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A physiological threshold for protection against menadione toxicity by human NAD(P)H:quinone oxidoreductase (NQO1) in Chinese hamster ovary (CHO) cells

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1) has often been suggested to be involved in cancer prevention by means of detoxification of electrophilic quinones. In the present study, a series of Chinese hamster ovary (CHO) cell lines expressing various elevated levels of human NQO1 were generated by stable transfection. The level of NQO1 over-expression ranged from 14 to 29 times the NQO1 activity in the wild-type CHO cells. This panel of cell lines, allowed investigation of the protective role of NQO1 in quinone cytotoxicity. It could be demonstrated that menadione toxicity was significantly reduced in all NQO1-transfected CHO clones compared to the wild-type cells, but the clones did not show differences in their level of protection against menadione. This observation pointed at a critical threshold concentration of NQO1 above which a further increase does not provide further protection against quinone cytotoxicity. Additional studies in which the NQO1 activity was inhibited by dicoumarol showed that only dicoumarol concentrations of about five times the EC_{50} for NQO1 inhibition were able to reduce NQO1 levels below the apparent threshold, making the cells more sensitive. The level of this threshold was estimated to be in the range of base line NQO1 activities observed in several tissues and species. Thus, the results of the present study indicate that beneficial effects of NQO1 induction by, for example, cruciferous vegetables might be absent or present depending on the NQO1 activity threshold for optimal protection and the basal level of NQO1 expression in the tissue and species of interest.

Keywords: NAD(P)H:quinone oxidoreductase; Menadione; Cytotoxicity; CHO cells; Threshold; Dicoumarol

1. Introduction

NQO1 (DT-diaphorase) (EC 1.6.99.2) is a widely distributed dimeric flavoprotein containing one molecule of FAD per subunit [1]. It catalyses the two-electron reduction of various quinones, quinone epoxides, azo dyes, and aromatic nitrocompounds [2–7] using NADH or NADPH as an electron donor. The reduction of electrophilic, redox-active quinones by the enzyme is believed to result in detoxification [8,9]. The role of NQO1 in chemoprotection is further supported by the increased incidence of carcinogenesis and xenobiotic-induced toxicity in individuals carrying a polymorphism in *NQO1*, resulting in decreased NQO1 activity [10]. On the other hand, NQO1 is supposed to be involved in the bioreductive activation of specific quinoid-type anticancer drugs, which in fact require a two-electron reduction to become active as a cytotoxic agent [10,11].

In some studies *in vitro* [12,13] as well as *in vivo* [13,14], an inverse correlation was established between cytotoxicity of quinones and the quinone reductase activity of the target organ/cells. In addition, comparison of quinone toxicity in different cell lines containing very low NQO1 activity (e.g. Caco-2), or expressing higher levels of NQO1 activity (e.g. HT-29 cells), revealed increased quinone toxicity in cells with very low quinone reductase

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Abbreviations: CHO, Chinese hamster ovary cells; NQO1, NAD(P)H:-quinone oxidoreductase 1; wt, wild-type; LDH, lactate dehydrogenase; DCPIP, 2,6-dichlorophenolindophenol.

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levels [15,16]. From a mechanistic point of view, however, the increased quinone toxicity towards cells with low or inhibited NQO1 activity does not necessarily imply that induced expression levels of the enzyme in cells already displaying moderate basal NQO1 activity will result in increased protection against quinone-mediated toxicity. Some studies have, indeed, questioned the correlation between increased NQO1 activity and reduced cytotoxicity *in vitro* [17]. Nevertheless, several studies have linked the protective effect against cancer of especially cruciferous vegetables to the induction and elevated levels of protective enzymes like NQO1 and glutathione *S*-transferases [18,19].

In the present study, the consequences of increased NQO1 levels for quinone-mediated toxicity were investigated in a series of CHO cell lines generated by stable transfection and expressing different elevated levels of NQO1, using menadione as the model compound.

2. Materials and methods

2.1. Chemicals

DMEM/F12 medium, HBSS, trypsin, foetal calf serum (FCS), gentamicin, fungizone and zeocin were purchased from Invitrogen. Standard chemicals were purchased from Merck. Menadione was from Sigma Chemical Co.

2.2. Cell lines

CHO-K1 (wild-type CHO) cells were purchased from the American Type Culture Collection (ATCC). CHO cells were cultured in DMEM/F12 medium supplemented with 10% FCS and 50 μg/mL gentamicin using a CO_2 incubator maintained at 37°, 5% (v/v) CO_2 and 100% relative humidity. CHO-hNQO1 cells were cultured in a similar way using the medium as described above for the CHO cells with the addition of 200 μg/mL zeocin. The cells were routinely subcultured twice a week.

2.3. Plasmids

A pVL1392 baculovirus expression vector (Pharmacia), containing full-length cDNA encoding human NADP(H):-quinone oxidoreductase 1 (hNQO1), was used as a template in a PCR reaction to amplify human NQO1 cDNA. The hNQO1 cDNA sequence from this vector was tailed by PCR with an upstream *Kpn*I restriction site and the hNQO1 ribosome binding region (bp 42–50 in GenBank/EMBL accession number J03934) [20,21], and with a downstream *Xba*I restriction site using the primers 5′-AGCGCCCCG-GACGGTACCAGAGCCATG-3′ and 5′-CCAGGCTAA-TCTAGATCATTTTCTAGCTTTGATCTG-3′ respectively, and subsequently inserted into the multiple cloning site of pcDNA3.1/Zeo(+) (Invitrogen) by standard subcloning procedures [22]. The resulting eukaryotic hNQO1 expression vector was called pCMV-hNQO1.1 and was maintained and amplified using Max Efficiency competent *Escherichia coli* DH5α bacterial cells purchased from Stratagene. The correct nucleotide sequence of the insert (containing bp 42–875 from J03934) was confirmed by bi-directional sequencing.

2.4. Stable transfection

CHO-K1 cells were seeded in 6-well tissue culture plates, containing 2 mL medium per well (α-MEM with 10% (v/v) FCS, 50 μg/mL gentamicin and 2.5 μg/mL fungizone), to reach approximately 50% confluence the next day. Subsequently, the cells were transfected during 24 hr with 1.5 μg of pCMV-hNQO1.1 and 1.5 μg of the β-galactosidase expression vector pSV-β-galactosidase (Promega) per well, using the calcium phosphate co-precipitation method as described [22]. To allow expression of the zeocin resistance trait and of β-galactosidase, the transfected cell population was incubated for another 24 hr. Subsequently, transfection efficiency was monitored by measuring β-galactosidase activity. Duplicate wells were trypsinised and stable transfectants isolated by diluting the contents of each well into three 75 cm^2 tissue culture dishes, holding 10 mL of selective medium (α-MEM with 10% (v/v) fetal calf serum, 50 μg/mL gentamicin, 2.5 μg/mL fungizone, and 200 μg/mL zeocin). After about three weeks colonies of zeocin-resistant cells appeared. Rounded, separate colonies, apparently originating from a single cell, were picked using a sterile Pasteur pipette, transferred to a 24-wells tissue culture dish, and further propagated. Eight zeocin-resistant clones thus obtained were screened for elevated NQO1 activity levels as described below, and six clones showing the highest levels of NQO1 over-expression were selected.

2.5. Measurement of the NQO1 activity

NQO1 activity was measured as the dicoumarol-inhibitable fraction of menadione or DCPIP reduction in the cell cytosol in the presence or absence of activators of NQO1 [1]. Cells were routinely trypsinised and resuspended in cold TE buffer (20 mM Tris–HCl containing 2 mM EDTA, pH 7.4). The cell lysate was stored at −80° until analysed for activity. Before measurement cells were disrupted in three cycles of freeze/thawing using liquid nitrogen and a 37° waterbath, and centrifuged for 5 min at 12,000 *g*. When menadione was used as an electron acceptor, the reaction mixture contained (final concentrations in 1 mL total volume) 33 mM potassium phosphate pH 7.4, 0.18 mM NADPH, cell lysate of approximately 0.3×10^6 cells, 20 μM menadione, with or without the activators bovine serum albumine (BSA, 0.2 mg/mL) and Tween-20 (0.01% (v/v)). The reaction was started by the addition of menadione added as 2 μL

from a 10 mM stock solution in DMSO. The oxidation of NADPH was followed spectrophotometrically at 340 nm ($\varepsilon = 6.3 \times 10^3\ M^{-1}\ cm^{-1}$). After 2 min NQO1 activity was stopped by adding 20 μM dicoumarol (5 μL from a 4 mM DMSO stock) to allow correction for non-specific NADPH oxidation. Activities are presented as μmol NADPH oxidised/min/mg protein. When DCPIP was used as the electron acceptor, reduction was measured as described [23] and was normalised to cytosolic protein. The reaction mixture contained in a final volume of 1 mL, 25 mM Tris–HCl (pH 7.4), 180 μM NADPH, with or without the addition of BSA (0.2 mg/mL) and Tween-20 (0.01% (v/v)) as NQO1 activators, 0 or 20 μM dicoumarol, and an appropriate amount of cell lysate. The reaction was started by the addition of 40 μM DCPIP (2 μL of 20 mM stock in DMSO). Reduction of DCPIP was measured at room temperature for 1–2 min at 600 nm ($\varepsilon = 21 \times 10^3\ M^{-1}\ cm^{-1}$) with or without 20 μM dicoumarol. NQO1 activity is considered to be the dicoumarol-inhibitable part of DCPIP reduction. Activities are presented as μmol DCPIP reduced/min/mg protein.

The significance of the NQO1 over-expression as observed in the transfected clones was tested using a Student's *t*-test for two-sample comparison.

Protein was measured using the BCA protein reagent kit from Pierce as described elsewhere [24].

2.6. Cytotoxicity

Cytotoxicity was measured using the LDH assay. To this end, cells were plated 24 hr prior to exposure in a 96-well plate at a density of 10^4 cells in 100 μL of culture medium per well. After careful removal of the culture medium, 100 μL exposure medium was added, consisting of culture medium containing the required concentration of menadione added as 0.5% (v/v) of a 200 times concentrated stock solution in DMSO. After 24 hr of exposure the medium was transferred to a new 96-well plate for LDH measurement in the medium. For measurement of intracellular LDH, cells were rinsed once with PBS, followed by addition of 100 μL of 0.5% Triton X-100 in PBS. Cell lysate and medium were kept at −80° until measuring LDH activity. After thawing, the samples were shaken for 10 min in an orbital shaker at 1000 rpm. Then 0.1 M potassium phosphate pH 7.4 containing 0.24 mM NADH (final concentration) was added, and the reaction was started by the addition of 0.77 mM (final concentration) sodium pyruvate. The NADH decrease was measured during 30 s in a Spectramax microplate reader (Molecular Devices) at 340 nm. Data were checked for linearity, and percent cytotoxicity values were calculated from the NADH oxidation rates using the following formula:

$$\%\,\text{LDH leakage} = \frac{\text{NADH decrease medium}}{\text{NADH decrease medium} + \text{NADH decrease cells}} \times 100.$$

3. Results

3.1. Expression of wild-type NQO1 in CHO cells

Table 1 shows the activities of NQO1 in the isolated transfectants, determined as the dicoumarol-inhibitable, NADPH-dependent menadione or DCPIP reduction capacity. In each isolated clone the activity was significantly higher than in the wild-type cell line, which showed a small background activity. The NQO1 activities differed amongst the clones; lowest activity was seen in CHO-hNQO1-1 and the highest in CHO-hNQO1-4 and CHO-hNQO1-5. NQO1 activity in the various clones amounted to 14–29 times the NQO1 activity in the CHO-wt cells. Addition of the activators BSA and Tween-20 during activity measurements led to significantly higher activities, with similar differences between wild-type and transfectants. Monitoring of the NQO1 activity of the various clones throughout several months of continuous culture revealed that the elevated levels remained stable over the test period, demonstrating stable expression of human NQO1 in these clones (Fig. 1).

Table 1
NQO1 activity[a] in CHO-wt cells and in the series of stable, human NQO1-transfected clonal cell lines

CHO cell line	Substrate				Fold over-expression (over CHO-wt)
	Menadione (μmol NADPH/min/mg protein)		DCPIP (μmol DCPIP/min/mg protein)		
	−Activation	+Activation	−Activation	+Activation	
CHO-wt	0.01 ± 0.001	0.04 ± 0.01	0.04 ± 0.01	0.19 ± 0.10	1
CHO-hNQO1-1	0.09 ± 0.01	1.0 ± 0.2	0.33 ± 0.07	2.0 ± 0.6	14 ± 6[b]
CHO-hNQO1-2	0.19 ± 0.01	1.8 ± 0.1	0.73 ± 0.27	3.3 ± 0.6	26 ± 10[b]
CHO-hNQO1-3	0.21 ± 0.03	1.4 ± 0.2	0.88 ± 0.13	4.1 ± 0.3	26 ± 6[b]
CHO-hNQO1-4	0.23 ± 0.03	1.7 ± 0.1	0.97 ± 0.28	3.6 ± 0.7	29 ± 9[b]
CHO-hNQO1-5	0.24 ± 0.03	1.4 ± 0.1	1.0 ± 0.3	4.2 ± 0.4	29 ± 7[b]

[a] Values are the mean ± SE of at least four independent cell samples.
[b] Using the Student's *t*-test, all transfectants were found to show significant ($P \leq 0.05$) over-expression of NQO1 as compared to CHO-wt cells.

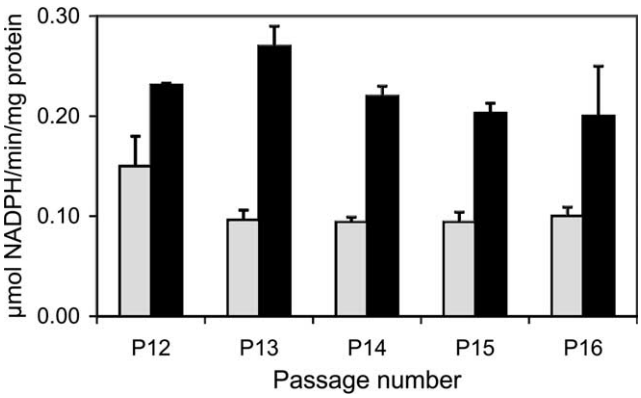

Fig. 1. NQO1 activities (measured using menadione as a substrate for NQO1, without NQO1 activators) in two different hNQO1-transfected clones observed throughout the consecutive passages used in the cytotoxicity assays. Light-shaded bar: CHO-hNQO1-1; dark bar: CHO-hNQO1-4. Average NQO1 activities of all CHO transfectants are presented in Table 1.

3.2. Cytotoxicity of menadione in the panel of cell lines

To clarify the relation between the NQO1 expression level and the cytotoxicity of quinones, the sensitivity of the wild-type and the five NQO1-transfected cell lines towards the model quinone menadione was determined (after 24 hr of exposure) using the LDH leakage assay. Fig. 2 shows typical 24 hr dose–response curves for menadione toxicity towards two clones of CHO-hNQO1 compared to CHO-wt cells. Table 2 presents the average EC_{50} values for the various CHO cells (wt cells and CHO-hNQO1 clones), defined as the menadione concentration resulting in 50% cell death. In all clones menadione toxicity appears to be significantly reduced compared to the CHO-wt cells. Ratios of EC_{50} values of transfected cell lines and the untransfected CHO-wt cell line were calculated to compare the level of protection of the different clones against menadione cytotoxicity. In spite of their differences in NQO1 levels, this ratio, EC_{50} CHO-hNQO1/EC_{50} CHO-wt, was similar for all transfected clones (Table 2). As a result, there was no proportional correlation between EC_{50} value and NQO1 activity.

Table 2
EC_{50} values for menadione cytotoxicity in the panel of CHO cell lines, expressed as the average ± SD from 2–4 independent dose–response curves

	EC_{50} (µM)	Ratio of EC_{50} CHO-wt/EC_{50} CHO-hNQO1
CHO-wt	20 ± 6	1
CHO-hNQO1-1	48 ± 1	2.4
CHO-hNQO1-2	45 ± 6	2.3
CHO-hNQO1-3	51 ± 6	2.6
CHO-hNQO1-4	45 ± 5	2.3
CHO-hNQO1-5	43 ± 9	2.3

3.3. Modulation of menadione cytotoxicity by dicoumarol

The effect of the NQO1 inhibitor dicoumarol on menadione cytotoxicity in CHO-wt cells and in the various CHO-hNQO1 transfectants is shown in Fig. 3. The presence of 20 µM dicoumarol restores the cytotoxic effect of 40 µM menadione in each CHO-hNQO1 clone to a level similar to that observed in the CHO-wt cells. Exposure to dicoumarol alone did not lead to cytotoxic effects (data not shown). Fig. 4 shows the effect of a range of concentrations of dicoumarol on the cytotoxicity of menadione for the CHO-hNQO1-1 cell line. Using a cytosolic sample, the actual inhibition of NQO1 by dicoumarol in CHO-hNQO1-1 was also determined (Fig. 5), revealing an *in vitro* EC_{50} value for NQO1 inhibition of about 0.1 µM. Comparison of this *in vitro* EC_{50} value to the results in Fig. 4, reveals that a concentration of more than five times this *in vitro* EC_{50} value is required to achieve almost complete abolition of the NQO1-mediated protection in the CHO-hNQO1-1 cells. Addition of dicoumarol at 0.1 µM, its *in vitro* EC_{50}

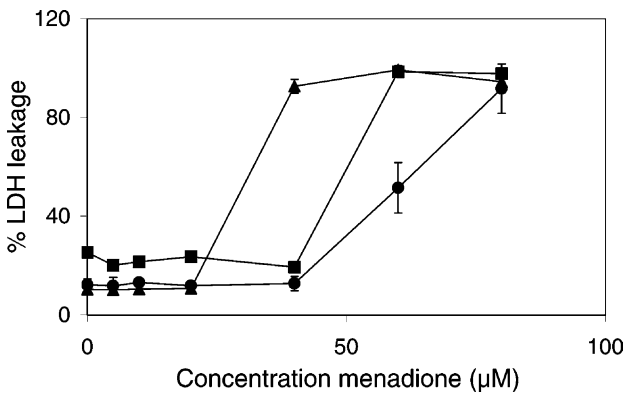

Fig. 2. Cytotoxicity of menadione in CHO-wt cells (▲), in CHO-hNQO1-1 cells (■) and in CHO-hNQO1-5 cells (●). Cytotoxicity was characterised by leakage of LDH in the culture medium measured after 24 hr of exposure as described in Section 2. Data are presented as mean ± SD and are from one representative experiment.

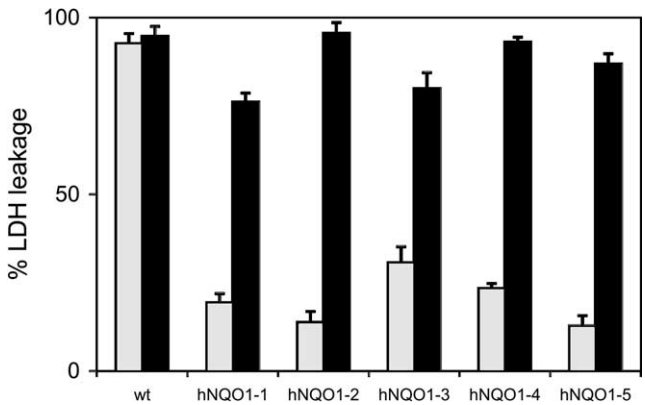

Fig. 3. Effect of 20 µM dicoumarol on the menadione toxicity in CHO-wt and CHO-hNQO1 transfectants. Cytotoxicity was measured after 24 hr exposure using the LDH leakage assay performed as described in Section 2. Data are presented as mean ± SD. Light-shaded bar: 0 µM dicoumarol; dark bar: 20 µM dicoumarol.

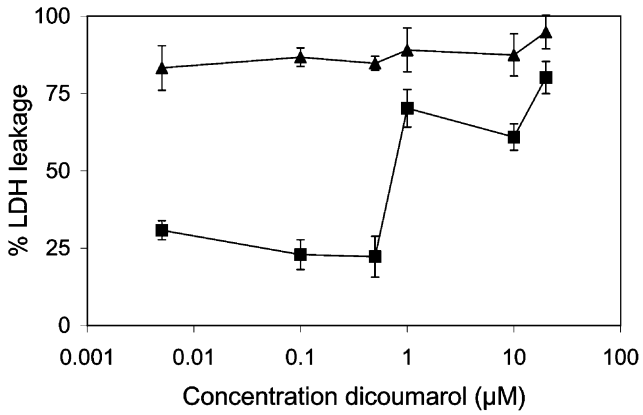

Fig. 4. Influence of varying concentrations of the NQO1 inhibitor dicoumarol on the cytotoxicity of 40 μM menadione (exposure time: 24 hr) in CHO-wt cells (▲) and in CHO-hNQO1-1 cells (■).

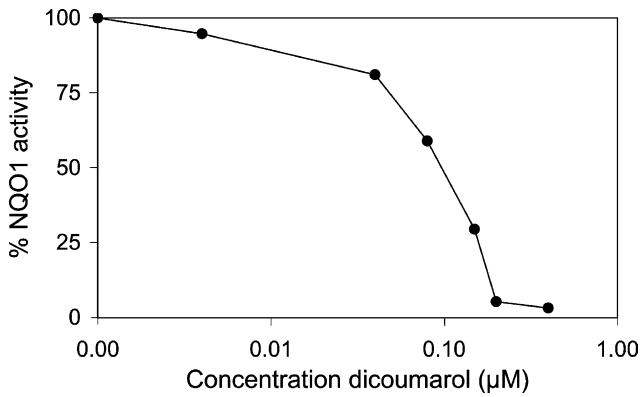

Fig. 5. Effect of increasing dicoumarol concentrations on NQO1 activity in a cytosolic sample of CHO-hNQO1-1 cells. The NADPH concentration in the incubations was 180 μM. Data are presented as percentage of the NQO1 activity without dicoumarol.

value, to the CHO-hNQO1-1 cells does not affect the NQO1-mediated protection of the cells towards menadione, and even at 0.5 μM dicoumarol, where NQO1 activity is expected to be below 25% of its uninhibited activity, the protection is still observed. This suggests that NQO1 activity in the CHO-hNQO1-1 cells has to be significantly reduced, before the NQO1-mediated protection against menadione is abolished. This implies a NQO1 threshold for optimal protection against 40 μM menadione of about 0.2–0.5 μmol DCPIP/min/mg protein; that is, between the level of wt-cells and 25% of the activity in CHO-hNQO1-1 cells.

4. Discussion

In the present study the consequences of increased NQO1 levels for quinone-mediated toxicity were investigated in a series of CHO cells, which were newly generated by stable transfection with human NQO1 cDNA. Together, the CHO parental cell line and the five different CHO-hNQO1 transfectants present a panel of cell lines which are of similar genetic and physiological background, but vary specifically in the level of NQO1 activity, thereby allowing a direct evaluation of the physiological role of the NQO1 enzyme. Clones with higher levels of NQO1 over-expression did not show increased protection against menadione cytotoxicity compared to clones with relatively lower levels of NQO1 over-expression. This lack of correlation between NQO1 activity and protection against quinone cytotoxicity points at an apparently maximal protection at NQO1 levels of 2 μmol DCPIP/min/mg protein measured in the presence of NQO1 activators. The wild-type cell line with an approximately 10-fold lower NQO1 activity is about 2.5 times more sensitive towards menadione cytotoxicity. These results indicate a threshold for NQO1-mediated protection against menadione between 0.2 and 2 μmol DCPIP/min/mg protein. Using the NQO1 inhibitor dicoumarol, the effect of lowering the NQO1 level in the CHO-hNQO1-1 cells on menadione sensitivity was investigated. The results obtained in these studies reveal a sharp threshold of the NQO1 activity which is effective for optimal protection against menadione-induced cell damage. When NQO1 levels fall below this threshold cells are no longer protected. Addition of dicoumarol at five times its *in vitro* EC_{50} value of 0.1 μM does not affect the protection of the hNQO1 cDNA-transfected cells against menadione toxicity (Fig. 4). Direct comparison of dicoumarol inhibition data *in vitro* to the *in vivo* environment of the cytotoxicity assay is hampered by the facts that (i) intracellular NADPH concentration may be lower [25] than the 180 μM NADPH present in the activity measurements, thus facilitating the intracellular competitive inhibition of dicoumarol with respect to NADPH, and also by the fact that (ii) dicoumarol concentrations in the cellular incubation may be lowered due to its binding to cell and medium components [26]. However, these two factors can be expected to counteract each other, indicating that, at 5 μM dicoumarol, which is five times the EC_{50} value (Fig. 4), a significant inhibition of cellular NQO1 can be expected, which is estimated to exceed 75%. That these cells appeared still optimally protected against 40 μM menadione in the presence of such a high concentration of dicoumarol, points at a threshold for optimal protection of about 0.2–0.5 μmol DCPIP/min/mg protein, measured in the presence of NQO1 activators. Using a panel of NQO1-transfected BE cells for anticancer drug activation by NQO1, Winski *et al.* [27] also reported a threshold NQO1 level for anticancer drug activation. This threshold level was measured for a different type of NQO1-mediated effect and in a different panel of cell lines, and amounted to 0.02 μmol DCPIP/min/mg protein. Mikami *et al.* [28] also mentioned a saturation of the activation of mitomycin C by NQO1, although they did not report a value for a possible threshold NQO1 level of maximal tumour drug activation. To our knowledge the present study is the first to report a NQO1 threshold for protection against quinone toxicity.

Table 3
NQO1 activities in different normal tissues from various species as reported in the literature

Species and tissue	Substrate/activators	NQO1 activity range	Units	Reference
Hamster kidney	Menadione/Triton X-100	0.2	μmol cytochrome c/min/mg protein	[34]
Human kidney	Menadione/Triton X-100	0.03	μmol cytochrome c/min/mg protein	[34]
Human lung and breast	Menadione/BSA	0.002–0.3	μmol cytochrome c/min/mg protein	[35]
Human breast, colon, kidney, liver, lung, and stomach	DCPIP/BSA	0.01–0.4	μmol NADPH/min/mg protein	[32]
Human breast, colon, kidney, liver, lung, and stomach	Menadione/BSA	0.002–0.1	μmol NADPH/min/mg protein	[32]
Human lung, colon, liver, and head/neck	DCPIP/no activators	0.002–0.1	μmol DCPIP/min/mg protein	[33]
Mouse liver and kidney	DCPIP/BSA, Tween-20	100–800	μmol DCPIP/min/mg protein	[13]
Mouse liver, kidney and heart	Thymoquinone/BSA	10–20	μmol NADPH/min/g tissue	[37]
Mouse liver and kidney	DCPIP/BSA, Tween-20	0.07–0.7	μmol DCPIP/min/mg protein	[38]
Rat liver and intestine	DCPIP/BSA, Tween-20	0.03–0.08	μmol DCPIP/min/mg protein	[30]
Rat liver and kidney	Menadione/Triton X-100	50–90	μmol cytochrome c/min/mg protein	[36]
Rat liver, lung, colon and kidney	DCPIP/BSA	0.04–0.2	μmol NADPH/min/mg protein	[32]
Rat liver, lung, colon and kidney	Menadione/BSA	0.03–0.4	μmol NADPH/min/mg protein	[32]

The reason why an increase in NQO1 beyond a threshold level has no further effect on protection against menadione toxicity may be related to full menadione conversion, and thus full protection, when an already optimal level of NQO1 activity is available.

In order to compare the protective NQO1 levels observed in the CHO-hNQO1 cell panel to levels relevant *in vivo* in mammalian cells and tissues, Table 3 summarises levels of NQO1 reported in the literature for different tissues in different species. These values show broad variation in NQO1 activities between the different studies. Comparison of these values to the threshold value of 0.2–0.5 μmol/min/mg protein found in the present study reveals that this threshold may be within the order of the NQO1 activity level found in several rodent and human tissues. Our results indicate that further induction above the threshold is futile, which would suggest that inducing NQO1 by dietary intake does not necessarily increase the protective effects of NQO1. Threshold levels for optimal protection by NQO1 against menadione cytotoxicity may differ between species and tissues, and may as well depend on the cytotoxic (or growth inhibitory) potency of the model compound studied and/or on the tissue concentration of the compound under investigation. Therefore, further studies seem to be required to better define the consequences of NQO1 thresholds for optimal protection against quinone cytotoxicity.

These observations are especially of interest because several literature reports [19,29] correlate the induction of NQO1 activity by cruciferous vegetables or antioxidants to a higher protection against quinone toxicity. Induction factors of 3–4 for NQO1 upon consumption of cruciferous vegetables have been reported [30]. These induction factors are often correlated to the protective effects of cruciferous vegetables in the process of carcinogenesis [31]. Regarding our results one can envision that it depends on the baseline NQO1 level, and also the threshold of NQO1 for optimal protection in the tissue and species under study, whether increased NQO1 activity would indeed result in an enhanced protective effect.

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