, Timson DJ, Siegel D, Ross D, Burke TR Jr, Stratford IJ, Bryce RA. Coumarin-based inhibitors of human NAD(P)H:quinone oxidoreductase-1. Identification, structure-activity, off-target effects and in vitro human pancreatic cancer toxicity. *J Med Chem.* 2007; 50:6316–6325. [PubMed: 17999461]
 39. Cavalier G, Amzel LM. Mechanism of NAD(P)H:quinone reductase: Ab initio studies of reduced flavin. *Proteins.* 2001; 43:420–432. [PubMed: 11340659]
 40. Zhou Z, Fisher D, Spidel J, Greenfield J, Patson B, Fazal A, Wigal C, Moe OA, Madura JD. Kinetic and docking studies of the interaction of quinones with the quinone reductase active site. *Biochemistry.* 2003; 42:1985–1994. [PubMed: 12590585]
 41. Suleman A, Skibo EB. A comprehensive study of the active site residues of DT-diaphorase: rational design of benzimidazoles as DT-diaphorase substrates. *J Med Chem.* 2002; 45:1211–1220. [PubMed: 11881990]
 42. Maiti A, Reddy PV, Sturdy M, Marler L, Pegan SD, Mesecar AD, Pezzuto JM, Cushman M. Synthesis of casimiroin and optimization of its quinone reductase 2 and aromatase inhibitory activities. *J Med Chem.* 2009; 52:1873–1884. [PubMed: 19265439]
 43. Colucci MA, Reigan P, Siegel D, Chilloux A, Ross D, Moody CJ. Synthesis and evaluation of 3-aryloxymethyl-1,2-dimethylindole-4,7-diones as mechanism-based inhibitors of

NAD(P)H:quinone oxidoreductase 1 (NQO1) activity. J Med Chem. 2007; 50:5780–5789.
[PubMed: 17944451]

Figure 1. Chemical structures of the indolequinones

Figure 2. NRH-dependent inhibition of NQO2 by indolequinones

Purified rhNQO2 was incubated with 1 μ M indolequinones either in the absence (closed bars) or presence (hatched bars) of NRH and after 5 min an aliquot was removed assayed for NQO2 catalytic activity. Results are expressed as percentage of NQO2 activity remaining compared to DMSO treated controls. Reaction conditions are described in Materials and Methods. Results represent the mean $\pm$ standard deviation of three independent determinations.

Figure 3. Kinetic analysis of the inhibition of NQO2 by indolequinones
 Concentration- and time-dependent inhibition of rhNQO2 by indolequinones **1** (A) and **5** (C). Results are expressed as percentage of NQO2 activity remaining compared to DMSO treated controls. Results represent the mean $\pm$ standard deviation of three independent determinations. Best-fit lines were derived from non-linear regression analysis. The resultant Kitz-Wilson plots were shown for indolequinone **1** (B) and **5** (D).

Figure 4. Comparison of indolequinones with known inhibitors of NQO2

Indolequinones **1**, **2**, and **5** were compared with previously reported NQO2 inhibitors quercetin, imatinib, resveratrol, chloroquine, and melatonin for their ability to inhibit rhNQO2 activity in a cell-free system. Inhibition reaction and activity measurement were carried out as described in Materials and Methods. Results are expressed as percentage of NQO2 activity remaining compared to DMSO treated controls. Results represent the mean $\pm$ standard deviation of three independent determinations.

Figure 5. Selective inhibition of NQO2 by indolequinones in K562 cells

K562 human leukemia cells were treated with indolequinones **1**, **2**, **4**, and **5** (100–500 nM) for 15 min after which the cells were recovered and NQO2 and NQO1 catalytic activities were measured as described in the Materials and Methods. Results are expressed as the percent activity remaining compared to DMSO-treated controls. **A**, percent NQO2 activity remaining following indolequinone treatment. **B**, percent NQO1 activity remaining following indolequinone treatment. Closed columns, 100 nM; striped columns, 250 nM; open columns, 500 nM. Results represent the mean $\pm$ standard deviation of three independent determinations. **Inset**, immunoblot analysis demonstrating NQO1 and NQO2

protein expression in K562 cells. Lane 1, rhNQO1 standard (10 ng); lane 2, rhNQO2 standard (10 ng); lane 3, K562 sonicate (20 μ g).

Figure 6. Structural frames from molecular docking simulations of the indolequinone ligands in the active site of NQO2 and NQO1

A–E representations of **1**(A), **2**(B), **3**(C), **5**(D), and **7**(E) in the quinone form (stick display style; carbon atoms colored salmon) in the reduced FAD (stick display style; carbon atoms colored yellow) site of NQO2; key amino acids (stick display style; carbon atoms colored green); and hydrogen bond contacts between the terminal amine of the aminoalkylamino chain and the active site (black dashed line) are displayed. F, molecular docking of indolequinone **1** into the active site of NQO1.

Figure 7. HPLC and LC/MS analysis of the adduction of the FAD cofactor in NQO2 by indolequinones

Incubation of NQO2 with indolequinone in the presence of NRH results in indolequinone adduction of FAD. (A) HPLC analysis (260 nm) of FAD extracted from NQO2 following incubation with indolequinone **5** in the absence (left panel) and presence (right panel) of NRH. ESI-LC/MS analysis of FAD adducts obtained from incubation with NQO2 and NRH were also shown for indolequinones **5** (B), **4** (C), and **2** (D). Detailed fragmentation analysis of the FAD adducts are shown in Supporting Information (Figures S2–4).

Scheme 1.

Proposed mechanism of FAD adduction by the indolequinone inhibitors of NQO2.

Table 1

Biochemical and physical characterization of the indolequinones.

Compound	R ¹	R ²	R ³	rhNQO2 inhibition at 1μM ^a	partition ratio ^b	IC ₅₀ ^c K562 cells	Binding Energy ^d kcal/mol
1	4-NO ₂ -C ₆ H ₄	NH ₂ (CH ₂) ₄ NH	H	96%	2.0	> 10uM	-21.6
2	2,4,6-F ₃ -C ₆ H ₂	NH ₂ (CH ₂) ₄ NH	H	96%	2.0	> 10uM	-30.2
3	2,4,6-F ₃ -C ₆ H ₂	H	NH ₂ (CH ₂) ₄ NH	33%	14.0	> 10uM	-18.0
4	2,4,6-F ₃ -C ₆ H ₂	NH ₂ (CH ₂) ₃ NH	H	97%	2.1	> 10uM	-21.7
5	2,4,6-F ₃ -C ₆ H ₂	(CH ₃) ₂ N(CH ₂) ₄ NH	H	98%	2.1	> 10uM	-26.5
6	2,4,6-F ₃ -C ₆ H ₂	H	(CH ₃) ₂ N(CH ₂) ₃ NH	73%	3.7	> 10uM	-12.8
7	2,4,6-F ₃ -C ₆ H ₂	(CH ₃) ₂ N(CH ₂) ₃ N(CH ₃)	H	5%	74	> 10uM	-13.1
8	C ₆ H ₅	(CH ₃) ₂ N(CH ₂) ₃ NH	H	NI ^e	NI ^e	> 10uM	-34.4
9	H	(CH ₃) ₂ N(CH ₂) ₃ NH	H	NI ^e	NI ^e	> 10uM	-25.9

^a rhNQO2 inhibition by 1 mM of indolequinone was also graphed and shown in Figure 2.

^b Partition ratio for each compound was determined as described in materials and methods.

^c IC50 values in K562 cells were determined using the MTT assay.

^d Binding energies in the form of ΔG value were calculated from molecular modeling simulations.

^e NI, no inhibition.