Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 346-351

# POSSIBLE ROLE OF DT-DIAPHORASE IN THE BIOACTIVATION OF ANTITUMOR QUINONES

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Received January 19, 1983

Menadione derivatives that are toxic to tumor cells are believed to be reduced intracellularly to species that react with DNA. In this communication, we report evidence that one of these derivatives, 3-bromomethylmenadione, is reduced by DT-diaphorases present in rat liver cytosol and in rat 9L brain tumor cells. Dicoumarol, an inhibitor of DT-diaphorases was found to inhibit both the reduction of 3-bromomethylmenadione and its mutagenicity to Salmonella typhimurium TA 97. Homogenates of rat 9L cells were found to contain relatively high levels of DT-diaphorase, suggesting that these tumor cells may be relatively sensitive to antitumor quinones that are activated by this enzyme.

The pyridine nucleotide oxidases known as DT-diaphorases (E.C.1.6.99.2) oxidize NADH and NADPH at equal rates, are uniquely sensitive to inhibition by dicoumarol, and transfer electrons to a variety of redox dyes, principally quinones (1). As quinone reductases, DT-diaphorases seem to be involved both in the detoxification of non-physiological quinones (2) and in the bioactivation of vitamin K (3). Since some analogs of vitamin $K_3$ (menadione) have been shown to be cytotoxic to tumor cells, we decided to investigate the possible role of DT-diaphorase in the bioactivation of these compounds. A representation of the hypothetical mechanism of bioactivation is shown in Figure 1.

The model compound selected for this study was 3-bromomethylmenadione (Figure 1) previously shown to be cytotoxic to adenocarcinoma 755 cells and sarcoma 180 cells (4). The bio-

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0006-291X/83/040346-06$01.50/0

activation of 3-bromomethylmenadione to the DNA-reactive species was studied indirectly by measuring the mutagenic activity of the quinone in the presence and in the absence of dicoumarol. The quinone reductase activity of tissue homogenates, measured spectrophotometrically with NADH as cofactor, was also investigated in the presence and absence of dicoumarol.

## METHODS

Dicoumarol and NADH were purchased from the Sigma Chemical Company, St. Louis, MO, menadione was purchased from Pfaltz and Bauer, Inc., Stamford, CT, and male Fisher 344 rats, 200-300 gm, were purchased from Charles River, Inc., Boston, MA. Twenty percent (w/v) homogenates of six pooled rat livers, five individual rat brains, and three cultures ($2 \times 10^8$ cells/culture) of rat 9L cells were prepared in 1.15 percent KCl with a Brinkmann Polytron tissue homogenizer. Rat liver cytosol was isolated from the liver homogenate by differential centrifugation. The 3-bromomethylmenadione was prepared by reacting menadione with formaldehyde and HBr gas as previously described (5). Salmonella typhimurium TA97 was generously provided by Dr. Bruce Ames.

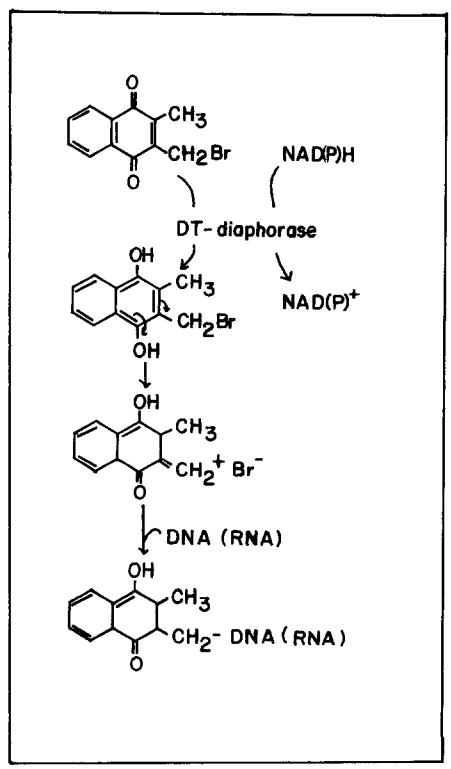


Figure 1. Bioactivation of 3-bromomethylmenadione. The production of the cytotoxic, DNA- reactive methide (4) may be catalyzed by DT-diaphorase, a pyridine nucleotide oxidase that reduces naphthoquinones (1).

The mutagenic activity of 3-bromomethylmenadione was assessed as follows: Salmonella typhimurium TA 97 (100 ul of a fully-grown culture) was added to test tubes containing 2.5 ml of molten top agar (6) and 0,2,4,8, or 16 ug of 3-bromomethylmenadione. A second series of tubes contained, in addition, 250 nmoles of dicoumarol, a specific inhibitor o DT-diaphorase (7). The contents of the tubes were poured onto minimal agar plates which were then incubated for 48 hr at 37°C. The number of revertant colonies on each plate was plotted as a function of the dose of 3-bromomethylmenadione.

The reduction of 3-bromomethylmenadione was assayed by monitoring the rate of NADH oxidation in the presence of tissue and quinone. Rat liver cytosol was added to 5 ml of 0.1 M tris-HCl buffer, pH 7.4, to a final concentration of 0.5 mg protein/ml. The mixture was divided between 2 cuvettes which were inserted in the sample and reference compartments of a Kontron Model 810 recording spectrophotometer. The monochromator was set at 340 nm and the cuvettes were allowed to warm to 37°C. NADH was added to the sample side to a final concentration of 0.15 mM. After starting the recorder, 3-bromomethylmenadione (final concentration, 64 uM) and dicoumarol (final concentration, 30 uM) were added sequentially, allowing several minutes in between for NADH oxidation to be recorded. The NADH oxidation rate, coupled to the quinone reduction rate, was calculated from the linear decrease in absorbance following quinone addition. (An initial absorbance increase was observed, due to absorption of 340 nm light by the quinone itself). The rates of 3-bromomethylmenadione reduction catalyzed by rat whole brain homogenates and rat 9L cell homogenates were also assayed by this spectrophotometric procedure.

## RESULTS AND DISCUSSION

The results of the bioactivation experiment (Figure 2A) indicated that 3-bromomethylmenadione was mutagenic in the absence, but not in the presence, of dicoumarol, a DT-diaphorase inhibitor. The antimutagenic effect of dicoumarol suggested that 3-bromomethylmenadione was activated by DT-diaphorases in S. typhimurium TA97. It was therefore of interest to investigate whether or not the reduction of 3-bromomethylmenadione by homogenates of mammalian tissues was also sensitive to dicoumarol.

When rat liver cytosol was used as the source of quinone reductase (Figure 2B), significant oxidation of NADH occurred after the quinone was added, but the rate declined to near zero after dicoumarol was added. When rat brain tumor cells (9L cells) were used (Figure 2C), similar effects were observed, indicating that these cells also contain dicoumarol-sensitive 3-bromomethylmenadione reductases.

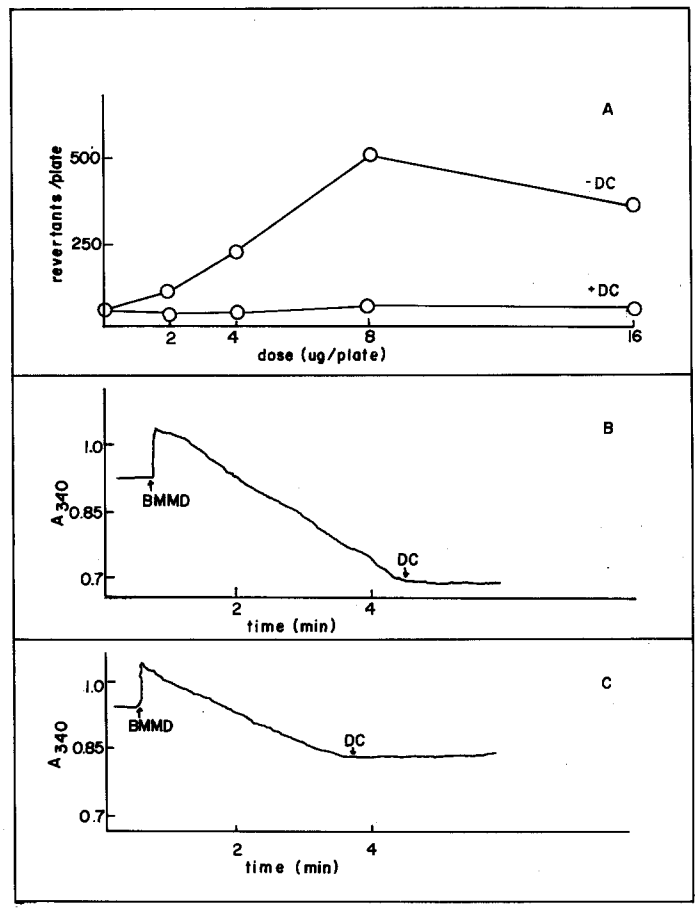


Figure 2. Effect of dicoumarol on the reduction of 3-bromomethylmenadione and on its activation to a mutagen. Figure 2A: various concentrations of 3-bromomethylmenadione were incubated with S.typhimurium TA97 in the presence and absence of dicoumarol (DC) as described in the methods section. Revertant ($his^-$) colonies were counted after 48 hours. Figure 2B: The oxidation of NADH catalyzed by rat liver cytosol was monitored spectrophotometrically in the presence of 3-bromomethylmenadione (BMMD) and dicoumarol (DC) which were added at the times indicated. Figure 2C: The oxidation of NADH catalyzed by rat 9L cell homogenate was monitored spectrophotometrically in the presence of BMMD and DC.

Although DT-diaphorase is widely distributed in the animal kingdom and can be found in most mammalian tissues, it seems to be particularly abundant in preneoplastic and neoplastic liver cells (8). Consistent with these findings, a comparison of the DT-diaphorase levels in rat 9L cell homogenates and rat whole brain homogenates (using 3-bromomethylmenadione as the quinone substrate); indicated that DT-diaphorase activity was remarkably

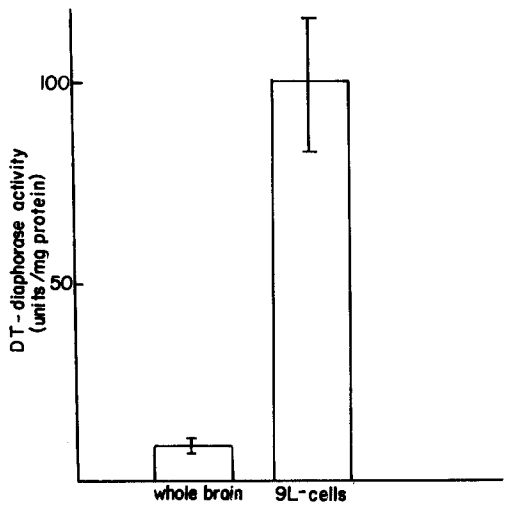


Figure 3. DT-diaphorase (3-bromomethylmenadione reductase) activity in rat 9L cells and rat brain. Triplicate cultures of rat 9L cells were grown in parallel to populations of about $2.0 \times 10^8$ cells/culture. The cells were isolated by centrifugation, washed, and homogenized with a Brinkman Polytron homogenizer. Aliquots of each homogenate were assayed for DT-diaphorase activity with 64 nm 3-bromomethylmenadione as substrate and 0.5 mM NADH as cofactor. Similarly, five whole brains obtained from male Fisher 344 rats, 200-300 gm were homogenized and assayed for DT-diaphorase (3-bromomethylmenadione reductase) activity.

high in the neoplastic tissue (Figure 3). Based on these results, one can rationally hypothesize that 3-bromomethylmenadione would be more toxic to the neoplastic 9L cells than to the non-neoplastic cells comprising the whole brain homogenate. A study of the relationship between cellular sensitivity to halogenated quinones and cellular levels of DT-diaphorase is presently underway.

## ACKNOWLEDGEMENT

This research was supported by Program Project Grant CA-13525 from the National Cancer Institute, U.S.A.

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