EXTENDED ABSTRACT

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Role of NAD(P)H:quinone oxidoreductase 1 polymorphism in breast cancer cell sensitivity to quinone-based chemotherapeutic agents

GLORIEUX Christophe[a], SANDOVAL Juan Marcelo[a,b], DEJEANS Nicolas[a,§], BUC CALDERON Pedro[a,b]

[a] *Université catholique de Louvain, Louvain Drug Research Institute, Toxicology and Cancer Biology Research Group, Brussels, Belgium.*

[b] *Facultad de Ciencias de la Salud, Universidad Arturo Prat, Iquique, Chile.*

Corresponding Author:
Pedro BUC CALDERON
Université catholique de Louvain, Louvain Drug Research Institute, Toxicology and Cancer Biology Research Group, Brussels, Belgium
pedro.buccalderon@uclouvain.be

Abstract

Altered antioxidant enzymes expression like NQO1 are associated with cancer cell sensitivity to chemotherapeutic drugs. We investigated mechanisms of resistance to pro-oxidant drugs by using oxidative stress-resistant MCF-7 breast cancer cells (Resox cells). Since NQO1 is frequently modified in tumors, at genomic or transcriptomic levels, we investigated the role of NQO1 modulation in breast cancer chemosensitivity. We found that both NQO1 polymorphism and NQO1 overexpression are main determinants for the classical resistance, such as that observed by using menadione and doxorubicin. NQO1 overexpression was associated with an increased sensitivity to β-lapachone, a compound whose therapeutic activity is triggered through a NQO1 dependent mechanism. Inversely, conditions leading to the decrease of NQO1 activity, such as NQO1*2 polymorphism, trigger quinone-based chemotherapies-sensitivity. This places NQO1 modulations as a potential link between redox alterations in cancers and chemoresistance.

Key words: NQO1, quinone, breast cancer

Introduction

NAD(P)H:quinone oxidoreductase 1 (NQO1) reduces quinone compounds facilitating their detoxification (1). Since NQO1 is frequently overexpressed in a variety of tumors (2,3), the use of bioactive quinones has been exploited therapeutically because they are activated by NQO1 (4,5).
A different NQO1 expression pattern (protein levels and enzyme activity) have been found in tumors and normal tissues (6), but in addition, the existence of a polymorphism has also been noted. Two single nucleotide mutations have been reported: the C609T polymorphism, corresponding to a Pro187Ser change in the enzyme and described as NQO1*2, and C465T polymorphism, corresponding to an Arg139Trp change in the enzyme and described as NQO1*3. These polymorphisms are associated with a decreased enzyme activity (7,8). Depending on both the genotype and the type of chemotherapeutic agent used, the final anticancer outcome can be dramatically influenced by NQO1.
Recently, we found that some antioxidant enzymes, including NQO1, were overexpressed in MCF-7 breast cancer cells chronically exposed to an oxidative stress, namely Resox cells (9). Moreover, a genomic gain of the chromosomal band 16q22 was detected in Resox cells as compared to parental MCF-7 cells (10). Here, we made a particular attention to the study of *NQO1* polymorphisms, using a model of NQO1-null MDA-MB-231 cells stably transfected with either *NQO1*1*, the wild-type form of *NQO1*, or the *NQO1*2* polymorphism.

Materials and Methods

Cell lines and culture conditions: The breast cancer cell line, MCF-7, was purchased from ECACC (Salisbury, UK).

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The Resox cell line was derived from MCF-7 cells which were rendered resistant to an oxidant treatment (9). The breast cancer MDA-MB-231 cell line was a kind gift from Pr. Akeila Bellahcene (Metastasis Research Laboratory, Giga Cancer, Liège, Belgium). Cells were maintained in DMEM supplemented with 10% fetal calf serum, in the presence of penicillin (100 U/ml) and streptomycin (100 µg/ml) from Gibco (Grand Island, NY, USA). Human Mammary epithelial (HMEC) 250MK cells were kindly provided by Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley CA). All the cultures were maintained at 37°C in 95% air/5% CO_2 with 100% humidity. Dicoumarol, sodium L-ascorbate, menadione sodium bisulfite, menadione, β-lapachone and doxorubicin hydrochloride were purchased from Sigma (St Louis, MO, USA). All other chemicals were ACS reagent grade.

Stable transfection: pKK233-2 plasmids containing human *NQO1*1* (wild-type), *NQO1*2* cDNA were kindly provided by Dr. David Ross (11). The different cDNAs were amplified by PCR using the following primers: Forward 5'-ccgaagcttgccatggtcggcagaagagc-3' and Reverse 5'-ccgggtacctcattttctagctttgatct-3' (Sigma, St Louis, MO, USA), cut by restriction enzymes HindIII and KpnI (Fermentas, Vilnius, Lithuania) and subsequently cloned into pcDNA3.1 plasmids from Invitrogen (Grand Island, NY, USA). MDA-MB-231 cells were transfected with 1 µg of the different plasmids and then selected for 4 weeks in the presence of 1000 µg/ml neomycin (Invivogen, San Diego, CA, USA). Stable transfecting clones were characterized based on NQO1 enzyme activity and NQO1 protein levels. Only clones with high NQO1 activity and similar NQO1 protein levels were chosen for further studies.

SiRNA transfection: Dharmafect reagent 1 was used for transfection of siRNA against NQO1 (ON-TARGET plus SMART pool siRNA) in all cell lines, according to the protocols provided by Dharmacon (Lafayette, CO, USA). Transfection was performed on cells at 50% confluence for 24 h, with a 0.1 µM siRNA solution. All experiments were performed 48 h after transfection.

Immunoblotting: The procedures for protein sample preparation from cancer cell cultures, protein quantification, immunoblotting and western blot analyses were performed as previously described (12). Mouse antibody against β-actin (ab6276) was from Abcam (Cambridge, UK) and mouse antibody against NQO1 (sc-32793) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

NQO1 activity: Enzyme activity was determined by measuring cytochrome C reduction in the presence of NADH as previously described (10). Results are expressed in nmol of cytochrome C reduced per min per mg of protein. An extinction coefficient for cytochrome C of 21.1 mM/cm was used in the calculations.

Cell survival assays: The effects of quinones on cell metabolic status was assessed by using a clonogenic assay according to Glorieux *et al* (10).

Real-time PCR: Total RNA was extracted with the TriPure reagent from Roche Applied Science Diagnostics (Mannheim, Germany). Experimental procedures including primer sequences were as previously reported by Glorieux *et al* (10).

Data analyses: All experiments were performed at least three times. Data were analyzed using either a one-way ANOVA or an unpaired t-test, using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The level of significance was set at $p < 0.05$.

Results

NQO1 was highly expressed in the breast cancer MCF-7 cells compared to normal human mammary epithelial 250MK cells (Fig. 1 A-C). Compared to parental MCF-7 cells, Resox cells have increased NQO1 mRNA and protein levels (Fig. 1 A-C). Sequencing of the complete open reading frame (ORF) of the human *NQO1* gene in the three cell lines confirmed that 250MK cells were homozygous *NQO1*1* (wild-type NQO1), whereas MCF-7 and Resox cells were heterozygous *NQO1*1*/*NQO1*2* (data not shown).

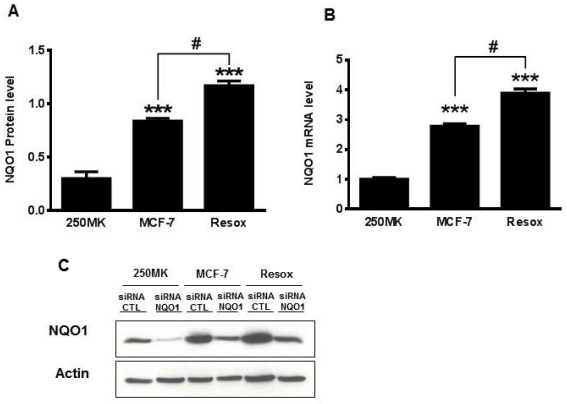

Fig.1 Basal levels of NQO1 expression in 250MK, MCF-7 and Resox cells.
(A) NQO1 protein levels normalized to β-actin. (B) NQO1 mRNA level. (C) NQO1 expression detected by immunoblotting. Data are means ± SEM from three separate experiments. #p-value < 0.05 vs MCF-7, ***p-value < 0.001 vs 250MK.

Figure 2 shows that 250MK cells were resistant to the pro-oxidant treatment, in contrast to MCF-7 and Resox cells. Pharmacological inhibition of NQO1, by using dicoumarol,

considerably increased the cytotoxicity in the three cell lines (Fig. 2A). These results were then confirmed by the use of specific siRNA (Fig. 2B).

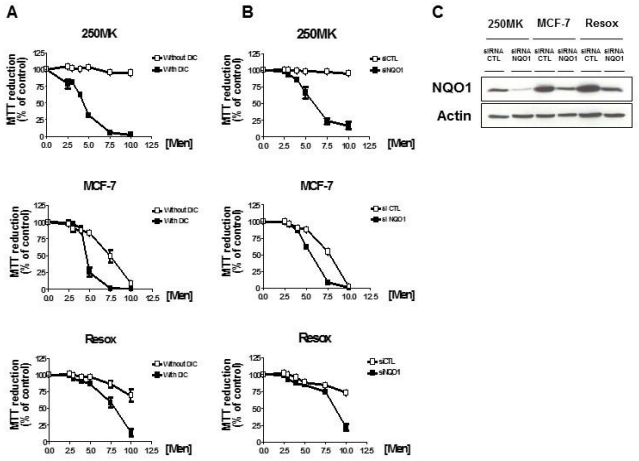

Fig.2. Sensitivity of mammary cells towards a pro-oxidant treatment. Cells were incubated for 24 h with indicated menadione (Men) concentrations associated with ascorbate (Asc) in a ratio Asc/Men 1:100. (A) Dicoumarol (DIC) was used at 25 µM. (B) For genetic invalidation cells were transfected for 48 hours with scrambled siRNA (siCTL) or specific siRNA against NQO1 mRNA (siNQO1). Cytotoxicity was evaluated using MTT assays. (C) NQO1 expression detected by immunoblotting. Data are means (% of control) ± SEM from three separate experiments.

Because MCF-7 cells are heterozygous *NQO1*1/NQO1*2* (19), we decided to study the importance of the *NQO1* polymorphism on the sensitivity of breast cancer cells to quinone-containing drugs. To this end, the wild-type form of *NQO1* (*NQO1*1*), or the variant form of the enzyme *NQO1*2*, were overexpressed in NQO1-null MDA-MB-231 cells.

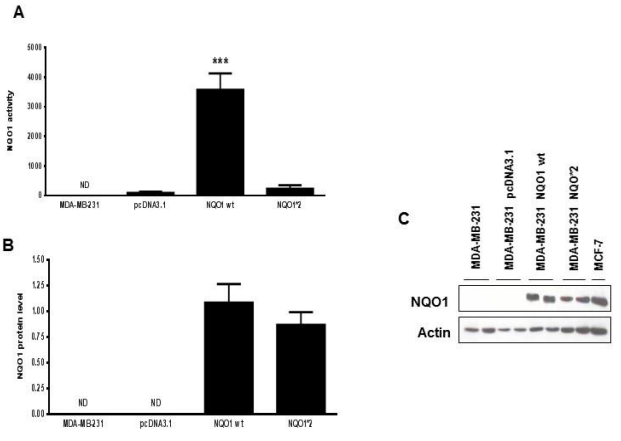

Fig.3. MDA-MB-231 cells overexpressing WT or C609T mutant forms of NQO1. (A) NQO1 enzymatic activity was measured by kinetic spectrophotometric assay. Results are expressed in nmol of cytochrome C reduced per minute per mg of protein. ***p-value < 0.001 vs pcDNA3.1. (B) NQO1 protein levels were quantified and normalized to β-actin. Data are means ± SEM. (C) NQO1 expression was detected by immunoblotting.

As shown in Fig. 3A, NQO1*1-overexpressing cells had about a 10-fold greater NQO1 activity than NQO1*2-overexpressing cells, despite the fact that both cells have similar NQO1 protein levels (Fig. 3 B-C). This result confirms that the presence of the polymorphism *NQO1*2* is associated with a decrease in NQO1 activity (8).

These newly generated cell lines were then exposed to menadione, doxorubicin and β-lapachone. Using a clonogenic survival assay (Table 1), we demonstrated that the expression of NQO1*1, the wild-type form with normal activity, was an important determinant of cancer cell resistance against menadione and doxorubicin. Moreover, our data confirmed that NQO1*1 expression was intrinsically related to cell sensitivity toward β-lapachone. In contrast, expression of the NQO1*2 variant, which presented virtually no NQO1 activity, had no significant influence on cell survival, compared to MDA-MB-231 cells expressing the empty vector. These data suggest that, beyond the expression of NQO1 itself, the *NQO1* polymorphism has a major influence on the sensitivity of cancer cells to some chemotherapeutic drugs.

Table 1. Sensitivity of MDA-MB-231 cells overexpressing WT or C609T mutant form of NQO1.

	pcDNA3.1	**NQO1 WT**	**NQO1*2**
Menadione (60 mM)	3.7 ± 3.2	23.8 ± 5.1**	5.5 ± 4.6
Doxorubicin (0.05 µM)	34.3 ± 8.5	58.6 ± 0.9**	40.4 ± 0.6
β-lapachone (1.5 µM)	87.1 ± 18.9	41.0 ± 12.9*	98.8 ± 18.0

Cytotoxicity was assessed by a clonogenic assay and results are expressed as percent of survival fraction. Cells were transfected with either the empty vector pcDNA3.1, NQO1 wild-type or NQO1*2. Data are means ± SEM from three separate experiments. *p-value < 0.05, **p-value < 0.01 as compared to MDA-MDB 231 (pcDNA3.1) cells.

Discussion

Since NQO1 is often increased in tumors as compared to healthy tissues (2,3), it emerges as an attractive target for cancer therapy, because it bioactivates compounds like β-lapachone leading to a selective cancer cells toxicity (5,13).

A polymorphism has been described for the *NQO1* gene (7), resulting in the production of three variants: *NQO1*1*, the wild-type form; *NQO1*2*, a variant with a C609T

substitution in exon 6; and *NQO1*3*, a variant with a C465T substitution in exon 4. Tumors with the *NQO1*2* polymorphism usually have low NQO1 protein levels: for example, MDA-MB-231 cells, which are homozygous *NQO1*2/NQO1*2*, have very low NQO1 proteins and are nearly undetectable by immunoblotting; thereby they are considered as NQO1-null (7). In contrast, MCF-7 cells, which are heterozygous *NQO1*1/NQO1*2*, have high NQO1 expression. Upon NQO1 inhibition, 250MK cells which were naturally resistant to the pro-oxidant treatment, were dramatically sensitized against this oxidant insult most likely explained because they are *NQO1*1* homozygous.
Due to the critical importance of *NQO1* polymorphism, both NQO1*1 and NQO1*2 isoforms were stably overexpressed in NQO1-null MDA-MB-231 cells. The overexpression of wild-type NQO1 made cells more resistant to menadione than cells transfected with pcDNA3.1, the empty vector. In contrast, the expression of NQO1*2 did not affect menadione cytotoxicity, most probably due to the strong decrease of enzyme activity showed by this variant isoform. Furthermore, we analyzed the impact of *NQO1* polymorphism on β-lapachone, which is bioactivated by the enzyme. Resox cells, with high NQO1 activity, were more sensitive to β-lapachone treatment and less sensitive to doxorubicin.

As conclusion we suggest that determining *NQO1* polymorphism may be important when considering the use of quinone-based chemotherapeutic drugs. Expression of the NQO1*2 isoform, for example, is not useful when using compounds such as β-lapachone, because they cannot be bioactivated by the NQO1*2 variant. However, compounds such as doxorubicin may be used in these circumstances because they are not detoxified by the NQO1*2 variant.

Acknowledgements

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