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EVIDENCE FOR THE METABOLISM OF MITOZANTRONE BY MICROSOMAL GLUTATHIONE TRANSFERASES AND 3-METHYLCHOLANTHRENE-INDUCIBLE GLUCURONOSYL TRANSFERASES

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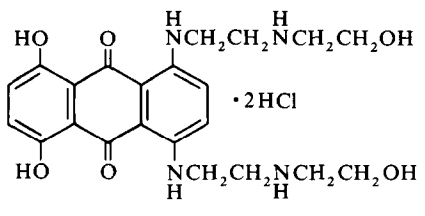
Abstract—The metabolism of mitozantrone, a chemotherapeutic agent used in the treatment of breast cancer, has been studied *in vitro* using rat liver subcellular fractions. This compound would appear to be metabolized by two interesting pathways. One involves conjugation with glucuronic acid, catalyzed most effectively by a 3-methylcholanthrene-inducible glucuronosyl transferase. The other pathway appears to be a glutathione conjugation reaction which requires prior metabolism by cytochrome P-450. The reaction with glutathione appears to be enzymatic as 1-chloro-2,4-dinitrobenzene was a potent inhibitor of this reaction. Liver cytosol did not enhance the microsomal rate of glutathione-conjugate formation, suggesting an important role for the microsomal glutathione transferases in the disposition of this compound. The relationship between these reactions and the mode of action of mitozantrone is discussed.

Mitozantrone (see below) is a promising new anticancer drug currently under clinical trial for the treatment of breast cancer [1]. The presence of the anthraquinone group makes this compound similar to the widely used anticancer drug doxorubicin. Mitozantrone does not appear to exhibit the same broad activity towards a wide range of cancers as doxorubicin, however, the toxic side-effects, particularly cardiotoxicity, are much less pronounced [2]. The relative lack of cardiotoxicity has been associated with the markedly reduced ability of the mitozantrone quinone grouping to "redox cycle", i.e. to generate free radical intermediates [3–5]. With a view to optimizing the clinical use of this compound, we have studied the pharmacokinetics and routes of metabolism of this compound in man [6]. In addition, we have investigated its mode of action and the enzyme systems responsible for its disposition using rat liver samples *in vitro*. The data described below indicate novel routes of metabolism and suggest that mitozantrone may prove an interesting model substrate for both the microsomal glutathione transferases as well as a specific substrate for a 3-methylcholanthrene-inducible glucuronosyl transferase.



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METHODS AND MATERIALS

Male Wistar rats (200 g) given food and water *ad libitum* were used. Animals receiving phenobarbital, 3-methylcholanthrene or Arochlor 1254 were treated as described previously [7]. Liver microsomal and cytosolic fractions were prepared by differential centrifugation using established procedures.

Incubations were carried out with hepatic microsomes (1 mg/ml) or cytosol (4 mg/ml) in 0.1 M phosphate buffer pH 7.4 in the presence or absence of NADPH (1 mM). Mitozantrone (final concentration 1 mM) was added as an aqueous solution. Other cofactors added to these incubation systems were either glutathione (4 mM), UDP-glucuronic acid (1 mM). Enzyme inhibitors, e.g. metyrapone (1 mM), trichloropropene oxide (0.1 mM) (TCPO), cyclohexene oxide (0.1 mM), or hexachloro-1:3-butadiene (1 mM) (HCBD), were added where required in aqueous solution or dissolved in 10 μl of ethanol. In certain experiments the microsomal samples were boiled for 2 min and then cooled to 4° before the addition of other components of the incubation. Incubations were carried out at 37° for periods up to 2 hr. For the majority of the experiments, the incubation time was 60 min. In some experiments, radioactive mitozantrone (0.2 μCi/ml incubation) or radioactive glutathione (1 μCi/ml incubation) were used. Following this incubation period, the microsomal protein was applied to a "SEP PAK" C_{18} cartridge which had been previously equilibrated with methanol (10 ml) followed by water (10 ml). The cartridge was washed with 10 mM HCl (10 ml), and mitozantrone, together with metabolites, eluted as a concentrated peak with methanol. The recovery of both mitozantrone and the metabolites using this

procedure was 80–90%. The samples were then evaporated to dryness under a stream of nitrogen and resuspended in 2 ml of ammonium formate (0.55 M): acetonitrile (73:27). Aliquots of this solution were applied to a 10 micron C_{18} reverse phase HPLC column, and mitozantrone and its metabolites eluted with ammonium formate (0.55 M): acetonitrile (73:27). The flow rate was 0.5 ml/min. Peaks were monitored either by their absorbance at 658 nm or in some experiments by their radioactivity either using a Berthold 503 radioactivity monitor or by scintillation counting of fractions collected into scintillation vials. The amount of radioactivity incorporated into the metabolites was used for their quantitation. Protein determinations were carried out by conventional procedures. Mitozantrone and [^{14}C]-mitozantrone (8.85 μCi/mg) labelled on the carbon chain were a generous gift from the Cyanamid Corporation (Pearl River, NY). [^{3}H]Glutathione (5 mCi/μmol) was purchased from New England Nuclear (Boston, MA). Hexachloro-1:3-butadiene, trichloropropene oxide, cyclohexene oxide and metyrapone were purchased from Aldrich and *N*-ethylmaleimide from Sigma. All other chemicals were of the highest purity available and were obtained from common commercial sources.

RESULTS

HPLC analysis of incubations of rat hepatic microsomal fractions, from rats treated with Arochlor 1254, with mitozantrone and UDPGA, gave a peak with a retention time of 5.8 min (Fig. 1). In this HPLC separation system the substrate mitozantrone had a retention time of approximately 11.5 min. The

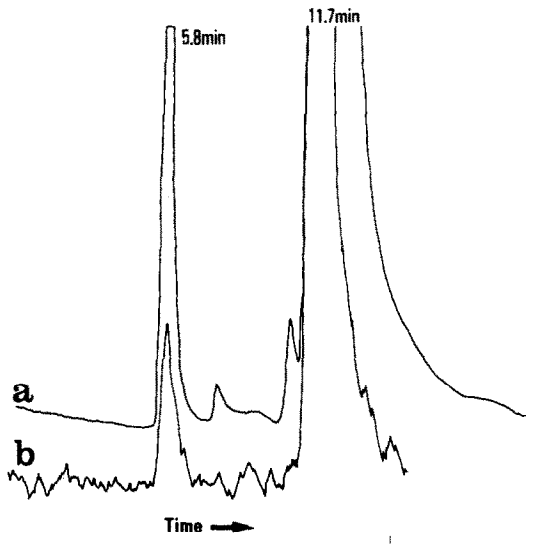

Fig. 1. HPLC trace obtained following incubation of mitozantrone with hepatic microsomal fractions from Arochlor 1254-treated rats. Incubations of microsomes (1 mg/ml) were in 0.1 M phosphate buffer pH 7.4 and [^{14}C]mitozantrone (1 mM, 0.2 μCi) and UDPGA (1 mM). Incubations were for 60 min at 37°. The preparation of the samples for HPLC analysis is described in the Materials and Methods section. The upper trace (a) represents products detected at 658 nm; the lower trace (b) is that of monitored radioactivity.

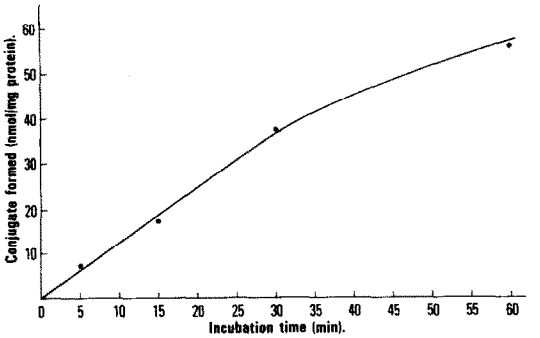

Fig. 2. Time course for the formation of the mitozantrone glucuronide conjugate. Incubation conditions were as described in Fig. 1. Metabolite was quantitated from the absorption peak at 658 nm.

formation of this peak was time-dependent and was linear for 30 min (Fig. 2). When either UDPGA was omitted from the incubation or the microsomes were boiled prior to incubation, no peak could be detected. Incubations with [^{14}C]mitozantrone showed conclusively that this peak was derived from this substrate as the HPLC trace for radioactivity could be superimposed on that obtained by monitoring absorbance at 658 nm (Fig. 1). In order to establish whether this metabolite was indeed a glucuronide conjugate of mitozantrone, the incubation medium was adjusted to pH 5.0 and β-glucuronidase (4000 units/ml) added. After 1 hr incubation, the mitozantrone metabolite was reduced by 88% (Fig. 3). The destruction of the metabolite by β-glucuronidase could be partially reversed by the addition of the β-glucuronidase inhibitor saccherolactone to these incubations (Fig. 3). The microsomal glucuronosyl transferase is well characterized as a latent enzyme which can be activated by partial solubilization of the microsomal membrane with detergents. Experiments to determine whether this

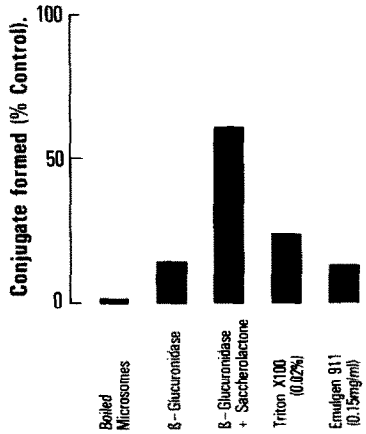

Fig. 3. Effect of various incubation conditions on mitozantrone-glucuronide formation in liver microsomal samples from Arochlor 1254-treated rats. Incubation conditions are those described in Fig. 1. Triton X100 or Emulgen 911 were added and the microsomal suspension vortexed prior to the addition of the other components. 100% values were between 0.85 and 1.28 nmol metabolite/min/mg protein.

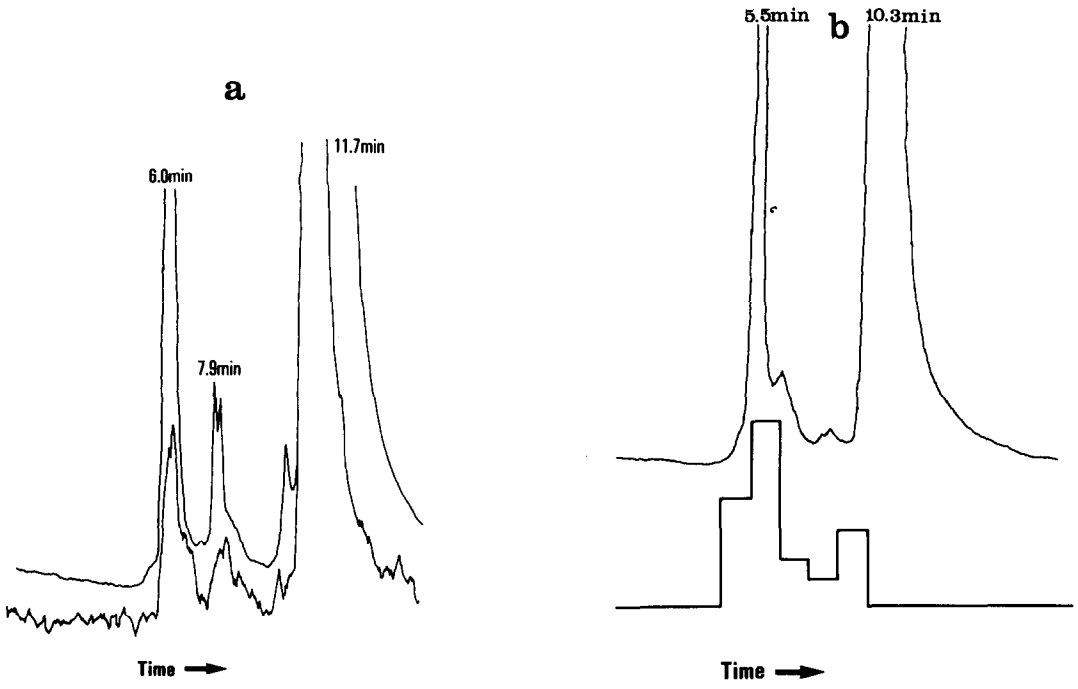

Fig. 4. HPLC trace obtained following incubation of mitozantrone and glutathione with hepatic microsomal fractions from Arochlor 1254 treated rats. Incubation conditions were as described in Fig. 1 with the exception that NADPH (1 mM) was included and [^{3}H] glutathione (4 mM, 1 μCi) was substituted for the UDPGA. (a) Incubations with [^{14}C]mitozantrone and unlabelled glutathione: absorption at 658 nm (upper trace), radioactivity (lower trace). (b) Incubations with [^{3}H]glutathione and unlabelled mitozantrone. Absorption at 658 nm (upper trace), radioactivity (lower trace).

is the case for the conjugation of mitozantrone are shown in Fig. 3. Treatment of microsomal fractions with Triton X100 or Emulgen 911 at concentrations known to activate UDP glucuronosyl transferase activity did not increase the formation of the mitozantrone metabolite, indeed both compounds inhibited significantly approximately 80% and 90% for Triton X100 and Emulgen 911 respectively.

On the premise that mitozantrone may also be oxidized by cytochrome P-450, microsomal samples were incubated with mitozantrone and NADPH. In this case no metabolites could be detected using the analysis procedure described above. However, when glutathione was added to these incubations, two metabolites could be detected with retention times ranging between 5.0–6.0 and 7.0–8.0 min (Fig. 4a). The retention times of the products and mitozantrone were always constant within an experiment but varied slightly on different days or with different HPLC columns. In view of the very low concentration of the second peak, it was difficult to quantitate accurately. However, the formation of both metabolites was time-dependent and in the case of the major product linear for approximately 60 min (Fig. 5). The metabolites were only formed in the presence of NADPH and glutathione. The use of radioactive substrate and glutathione confirmed the presence of both of these components in the products formed (Figs 4a and b). These findings have been confirmed in several further experiments. Boiled microsomes had no activity, indicating an enzymatic reaction (Fig. 6). It was interesting that the addition of hepatic cytosol did not increase the rate of conjugate formation. The effect of various other compounds on the formation of the major metabolite is shown in Fig. 6. Metyrapone, an inhibitor of cytochrome P-450, was a potent inhibitor of metabolite formation (95% inhibition at 1 mM). In addition, in incubations carried out in a carbon monoxide: oxygen atmosphere (80:20) an 88% inhibition of metabolite formation was observed. 1-Chloro-2,4-dinitrobenzene, a substrate for the microsomal and cytosolic glutathione transferases also inhibited approximately 95%. However, hexachloro-1:3-butadiene, a good substrate for a microsomal glutathione transferase [8], was not a very potent inhibitor of metabolite production. Cyclohexene oxide and trichloropropene oxide were added to the incu-

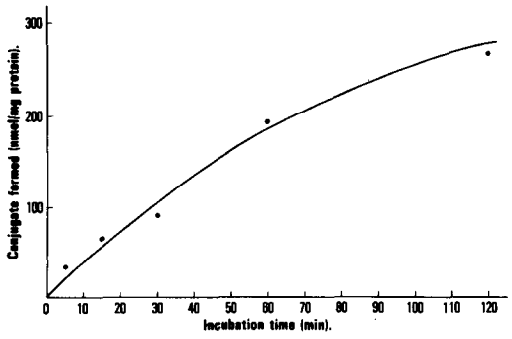

Fig. 5. Time course for the formation of the major metabolite formed on incubation of hepatic microsomes with mitozantrone, NADPH and glutathione. Experimental details are given in Figs. 1 and 4.

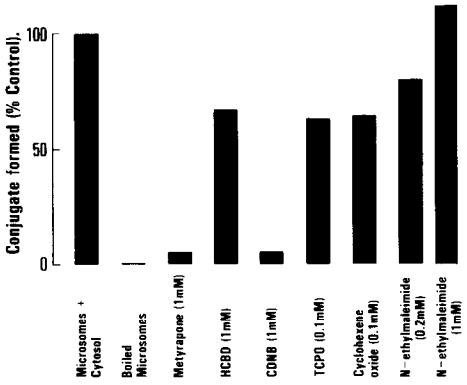

Fig. 6. Effect of various incubation conditions on mitozantrone-glutathione conjugate formation. Experimental details are given in Fig. 4. All metabolites were quantitated from the absorption at 658 nm. When added, the hepatic cytosol concentration was 4 mg/ml. Hexachloro-1:3-butadiene (HCBD), trichloropropene oxide (TCPO) and cyclohexene oxide were added in 10 μl of ethanol. *N*-ethylmaleimide was added 1 min prior to the addition of glutathione and the other components of the incubation. Incubations were for 60 min. 100% values were between 1.7 and 2.7 nmol metabolite/min/mg protein.

bations because they are both well characterized inhibitors of microsomal epoxide hydrolase [9]. This enzyme would compete with glutathione for any epoxide intermediates should they be the reactive species formed. However, the addition of these compounds inhibited rather than enhanced conjugate formation. *N*-ethylmaleimide has been shown to activate a microsomal glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene [10]. In experiments here no activation was observed.

Most of the hepatic microsomal drug metabolizing enzymes can be induced by treating the animals with either phenobarbital, 3-methylcholanthrene or Arochlor 1254. The effect of these agents on mitozantrone metabolism is shown in Table 1. It was very interesting that control microsomes had very low glucuronide-conjugating activity and that this activity was not induced by phenobarbital but was increased up to 10-fold by treatment with either Arochlor 1254 or 3-methylcholanthrene. This was not the case for the formation of the glutathione conjugates where inducing agents had no effect on the rates of formation of the metabolites.

DISCUSSION

This study was designed to identify the pathways of metabolism of mitozantrone in the rat to gain an insight into both its probable routes of metabolism in man as well as the factors which may affect its therapeutic efficiency. However, in addition to these data, the interesting metabolic pathways observed for this compound may make it a useful model substrate in drug metabolism studies.

Following i.v. infusion, mitozantrone has been shown to be rapidly removed from the circulation and distributed through the tissues [6, 11]. Only a small percentage (approximately 10%) is excreted within 24 hr in the urine. Using the same HPLC system as that described here, we have identified two urinary metabolites [6]. However, it has not yet been established whether these are derived from glucuronide or glutathione metabolites. To date we have also been unable to detect these metabolites when human liver instead of rat liver microsomal samples were used in the *in vitro* incubation systems. This may be attributable to the much lower activity of human liver, particularly in catalyzing glucuronidation reactions. The mechanism of antitumour action of mitozantrone is not understood. Compared with doxorubicin, this drug appears to cause less cardiotoxicity [2]. This may well be related to its reduced ability to redox cycle effectively [3–5] and generate the superoxide and hydroxyl radicals implicated in the cardiotoxicity of doxorubicin. Mitozantrone has been shown to interact very poorly with cytochrome P-450 reductase, an enzyme capable of catalyzing one electron reduction reactions which result in redox cycling [3, 5]. The NADPH-dependent metabolism of mitozantrone would therefore not appear to be via this flavoprotein but rather via cytochrome P-450. This conclusion is supported by the finding that metyrapone as well as carbon monoxide, potent inhibitors of cytochrome P-450, were potent inhibitors of mitozantrone metabolism.

There would appear to be at least two major pathways for the metabolism of mitozantrone, the first being a direct conjugation reaction with glucuronic acid catalyzed in a relatively specific manner by a 3-methylcholanthrene-inducible glucuronosyl transferase. The high specificity of this reaction would make it a useful marker substrate for this form of glucuronosyl transferase. It has previously been shown that benzo(*a*)pyrene quinones are also excel-

Table 1. Effect of hepatic inducing agents on the metabolism of mitozantrone in rat liver microsomal fractions

	Conjugate formed (nmol/min/mg protein)		
		Glutathione	
Inducer	Glucuronide	Peak A	Peak B
Untreated	0.09	2.26	0.46
Phenobarbital	0.06	2.12	0.33
3-Methylcholanthrene	0.75	1.98	0.50
Arochlor 1254	1.09	2.22	0.37

Incubation conditions and the detection of metabolites were as described in Materials and Methods and Figs 1 and 4. This experiment was carried out three times with essentially the same results.

lent substrates for this enzyme [12]. The glucuronidation of mitozantrone could not be increased by detergent treatment of the microsomal membranes, indicating that the activity of this glucuronosyl-transferase is not latent [13].

The formation of the glutathione conjugates appears to be the result of a cytochrome P-450-mediated oxidation followed by a glutathione transferase-mediated conjugation reaction. The fact that two conjugates appear to be formed is very intriguing. The glutathione conjugation would appear to be enzymic as 1-chloro-2,4-dinitrobenzene was a potent inhibitor of the reaction. Both membrane-bound microsomal glutathione transferases as well as cytosolic enzymes have been characterized [10, 14]. The finding that the addition of cytosol to the incubation medium did not enhance the rate of product formation suggests that the microsomal enzyme may have an important function in this case. However, if the cytochrome P-450-mediated reaction was the rate-limiting factor in metabolite formation, then there could possibly be sufficient contamination of the microsomal fraction with cytosol to catalyze the reaction. All the microsomal samples used in this study were washed at least twice and the contamination by cytosol would be very small. An indication that the cytochrome P-450-mediated reaction was not rate-limiting was the fact that metabolite formation was not increased by cytochrome P-450 inducers known to increase the metabolism of a wide range of cytochrome P-450 substrates. Recent studies have shown that there may be more than one species of microsomal glutathione transferase, one which has high activity towards hexachloro 1:3-butadiene [8, 15] and one with high affinity for 1,chloro-2,4-dinitrobenzene [10]. Hexachloro-1:3-butadiene was a poor inhibitor of conjugate formation which may indicate that the latter enzyme is that with high activity towards the mitozantrone. The reason why the epoxide hydrolase inhibitors trichloropropene oxide and cyclohexene oxide inhibited conjugate formation is probably due to their inhibition of the glutathione transferase reaction. The requirement for two enzyme-catalyzed steps in the formation of the glutathione conjugates makes it a difficult system to study and the relative importance of both cytochrome P-450 isozymes and various glutathione transferases still requires clarification.

The mechanism of the antitumour action of mitozantrone is unknown; however, it would not appear to be via redox cycling and subsequent generation of superoxide radicals. The formation by cytochrome P-450 of products that are conjugated to glutathione is very often associated with the formation of electrophilic cytotoxic intermediates [16]. The cytochrome P-450-mediated generation of toxic metabolites within the tumour cell would be a possible alternative mechanism of action. Mitozantrone is an active agent in the treatment of breast cancer and it is interesting to note that the MCF7 human breast cancer cell line has been shown to contain an inducible cytochrome P-450 system [17] and is also highly sensitive to mitozantrone (unpublished). Independent of whether the cytotoxic action of this compound requires cytochrome P-450, it is clear that both glucuronidation and glutathione conjugation may represent important detoxification pathways for mitozantrone. The concentrations of the enzymes involved in these reactions within tumour cells may be an important factor in the therapeutic effectiveness of this compound.

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