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PRELIMINARY COMMUNICATION

ANTHRACENEDIONE ACTIVATION BY NADPH-CYTOCHROME P-450 REDUCTASE; COMPARISON WITH ANTHRACYCLINES*

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Metabolic activation of free radical semiquinone intermediates with subsequent formation of superoxide has been demonstrated for a wide variety of quinone-containing antineoplastic agents (1). NADPH-cytochrome P-450 reductase (EC 1.6.2.4) has been established as the flavoprotein which most effectively catalyzes this one-electron transfer from NADPH to oxidized quinone, forming the semiquinone free radical (2,3) which subsequently reacts with molecular oxygen to form superoxide with concomitant regeneration of the oxidized quinone. Both detergent-solubilized NADPH-cytochrome P-450 reductase and trypsin-solubilized cytochrome c reductase catalyze drug-stimulated NADPH oxidation and semiquinone free radical formation (2,4). Formation of the semiquinone free radical and resultant activated oxygen species has been implicated in the etiology of anthracycline-induced cardiotoxicity, as well as in the ability of these agents to bind to DNA and produce cytotoxicity (1,5-7).

The anthracenedione 1,4-dihydroxy-5,8-bis[[(2-hydroxyethyl)amino]ethyl]amino-9,10-anthracenedione (NSC 279836, DHAQ) and its analog 1,4-bis[2-[(2-hydroxyethyl)amino]ethyl]-amino-9,10-anthracenedione diacetate (NSC 287513, HAQ) are two new quinone-containing antineoplastic agents (8). DHAQ has anti-tumor activity equal to or superior to that of Adriamycin (ADM) in several hematopoietic and solid animal tumor models (9-12). Studies using the rat cardiotoxicity model have shown that both DHAQ and HAQ are significantly less cardiotoxic than ADM (9). The dihydrochloride salt of DHAQ (Mitoxantrone) is currently in Phase I and II clinical trials, which have failed to detect any evidence of cardiotoxicity (13,14).

In order to evaluate the metabolic activation of DHAQ or HAQ, we have examined the reduction of these drugs by homogenous NADPH-cytochrome P-450 reductase and rabbit liver microsomes and compared these results with those obtained for ADM and daunorubicin (DNR). The results presented herein show that cytochrome P-450 reductase catalyzed metabolic activation occurs to a substantially smaller degree for DHAQ and HAQ as compared to ADM and DNR, and that HAQ inhibits P-450 reductase activity and oxidative microsomal drug metabolism.

METHODS

Adriamycin, daunorubicin, DHAQ and HAQ were supplied by the Natural Products Branch and Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, and were dissolved in water before use. Detergent solubilized NADPH-cytochrome P-450

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reductase was prepared from liver microsomes of phenobarbital-induced New Zealand rabbits using a 2',5'-ADP agarose affinity column as described previously (15). The enzyme was homogenous as evidenced by the presence of a single band using sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis. Metabolic incubations contained 0.1 M phosphate buffer (pH 7.7), 0.1 mM Na_2EDTA, 0.1 mM NADPH, 0.1 mM quinone drug, and 0.2 to 14 µg/ml enzyme in a final volume of 1.0 ml. NADPH oxidation was measured at 340 nm using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. Superoxide formation was determined by the reduction of acetylated ferricytochrome c (final concentration 17 µM), monitored at 550 nm using an extinction coefficient of 19.6 $mM^{-1}cm^{-1}$ (16). All reactions were carried out in a Cary 219 spectrophotometer at 37°, and were initiated by the addition of enzyme following a 3-min preincubation period.

RESULTS

Spectrophotometric tracings illustrating the stimulation of NADPH consumption by P-450 reductase in the presence of quinone acceptors are shown in Fig. 1A. ADM and DNR produced a marked increase in the rate of NADPH oxidation, as has been reported previously (4). The rates for ADM and DNR of 13.2 and 10.1 µmoles/min/mg protein, respectively, represent 130- and 100-fold increases over the basal rate of 0.1 µmole/min/mg measured in the absence of drug and are in agreement with those reported previously (1,3,4). In contrast, DHAQ addition stimulated NADPH consumption only 21-fold to 2.1 µmoles/min/mg while the rate in the presence of HAQ was only 0.3 µmole/min/mg.

Similar results were obtained for the reduction of acetylated ferricytochrome c, used as an indicator of superoxide generation (Fig. 1B). ADM and DNR caused the reduction of acetylated ferricytochrome c at the rate of 8.9 and 8.6 µmoles/min/mg, respectively, whereas the rate in the presence of DHAQ was only 1.8 µmoles/min/mg. Furthermore, HAQ reduced the

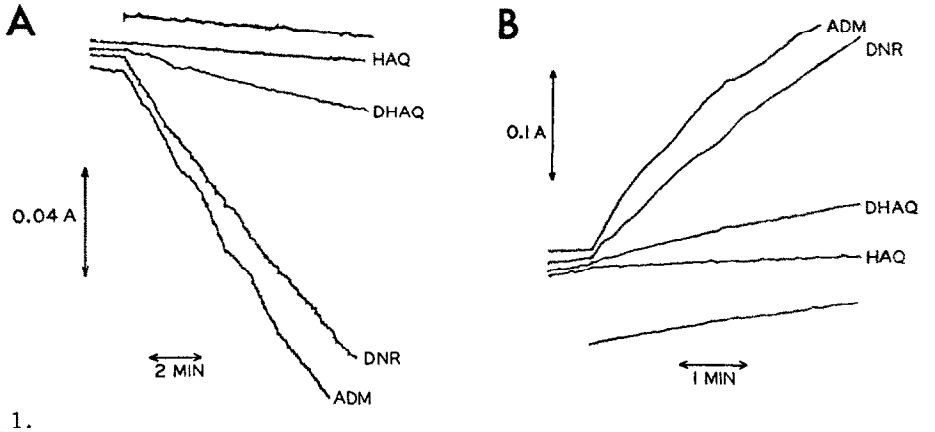

Figure 1.

(A) Quinone drug stimulation of NADPH oxidation. Typical spectrophotometric record illustrates the rate of NADPH oxidation by P-450 reductase in the presence of ADM (13.2 µmoles/min/mg), DNR (10.1 µmoles/min/mg), DHAQ (2.1 µmoles/min/mg) and HAQ (0.3 µmole/min/mg), each present at 100 µM. The unlabeled record shows the basal rate of NADPH oxidation measured in the absence of drug (0.1 µmole/min/mg). The reaction was initiated by the addition of enzyme (arrow).
(B) Quinone drug stimulation of superoxide formation. The increase in absorbance at 550 nm reflects the reduction of acetylated ferricytochrome c (17 µM). The initial slope of the reaction was used to calculate the rate of superoxide formation. Results of a typical experiment show the rate of acetylated ferricytochrome c reduction by P-450 reductase in the presence of ADM (8.9 µmoles/min/mg), DNR (8.6 µmoles/min/mg), DHAQ (1.8 µmoles/min/mg) and HAQ (0.6 µmole/min/mg), each present at 100 µM. The bottom record depicts the basal rate in the absence of drug (1.0 µmole/min/mg).

basal rate of 1.0 μmole/min/mg to only 0.6 μmole/min/mg. Although the rates of NADPH consumption appear greater than those of superoxide production, these differences may be attributed to suboptimalization of acetylated ferricytochrome c concentration, non-stoichiometric reduction of the acetylated ferricytochrome c, or formation of other intermediates (e.g. hydrogen peroxide or other drug metabolites).

Similar results were observed when the above experiments on quinone stimulation of NADPH oxidation and superoxide generation were repeated using rabbit liver microsomes from phenobarbital-induced animals. The activation of ADM and DNR was again significantly greater than that of DHAQ, while HAQ diminished basal NADPH consumption and superoxide formation. Differences in HAQ inhibition of NADPH oxidation and superoxide formation between purified P-450 reductase and microsomes are small, and may result from the effects of HAQ on other enzyme components present in microsomes.

The results of the NADPH oxidation and superoxide generation experiments suggest that, in contrast to the anthracyclines, DHAQ and HAQ are poor substrates for P-450 reductase and that HAQ may even inhibit the activity of this enzyme. To further investigate this behavior, the activity of P-450 reductase toward known substrates in the presence and absence of HAQ was measured. Table 1 shows that menadione reduction, as measured by NADPH consumption, was decreased from 19.4 to 11.2 μmoles/min/mg in the presence of 500 μM HAQ. Cytochrome c reduction was similarly diminished by HAQ, from 31 to 24 μmoles/min/mg in the presence of 250 μM HAQ. As would be expected from data showing inhibition of P-450 reductase, HAQ has also been shown to inhibit microsomal N-demethylase and O-dealkylase activity. For example, the N-demethylation of N,N-dimethylaniline and O-dealkylation of p-nitroanisole are inhibited approximately 40% and 20%, respectively, by 2 mM HAQ. Further studies, however, are required to characterize the type and mechanism of HAQ inhibition of microsomal drug metabolism.

Table 1. Anthracenedione inhibition of NADPH-cytochrome P-450 reductase activity

Substrate	Additions	Activity (μmoles/min/mg)
Menadione*	None	19.4
	500 μM HAQ	11.2
Cytochrome c†	None	31.4
	250 μM HAQ	24.2

*Measured by the oxidation of NADPH using 100 μM menadione under conditions described in Methods.

†Formation of reduced cytochrome c was measured directly at 550 nm using an extinction coefficient of 21 $mM^{-1}cm^{-1}$. The concentration of oxidized cytochrome c was 40 μM. The large absorbance of HAQ at 550 nm interfered with measurement of cytochrome c reduction and precluded use of higher concentrations to maximally inhibit enzyme activity.

DISCUSSION

The metabolism of ADM and DNR by P-450 reductase, as indicated by stimulation of NADPH oxidation, e.p.r. measurement of semiquinone formation and generation of superoxide, has been shown for microsomes, sarcosomes, submitochondrial particles, and purified enzyme (1-4). Our results agree with these previous reports. In contrast, the anthracenedione DHAQ

stimulates NADPH oxidation and superoxide generation only slightly, 5-fold less than that produced by the anthracyclines. HAQ was an even poorer substrate for P-450 reductase, and appears to be an inhibitor of this enzyme.

Several explanations for the diminished activation of the anthracenediones may be proposed. The possibility that these compounds simply do not interact with the enzyme is not supported by the activity demonstrated toward DHAQ. Additionally, the data of Table 1 show that HAQ is able to inhibit normal catalytic activity of P-450 reductase, also suggesting that this class of substrates does interact with the enzyme. Molecular level studies show that DHAQ and HAQ interact with FMN and FAD in a manner similar to ADM and DNR (17-19), however with association constants 2-3 times greater (18). Furthermore, spectral titrations have revealed that DHAQ does indeed interact with P-450 reductase (unpublished observation). Thus, it appears that DHAQ and HAQ may interact with the catalytic site(s) of P-450 reductase, but are substantially less activated.

Single-electron reduction potentials of DHAQ and HAQ may also serve as a basis for explanation of the above results. Powis and Appel (20) showed that, for a series of quinones, metabolism by P-450 reductase was more closely related to the single-electron reduction potential of the substrate than to structural features or lipophilicity. Thus another possibility for lack of DHAQ or HAQ activation is that DHAQ or HAQ may possess reduction potentials that lie outside the range for the enzyme. These reduction potentials, however, have not been reported.

Our results suggest that DHAQ and HAQ are bioactivated at a substantially smaller rate than ADM or DNR, that HAQ inhibits NADPH-cytochrome P-450 reductase activity, and that HAQ also inhibits microsomal oxidative drug metabolism. Further investigations are currently in progress to assess the nature of anthracenedione metabolism by several organ fractions, including cardiac sarcosomes and liver microsomes, as well as the mechanism of inhibition of drug metabolism.

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