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NRH:quinone oxidoreductase 2 (NQO2) catalyzes metabolic activation of quinones and anti-tumor drugs

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BMY25067, analogue of mitomycin C
BP-3,6-quinone, benzo(a)pyrene-3, 6-quinone
CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide also known as tretazicar
CHO, Chinese hamster ovary cells
EO9, indoloquinone
Menadione, 2-methyl-1,4-naphthoquinone
MMC, mitomycin C
mNQO2-rec, histidine tagged mouse NQO2 protein
NQO1, NAD(P)H:quinone oxidoreductase 1
NQO2, dihydronicotinamide riboside:quinone oxidoreductase 2

ABSTRACT

NRH:quinone oxidoreductase 2 (NQO2) is a cytosolic flavoprotein that utilizes NRH as electron donor. The present studies investigate the role of NQO2 in metabolic detoxification/activation of quinones and quinone based anti-tumor drugs. Chinese hamster ovary (CHO) cells stably overexpressing cDNA derived mouse NQO2 and mouse keratinocytes from DMBA-induced skin tumors in wild-type and NQO2-null mice were generated. The CHO cells overexpressing NQO2 and mouse keratinocytes expressing or deficient in NQO2 were treated with varying concentrations of mitomycin C (MMC), CB1954, MMC analog BMY25067, EO9, menadione and BP-3,6-quinone, in the absence and presence of NRH. The cytotoxicity of the drugs was evaluated by colony formation. The CHO cells overexpressing higher levels of mouse NQO2 showed significantly increased cytotoxicity to menadione, BP-3,6-quinone and to the anti-tumor drugs MMC and CB1954 when compared to CHO cells expressing endogenous NQO2. The cytotoxicity increased in presence of NRH. Similar results were also observed with BMY25067 and EO9 treatments, but to a lesser extent. The results on keratinocytes deficient in NQO2 supported the data from CHO cells. The inclusion of NRH had no effect on cytotoxicity of quinones and drugs in keratinocytes deficient in NQO2. Mouse NQO2 protein was expressed in bacteria, purified and used to study the role of NQO2 in MMC-induced DNA cross-linking. Bacterially expressed and purified NQO2 efficiently catalyzed MMC activation that led to DNA cross-linking. These results concluded that NQO2 plays a significant role in the metabolic activation of both quinones and anti-tumor drugs leading to cytotoxicity and cell death.

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NRH, dihydronicotinamide riboside [nicotinamide riboside (reduced)]
2,6-DCPIP, 2,6-dichlorophenolindophenol

1. Introduction

Quinone oxidoreductases (NQO1 and NQO2) are flavoproteins that catalyze reductive metabolism of quinones and its derivatives [1–4]. The dicoumarol sensitive NQO1 is a cytosolic protein of 274 amino acid residues (MW = 30,880) that catalyzes the two-electron reduction and detoxification of quinones [1–3]. NQO1 activity is ubiquitously present in all tissues types [1–3]. It has been shown that the two-electron reduction of quinones, catalyzed by NQO1, competes with the one-electron reduction catalyzed by cytochromes P450 and P450 reductase. The two-electron reduction produces a relatively stable hydroquinone that is removed by conjugation with glutathione, UDP-glucuronic acid and other compounds, avoiding the formation of free radicals (semiquinones) and highly reactive oxygen species (ROS). Consequently, this mechanism protects the cells against the adverse effects of quinones and their derivatives [1–3]. NQO1 activity has been shown to prevent the formation of highly reactive quinone metabolites [5,6], to detoxify benzo(a)pyrene quinones [7,8], and to reduce chromium (VI) toxicity [9]. NQO1 was also shown to decrease benzo(a)pyrene and benzo(a)pyrene quinone induced-mutagenicity [10,11]. In summary, NQO1 is generally accepted as protective against toxicity due to exposure to xenobiotics and drugs. Mice lacking a functional NQO1 gene (NQO1−/−) were born normal and reproduced the same as the wild-type mice [12]. However, NQO1−/− mice exhibited myeloid hyperplasia of bone marrow [13] and increased toxicity when administered menadione, compared with the wild-type mice [12]. NQO1−/− mice also demonstrated increased susceptibility to develop skin tumors in response to benzo(a)pyrene and DMBA [14,15]. Besides its protective activity, NQO1 has been shown to activate quinones in many instances [16–22]. The hydroquinones produced by NQO1 can auto-oxidize to generate ROS or directly alkylate DNA [16–22]. In these cases, NQO1 catalyzes activation of such compounds to their ultimate toxic forms. This property of NQO1, along with the observation that NQO1 is expressed at higher levels in certain tumor types, has been used to develop bioreductive chemotherapeutic agents [16–22]. Though, the role of NQO1 in the activation of drugs is controversial [22,23], especially regarding mitomycin C activation [23].

NRH:quinone oxidoreductase 2 (NQO2) is a second member of this quinone oxidoreductase family of proteins. Recently, the cDNA and gene encoding NQO2 were isolated from human liver [24,25]. The human NQO2 gene encodes a protein of 231 amino acids (MW = 25,956). The human NQO2 carboxy-terminus is 43 amino acids shorter than the human, rat and mouse NQO1. The human NQO2 cDNA and protein are 54 and 49% similar to the human liver cytosolic NQO1 cDNA and protein, respectively [24]. The human NQO2 gene has been precisely localized to chromosome 6p25 and its gene locus is highly polymorphic [26]. The cofactor requirement for protein activity is very selective, pointing out dihydronicotinamide riboside (NRH) for NQO2, and NAD(P)H for NQO1 as an electron donor [27,28]. Although NQO2 is resistant to typical activity inhibitors of NQO1, such as dicoumarol, cibacron blue, and phenindone, NQO2 is inhibited by flavones such as quercetin [27]. Benzo(a)pyrene is another known inhibitor of NQO2 [28]. Even though overlapping substrate specificities have been observed for NQO1 and NQO2, such as for CB1954 activation, significant differences exist in relative affinities for the various substrates [27–29]. Analysis of the crystal structure of NQO2 revealed that NQO2 contains a specific metal binding site, which is not present in NQO1 [30]. There is only limited information available on the role of NQO2 in metabolism and detoxification and/or activation of quinones and anti-tumor drugs. Similar to NQO1−/− mice, myeloid hyperplasia of bone marrow was detected in NQO2−/− mice [31]. In contrast to NQO1−/− mice, NQO2−/− mice showed decreased sensitivity to menadione induced-hepatic toxicity suggesting that NQO2 catalyzed menadione activation [12,31]. NQO2 is also shown as melatonin binding site MT3 [32].

In the present report, we studied the role of NQO2 in the metabolic detoxification and/or activation of quinones (menadione and BP-3,6-Q) and anti-tumor drugs (CB1954, mitomycin C, MMC analog BMY25067 and EO9). Chinese hamster ovary (CHO) cells stably overexpressing cDNA derived mouse NQO2 and wild-type CHO cells and skin cells expressing or deficient in NQO2 were treated with the various quinones and anti-tumor drugs and the cytotoxicity was evaluated by colony formation. The results showed that NQO2 activated both quinones (menadione and BP-3,6-Q) and anti-tumor drugs (CB1954 and mitomycin C) and to a lesser extent MMC analogue BMY25067 and EO9. Bacterially expressed and purified NQO2 also catalyzed MMC activation leading to DNA cross-linking.

2. Materials and methods

2.1. Materials

The Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (ATCC, CRL9096) (Rockville, MD, USA). Cell culture reagents were obtained from Gibco-BRL (Gaithersburg, MD, USA). Mitomycin C (MMC) was a gift from Bristol Myers Squibb (Princeton, NJ, USA), and was also purchased from Sigma–Aldrich Chemical Company (MO, USA). Plasmid DNA isolation kits and gel extraction kits were purchased from Qiagen Inc. (Valencia, CA). Protein concentration was evaluated by Bradford's method using Bio-Rad protein assay kit. All other reagents used in the experiments were of highest purity available commercially.

2.2. Cell culture

The CHO/DHFR$^-$ cells were grown in monolayer culture in Iscove's modified Dulbeccos's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.1 mM hypoxanthine/0.016 mM thymidine, and 10% fetal bovine serum. The cells were grown in 5% CO_2 at 37 °C.

2.3. CHO/DHFR$^-$ cells permanently overexpressing cDNA derived NQO2

Mouse NQO2 cDNA was subcloned in pcDNA3.1/V5/His-Topo vector (Invitrogen, Carlsbad, CA) to generate pcDNA-mNQO2 plasmid. The full-length mouse NQO2 cDNA fragment was isolated by digestion of original plasmid pCR2.1/Topo vector with HindIII/XhoI and agarose gel electrophoresis [31]. The 1.2-kb NQO2 cDNA was ligated into HindIII/XhoI site of pre-digested pcDNA3.1/V5/His-Topo vector. The resulting recombinant clones were grown and verified by restriction digestion and sequencing. The nucleotide sequence matched 100% to the published mouse NQO2 sequence (accession number AF252260). The pcDNA-NQO2 plasmid (5.0 μg/dish) was transfected into CHO cells using Lipofectin reagent (Invitrogen, Carlsbad, CA). Seventy-two hours after transfection, the cells were washed with 1X PBS without calcium and magnesium, trypsinized and plated in medium containing 400 μg/ml G418 by previously described procedures [7]. Several individual clones were grown in 24 well plates containing 400 μg/ml of G418. All the selected clones were analyzed for NQO2 protein by Western analysis and NQO2 activity measured by procedures as described [24]. Selected clones were grown in medium deficient in G418 for 4 weeks without significant loss of NQO2 overepression.

Transfected and untransfected CHO cells were washed 3× with PBS and scraped in 5 ml PBS and centrifuged at 3000 × *g* (15 min/4 °C) to prepare cell lysate. The pellet was resuspended in 1 ml homogenate buffer (0.25 M sucrose, 0.05 M Tris pH 7.4, 1 mM EDTA, 2 μg/ml antipain, 2 μg/ml leupeptin). The cells were downced manually 20 times and vortex for 1 min and the cell lysate was centrifuged at 5000 × *g* for 15 min at 4 °C. The cell lysate was concentrated in Centricon Y-10 by centrifugation at 3500 rpm at 4 °C and saved for further protein analysis by 10% SDS-PAGE and Western analysis.

2.4. Establishment and culture of WT and NQO1−/− keratinocytes from 7,12-dimethylbenzanthracene-induced skin tumors in mice

Wild-type and NQO2−/− keratinocytes were established from 7,12-dimethylbenzanthracene-induced skin tumors by standard procedures [33]. The tumors were removed and placed in high (200 mM) calcium (HiCa) supplemented with antibiotics and antimytotic agents. The tumors were transferred in a dish and minced in collagenase and contents transferred to 50 ml tube. The contents were incubated at 37 °C for 2 h with agitation. The supernatant removed after clumps were settled. 10 ml HiCa added to the clumps, let settle for 5 min and supernatant removed. The HiCa treatment was repeated. HiCa/KGF (keratinocyte growth factor) was added to the clumps and plated in the four corner wells of collagen (Vitrogen)-coated 12-well plate. The medium was replaced with LoCa (50 mM)/KGF. The medium replaced every 2–3 days. The growing keratinocyte cells were collected and cultured in DMEM medium supplemented with 10% FCS and antibiotics. The karyotyping analysis did not reveal loss/gain or translocation of chromosome(s) in established skin tumor cells (data not shown).

2.5. NQO1 and NQO2 activity

NQO1 and NQO2 enzymatic activities in CHO or mouse keratinocyte cell lysate or in bacterially expressed and purified fractions were determined by previously described procedures [23]. Briefly, NQO1 activity was measured by following the NADH-dependent reduction of 2,6-dichloroindophenol (2,6-DCPIP) at 600 nm in a Beckman spectrophotometer. The freshly prepared NRH was used as electron donor to measure NQO2 activity. Reaction medium for measurement of NQO2 activity contained: 25 mM Tris–HCl (pH 7.4), 5 μM FAD, 0.18 mg/ml BSA, 0.01% Tween 20, 50 μM 2,6-DCPIP, 0.2–1 mg total protein, and 200 μM NADH (or NRH as required). The reduction of DCPIP was followed at 600 nm.

2.6. Cytotoxicity assays

The wild-type control CHO cells and the CL5 cells expressing higher levels of cDNA-derived NQO2 were used. The wild-type and NQO2−/− keratinocyte cells were also used. All types of cells were grown in dishes without methotrexate. The various cells were treated with different concentrations of quinones and drugs in the absence and presence of 100 μM NRH for either 72 h (menadione and CB1954) or 24 h (BP-3,6-Q, MMC, BMY25067 and EO9). The control cultures received DMSO (0.1% final) and/or 100 μM NRH when required. The cell culture media used during drugs treatment did not contain fetal bovine serum (FBS). Cell growth/survival was measured by colony formation assay by procedures as described [34,35]. Briefly, the cells were grown in monolayer, trypsinized, counted by Coulter Counter and plated at a density of 200 cells/60 mm petridish in medium containing the various drug concentrations. After 72 or 24 h, the medium containing the drug for treatment was replaced with fresh medium (with FBS) without the drug in study. The cells were allowed to grow for 10–14 days to form colonies, which were stained and counted [35]. IC_{50} of the drug concentration that reduces colony formation or cell number by 50% relative to control, was determined.

2.7. Bacterial expression of mouse NQO2

The full-length mNQO2 cDNA was cut out by digestion of pcDNA-mNQO2 with HindIII and XhoI. The 1.2-kb NQO2 cDNA fragment was ligated into HindIII/XhoI site of pre-digested pProEx-HTA vector. The *E. coli* DH5(was transfected with recombinant plasmid and plated on LB agar plates containing 50 μg/ml ampicillin. Resulting ampicillin-resistant clones were selected, verified by digestion with restriction enzymes and by DNA sequencing. An overnight culture of DH5α *E. coli* (pProEx-HTA-mNQO2) was diluted 1:50 (with 100 (g/ml ampicillin) in LB media, grown at 37 °C with vigorous shaking until OD_{600} of 0.6 was reached and induced for another 4–5 h by addition of 1 mM of isopropyl β-D-thiogalactoside (IPTG).

The cells were harvested by centrifugation at 4000 × *g* for 20 min at 4 °C and frozen. Bacteria were lysed by freeze/thaw in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0), followed by sonication. The lysate was cleared by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant was filtered through 1.2 μm Acrodiscs (Gelman Sciences) and applied to a column of Ni-NTA-Agarose to purify 6xHis-tagged-mNQO2. The column was washed with 50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole (pH 8.0), and then eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole (pH 8.0). Fractions including 6xHis-tagged-mNQO2 were dialyzed extensively against 0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris pH 7.4. To remove the 6xHis affinity tag, the dialyzed fractions were incubated overnight at 4 °C with rTEV protease (Gibco Life Technologies), 1 mM DTT, 0.5 mM EDTA, 50 mM Tris–HCl (pH 8.0). The reaction solution was dialyzed against 0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris pH 7.4, concentrated in Centricon Y-30 and the bacterial expressed mouse mNQO2 was maintained at 4 °C for further analysis.

2.8. Role of NQO2 in MMC activation and DNA cross-linking

MMC-induced DNA cross-linking assays were performed by procedure as described [7,22]. Briefly, the two strands of the twenty-three base pairs of oligonucleotides containing MMC binding site were selected from plasmid pBR322, synthesized and used for the cross-linking experiments. The nucleotide sequence of the 23 base pair oligonucleotide is as shown below:

```
5′-CTA CAT CGT GTC ATG CAC AGG AT
     T GTA GCA CAG TAC GTG TCC TAG A-5′
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Similarly, the two strands of the oligonucleotides containing mutated MMC binding site were synthesized. The nucleotide sequence of mutant oligonucleotide is shown below. The mutated nucleotides are labeled with '*'.

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          **     *      *      *
5′-CTA CAT ATT GTA ATG AAC AGT AT
     T GTA TAA CAT TAC TTG TCA TAG A-5′
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The complementary strands were mixed in equal amounts and annealed by heating to 70 °C for 15 min and slowly cooling to room temperature. The 3′-end of the top strand was selectively labeled by DNA polymerase I large (Klenow) fragment (Promega, Madison, WI) in the presence of [α-^{32}P]dCTP (NEN, Boston, MA). The oligonucleotides were purified on a 15% non-denaturing polyacrylamide gel, eluted, and concentrated by ethanol precipitation. The ^{32}P-labeled oligonucleotides were used in MMC-induced DNA cross-linking by incubation with the various concentrations of the bacterially expressed and purified mNQO2 protein, 100 mM phosphate buffer (pH 5.8), 1 mM NRH, 150 μM MMC, 5 μM FAD, 0.01% Tween 20 and 0.18 mg bovine serum albumin (BSA), unless specified differently. The cytosol prepared from NQO1−/− mouse testis was used as positive control. Reactions were incubated for 1 h at 37 °C and terminated by the addition of ethanol, 10 mM $MgCl_2$, 1.5 M ammonium acetate. The samples were frozen at −70 °C and DNA collected by centrifugation at 13,700 rpm for 30 min at 4 °C. The supernatant was removed and the precipitated oligonucleotides were centrifuged in Speed-Vac until dry. The precipitated oligonucleotides were resuspended in DNA sequencing dye containing formamide and denatured by heating at 95 °C for 15 min, and then rapidly cooled on ice. The samples were analyzed on a 15% denaturing polyacrylamide gel containing 8 M urea, and cross-linked and unmodified oligonucleotides were detected by autoradiography performed on Kodak Bio-max MS films using intensifying screens at −70 °C for 18 h.

2.9. Statistical analysis

Results are expressed as mean ± S.D. Two-sided Fischer's exact test was used to determine statistical significance of cytotoxicity assays. In all statistical tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Generation of CHO cells overexpressing cDNA-derived mouse NQO2

Six CHO cell clones stably expressing varying amounts of cDNA derived mouse NQO2 protein were isolated (Fig. 1). The analysis of NQO2 activity revealed 20–80% increase in NQO2 activity among the selected CHO cell clones as compared to wild-type CHO cells (Fig. 1A). The CHO cell clones were also analyzed for NQO1 activity. The results demonstrated that NQO1 activity remained unaltered in CHO clones overexpressing NQO2 (data not shown). Western blot analysis showed an increase in NQO2 protein levels in all the selected clones (Fig. 1B). Interestingly, the increases in NQO2 protein were proportional to increases in NQO2 activity in respective CHO cell clones. Apparently, the mouse NQO2 expressed from its cDNA ran slightly slower than the endogenous NQO2 from the CHO cells, almost generating a second band in SDS-PAGE (Fig. 1B). This effect is most likely due to the 6xHis-affinity tag and consequent small increase in the recombinant protein size. Western analysis also revealed that NQO1 protein remained unaltered in CHO cells overexpressing NQO2 (Fig. 1B). We made several attempts to obtain CHO cells overexpressing higher than 80% of increase in NQO2 activity.

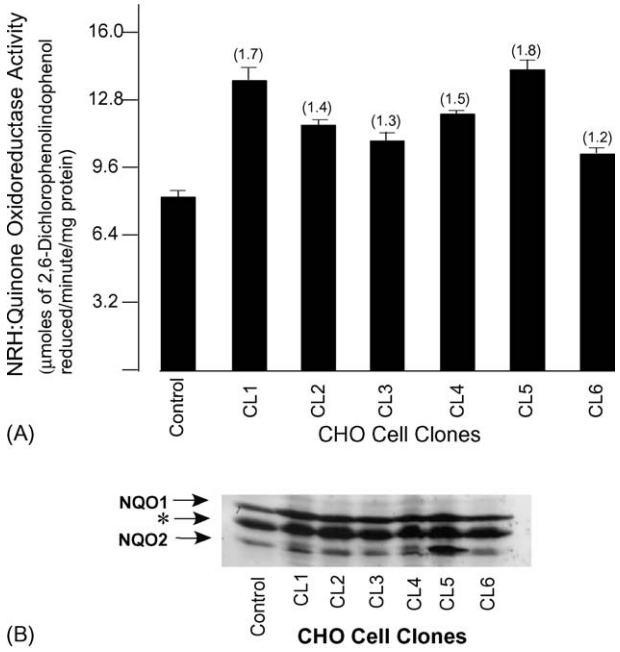

Fig. 1 – Characterization of CHO cells stably overexpressing mouse NQO2. (A) NQO2 activity. Wild-type CHO cells (Control) and different clones of CHO cells stably overexpressing NQO2 (CL1–CL6) were homogenized and analyzed for NQO2 activity in the absence and presence of benzo(a)pyrene. The benzo(a)pyrene inhibitable NQO2 activity is shown. (B) Western analysis. The cytosolic proteins from wild-type (control) and NQO2 overexpressing CHO cells were separated on SDS-PAGE, transferred on nitocellulose membrane and probed with polyclonal antibody against full-length rat NQO1 protein. Western blot was developed with ECL (Amersham Pharmacia) reagents. Anti-NQO1 polyclonal antibody against full-length rat NQO1 protein is known to cross-react with both mouse NQO1 and NQO2 proteins in Western analysis [12,31]. This antibody also detects an unspecific band represented by '*' and has been previously reported [12,31].

However, these attempts did not result in higher expression of NQO2 because of unknown reasons. Therefore, the CHO cell clone CL5, expressing 80% higher amount of NQO2 protein and activity than wild-type CHO cells, was chosen for cytotoxicity studies.

3.2. *Generation and characterization of wild-type WT and NQO2−/− keratinocyte cells*

The wild-type and NQO2−/− keratinocyte cells were cultured and analyzed for NQO2 and NQO1 proteins by Western and NQO2/NQO1 activity. The results are shown in Fig. 2. Western analysis revealed presence of NQO2 protein in WT cells. NQO2 protein was absent in NQO2−/− cells. NQO2 activity was significantly reduced in NQO2−/− cells. The residual NQO2 activity in NQO2−/− cells was not due to NQO2 protein since Western analysis demonstrated absence of NQO2 protein in NQO2−/− cells. The small amount of NQO2 activity observed in NQO2−/− cells might be due to NQO1 cross-activity. NQO1 activity did not alter and remained same as wild-type in NQO2−/− cells.

3.3. *Cytotoxicity of CHO cells to quinones and anti-tumor drugs*

The wild-type CHO (WT-CHO) and CHO cells overexpressing NQO2 (NQO2-CHO) were treated with different concentrations of quinones and anti-tumor drugs and cytotoxicity determined by colony formation assays. All the quinones and anti-tumor drugs showed toxicity in WT-CHO and NQO2-CHO cells. However, the magnitude of cytotoxicity differed between the two CHO cell types. The IC_{50} for menadione decreased from 12.5 μM for WT-CHO cells to 5.5 μM for NQO2-CHO cells ($P > 0.001$, Fig. 3A, Table 1). This decrease in IC_{50} for menadione was directly proportional to 2-fold increase in NQO2-CHO cells. Inclusion of NRH in the cell culture medium caused a further decrease of 5.5-fold in IC_{50} of menadione for NQO2-CHO cells (Fig. 3A, Table 1). This led to a 10-fold increase in cytotoxicity when compared to WT-CHO cells (Table 1). Similar results were observed with BP-3,6-quinone (Fig. 3B, Table 1). However, the addition of NRH was less effective in increasing cytotoxicity of BP-3,6-Q (Table 1). Nevertheless, it is important to note that CHO cells are approximately 100 times more sensitive to BP-3,6-quinone than menadione concentrations.

The NQO2 was found highly efficient in metabolic activation of anti-tumor drugs leading to cytotoxicity, especially in the presence of exogenous NRH (Fig. 4A, B–D). The NQO2-CHO cells showed increased sensitivity to CB1954 when compared to WT-CHO cells (Fig. 4A, Table 1). This resulted in a decrease in IC_{50} of CB1954 from 130 to 62.5 μM ($P > 0.001$). It may be noteworthy that this decrease was also directly proportional to the 2-fold increase in NQO2 in NQO2-CHO (CL5) cells (Fig. 1). Both WT-CHO and NQO2-CHO cells showed 5–6 times higher cytotoxicity of CB1954 in the presence of NRH (Fig. 4A; Table 1). This resulted in a 15-fold increase in sensitivity of NQO2-CHO cells to CB1954, when compared to WT-CHO cells without NRH (Fig. 4A, Table 1). Similar results were also observed with a second anti-tumor drug, MMC (Fig. 4B). The NQO2-CHO cells were 50% more sensitive to mitomycin C than WT-CHO cells. The inclusion of NRH led to 10-fold decrease in IC_{50} of MMC for NQO2-CHO cells, as compared to only 4-fold decrease for WT-CHO cells (Table 1, $P > 0.001$). The treatment of WT-CHO and NQO2-CHO cells with MMC analogue BMY25067 and EO9 also showed cytotoxicity in the absence and presence of NRH (Fig. 2C and D, Table 1). However, the magnitude of cytotoxicity and effect of NRH were significantly lower, when compared with CB1954 and MMC. Interestingly, EO9 was the most cytotoxic among the anti-tumor drugs used in the present studies.

3.4. *Cytotoxicity of WT and NQO2−/− keratinocyte cells to quinones and anti-tumor drugs*

The data on WT and NQO2−/− keratinocyte cells largely supported observations of quinone and anti-tumor drug cytotoxicity in CHO cells as described above (Figs. 5 and 6; Table 2). Menadione cytotoxicity observed with WT cells

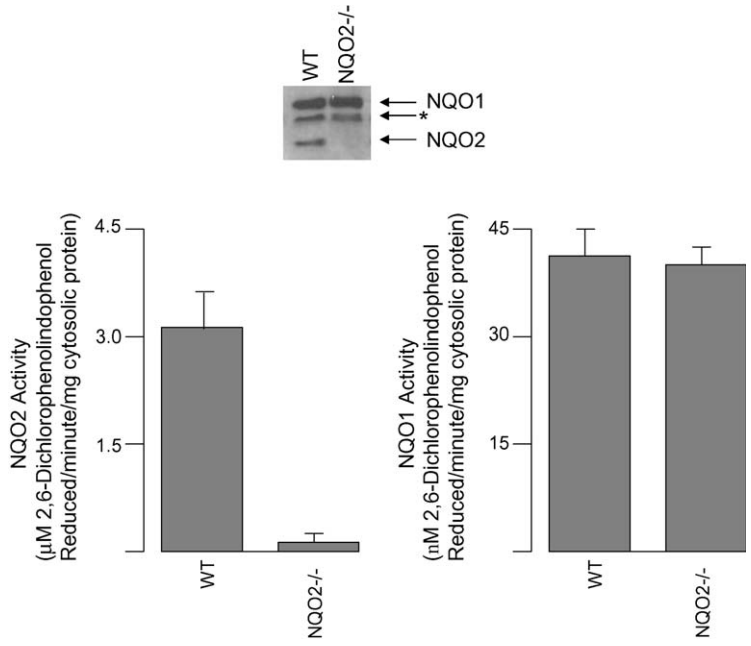

Fig. 2 – Characterization of wild-type and NQO2−/− skin cells. Western analysis (upper panel). The cytosolic proteins from wild-type (WT) and NQO2−/− cells were separated on SDS-PAGE, transferred on nitocellulose membrane and probed with polyclonal antibody against mouse NQO2 and NQO1 protein. Western blot was developed with ECL (Amersham Pharmacia) reagents. NQO1 polyclonal antibody against full-length NQO1 protein is known to cross-react with both NQO1 and NQO2 proteins in Western analysis [12,31]. This antibody also detects an unspecific band represented by '*' and has been previously reported [12,31]. NQO2/NQO1 activity (lower panel). Wild-type (WT) and NQO2−/− cells were homogenized and analyzed for NQO2 activity in the absence and presence of benzo(a)pyrene and NQO1 activity in absence and presence of dicoumarol. The benzo(a)pyrene inhibitable NQO2 and dicoumarol inhibitible NQO1 activity is shown.

increased in presence of NRH. WT cells expressing NQO2 showed IC50 of 21 μM as compared to 7.5 μM in presence of NRH ($P > 0.001$) (Fig. 5A; Table 2). NQO2−/− cells deficient in NQO2 demonstrated significant resistance to menadione cytotoxicity. In addition, the presence of NRH had more or less no effect on cytotoxicity of menadione in NQO2−/− cells. The IC50 for menadione was 28–29 μM in absence and presence of NRH (Table 2). The treatment of cells with BP-3,6-quinone showed similar results as menadione (Fig. 5B; Table 2). Exposure of WT and NQO2−/− cells to anti-tumor drugs CB1954 and MMC demonstrated significant resistance of NQO2−/− cells to cytotoxicity from drugs, as compared to WT cells (Fig. 6; Table 2). The inclusion of NRH increased cytotoxicity of anti-tumor CB1954 and MMC drugs in WT type cells. However, NRH had no effect on cytotoxicity of anti-tumor drugs in NQO2−/− cells. The IC50 for CB1954 and MMC significantly increased in NQO2−/− cells, as compared to WT cells in presence of NRH ($P > 0.001$).

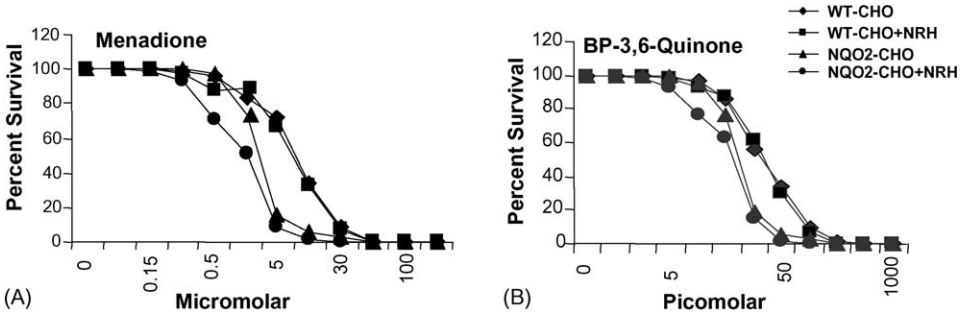

Fig. 3 – Cytotoxicity of quinones in CHO cells. Wild-type CHO cells (WT-CHO) expressing endogenous levels of NQO2 and clone CHO cell clone CL5 cells (NQO2-CHO) overexpressing cDNA derived mouse NQO2 protein were treated with various concentrations of menadione and benzo(a)pyrene-3,6-quinone (BP-3,6-quinone) in the absence or presence of NRH. The cells were washed after treatment and allowed to grow in normal medium for 10–14 days to form colonies. The colonies were stained and counted by procedures as described in Section 2. Data are representative of mean from three independent analyses.

Table 1 – IC_{50} of quinones and anti-tumor drugs for CHO cells

	CHO-WT	CHO-WT + NRH	CHO-NQO2	CHO-NQO2 + NRH
Menadione (μM)	12.50	10.00	5.50	1.00
BP-3,6-quinone (nM)	0.130	0.150	0.042	0.034
CB1954 (μM)	130.0	25.40	62.50	9.06
MMC (μM)	0.059	0.014	0.041	0.004
BMY25067 (μM)	0.115	0.090	0.086	0.081
EO9 (nM)	0.008	0.002	0.008	0.006

3.5. *Characterization of bacterially expressed NQO2 protein and its role in mitomycin C-induced DNA cross-linking*

Bacterially expressed mouse NQO2 (mNQO2-rec) was purified by affinity chromatography. This led to greater than 500-fold purification of NQO2 from the bacterial extract, as shown by SDS-PAGE analysis (Fig. 7A, lane 3). The protease treatment removed the 6xHis-affinity tag and spacer region from mNQO2-rec protein, resulting in a final protein of 26 kDa (Fig. 7A, lane 4). The antibodies against rat NQO1 protein are known to cross-react with NQO2 protein because of high amino acid similarity between the two proteins. The NQO1 antibodies cross-reacted with bacterially expressed NQO2 protein (Fig. 7B). The purified mNQO2-rec protein efficiently catalyzed reduction of 2,6-DCPIP (Fig. 7C). Twenty micromolar benzo(a)pyrene inhibited more than 90% of the bacterially expressed and purified NQO2 activity (Fig. 7C).

The bacterially expressed and purified NQO2 activity catalyzed metabolic activation of mitomycin C that led to DNA cross-linking. Interestingly, 1 μg of mNQO2-rec protein, that efficiently catalyzed reduction of 2,6-DCPIP (Fig. 7C), failed to catalyze MMC-induced DNA cross-linking (Fig. 7D). Increasing the purified NQO2 protein to 5 μg activated MMC leading to DNA cross-linking (Fig. 7D). On the other hand, a further 10-fold increase in mNQO2-rec protein concentration did not demonstrate a proportional increase in MMC-induced DNA cross-linking (Fig. 7D). This plateau suggested that the enzymatic reaction is saturated due to substrate amount (either MMC or DNA binding sites). Addition of cofactors like FAD, BSA and Tween 20, had no effect on the MMC bioactivation induced by purified mNQO2-rec. These results are more evident in case of the MMC-induced DNA cross-linking by testis cytosol, known to contain higher amounts of NQO2 [13]. In this case, addition of cofactors even reduced the MMC-induced DNA cross-linking detected. The mutation of the MMC-binding site in oligonucleotide led to a significant decrease in the MMC activation by NQO2 (Fig. 7D), suggesting that MMC activation by mNQO2-rec is specific to the DNA binding site, as previously observed for MMC activation by NQO1.

4. Discussion

The oxidative metabolism of xenobiotics and drugs generate electrophiles and reactive oxygen species (ROS) [36]. Both

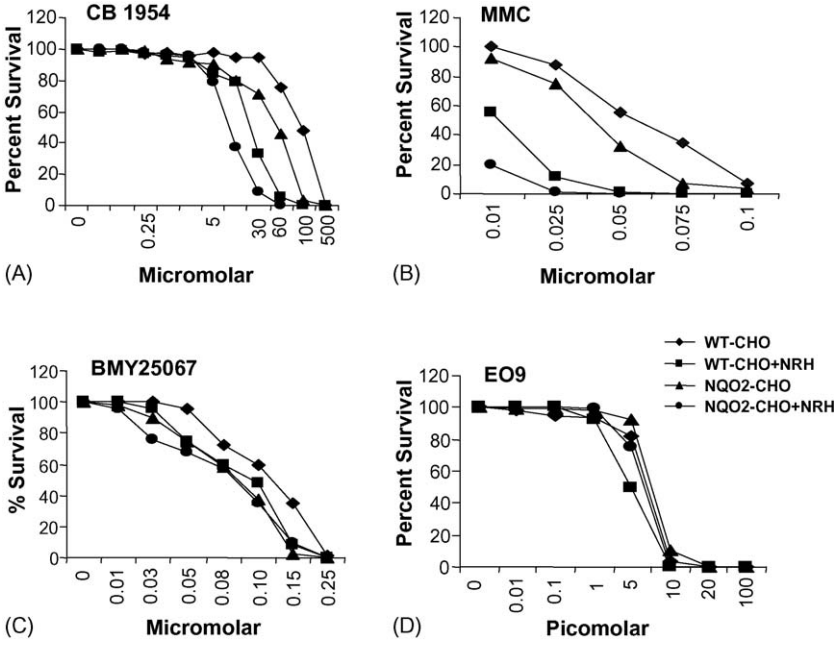

Fig. 4 – Cytotoxicity of anti-tumor drugs in CHO cells. Wild-type CHO cells (WT) and NQO2-CHO cells were treated with various concentrations of anti-tumor drugs in the absence or presence of NRH. The cells were washed after treatment and allowed to grow in normal medium for 10–14 days to form colonies. The colonies were stained and counted by procedures as described in materials and methods. Data are representative of mean from three independent analyses.

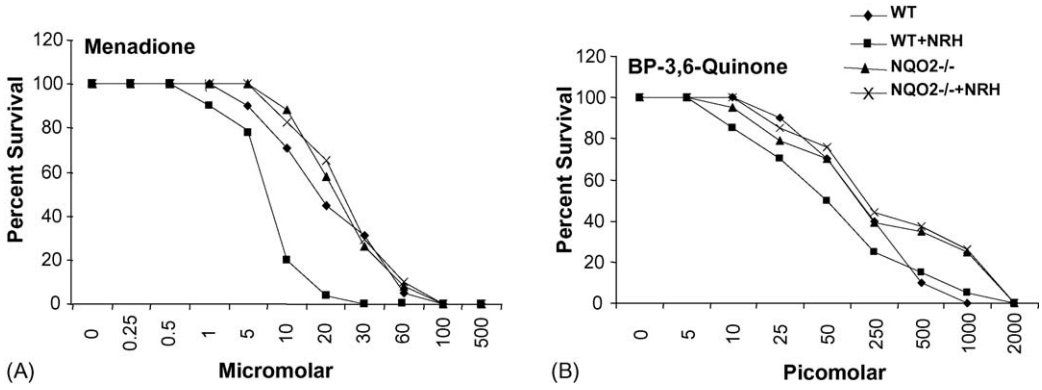

Fig. 5 – Cytotoxicity of quinones in mouse keratinocytes. Wild-type (WT) and NQO2−/− keratinocyte cells were treated with various concentrations of menadione and BP-3,6-quinone in the absence or presence of NRH. The cells were washed after treatment and allowed to grow in normal medium for 10–14 days to form colonies. The colonies were stained and counted by procedures as described in Section 2. Data are representative of mean from three independent analyses.

electrophiles and ROS attack cellular macromolecules, including DNA, which leads to oxidative stress, premature aging, neuro-degenerative diseases, arthritis, arteriosclerosis, inflammatory responses, and induction/promotion of tumors. Therefore, oxidative stress has a profound impact on the survival of all living organisms [37]. It is now clearly established that ROS also activate a group of cellular enzymes, including NQO1, which either prevent the generation of ROS or detoxify ROS, leading to protection of cells against damage due to oxidative stress [38]. It may be noteworthy that the concentration at which ROS provide protection to a cell might be only slightly lower than the concentration at which damage occurs. Therefore, the cellular defense mechanisms must be tightly regulated to deal with the constant environmental changes.

In vivo studies have shown that NQO1 protects against myelogenous hyperplasia and oxidative stress due to exposure to quinones including menadione and BP-3,6-quinone [1–3,13]. It also protects against benzo(a)pyrene and DMBA induced skin carcinogenesis [14,15]. In addition, NQO1 has also been shown to play a role in metabolic activation of anti-tumor drugs including CB1954, MMC and EO9 [16–22]. The high magnitude of sequence homology between NQO1 and NQO2 suggested that NQO2 might have similar functions as NQO1. The studies with NQO2-null mice in part supported this idea, since the loss of NQO2 was also found associated with myelogenous hyperplasia of bone marrow [13]. However, the loss of NQO2 protected against menadione induced oxidative stress and hepatic damage indicating a role of NQO2 in metabolic activation of menadione [13]. This was in contrast to the role of NQO1 in detoxification of menadione and prevention of menadione-induced oxidative stress and hepatic damage. Therefore, this prompted us to perform studies to further investigate the role of NQO2 in bioactivation of quinones in general and anti-tumor drugs.

We successfully generated CHO cells overexpressing mouse NQO2 protein and activity. We also successfully established skin tumor cells from wild-type mice expressing NQO2 and NQO2−/− mice deficient in NQO2. The CHO cells overexpressing NQO2 demonstrated higher cytotoxicity due

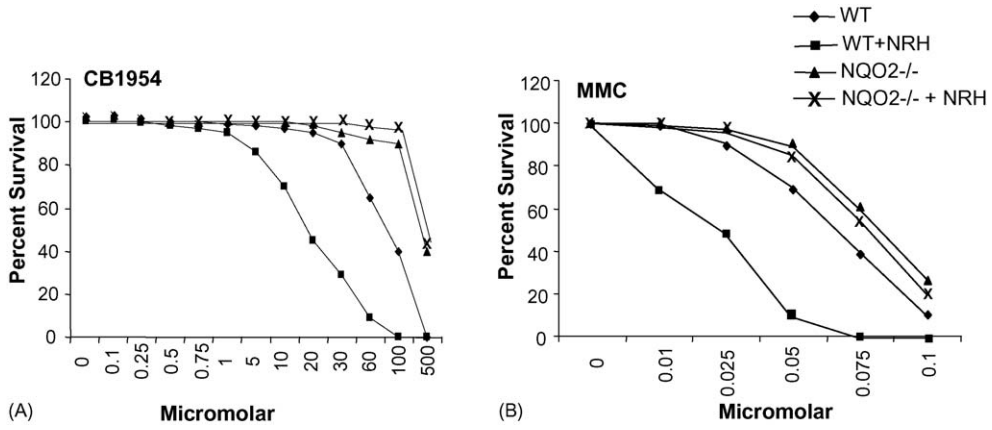

Fig. 6 – Cytotoxicity of anti-tumor drugs in mouse keratinocytes. Wild-type (WT) and NQO2−/− mouse keratinocyte cells were treated with various concentrations of CB1954 and mitomycin C (MMC) in the absence or presence of NRH. The cells were washed after treatment and allowed to grow in normal medium for 10–14 days to form colonies. The colonies were stained and counted by procedures as described in Section 2. Data are representative of mean from three independent analyses.

Table 2 – IC_{50} of quinones and anti-tumor drugs for wild-type (WT) and NQO2−/− keratinocytes

	WT	WT + NRH	NQO2−/−	NQO2−/− + NRH
Menadione (μM)	21.0	7.5	28.0	29.0
BP-3,6-quinone (nM)	250	60	240	250
CB1954 (μM)	95	20	500	500
MMC (μM)	0.065	0.025	0.080	0.085

to exposure to quinones and anti-tumor drugs, as compared to wild-type CHO cells. Menadione and BP-3,6-Q, both showed increased cytotoxicity in CHO cells overexpressing NQO2. Similar results were observed with anti-tumor drugs CB1954 and MMC. Interestingly, the cytotoxicity of quinones and anti-tumor drugs were significantly higher in the presence of NRH. This was observed for both wild-type and NQO2 overexpressing CHO cells. However, the magnitude of increase in cytotoxicity was significantly higher in case of CHO cells overexpressing NQO2. The cytotoxicity studies with WT and NQO2−/− cells supported quinone and anti-tumor drug cytotoxicity observations in CHO cells. NQO2−/− cells deficient in NQO2 demonstrated NRH independent resistance to menadione and BP-3,6-quinone and anti-tumor drugs CB1954 and MMC. NRH is an electron donor for NQO2 and is known to freely diffuse in the cells [29]. The intracellular level of NRH is low and exogenous addition of NRH might have activated NQO2 leading to increased metabolic activation of quinones and anti-tumor drugs and increased cytotoxicity in CHO cells expressing endogenous and overexpressed NQO2 and wild-type skin cells [29]. The NQO2 mediated quinone and drug cytotoxicity was also evident from observations of the loss of effect of NRH on cytotoxicity in absence of NQO2 in NQO2−/− cells. The role of NQO2 in metabolic activation of CB1954 is in agreement and supported by previous report [29]. Previous studies [39] and studies in this report (data not shown) also revealed that NRH could be replaced with co-substrate EP0152R (1-(carbamoylmethyl)-3-carbamoylpiridinium iodide) for similar effects as described above.

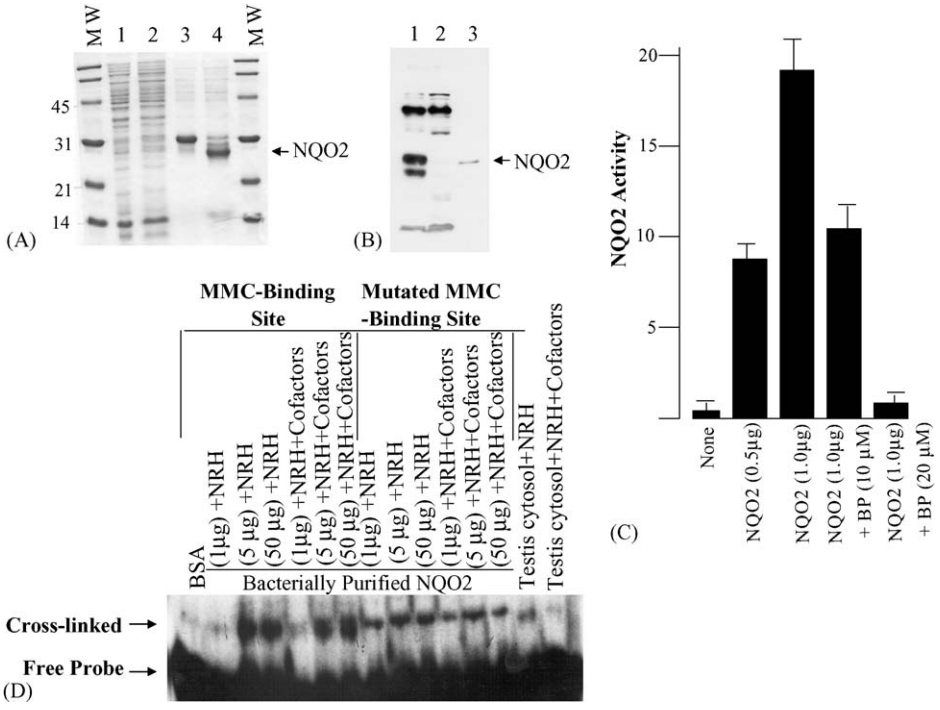

Fig. 7 – Characterization of bacterially expressed and purified mouse NQO2 protein and mitomycin C-induced DNA cross-linking. (A) SDS-PAGE: the *E. coli* extract (lane 1); *E. coli* extract transformed with NQO2 cDNA (lane 2); affinity purified NQO2 with 6xHis-affinity tag (lane 3); NQO2 digested with rTEV protease to remove 6xHis-affinity tag (lane 4) were run on SDS-PAGE and silver stained. MW, molecular weight markers. (B) Western analysis. The *E.coli* transformed with NQO2 cDNA and IPTG-induced (lane 1); *E.coli* transformed with NQO2 cDNA but not IPTG-induced (lane 2); affinity purified NQO2 (lane 3) were separated on SDS-PAGE and probed with antibody against rat NQO1 to identify bacterially expressed NQO2. (C) NQO2 activity. Bacterially expressed and purified mouse NQO2 activity was analyzed for NQO2 activity in absence and presence of benzo(a)pyrene. The benzo(a)pyrene inhibitable NQO2 activity is shown. The data represent mean ± S.E. from three independent analyses. (D) Mitomycin C-induced DNA cross-linking. Bacterially expressed and purified mNQO2 protein was incubated with ^{32}P-labeled oligonucleotide containing normal and mutated mitomycin C binding site, NRH and mitomycin C, in absence and presence of cofactors (BSA, Tween20, FAD). The samples were analyzed on 15% denaturing SDS-PAGE and autoradiographed. The cross-linked and free oligonucleotides are shown.

The various results clearly establish that NQO2 catalyzes metabolic activation of quinones that are detoxified by NQO1. This raised an interesting question regarding the differential response of the two highly homologous NQO1 and NQO2 proteins to quinones. It has been suggested that NQO2 catalyzes four-electron reduction of its substrates [26]. The metabolic products generated may undergo further enzymatic metabolism or self re-arrangement to produce electrophiles and/or ROS that attack macromolecules leading to cell cytotoxicity. The four-electron reducing properties of NQO2 might be responsible for its differences with NQO1 that prefers two-electron reduction of its substrates. NQO2 may also have properties to detoxify some unknown compounds and protect cells against their toxicity. However, the detoxification role of NQO2 has not yet been found. The results also establish that NQO2, like NQO1 activates anti-tumor drugs leading to cytotoxicity and cell death. The chemistry of NQO2 catalyzed reactions and the identities of metabolic products are worthy of further investigation.

This is the first report that shows a role of cytosolic NQO2 in metabolic activation of MMC leading to cytotoxicity. The increase in NQO2 levels in CHO cells showed a proportional increase in MMC cytotoxicity. This effect was several times higher in the presence of NRH. The studies with NQO2−/− cells also demonstrated resistance to MMC cytotoxicity that was independent of NRH. Further studies with bacterially expressed and purified NQO2 demonstrated that NQO2 activated MMC cross-linked DNA. Therefore, it is reasonable to suggest that NQO2 metabolically activates MMC leading to cross-linking of DNA that contributes to MMC cytotoxicity.

Our data further indicated that NQO2 catalyzes limited activation of MMC analogues BMY25067 and EO9 leading to increased cytotoxicity in CHO cells overexpressing NQO2. EO9 is structurally related to MMC, is less myelosupressive and is a very good substrate for NQO1 [40,41]. BMY25067 is also structurally related to MMC, but water insoluble and a less efficient substrate for NQO1. These results suggest that the NQO2 overlaps but also differs from NQO1 in selection of drugs for catalysis. These results also indicate that the chemical structure of the drugs might be an important factor in their metabolism by NQO2 and/or NQO1.

In conclusion, NQO2 is a metabolically activating enzyme. It catalyzes activation of quinones and anti-tumor drugs leading to cytotoxicity and cell death. This property of NQO2 suggests that NQO2 might be an interesting target for future research related to development of chemotherapeutic drugs.

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