Biochemical Pharmacology, Vol. 57, pp. 27–37, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00288-3

Quinone Toxicity in DT-Diaphorase-Efficient and -Deficient Colon Carcinoma Cell Lines

Jurgen M. Karczewski, Janny G. P. Peters and Jan Noordhoek*
Department of Toxicology, University of Nijmegen, NL-6500 HB Nijmegen, The Netherlands

ABSTRACT. The human colon carcinoma cell lines Caco-2 and HT-29 were exposed to three structurally related naphthoquinones. Menadione (MEN), 1,4-naphthoquinone (NQ), and 2,3-dimethoxy-1,4-naphthoquinone (DIM) redoxcycle at similar rates, NQ is a stronger arylator than MEN, and DIM does not arylate thiols. The Caco-2 cell line was particularly vulnerable to NQ and MEN and displayed moderate toxic effects of DIM. The HT-29 cell line was only vulnerable to NQ and MEN after inhibition of DT-diaphorase (DTD) with dicoumarol, whereas dicoumarol did not affect the toxicity of quinones to Caco-2 cells. DTD activity in the HT-29 and Caco-2 cell lines, as estimated by the dicoumarol-sensitive reduction of 2,6-dichlorophenolindophenol, was 393.7 ± 46.9 and 6.4 ± 2.2 nmol NADPH.min^{-1}.mg protein^{-1}, respectively. MEN depleted glutathione to a small extent in the HT-29 cell line, but a rapid depletion similar to Caco-2 cells was achieved when dicoumarol was added. The data demonstrated that the DTD-deficient Caco-2 cell line was more vulnerable to arylating or redoxcycling quinones than DTD-expressing cell lines. Exposure of the Caco-2 cell line to quinones produced a rapid rise in protein disulphides and oxidised glutathione. In contrast to NQ and DIM, no intracellular GSSG was observed with MEN. The relatively higher levels of ATP in MEN-exposed cells may account for the efficient extrusion of intracellular GSSG. The reductive potential of the cell as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction was only increased by MEN and not with NQ and DIM. We conclude that arylation is a major contributing factor in the toxicity of quinones. For this reason, NQ was the most toxic quinone, followed by MEN, and the pure redoxcycler DIM elicited modest toxicity in Caco-2 cells. BIOCHEM PHARMACOL **57**;1:27–37, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. colon carcinoma cell line; Caco-2; oxidative stress; menadione; glutathione; DT-diaphorase

Quinones such as streptonigrin, mitomycine, and diaziquone are widely used in the treatment of gastrointestinal malignancies. Drug resistance, either intrinsic or acquired, severely limits the effectiveness of chemotherapy. Research has been focused on increased detoxification by glutathione and glutathione-dependent enzymes such as glutathione *S*-transferases [1–3] and the expression of mdr-1/P-glycoprotein [4]. Antitumour quinones require enzymatic reduction prior to eliciting their antitumour activity. One of the major reducing enzymes is DTD† and the level of this enzyme varies between different tumour tissues [5, 6]. The majority of DTD activity in human tissues is the product of the dioxine-inducible gene *NQO$_1$* [7]. DTD exclusively catalyses the 2-electron reduction of quinones which is necessary for the activation of streptonigrin, mitomycine, and the more novel drug EO9 [5, 8, 9]. After possible rearrangement reactions, the hydroquinone may become a reactive alkylating agent or autoxidise, leading to reactive oxygen species and toxicity.

MEN is a vitamin K_3 analogue and has been used in antitumour therapy [8]. MEN and other naphthoquinones can undergo a one- and two-electron reduction which gives rise to the semiquinone and the hydroquinone, respectively. Both can reduce molecular oxygen to the superoxide anion radical, thereby regenerating the parent quinone [10].

Besides redoxcycling, quinones can arylate to nucleophilic moieties of the cell but this process depends on the substituents of the molecule [11, 12]. In this study, we used the related quinones NQ, MEN, DIM which were all able to redox cycle and cause oxidative stress and thiol oxidation in hepatocytes [11–13]. However, the quinones differ in their ability to arylate. NQ is a strong arylator compared to MEN while DIM is not capable of arylation due to its substituents at the C_2 and C_3. In contrast to streptonigrin, mitomycine, and EO9, the hydroquinone of naphthoquinones is more stable than the semiquinone and is a target for detoxification reactions such as glucuronidation or sulphatation [10, 14, 15]. For these reasons, the DTD-mediated two-electron reduction is generally considered to be a detoxification reaction.

* Corresponding author: Dr. Jurgen Karczewski, Department of Toxicology, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands. Tel. 31 24 361366; FAX 31 24 3541802; E-mail: J.Karczewski@toxi.kun.nl

† *Abbreviations:* DTD, DT-diaphorase; MEN, menadione; NQ, 1,4-naphthoquinone; DIM, 2,3-dimethoxy-1,4-naphthoquinone; RSSG, protein-bound glutathione; DTT, dithiothreitol; mBBr, monobromobimane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; and TCA, trichloric acid.

Received 24 October 1997; accepted 17 June 1998.

MEN has a wide range of effects on cells. Exposure of hepatocytes to MEN perturbs the cytoskeleton of the cell, which can be recognised as membrane blebbing [10]. The bleb formation is associated with an altered thiol and Ca^{2+} homeostasis [10, 16–20]. The semiquinone of MEN and the reactive oxygen species generated by MEN were found to interact with the DNA of hepatocytes [20–23], Chinese hamster ovary cells [24] and leukemic K562 cells [25]. Further, quinones interfere with the energy production of the cell. Redegeld *et al.* [26] showed a loss of ATP preceding cell death in MEN-treated hepatocytes.

Most studies on naphthoquinone toxicity have been performed in rat hepatocytes and nothing is known about the toxic response of human intestinal tissue. In this report, we show that two human colon carcinoma cell lines differ in sensitivity to naphthoquinones due to differences in expression of DTD. The DTD-deficient Caco-2 cell line proved to be vulnerable to all quinones in contrast to the DTD-efficient HT-29 cell line, in which toxicity and glutathione depletion only occurred after inhibition of DTD with dicoumarol. A comparison between glutathione status, ATP, protein thiol, and MTT reduction in Caco-2 cells was made to study the behaviour of arylation and/or redox cycling quinones. We conclude that arylation is a major contributing factor in the toxicity of these quinones. For this reason, NQ was the most toxic quinone, followed by MEN, and the pure redoxcycler DIM was only modestly toxic to Caco-2 cells.

MATERIALS AND METHODS

Chemicals

NQ and menadione were purchased from Aldrich. DIM was prepared according to [13]. The identity was checked with mass spectrometry. mBBr was from Calbiochem. Other chemicals and enzymes were obtained from Sigma. All chemicals were of analytical grade. All cell culture materials were purchased from ICN, except for flasks and tissue culture plates (Greiner) and gentamicine (Centrafarm).

Cell Culture and Incubation

The Caco-2 and HT-29 cell lines were kindly provided by Dr. W. Peters (Dept. of Gastroenterology, University Hospital Nijmegen, Nijmegen, the Netherlands). Caco-2 and HT-29 cells were maintained at 37° in Dulbecco's modified Eagle's medium, containing 10% (v/v) fetal bovine serum, 10 mM of HEPES, 1% nonessential amino acids, 5 mM of L-glutamine, and 5 μg/mL of gentamicine in an atmosphere containing 5% CO_2. Cells (1×10^5) were seeded in 24-well culture plates and became confluent after 4 days. Cell cultures were supplied with medium every second day and used on day 7 of culture. Incubations with menadione were performed in 24-well tissue plates in an atmosphere as described above. Cells were washed with Krebs–Henseleit buffer (KH, formulation in mM: 1.2 $MgSO_4.7H_2O$, 2.5 $CaCl_2.2H_2O$, 4.7 KCl, 94 NaCl, 11.6 D-glucose, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 5 L-glutamine, pH 7.4) and supplied with 0.5 mL of KH. MEN, NQ, and DIM were dissolved in dimethylsulfoxide and control cells were treated with equal volumes of the solvent (0.3% of final volume).

Neutral Red Uptake Assay

Viability was determined according to [27]. Briefly, after incubation the monolayer was washed with KH. Fresh complete Dulbecco's modified Eagle's medium with 50 μg/mL of neutral red was added to the wells. After 30 min of incubation at 37°, cells were washed rapidly with 40% formaldehyde-10% $CaCl_2$ to remove extraneously adhering, unincorporated dye. Neutral red was extracted with 500 μL of 1% acetic acid-50% ethanol and 150 μL was transferred to a 96-well titreplate. The plate was measured on a Thermomax microplate reader (Molecular Devices) equipped with a 550 nm filter. The readings were expressed as percentages of the nonexposed cells.

DTD Activity

Activity of DTD was determined according to [28]. Briefly, 100 μL of cell homogenate was added to 800 μL of PBS pH 7.4 containing 50 μM 2,6-dichlorophenolindophenol, 0.23 mg of BSA/mL and 0.01% Tween 20. The reaction was started by adding 100 μL of 2 mM NADPH. The reaction rate was monitored for 2 min at 37° in a Perkin Elmer Lambda 15 UV/VIS spectrophotometer (Perkin Elmer) by measuring the decrease in absorbance at 600 nm due to reduction of the substrate and of NADPH at 340 nm. DTD activity was determined in the absence and presence of 50 μM dicoumarol, a highly potent inhibitor of DTD. The cell homogenates were incubated with dicoumarol for 2 min prior to the enzyme assay.

Cellular ATP Content

Cells were treated with 300 μL of 5% (v/v) perchloric acid and neutralised with 700 μL of 0.8 M potassium phosphate buffer. Insoluble material was removed by centrifugation (5 min, 13,000 *g*) and stored at −80° until use. ATP was separated using a Spectra-Physics HPLC system consisting of an SP8800 ternary pump, an SP8875 autosampler, an SP4600 integrator, and a Merck 100 RP-18 column (LiChroCART 125-4 Lichrospher). Twenty μL of supernatant was injected on the column at 40° and separated at a flow of 1 mL/min. The elution buffer was a 0.1 M potassium phosphate buffer, pH 6.0 that was filtered through a 0.45 mm filter before use. ATP was detected by a Kratos Spectroflow 773 UV detector at 260 nm. ATP content was expressed as nmol/mg protein.

GSH and Extracellular (GSH + GSSG)

GSH was determined by HPLC after derivatisation with mBBr as described in [29]. After incubation, cells were washed with KH and 100 μL of aqua pure was added to the well. GSH was derivatised by adding 100 μL of 2 mBBr dissolved in 50 mM *N*-ethylmorpholine and incubating this mixture for 5 min in the dark. Protein was denatured by inclusion of 25 μL of 40% TCA and removed by centrifugation (5 min, 13,000 *g*). For determination of RSSG, 500 μL of cell material was precipitated with 25 μL of 40% TCA and separated by centrifugation. The pellet was washed 3 times with 500 μL of 5% TCA and resuspended in 100 μL of 1% SDS in PBS. After neutralisation with 10 μL of saturated $NaHCO_3$, mixed disulphides were reduced with 5 μL of 25 mM DTT. After 45 min incubation, GSH was labelled with 100 μL of 5 mM mBBr and measured as described. In order to measure total GSH (GSH + GSSG + RSSG), 100 μL of cell material was treated with 10 μL of 20% TCA to inactivate GSH-metabolising enzymes. After buffering with 100 μL of 1 M phosphate, 100 μL of sample was reduced with 10 μL of 25 mM DTT and labelled with 100 μL of 10 mM mBBr. GSSG was calculated with the formula: GSSG = TGSH − GSH − RSSG. For the determination of extracellular and total extracellular glutathione (GSH + GSSG), 250 μL aliquots of KH buffer were taken at the appropriate time from the culture plate. For the estimation of total extracellular GSH, 100 μL of KH was reduced with 10 μL of 25 mM DTT for 45 minutes at room temperature. The mixture was labelled with 100 μL of 5 mM mBBr and deproteinised with 10 μL of 40% TCA.

Twenty μL samples of glutathione derivatives were separated by HPLC using a Merck 100 RP-18 column at 40° and a flow rate of 1 mL/min. Buffer A was 128 mL of methanol and 2.5 mL of acetic acid diluted to 1 L with aqua pure, adjusted to pH 3.9 with 5 N of NaOH. Buffer B was 900 mL of methanol and 2.5 mL of acetic acid, diluted to 1 L with aqua pure. A linear gradient from 0% B at 0 min, 50% B at 8 min to 0% B at 17 min was used to elute the glutathione derivatives that were detected using a Shimadzu RF-530 fluorescence detector at λ_{ex} = 385 nm and λ_{em} = 480 nm. Glutathione content was expressed as nmole/mg protein.

Protein thiols

Protein thiols were determined using Ellman's reagent [30] according to [31] and modified by [32]. Briefly, 500 μL of cell lysate was precipitated with 100 μL of 3.5% $HClO_4$ and pelleted (5 min, 500 *g*). The pellet was washed twice with 500 μL of 5% TCA and resuspended in 1 mL of PBS/EDTA (5 mM)/SDS (0.5% w/v). Ten μL of 1 N NaOH was added to clarify the mixture. Sample or glutathione standard (150 μL) was incubated with 20 μL of Ellman's reagent for 30 min at 37° and the optical density of the mixture was measured at 405 nm. A correction for the background was performed by inclusion of 30 μL of 100 nM NEM. The data was expressed as nmol SH/mg protein.

MTT Reduction Assay

After incubations with quinones, cells were washed twice with KH and 500 μL of PBS containing 1 mg/mL of MTT was added to each well. After 1 hr incubation at 37° and 5% CO_2, cells were washed again with KH and the formed formazan was extracted from the cells with 500 μL of isopropanol. The optical density of the extract was determined in a microtitre plate at 550 nm. The data were expressed as percentages of the values of the controls at t = 0 hr.

Cellular Protein

Cellular protein was quantified with the Bradford method [33] using BSA as a standard.

Statistical Analysis

Results are expressed as means ± SD of three to six independent experiments. Statistical significance between two groups was determined by means of an unpaired Student's *t*-test. Statistical differences between groups were determined by means of a one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. A probability of $P \leq 0.05$ was considered significant.

RESULTS

Caco-2 and HT-29 colon carcinoma cells reached confluence after 5–6 days and were used on day 7 and 8 of culture. No significant differences in neutral red uptake, intracellular ATP and glutathione levels, MTT reduction, and DTD activity were observed between cells on day 7 and day 8 of culture. Caco-2 and HT-29 cells were exposed to 50 μM of NQ, 100 μM of MEN, and 300 μM of DIM for 2 and 4 hr with or without 100 μM of dicoumarol, a potent inhibitor of DTD. Dicoumarol alone had no effect on Caco-2 cells, but the HT-29 cell line displayed a higher neutral red uptake compared to the control cells (Fig. 1). NQ caused an sharp drop in viability of Caco-2 cells, while this effect was not observed with HT-29 cells. Dicoumarol had no effect on the viability of NQ-exposed Caco-2 cells but had a deteriorating effect on the viability of NQ-treated HT-29 cells. Menadione displayed a similar effect on the viability of both cell lines. The effect of the inhibitor dicoumarol was in accordance with the effect observed with NQ. DIM, which is unable to arylate due to the substituents at the 3′ and 4′ position, was not toxic to HT-29 cells and moderately toxic to Caco-2 cells. Again, no effect of dicoumarol was observed in combination with DIM in the Caco-2 cell line. Dicoumarol combined with DIM resulted in increased

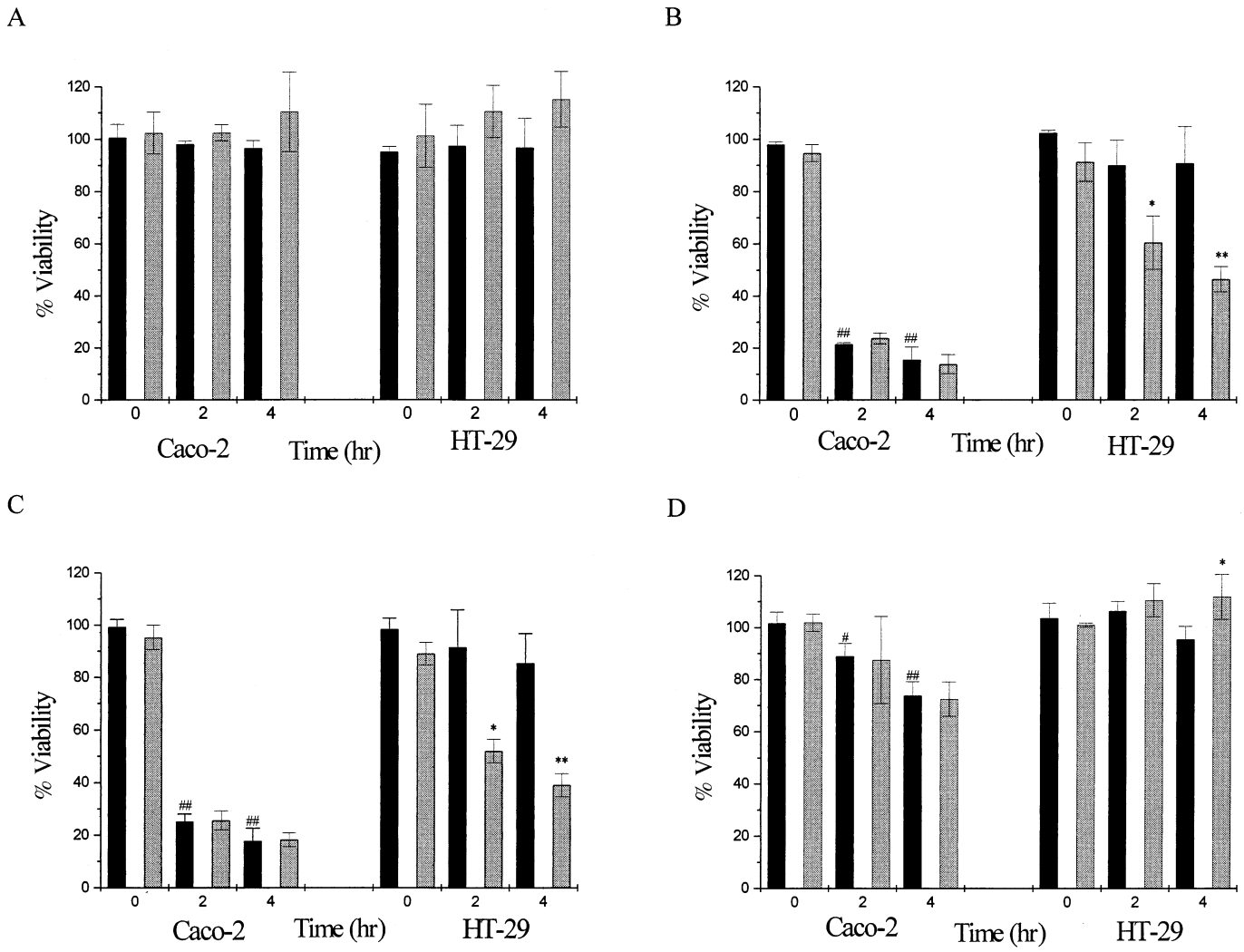

FIG. 1. Effect of quinones on the viability of colon carcinoma cells. Caco-2 and HT-29 cells were untreated (A) or exposed to 50 μM of NQ (B), 100 μM of MEN (C), or 300 μM of DIM (D) without (▨) or with 100 μM of dicoumarol (▩). Shown are the means ± SD of three independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$, effect of dicoumarol on quinone-exposed cells compared to treatment with quinone only; #: $P \leq 0.05$, ##: $P \leq 0.01$, effect of quinone on cells compared to untreated cells).

neutral red uptake compared to DIM-exposed HT-29 cells. Exposure to dicoumarol alone did not have an effect on the viability of cells (data not shown).

Since dicoumarol had no effect on the toxic action of NQ or MEN in Caco-2 cells, the activity of DTD was examined in both cell lines using 2,6-dichlorophenolindophenol as substrate. The HT-29 cell line was reported to contain a functional gene and normal expression of DTD [5, 6]. Table 1 shows that the greater part of the NADPH-oxidising activity in the HT-29 cell line was dicoumarol-sensitive. This is in contrast to the Caco-2 cell line, where very low levels of 2,6-dichlorophenolindophenol-reducing activity were observed. The reducing activity in the Caco-2 cell line was for the most part not inhibited by dicoumarol, which is a strong indication that DTD is not functional in the Caco-2 cell line.

Since the cell lines differed in dicoumarol-sensitive DTD activity, the effect of dicoumarol on menadione-induced glutathione depletion was determined in the Caco-2 and HT-29 cell lines. Figure 2A shows a gradual decline in levels of reduced GSH in the HT-29 cell line, probably due to intracellular consuming processes. Some of these processes seemed to be dicoumarol-sensitive, since dicoumarol-treated cells contained more reduced GSH than did control cells. Although MEN was not able to induce a decrease in viability, the quinone clearly decreased the intracellular levels of glutathione. This decrease was strongly enhanced

TABLE 1. Comparison of DTD activity between colon carcinoma cell lines

Cell line	Without dicoumarol	With dicoumarol	Dicoumarol-inhibitable activity
HT-29	445.7 ± 41.7	52.0 ± 5.8	393.7 ± 46.9
Caco-2	29.9 ± 1.1**	23.5 ± 1.9**	6.4 ± 2.2**

DTD activity was expressed in the oxidation of nmol NADPH.min^{-1}.mg protein^{-1}. Experiments were performed in quadruplicate.

** $P \leq 0.01$ when the Caco-2 cell line was compared to the HT-29 cell line.

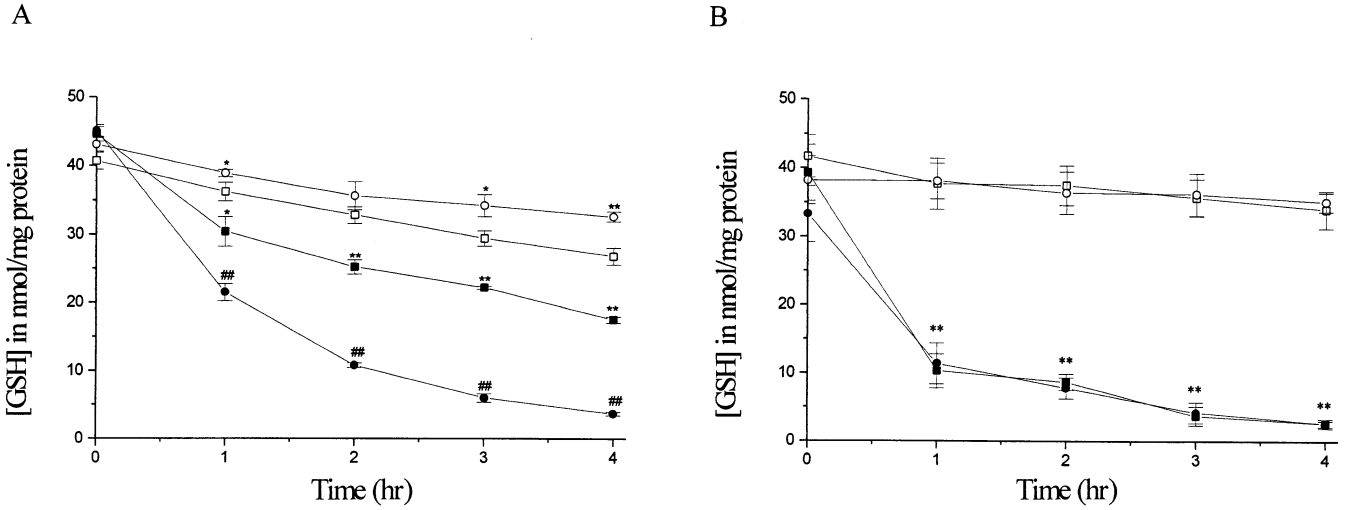

FIG. 2. Effect of menadione and dicoumarol on the glutathione content of HT-29 (A) and Caco-2 cells (B). HT-29 and Caco-2 cells were untreated (□) or exposed to 100 μM of dicoumarol (○), 100 μM of MEN (■), or to a combination of MEN and dicoumarol (●). Glutathione was extracted and measured as described in Materials and Methods. Shown are the means ± SD of three independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to untreated cells; ##: $P \leq 0.01$: compared to menadione-exposed cells).

in the presence of dicoumarol. Figure 2B shows that MEN rapidly depleted the levels of reduced GSH in the Caco-2 cell line. Dicoumarol did not increase this depletion nor affect the levels of GSH in control cells.

HT-29 cells seem to metabolise quinones primarily via two-electron reduction pathways without being really affected. In the Caco-2 cell line, quinones enter redoxcycling primarily via one-electron reduction. Based on the results achieved with the Caco-2 cell line, it was concluded that quinones elicit toxicity via the parent quinone and its corresponding semiquinone. To investigate whether quinones are toxic to cells via arylation or redoxcycling, the distribution of glutathione was determined in NQ-, MEN-, and DIM-treated Caco-2 cells.

Control Caco-2 cells display high levels of reduced glutathione and small amounts of intracellular oxidised free glutathione (Fig. 3A). All quinones affected the distribution of glutathione, but in different manners (Fig. 3B, C and D). Exposure to NQ and MEN rapidly depleted cells of reduced glutathione, while DIM caused a more gradual decline. Formation of mixed disulphides in Caco-2 cells occurred with all three quinones. All quinones in this study induced the formation of free oxidised glutathione. With MEN, only a small amount of free oxidised glutathione was found within the cell at t = 1 hr, the greater part of GSSG being retrieved in the incubation buffer. In contrast to MEN, free intracellular GSSG was found in cells exposed to NQ and DIM. No extracellular glutathione was found with DIM, but large amounts of extracellular GSSG were observed in incubations with NQ and MEN. In these experiments, only oxidised glutathione was found in the incubation buffer of quinone-treated cells. GSSG appeared at t = 1 hr in the incubation buffer with MEN and somewhat later with NQ. Both NQ and MEN caused an initial decrease in the total amount of glutathione. Excessive formation of intracellular GSSG is accompanied by formation of protein-associated disulphides using all three quinones (Fig. 3). Further, arylating quinones like NQ and MEN can bind directly to cellular protein thiols. Both mechanisms lead to depletion of cellular thiols and may have deteriorating effects on the viability of cells. Figure 4A shows that both NQ and MEN rapidly depleted cellular protein thiols and that DIM had only a significant effect on protein thiols after 4 hr of exposure. Figure 4B shows that protein thiols were arylated at a similar rate by the NQ and MEN.

The viability of cells is closely related to the cellular content of energy-rich components like ATP. Enzymes of energy-producing pathways such as glycolysis and the TCA cycle contain crucial thiols and are particularly vulnerable to the actions of quinones [26, 34]. To investigate the involvement of ATP in the toxicity of the three quinones, ATP was measured throughout the incubation. Figure 6 shows that NQ caused a fast drop in cellular ATP with as much as 16% left after 4 hr. MEN showed a more gradual loss of ATP with the depletion after 4 hr being comparable with NQ. The moderately toxic quinone DIM showed a small depletion of ATP after 4 hr of incubation.

Although MEN showed a similar depletion of protein thiol to NQ (Fig. 4), MEN-treated cells contained more ATP than the NQ-exposed cells. The MTT assay is associated with the activity of mitochondrial enzyme succinate dehydrogenase and was used to estimate the activity of mitochondria during exposure to quinones. As expected, control cells displayed an unaltered reduction of MTT during incubation. Exposure to DIM had no effect on the cellular reduction of MTT when compared to controls. NQ, however, caused a sharp drop in MTT reduction after 2 hr of incubation. Interestingly, incubation with MEN resulted in an initial rise in MTT reduction and then a decline to 50% of the controls.

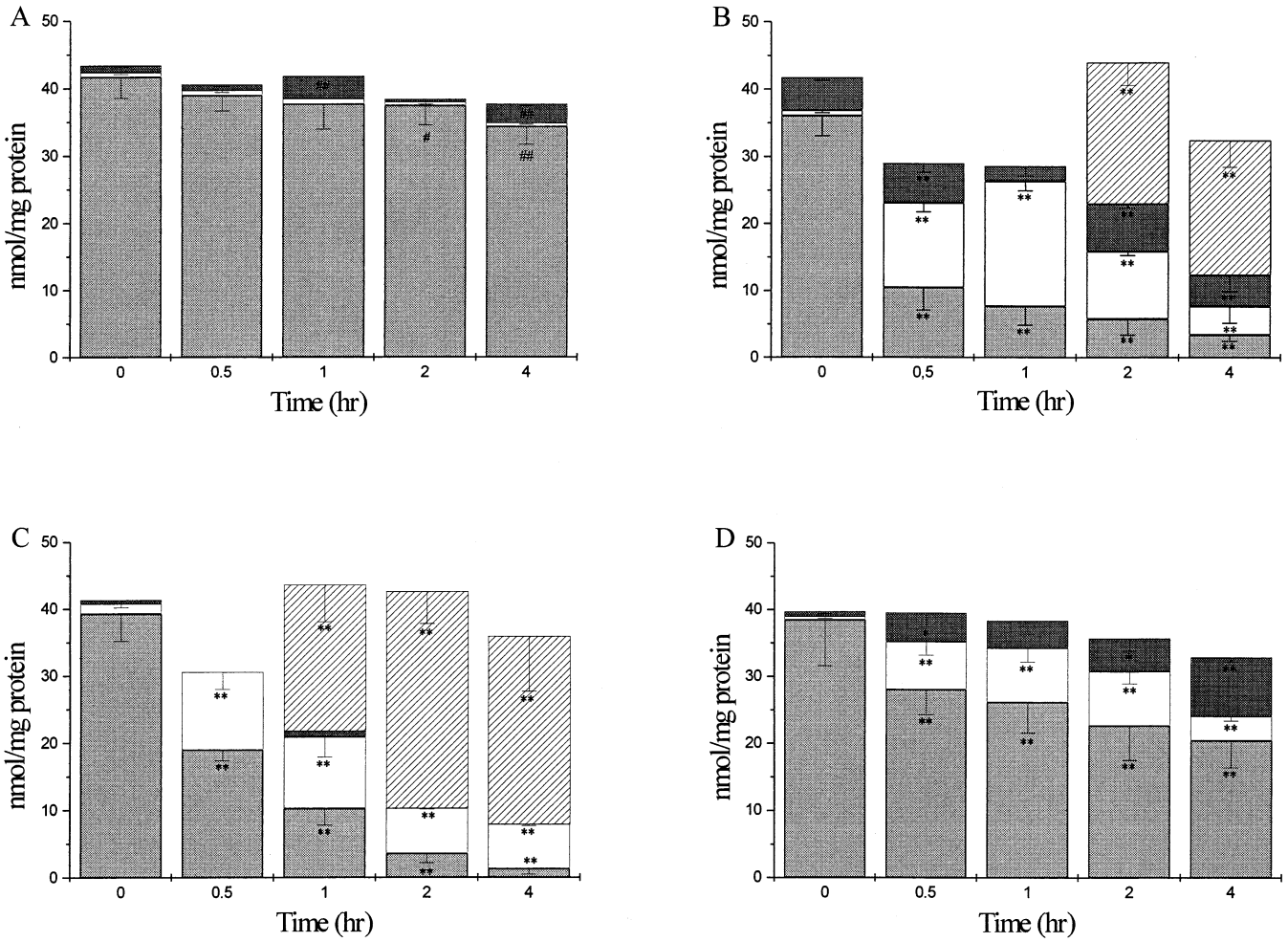

FIG. 3. Effect of quinones on the distribution of glutathione in the Caco-2 cell line. Caco-2 cells were exposed to KH buffer alone or exposed to KH containing 50 μM of NQ (B), 100 μM of MEN (C), or 300 μM of DIM (D). Depicted are reduced glutathione (▨), protein-bound glutathione (□), oxidised glutathione (▩), and extracellular oxidised glutathione (▨). Shown are the means ± SD of four independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$, effect of exposure to quinones on cells compared to control cells; #: $P \leq 0.05$, ##: $P \leq 0.01$, effect of incubation on control cells).

DISCUSSION

The behaviour of quinones with regard to their toxicity is strongly related to the biochemical constitution of the exposed cell and the chemical structure of the quinone. Naphthoquinones such as MEN have been studied extensively for their ability to induce oxidative stress in hepatocytes [10, 26, 32], cardiomyocytes [35], and isolated intestinal cells [36]. Quinones exert their toxic effect by redoxcycling and arylation. Redoxcycling involves one-electron reduction pathways catalysed by microsomal NADPH cytochrome P450 reductase, microsomal NADH cytochrome b_5 reductase, mitochondrial NADH ubiquinone oxidoreductase and gives rise to a semiquinone. Cytosolic DTD reduces the quinone to a hydroquinone via a two-electron reduction. Superoxide anion radical and singlet oxygen are formed during the oxidation process of the hydroquinone and semiquinone [8]. The arylation reaction between quinones and nucleophiles such as GSH is a 1,4-reductive addition of the Michael type which reduces the quinone to its corresponding glutathionyl hydroquinone [37].

The quinones MEN, NQ, and DIM were used in this study because of their differences in eliciting toxicity. NQ and MEN are both capable of arylation, although MEN is a lesser arylator than NQ due to its methyl substituent in the quinoid ring [38]. Because of this methyl substituent, MEN ($E(Q/Q^{\cdot -} = -203$ mV) has a lower redox potential than NQ ($E(Q/Q^{\cdot -} = -140$ mV) and is for this reason a better redoxcycler [8]. The one-electron reduction potential of DIM was estimated at −183 mV [39], signifying that it has similar redoxcycling capabilities as menadione as confirmed in studies using hepatocytes [13].

Two human colon carcinoma cell lines were used in this comparative study in which the three quinones caused different effects on viability. None of the quinones had any effect on the viability of HT-29 cells, whereas MEN and especially NQ caused a rapid decrease in viability of the Caco-2 cell line. In the HT-29 cell line, the two quinones

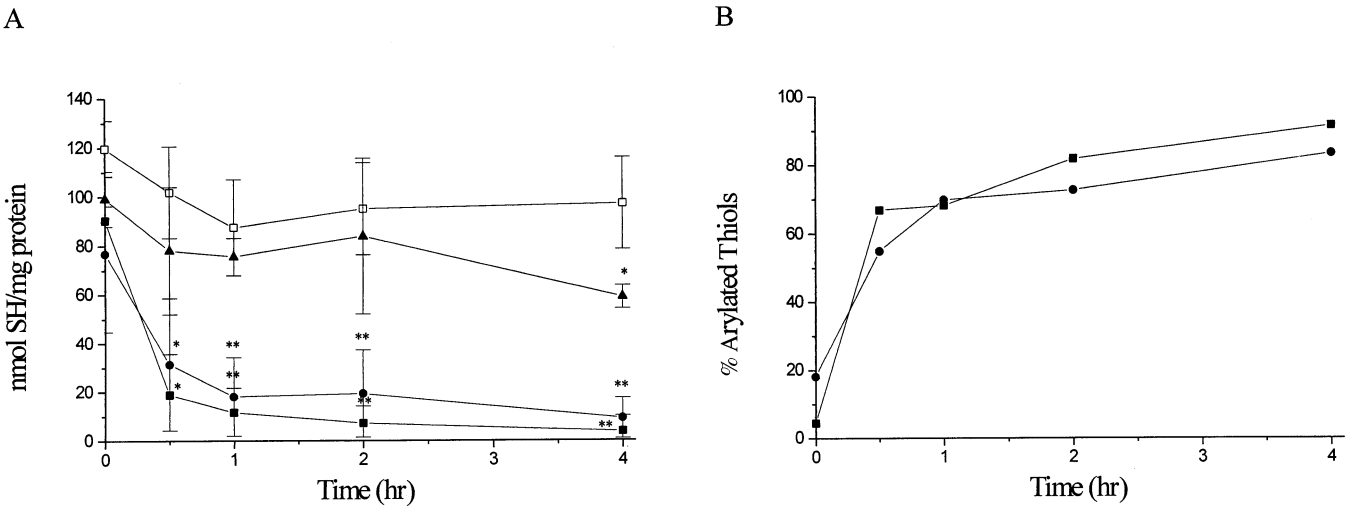

FIG. 4. Effect of quinones on protein thiols of Caco-2 cells. Caco-2 cells were untreated (□) or exposed to 50 μM of NQ (■), 100 μM of MEN (●), or 300 μM of DIM (▲). Graph A shows the actual cellular protein thiols that were determined as described in Materials and Methods. Shown are the means ± SD of three independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to untreated cells). The percentages of alkylated thiols presented in graph B were calculated by subtracting the nmol of protein-associated glutathione (Fig. 3) from the data in graph A.

were only toxic when DTD was inhibited by dicoumarol, which indicates a difference in DTD activity between the two cell lines. Naphthoquinones are a substrate for human DTD. A comparative study between human and rat hepatoma cells showed no difference in activity between species [40]. Another study compared the MEN-reducing activity of purified rat and human DTD. The DTD was purified from rat liver cytosol and from *Escherichia coli* in which rat and human lung tumour diaphorase complementary DNA was expressed. The enzyme kinetics of MEN reduction were similar for the three enzyme preparations [41]. Measurement of DTD confirmed a very low activity in Caco-2 cells in comparison with HT-29 cells. Loss of DTD activity has been documented in several studies with tumour material. A study with 10 human colon carcinoma cell lines shows that seven lines expressed lower or no DTD activity compared to the HT-29 cell line [6]. HT-29 cells seem to metabolise quinones primarily via two-electron reduction pathways which convert quinones to hydroquinones. Superoxide dismutase prevents the hydroquinone from autoxidising and thus stabilises the hydroquinone. [39, 42–44]. The manganese-containing form of superoxide dismutase was detected in HT-29 cells [45] while CuZn superoxide dismutase was demonstrated in the Caco-2 cell line [46]. The hydroquinone is excreted from the cell after glucuronidation or sulphation reactions [14, 15]. Caco-2 cells appeared to be deprived of DTD activity and quinones redoxcycle only via one-electron reduction. The absence or inhibition of DTD activity had a detrimental effect on the

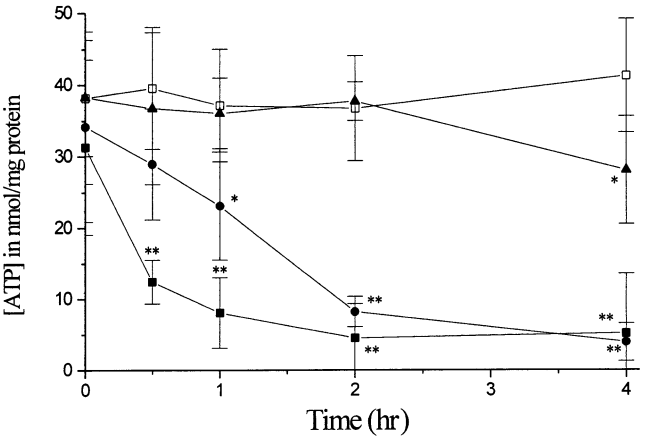

FIG. 5. Effect of quinones on intracellular ATP content of Caco-2 cells. Caco-2 cells were untreated (□) or exposed to 50 μM of NQ (■), 100 μM of MEN (●), or 300 μM of DIM (▲). ATP was extracted and measured as described in Materials and Methods. Shown are the means ± SD of four independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to untreated cells).

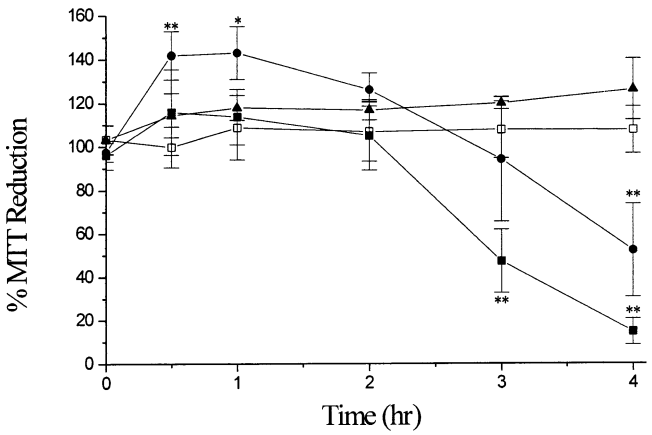

FIG. 6. Effect of quinones on cellular MTT reduction of Caco-2 cells. Caco-2 cells were untreated (□) or exposed to 50 μM of NQ (■), 100 μM of MEN (●), or 300 μM of DIM (▲). Shown are the means ± SD of three independent experiments (*: $P \leq 0.05$, **: $P \leq 0.01$ compared to untreated cells).

cellular GSH content of quinone-exposed cells. MEN caused a moderate depletion of glutathione in the absence of dicoumarol, but induced an intensive exhaustion in combination with dicoumarol in HT-29 cells. Since a hydroquinone does not conjugate to GSH or arylate to protein thiols [44], a large amount of MEN is not able to form conjugates with GSH in HT-29 cells. The inactivation of DTD allows MEN to enter the one-electron reduction pathways with consequent consumption of GSH by both arylation and redoxcycling as occurred in Caco-2 cells. Some information does exist on the one-electron reduction pathway capabilities of both cell lines. The HT-29 cell line has a cytochrome P450 reductase activity of 3 nmol/min/mg [5, 47]. Schiemdlin-Ren *et al.* [48] demonstrated the presence of cytochrome P450 reductase with immunoblots of Caco-2 homogenates. Rossi *et al.* [49] found a cytochrome P450 reductase activity of 5.01 ± 0.61 nmol/min/10^6 cells in the Caco-2 cell line. Based on our protein measurements (1.86 ± 0.05 mg/10^6 Caco-2 cells), the cell lines are expected to have similar cytochrome P450 reductase activities. Some caution must be exercised concerning the use of dicoumarol, since this inhibitor was found to inhibit several other enzymes (e.g. glutathione transferase) and has a disturbing effect on mitochondrial oxidative phosphorylation [40]. The Caco-2 cell line was reported to have an activity 0.6–5.4 μmol/min/mg [50]. The transferase activity of HT-29 cells is 0.3 nmol/min/mg [47], 100 nmol/min/mg [5, 51], and 200 nmol/min/mg [52–54]. The glutathione levels in dicoumarol-treated HT-29 cells were higher than in control cells, and this may indicate the inhibitory effect of dicoumarol on the glutathione transferases, although this effect was not visible in the Caco-2 cell line. The dicoumarol-induced increased depletion of GSH in MEN-treated HT-29 cells cannot be explained by this inhibiting effect, since dicoumarol did not affect the rate of MEN-induced GSH depletion in Caco-2 cells.

The toxicity of the different arylation and redoxcycling quinones was studied in more detail with the Caco-2 cell line. Formation of mixed disulphides and intra- and extracellular GSSG is an indication of oxidative stress. Exposure to the three quinones resulted in different patterns of glutathione distribution. NQ and MEN rapidly depleted the reduced GSH pool while DIM showed a more gradual decrease, indicating that arylation is a major factor in the quinone-mediated decrease of GSH. During the first period of exposure, a decrease in the total amount of glutathione with a subsequent restoration to its initial levels occurred only with the arylating quinones. Since the conjugation of GSH to quinones is irreversible, the supplement of total GSH must be the result of *de novo* synthesis of GSH. In all cases, protein-associated glutathione appeared within 30 min of exposure. Similar observations were made with menadione- and *t*-butyl hydroperoxide-treated hepatocytes [55, 56]. Birge *et al.* [57] suggested that thiolation of protein thiols is a protective mechanism, since the same proteins arylated by paracetamol are thiolated by glutathione during paracetanol-induced oxidative stress. However, it is more likely that the thiolation of proteins occurred as the result of increased GSSG formation, since with the nonarylating quinone DIM similar high levels of protein disulphides were observed. Extracellular GSSG was found after 1 hr of MEN exposure, but no intracellular GSSG was observed; this is in contrast to NQ where relatively high levels of intracellular GSSG were found. The translocation of GSSG in hepatocytes was found to be ATP-dependent [58, 59] and this transport could be blocked with the thiol-alkylating agent NEM, indicating that the translocator possesses thiol groups essential for its activity [60]. NQ did not irreversibly block GSSG export but only delayed the process. NQ induced a more rapid depletion of cellular ATP than MEN, and the lack of ATP after NQ treatment may contribute to the stronger accumulation of intracellular GSSG. Another explanation comes from the finding that glutathione-S-conjugates are responsible for the accumulation of GSSG by inhibiting GSSG reductase [15, 61]. Further, MEN has been shown to directly inhibit glutathione reductase [55]. Since NQ is stronger arylator than MEN, it is conceivable that direct and indirect inhibition of glutathione reductase occurred earlier with NQ. Although MEN and DIM have similar redoxcycling capabilities, the higher rate of GSSG formation and stronger toxicity observed with MEN may be ascribed to the MEN-induced inhibition of glutathione reductase.

NQ and MEN rapidly depleted the cellular ATP content of Caco-2 cells. Quinones and activated oxygen species are able to interfere with cellular energy-producing enzymes. Hyslop *et al.* [34] showed that hydrogen peroxide inactivates both the glycolytic and mitochondrial pathways by interfering with GAPDH and ATP-ases, respectively. Redegeld *et al.* [26] have shown that GAPDH was 80% inhibited by menadione (150–200 μM). The differences between ATP depletion by NQ and MEN are not simply attributable to the differences in arylation of protein thiols. The percentage of arylated protein thiols did not differ during exposure to NQ and MEN. The higher levels of ATP in MEN-treated cells corresponded with an initial increase of MTT reduction. Although the mechanism of MTT reduction is still unclear, several cellular reductive pathways are involved. Earlier work showed the role of mitochondrial succinate dehydrogenase [62] and in HeLa cells, MTT was reduced by superoxide anion radicals derived from mitochondria or xanthine oxidase [63]. The initial rise of MTT reduction in the presence of menadione may reflect the ability of menadione to interfere with the electron current in the electron transport chain in mitochondria [64]. Berridge and Tan [65] showed that most cellular MTT reduction occurs outside the mitochondrial inner membrane and involves NADH- and NADPH-dependent mechanisms. A further alternative explanation may be an increased production of NADPH as the result of the activation of glucose-6-phosphate dehydrogenase. Enzymes which are involved in the hexose monophoshate shunt (HMPS) such as glucose-6-phosphate dehydrogenase are upregulated upon S-thiolation [66, 67]. This can explain

the 600% rise in HMPS activity in 50 μM of MEN-exposed Caco-2 cells in the experiments of Baker and Baker [68]. This increased reductive potential may have beneficial effects on the elimination of GSSG and semiquinones.

Our results confirm that the arylation capacity is a determining factor in the cytotoxic potential of quinones [12]. The redoxcycling and arylating quinones NQ and MEN were more toxic than the redoxcycler DIM. Whether a quinone can elicit toxicity is dependent on the presence of reducing enzymes like DTD. The Caco-2 cell line displays a very low DTD activity and was therefore more susceptible to quinone toxicity than the HT-29 cell line. The presence of DTD is of interest for cancer therapy. The cytotoxicity and DNA cross-linking ability of mitomycin C and diaziquone are dependent on a two-electron reduction by DTD, and the HT-29 cell line was found to be more sensitive than the DTD-deficient or low-level-expressing colon carcinoma cell line BE [5, 6]. On the other hand, this paper shows that redoxcycling and arylating naphthoquinones were toxic to cells when DTD activity was inhibited or missing. The determination of the enzymatic capabilities of a tumour may provide an indication for the choice of an anticancer drug.

This work was supported by a grant from the Dutch Organization for Scientific Research (NWO), The Hague, The Netherlands (Grant no. 902-22-112).

References

1. Lai GM, Moscow JA, Alvarez MG, Fojo AT and Bates SE, Contribution of glutathione and glutathione-dependent enzymes in the reversal of adriamycin resistance in colon carcinoma cell lines. *Int J Cancer* **49:** 688–695, 1991.
2. Peters WH and Roelofs HM, Biochemical characterization of resistance to mitoxantrone and adriamycin in Caco-2 human colon adenocarcinoma cells: A possible role for glutathione S-transferases. *Cancer Res* **52:** 1886–1890, 1992.
3. Djuric Z, Corbett TH, Valeriote FA, Heilbrun LK and Baker LH, Detoxification ability and toxicity of quinones in mouse and human tumor cell lines used for anticancer drug screening. *Cancer Chemother Pharmacol* **36:** 20–26, 1995.
4. Alvarez M, Paull K, Monks A, Hose C, Lee JS, Weinstein J, Grever M, Bates S and Fojo T, Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen. *J Clin Invest* **95:** 2205–2214, 1995.
5. Siegel D, Gibson NW, Preusch PC and Ross D, Metabolism of mitomycin C by DT-diaphorase: Role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50:** 7483–7489, 1990.
6. Traver RD, Horikoshi T, Danenberg KD, Stadlbauer TH, Danenberg PV, Ross D and Gibson NW, NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: Characterization of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. *Cancer Res* **52:** 797–802, 1992.
7. Joseph P, Xie T, Xu Y and Jaiswal AK, NAD(P)H:quinone oxidoreductase1 (DT-diaphorase): expression, regulation, and role in cancer. *Oncol Res* **6:** 525–532, 1994.
8. O'Brien P, Molecular mechanisms of quinone cytotoxicity. *Chem-Biol Interact* **80:** 1–41, 1991.
9. Ross D, Beall H, Traver RD, Siegel D, Phillips RM and Gibson NW, Bioactivation of quinones by DT-diaphorase, molecular, biochemical, and chemical studies. *Onco Res* **6:** 493–500, 1994.
10. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. *J Biol Chem* **257:** 12419–12425, 1982.
11. Miller M, Rodgers A and Cohen GM, Mechanisms of toxicity of naphthoquinones to isolated hepatocytes. *Biochem Pharmacol* **35:** 117–1184, 1986.
12. Toxopeus C, Holsteijn I, Thuring JW, Blaauboer BJ and Noordhoek J, Cytotoxicity of menadione and related quinones in freshly isolated hepatocytes: Effects on thiol homeostasis and energy charge. *Arch Toxicol* **67:** 674–679, 1993.
13. Gant TW, Ramakrishna Rao DN, Mason RP and Cohen GM, Redox cycling and sulphydryl arylation: Their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem-Biol Interact* **65:** 157–173, 1988.
14. Lind C, Hochstein P and Ernster L, DT-diaphorase as a quinone reductase. A cellular control device against semiquinone and superoxide formation. *Arch Biochem Biophys* **216:** 178–185, 1982.
15. Akerboom T, Bultmann T and Sies H, Inhibition of biliary taurocholate excretion during menadione metabolism in perfused rat liver. *Arch Biochem Biophys* **263:** 10–18, 1988.
16. DiMonte D, Ross D, Bellomo G, Eklöw L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235:** 334–342, 1984.
17. DiMonte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alterations in Ca^{2+} homeostasis. *Arch Biochem Biophys* **235:** 343–350, 1984.
18. Mirabelli F, Salis A, Perotti M, Taddei F, Bellomo G and Orrenius S, Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulphydryl groups in the cytoskeletal proteins. *Biochem Pharmacol* **37:** 3423–3427, 1988.
19. Mirabelli F, Salis A, Marinoni V, Finardi G, Bellomo G, Thor H and Orrenius S, Menadione-induced bleb formation in hepatocytes is associated with the oxidation of thiol groups in actin. *Arch Biochem Biophys* **264:** 261–269, 1988.
20. Nicotera P, Moore M, Mirabelli F, Bellomo G and Orrenius S, Inhibition of hepatocyte plasma membrane Ca^{2+}-ATPase activity by menadione metabolism and its restoration by thiols. *FEBS Lett* **181:** 149–153, 1985.
21. Cantoni O, Fiorani M, Cattabeni F and Bellomo G, DNA breakage caused by hydrogen peroxide produced by the metabolism of 2-methyl-1,4-naphthoquinone (menadione) does not contribute to the toxicity of the cytotoxic action of the quinone. *Biochem Pharmacol* **42:** S220–S222, 1991.
22. Fischer-Nielsen A, Corcoran GB, Poulsen HE, Kamendulis LM and Loft S, Menadione-induced DNA fragmentation without 8-oxo-2′deoxyguanosine formation in isolated rat hepatocytes. *Biochem Pharmacol* **49:** 1469–1474, 1995.
23. Morrison H, Jernström B, Nordenskjöld H, Thor H and Orrenius S, Induction of DNA damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes. *Biochem Pharmacol* **33:** 1763–1769, 1984.
24. Martins EAL and Meneghini R, DNA damage and lethal effects of hydrogen peroxide and menadione in Chinese hamster cells: Distinct mechanisms are involved. *Free Radical Bio Med* **8:** 433–440, 1990.
25. Morgan WA, Harley JA and Cohen GM, Quinone-induced DNA single strand breaks in rat hepatocytes and human chronic myelogenous leukaemic K562 cells. *Biochem Pharmacol* **44:** 215–221, 1992.

26. Redegeld FAM, Moison RMW, Barentsen HM, Koster AS and Noordhoek J, Interaction with cellular ATP generating pathways mediates menadione-induced cytotoxicity in isolated rat hepatocytes. *Arch Biochem Biophys* **280:** 130–136, 1990.
27. Borenfreund E and Puerner JA, Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicol Lett* **24:** 119–124, 1985.
28. Fisher GR and Gutierrez PL, Free radical formation and DNA strand breakage during metabolism of diaziquone by NAD(P)H quinone-acceptor oxidoreductase (DT-diaphorase) and NADPH-cytochrome C reductase. *Free Radical Bio Med* **10:** 359–370, 1991.
29. Cotgreave IA and Moldeus P, Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol component of biological systems. *J Biochem Bioph Meth* **13:** 231–249, 1986.
30. Ellman G, Tissue sulfhydryl groups. *Arch Biochem Biophys* **82:** 70–77, 1959.
31. Sedlak J and Lindsay RH, Estimation of total, protein-bound, nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25:** 192–205, 1968.
32. Toxopeus C, Holsteijn I, De Winther MPJ, Dobbelsteen D, Horbach JGJM, Blaauboer BJ and Noordhoek J, Role of thiol homeostasis and adenine nucleotide metabolism in the protective effects of fructose in quinone-induced cytotoxicity in rat hepatocytes. *Biochem Pharmacol* **48:** 1683–1692, 1994.
33. Bradford MM, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
34. Hyslop PA, Hinshaw DB, Halsey WA, Schraufstätter IU, Sauerheber RD, Spragg RG, Jackson JH and Cochrane CG, Mechanisms of oxidant-mediated cell killing: The glycolytic and mitochondrial pathways of ADP phosphorylation are major targets of H_2O_2-mediated injury. *J Biol Chem* **263:** 1655–1675, 1988.
35. Tzeng WF, Lee JL and Chiou TJ, The role of lipid peroxidation in menadione-mediated toxicity in cardiomyocytes. *J Mol Cell Cardiol* **27:** 1999–2008, 1995.
36. Lash LH, Hagen TM and Jones DP, Exogenous glutathione protects intestinal epithelial cells from oxidative injury. *Proc Natl Acad Sci USA* **83:** 4641–4645, 1986.
37. Finely KT, The addition and substitution chemistry of quinones. In: *The Chemistry of Quinoniod Compounds* (Ed. Patai S), pp. 877–1144. John Wiley & Sons, London, 1974.
38. Wilson I, Wardman P, Lin TS and Sartorelli AC, Reactivity of thiols towards derivatives of 2-methyl and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents. *Chem-Biol Interact* **61:** 229–240, 1987.
39. Buffinton GD, Öllinger K, Brunmark A and Cadenas E, DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates. *Biochem J* **257:** 561–571, 1989.
40. Flowers Geary L, Harvey RG and Penning TM, Cytotoxicity of polycyclic aromatic hydrocarbon o-quinones in rat and human hepatoma cells. *Chem Res Toxicol* **6:** 252–260, 1993.
41. Beall HD, Mulcahy RT, Siegel D, Traver RD, Gibson NW and Ross D, Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. *Cancer Res* **54:** 3196–3201, 1994.
42. Öllinger K, Buffington GD, Ernster L and Cadenas E, Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chem-Biol Interact* **73:** 53–76, 1990.
43. LLopis J, Ernster L and Cadenas E, Effect of glutathione on the redox transitions of naphthohydroquinone derivatives formed during DT-diaphorase catalysis. *Free Rad Res Comm* **8:** 271–285, 1990.
44. Cadenas E, Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem Pharmacol* **49:** 127–140, 1995.
45. Wong GHW and Goeddel DV, Induction of manganous superoxide dismutase by tumor necrosis factor: Possible protective mechanism. *Science* **242:** 941–944, 1988.
46. Baker SS and Baker RDJ, Antioxidant enzymes in the differentiated Caco-2 cell line. *In Vitro Cell Dev-An* **28A:** 643–647, 1992.
47. Ciaccio PJ, Stuart JE and Kenneth DT, Overproduction of a 37.5-kDa cytosolic protein structurally related to prostagladin F synthase in ethacrynic acid-resistant human colon cells. *Am Soc Pharmacol Exp Ther* **43:** 845–853, 1993.
48. Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS and Watkins PB, Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alpha,25-dihydroxyvitamin D3. *Mol Pharmacol* **51:** 741–754, 1997.
49. Rossi L, De Angelis I, Pedersen JZ, Marchese E, Stammati A, Rotilio G and Zucco FN, [5-nitro-2-furfurylidene]-3-amino-2-oxazolidinone activation by the human intestinal cell line Caco-2 monitored through noninvasive electron spin resonance spectroscopy. *Mol Pharmacol* **49:** 547–555, 1996.
50. Peters WH and Roelofs HM, Time-dependent activity and expression of glutathione S-transferases in the human colon adenocarcinoma cell line Caco-2. *Biochem J* **264:** 613–616, 1989.
51. Tew KD, Bomber AM and Hoffman SJ, Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug-resistant and -sensitive cell lines. *Cancer Res* **48:** 3622–3925, 1988.
52. Lewis AD, Forrester LM, Hayes JD, Wareing CJ, Carmichael J, Harris AL, Mooghen M and Wolf CR, Glutathione S-transferase isoenzymes in human tumours and tumour-derived cell lines. *Br J Cancer* **60:** 327–331, 1989.
53. Kuzmich S, Vanderveer LA, Walsh ES, LaCreta FP and Tew KD, Increased levels of glutathione S-transferase (pi) transcripts as a mechanism of resistance to ethacrynic acid. *Biochem J* **281:** 219–224, 1992.
54. Perry RR, Kang Y and Greaves B, Biochemical characterization of a mitomycin C resistant colon cancer cell line variant. *Biochem Pharmacol* **46:** 1999–2005, 1993.
55. Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. A study with isolated hepatocytes and menadione (2-methyl-1,4-naphthoquinone). *Biochem Pharmacol* **36:** 1313–1320, 1987.
56. Chai YC, Hendrich S and Thomas JA, Protein S-thiolation in hepatocytes stimulated by *t*-butyl hydroperoxide, menadione, and neutrophils. *Arch Biochem Biophys* **310:** 264–272, 1994.
57. Birge RB, Bartolone JB, Cohen SD, Khairallah EA and Smolin LA, A comparison of protein S-thiolated by glutathione or those arylated by acetaminophen. *Biochem Pharmacol* **42:** S197–S207, 1991.
58. Meister A and Anderson ME, Glutathione. *Annu Rev Biochem* **52:** 711–760, 1983.
59. Fernandez-Checa JC, Ren C, Aw TY, Ookhtens M and Kaplowitz N, Effect of membrane potential and cellular ATP on glutathione efflux from isolated rat hepatocytes. *Am J Physiol* **255:** G403–G408, 1988.
60. Griffiths JC, Sies H, Meier PJ and Akerboom TMP, Inhibition of taurocholate efflux from rat hepatic canalicular membrane vesicles by glutathione disulfide. *FEBS Lett* **213:** 34–38, 1987.
61. Bilzer M, Krauth-Siegel RL, Schirmer RH, Akerboom TPM, Sies H and Schulz GE, Interaction of a glutathione-S-conjugate with glutathione reductase. Kinetic and X-ray crystallographic studies. *Eur J Biochem* **138:** 373–378, 1984.
62. Lippold HJ, Quantitative succinic dehydrogenases histochemistry. *Histochemistry* **76:** 381–405, 1982.

63. Burdon RH, Gill V and Rice-Evans C, Reduction of a tetrazolium salt and the superoxide generation in human tumor cell (HeLa). *Free Rad Res Com* **18:** 369–380, 1993.
64. Colpa-Boonstra JP and Slater EC, The possible role of vitamin K in the respiratory chain. *Biochim Biophys Acta* **27:** 122–133, 1958.
65. Berridge MV and Tan AS, Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and the involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* **303:** 474–482, 1993.
66. Eggleston LV and Krebs HA, Regulation of the pentose phosphate cycle. *Biochem J* **138:** 425–435, 1974.
67. Burchell A and Burchell B, Stabilization of partially purified glucose-6-phosphatase by fluoride. Is enzyme inactivation caused by dephosphorylation? *FEBS Lett* **118:** 180–184, 1980.
68. Baker S and Baker RD, Caco-2 cell metabolism of oxygen-derived radicals. *Digest Dis Sci* **38:** 2273–2280, 1993.