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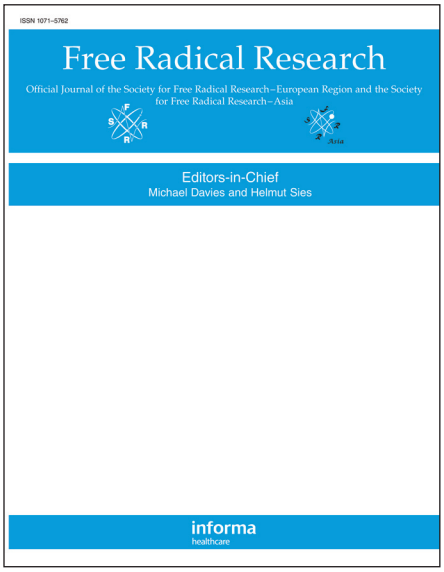

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Evidence for NQO1 and NQO2 Catalyzed Reduction of *ortho*- and *para*-Quinone Methides

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Abstract

NAD(P)H:quinone oxidoreductase (NQO1) and NRH:quinone oxidoreductase 2 (NQO2) catalyze the two-electron reduction of quinones and thereby prevent generation of toxic radicals. Quinone methides (QM) covalently react with cellular macromolecules to form DNA adducts and/or protein conjugates resulting in toxicity and carcinogenesis. Based on similar structural features of quinones and QM's, it is logical to assume that NQO1 and/or NQO2 could also catalyze the two-electron reduction of QM's. However, hitherto the reduction of QM's, as both endogenous and/or exogenous biological substrates, by either NQO1/NQO2 has never been demonstrated. Here we show for the first time that both NQO1 and NQO2 can catalyze the reduction of electrophilic *ortho*-/*para*-QM's. The involvement of the enzyme in the reduction of *p*-cresol-quinone-methide (PCQM) and *o*-cresol-quinone-methide (OCQM) was demonstrated by reappearance of NQO1/NQO2-FAD peak at 450 nm after addition of the QM's to the assay mixture. Further reduction of methides by NQO1/NQO2 was confirmed by analyzing the assay mixture by tandem mass spectrometry. Preliminary kinetic studies show that NQO2 is faster in reducing QM's than its homolog NQO1, and moreover, *ortho*-QM's are reduced faster than *para*-QM's. Enzyme-substrate docking studies showed results consistent with enzyme catalysis. Thus, NQO1/NQO2 can play a significant role in deactivation of QM's.

Evidence for NQO1 and NQO2 Catalyzed Reduction of *ortho*- and *para*-Quinone Methides

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Short title: NQO1 and NQO2 are quinone methide reductase

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Abstract

NAD(P)H:quinone oxidoreductase (NQO1) and NRH:quinone oxidoreductase 2 (NQO2) catalyze the two-electron reduction of quinones and thereby prevent generation of toxic radicals. Quinone methides (QM) covalently react with cellular macromolecules to form DNA adducts and/or protein conjugates resulting in toxicity and carcinogenesis. Based on similar structural features of quinones and QM's, it is logical to assume that NQO1 and/or NQO2 could also catalyze the two-electron reduction of QM's. However, hitherto the reduction of QM's, as both endogenous and/or exogenous biological substrates, by either NQO1/NQO2 has never been demonstrated. Here we show for the first time that both NQO1 and NQO2 can catalyze the reduction of electrophilic *ortho*-/*para*-QM's. The involvement of the enzyme in the reduction of *p*-cresol-quinone-methide (PCQM) and *o*-cresol-quinone-methide (OCQM) was demonstrated by reappearance of NQO1/NQO2-FAD peak at 450 nm after addition of the QM's to the assay mixture. Further reduction of methides by NQO1/NQO2 was confirmed by analyzing the assay mixture by tandem mass spectrometry. Preliminary kinetic studies show that NQO2 is faster in reducing QM's than its homolog NQO1, and moreover, *ortho*-QM's are reduced faster than *para*-QM's. Enzyme-substrate docking studies showed results consistent with enzyme catalysis. Thus, NQO1/NQO2 can play a significant role in deactivation of QM's.

Key words: NQO1; NQO2; *ortho*-quinone methide; *para*-quinone methide; mass spectrometry; enzyme kinetics; substrate binding

Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase 1; BNAH, benzyldihydronicotinamide riboside; NQO2, NRH:quinone oxidoreductase 2; NRH, dihydronicotinamide riboside; PCQM, *p*-cresol quinone methide; OCQM, *o*-cresol quinone methide; MD,Menadione

Introduction

NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase) and NRH:quinone oxidoreductase 2 (NQO2) enzymes have unique ability to catalyze the two-electron reduction of quinones to hydroquinones, thereby prevents generation of toxic semiquinone radicals, reactive oxygen species and alkylation [1-5]. Recently expression levels or single nucleotide polymorphisms in NQO1 have been shown to be associated with numerous cancers, viz. breast [6], prostate [7], colorectal [8], esophageal [9], gastric [10], osteocarcinoma [11], skin [12], acute myeloid leukemia (AML) [13] as well as cardiovascular diseases [14], Parkinson's disease [15] and diabetes [16]. Similarly, expression levels or single nucleotide polymorphisms in NQO2 are shown to be associated with AML [13], breast [17], prostate [18] and esophageal [9] cancer as well as cardiovascular diseases [14],

Parkinson's disease [19] and schizophrenia [20]. Furthermore, both NQO1 and NQO2 are proposed as strong prognostic and predictive indicators of breast cancer [21]. NQO1 has been extensively studied since 1960 when it was first identified by Ernster et al. and named as DT-diaphorase [22]. NQO2 was discovered by Liao and Williams-Ashman in 1961, but its exact biological role is still being determined [23]. NQO1 and its homolog NQO2 are ubiquitous among various tissues, including liver, kidney, brain, heart, and lung [24, 25]; in addition, many solid tumors including thyroid, adrenal, breast, ovarian, cornea, colon, liver, and non-small cell lungs cancers express NQO1. The high level of sequence homology between NQO1 and NQO2 suggests that NQO2 may have overlapping substrate specificities and similar functions to that of NQO1 [26, 27]. NQO2 is different from NQO1, however, in its cofactor requirements, using a metabolic product of NAD(P)H, dihydronicotinamide riboside (NRH), rather than NAD(P)H as an electron donor. Another major difference between NQO2 and NQO1 is that NQO2 is resistant to typical inhibitors of NQO1, such as dicumarol. The crystal structure analysis of NQO2 shows that it contains a specific metal binding site that is not present in NQO1 [28, 29]. Gathering evidence suggest that in addition to two-electron reduction of quinone compounds, NQO1 is involved in scavenging of superoxide anion radicals, maintenance of endogenous antioxidants, 20S proteasome pathway and stabilization of p53 protein [21]. Owing to their broad substrate specificity, NQO1 and NQO2 play an important role in the detoxification of various endogenous and exogenous quinones [30-32], including estrogen quinones [33, 34] which is consistent with their association with many diseases. However, hitherto the reduction of quinone methides (QM), as both endogenous and exogenous biological substrates, by either NQO1 or NQO2 has never been demonstrated.

Quinone methides are structurally analogous to quinones, with one of the carbonyl groups replaced by a methylene group. Due to this, QM's are more polarized and reactive than quinones, with rearomatization being a key driving force. QM's are known to interact with biological macromolecules resulting in formation of DNA adducts and/or protein conjugates (Fig. 1) [35-38]. Like quinones, QM's are also known to undergo redox-cycling and generate reactive oxygen species. QM intermediates react with glutathione, resulting in glutathione depletion, which can cause cell death. Previous studies with **p**-cresol, **o**-cresol, and tamoxifen have demonstrated the enzyme-catalyzed formation of QM's [36-38], which covalently interact with DNA resulting in formation of DNA adducts. Due to their cytotoxicity, molecules that have the QM moiety or that can produce QM intermediates upon biological activation are being synthesized or extracted from natural products and are tested as potential chemotherapeutic drugs against cancer [39].

Taking into account the similar structural features of quinones and quinone methides, it is logical to assume that NQO1 and/or NQO2 could also catalyze the two electron reduction of endogenous or exogenous QM's. In fact QM's have never

been investigated as substrates for NQO1 and NQO2. In this study, we used strategies to ascertain the role of NQO1 and NQO2 in catalyzing the reduction of *ortho*- and *para*-quinone methides. Both **o**-cresol and **p**-cresol have minimal structural features needed to form the basic backbone of *ortho*- and *para*-quinone methide respectively, and hence they were used in this study. For the first time, we have established that QM's are biological substrates of NQO1 and NQO2.

EXPERIMENTAL PROCEDURES

NQO1 (expressed in *E. coli,* lyophilized powder), NQO2 (expressed in *E. coli)*, NADH, menadione (MD), *p*-cresol and *o*-cresol were purchased from Sigma Chemical Co. (St. Louis, MO). BNAH was purchased from TCI America (Portland, OR). Activated MnO_2 was obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents used were HPLC grade and all other chemicals were of the highest grade available. *p*-cresol quinone methide (PCQM) and *o*-cresol quinone methide (OCQM) were freshly synthesized using a reported procedure [36, 37]. The identity of both quinone methides was confirmed by UV and MS/MS (Fig. 2).

Determination of NQO1 and NQO2 activity using UV-Vis spectroscope

The anaerobic reduction and oxidation of protein-bound FAD were monitored at 450 nm in the absence and presence of the cofactor, and substrate using a UV-Vis spectrophotometer (Evolution 300, Fisher Scientific). Nitrogen gas was bubbled continuously for 2 h in the buffer, and organic solvents were degassed to ensure anaerobic conditions. The reduction of protein-bound FAD was monitored between 280 nm and 700 nm. First, the protein-bound FAD was reduced by NADH, and then the reduced flavin was used to carry out the reduction of the substrate.

10U of NQO1 was added to 1mL buffer (25 mM Tris-HCl, pH 6.8) containing 0.7 mg/ml BSA in a 1.5 ml cuvette, and a spectrum was recorded. This was followed by the addition of 0.9 eq NADH to the same cuvette, and then the reoxidation of the protein-bound flavin was carried out by the addition of PCQM or OCQM (1.2eq). Spectra were recorded after each addition. Exactly same procedure was followed for NQO2 assay, except BNAH was used as the cofactor. The assay mixtures were passed through 5000MW cutoff filters and directly injected into the mass spectrometer for product analysis.

Control spectra were recorded separately of NADH (0.9 eq), BNAH (0.9 eq), and 1.2 eq of PCQM and OCQM added at room temperature to 1mL buffer (25 mM Tris-HCl, pH 6.8) containing 0.7 mg/ml BSA in a 1.5ml cuvette. As a control, to determine whether *p*-cresol or *o*-cresol regenerates the protein-bound reduced FAD, a separate assay was performed with the cresol isoforms as the substrates.

MS/MS analysis of quinone methide reduction

Xevo-TQ triple quadrupole mass spectrometer (Waters, Milford, MA, USA) recorded MS/MS spectra using Electron Spray Ionization (ESI) in positive mode, capillary voltage of 3.0 kV, extractor cone voltage of 3V and detector voltage of 500 V. Desolvation gas flow was maintained at 600L/h. Source temperature and desolvation temperatures were set at 150 and 350° C, respectively. The collision energy was varied to optimize daughter ions. The acquisition range was 20-500 Da. The test samples, pure standards of *ortho-/ para*-cresol, both QM's and the enzyme assay mixtures, were introduced to the source at a flow rate of 5µl/min by using methanol: water (1:1) and 0.1% formic acid mixture as the carrier solution and the MS and MS/MS spectra were recorded.

Kinetic studies

Kinetic measurements were carried out at 25°C in 1 ml of 25 mM Tris-HCl, pH 6.8, containing 0.7 mg/ml BSA in a 1.5-ml cuvette using a UV spectrophotometer. Reduction of the substrate, PCQM, OCQM and MD, by reduced NQO1/NQO2 was assayed at 450 nm. In these cases, the kinetic studies were carried out by assaying 0.4 µM NQO1/NQO2 at increasing concentrations of PCQM, OCQM, MD. Km values were calculated with Origin software (Origin 8.5.0, Northampton, MA).

Substrate docking study

Substrate docking was performed with SwissDock [40, 41] using PDB's for NQO1 (1D4A) [42] and NQO2 (1QR2) [28] along with PCQM (CID: 136328) [43] and OCQM (CID: 13265823) [44] as inputs for docking. Zinc atoms were deleted from NQO2 PDB. Only one subunit of NQO1 PDB was utilized for docking. Solutions were chosen based on reported energy values and proximity to and correct alignment of the substrate with the FAD isoalloxazine ring and orientation of the alkene and N-5, based on previously reported crystal structure of menadione with NQO2 (PDB 2QR2, [28]). Docking results were visualized with UCSF Chimera [45].

Results

We have investigated the NQO1- and NQO2-catalyzed reduction of both *ortho-* and *para*-quinone methides by using UV and tandem mass spectrometry.

Determination of methide reductase activity of NQO1 & NQO2

We took advantage of the ping-pong mechanism of enzyme catalysis [33, 34], and divided the assay into two steps. First, FAD-NQO1/ FAD-NQO2 were reduced with either NADH or BNAH, and second, the reduced enzyme was further used to carry out the reduction of the substrate. The reduction of methide substrates by the NQO1/ NQO2 enzymes was studied by monitoring the redox

cycle of FAD in the active site of NQO1/ NQO2. The UV-Vis absorption spectrum of native (oxidized) NQO1 is shown in Fig.3. The stoichiometry of cofactor, NADH was adjusted to 0.9 eq to avoid spontaneous reduction of the substrate by excess cofactor. Upon addition of NADH to the assay mixture containing NQO1 the FAD absorption peak at 450 nm completely disappeared, due to its reduction to FADH2 (Fig. 3 Ab & Bb). Next, 1.2 eq of PCQM or OCQM was added to the assay mixture, which caused the peak at 450 nm to reappear (Fig. 3 Ac & Bc). Excess PCQM and OCQM (1.2 eq) was used in the assay so that it could outnumber any unreacted NADH and sufficient molecules would be available for enzyme catalysis. Control experiments were performed in which excess *p*-cresol or *o*-cresol was added to assay mixture containing reduced NQO1 (Fig. 3 C & D). Addition of either *p*-cresol or *o*-cresol did not result in reappearance of peak at 450 nm confirming that NQO1 used only methides as a substrates and reduction of methide was indeed catalyzed by NQO1.

Similarly, The UV-Vis absorption spectrum of native (oxidized) NQO2 is shown in Fig.4. The stoichiometry of cofactor, BNAH, was adjusted to 0.9 eq to avoid spontaneous reduction of the substrate by excess cofactor. Upon addition of BNAH to the assay mixture containing NQO2, the FAD absorption peak at 450 nm completely disappeared, due to its reduction to FADH2 (Fig. 4 Ab & Bb). Next, 1.2 eq of PCQM and OCQM was added to the reaction mixture, which caused the peak at 450 nm to reappear (Fig. 4 Ac & Bc). Like the NQO1 assay, excess PCQM and OCQM (1.2 eq) was used in the NQO2 assay so that it could outnumber any unreacted BNAH and sufficient molecules would be available for enzyme catalysis. Control experiments were performed in which excess *p*-cresol or *o*-cresol was added to assay mixture containing reduced NQO2 (Fig. 4 C & D). Addition of either *p*-cresol or *o*-cresol did not result in reappearance of peak at 450 nm confirming that NQO2 used only methides as a substrates and reduction of methide was indeed catalyzed by NQO2.

Both these observations are consistent with the regeneration of FAD from $FADH_2$ after transfer of hydride. However, it is reasonable to assume that an increase in the peak at 450 nm reports on the reduction of the substrate, because it is the immediate product of hydride transfer from $FADH_2$. At the completion of the substrate reduction, the spectrum of FAD was completely restored in both NQO1 and NQO2 (Fig. 3&4).

Furthermore, to confirm the reduction of PCQM and OCQM by NQO1, the assay mixtures were passed through a filter and subjected to MS and MS/MS analysis. Assay mixture containing PCQM/OCQM and NQO1 showed the presence of MS peak at 108.9 (Fig. 5B) and 109 (Fig. 5E) m/z respectively, which is consistent with the M+1 of molecular mass of *p*-cresol or *o*-cresol (Fig. 2A & 2D). Further parent peaks, 108.9 and 109 m/z, were fragmented resulting in daughter ions at 92.7, 56.6 and 92.8, 56.3 m/z respectively (Figure 5B &5E). The fragmentation patterns matched well with that of standard *p*-cresol or *o*-cresol. To

confirm the reduction of PCQM and OCQM by NQO2, the assay mixtures were passed through a filter and subjected to MS and MS/MS analysis. Assay mixture containing PCQM/OCQM and NQO2 showed the presence of MS peak at 108.8 and 109 m/z respectively, which is consistent with the M+1 of molecular mass of *p*-cresol or *o*-cresol (Fig. 2A & 2D). Further parent peaks, 108.8 and 109 m/z, were fragmented resulting in daughter ions at 93, 56.2 and 92.8, 56.4 m/z respectively (Figure 5C &5F). The fragmentation patterns matched well with that of standard *p*-cresol or *o*-cresol.

Kinetic studies

The Km values for reduction of PCQM, OCQM and MD were determined to be 0.474, 0.242, and 0.403 μM respectively for NQO1 mediated catalysis and 0.242, 0.192, and 0.354 μM respectively for NQO2 mediated catalysis (Fig. 6). As expected in enzyme catalyzed reaction, the graph of velocity against substrate concentration was non-linear for all above assays (Fig. 6). When compared among themselves the rates of the catalysis were following order NQO2-OCQM > NQO1-OCQM > NQO2-PCQM > NQO2-MD > NQO1-MD > NQO1-PCQM.

Substrate docking study

The outputs from these four combinations appeared in proximity to the FAD isoalloxazine ring, (Fig. 7), and were oriented to align the site of nucleophilic attack (alkene) and N-5 of the FAD isoalloxazine ring. The chosen docking solutions had NQO1 with OCQM had a total Energy = 13.66, ΔG = -5.49, and a distance of 1.87 Å

NQO1 with PCQM had a total Energy = 12.52, ΔG = -5.2, and a distance of 2.05 Å.

NQO2 with OCQM had a total Energy = 11.98, ΔG = -5.66, and a distance of 2.42

Å. NQO2 with PCQM had a total Energy = 10.55, ΔG = -5.48, and a distance of 4.71

Å. The crystal structure of NQO2 and MD (PDB 2QR2, [28]) had a distance of 4.23Å

(Table.1, Fig. 7).

In summary, UV-Vis and mass spectrometry as well as the docking studies of the enzyme–substrate complex revealed the ability of NQO1 and NQO2 to reduce the *ortho*- as well as *para*- quinone methide backbones. Kinetic studies revealed that NQO1 and NQO2 reduced *para*-quinone methides slower than *ortho*-quinone methides.

Discussion

For the first time, we have demonstrated the NQO1- and NQO2-catalyzed reduction of both *ortho*- and *para*-quinone methides by using UV-Vis spectroscopy and tandem mass spectroscopy. These studies were further supported by docking studies of the enzyme–substrate complex. The NQO1 and NQO2 activity assays were carried out in two stages. First, in the absence of oxygen, NQO1/NQO2 was stoichiometrically reduced with NADH/BNAH, which transferred hydride to the FAD bound to the enzyme (Fig. 8). It has been suggested that the negative charge generated by hydride transfer is delocalized to the oxygen of the isoalloxazine of FAD, which takes a proton from the neighboring amino acid, tyrosine. After hydride transfer, the resulting NAD+ leaves and makes the binding pocket available for substrate binding [46]. Fig. 3 & 4 reflect that the NQO1/NQO2 enzymes are readily reduced by NADH and BNAH respectively, irrespective of a possible need for the presence of the substrate as an activating effector. In the next step, hydride transfer from reduced flavin to *ortho*-/*para*-quinone methide is carried out (Fig. 8). This transfer is primarily dependent upon the correct orientation and approach of the C-6/8 of the quinone methide, PCQM/OCQM, and N-5 of the isoalloxazine ring. The reoxidation of FAD after addition of PCQM/OCQM, confirming the reduction of PCQM/OCQM in the binding pocket of NQO1/NQO2, is shown in Fig. 3 & 4. After this reduction, the resulting hydro-quinone methide tautomerizes to methylphenol, *p*-cresol/*o*-cresol (Fig. 8) and the isoalloxazine of FAD returns to the oxidized quinoid form. The regeneration of FAD implies that PCQM/OCQM has reached the binding pocket and did not alkylate the binding site. It is possible that some part of QM's, due to its high reactivity, could covalently interact with minor portion of NQO1/NQO2, rendering them inactive. However, assay conditions such as almost instantaneous recording of the UV spectrum after addition of substrate keeps the major portion of the enzyme intact, which is reflected in Mechalis –Menton kinetics. Subsequent tandem mass spectrometry analysis of the reaction mixtures that were used for UV assays were performed to investigate the catalytic products of NQO1 and NQO2 enzyme reductions. MS/MS results clearly demonstrates the presence of *p*-cresol/*o*-cresol in the respective assay mixtures confirming the reduction of PCQM/OCQM (Fig. 5).

To provide additional evidence for enzyme involvement in the reduction and to understand the comparative activities of NQO1 and NQO2, we used the ping-pong mechanism of enzyme catalysis [33, 34] to determine the Km values for PCQM, OCQM, and MD. Our results suggest that NQO2-catalyzed reaction is slightly faster for all three substrates, PCQM, OCQM, and MD, than NQO1 (Fig. 6). The binding pockets of NQO1 and NQO2 are similar to each other with the exception of three residues, residue # 126, 128, and 131, which allow the NQO2 binding pocket to be slightly bigger and hydrophobic than that of NQO1 [28, 47]. The larger size and hydrophobicity of the NQO2 binding site might explain the

higher affinity of all three substrates, (PCQM, OCQM, and MD) for NQO2 than for NQO1. The substrate with the highest affinity for both enzymes was OCQM (Fig. 6), which is not surprising because OCQM is more electrophilic than PCQM based on strain in ring structure due to the *ortho*- positioned alkene. Both NQO1 and NQO2 reduced *para*-quinone methides slower than *ortho*-quinone methides. The *para*-containing substrates, PCQM and MD, had similar affinities for NQO1 and NQO2. The results show that NQO2 has a slightly higher affinity for PCQM than MD. However, NQO1 appears to have a higher affinity for MD than PCQM (Fig. 5). These results also suggest that for NQO2, QM's appears to be better substrates than quinones, whereas for NQO1 the distinction is not clear.

NQO1 and NQO2 both contain one FAD binding site per subunit [28, 42]. The FAD sits in the floor of the binding pocket, and the isoalloxazine ring of the FAD is responsible for the stacking of planar aromatic substrates that insert into the active site [48, 49]. The hydrophobic pocket along with π-stacking of the isoalloxazine ring of NQO1 and NQO2 could provide a region with high affinity for ligands such as PCQM and OCQM (Fig. 7) [19]. Docking studies show that both PCQM and OCQM are aligned over the isoalloxazine ring in the binding pocket of NQO1/NQO2, which is consistent with previous reports [14, 19]. There were no direct contacts observed between the substrate and enzyme, following previous reports in the menadione NQO2 crystal structure [28]. We chose docking outputs that oriented the alkene group of both PCQM and OCQM near the N-5 of the isoalloxazine ring in order to facilitate reduction by respective 1,6 [36] and 1,8 [50] Michael's addition reaction (Fig. 8). The optimal distance for direct hydride transfer from the N-5 of the isoalloxazine ring of FAD to the substrate has been shown to be within 10 Å [51]. For example, the distance between NADH and N-5 of NQO1 has been previously reported to be 4.02 Å [19, 42] and the distance between MD and the N-5 of NQO2 was found to be 4.23 Å (Table 1, Fig. 7, [28]). In the current study the distance between PCQM and N-5 of NQO1 was found to be 4.71 Å, whereas it was 1.87 Å in case of NQO2. For OCQM the distance was found to be 2.05 Å for NQO1 and 2.42 Å in the case of NQO2. All these distances fall within needed 10 Å range, suggesting that these docking solutions position the N-5 at a favorable distance for hydride transfer (Table 1, Fig. 7). Additionally, a similar planar orientation over the isoalloxazine ring π-system has been observed, though with some slight deviations for some solutions (Fig. 7). Due to their reasonably wide binding site, both NQO1 and NQO2 could accommodate structurally diverse endogenous and exogenous quinone methides.

It has been well documented that alkyl phenol group present in endogenous molecules, environmental toxicants, carcinogens and chemotherapeutic drugs could undergo enzymatic oxidation by peroxidases and P450 enzymes to form transient radical species, which can further oxidize to quinone methides [39]. Quinone methides are known electrophilic species that covalently react with cellular macromolecules to form DNA adducts and/or protein conjugates [35-39]. The

covalent binding of quinone methides have been shown to lead to various forms of cytotoxicity, immunotoxicity, and carcinogenesis [35-39]. The UV spectra obtained indicate that both NQO1 and NQO2 accept QM's a substrate (Fig. 3&4). Our results suggest that NQO1/NQO2 can catalyze reduction of a variety of both *ortho*- and *para*-quinone methides and thereby provide protection against these reactive species.

On the contrary, the distribution of NQO1 and NQO2 in many tumors could have a potential impact on the development and efficacy of QM generating or containing anticancer agents. Many solid tumors including thyroid, adrenal, breast, ovarian, cornea, colon, liver, and non-small cell lungs cancers express the NQO1 gene [52- 53]. Expression of the NQO2 gene is high in normal human liver and skeletal muscle [24, 56, 57], and minimal in the kidney, heart, pancreas, brain, and red blood cells [58]. There is growing interest in the possible use of QM containing molecules as promising chemotherapeutic drugs [39]. Recently, numerous synthetic QM DNA alkylating agents that can form nucleobase adducts have been developed as probable antitumor agents [39, 59]. The ability of NQO1 and NQO2 to reduce QM's may have significant implications against the efficacy of potential QM based anticancer drugs currently under development.

In conclusion, *ortho*- and *para*-QM's are substrates of NQO1 as well as NQO2, and NQO1/NQO2 can play significant role in deactivation of *ortho*- and *para*-QM's that are formed from endogenous molecules, environmental toxicants, and carcinogens that might lead to toxicity. These findings may also have broad implications for the efficacy of potential QM based anticancer drugs that are currently under development.

Acknowledgments

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References

[1] Dinkova-Kostova, A. T.; Talalay, P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. *Archives of biochemistry and biophysics* **501:**116-123; 2010.

[2] Ross, D.; Siegel, D. NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), functions and pharmacogenetics. *Methods in enzymology* **382:**115-144; 2004.

[3] Iyanagi, T. On the mechanisms of one- and two- electron transfer by flavin enzymes. . *Chem. Scr* **27A:**31-36; 1987.

[4] Leung, K. K. K.; Litchfield, D. W.; Shilton, B. H. Flavin adenine dinucleotide content of quinone reductase 2: Analysis and optimization for structure–function studies. *Analytical Biochemistry* **420:**84-89; 2012.

[5] Dinkova-Kostova, A. T.; Talalay, P. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. *Free Radical Biology and Medicine* **29:**231-240; 2000.

[6] Jamshidi, M.; Bartkova, J.; Greco, D.; Tommiska, J.; Fagerholm, R.; Aittomaki, K.; Mattson, J.; Villman, K.; Vrtel, R.; Lukas, J.; Heikkila, P.; Blomqvist, C.; Bartek, J.; Nevanlinna, H. NQO1 expression correlates inversely with NFkappaB activation in human breast cancer. *Breast cancer research and treatment* **132:**955-968; 2012.

[7] Stoehr, C. G.; Nolte, E.; Wach, S.; Wieland, W. F.; Hofstaedter, F.; Hartmann, A.; Stoehr, R. NAD(P)H:Quinone Oxidoreductase 1 (NQO1) P187S Polymorphism and Prostate Cancer Risk in Caucasians. *International journal of molecular sciences* **13:**10959-10969; 2012.

[8] Chen, J.; Lin, Y.; Zhang, R.; Huang, Z. J.; Pan, X. G. Contribution of NAD(P)H quinone oxidoreductase 1 (NQO1) Pro187Ser polymorphism and risk of colorectal adenoma and colorectal cancer in Caucasians: a meta-analysis. *Archives of medical research* **43:**58-66; 2012.

[9] Malik, M. A.; Zargar, S. A.; Mittal, B. Role of NQO1 609C>T and NQO2 -3423G>A gene polymorphisms in esophageal cancer risk in Kashmir valley and meta analysis. *Molecular biology reports* **39:**9095-9104; 2012.

[10] Zhang, Y.; Wang, Z. T.; Zhong, J. Meta-analysis demonstrates that the NAD(P)H: quinone oxidoreductase 1 (NQO1) gene 609 C>T polymorphism is associated with increased gastric cancer risk in Asians. *Genetics and molecular research: GMR* **11:**2328-2337; 2012.

[11] Windsor, R. E.; Strauss, S. J.; Kallis, C.; Wood, N. E.; Whelan, J. S. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. *Cancer* **118:**1856-1867; 2012.

[12] Patrick, B. A.; Jaiswal, A. K. Stress-induced NQO1 controls stability of C/EBPalpha against 20S proteasomal degradation to regulate p63 expression with implications in protection against chemical-induced skin cancer. *Oncogene* **31:**4362-4371; 2012.

[13] Dunna NR, A. C., Vure S, Sailaja K, Surekha D, Raghunadharao D, Rajappa S, Vishnupriya S. NQO1*2 [NAD(P)H: quinone

oxidoreductase 1 polymorphism and its influence on acute leukemia risk. *Biology and Medicine* **3:**19-25; 2011.

[14] Zhu, H.; Li, Y. NAD(P)H: quinone oxidoreductase 1 and its potential protective role in cardiovascular diseases and related conditions. *Cardiovascular toxicology* **12:**39-45; 2012.

[15] van Muiswinkel, F. L.; de Vos, R. A.; Bol, J. G.; Andringa, G.; Jansen Steur, E. N.; Ross, D.; Siegel, D.; Drukarch, B. Expression of NAD(P)H:quinone oxidoreductase in the normal and Parkinsonian substantia nigra. *Neurobiology of aging* **25:**1253-1262; 2004.

[16] Ramprasath, T.; Murugan, P. S.; Kalaiarasan, E.; Gomathi, P.; Rathinavel, A.; Selvam, G. S. Genetic association of Glutathione peroxidase-1 (GPx-1) and NAD(P)H:Quinone Oxidoreductase 1(NQO1) variants and their association of CAD in patients with type-2 diabetes. *Molecular and cellular biochemistry* **361:**143-150; 2012.

[17] Hubackova, M.; Vaclavikova, R.; Ehrlichova, M.; Mrhalova, M.; Kodet, R.; Kubackova, K.; Vrana, D.; Gut, I.; Soucek, P. Association of superoxide dismutases and NAD(P)H quinone oxidoreductases with prognosis of patients with breast carcinomas. *International journal of cancer. Journal international du cancer* **130:**338-348; 2012.

[18] Mandal, R. K.; Nissar, K.; Mittal, R. D. Genetic variants in metabolizing genes NQO1, NQO2, MTHFR and risk of prostate cancer: a study from North India. *Molecular biology reports* **39:**11145-11152; 2012.

[19] Fu, Y.; Buryanovskyy, L.; Zhang, Z. Quinone reductase 2 is a catechol quinone reductase. *The Journal of biological chemistry* **283:**23829-23835; 2008.

[20] Harada, S.; Tachikawa, H.; Kawanishi, Y. A possible association between an insertion/deletion polymorphism of the NQO2 gene and schizophrenia. *Psychiatric genetics* **13:**205-209; 2003.

[21] Fagerholm, R.; Hofstetter, B.; Tommiska, J.; Aaltonen, K.; Vrtel, R.; Syrjakoski, K.; Kallioniemi, A.; Kilpivaara, O.; Mannermaa, A.; Kosma, V. M.; Uusitupa, M.; Eskelinen, M.; Kataja, V.; Aittomaki, K.; von Smitten, K.; Heikkila, P.; Lukas, J.; Holli, K.; Bartkova, J.; Blomqvist, C.; Bartek, J.; Nevanlinna, H. NAD(P)H:quinone oxidoreductase 1 NQO1*2 genotype (P187S) is a strong prognostic and predictive factor in breast cancer. *Nature genetics* **40:**844-853; 2008.

[22] Ernster, L.; Lindberg, O. Animal mitochondria. *Annual review of physiology* **20:**13-42; 1958.

[23] Liao, S.; Williams-Ashman, H. G. Enzymatic oxidation of some non-phosphorylated derivatives of dihydronicotinamide. *Biochemical and biophysical research communications* **4:**208-213; 1961.

[24] Long, D. J., 2nd; Jaiswal, A. K. NRH:quinone oxidoreductase2 (NQO2). *Chemico-biological interactions* **129:**99-112; 2000.

[25] Nosjean, O.; Nicolas, J. P.; Klupsch, F.; Delagrange, P.; Canet, E.; Boutin, J. A. Comparative pharmacological studies of melatonin receptors: MT1, MT2 and MT3/QR2. Tissue distribution of MT3/QR2. *Biochemical pharmacology* **61:**1369-1379; 2001.

[26] Jaiswal, A. K.; Burnett, P.; Adesnik, M.; McBride, O. W. 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* **29:**1899-1906; 1990.

[27] Wu, K.; Knox, R.; Sun, X. Z.; Joseph, P.; Jaiswal, A. K.; Zhang, D.; Deng, P. S.; Chen, S. Catalytic properties of NAD(P)H:quinone oxidoreductase-2 (NQO2), a dihydronicotinamide riboside dependent oxidoreductase. *Archives of biochemistry and biophysics* **347:**221-228; 1997.

[28] Foster, C. E.; Bianchet, M. A.; Talalay, P.; Zhao, Q.; Amzel, L. M. Crystal structure of human quinone reductase type 2, a metalloflavoprotein. *Biochemistry* **38:**9881-9886; 1999.

[29] AbuKhader, M.; Heap, J.; De Matteis, C.; Kellam, B.; Doughty, S. W.; Minton, N.; Paoli, M. Binding of the anticancer prodrug CB1954 to the activating enzyme NQO2 revealed by the crystal structure of their complex. *Journal of medicinal chemistry* **48:**7714-7719; 2005.

[30] Ross, D.; Siegel, D. NAD(P)H:Quinone Oxidoreductase 1 (NQO1, DT-Diaphorase), Functions and Pharmacogenetics. In: Helmut, S.; Lester, P., eds. *Methods in enzymology*: Academic Press; 2004: 115-144.

[31] Kiyohara, C.; Yoshimasu, K.; Takayama, K.; Nakanishi, Y. NQO1, MPO, and the risk of lung cancer: a HuGE review. *Genetics in medicine: official journal of the American College of Medical Genetics* **7:**463-478; 2005.

[32] Bianco, N. R.; Chaplin, L. J.; Montano, M. M. Differential induction of quinone reductase by phytoestrogens and protection against oestrogen-induced DNA damage. *The Biochemical journal* **385:**279-287; 2005.

[33] Gaikwad, N. W.; Rogan, E. G.; Cavalieri, E. L. Evidence from ESI-MS for NQO1-catalyzed reduction of estrogen ortho-quinones. *Free radical biology & medicine* **43:**1289-1298; 2007.

[34] Gaikwad, N. W.; Yang, L.; Rogan, E. G.; Cavalieri, E. L. Evidence for NQO2-mediated reduction of the carcinogenic estrogen ortho-quinones. *Free radical biology & medicine* **46:**253-262; 2009.

[35] Thompson, D. C.; Thompson, J. A.; Sugumaran, M.; Moldeus, P. Biological and toxicological consequences of quinone methide formation. *Chemico-biological interactions* **86:**129-162; 1993.

[36] Gaikwad, N. W.; Bodell, W. J. Formation of DNA adducts by microsomal and peroxidase activation of p-cresol: role of quinone methide in DNA adduct formation. *Chemico-biological interactions* **138:**217-229; 2001.

[37] Gaikwad, N. W.; Bodell, W. J. Formation of DNA adducts in HL-60 cells treated with the toluene metabolite p-cresol: a potential biomarker for toluene exposure. *Chemico-biological interactions* **145:**149-158; 2003.

[38] Gaikwad, N. W.; Bodell, W. J. Peroxidase-mediated dealkylation of tamoxifen, detected by electrospray ionization-mass spectrometry, and activation to form DNA adducts. *Free radical biology & medicine* **52:**340-347; 2012.

[39] Dufrasne, F.; Gelbcke, M.; Neve, J.; Kiss, R.; Kraus, J. L. Quinone methides and their prodrugs: a subtle equilibrium between cancer promotion, prevention, and cure. *Current medicinal chemistry* **18:**3995-4011; 2011.

[40] Grosdidier, A.; Zoete, V.; Michielin, O. Fast docking using the CHARMM force field with EADock DSS. *Journal of computational chemistry*; 2011.

[41] Grosdidier, A.; Zoete, V.; Michielin, O. SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic acids research* **39:**W270-277; 2011.

[42] Faig, M.; Bianchet, M. A.; Talalay, P.; Chen, S.; Winski, S.; Ross, D.; Amzel, L. M. Structures of recombinant human and mouse NAD(P)H:quinone oxidoreductases: species comparison and structural changes with substrate binding and release. *Proceedings of the National Academy of Sciences of the United States of America* **97:**3177-3182; 2000.

[43] National Center for Biotechnology Information. PubChem Compound Database; CID=136328, h. p. n. n. n. g. s. s. c. c. l. e. r. a. N., 2012).

[44] National Center for Biotechnology Information. PubChem Compound Database; CID=13265823, h. p. n. n. n. g. s. s. c. c. l. e. r. a. N., 2012).

[45] Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera--a

visualization system for exploratory research and analysis. *Journal of computational chemistry* **25:**1605-1612; 2004.

[46] Li, R.; Bianchet, M. A.; Talalay, P.; Amzel, L. M. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proceedings of the National Academy of Sciences of the United States of America* **92:**8846-8850; 1995.

[47] Vella, F.; Ferry, G.; Delagrange, P.; Boutin, J. A. NRH:quinone reductase 2: an enzyme of surprises and mysteries. *Biochemical pharmacology* **71:**1-12; 2005.

[48] Calamini, B.; Santarsiero, B. D.; Boutin, J. A.; Mesecar, A. D. Kinetic, thermodynamic and X-ray structural insights into the interaction of melatonin and analogues with quinone reductase 2. *The Biochemical journal* **413:**81-91; 2008.

[49] Bianchet, M. A.; Erdemli, S. B.; Amzel, L. M. Structure, function, and mechanism of cytosolic quinone reductases. *Vitamins and hormones* **78:**63-84; 2008.

[50] Liu, Y.; Rokita, S. E. Inducible alkylation of DNA by a quinone methide-peptide nucleic acid conjugate. *Biochemistry* **51:**1020-1027; 2012.

[51] Basner, J. E.; Schwartz, S. D. Donor−Acceptor Distance and Protein Promoting Vibration Coupling to Hydride Transfer: A Possible Mechanism for Kinetic Control in Isozymes of Human Lactate Dehydrogenase. *The Journal of Physical Chemistry B* **108:**444-451; 2003.

[52] Belinsky, M.; Jaiswal, A. K. NAD(P)H:quinone oxidoreductase1 (DT-diaphorase) expression in normal and tumor tissues. *Cancer metastasis reviews* **12:**103-117; 1993.

[53] Joseph, P.; Xie, T.; Xu, Y.; Jaiswal, A. K. NAD(P)H:quinone oxidoreductase1 (DT-diaphorase): expression, regulation, and role in cancer. *Oncology research* **6:**525-532; 1994.

[54] Jaiswal, A. K. Human NAD(P)H:quinone oxidoreductase2. Gene structure, activity, and tissue-specific expression. *The Journal of biological chemistry* **269:**14502-14508; 1994.

[55] Siegel, D.; Ross, D. Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. *Free Radical Biology and Medicine* **29:**246-253; 2000.

[56] Jaiswal, A. K. Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free radical biology & medicine* **29:**254-262; 2000.

[57] Strassburg, A.; Strassburg, C. P.; Manns, M. P.; Tukey, R. H. Differential gene expression of NAD(P)H:quinone oxidoreductase

and NRH:quinone oxidoreductase in human hepatocellular and biliary tissue. *Molecular pharmacology* **61:**320-325; 2002.

[58] Graves, P. R.; Kwiek, J. J.; Fadden, P.; Ray, R.; Hardeman, K.; Coley, A. M.; Foley, M.; Haystead, T. A. Discovery of novel targets of quinoline drugs in the human purine binding proteome. *Molecular pharmacology* **62:**1364-1372; 2002.

[59] Zhou, Q.; Qu, Y.; Mangrum, J. B.; Wang, X. DNA alkylation with N-methylquinolinium quinone methide to N2-dG adducts resulting in extensive stops in primer extension with DNA polymerases and subsequent suppression of GFP expression in A549 cells. *Chemical research in toxicology* **24:**402-411; 2011.

Figure Legends

Figure 1

Proposed biological mechanisms of quinone methide formation and toxic pathways.

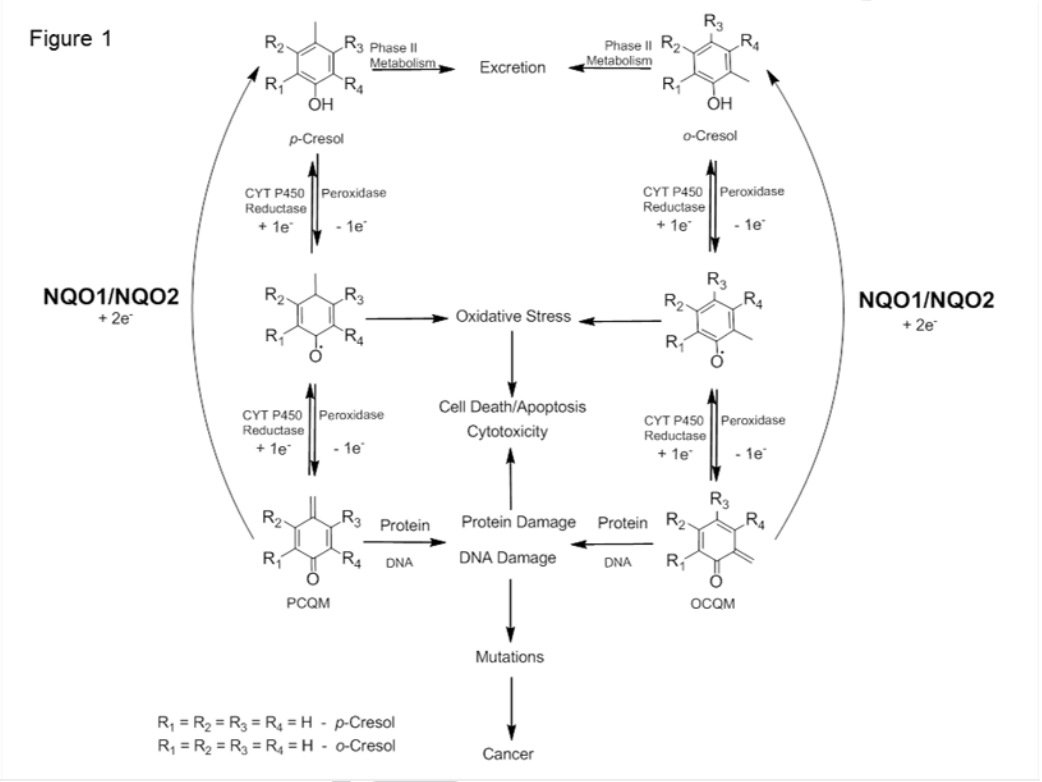

Figure 2

ESI-MS/MS spectra of synthesized PCQM (A) and OCQM (B) substrates.

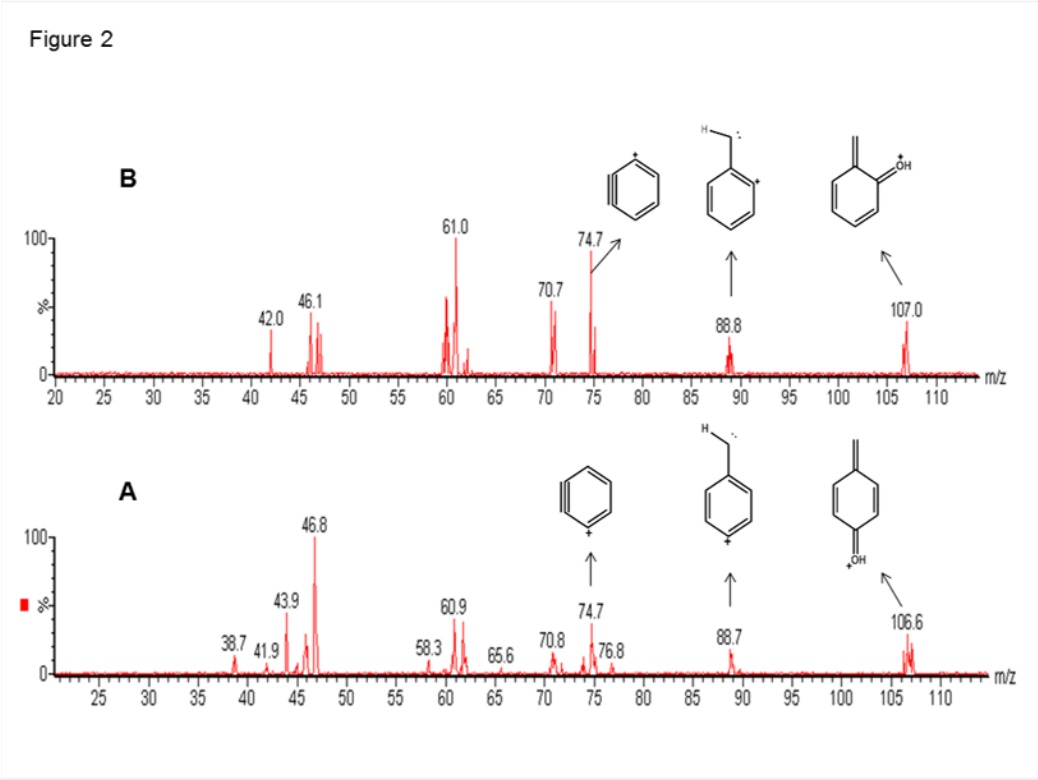

Figure 3

Spectral changes of NQO1 following addition of NADH and substrate, PCQM (A) and OCQM (B). The absorption spectra were measured after sequential addition of NQO1, NADH and substrate to 1 ml of 25 mM Tris-HCl, pH 6.8 at room temperature. The concentration of the enzyme was 10 U NQO1 (spectra a). The spectra after addition of 0.9 eq NADH (spectra b) and 1.2 eq substrate (spectra c), in that order. The control spectra (C & D) were recorded by using p-cresol or o-cresol as a substrates. The absorption spectra were measured after sequential addition of 10 U NQO1 and 0.9 eq NADH to 1 ml of 25 mM Tris-HCl, pH 6.8 at room temperature (spectra d). Addition of either 1.2 eq of p-cresol (C) or o-cresol (D) to assay mixture did not cause reoxidation of NQO1 (spectra e).

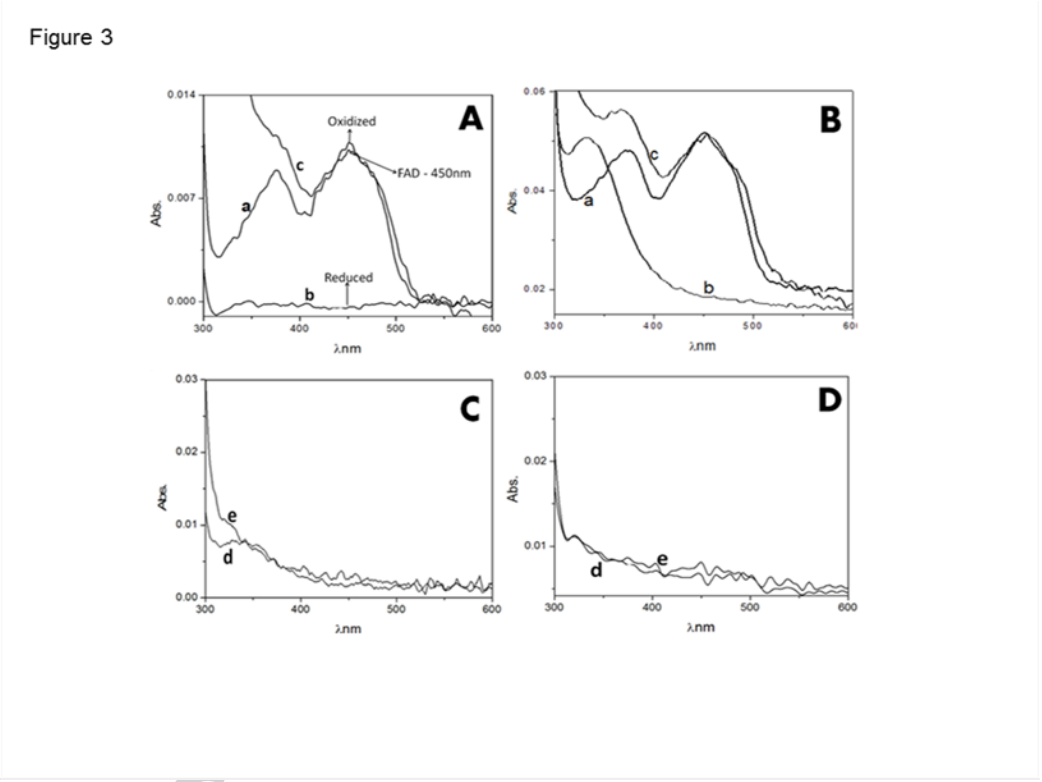

Figure 4

Spectral changes of NQO2 following addition of BNAH and substrate, PCQM (A) and OCQM (B). The absorption spectra were measured after sequential addition of NQO2, BNAH and substrate to 1 ml of 25 mM Tris-HCl, pH 6.8 at room temperature. The concentration of the enzyme was 10 U NQO1 (spectra a). The spectra after addition of 0.9 eq BNAH (spectra b) and 1.2 eq substrate (spectra c), in that order. The control spectra (C & D) were recorded by using *p*-cresol or *o*-cresol as a substrates. The absorption spectra were measured after sequential addition of 10 U NQO2 and 0.9 eq BNAH to 1 ml of 25 mM Tris-HCl, pH 6.8 at room temperature (spectra d). Addition of either 1.2 eq of *p*-cresol (C) or *o*-cresol (D) to assay mixture did not cause reoxidation of NQO2 (spectra e).

Figure 4

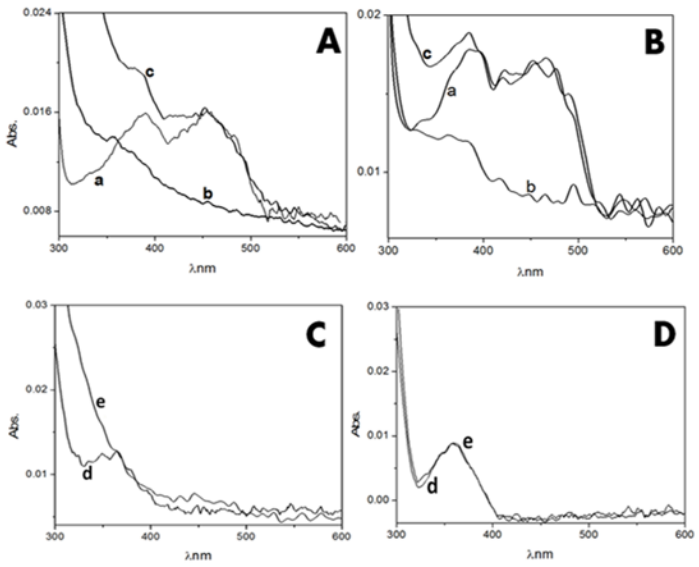

Figure 5

ESI-MS/MS spectra of p-cresol standard (A) and o-cresol standard (D). MS/MS analysis of the assay mixture containing NQO1, NADH and PCQM/OCQM clearly shows formation of p-cresol (B) and o-cresol (E) respectively. Similarly, MS/MS analysis of the assay mixture containing NQO2, BNAH and PCQM/OCQM clearly shows formation of p-cresol (C) and o-cresol (F) respectively.

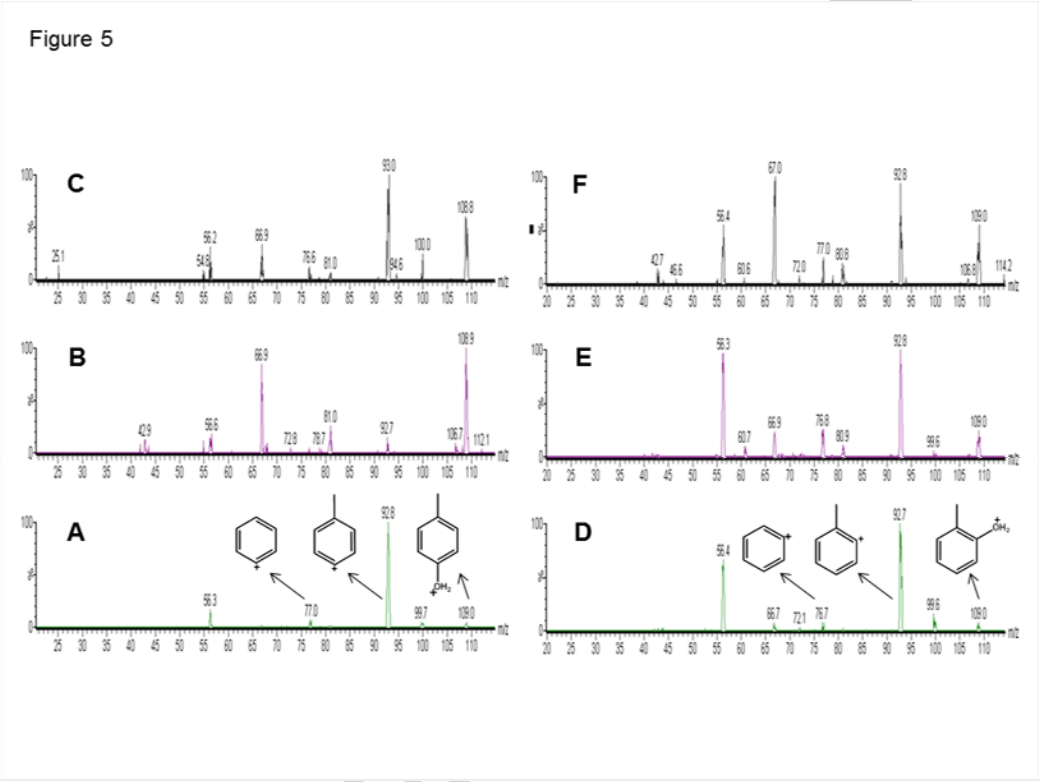

Figure 6

Michaelis-Menten plots for the kinetics of NQO1 (A & B) and NQO2 (C & D) catalysis. Reduction of PCQM (A), OCQM (B) and MD (E) by NQO1 enzyme and reduction of PCQM (C), OCQM (D) and MD (F) by NQO2 enzyme, respectively.

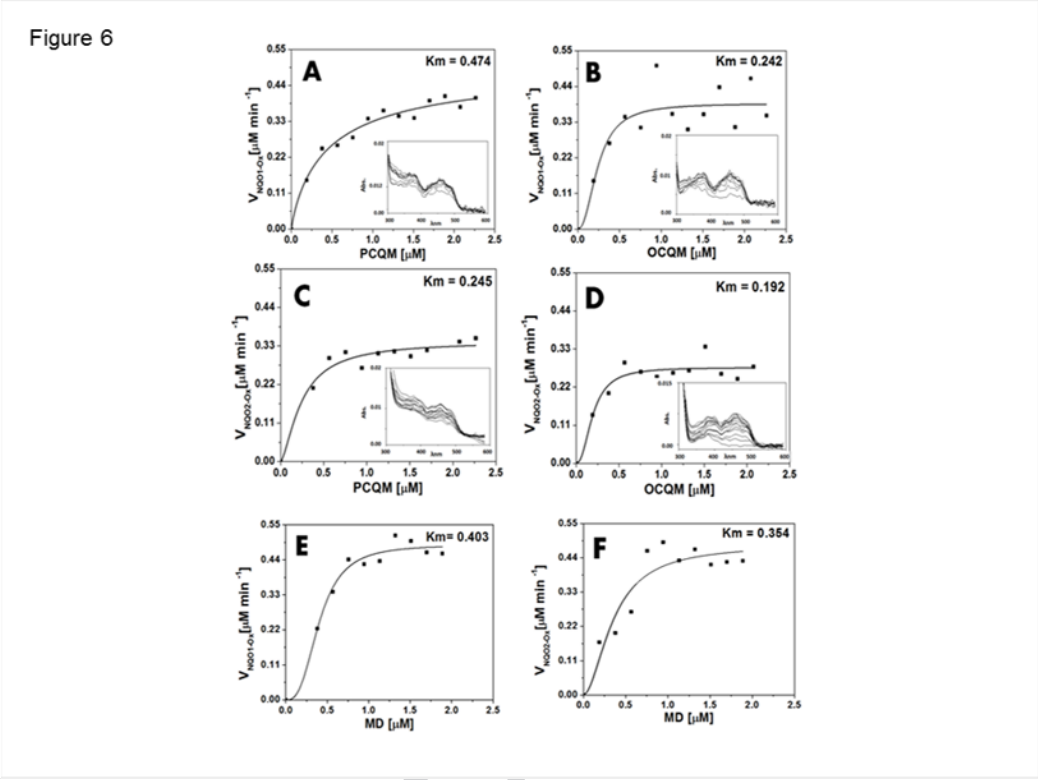

Figure 7

Substrate binding with NQO1 (light grey) and NQO2 (dark grey). (**A**) PCQM (orange) docking to NQO1. (**B**) OCQM (yellow) docking to NQO1. (C) PCQM docking with NQO2. (**D**) OCQM docking to NQO2. (**E**) MD (green) bound to NQO2 (28). All substrate and enzymes docking was performed using Swissdock online docking (11), and created using Chimera (45).

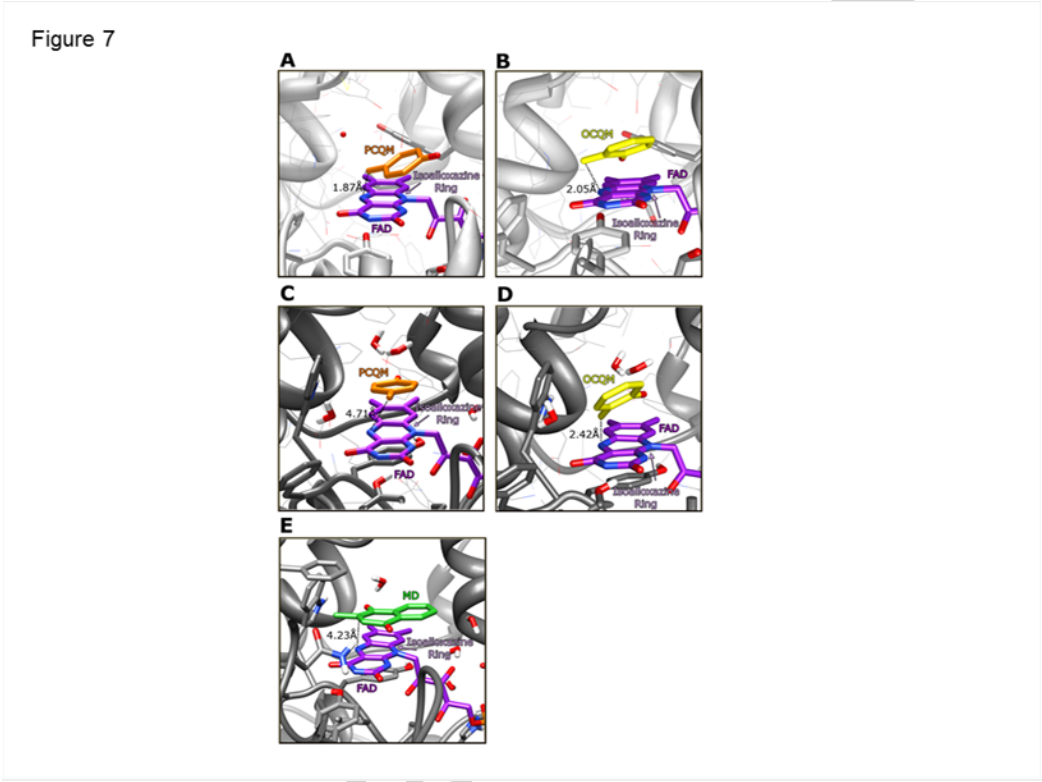

Figure 8

Proposed mechanism for reduction of quinone methides by NQO1 and NQO2.

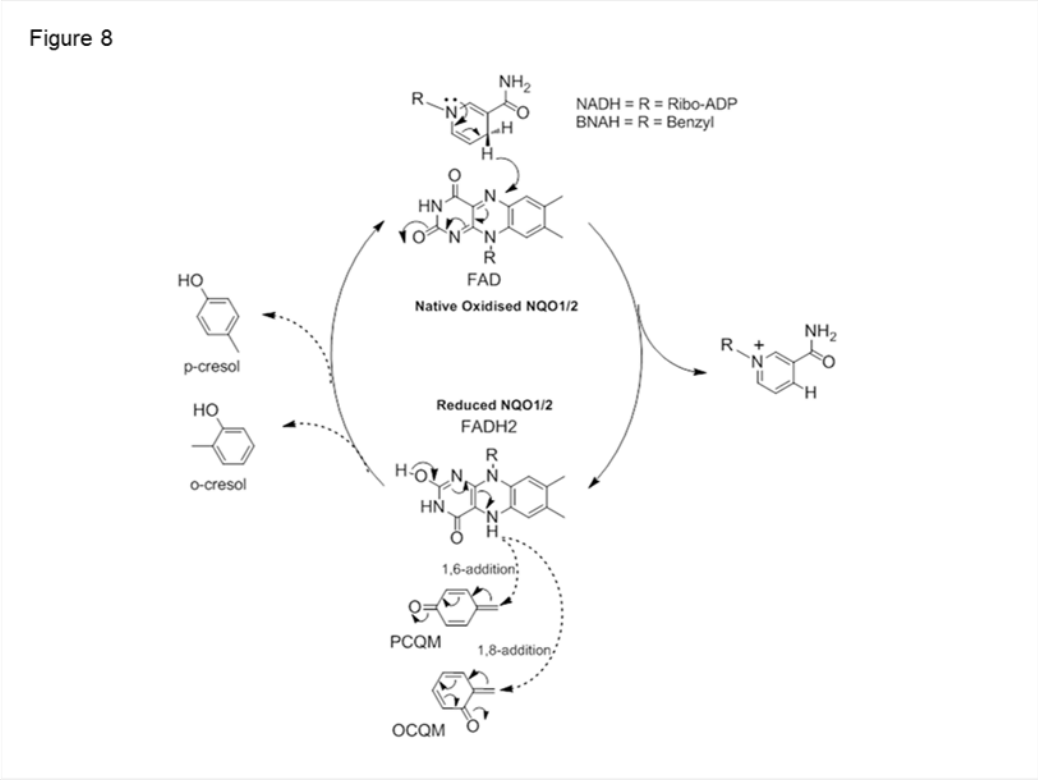

Table Legend

Table 1

Energies of docking solutions and measured distances from FAD

Substrate	Total Energy[α]	ΔG[α]	Distance from N-5(FAD)[β]
	NQO1		
PCQM	12.52	-5.20	1.87 Å
OCQM	13.66	-5.49	2.05 Å
	NQO2		
PCQM	10.55	-5.48	4.71 Å
OCQM	11.98	-5.66	2.42 Å
MD			4.23 Å

Values calculated using SwissDock[α] and Chimera[β] for NQO1 and NQO2 with PCQM, OCQM,or MD.