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NAD(P)H:quinone oxidoreductase 1 Arg139Trp and Pro187Ser polymorphisms imbalance estrogen metabolism towards DNA adduct formation in human mammary epithelial cells

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ABSTRACT

Estrogens (estrone, E_1; estradiol, E_2) are oxidized in the breast first to catechols and then to form two *ortho*-quinones ($E_{1/2}$-3,4-Q) that react with DNA to form depurinating adducts, which lead to mutations associated with breast cancer. NAD(P)H:quinone oxidoreductase 1 (NQO1) reduces these quinones back to catechols, and thus may protect against this mechanism. We examined whether the inheritance of two polymorphic variants of NQO1 (Pro187Ser or Arg139Trp) would result in poor reduction of $E_{1/2}$-3,4-Q in normal human mammary epithelial cells (MCF-10F) and increased depurinating adduct formation. An isogenic set of stably transfected normal human breast epithelial cells (MCF-10F) that express a truncated (135Stop), the wild-type, the 139Trp variant or the 187Ser variant of human NQO1 cDNA was constructed. MCF-10F cells showed a low endogenous NQO1 activity. NQO1 expression was examined by RT-PCR and Western blotting, and catalytic activity of reducing E_2-3,4-Q to 4-hydroxy$E_{1/2}$ and associated changes in the levels of quinone conjugates (4-methoxy$E_{1/2}$, 4-OH$E_{1/2}$-2-glutathione, 4-OH$E_{1/2}$-2-Cys and 4-OH$E_{1/2}$-2-*N*-acetylcysteine) and depurinating DNA adducts (4-OH$E_{1/2}$-1-N3Ade and 4-OH$E_{1/2}$-1-N7Gua) were examined by HPLC with electrochemical detection, as well as by ultra-performance liquid chromatography with tandem mass spectrometry. The polymorphic variants transcribed comparably to the wild-type NQO1, but produced ~2-fold lower levels of the protein, suggesting that the variant proteins may become degraded. $E_{1/2}$-3,4-Q toxicity to MCF-10F cells (IC50 = 24.74 μM) was increased (IC50 = 3.7 μM) by Ro41-0960 (3 μM), a catechol-*O*-methyltransferase inhibitor. Cells expressing polymorphic NQO1 treated with E_2-3,4-Q with or without added Ro41-0960, showed lower ability to reduce the quinone (~50% lower levels of the free catechols and ~3-fold lower levels of methylated catechols) compared to the wild-type enzyme. The increased availability of the quinones in these cells did not result in greater glutathione conjugation. Instead, there was increased (2.5-fold) formation of the depurinating DNA adducts. Addition of Ro41-0960 increased the amounts of free catechols, quinone conjugates and depurinating DNA adducts. NQO1 polymorphic variants (Arg139Trp and Pro187Ser) were poor reducers of estrogen-3,4-quinones, which caused increased formation of estrogen-DNA adduct formation in MCF-10F cells. Therefore, the inheritance of these NQO1 polymorphisms may favor the estrogen genotoxic mechanism of breast cancer.

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Abbreviations: COMT, catechol *O*-methyltransferase; MTT, 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide; NQO1, NAD(P)H:quinone oxidoreductase 1; Ro41-0960, 3,4-dihydroxy-5-nitro-2′-fluorobenzophenone; E_1, estrone; E_2, estradiol; $E_{1/2}$, estrone and estradiol; 4-OHE_2, 4-hydroxyestradiol; 4-OC$H_3E_{1/2}$, 4-methoxy(estrone and estradiol); 4-OH$E_{1/2}$-2-SG, 4-hydroxy(estrone and estradiol)-2-glutathione; 4-OH$E_{1/2}$-Cys, 4-hydroxy(estrone and estradiol)-2-cysteine; 4-OHE_2-2-NacCys, 4-hydroxy(estrone and estradiol)-2-*N*-acetylcysteine; 4-OH$E_{1/2}$-1-N3Ade, 4-hydroxy(estrone and estradiol)-1-N3-adenine; 4-OH$E_{1/2}$-1-N7Gua, 4-hydroxy(estrone and estradiol)-1-N7-guanine.

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1. Introduction

The NQO1 gene codes for a flavoprotein enzyme that catalyzes the reduction of various endogenous and exogenous quinones and quinone imines to protect cells from DNA damage that causes toxicity, and induces mutations and cancer [1,2]. Recent studies suggest that NQO1 may have a protective role against the initiation of breast cancer [3–5]. Natural estrogens can be oxidized to *ortho*-quinones in the breast, and then react with DNA to form depurinating adducts, leading to mutations that could initiate breast cancer [6,7]. NQO1 and NQO2 reduce estrogen *ortho*-quinones back to catechols by a two-electron transfer mechanism, without forming the toxic semiquinone [8,9]. The catechol thus formed may largely become conjugated for detoxification ([10] and this work). Consistent with the idea that NQO1 could protect against the initiation of breast cancer, it was observed that NQO1 RNA levels in breast cancer-adjacent 'normal' tissue can be 10–1000-fold lower compared to breast tissue of control women [5]. Such downregulation of NQO1 in pre-cancerous tissue would favor the oxidation of estrogens to *ortho*-quinones and may favor the initiation of breast cancer. Certain exogenous and dietary factors may protect against this defect by upregulating NQO1 gene expression [11–17].

Similar to the effect of NQO1 downregulation in precancerous breast tissue, polymorphic variants of NQO1 that have decreased catalytic activity may increase the risk of breast cancer. An exon 6 C609T polymorphism in NQO1 that results in Pro187Ser alteration is known to produce an enzyme that shows negligible/very low activity, and it may increase the risk of breast cancer [4,18–20]. The inheritance of the 187Ser/Ser allele can vary among ethnic groups. For example, among Whites, it appears in 1–2% of controls and 4–8% of breast cancer patients, and among Asians it appears in 14–20% of controls and breast cancer patients [4,18,21]. An additional NQO1 polymorphic variant (a C465T mutation in exon 4 that causes an Arg139Trp change) has been reported in the literature [22,23]. It is not well studied, but may appear at 2% frequency in the tested populations (http://www.genecards.org/cgi-bin/carddisp.pl?gene=NQO1&search=NQO1). The relationship of Arg139Trp to breast cancer is not known. However, some studies suggest that compared to the wild-type protein, the 139Trp variant also has a lower activity of reducing the anti-tumor quinone, mitomycin C [22,24]. Therefore, it is possible that the inheritance of 139Trp and 187Ser variants may increase the risk of breast cancer.

To study the impact of these two NQO1 polymorphisms on estrogen metabolism in breast cells, we constructed NQO1 cDNA clones that express these variants in a normal human breast epithelial cell line (MCF-10F). This cell line is an important model for studying the role of estrogen genotoxicity in breast cancer initiation [25–27]. It expresses a low level of the endogenous NQO1 protein and shows very little catalytic activity (this work). In this study, we examined these isogenic NQO1 cells to evaluate the abilities of the 139Trp and 187Ser variants to reduce estradiol-3,4-quinone to the 4-catechol, and their impact on estrogen-DNA adduct formation.

2. Materials and methods

2.1. NQO1 cDNA constructs and transfection of MCF-10F cells

A wild-type NQO1 cDNA cloned between the *Eco*RI-*Sal*I sites of the plasmid pCMV6-XL5 was purchased from Origene Technologies (Cat No. SC119599, Rockville, MD). This cDNA (transcript variant 1, GenBank Accession No. NM_000903) corresponds to the longest of the three known alternative transcripts [28]. The insert (1147 bp) contained the complete NQO1 coding sequence (745 bp) flanked by a 141 bp 5′ UTR and a 261 bp 3′ UTR sequence. Following validation of the NQO1 DNA sequence (with vector primer v1.5 and vector primer XL39, Origene), this plasmid was used as a template for generating three mutant clones; one contained an AAA^{Lys} 135 TAA^{Stop} mutation in exon 4 of the NQO1 gene (Entrez GeneID: 1728), a second contained the polymorphic CGG^{Arg} 139 TGG^{Trp} mutation in exon 4, and a third contained the polymorphic CCT^{Pro} 187 TCT^{Ser} mutation in exon 6. The mutations were introduced by using the Quick Change XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: K135X Forward (5′ CGCTGCCATGTATGACTAAGGACCCTTCCGGAG), K135X Reverse (5′ CTCCGGAAGGGTCCTTAGTCATACATGGCAGCG), R139W Forward (5′ GACAAAGGACCCTTCTGGAGTAAGAAGGCAGTG), R139W Reverse (5′ CACTGCCTTCTTACTCCAGAAGGGTCCTTTGTC), P187S Forward (5′ TGTGGCTTCCAAGTCTTAGAATCTCAACTGACATATAGCATTGGG), P187S Reverse (5′ CCCAATGCTATATGTCAGTTGAGATTCTAAGACTTG-GAAGCCACA), as described by the vendor. Following mutagenesis, the NQO1 insert was PCR amplified with a pair of primers with built-in *Sna*BI and *Sal*I restriction sites (Forward: 5′ GTTACG-TATTCGGCACGAGGTTG, containing a *Sna*BI site; Reverse: 5′ AGCAGTCGACGGAAGCCTGGAAAGATACCC, containing a *Sal*I site) for convenient cloning of the NQO1 cDNA into the *Sna*BI-*Sal*I sites of the mammalian retroviral expression vector pBABE-puro [29] (map and sequence from: http://www.stewartlab.net/index-2-plasmidmaps.html and http://www.addgene.org/pgvec1?f=c&cmd=findpl&identifier=1764), kindly provided by Dr. Kay-Uwe Wagner, University of Nebraska Medical Center, Omaha, NE. A fourth pBABE-puro construct was similarly prepared with the wild-type NQO1 insert. The size of the NQO1 inserted in pBABE-puro was 1031 bp. The pBABE-puro: NQO1 constructs were transformed in *E. coli* One Shot ® TOP10 cells (Invitrogen, Carlsbad, CA), harvested with a recommended kit (Endofree Plasmid Maxi kit, Qiagen Inc., Valencia, CA), and verified by sequencing with primers located within the NQO1 ORF (Forward: 5′ ACGCTGC-CATGTATGACAAAG, Reverse: 5′ GATCCCTTGCAGAGAGTACATG). All primers were synthesized in the Eppley Institute Molecular Biology Core Facility, University of Nebraska Medical Center, Omaha, NE, and DNA sequencing reactions were conducted in the Genomics Core Research Facility, University of Nebraska, Lincoln, NE.

The purified pBABE-puro: NQO1 recombinant plasmids, as well as the empty pBABE-puro vector, were linearized with *Not*I, extracted with phenol:chloroform, precipitated with ethanol, resuspended in H_2O, electophoresed in a low-melting point agarose gel, extracted with Gelase (Epicentre Biotechnologies, Madison, WI) and re-purified with a spin column (QIAquick PCR purification kit, Qiagen). The purified linear DNAs (5 μg) were electro-transfected into 10 million MCF-10F cells (CRL-10318, ATCC, Rockville, MD) using a Nucleofector Device (Amaxa GmbH, Köln, Germany). For use in transfection, exponential cells [grown to 50% confluence in DMEM/Ham's F-12 50/50 culture medium (Mediatech Inc., Herndon, VA), supplemented with 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, 100 μg/mL penicillin/streptomycin mixture (all from Sigma) and 5% horse serum (Hyclone)] were harvested by centrifugation ($300 \times g$ for 5 min), washed with PBS supplemented with 0.5% bovine serum albumin (Sigma Chemical Co., St Louis, MO), resuspended in 100 μL of solution T (Cell Line Nucleofector kit T, Amaxa), transferred to the supplied cuvette, and pulsed at the electrical setting T-24. The cells were gently pipetted out and resuspended into 500 μL of pre-warmed supplemented Nucleofector solution (Amaxa) and then transferred into six-well plates containing 2.0 mL of culture medium for incubation at 37 °C with 5% CO_2. The transfected cells were initially enriched, and then individual clones were isolated by growth in culture medium supplemented with 2.5 μg/mL puromycin (Sigma). These procedures generated an isogenic set of cell lines: MCF-10F, MCF-10F pBABE-puro (empty vector), MCF-10F NQO1 WT, MCF-10F NQO1 135Stop, MCF-10F NQO1 139Trp and MCF-10F NQO1 187Ser.

2.2. RT-PCR and Western blot analysis

To characterize the expression of the NQO1 cDNAs, RNA extracted (RNeasy Mini kit, Qiagen) from two randomly selected clones of the various isogenic NQO1 cells were analyzed by RT-PCR. The forward primer was located 45 bp upstream of the NQO1 insert within the viral *gag* gene (5′ CTCAATCCTCCCTTTATCCAG) in pBABE-puro, and the reverse primer (5′ TGAACACTCGCTCAAACCAG) was located within the NQO1 ORF (between codons 115 and 122). RT-PCR of this segment produced a 573 bp DNA. For comparison, GAPDH expression was similarly analyzed by RT-PCR using previously described primers (Forward: 5′ ACGCATTTGGTCGTATTGGG and Reverse: 5′ TGATTTTGGAGGGATCTCGC), which generated a 230 bp DNA [30].

Whole-cell protein extracts were prepared by three freeze-thaw cycles of cells suspended in RIPA buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, pH 7.5) supplemented with a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail tablet, Roche Diagnostics GmbH, Mannheim, Germany). The protein content in the extracts was quantified by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Aliquots of the extracts (equivalent to 10 μg protein) were fractionated by 12% SDS-PAGE, electroblotted to PVDF membrane (Millipore Corporation, Bedford, MA) and analyzed for NQO1 protein level with a polyclonal antibody (NB100-1005, goat polyclonal anti-NQO1, Novus Biologicals Inc., Littleton, CO), and after stripping the membranes (Restore Plus Western Blot Stripping Buffer, Pierce), analyzed again for β-actin levels with a mouse monoclonal antibody (A5441, Sigma). The blots were processed with anti-goat or anti-mouse HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and developed with a chemiluminescence substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce). As described by the vendor, the NQO1 antibody was raised against its C-terminal amino acids (267–274), and it cross reacts with the human protein, detecting all three variant forms (30.9, 27.3 and 26.4 kDa).

2.3. Estrogen cytotoxicity

Exponentially growing MCF-10F cells in the DMEM/F-12 medium (described above) were seeded at a density of 5000 cells/well in 96-well plates. After a day (day 0), cells in one 96-well plate were counted by the MTT [3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl-tetrazolium bromide, Sigma] assay (see below), while other cells were treated with estradiol-3,4-quinone (E_2-3,4-Q, 10-50 μM) and incubated for 24 h. The estrogen solutions were prepared in acetonitrile (final concentration 0.007%). Following this incubation, cells were rinsed with PBS (Invitrogen, Carlsbad, CA), fresh medium added, and the cells were cultured for another 3 days. Cell numbers at days 1–3 were determined by the MTT assay. The effect of the catechol *O*-methyltransferase (COMT) inhibitor, Ro41-0960 (3,4-dihydroxy-5-nitro-2′-fluorobenzophenone, 3 μM, Sigma) [31,32] on the growth of the estrogen-treated cells was similarly determined.

In the MTT assay, the media in the 96-well plate cultures were replaced with 100 μL fresh medium containing 25 μL MTT (5 mg/mL in PBS), and incubated for 2 h at 37 °C to allow the reduction of MTT by metabolically active cells to form a purple formazan precipitate. The precipitate was then solubilized by adding 100 μL of 20% SDS in 1:1 DMF:H_2O (pH 4.7), and incubating overnight at 37 °C. The purple color was read at 570 nm in a μQuant microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) and analyzed by using KCjunior (version 1.41) software. The absorbance values were converted into cell numbers using a standard curve constructed by plotting MTT assay absorbance against cell counts. Linear interpolation was used to estimate the IC50. Survival estimates are presented using means and 95% confidence intervals.

2.4. Analysis of estrogen metabolites and DNA adducts

Cells (2×10^5) were grown for 48 h in DMEM/F-12 medium (described above), and then adapted for 72 h in phenol red-free DMEM/F-12 50/50 culture medium (Mediatech) similarly supplemented, except replacing the horse serum with 5% charcoal-stripped fetal bovine serum (HyClone). The adapted cells (20×10^6) were treated for 24 h either with E_2-3,4-Q (10 μM) alone, with Ro41-0960 alone (3 μM) or with E_2-3,4-Q (10 μM) and R41-0960 (3 μM, 1 h pre-treatment). Following the treatments, the culture media were harvested and supplemented with 3 μg of 2-hydroxy 3-methoxyestrone (internal standard for assessing sample recovery) and 2 mM ascorbic acid (to prevent the possible decomposition of the compounds) and processed immediately. Media from MCF-10F cells treated with 10 μL acetonitrile (solvent) were used as controls. Catecholestrogens and their conjugates, as well as the estrogen quinone conjugates and DNA adducts are efficiently secreted to the media, but a portion of these compounds is retained by the cells. We analyzed the secreted fractions in the culture media, because cell lysates can rapidly decompose the quinone conjugates and make it difficult to conduct these studies.

The harvested media were partially purified by passing through Varian C8 Certify II cartridges (Varian, Harbor City, CA) pre-equilibrated by sequentially passing 1 mL of methanol, distilled water, and potassium phosphate buffer (100 mM, pH 8). The collected media (40 mL) were adjusted to pH 8 with 1 mL of 1 M potassium phosphate buffer, and passed through these cartridges. The retained analytes in the cartridges were washed with the above phosphate buffer, eluted with 8:1:1:0.1 of methanol:acetonitrile:water:trifluoroacetic acid, and processed by passing through a 5000/10,000 cut-off filter (Millipore Corporation, Burlington, MA).

The resulting samples were analyzed in an HPLC apparatus equipped with a multi-channel electrochemical detector (Model 580 solvent delivery modules, Model 540 auto-sampler fitted with a 12-channel CoulArray electrochemical detector, Environmental Sciences Association, Chelmsford, MA). The analytes and adducts were separated with solvent gradients generated by solvent A [15:5:10:70 of acetonitrile:methanol:CAA buffer (5.25% citric acid, 3.85% ammonium acetate, 11.5% acetic acid):water] and solvent B [50:20:10:20 of acetonitrile:methanol:CAA buffer:water]. The samples were injected into a Phenomenex Luna-2 C-18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, Torrence, CA), and eluted isocratically (90% solvent A:10% solvent B) for 10 min, then by a linear gradient (up to 90% solvent B) in the next 35 min, at a flow rate of 1 mL/min. The 12 coulometric electrodes were set at potentials of −35, 10, 70, 140, 210, 280, 350, 420, 490, 550, 620 and 690 mV. The analyte and adduct peaks were identified by their retention times and peak height ratios between the dominant peak and the peaks in the two adjacent channels. The data were quantified by comparison with known amounts of standards. The results were compared between groups using the Mann-Whitney test.

The results were confirmed by analysis on a MicroMass QuattroMicro triple stage quadrupole mass spectrometer attached to a Waters Acquity UPLC (Waters, Milford, MA) as described previously [33].

3. Results and discussion

The scheme for generating the NQO1 cDNA constructs in the pBABE-puro vector is shown in Fig. 1. These constructs were

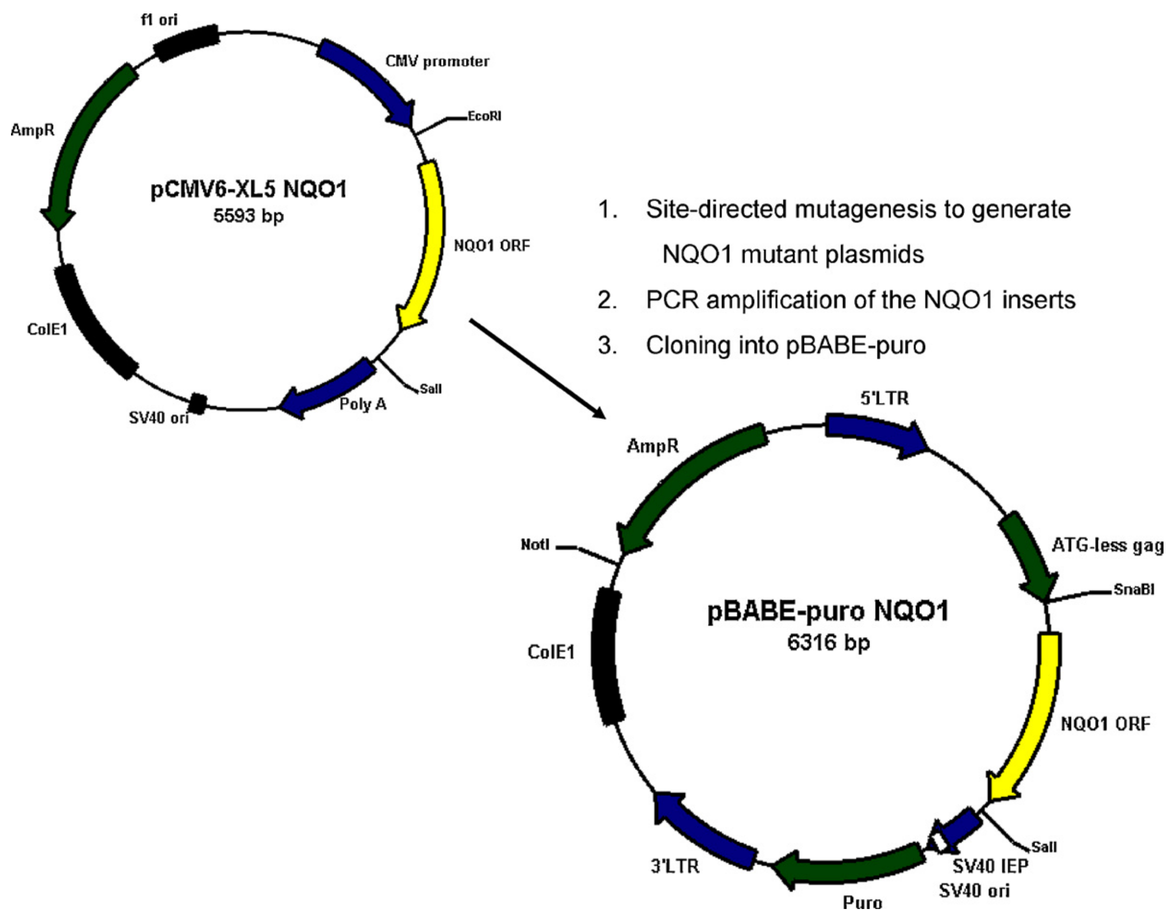

Fig. 1. Generation of NQO1-expressing retroviral vector constructs. A recombinant plasmid (pCMV6-XL5 NQO1) containing the wild-type NQO1 cDNA was mutagenized and inserted in pBABE-puro retroviral vector [29]. In this construct, the NQO1 cDNA is transcribed by a promoter present in the 5′LTR. The selectable marker (puromycin) is under the control of the SV40 early promoter.

linearized with *Not*I, transfected into MCF-10F cells and clonal cells selected for puromycin resistance.

3.1. Expression of ectopic NQO1 in MCF-10F cells

RT-PCR analysis of RNA extracted from the clonal cells (Fig. 2) indicated that the NQO1 constructs are efficiently expressed in MCF-10F cells. As is generally observed from ectopic expression, the levels of NQO1 transcription varied within the pairs of the clonal cells. For example, NQO1 transcript levels in WT-2 was 21% more than WT-3 (NQO1:GAPDH ratios were 3.07 and 2.52 respectively), 135Stop-4 was 7% lower than 135Stop-5 (1.59 and 1.71), 139Trp-4 was 12% higher than 139Trp-6 (2.20 and 1.96), and 187Ser-2 was 22% lower than 187Ser-3 (1.96 and 2.53). However, NQO1 transcript levels in two mutant clones (139Trp-4 and 187Ser-3) were comparable to a wild-type clone (WT-3), which suggests that the mutations *per se* did not affect the transcription of NQO1 cDNA.

Western blot analysis (Fig. 3) indicated that MCF-10F cells have low endogenous NQO1 protein. The MCF-10F NQO1 protein level was comparable to that in Caco-2 cells, and was approximately 10-fold lower NQO1 protein than in HeLa S3 and HT-29 cells (Supplement 1). The Caco-2 are human colon cancer epithelial cells, known to be homozygous for the NQO1 187Ser allele. Caco-2 is deficient in NQO1, because the 187Ser variant has poor enzymatic activity [34] and can be degraded by proteasomal mechanisms [35]. HT-29 are also human colorectal adenocarcinoma epithelial cells, but they are homogyzous for wild-type NQO1 [35]. Introduction of the pBABE-puro: wild-type NQO1 cDNA in MCF-10F cells elevated NQO1 protein levels by approximately 10-fold (Fig. 3), i.e., the ectopic expression of NQO1 cDNA brought this protein up to HT-29 and HeLa S3 levels (Supplement 1).

As expected, the NQO1 antibody (specific for the NQO1 C-terminal dodecapeptide) did not detect the truncated NQO1 protein in MCF-10F cells expressing the 135Stop mutant (expected protein size = 14.85 kDa) (Fig. 3). Densitometry indicated that NQO1 protein levels in the 139Trp and 187Ser variant cells were respectively, ~2.8-fold and ~1.8-fold lower than the wild-type. Expression of these polymorphic variants in Caco-2 cells also showed lower levels than in wild-type (Supplement 1). Considering the similarities of the RT-PCR and Western blot results, the possibility is raised that like 187Ser [35], the 139Trp variant may also be susceptible to post-translational changes.

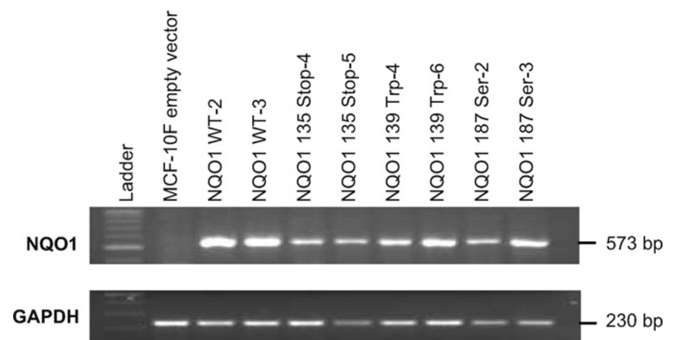

Fig. 2. RT-PCR analysis of NQO1 transcription in the MCF-10F host cells.

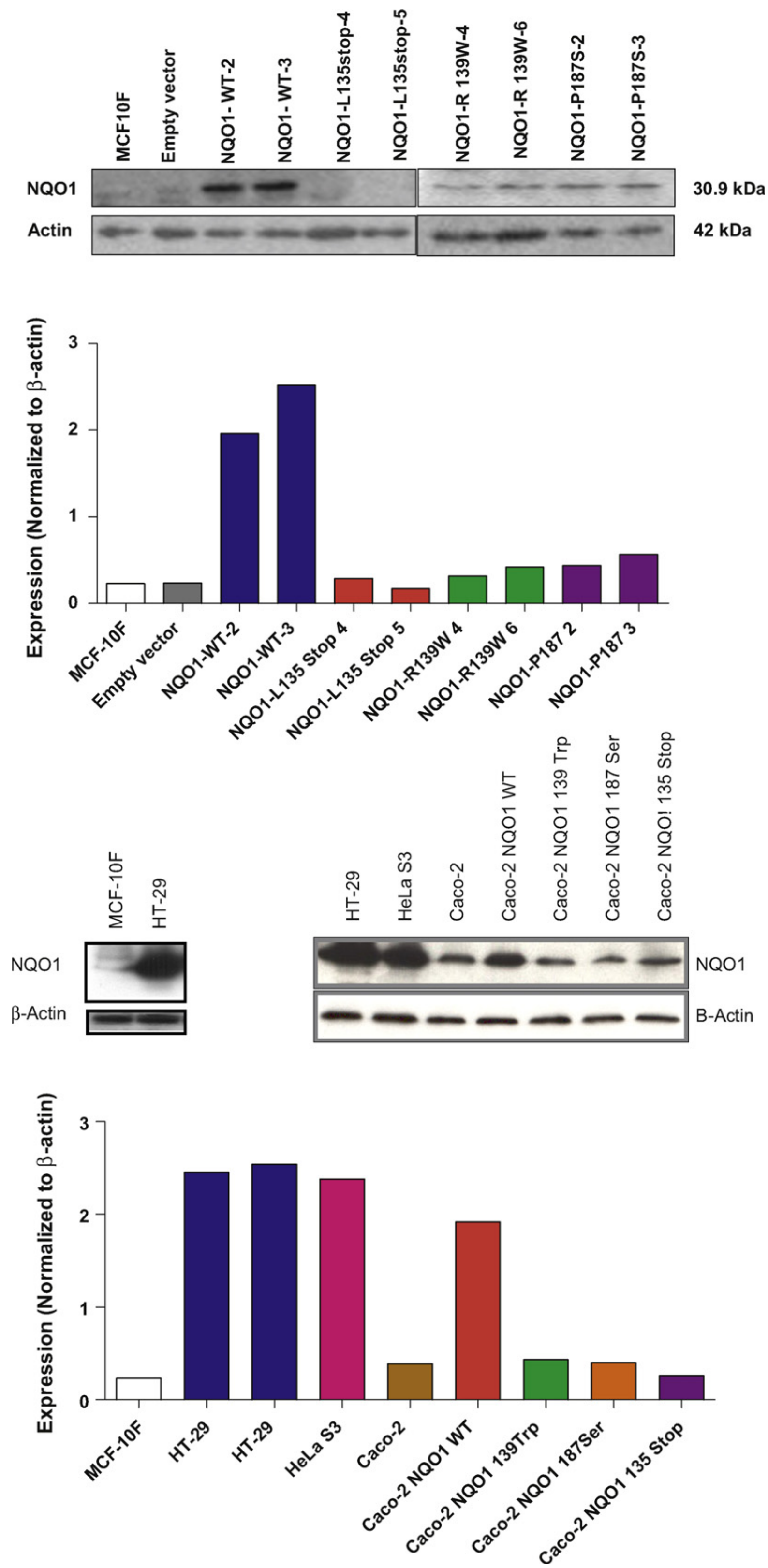

Fig. 3. Top: Western blot analysis of NQO1 protein levels in MCF-10F host cells. Bottom: Western blot analysis of endogenous NQO1 levels in MCF-10F, HT-29, HeLa S3, Caco-2 cells, as well as in Caco-2 cells transfected with NQO1 constructs. MCF-10F and Caco-2 cells showed comparable levels of NQO1 protein.

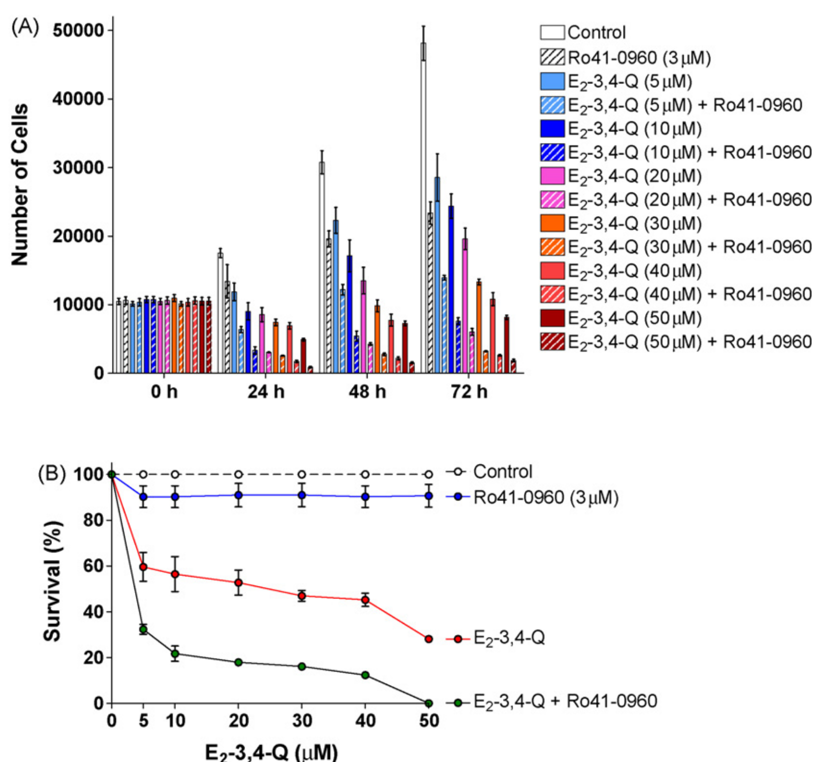

Fig. 4. E2-3,4-Q-induced MCF-10F cytotoxicity can be increased by a COMT inhibitor (Ro41-0960). (A) Dose and time-course of cytotoxicity. (B) Re-plot of the 24 h data for determining 50% killing dose.

3.2. Estrogen toxicity in MCF-10F cells

E_2-3,4-Q toxicity to the MCF-10F (host) cells was examined to identify a suitable dose for studying estrogen metabolism. These studies were conducted in the presence or absence of a known inhibitor (Ro41-0960) of catechol-*O*-methyltransferase [31,32] to determine the contributions of the 4-hydroxyestrogens (4-$OHE_{1/2}$, endogenous as well as those produced by NQO1 from the added E_2-3,4-Q) to toxicity.

The toxicity time-course is shown in Fig. 4A. Cells were treated (0 h) and followed for 72 h. E_2-3,4-Q showed a narrow spectrum (0–50 μM) of cytotoxicity. The untreated (control) cells showed normally growing cells, and all treatments including Ro41-0960 (3 μM) alone and E_2-3,4-Q (5-50 μM) with or without Ro41-0960 (3 μM) affected cell numbers in the 24-72 h period. At the low doses of E_2-3,4-Q (5 and 10 μM), cell numbers showed an initial decline (24 h), and then increased at a lower rate than control (48–72 h). At high doses (40 and 50 μM), cells showed poor growth recovery beyond 24 h. Addition of Ro41-0960 with E_2-3,4-Q increased the initial toxicity (24 h) and also inhibited the growth recovery. Since E_2-3,4-Q- and E_2-3,4-Q/Ro41-0960-treated cells showed a linear decline up to 24 h, the data at the 24 h time point was re-plotted in a survival curve to determine the 50% killing dose (IC50) (Fig. 4B). The estimated IC50 values for E_2-3,4-Q was 24.74 μM and E_2,3,4-Q + Ro41-0960 was 3.70 μM.

Estrogen cytotoxicity may occur by multiple mechanisms. The two better known mechanisms include oxidative stress generated by estrogen semiquinones, and estrogen quinone reaction with DNA [36] and proteins [37,38] (Fig. 5). $E_{1/2}$-3,4-Q has been thought to induce cytotoxicity primarily through its reactions with DNA and proteins. This conclusion was reached because E_2-3,4-Q has a relatively short half-life ($t_{1/2} \approx 45$ min at pH 7); it self-polymerizes to form inert compounds ([39] and unpublished results) and in the cell, its reaction with DNA and proteins is rapid. Furthermore, additives such as *N*-acetylcysteine and cysteine protect cells from estrogen quinone toxicity by forming conjugates [40] (Fig. 5), whereas ascorbic acid, which does not form a quinone conjugate, protects cells by minimizing endogenous oxidation of 4-$OHE_{1/2}$ to $E_{1/2}$-3,4-Q [41]. In particular, cells such as MCF-10F which have low endogenous levels of NQO1 would be expected to convert only a portion of the added E_2-3,4-Q to 4-OHE_2 that can then be oxidized to the toxic semiquinone. Therefore, E_2-3,4-Q toxicity in MCF-10F cells should be a good model to test this idea.

In our studies, E_2-3,4-Q induced an acute toxicity to MCF-10F cells (Fig. 4A), which is consistent with the idea that E_2-3,4-Q-DNA and protein adducts are major contributors to the observed toxicity. However, when Ro41-0960 was added with E_2-3,4-Q, there was a ~3-fold increase in acute cytotoxicity (Fig. 4B). For example, 24 h after treatment with 40 μM E_2-3,4-Q, 45.2% of MCF-10F cells survived, whereas Ro41-0960 supplementation further reduced the survival to 12.4% (i.e., a 3.6-fold reduction). In contrast, without exogenously added E_2-3,4-Q, Ro41-0960 showed only a marginal increase (~10%) of toxicity. Ro41-0960 blocks COMT, preventing it from methoxylating the catechols (e.g., 4-$OHE_{1/2}$ to 4-$OCH_3E_{1/2}$, Fig. 5), and may thus increase the availability of E_2-3,4-Q for biological reactions leading to toxicity. If so, NQO1 may have a critical influence on reverting estrogen metabolism leading to toxicity.

Next, we examined whether the study of estrogen metabolites, conjugates and DNA adducts in breast epithelial cells expressing wild-type or truncated or polymorphic NQO1 cDNAs and treated with E_2-3,4-Q with or without Ro41-0960 can provide clues towards understanding how NQO1 impacts estrogen metabolism.

3.3. Influence of NQO1 on E_2-3,4-Q metabolism and DNA adduct formation

In MCF-10F cells, estrogens are oxidatively metabolized to quinones by cytochrome P450 and peroxidases (Fig. 5). The catechols and the quinones can be shunted off this pathway by conjugation to protect cells from DNA adduct formation. NQO1

exerts its protective function by reducing the quinones back to catechols. RT-PCR (Fig. 2) and Western blot (Fig. 3) results suggest that MCF-10F cells are poor in NQO1. However, cytotoxicity studies (Fig. 4) suggest that despite the low levels of NQO1, these cells have significant protection against estrogen cytotoxicity.

To determine the role of endogenous NQO1 on estrogen metabolism in MCF-10F cells and to study the effects of overexpressing wt or polymorphic variants of NQO1, we treated these cells with 10 μM E_2-3,4-Q for 24 h, with or without 3 μM Ro41-0960, and examined the culture media for estrogen metabolites (4-OHE$_{1/2}$), conjugates (4-OCH$_3$E$_{1/2}$, 4-OHE$_{1/2}$-2-SG, 4-OHE$_{1/2}$-2-Cys and 4-OHE$_2$-2-NAcCys) and DNA adducts (4-OHE$_{1/2}$-1-N3Ade and 4-OHE$_{1/2}$-1-N7Gua) (Fig. 6 and Table 1). At the 10 μM dose, E_2-3,4-Q induced 56.5% killing of MCF-10F cells at 24 h (Fig. 4B). In both MCF-10F cells and human breast, E_1 and E_2 metabolites are easily interconverted, therefore, the treatment with E_2-3,4-Q results in metabolites, conjugates and adducts from both estrogens [32]. Since breast cells have high levels of COMT, it is typical to observe that most of the 4-OHE$_{1/2}$ is present as its conjugate (4-OCH$_3$E$_{1/2}$), and since estrogen quinones are readily conjugated with glutathione, the major fraction of these quinones are detected as conjugates (4-OHE$_{1/2}$-2-SG and its derivatives 4-OHE$_{1/2}$-2-Cys and 4-OHE$_2$-2-NAcCys) [32]. Additionally, E$_{1/2}$-3,4-Q reacts with DNA, which almost quantitatively (>99.9%) form depurinating adducts (4-OHE$_{1/2}$-1-N3Ade and 4-OHE$_{1/2}$-1-N7Gua) [42].

Treatment of the control group [MCF-10F, MCF-10F transfected with pBABE-puro (empty vector) or MCF-10F transfected with pBABE-puro containing the truncated NQO1 cDNA constructs (NQO1 135Stop-4 and 135Stop-5)] with 10 μM E_2-3,4-Q resulted in comparable profiles of estrogen metabolites, conjugates and adducts. The addition of Ro41-0960 (3 μM) in these experiments resulted in a drastic (∼98%) reduction in the levels of the methoxy conjugates, accompanied by an increase in the levels of free 4-OHE$_{1/2}$ by ∼2-fold (Table 1). In the Ro41-0960-treated control group, cells showed elevated overall levels of quinone conjugates [35% (MCF-10F, 0.51–0.84, $p = 0.09$), 48% (MCF-10F empty vector, 0.54–0.82, $p = 0.08$), 49% (135Stop-4, 0.48–0.76, $p = 0.27$) and 16% (135Stop-5, 0.38–0.52, $p = 0.13$)]. In addition, Ro41-0960 increased the levels of both DNA adducts (∼4-fold) in all control cells. The levels of the individual DNA adducts (N3Ade and N7Gua) were comparable. These results also suggest that the truncation of NQO1 at codon 135 results in the loss of catalytic activity.

In comparison to the negative control group, E_2-3,4-Q treatment of MCF-10F cells transfected with wild-type NQO1 cDNA (NQO1 WT-2 and WT-3) resulted in major changes in the estrogen metabolic profile. Specifically, compared to the controls, 4-OHE$_{1/2}$ levels were increased by 20% (WT-2) or 72% (WT-3) (1.42 in control vs. 1.71 or 2.44 in the WT cells, $p = 0.01$), and 4-OCH$_3$E$_{1/2}$ levels increased by 2.4-fold (WT-2) or 2.5-fold (WT-3) (28.4 in control vs. 68.92 or 71.71 in the WT cells, $p = 0.003$), accompanied by a lowering in the levels of quinone conjugates [1.88 in the controls vs. 1.17 (WT-2) or 1.35 (WT-3)] and DNA adducts 37% (WT-2) and 30% (WT-3) [0.27 in controls vs. 0.17 (WT-2) or 0.19 (WT-3)]. These results suggest that expression of wt NQO1 effectively reduced the exogenously added E_2-3,4-Q, forming increased levels of the catechols and their conjugates, leaving a smaller portion of the quinone to conjugate with glutathione and estrogen-DNA adducts. The addition of Ro41-0960 in these experiments brought about a trend of alterations in the metabolism profile comparable to the negative controls. Specifically, Ro41-0960 increased the levels of the free 4-OHE$_{1/2}$ by 28% (WT-2)(1.71 vs. 2.19, $p = 0.14$) or 39% (WT-3)(2.44 vs. 3.40, $p = 0.14$) and decreased 4-OCH$_3$E$_{1/2}$ by 99.1% (WT-2) (68.92 vs. 0.66, $p = 0.14$) or 99.5% (WT-3) (71.71 vs. 0.41, $p = 0.14$). Compared to control, the overall levels of the quinone conjugates increased from the Ro41-0960 treatment by 77% (WT-2, 1.18 vs. 2.07) or 58% (WT-3, 1.35–2.13).

As in the control cells, Ro41-0960 treatment to these cells, however, resulted in ∼4-fold increase in DNA adduct formation (WT-2, 0.17–0.70 and WT-3, 0.19–0.72) (Table 1). Thus, with COMT inhibition by Ro41-0960, wt NQO1 was able to reduce a greater fraction

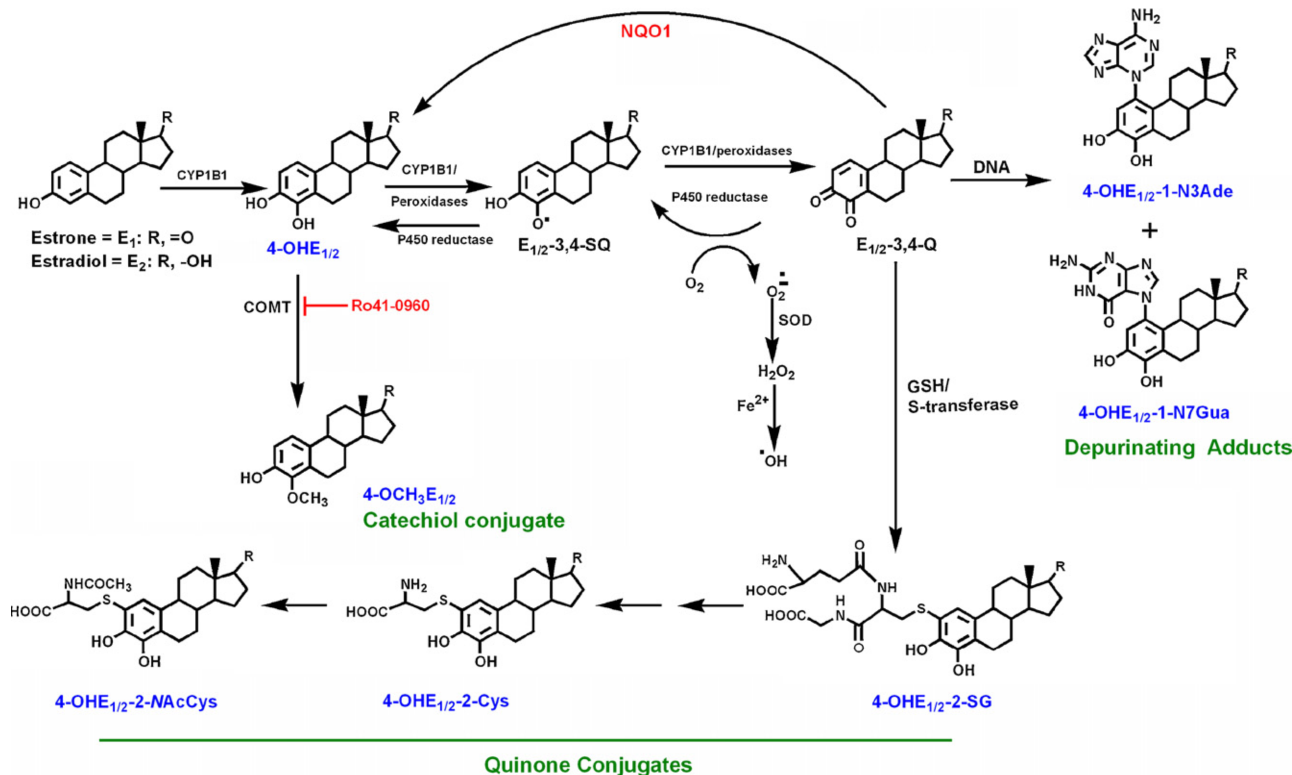

Fig. 5. Estrogen genotoxicity pathway in MCF-10F cells.

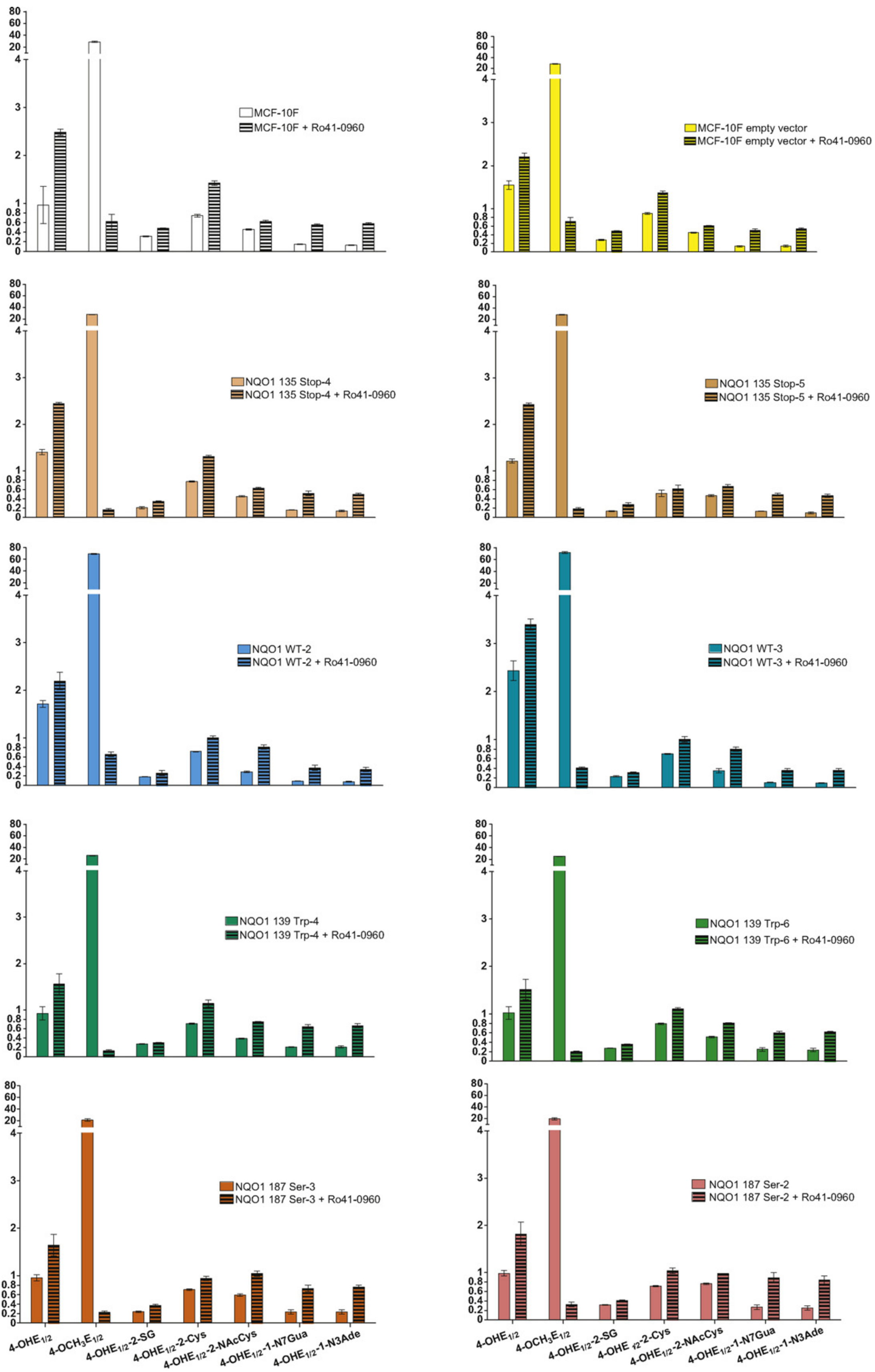

Fig. 6. Analysis of estrogen metabolism and DNA adduct formation in MCF-10F cells expressing wild-type and polymorphic NQO1 variants.

Table 1
NQO1 polymorphisms and COMT inhibition imbalance estrogen metabolism in human mammary epithelial cells.

Cells treated with 10 μM E_2-3,4-Q		Negative controls				Positive control		Polymorphisms			
Analytes (pmol/10^6 cells)	Ro41-0960 (μM)	MCF-10F host	MCF-10F empty vector	NQO1 135Stop		NQO1 WT		NQO1 139 Trp		NQO1 187 Ser	
				Clone 4	Clone 5	Clone 2	Clone 3	Clone 4	Clone 6	Clone 2	Clone 3
Catechol and conjugates 4-$OHE_{1/2}$, 4-$OCH_3E_{1/2}$ (group total)	–	1.53 ± 0.31, 28.95 ± 1.56 (30.51)	1.55 ± 0.17, 28.60 ± 0.61 (30.05)	1.40 ± 0.10, 27.71 ± 0.74 (29.11)	1.21 ± 0.08, 28.35 ± 0.73 (29.56)	1.71 ± 0.12, 68.92 ± 1.95 (70.63)	2.44 ± 0.35, 71.71 ± 2.33 (74.15)	0.93 ± 0.24, 25.66 ± 0.97 (26.59)	1.02 ± 0.23, 24.91 ± 0.67 (25.93)	0.99 ± 0.10, 19.46 ± 3.45 (20.45)	0.95 ± 0.01, 21.04 ± 3.59 (21.99)
	3	2.49 ± 0.11, 0.62 ± 0.26 (3.11)	2.21 ± 0.15, 0.71 ± 0.16 (2.92)	2.44 ± 0.05, 0.17 ± 0.04 (2.61)	2.43 ± 0.07, 0.18 ± 0.04 (2.61)	2.19 ± 0.03, 0.66 ± 0.09 (2.85)	3.40 ± 0.20, 0.41 ± 0.03 (3.81)	1.56 ± 0.38 0.12 ± 0.04 (1.68)	1.51 ± 0.37, 0.20 ± 0.03 (1.71)	1.82 ± 0.44, 0.33 ± 0.09 (2.15)	1.64 ± 0.40, 0.23 ± 0.05 (1.87)
Quinone conjugates (4-$OHE_{1/2}$-2-SG, 4-$OHE_{1/2}$-2-NAcCys, 4-$OHE_{1/2}$-2-Cys) (group total)	–	0.32 ± 0.02, 0.46 ± 0.03, 0.74 ± 0.05 (1.52)	0.28 ± 0.03, 0.45 ± 0.02, 0.89 ± 0.04 (1.62)	0.21 ± 0.04, 0.45 ± 0.02, 0.77 ± 0.03 (1.43)	0.14 ± 0.07, 0.47 ± 0.03, 0.52 ± 0.12 (1.13)	0.19 ± 0.01, 0.29 ± 0.03, 0.70 ± 0.02 (1.18)	0.23 ± 0.02, 0.35 ± 0.08, 0.77 ± 0.03 (1.35)	0.27 ± 0.01, 0.39 ± 0.02, 0.71 ± 0.02 (1.37)	0.30 ± 0.05, 0.51 ± 0.03, 0.79 ± 0.03 (1.60)	0.32 ± 0.02, 0.76 ± 0.03, 0.71 ± 0.02 (1.79)	0.24 ± 0.03, 0.59 ± 0.05, 0.71 ± 0.03 (1.54)
	3	0.48 ± 0.1, 0.62 ± 0.05, 1.43 ± 0.09 (2.53)	0.48 ± 0.02, 0.61 ± 0.01, 1.37 ± 0.07 (2.46)	0.34 ± 0.02, 0.63 ± 0.02, 1.31 ± 0.04 (2.28)	0.27 ± 0.07, 0.67 ± 0.06, 0.61 ± 0.13 (1.55)	0.26 ± 0.10 0.81 ± 0.07, 1.00 ± 0.07 (2.07)	0.32 ± 0.02, 0.80 ± 0.08, 1.01 ± 0.10 (2.13)	0.30 ± 0.02, 0.75 ± 0.02, 1.14 ± 0.13 (2.19)	0.35 ± 0.02, 0.81 ± 0.02, 1.10 ± 0.06 (2.26)	0.41 ± 0.03, 0.98 ± 0.01, 1.04 ± 0.10 (2.43)	0.37 ± 0.04, 1.04 ± 0.09, 0.94 ± 0.09 (2.35)
DNA adducts 4-$OHE_{1/2}$-1-N7Gua, 4-$OHE_{1/2}$-1-N3Ade (group total)	–	0.15 ± 0.01, 0.13 ± 0.01 (0.28)	0.13 ± 0.02, 0.14 ± 0.03 (0.27)	0.16 ± 0.01, 0.14 ± 0.03 (0.30)	0.13 ± 0.01, 0.10 ± 0.03 (0.23)	0.09 ± 0.01, 0.08 ± 0.02 (0.17)	0.10 ± 0.01, 0.09 ± 0.01 (0.19)	0.21 ± 0.01, 0.21 ± 0.04 (0.42)	0.25 ± 0.07, 0.24 ± 0.07 (0.49)	0.27 ± 0.09, 0.25 ± 0.08 (0.52)	0.24 ± 0.07, 0.24 ± 0.07 (0.48)
	3	0.54 ± 0.02, 0.58 ± 0.02 (1.12)	0.50 ± 0.06 0.54 ± 0.03 (1.04)	0.52 ± 0.09, 0.50 ± 0.04 (1.02)	0.49 ± 0.05, 0.47 ± 0.06 (0.96)	0.37 ± 0.10, 0.33 ± 0.07 (0.70)	0.36 ± 0.07, 0.36 ± 0.06 (0.72)	0.65 ± 0.07, 0.66 ± 0.07 (1.31)	0.60 ± 0.05, 0.62 ± 0.02 (1.22)	0.89 ± 0.19, 0.85 ± 0.15 (1.74)	0.73 ± 0.12, 0.76 ± 0.06 (1.49)

of the exogenously added E_2-3,4-Q, which increased the levels of 4-$OHE_{1/2}$, but also allowed greater formation of quinone conjugates (under these conditions, quinone conjugates increased by 16–49% in control cells and 58–77% in WT-2 and WT-3). In WT-2 and WT-3 cells, even though Ro41-0960 increased DNA adduct levels by 4-fold, the absolute levels of these adducts were 55% lower than in the control cells (0.71 vs. 1.03), suggesting that wtNQO1 can protect breast epithelial cells from estrogen-DNA adduct formation.

Compared to cells expressing wild-type NQO1, cells that expressed the polymorphic variants (139Trp or 187Ser) showed lower levels of 4-$OHE_{1/2}$ and 4-$OCH_3E_{1/2}$. Specifically, NQO1 139Trp-4 showed 55% [2.07 (average of 4-$OHE_{1/2}$ levels in WT-2 and WT-3, 1.71 and 2.44) vs. 0.93] and 139TRp-6 showed 51% (2.07 vs. 1.02) lower levels of 4-$OHE_{1/2}$, and 2.7-fold [70.31 (average of 4-$OCH_3E_{1/2}$ levels in WT-2 and WT-3, 68.92 and 71.71) vs. 25.66] and 2.8-fold (70.31 vs. 24.91) lower levels of 4-$OCH_3E_{1/2}$ than the wild-type (Table 1). Similarly, NQO1 187Ser-2 showed 52% (2.07 vs. 0.99) and 187Ser-3 showed 54% (2.07 vs. 0.95) lower levels of 4-$OHE_{1/2}$, and 3.6-fold (70.31 vs. 19.46) and 3.3-fold (70.31 vs. 21.04) lower levels of 4-$OCH_3E_{1/2}$ than the wild-type. Since the Western blot results indicate that 139Trp and 187Ser variants carry lower levels of NQO1 protein than the wild-type (2.78-fold and 1.8-fold respectively) (Fig. 3), the apparent lowering of the reduction of E_2-3,4-Q could not be readily interpreted as a consequence of poor catalytic activity of these variants. On the other hand, it is known that compared to the wild-type, both 139Trp and 187Ser variants show poor quinone-reducing activity [22,24]. Since the levels of 139Trp-4 and -6 proteins are comparable to the 187Ser-2 and -3 proteins, and both showed similar levels of 4-$OHE_{1/2}$, it is likely that both polymorphisms have poorer ability to reduce E_2-3,4-Q than the wild-type enzyme.

The 139Trp and 187Ser variants showed comparable quinone conjugate levels as in the negative and positive control cells (average values were 1.575 for the polymorphic, 1.425 for the negative and 1.265 for the positive). Addition of Ro41-0960 increased quinone conjugates to ~55.6 ± 10% [54.7% in the negative controls (1.425 vs. 2.205), 66% in the positive controls (1.265 vs. 2.1) and 46% in the polymorphic variants (1.575 vs. 2.30)]. On the other hand, the 139Trp and 187Ser variants showed greater levels of estrogen-DNA adducts than either negative controls or the wild-type clones. For example, compared to wild-type clones (positive controls), DNA adduct levels were 2.3-fold higher for 139Trp-4 (0.18 vs. 0.42) and 2.7-fold higher for 139Trp-6 (0.18 vs. 0.49), and 2.9-fold higher for 187Ser-2 (0.18 vs. 0.52) and 2.7-fold higher for 187Ser-3 (0.18 vs. 0.48). These results suggest that both polymorphisms could allow greater formation of DNA adducts in estrogen-exposed breast epithelial cells. Similar to results obtained with the control cells, addition of Ro41-0960 increased the levels of DNA adducts by a further ~3-fold. For example, adduct levels increased by 3.1-fold for 139Trp-4 (0.42–1.31), 2.5-fold for 139Trp-6 (0.49–1.22), and 3.3-fold for 187Ser-2 (0.52–1.74) and 3.1-fold for 187Ser-3 (0.48–1.49).

Overall, factors that increased the levels of E_2-3,4-Q led to greater formation of DNA adducts. This suggests that polymorphic variants of NQO1 with diminished capacity to reduce E1(E2)-3,4-Q could be associated with increased estrogen-DNA adduct formation and, consequently, increased risk of developing cancer.

4. Conclusions

Both 139Trp and 187Ser variants of NQO1 showed lower activity in reducing $E_{1/2}$-3,4-Q than the wild-type protein, which increased the availability of these quinones for reaction with DNA and formation of depurinating adducts. The mechanisms by which these polymorphic variants suffer loss in catalytic activity are not well understood. However, it is known that NQO1 (UniProtKB/Swiss-Prot entry: P15559) is an interlocked dimer of two identical

subunits of 274 amino acid polypeptides, both non-covalently attached to a prosthetic FAD group [43–45]. NQO1 is thought to have two domains: a catalytic domain (amino acids 1–220) and a small C-terminal domain. The catalytic domain is a large pocket at the dimer interface and has three functional regions: (1) FAD-binding, (2) NAD(P)-binding and (3) NAD(P)H/substrate-binding. NQO1 catalyzes in two-steps: first, the cofactor (NADH/NADPH) reduces the prosthetic FAD to $FADH_2$, and second, a hydride transfer from the $FADH_2$ to the substrate quinones by a 1,4-Michael addition mechanism [8,45]. The reported catalytically involved amino acids include: Gln104, Tyr128, Tyr155 and His161 [1,43,45,46]. The 139Trp (Swiss Prot variant: VAR_016170) and 187Ser (VAR_008384) variants are yet to be studied in detail, but available evidence suggests that 187Ser alters the conformation of a β-sheet, which lowers FAD-binding and results in very poor enzyme activity [1,43].

The 187Ser variant may undergo post-translational inactivation in human cells as evidenced by its not being detectable in tissues of homozygous individuals [34]. Since the levels of expression and activity of the 187Ser and 139Trp variants show a similar pattern, this raises the possibility that the latter variant may also undergo post-translational changes.

The observation that the polymorphisms may lead to increased formation of depurinating estrogen-DNA adducts suggests that they may increase mutagenesis in estrogen-exposed breast epithelial cells [6,7]. While this idea remains to be examined in the context of breast cancer, one study reported that the 187Ser variant significantly increased the incidence of, and the 139Trp variant shows a possible association with transversion mutations in the p53 gene in bladder cancer [47]. Such reports give credence to the idea that the NQO1 Arg139Trp and Pro187Ser polymorphisms may increase breast cancer risk.

The estrogen genotoxicity mechanism is mediated by a host of phase I and phase II enzymes, and alterations in several of these enzymes could impact the risk of breast cancer [48]. Upregulation of 'activating' genes (CYP19, CYP1B1) that convert estrogens to $E_{1/2}$-3,4-Q and downregulation of 'protective' genes (COMT, NQO1) [5], as well as inheritance of deleterious polymorphic mutations in these genes [4,49–52] will favor estrogen genotoxicity. If sufficient levels of estrogens are present in the breast, the present study suggests that the inheritance of NQO1 Arg139Trp or Pro 187Ser polymorphisms would favor increased estrogen genotoxicity. The NQO1 polymorphisms represent one of these factors of breast cancer risk, but it remains to be determined whether they would indeed play a critical role. Our studies have demonstrated that the estrogen-induced depurinating DNA adducts are excreted in the human urine, and can serve as biomarkers of breast cancer risk [33,53]. We think that a parallel study of urine-excreted depurinating adducts and the inheritance of the Arg139Trp and Pro187Ser polymorphisms in control, high risk and breast cancer patients will reveal the true impact of NQO1 in breast cancer.

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