Biochemical Pharmacology, Vol. 37, No. 12, pp. 2451–2459, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

DT-DIAPHORASE ACTIVITY AND THE CYTOTOXICITY OF QUINONES IN C3H/10T1/2 MOUSE EMBRYO CELLS

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(*Received* 19 *May* 1987; *accepted* 24 *November* 1987)

Abstract—A permanent mouse fibroblast cell line derived from C3H mouse embryos, C3H/10T1/2 C18, was used to study the cytotoxicity of some model quinones under conditions in which DT-diaphorase (EC 1.6.99.2) activity was induced or inhibited. Sudan III [1-[[4-(phenylazo)phenyl]azo]-2-naphthalenol] and 3-methylcholanthrene (MCA), but not butylated hydroxyanisole (BHA), induced DT-diaphorase in a concentration-dependent manner. Induction of DT-diaphorase activity was dependent upon new RNA and protein synthesis, as shown by experiments employing actinomycin D and cycloheximide respectively. Induction of DT-diaphorase by Sudan III or MCA was associated with protection against the cytotoxicity of quinones as measured by a colony survival assay. When control and induced cells were also exposed to dicoumarol, a specific and potent inhibitor of DT-diaphorase, the cytotoxicity of the quinones in both control and induced cells was enhanced markedly. The results support the hypothesis that DT-diaphorase competes with one-electron quinone-reducing enzymes (such as cytochrome P-450 reductase) which generate auto-oxidizable semiquinones and forms more stable hydroquinones as an initial step in the detoxification of quinones in 10T1/2 cells.

Quinones are widely distributed in nature in both plant and animal kingdoms [1]. Their capacity to participate in oxidation–reduction reactions, as well as their hydrophobicity, permit them to play essential roles in electron transport processes associated with respiration and photosynthesis in biological membranes. Of equal significance is the fact that quinones are ubiquitous environmental pollutants formed during combustion of organic matter. Their formation is implicated in the toxicity and possibly carcinogenicity of many disparate chemical compounds [2]. The formation of reactive oxygen species in the presence of quinones has been demonstrated [3], and the cytotoxicity of a number of quinones has been related to their ability to participate in redox-cycling in target cells [4]. For example, the cardiotoxicity induced by the quinoid antineoplastic drug adriamycin has been related to its one-electron reduction by subcellular enzymes [5, 6] and subsequent capacity to form oxygen radicals. Similar mechanisms have been proposed for menadione-induced erythrocyte toxicity [7] and hepatotoxicity [8].

Reduction of quinones at the expense of cellular reducing equivalents leads to formation of semiquinones and hydroquinones [9]. The former substances are known to react rapidly with oxygen to form the superoxide anion radical, singlet molecular oxygen, hydrogen peroxide, and possibly other reactive chemical species [10]. These non-stoichiometric products of redox-cycling may, depending on the sites at which they are formed, be responsible for the toxic and/or therapeutic effects of quinones [11]. Interestingly, a corollary of their potential toxicity has been in the recognition and development of quinones as useful therapeutic agents [12].

The one-electron reduction of quinones is competitive with a two-electron reduction, uniquely catalyzed by the flavoprotein DT-diaphorase, to form the corresponding hydroquinone [13]. This reaction likely represents a detoxification pathway for quinones, because hydroquinones are relatively stable with respect to auto-oxidation, and may undergo conjugation with glucuronate and other water-soluble ligands [14]. These latter reactions may terminate the ability of quinones to participate in redox-cycling and facilitate their physiological disposition.

We have studied the role of DT-diaphorase in quinone detoxification and the role of quinones in cytotoxicity and genotoxicity in C3H/10T1/2 C18 (10T1/2) cells. Several properties of these cells make them uniquely suitable for studying the cytotoxicity and genotoxicity of quinones. They possess cytochrome P-450 reductase [15], an enzyme which catalyzes the one-electron reduction of quinones [16]. As shown in the present study, 10T1/2 cells also possess DT-diaphorase activity. Both enzymes can be preferentially induced or inhibited with a number of known inducers and inhibitors. In addition, these cells have been used to study mutation and morphological transformation induced by polycyclic aromatic hydrocarbons and aromatic amines. It has been

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shown that benzo[a]pyrene (BaP*) and N-acetoxyacetylaminofluorene (NAcO-AAF) induce both morphological transformation and mutation to ouabain resistance over the same concentration ranges [17], suggesting that mutation may play a role in the process of transformation induced by BaP and NAcO-AAF [reviewed in Refs. 18 and 19]. Hence, we initiated studies of quinoid compounds as potential cytotoxic agents, carcinogens, and mutagens. In the present report, we describe experiments in which 10T1/2 cells were treated with several compounds known to induce DT-diaphorase in whole animals. Optimal conditions for inducing DT-diaphorase activity in 10T1/2 cells were determined, and the cytotoxicity of several quinones was then determined in control cells, induced cells, and cells pretreated with the DT-diaphorase inhibitor, dicoumarol.

MATERIALS AND METHODS

Menadione, 2,6-dichlorophenolindophenol (DCPIP), NADH, NADPH, Tris–HCl, benz[a]anthracene (BA), butylated hydroxyanisol (BHA), reduced glutathione, oxidized glutathione, 1-chloro-2,4-dinitrobenzene, and Sudan III were purchased from the Sigma Chemical Co., St. Louis, MO. Dicoumarol was purchased from the Nutritional Biochemical Corp., Cleveland, OH. 1,2-Naphthoquinone, 1,4-naphthoquinone, and duroquinone were purchased from the Aldrich Chemical Co. Milwaukee, WI. Daunomycin was a gift from Dr. Kenneth Chan, School of Pharmacy, University of Southern California, Los Angeles, CA. Basal Eagle's medium heat-inactivated fetal calf serum, and trypsin were purchased from the Grand Island Biological Co., Grand Island, NY. 3-Methylcholanthrene (MCA) was purchased from the Eastman Kodak Co., Rochester, NY. Plasticware for cell culture was purchased from Corning Glass Work, Corning, NY.

* Abbreviations: BaP, benzo[a]pyrene; MCA, 3-methylcholanthrene; BHA, butylated hydroxyanisole; NAcO-AAF, N-acetoxy-acetylaminofluorene; DCPIP, 2,6-dichlorophenolindophenol; BA, benz[a]anthracene; and AHH, aromatic hydrocarbon hydroxylase.

Cells and cell culture

A description of the C3H/10T1/2 cell line and of procedures for the routine handling and subculturing of the C3H/10T1/2 cells, which were originally established from embryos of C3H mice, is given in Ref. 20, with the more recent modifications in Ref. 19. 10T1/2 Cells were cultured in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf

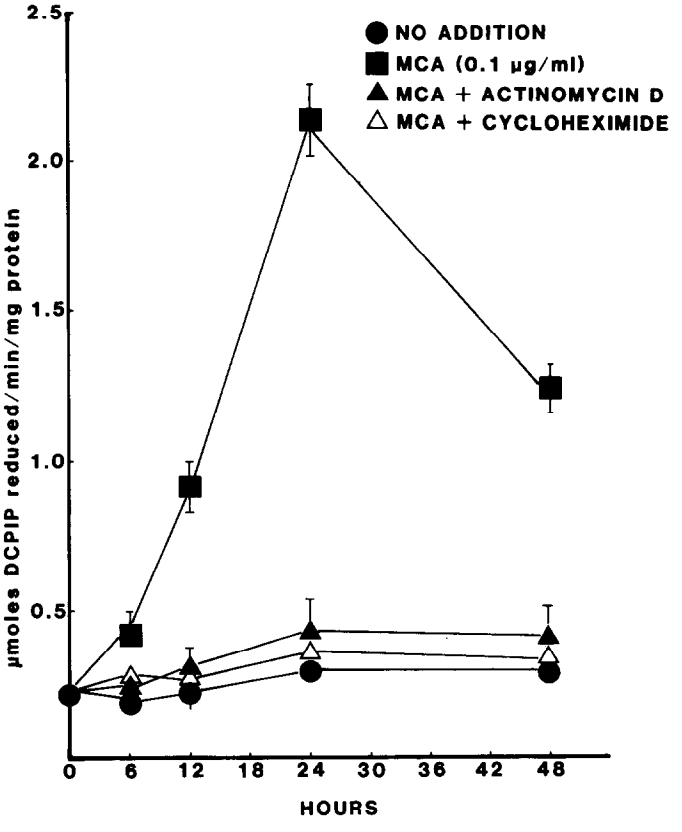

Fig. 1. Effect of 3-methycholanthrene, a polycyclic hydrocarbon inducer, on DT-diaphorase activity in 10T1/2 cells. Cells were grown in control medium alone and in medium containing 3-methylcholanthrene, actinomycin D and 3-methylcholanthrene, or cycloheximide and 3-methylcholanthrene. The concentrations used were: 3-methylcholanthrene, 0.1 μg/ml; cycloheximide, 10 μg/ml; and actinomycin D, 1 μg/ml. Each experiment was done in quadruplicate, and each point represents the mean ± standard deviation of three experiments.

serum, without antibiotics, and all cells used in this study were between the 7th and 15th passage. Cells were tested for Mycoplasma contamination at 6- to 8-week intervals by growth on Mycoplasma agar throughout the course of these studies and were negative [21].

Cytotoxicity assays

Cytotoxicity assays using logarithmically growing cells were performed by measuring reduction in plating efficiency of treated cells [17]. Briefly, 200 cells were seeded in 5 ml medium per 60-mm tissue culture dish, allowed to attach and recover for 24 hr, and then treated with the appropriate quinone in acetone (0.2% acetone in the medium). This amount of acetone caused no cytotoxicity by itself. Cells were exposed to the quinone compounds for 24 hr, and the medium was then removed and replaced with fresh medium. At day 10 post-seeding, the medium was removed, and the colonies were fixed with methanol, stained with Giemsa, and counted under a dissecting microscope. Only colonies containing 20 or more cells were scored as viable. Each determination represents the average ± standard deviation of values from five dishes.

Preparation of cells for enzyme assays

Cells to be assayed for enzyme activities were seeded at 10^5 cells in 5 ml medium per 60-mm tissue culture dish. Inducing agents were added at different times throughout the growth curve in the presence or absence of either actinomycin D or cycloheximide.

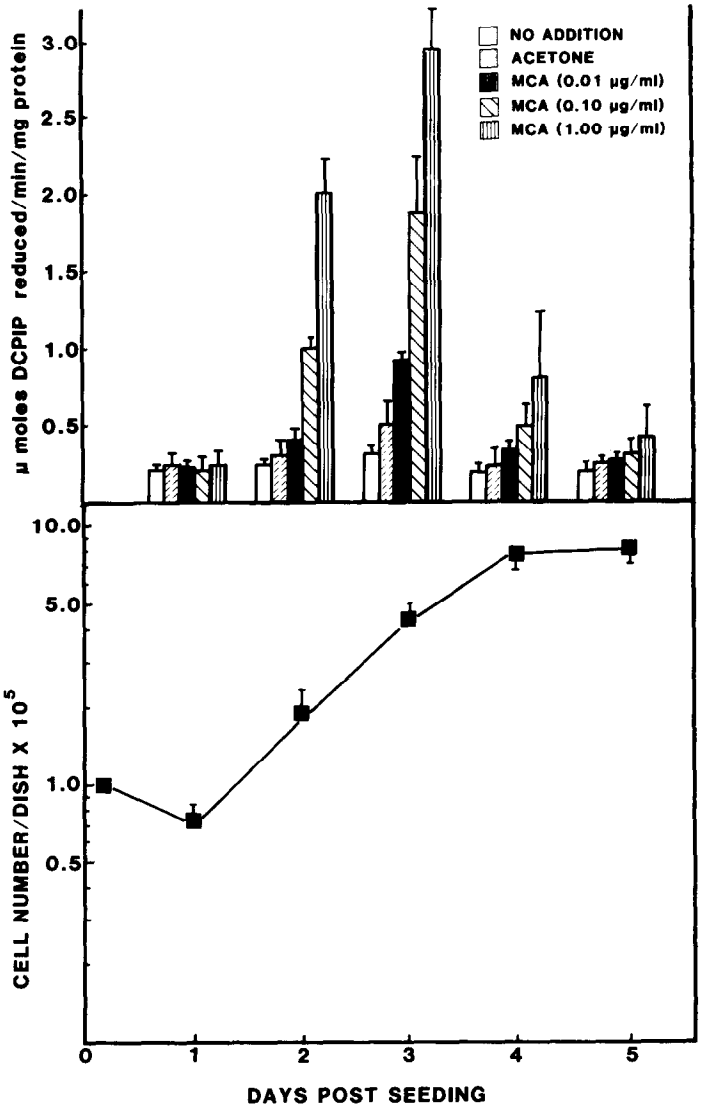

Fig. 2. Inducibility of DT-diaphorase activity by MCA during the growth of 10T1/2 cells. Each day, five 60-mm dishes were treated with acetone at a final concentration of 0.25%, and with 3-methylcholanthrene (dissolved in acetone): 0.01, 0.1 and 1.0 μg/ml. After 24 hr, cells were harvested, counted, and assayed as described under Materials and Methods. Bottom panel: (■—■) $\log_{10}$ average cell number per 60-mm dish for all five dishes harvested on that day.

At different time points, the medium was removed and the cells were washed with phosphate-buffered saline, harvested by scraping with a rubber policeman in the absence of trypsin, and centrifuged at 1000 *g* for 5 min. The cell pellets were suspended in cold 10 mM Tris buffer (pH 7.4) at approximately 10^6 cells/ml. Cells were lysed in the same buffer by sonicating them three times for 45 sec, each time at a power of 100 W in a W-225R Cell Disruptor (Ultrasonics Inc.). There was a 45-sec period between each sonication period to allow dissipation of heat. During sonication the samples were kept at 4° by a circulating water bath. Cell lysates were frozen in liquid nitrogen and stored at −80° until enzyme activities were assayed. There was no measurable loss of enzymatic activity in cell lysates stored under these conditions for up to 60 days.

Assay of DT-diaphorase activity. The dicoumarol-sensitive rate of reduction of DCPIP (40 μM) by NADPH (250 μM) in a 3-ml assay system at pH 7.4 was measured as described [22], at 600 nm in a Perkin–Elmer 552-UV/Vis-Spectrophotometer. Specific activities are expressed as micromoles of DCPIP reduced per minute per milligram of protein. Protein concentrations were measured by the method of Lowry *et al.* [23], with bovine serum albumin as a standard.

Assay of aryl hydrocarbon hydroxylase (AHH) activity. The hydroxylation of benzo[*a*]pyrene by cell lysates (10^6 cells/assay) was measured fluorometrically as previously described [24] in an Aminco–Bowman spectrophotofluorometer, at 396 nm excitation and 522 nm emission, except that 2 ml of organic phase was extracted with 2 ml of 1 M sodium hydroxide. In the absence of substrate, cell lysate, or NADPH, the hydroxylation of benzo[*a*]pyrene occurred to the extent of less than 4% that of the complete system in which all factors were present. Activities are expressed as aryl hydrocarbon hydroxylase units. One unit of aryl hydrocarbon hydroxylase catalyzes the formation of phenolic products with the fluorescence equivalent to 1 pmol of 3-hydroxybenzo[*a*]pyrene in 30 min [24].

Assay of glutathione reductase. The rate of oxidation of NADPH by oxidized glutathione at 25° was used as a standard measure of enzymatic activity as described [25]. The oxidation of 1 μmol of NADPH per min is used as a unit of glutathione reductase activity. Activities are expressed as units per mg protein in the cell lysate.

Assay of glutathione transferase(s). Glutathione transferase activity was measured as previously described [26], using 1-chloro-2,4-dinitrobenzene (1 mM) as the acceptor. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min. Activities are expressed as units per mg protein in the cell lysate.

RESULTS

Characterization of DT-diaphorase activity in 10T1/2 *cells*

The kinetic properties of DT-diaphorase activity in 10T1/2 cell lysates with respect to NAD(P)H and quinone substrates yielded estimated Michaelis constants (K_m) for NADH and NADPH of 74 and 27 μM respectively. The inhibition constants (K_i) for dicoumarol with NADH and NADPH were 22 and 66 nM respectively. As in the case of rat liver cytosolic enzyme, the inhibition of DT-diaphorase by dicoumarol was competitive with respect to the reduced pyridine nucleotide. These values are very similar to values reported for the purified enzyme from rat liver cytosol [27], indicating the similarity of the enzymatic activity of DT-diaphorase in 10T1/2 cells to that of the purified rat liver cytosolic enzyme.

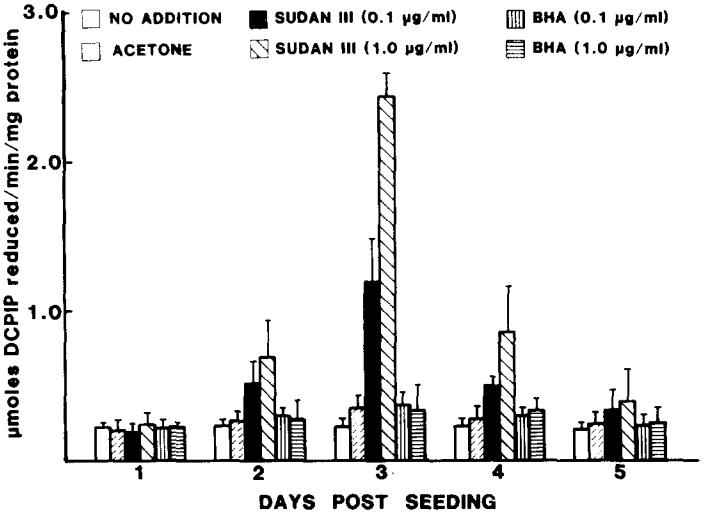

Fig. 3. Inducibility of DT-diaphorase activity by Sudan III during the growth of 10T1/2 cells. Each day, five 60-mm dishes were treated with acetone at a final concentration of 0.25%, with Sudan III: 0.1 and 1.0 μg/ml or with butylated hydroxyanisole: 0.1 and 1.0 μg/ml. After 24 hr, cells were harvested, counted, and assayed as described in Materials and Methods.

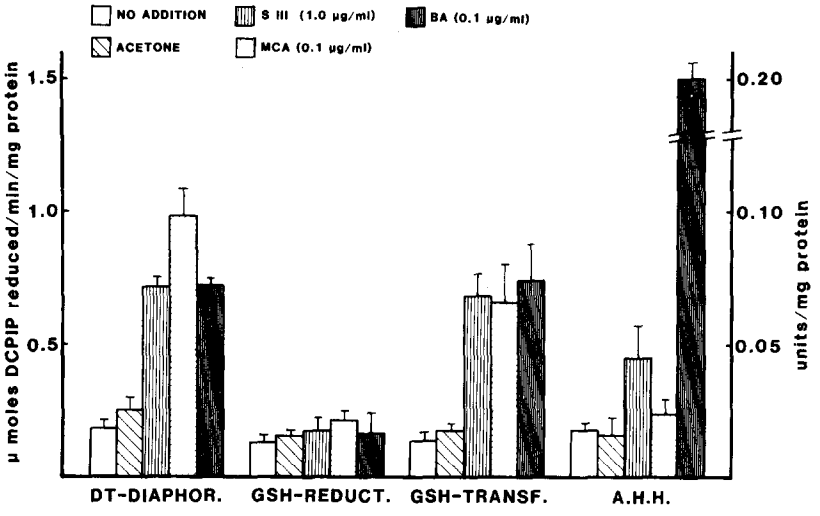

Fig. 4. Activities of DT-diaphorase. GSH-reductase, GSH-transferase(s) and aromatic hydrocarbon hydroxylase in 10T1/2 cells. The bars indicate control cells; addition of 0.25% acetone; Sudan III, 1.0 μg/ml; 3-methylcholanthrene, 0.1 μg/ml; and benz[*a*]anthracene, 0.1 μg/ml. Cells were treated for 24 hr, then harvested, counted, and assayed as described in Materials and Methods. All four enzyme activities were measured on each single dish. Each experiment was done in quadruplicate, and the results are means ± standard deviation of four experiments.

Characterization of DT-diaphorase induction in 10T1/2 cells

Previous reports have shown that treatment of rats with MCA, an inducer of the microsomal aryl hydrocarbon hydroxylase system [28], causes a 4- to 5-fold increase in liver DT-diaphorase activity [29] in both the cytosolic and microsomal fractions. Treatment of growing 10T1/2 cells with MCA caused an increase in DT-diaphorase activity as early as 6 hr, and the activity reached a maximum at 24 hr and then declined. The maximal induction at 24 hr was 9-fold over the basal level (Fig. 1). Addition of cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml) simultaneously with the inducer completely inhibited the increase in DT-diaphorase activity, indicating that syntheses of both new RNA and protein are necessary for induction of DT-diaphorase activity in 10T1/2 cells (Fig. 1), as has been reported previously [30–32].

In the early phases of our investigations with inducers of DT-diaphorase, we noted a large variability in the absolute values of DT-diaphorase activity within the same passage of logarithmically growing cells. To control this variability, we undertook a thorough study of the variation of basal and MCA-induced DT-diaphorase levels during the growth of cell cultures from the logarithmic phase of growth through confluence. Each day after seeding, cultures that had been treated 24 hr previously with MCA were assayed for DT-diaphorase activity. We found the inducibility by MCA to be a function of the growth status of the cells, as illustrated in Fig. 2. The inducibility of DT-diaphorase increased from day 1 to day 3 post-seeding, when cells were in the log phase of growth, attaining a maximal of a 6-fold increase in inducible enzyme levels on day 3. As the cultures entered confluence (day 4), the inducibility of DT-diaphorase by MCA decreased to basal levels on day 5 post-seeding. At each time point, the extent of induction of DT-diaphorase was dose dependent with regard to the inducer.

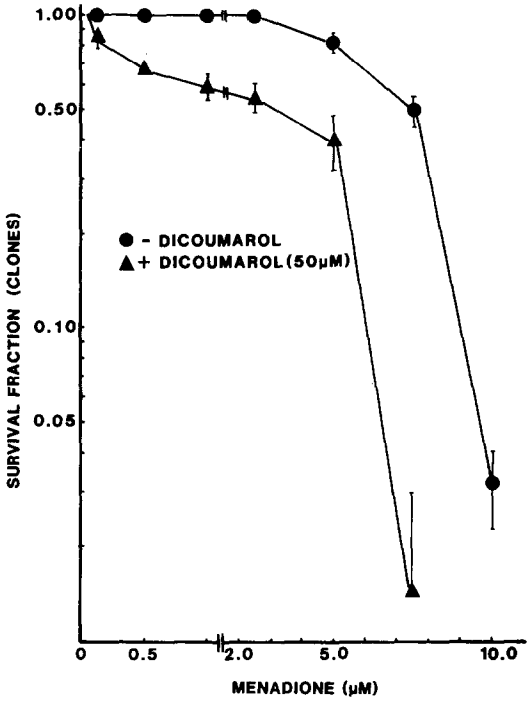

Fig. 5. Normalized growth curves of 10T1/2 cells treated with increasing concentrations of menadione, for 24 hr, in the absence and presence of 50 μM dicoumarol.

The same experimental design was repeated using BHA and Sudan III. Both compounds have been shown previously to induce DT-diaphorase activity in rodent livers [33, 34]. Treatment of 10T1/2 cells with Sudan III (1.0 μg/ml) induced DT-diaphorase activity up to 5-fold at day 3, when cells were treated during the logarithmic phase of growth, and again the inducibility decreased down to basal levels as cells became confluent on day 5 (Fig. 3). Unexpectedly, butylated hydroxyanisole did not cause measurable induction of DT-diaphorase in 10T1/2 cells (Fig. 3). Although not shown in the figure, this was true at concentrations as high as 5 μg/ml.

Activities of other drug-metabolizing enzymes

Many substances that protect animals against the cytotoxicity and carcinogenicity of various chemicals alter the metabolic fates of these compounds [35, 36] by modulating the activities of either or both phase I and phase II drug-metabolizing enzymes [37, 38]. The induction of such enzymes that detoxify electrophilic metabolites of carcinogens may interrupt the processes of cytotoxicity and neoplastic transformation. We therefore determined the levels of various phase I and phase II drug-metabolizing enzymes in both control and treated cells. Figure 4 shows that all three compounds tested (Sudan III at 1.0 μg/ml, benz[*a*]anthracene at 0.1 μg/ml and 3-methylcholanthrene at 0.1 μg/ml) actively induced both DT-diaphorase and glutathione transferase(s) activities. None of the compounds tested had any significant effect on glutathione reductase levels. Sudan III induced AHH activity almost 2-fold, whereas benz[*a*]anthracene induced the activity

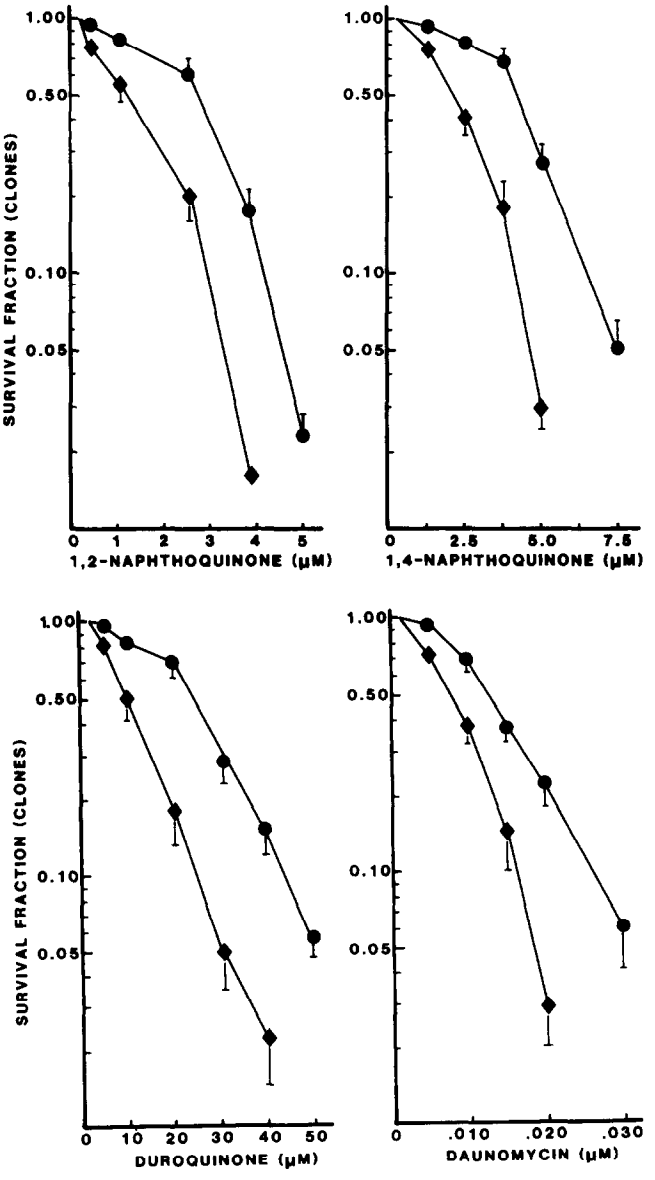

Fig. 6. Normalized growth curves of 10T1/2 cells treated with several quinones, for 24 hr, in the absence (●—●) and presence of dicoumarol (◆—◆).

about 10-fold. In agreement with earlier reports [39], there was a complete absence of induction of AHH in 10T1/2 cells by 3-methylcholanthrene, a potent inducer of AHH in rodent liver [28]. There was no correlation between the abilities of BA and MCA to induce aromatic hydrocarbon hydroxylase and DT-diaphorase in 10T1/2 cells.

Effect of dicoumarol on the cytotoxicity of quinones

Treatment of 10T1/2 cells with menadione caused a dose-dependent decrease in the survival fraction with increasing concentrations of menadione (Fig. 5). Treating cells with dicoumarol 10 min prior to addition of menadione, to inhibit DT-diaphorase, rendered non-cytotoxic concentrations of menadione ($< 5\ \mu M$) toxic, and enhanced menadione cytotoxicity at all concentrations of menadione used. Identical results were obtained using concentrations of dicoumarol as low as $5\ \mu M$ (not shown). Similarly, pretreating 10T1/2 cells with dicoumarol markedly enhanced the cytotoxicity of four other quinones: duroquinone, 1,2-naphthoquinone, daunomycin, and 1,4-naphthoquinone (Fig. 6). These data therefore suggest that inhibition of DT-diaphorase by dicoumarol enhances the cytotoxicity of these quinones, as indicated by a shift of the curves to lower concentrations for each quinone.

Induction of DT-diaphorase and its effect on the cytotoxicity of quinones

Treatment of 10T1/2 cells with Sudan III for 24 hr prior to addition of menadione completely abolished menadione cytotoxicity up to a concentration of $5\ \mu M$ and inhibited the cytotoxicity of menadione at concentrations higher than $5\ \mu M$ (Fig. 7). Again, $50\ \mu M$ dicoumarol enhanced the cytotoxicity of menadione. Moreover, the sensitivity of induced cells was increased and the survival curve was identical to that of uninduced cells.

The same patterns of protection were seen when cells were treated with MCA for 24 hr prior to the addition of four quinones: 1,2-naphthoquinone, duroquinone, 1,4-naphthoquinone, or menadione (Fig. 8). As shown in this figure, the survival curves of 10T1/2 cells treated with these four quinones were also shifted to higher values when cells were pretreated with MCA ($0.1\ \mu g/ml$) for 24 hr prior to addition of quinones. (Although the data are not shown, similar patterns of protection were also observed in the case of daunomycin.) Again, for each quinone, dicoumarol decreased the survival fraction of MCA-induced and uninduced cells to the same level. These results suggest that Sudan III and MCA protect 10T1/2 cells against quinone cytotoxicity by inducing DT-diaphorase.

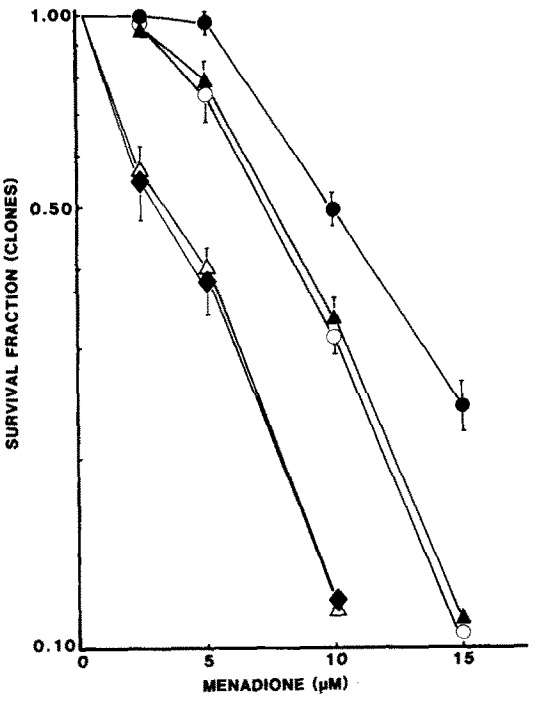

Fig. 7. Effect of Sudan III on normalized growth curves of 10T1/2 cells. Cells were either not treated or pretreated with 0.25% acetone or Sudan III ($1.0\ \mu g/ml$) for 24 hr. Then increasing concentrations of menadione were added to all groups both in the absence and presence of $50\ \mu M$ dicoumarol. Key: control cells (○—○); cell pretreated with: 0.25% acetone (▲—▲), Sudan III (●—●), acetone and dicoumarol (◆—◆), or Sudan III and dicoumarol (△—△).

DISCUSSION

The flavoprotein DT-diaphorase has been implicated in the detoxification of many quinones [13, 40]. In this study, increases in DT-diaphorase activity were obtained with MCA and Sudan III. Both compounds caused a 5- to 10-fold increase in enzyme activity. Consistent with other studies [30–32], we showed that actinomycin D and cycloheximide inhibited this increased enzyme activity, indicating that induction of DT-diaphorase requires new RNA and protein synthesis and, is likely a *bona fide* induction of new enzyme synthesis. Interestingly, BHA, which had been reported to induce the diaphorase in animals [41], had no such effect in cultured 10T1/2 cells. It should be noted that, at the concentrations of inducers used (0.1 to $5\ \mu g/ml$), no measurable cytotoxicity was observed. It is presently unresolved in the literature whether BHA or one (or more) of its metabolites is involved in induction of DT-diaphorase *in vivo*. Armstrong and Wattenberg [42] recently reported that, in isolated rat liver microsomes, the metabolism of BHA produces two major products. A characteristic of both metabolites is their potential for redox reactions. It has been suggested that the capacity to form such metabolites is essential for signaling the induction of DT-diaphorase [43]. The inability of BHA to induce DT-diaphorase in 10T1/2 cells may be a result of differences in its metabolism in 10T1/2 cells compared to that in hepatic microsomes.

Similar to the situation in hepatocytes. BA induces both AHH and DT-diaphorase in 10T1/2 cells. However, another striking difference between 10T1/2 cells and hepatocytes *in situ* [44] or in culture [43], is the lack of correlation in the 10T1/2 cells between induciblities of AHH and DT-diaphorase by MCA. The reason for the lack of induction of the aromatic

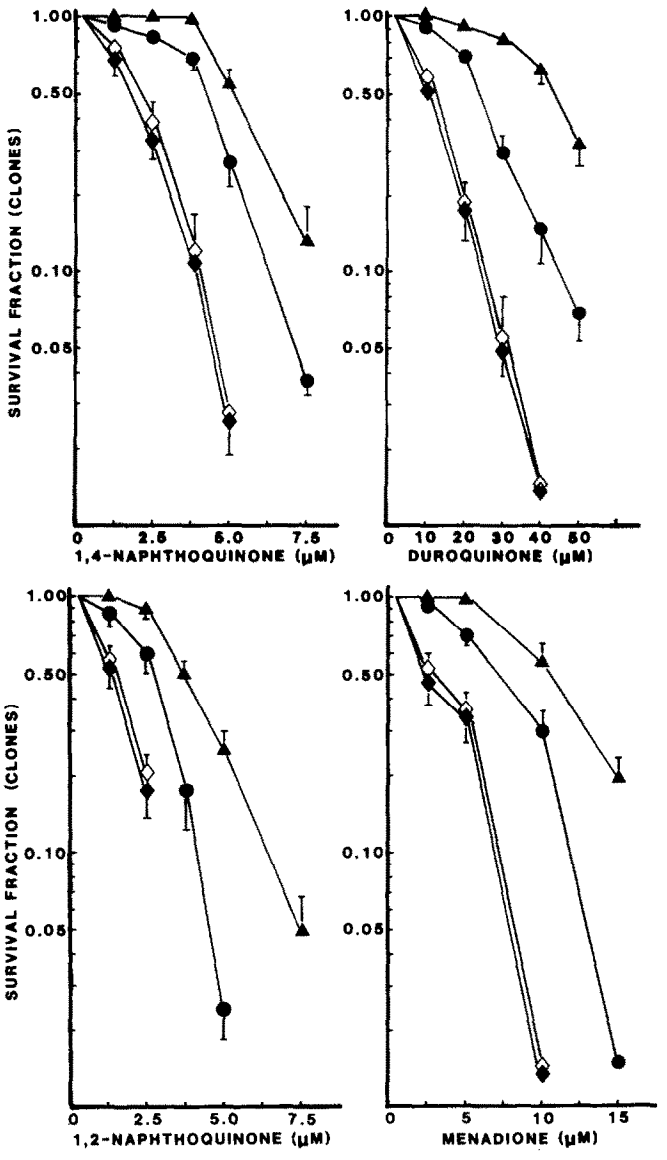

Fig. 8. Effect of MCA on normalized growth curves of 10T1/2 cells. Cells were either not treated or pretreated with 3-methylcholanthrene (0.1 μg/ml) for 24 hr. Then increasing concentrations of several quinones were added to both groups, both in the absence or presence of 50 μM dicoumarol. Key: control cells (●—●); cells pretreated with 3-methylcholanthrene (▲—▲), dicoumarol (◇—◇), or 3-methylcholanthrene and dicoumarol (◆—◆).

hydrocarbon hydroxylase by MCA in 10T1/2 cells may be that the receptor responsible for the induction of this enzyme differs from that in liver cells in that it does not interact with MCA, although it does interact with BA. However, these cells still may possess the ability to metabolize MCA via cytochrome P-448, which is believed to be a prerequisite for the induction of DT-diaphorase [43]. A further investigation of this phenomenon may provide important insight into the mechanisms of inducation of DT-diaphorase.

The anticoagulant dicoumarol was utilized to inhibit DT-diaphorase. Although this agent is known to uncouple oxidative phosphorylation [45] and to inhibit a dicoumarol-sensitive form of UDP-glucuronosyltransferase (UDPGT) [46], it has been commonly used as a presumptive inhibitor of DT-diaphorase in several cell systems [7, 47]. In our experiments, we observed no cytotoxicity with concentrations of dicoumarol as high as 50 μM, suggesting that uncoupling was not a major factor in enhancing the sensitivity of 10T1/2 cells to quinones at the concentrations employed. On the other hand, inhibition of UDPGT may well contribute to the altered sensitivity to quinones. Even if such inhibition takes place, the two-electron reduction of the quinones would be a prerequisite for conjugation with glucuronide. An effect of dicoumarol on both

enzymes might serve to enhance further the cytotoxicity of quinones.

We have shown in these experiments that the cytotoxicity of several quinones in 10T1/2 cells can be reduced markedly by pretreatment with MCA and SIII, which induce DT-diaphorase. Additionally, prior treatment with inducing agents increased glutathione transferase(s) (GT) and AHH (in the case of Sudan III) activities. Although both DT-diaphorase and glutathione transferase(s) may be involved in quinone detoxification, the effect of dicoumarol suggests that protection against quinone cytotoxicity after treatment with inducers results primarily from the higher levels of DT-diaphorase and not glutathione transferases. It should be noted that these alterations were not present if the cells were treated with dicoumarol (Figs. 7 and 8). Dicoumarol abolished the effects of inducing agents and caused an increase in sensitivity to quinones which was identical in both induced and uninduced cells.

We have shown here that manipulation of DT-diaphorase activity altered the sensitivity of C3H/10T1/2 mouse embryo fibroblasts to the cytotoxic effects of several common quinones. These experiments are supportive of the hypothesis that DT-diaphorase plays a key role in inhibiting quinone cytotoxicity. They also illustrate the utility of 10T1/2 cells as a model system for further investigation of the role of this unique flavoprotein in inhibiting cytotoxicity and neoplastic transformation induced by certain quinones.

Acknowledgements—This work was supported, in part, by grants from the NIH (CA41277 to J.R.L., ES-03816 to P.H.) and from the Swedish Natural Science Research Council, Pharmacia AB, Uppsala, Sweden, and The National Foundation for Cancer Research, Washington DC (to L.E.).

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