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Inhibition of Microsomal Oxidative Drug Metabolism by 1,4-Bis {2-[(2-hydroxyethyl)amino]-ethylamino}-9,10-anthracenedione Diacetate, a New Antineoplastic Agent

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SUMMARY

The effects of 1,4-bis{2-[(2-hydroxyethyl)amino]-ethylamino}-9,10-anthracenedione diacetate (HAQ) on rabbit liver microsomal oxidative drug metabolism were investigated. HAQ was found to inhibit *O*-dealkylase and *N*-demethylase activities in phenobarbital-induced microsomes, and aryl hydrocarbon hydroxylase activity in β-naphthoflavone-induced microsomes. The inhibition was noncompetitive with respect to substrate concentration, with inhibitory constant (K_i) values of 2.9, 2.6, and 3.0 mM for *p*-nitroanisole, *N,N*-dimethylaniline, and benzo[*a*]pyrene, respectively. In contrast, HAQ failed to inhibit *p*-nitroanisole metabolism when the reaction was supported with cumene hydroperoxide. HAQ also inhibited basal and substrate-stimulated microsomal NADPH oxidation. The degree of inhibition of NADPH oxidation and product formation were comparable. These data, in conjunction with the results of previous studies, suggest that HAQ inhibits electron transfer by microsomal NADPH-cytochrome P-450 reductase, diminishing electron flow to cytochrome P-450 and thereby inhibiting substrate metabolism. This mechanism differs markedly from that for inhibition of drug metabolism by other quinones, such as menadione, in which accelerated electron flow through P-450 reductase to the quinone diverts reducing equivalents from cytochrome P-450.

INTRODUCTION

The cumulative dose-dependent cardiotoxicity associated with the anthracycline antineoplastic agent Adriamycin has stimulated development of structurally similar quinone-containing agents with the objective of identifying a compound which possesses comparable antitumor efficacy, but with diminished cardiotoxicity. HAQ[2] is a new anthracenedione antineoplastic agent which displays excellent activity *in vivo* against several transplantable mouse tumor models (1–3). HAQ is significantly less cardiotoxic, however, than both Adriamycin and daunorubicin, as demonstrated by Cheng *et al.* (2) using the rat cardiotoxicity model. HAQ is also less cardiotoxic than its hydroxylated derivative, DHAD (mitoxantrone), currently employed in several Phase I and II clinical trials (4, 5).

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[2] The abbreviations used are: HAQ, 1,4-bis{2-[(2-hydroxyethyl)amino]-ethylamino}-9,10-anthracenedione diacetate (NSC 287513); DHAD, 1,4-dihydroxy-5,8-bis{2-[(2-hydroxyethyl)amino]-ethylamino}-9,10-anthracenedione dihydrochloride (NSC 301739).

Several investigators have demonstrated that quinone-containing antineoplastic agents serve as substrates for flavoprotein reductases such as NADPH-cytochrome P-450 reductase and mitochondrial NADH dehydrogenase (6–9). In a preliminary communication we showed that, in contrast to the anthracyclines Adriamycin and daunorubicin, the anthracenediones HAQ and DHAD undergo substantially less reductive metabolism by purified NADPH-cytochrome P-450 reductase (10). These studies also demonstrated that HAQ effectively inhibited the activity of this enzyme toward oxidized acceptors such as cytochrome *c* and menadione.

The critical role of cytochrome P-450 reductase in electron transfer between NADPH and cytochrome P-450 in the microsomal mixed-function oxidase system suggests that inhibition of this enzyme by HAQ may adversely affect microsomal drug metabolism. Indeed, initial investigations provided evidence that such inhibition did occur (10). In the present studies, the effect of HAQ on hepatic microsomal oxidative drug metabolism has been characterized. Results show that HAQ noncompetitively inhibits *N,N*-dimethylaniline *N*-demethylase, *p*-nitroanisole *O*-dealkylase, and aryl hydrocarbon hydroxylase activities of rabbit liver microsomal preparations and suggest that this inhibition results from the effects of HAQ on microsomal NADPH-cytochrome P-450 reductase activity.

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MATERIALS AND METHODS

HAQ was supplied by Mr. Len Kedda, of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). The drug exhibited a single peak following reverse-phase high-pressure liquid chromatography on a Whatman Partisil PXS 10/25 ODS-3 column using a mobile phase of 4.4 M ammonium formate (pH 4.3):acetonitrile:water (2:1:1) (11), and was used without further purification. Stock solutions (5 mM) were freshly prepared in deionized water. Renex 690 was the gift of ICI Americas (Wilmington, Del.). *N,N*-Dimethylaniline and benzo[*a*]pyrene were obtained from Aldrich Chemical Company (Milwaukee, Wisc.), and *p*-nitroanisole was obtained from Eastman Chemical Company (Rochester, N. Y.). All other chemicals were reagent-grade and obtained from commercial sources.

Preparation of microsomes. New Zealand White male rabbits (2.0–2.5 kg) were obtained from Lesser's Rabbitry (Union Grove, Wisc.) and maintained on standard laboratory food and water *ad libitum*. Animals were induced by drinking water containing 0.1% sodium phenobarbital for 5 days, or by i.p. injection of β-naphthoflavone (80 mg/kg) 48 hr prior to sacrifice. The rabbits were fasted for 24 hr and killed by cervical dislocation; their livers were perfused *in situ* with cold isotonic saline prior to excision. Pyrophosphate-extracted microsomes were prepared as described previously (12, 13) and stored at −85° in 50 mM Tris acetate buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol. Protein content was determined according to the method of Lowry *et al.* (14), using bovine serum albumin as a standard.

Cytochrome P-450 content was determined by the method of Omura and Sato (15). In the presence of HAQ, addition of dithionite to microsomes reduced the quinone, as well as P-450, interfering with quantitation of the cytochrome at 450 nm. To quantitate P-450 in the presence of HAQ, microsomes were centrifuged, washed once with 0.1 M potassium phosphate buffer, and applied to a column (0.4 × 20 cm) of Amberlite XAD-2 (20–50 mesh) resin (16). The microsomes were eluted from the column with phosphate buffer while the HAQ remained adsorbed onto the resin. The P-450 and protein contents of the eluted microsomes were then determined.

Microsomal incubations and drug metabolism. Microsomal incubations were performed at 37° in an oscillating water bath, using a reaction mixture which contained 0.1 M potassium phosphate buffer (pH 7.5), 1 mM NADPH, 10–300 μM *p*-nitroanisole, or 50–800 μM dimethylaniline, and 1 mg of microsomal protein in a final volume of 1.0 ml. Reactions were initiated after a 2-min preincubation period by the addition of NADPH and terminated after 10 min by the addition of 0.25 ml of cold 20% trichloroacetic acid. Aryl hydrocarbon hydroxylase activity was determined under similar conditions, using a 1.0-ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.25), 360 μM NADPH, 1–100 μM benzo[*a*]pyrene, and 1 mg of protein. The reaction was terminated after 10 min by the addition of 1.0 ml of cold acetone. Reaction rates were linear over the time period employed.

Conventional methods for the determination of *N,N*-dimethylaniline and *p*-nitroanisole dealkylation, and of benzo[*a*]pyrene hydroxylase activities were complicated by the presence of HAQ. Although a limited amount of HAQ was removed with the precipitated microsomal protein, a considerable amount remained in the supernatant. HAQ has a large absorbance at both 412 and 420 nm, the wavelengths used to quantitate the Nash adduct of formaldehyde and *p*-nitrophenol, respectively (Fig. 1). The intense broad absorption spectrum of HAQ precluded product quantitation at other wavelengths and necessitated modification of standard procedures.

N,N-Dimethylaniline *N*-demethylase activity was measured by quantitation of formaldehyde production according to the method of Nash (17). After termination of the reaction with trichloroacetic acid and pelleting the microsomal protein, 0.75 ml of the supernatant was mixed with 0.3 ml of double-strength Nash reagent and the mixture was incubated at 37° for 45 min. The absorbance at 412 nm, reflecting contributions from both the Nash formaldehyde adduct and HAQ, and at 648 nm, the isosbestic point of HAQ, was recorded. The absorbance at 648 nm was used to calculate the HAQ concentration, using an extinction coefficient of 7.44 mM^{-1} cm^{-1}. This calculated HAQ concentration was then used in conjunction with a standard curve (Fig. 3) to assess the HAQ contribution to A_{412}, which was then subtracted from the observed A_{412} to yield the net absorbance of the Nash adduct. Formaldehyde production was then determined from a standard curve. The absorbance of the Nash adduct at 648 nm was negligible, and hence did not interfere with HAQ quantitation.

p-Nitroanisole *O*-demethylase activity was assayed by quantitation of *p*-nitrophenol production after 10-min microsomal incubations, using a modification of previous procedures (18). Following the precipitation of microsomal protein, 0.5 ml of 4 M KOH was added to the supernatant. The mixture was vigorously extracted three times with 1 ml of chloroform, which removed all remaining HAQ. The absorbance of the aqueous layer was recorded at 420 nm, and product formation was determined from a standard curve. The three chloroform extractions did not remove any *p*-nitrophenol from the aqueous layer.

Hydroperoxide-dependent dealkylation of *p*-nitroanisole was measured in a Cary 219 spectrophotometer at 30° using the method of Koop and Hollenberg (19). The 1.0 ml-reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.70), 100 μM EDTA, 3 mM *p*-nitroanisole, 1 mM cumene hydroperoxide or NADPH, and 0.5 mg of microsomal protein. The reaction was initiated by the addition of NADPH or cumene hydroperoxide, and *p*-nitrophenol formation at 403 nm was monitored with time. Specific activity was calculated using an extinction coefficient for *p*-nitrophenol of 14.9 mM^{-1} cm^{-1} (19). Hydroperoxide-supported *p*-nitrophenol formation was also determined using a 5-min microsomal incubation followed by extraction with chloroform as described above, with the substitution of 1 mM cumene hydroperoxide for NADPH.

Aryl hydrocarbon hydroxylase activity was determined essentially as described by Nebert and Gelboin (20). Extraction of the acetone-precipitated reaction mixture with hexane effectively removed benzo[*a*]pyrene and its metabolites while HAQ remained in the aqueous acetone

phase. Fluorescence of the alkali-extracted metabolites was determined by using a Farrand Mark I spectrofluorimeter with excitation at 396 nm and emission at 522 nm. Product formation was quantitated by using a standard curve prepared by adding 3-hydroxybenzo[*a*]pyrene standard (obtained from the National Institutes of Health) to the reaction mixture in place of benzo[*a*]pyrene.

Endogenous and substrate-stimulated rates of microsomal NADPH oxidation were determined in a medium containing 0.1 M phosphate buffer (pH 7.7), 100 μM EDTA, 100 μM NADPH, and 215 μg of microsomal protein. NADPH oxidation was monitored at 340 nm, using an extinction coefficient of 6.22 mM^{-1} cm^{-1} following addition of NADPH after a 2-min preincubation period.

Spectral titrations characterizing the behavior of HAQ were performed at 25° in a Cary 219 spectrophotometer operating in the autoslit mode. Aliquots (1–10 μl) of a stock solution of HAQ (5 mM) were added stepwise to 0.1 M potassium phosphate buffer (pH 7.5). Final concentrations were corrected for dilution.

Statisitcal comparison of the data was performed using a one-way analysis of variance.

RESULTS

Characteristics of HAQ. HAQ is a basic molecule which is freely soluble in water or buffer in the concentration range employed (1 μM–5 mM). The UV-visible absorption spectrum displayed three distinct peaks at 258 nm, 583 nm, and 627 nm (Fig. 1). The absorbance of the three peaks varied, depending on pH and concentration (Fig. 2). The extinction coefficient of the peak at 627 nm was particularly sensitive to drug concentration, suggesting self-association of drug molecules. This self-association has been confirmed by NMR spectroscopy (22). The existence of a wavelength at which the extinction coefficient did not change with concentration (isosbestic point at 648 nm; $\epsilon = 7.44$ cm^{-1} mM^{-1}) allowed the spectrophotometric determination of HAQ concentrations in solution (Fig. 2). At this wavelength, solutions in the range 5–250 μM obeyed Beer's Law. The absorbance of an HAQ solution at any other wavelength could then be determined from a standard curve, such as the one shown in Fig. 3, which was used in the formaldehyde assay of demethylase activity to calculate the absorbance of HAQ at 412 nm.

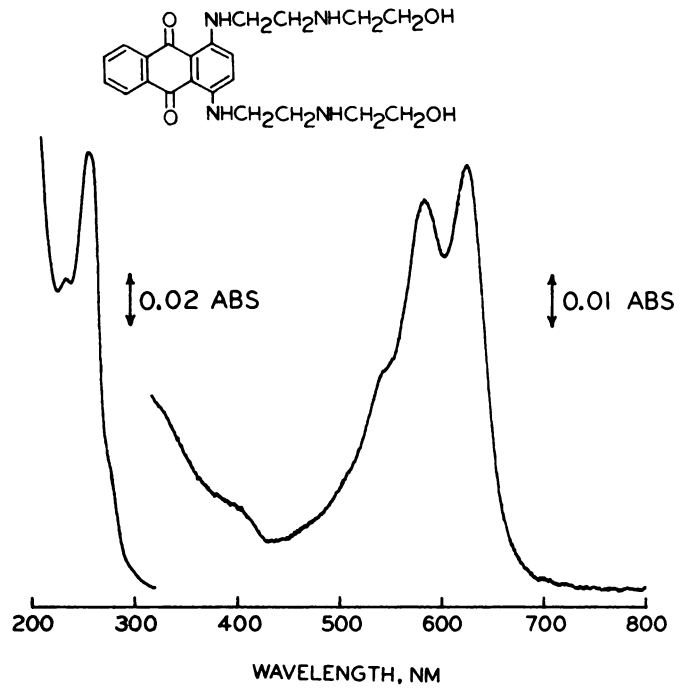

FIG. 1. *UV-visible absorption (ABS) spectrum of 5 μM HAQ in 100 mM potassium phosphate buffer (pH 7.5) obtained in a 1.0-cm path length cuvette at room temperature*

Three distinct absorption maxima occur at 258 nm, 583 nm, and 627 nm with minor shoulders at 234 nm and 522 nm. The structure of HAQ is shown at the *top*.

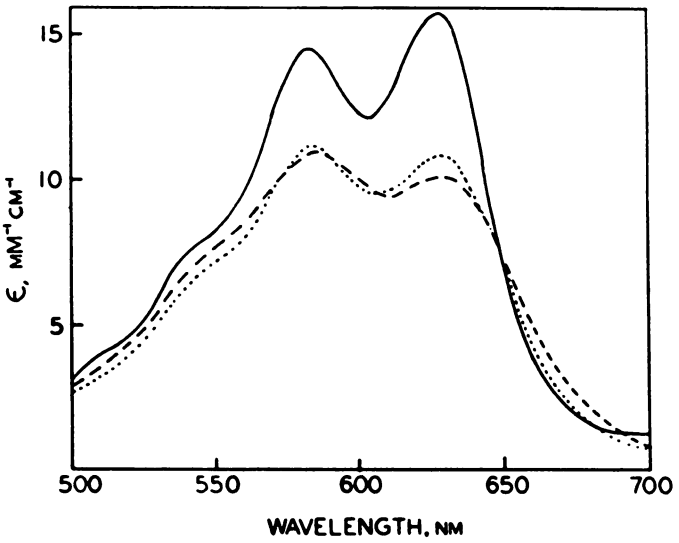

FIG. 2. *Visible absorption spectra of HAQ at concentrations of 10 μM (——), 75 μM (· · · ·), and 215 M (– – –)*

The extinction coefficient at 648 nm (7.44 mM^{-1} cm^{-1}) remained constant over the concentration range 5–250 μM and provided a wavelength at which to determine the concentration of an HAQ solution. The increase in relative absorbance of the peak at 627 nm ($\epsilon_{627}/\epsilon_{583}$) indicated self-association of HAQ in solution and was similar to the increase observed with mitoxantrone (21). Self-association of HAQ and mitoxantrone in solution has been confirmed and characterized by proton NMR spectroscopy (22).

Microsomal drug metabolism. HAQ was effective in inhibiting basal and substrate-stimulated NADPH oxidation. The rate of microsomal NADPH oxidation in the absence of substrate was decreased from 4.3 to 3.3 nmoles/min in the presence of 200 μM HAQ (Table 1). The addition of *p*-nitroanisole, *N,N*-dimethylaniline, or benzo[*a*]pyrene stimulated the rate of NADPH oxidation, as expected. NADPH oxidation in the presence of dimethylaniline, benzo[*a*]pyrene, or *p*-nitroanisole was inhibited 21%, 29%, and 65%, respectively, when 200 μM HAQ was present (Table 1).

HAQ also inhibited product formation from these substrates in microsomal incubations. Figure 4 shows the saturation curve for demethylation of *p*-nitroanisole by phenobarbital-induced microsomes, and the effect of various concentrations of HAQ on product formation. The apparent K_m for *p*-nitroanisole demethylation, 0.03 mM, was unchanged by the addition of HAQ, which produced pure noncompetitive inhibition (Fig. 4B). Linear replots

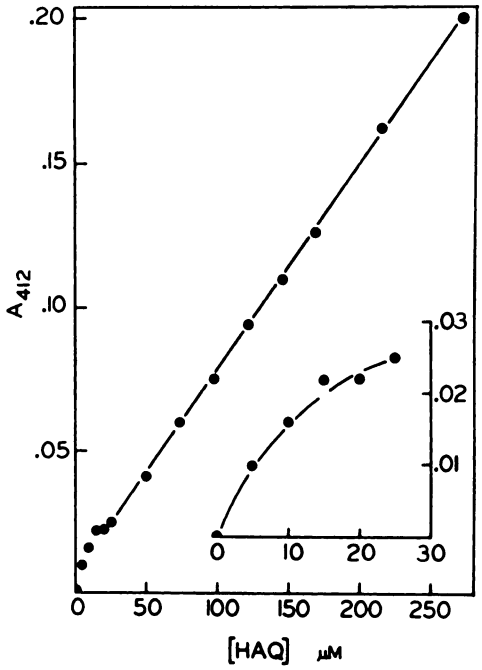

FIG. 3. *Standard curve of the absorbance at 412 nm versus concentration of HAQ*

The relationship was linear over the range 30–250 μM HAQ but deviated from linearity at lower concentrations. The absorbance in the range 0–30 μM HAQ is shown expanded in the *inset*. This curve was used in the modified assay of *N*-demethylase activity to quantitate the contribution of HAQ absorbance at 412 nm to that of the Nash formaldehyde adduct.

of slope^{-1} or V_{max}^{-1} (from the Lineweaver-Burk plot) versus HAQ concentration (data not shown) confirmed the pure noncompetitive inhibition in contrast to partial noncompetitive inhibition. Plotting the reciprocal of rate versus HAQ concentration also showed pure noncompetitive inhibition, and yielded K_i = 2.9 mM (*inset*, Fig. 4B).

The *N*-demethylation of *N,N*-dimethylaniline was also inhibited by HAQ in phenobarbital-induced microsomes. Figure 5A displays the saturation curve for formaldehyde formation and the inhibitory effect of various HAQ concentrations. The noncompetitive nature of the inhibition is demonstrated by the double-reciprocal plot in Fig. 5B. Linear replots of the slopes and intercepts from Fig. 5B, as well as the Dixon plot (*inset*) confirmed noncompetitive inhibition, and gave K_i = 2.6 mM. *N*-Demethylase activity was not affected by addition of 2 or 6 mM acetate, indicating that the acetate anion of HAQ was not responsible for inhibition of drug metabolism activity.

The effect of HAQ on aryl hydrocarbon hydroxylase activity in β-naphthoflavone-induced microsomes was also studied. Figure 6A shows the saturation curve for product formation in the absence and presence of 1 or 3 mM HAQ. The inhibition by HAQ was again noncompetitive, with a K_i of 3.0 mM (Fig. 6B and *inset*).

In order to determine whether metabolites of HAQ or the parent molecule were responsible for the inhibition

TABLE 1

Effect of HAQ on basal and substrate-stimulated NADPH oxidation

NADPH oxidation was determined as described under Materials and Methods, using 215 μg of protein from phenobarbital-induced microsomes. Results are expressed as the mean ± standard error of five determinations.

Substrate	NADPH oxidation		% Inhibition
	0 μM HAQ	200 μM HAQ	
	nmoles/min		
Buffer	4.30 ± 0.19	3.30 ± 0.14[a]	23
100 μM *p*-nitroanisole	5.74 ± 0.43	2.03 ± 0.17[a]	65
250 μM *N,N*-dimethylaniline	9.39 ± 0.23	7.40 ± 0.38[a]	21
30 μM benzo[*a*]pyrene	7.04 ± 0.43	5.03 ± 0.24[a]	29

[a] Significantly different from the absence of HAQ ($p < 0.01$).

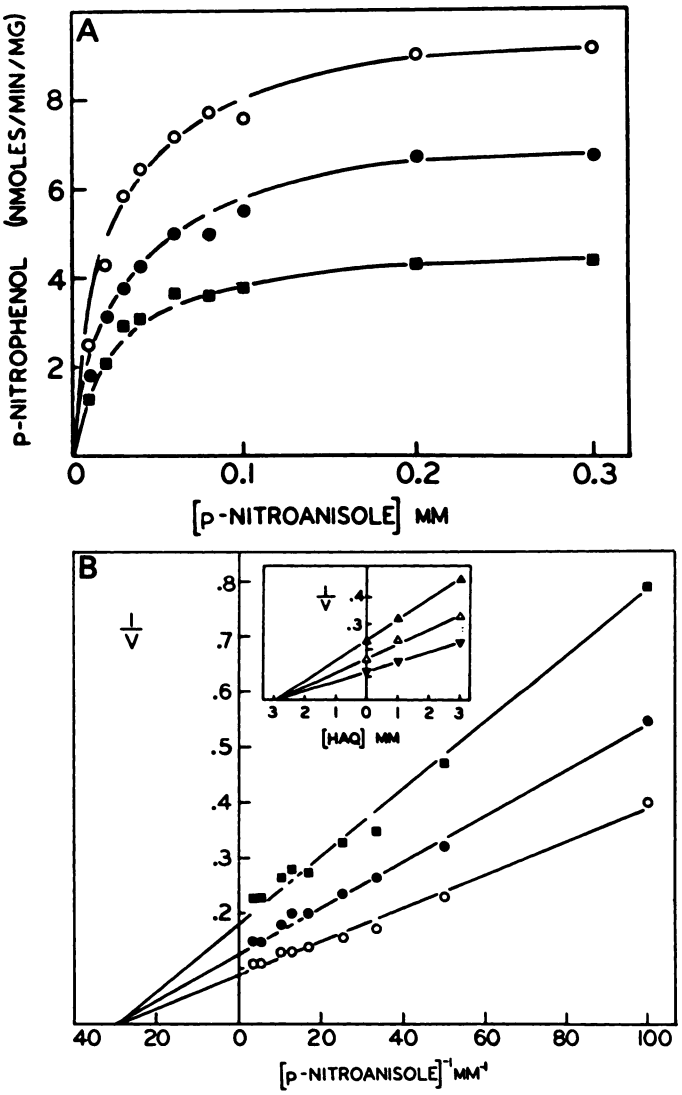

FIG. 4. *Inhibition of p-nitroanisole O-demethylation by HAQ*

Rates of *p*-nitrophenol production were assayed as described under Materials and Methods, using the method of Shigematsu *et al.* (18) with phenobarbital-induced microsomes and a 10-minute incubation period.

A. Saturation kinetics of *p*-nitrophenol formation in the absence (○) or presence of 1 mM (●) or 3 mM (■) HAQ.

B. Double-reciprocal representation of product formation in the absence or presence of HAQ. The *inset* shows a Dixon plot of *p*-nitrophenol formation from 0.02 mM (▲), 0.04 mM (△), or 0.2 mM (▼) *p*-nitroanisole. Each data point represents the mean of six to nine determinations with a standard error of less than 6%.

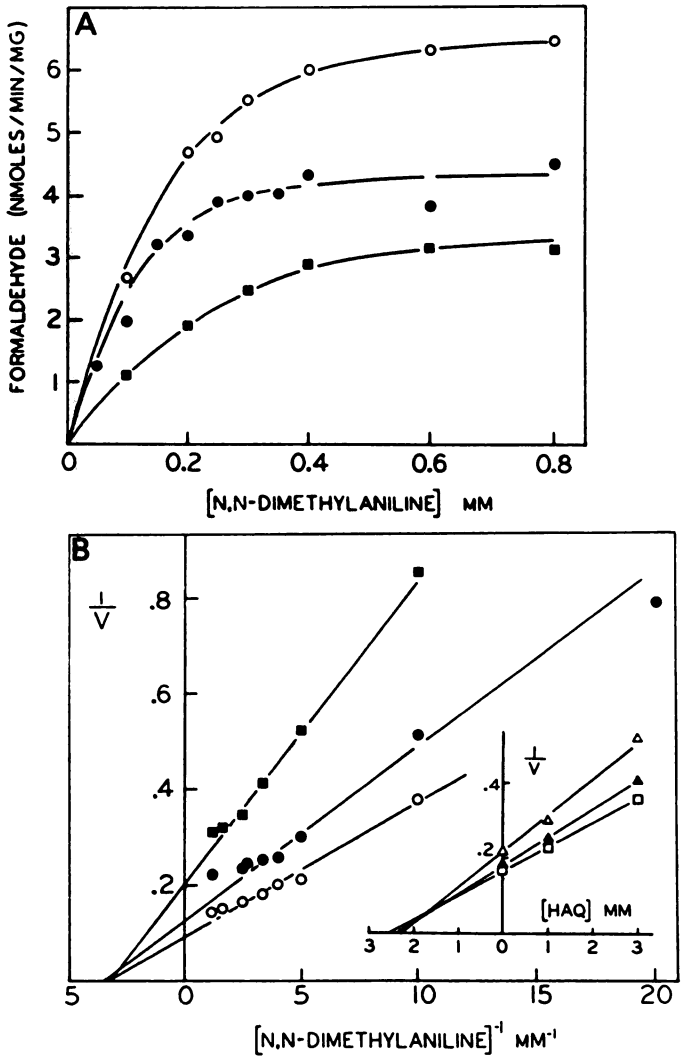

FIG. 5. *Inhibition of N,N-dimethylaniline N-demethylation by HAQ*

Rates of formaldehyde production were assayed as described under Materials and Methods, using microsomes from phenobarbital-induced rabbits.

A. Saturation plot of formaldehyde formation in the absence (○) or presence of 1 mM (●) or 3 mM (■) HAQ.

B. Lineweaver-Burk plot of formaldehyde production in the absence or presence of HAQ. The *inset* shows a Dixon-type representation of formaldehyde production from 0.2 mM (△), 0.3 mM (▲), or 0.4 mM (□) dimethyaniline. Each data point represents the mean of 6–12 determinations with a standard error of less than 8%.

of drug metabolism, HAQ was incubated with microsomes for 10 min prior to addition of *N,N*-dimethylaniline (Table 2). In the absence of HAQ, preincubation had little effect on the rate of formaldehyde production. As expected (Fig. 5A), inclusion of 2 mM HAQ decreased formaldehyde production, from 6.65 to 5.65 nmoles/min/mg. Ten-minute preincubation of HAQ with microsomes and NADPH enhanced the HAQ inhibition of demethylase activity by 21%. However, a supplemental addition of 0.5 mM NADPH immediately prior to addition of dimethylaniline negated the effects of preincubation. These results suggest that, although prior incubation of HAQ with microsomes does enhance the HAQ inhibition of demethylase activity, this effect may be due to elevated NADP/NADPH ratios. It appears, therefore, that the parent drug, rather than metabolites of HAQ, is responsible for the inhibitory effect on drug metabolism.

To investigate the possibility that HAQ inhibition of drug metabolism was the result of inactivation of cytochrome P-450, levels of the cytochrome were monitored before and after a 10-min incubation. Under conditions identical with those employed in the assays (Figs. 4–6), inclusion of HAQ did not detectably increase the levels of cytochrome P-420 (data not shown). Furthermore, addition of HAQ to microsomes produced no difference spectrum in the Soret region, suggesting that HAQ did not interact with cytochrome P-450 (data not shown).

Organic hydroperoxides can substitute for NADPH and oxygen to support liver microsomal cytochrome P-

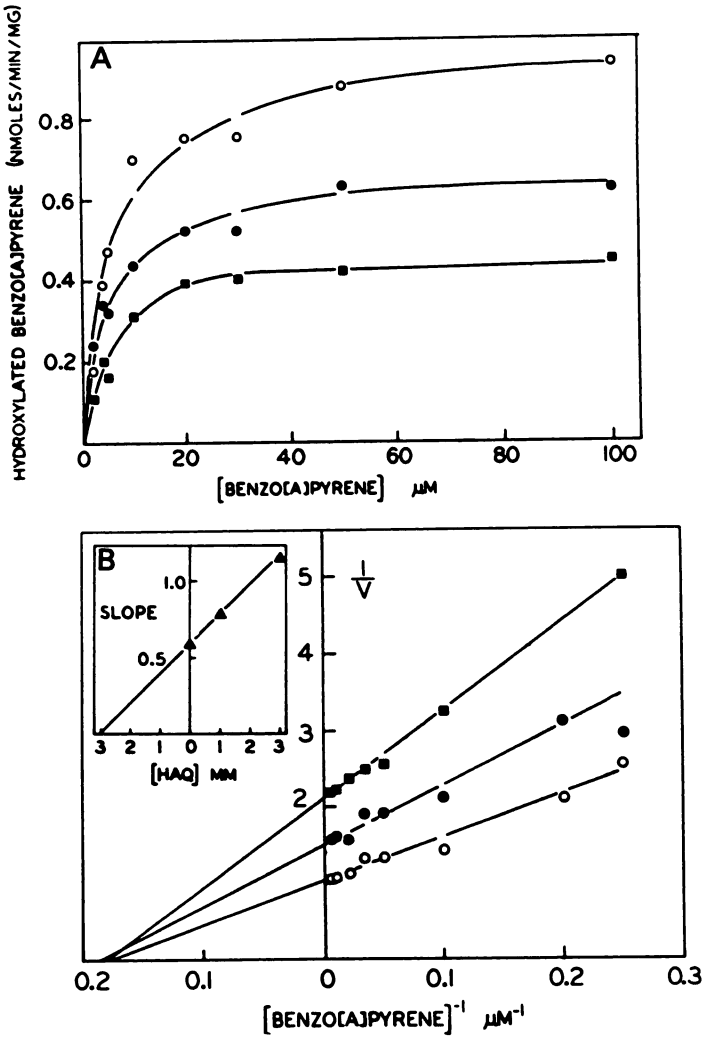

FIG. 6. *Inhibition of aryl hydrocarbon hydroxylase activity by HAQ.*

Total hydroxylated benzo[*a*]pyrene metabolite formation catalyzed by β-naphthoflavone-induced microsomes was determined as described under Materials and Methods.

A. Saturation behavior of benzo[*a*]pyrene metabolism in the absence (○) or presence of 1 mM (●) or 3 mM (■) HAQ.

B. Lineweaver-Burk representation of hydroxylated product formation. The *inset* shows a replot of slope *versus* HAQ concentration. Each data point represents the mean of 5–10 determinations with a standard error of less than 10%.

TABLE 2

Effect of microsomal preincubation in the absence and presence of HAQ on inhibition of N-demethylase activity

Microsomes (1 mg/ml) from phenobarbital-induced rabbits were incubated at 37° with buffer and 1.0 mM NADPH in the absence and presence of 2 mM HAQ for the time indicated. At the conclusion of the preincubation period, 0.8 mM *N,N*-dimethylaniline was added to start the reaction. Formaldehyde production was determined after 10 min as described under Materials and Methods and expressed as mean ± standard error of the number of determinations indicated in parentheses.

HAQ	Preincubation	Supplemental addition[a]	Formaldehyde production	% Inhibition
mM	*min*		*nmoles/min/mg*	
0	0	None	6.65 ± 0.07 (24)	—
0	10	None	6.11 ± 0.08 (12)	8
0	10	0.5 mM NADPH	6.43 ± 0.17 (12)	3
2	0	None	5.65 ± 0.09 (24)	—
2	10	None	4.46 ± 0.09 (12)[b]	21
2	10	0.5 mM NADPH	5.25 ± 0.10 (12)	6

[a] Additional NADPH (0.5 mM) was added to the reaction mixture at the conclusion of the preincubation period, immediately prior to addition of dimethylaniline. Supplemental NADPH was added to compensate for NADPH consumption which occurred during the preincubation period.

[b] Significantly different from $t = 0$ in the presence of 2 mM HAQ ($p < 0.005$).

TABLE 3

Effect of HAQ on cumene hydroperoxide supported p-nitroanisole O-dealkylation

p-Nitrophenol formation was determined at 30° by the increase in absorbance at 403 nm. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.70), 0.1 mM EDTA, 3 mM *p*-nitroanisole, 538 μg of microsomal protein, and 1 mM NADPH or cumene hydroperoxide (added to initiate the reaction). Results are expressed as the mean ± standard error of the number of determinations indicated in parentheses.

Cofactor	HAQ	*p*-Nitrophenol formation	% Inhibition
	μM	*nmoles/min/mg*	
NADPH	0	3.09 ± 0.03 (10)	—
	500	2.65 ± 0.02 (6)[a]	17%
Cumene hydroperoxide	0	9.4 ± 1.1 (7)	—
	500	14.5 ± 0.6 (5)[a]	(55%)[b]

[a] Significantly different in the presence of HAQ ($p < 0.001$).

[b] Percentage stimulation.

450-catalyzed oxidation reactions. The effect of HAQ on cumene hydroperoxide supported formation of *p*-nitrophenol in hepatic microsomes was initially estimated by using 5-min microsomal incubations and the method of Shigematsu *et al.* (18). HAQ was observed to stimulate the rate of *p*-nitrophenol formation. A second, more direct, spectrophotometric estimation of product formation was then employed to confirm the results, as summarized in Table 3. Using NADPH as a source of reducing equivalents, product formation was inhibited 17% in the presence of 0.5 mM HAQ. In contrast, when cumene hydroperoxide was used in place of NADPH, HAQ failed to inhibit microsomal activity and actually stimulated *p*-nitrophenol formation, as observed using 5-min incubations.[3] Such stimulation of product formation may result from either HAQ-enhanced solubility of the extremely hydrophobic hydroperoxide or a detergent-like effect of HAQ on the microsomal suspension.

[3] Only the results obtained using the spectrophotometric assay are presented for the sake of brevity.

DISCUSSION

HAQ is a new anthracenedione antineoplastic agent that has shown promising antitumor efficacy with no evidence of causing the fatal cardiomyopathy which has severely limited the use of the anthracycline agents Adriamycin and daunorubicin. Preliminary studies demonstrated that HAQ inhibited microsomal NADPH oxidation and acetylated cytochrome *c* reduction, and diminished the NADPH-cytochrome P-450 reductase-catalyzed reduction of menadione and native cytochrome *c* (10).

The present studies have revealed that HAQ noncompetitively inhibits the hepatic microsomal oxidative metabolism of several different classes of substrates. For all three of the compounds studied, comparable inhibition of product formation occurred at equimolar HAQ concentrations with inhibitory constant values, K_i, of 2.6 mM, 2.9 mM, and 3.0 mM obtained using dimethylaniline, *p*-nitroanisole, and benzo[*a*]pyrene, respectively. The concentration of HAQ required to produce 50% inhibition was unrelated to the apparent substrate K_m, which was 0.3 mM for dimethylaniline, 30 μM for *p*-nitroanisole and 6 μM for benzo[*a*]pyrene, suggesting that HAQ did not interfere with substrate binding. HAQ was equieffective in inhibiting the mixed-function oxidase activity of either phenobarbital or β-naphthoflavone-induced hepatic microsomes. The inhibitory effects of HAQ were attributed to the parent molecule rather than any active mtabolites.

In addition to inhibiting product formation, HAQ also inhibited basal and substrate-stimulated microsomal NADPH oxidation (Table 1). For dimethylaniline and benzo[*a*]pyrene, the degree of inhibition of NADPH oxidation was comparable to that of product formation. For example, at a ratio of 0.9 μmole of HAQ per milligram of protein (200 μM HAQ with 215 μg of microsomal protein per milliliter), the oxidation of NADPH in the presence of 250 μM dimethylaniline was inhibited 21% (Table 1). Similarly, at a ratio of 1.0 μmole of HAQ per milligram of protein (1 mM HAQ and 1 mg of protein per milliliter), formaldehyde production from the same concentration of dimethylaniline was inhibited 20% (Fig. 4). Furthermore, basal, benzo[*a*]pyrene-stimulated and dimethylaniline-stimulated NADPH oxidation were comparably inhibited by 200 μM HAQ.

The results of the present studies in conjunction with previous observations (10) suggest that the inhibition of microsomal mixed-function oxidase activity by HAQ is the result of inhibition of microsomal NADPH-cytochrome P-450 reductase activity. HAQ inhibition of electron flow through P-450 reductase to cytochrome P-450 would be expected to be manifested as noncompetitive inhibition with respect to substrate concentration. The failure of HAQ to alter either substrate binding to cytochrome P-450 or the levels of the cytochrome in the microsomal suspension and the inability of HAQ to elicit a P-450 difference spectrum suggest that HAQ does not act on cytochrome P-450. Additional evidence for HAQ inhibition of NADPH-cytochrome P-450 reductase activ-

ity may be derived from the inhibitory effects of HAQ on purified P-450 reductase activity toward known electron acceptors (10).

Further support for the concept that HAQ acts mechanistically at a P-450 reductase-dependent step derives from studies on the effect of HAQ on organic hydroperoxide-supported microsomal metabolism. Cytochrome P-450 can use organic hydroperoxides as a source of reducing equivalents and oxygen to support *N*- and *O*-dealkylation reactions, eliminating the requirement for NADPH, cytochrome P-450 reductase, and molecular oxygen (19, 23–25). We examined the effect of HAQ on the cumene hydroperoxide-supported *O*-demethylation of *p*-nitrophenol. When NADPH was used to support the reaction, *p*-nitrophenol formation was decreased 17% by 0.5 mM HAQ, whereas no inhibition was observed when the reaction was supported by cumene hydroperoxide. Thus, HAQ failed to inhibit microsomal oxidative activity when P-450 reductase was not required.

Although inhibition of product formation from *p*-nitroanisole was comparable to that of the other substrates, inhibition of NADPH oxidation in the presence of *p*-nitroanisole was more than 2-fold greater as compared with the other two substrates, as well as to the degree of *p*-nitrophenol formation. These results may be clearly understood by examining the pathways of *p*-nitroanisole metabolism. NADPH oxidation in the presence of *p*-nitroanisole represents electron flow through P-450 reductase for both *p*-nitrophenol formation and aerobic reduction of the nitro group to the nitro-anion free radical. Consequently, HAQ inhibition of P-450 reductase activity would diminish NADPH oxidation more than *p*-nitrophenol formation. Furthermore, such inhibition would diminish NADPH oxidation in the presence of *p*-nitroanisole more than in the presence of either dimethylaniline or benzo[*a*]pyrene.

Inhibition of microsomal oxidative drug metabolism by quinone-containing compounds is a well-known phenomenon, first reported by Gillette *et al.* (26) for menadione inhibition of antipyrene demethylation. It has also been reported by Shen *et al.* (27) that benzo[*a*]pyrene 1,6-, 3,6-, and 6,12-quinones inhibited the microsomal mixed-function oxidation of benzo[*a*]pyrene and *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene in a noncompetitive manner.

Quinone inhibition of microsomal metabolism has been proposed to occur via an electron shunt. Menadione accelerates the flow of reducing equivalents through P-450 reductase to molecular oxygen, thereby decreasing electron flow to cytochrome P-450. Although substrate hydroxylation is decreased or prevented by menadione, microsomal NADPH oxidation, oxygen consumption, and hydrogen peroxide production are stimulated (26). Cytochrome *c* and paraquat similarly inhibit microsomal metabolism by diversion of reducing equivalents away from cytochrome P-450 (26, 28). Preliminary experiments suggest that the ring-hydroxylated HAQ derivative, DHAD, also inhibits drug metabolism by this mechanism (29).

A similar shunt mechanism for the noncompetitive inhibition of benzo[*a*]pyrene and 7,8-dihydrodiol metabolism by benzo[*a*]pyrene 1,6-, 3,6-, and 6,12-quinones has been proposed (27). Supporting this conclusion was the observation that the benzo[*a*]pyrene quinones were reduced under aerobic and anaerobic conditions by both microsomes and purified P-450 reductase. Paradoxically, however, the extent of inhibition of benzo[*a*]pyrene hydroxylation by 1,6-, 3,6-, and 6,12-quinones was inversely proportional to their rate of anaerobic reduction. The effect of the quinones on NADPH oxidation or rates of aerobic reduction were not reported.

In contrast to the above compounds, the inhibition by HAQ of mixed-function oxidase activity apparently does not occur by diversion of reducing equivalents from cytochrome P-450, with accelerated electron flow through P-450 reductase. Rather, HAQ appears to diminish electron flow through cytochrome P-450 reductase, and thus to P-450. Microsomal NADPH oxidation was decreased by HAQ both in the absence and presence of substrate (Table 1). HAQ also inhibited superoxide generation in microsomes as well as hydrogen peroxide formation catalyzed by purified cytochrome P-450 reductase.[4] Elucidation of the mechanism by which HAQ inhibits electron transfer by P-450 reductase requires further investigation.

The inhibitory properties of HAQ toward drug metabolism may have important therapeutic consequences, and are unique among antineoplastic agents which inhibit drug metabolism in that HAQ appears not to require prior bioactivation for inhibitory effects. Several other antineoplastic agents have also been shown to inhibit oxidative drug metabolism in microsomes. Both procarbazine and hexamethylmelamine produced mixed inhibition of *p*-nitroanisole *O*-demethylation (30). The inhibition of drug metabolism by cyclophosphamide has also been extensively studied both *in vitro* and *in vivo* (30–32). All of these agents require bioactivation by the mixed-function oxidase system for cytotoxic activity (33), and the active metabolites may also mediate the inhibition of mixed-function oxidase activity. For example, inhibition of *p*-nitroanisole demethylation by procarbazine was found to be mediated by a metabonate (30). Furthermore, activation of cyclophosphamide was necessary for inhibitory effects on drug metabolism. The cyclophosphamide metabolite acrolein inhibited both microsomal and purified NADPH-cytochrome P-450 reductase, whereas the parent compound had no effect on reductase activity (32).

In contrast, HAQ apparently does not require bioactivation for inhibitory activity toward purified and microsomal P-450 reductase. We have shown that HAQ is a poor substrate for P-450 reductase and microsomes (10). Furthermore, HAQ inhibits endogenous microsomal NADPH consumption (Table 1), suggesting little microsomal metabolism. As might be expected, HAQ also inhibits endogenous and Adriamycin-stimulated microsomal lipid peroxidation.[4]

Inhibition of mixed-function oxidase activity by HAQ *in vivo* may have important consequences that could affect the therapeutic use of this drug. Several antineoplastic agents (for example, cyclophosphamide) require mixed-function oxidase activity for metabolism to a cytotoxic species (33). Since many antineoplastic agents are combined in multidrug regimens, use of HAQ in such a

[4] E. D. Kharasch and R. F. Novak, in preparation.

situation may result in drug interactions that will decrease the therapeutic effect. Such effects depend on sufficient inhibitory hepatic concentrations of the drug. Lu and Loo (34) have demonstrated that HAQ is rapidly accumulated in the liver of dogs, reaching a concentration of 40 μg/g (equivalent to 0.1 mM) 5 hr after intravenous administration of 15 mg/kg. Current research is directed toward investigating the inhibitory effects of HAQ on drug metabolism *in vivo*, as well as the molecular mechanism of HAQ inhibition of P-450 reductase activity.

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