MECHANISM OF INHIBITION OF HEPATIC BIOACTIVATION OF PARACETAMOL BY DIMETHYL SULFOXIDE

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CONTENTS

		Page
	SUMMARY	414
I.	INTRODUCTION	412
II.	MATERIALS AND METHODS	415
	2.1 In vitro Studies on Peroxidation and DMNA Metabolism	415
	2.2 In Vivo Studies on Paracetamol Metabolism	416
III.	RESULTS	417
IV.	DISCUSSION	421
V.	REFERENCES	423

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SUMMARY

Prior work has shown that DMSO inhibits paracetamol hepatotoxicity. In this paper we show that DMSO and its reduced metabolite dimethyl sulfide (DMS) can inhibit *in vitro* hepatic dimethylnitrosamine N-demethylase. We also show that DMSO can inhibit in vivo production of glutathione conjugates of paracetamol. Glutathione is known to conjugate the bioactivated form of paracetamol. Also, the isozyme of cytochrome P-450 responsible for dimethylnitrosomine N-demethylase, cytochrome P-450j, is thought responsible for paracetamol bioactivation. We therefore propose that DMSO inhibits paracetamol hepatotoxicity due to inhibition of cytochrome P-450j-dependent paracetamol bioactivation by DMSO and its metabolite DMS.

I. INTRODUCTION

Dimethyl sulfoxide (DMSO) is frequently used as a solvent for research chemicals because it is an aprotic dipole, soluble in aqueous and organic media. However, DMSO is not without pharmacological effects itself, even at relatively low doses. We have reported that in mice, DMSO (4 g/kg s-c) protects against paracetamol hepatotoxicity while having no effect on pulmonary or nasal toxicity /1/. We have found this organ specificity in rats also (unpublished data) and others have reported protection against hepatotoxicity in hamsters as well as mice /2,3/.

Normally greater than 95% of a dose of paracetamol is conjugated to glucuronic acid or sulfate prior to excretion. The remainder is bioactivated by cytochrome P-450 to a toxic reactive intermediate, possibly the quinoneimine /4/, which is able to covalently bind lipids and proteins. In the presence of glutathione (GSH), the reactive intermediate preferentially binds GSH to form a non-toxic conjugate which is further metabolized and excreted harmlessly /5/.

One of the better-known properties of DMSO is its reaction with hydroxyl radicals /6/, thereby inhibiting hydroxyl radical-induced lipid peroxidation. However, a primary product of this reaction is the methyl radical, which abstracts an electron from the environment to form methane /7/. Since unsaturated lipids are the most available source of electrons, methyl radicals also cause lipid peroxidation,

even though they are 10^4 less reactive than the hydroxyl radical /7/. Thus, unless hydroxyl radical production were a rate-limiting step, no effect on lipid peroxidation would ensue from the DMSO reaction. DMSO protects against radiation toxicity /8/, where the toxic response is initiated by radiolysis of cell water to produce hydroxyl radicals. Thus hydroxyl radicals play a central role in radiation toxicity, and replacing them with the less reactive methyl radicals can slow the rate of lipid peroxidation. It has been suggested that this may also be the mechanism of DMSO protection against paracetamol hepatotoxicity /2/. This theory is based on the supposition that reactive intermediates of paracetamol metabolism cause the production of reactive oxygen species, including hydroxyl radicals, and that these, by initiation of lipid peroxidation, are responsible for toxicity. However, while peroxidation has been found to occur in tissues after paracetamol poisoning /9/, there is no evidence that lipid peroxidation has any central role in paracetamol hepatotoxicity. Indeed, in an *in vitro* system, paracetamol actually inhibits lipid peroxidation /10/. Furthermore, this theory does not explain the organ specificity of DMSO protection. This paper reports that while only poorly inhibiting microsomal lipid peroxidation, DMSO significantly inhibits paracetamol bioactivation to the toxic reactive intermediate. We therefore propose that DMSO protects against paracetamol hepatotoxicity by its inhibitory effect on bioactivation.

II. MATERIALS AND METHODS

2.1 In vitro studies on peroxidation and DMNA metabolism

Female Swiss-Webster mice, weighing 20-25 g, were killed by cervical dislocation. Livers were excised rapidly, homogenized in ice-cold isotonic KCl, and fresh hepatic microsomes prepared by differential centrifugation, as previously described /11/. Microsomes were resuspended in 0.2 M Tris buffer, pH 7.4 and protein estimated by the method of Lowry et al. /12/. Microsomal lipid peroxidation was determined by incubating fresh hepatic microsomes (mg/ml) with an NADPH generating system (NADP, 0.4 mM; Glucose-6-phosphate, 4 mM; Glucose-6-phosphate dehydrogenase, 2 U/ml) in 0.1 M Tris buffer, pH 7.4, and removing aliquots into trichloracetic

acid at timed intervals, for reaction with thiobarbituric acid /13/. Some incubations contained DMSO, 5, 10, or 20 mM, some contained dimethylsulfide (DMS), 0.1, 0.2, or 1 mM. The metabolism of dimethylnitrosamine (DMNA) was carried out in a similar incubation medium, except that DMNA, 0.1 - 100 mM, was also present. The reaction was stopped by addition of zinc sulphate followed by barium hydroxide, centrifuged, and the clear supernatant analyzed for formaldehyde by the method of Nash /14/.

2.2 In vivo studies on paracetamol metabolism

The effect of DMSO on *in vivo* metabolism of paracetamol was studied in female Sprague-Dawley rats weighing 200-220g. The rats were anaesthetized with pentobarbital (50 mg/kg) and their bile ducts cannulated as previously described /15/. Rats were administered a subtoxic dose of paracetamol (100 mg/kg) by gavage, and bile samples collected in 15 min aliquots over 2 hours. Some animals received DMSO subcutaneously, 4 g/kg as a 50% solution in KCl, within 15 minutes of receiving paracetamol. Alteration in bile flow due to hypothermia was avoided by maintaining rectal temperature at 37°C with the use of a heating pad. Bile weight in g/15 min period was 0.20 $\pm$ 0.04 and 0.23 $\pm$ 0.03 for paracetamol alone and paracetamol plus DMSO, respectively. At death (2 hours) urine was collected from the bladder of each animal. Bile samples were diluted in water 50-fold, and frozen until analyzed by HPLC. Urine samples were deproteinized with methanol (final concentration 50%) and similarly diluted and stored. Metabolites of paracetamol were separated on a Whatman Partisphere C18-5μ reverse phase column (12.5 cm x 4 mm). using a mobile phase of 1% acetic acid: methanol: ethylacetate, 90:15:0.1, and a flow rate of 1.4 ml/min, as described by Howie and co-workers /16/. A Perkin-Elmer series 4 HPLC was attached to a Hitachi UV detector, and the metabolites quantified by their absorbance at 250 nm /17/. Paracetamol standards added to control bile samples prior to dilution and freezing gave greater than 95% recovery. Retention times were: sulphate, 1.3 min; glucuronide, 1.8 min; parent compound, 3.2 min; GSH conjugate, 5.5 min; mercapturic acid, 6.5 min.

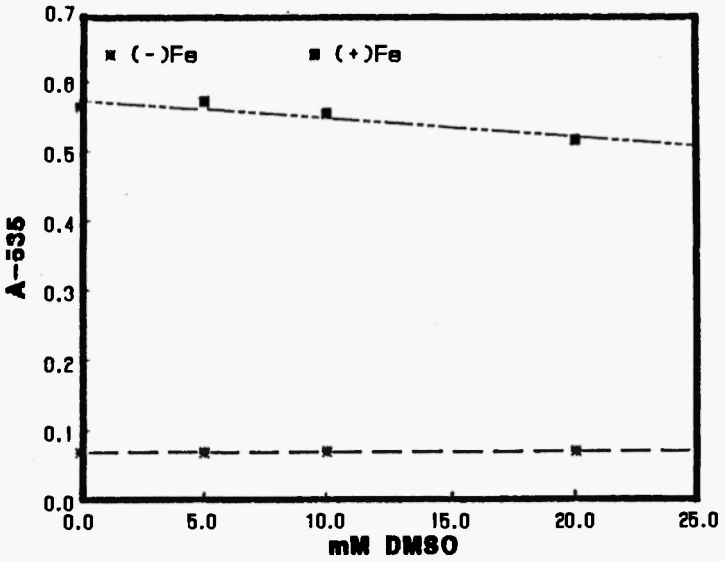

Fig. 1: The effect of DMSO on hepatic microsomal lipid peroxidation. Mouse hepatic microsomes were incubated for 10 min with an NADPH generating system, and malondialdehyde estimated as described under methods, in the presence or absence of 20μm iron sulfate. DMSO, 5, 10, or 20 mM, was added prior to NADP. Values represent the mean of triplicate determinations at each substrate concentration.

III. RESULTS

As shown in Figure 1, DMSO was very slightly inhibitory to mouse hepatic microsomal lipid peroxidation, with a Ki of 106 mM. Conversely, DMSO strongly inhibited microsomal DMNA demethylase. When the DMNA substrate concentration was varied between 0.1 and 1 mM DMNA, DMSO was found to inhibit microsomal DMNA demethylase with a Ki of 0.93 mM, Figure 2a. However, when the DMNA substrate concentration ranged between 10 and 50 mM DMNA, DMSO was without inhibitory effect, Figure 2b. Similar results were obtained for DMS inhibition of DMNA demethylase, with a Ki of 0.0175 mM for the low Km demethylase activity (Figure 3a) and no inhibition of the high Km demethylase activity (Figure 3b). The inhibitory effect of DMSO and DMS

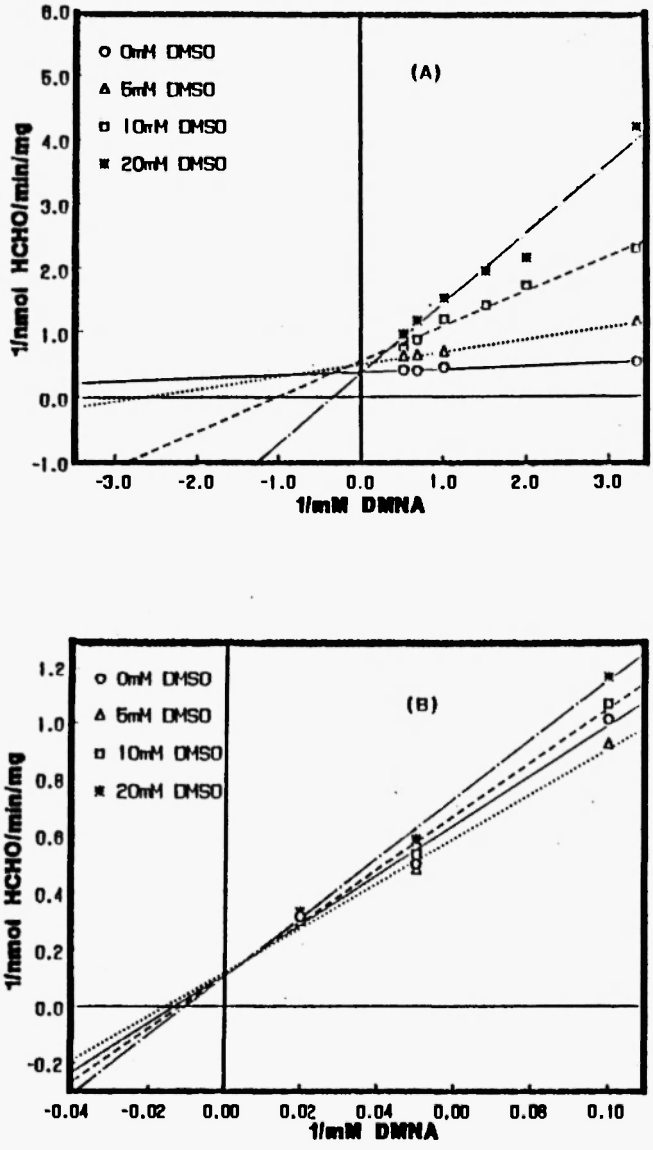

Fig. 2: Lineweaver-Burk plots of the inhibition of DMNA demethylation by DMSO as a function of substrate concentration. incubation conditions are described under methods. Formaldehyde production for the low Km activity (2a, DMNA concentrations 0.3.2 mM) was subtracted from total activity to obtain the high Km activity (2b).

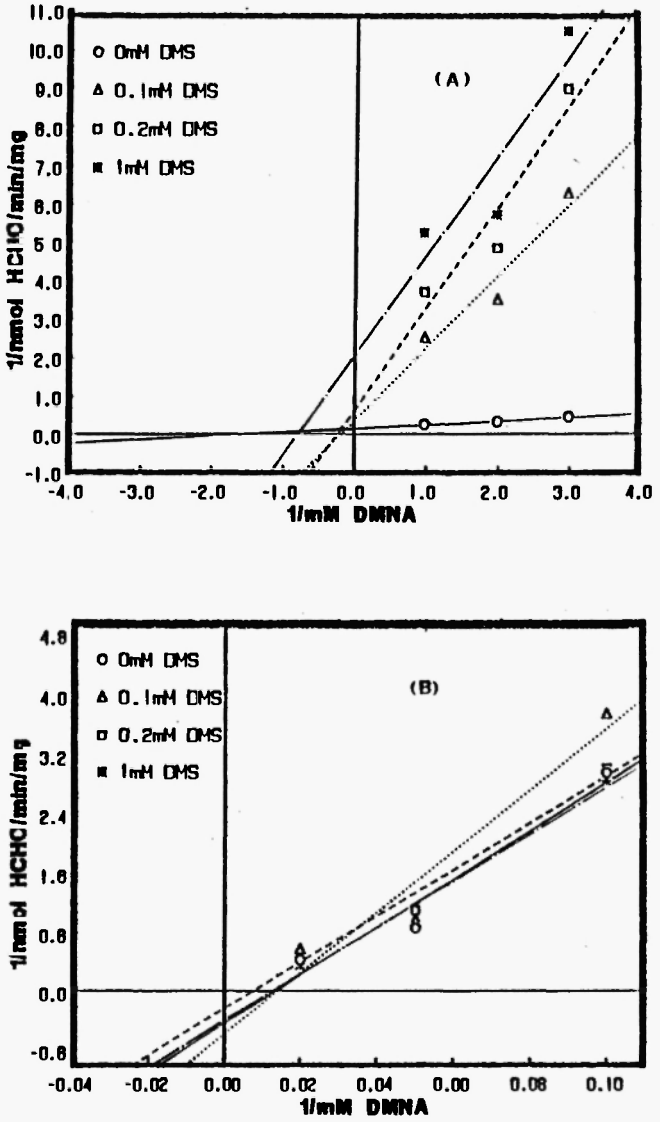

Fig. 3: Lineweaver-Burk plots of the inhibition of DMNA demethylation by DMS as a function of substrate concentration. Incubation conditions are described under methods. Formaldehyde production for the low Km activity (3a, DMNA concentrations 0.3.2 mM) was subtracted from total activity to obtain the high Km activity (3b).

appeared to be directed solely towards the high-affinity, low Km enzyme activity, which activity is carried out by a single isozyme of cytochrome P-450, isozyme j /18/. Since this isozyme has been associated with paracetamol bioactivation in the rat /19/, it was necessary to determine whether DMSO could depress the rate of paracetamol bioactivation in the whole animal.

At the subtoxic dose of paracetamol used (100 mg/kg), biliary production of metabolites did not vary significantly with time from 30 min after dosing to 90 min after dosing. After this, metabolite production decreased. Comparing the biliary metabolite profiles generated from rats receiving paracetamol alone, or paracetamol followed by DMSO, there was a significant decrease in biliary output of the GSH conjugate in rats receiving DMSO, Table 1. However, there was little change in the biliary output of conjugated products (sulphate plus glucuronide). These data point to an inhibition of paracetamol bioactivation. While urine was analyzed at only one timepoint (2 hours), urinary production of the GSH conjugate and its metabolites was similarly depressed to approximately 40% by DMSO treatment (data not shown). Although both rat and mouse exhibit hepatotoxicity in response to paracetamol bioactivation, the mouse bioactivates a far larger proportion of the dose than the rat /20/ most probably indicative of a greater proportion of cytochrome P-450j. We are currently repeating in vivo studies using the mouse.

TABLE 1

Effect of DMSO (4 g/kg) on in vivo Paracetamol Metabolism in Rats

	Biliary Metabolism[1]		
	Sulfate	Glucuronide	GSH Conjucate
Control	14.6 ± 0.02	54.6 ± 1.13	26.0 ± 0.51
DMSO	5.2 ± 0.76	70.1 ± 2.23	9.1 ± 1.5

[1] μg/ml; mean ± S.E. of 4 separate samples. Biliary flow averaged 0.27 and 0.26 ml/15 min for control and DMSO-treated rats, respectively.

[2] GSH conjugates were 27.3% of the total metabolites in the control group and 10.8% of the total metabolites in the DMSO-treated group.

IV. DISCUSSION

When one considers not only the poor inhibition of microsomal lipid peroxidation, but also the organ specificity of DMSO protection, it appears unlikely that DMSO protects against paracetamol hepatotoxicity by acting as a free radical scavenger.

We have found that for a given dose of paracetamol, the loss of GSH was significantly less from the livers of rats or mice receiving paracetamol and DMSO than from those receiving paracetamol alone /1/. Such an effect could be due to an increased rate of GSH synthesis, or a decreased rate of utilization. However, treating mice with Buthione Sulfoximine (an inhibitor of GSH synthesis) while significantly decreasing hepatic GSH levels, did not obliterate the protective effect of DMSO /1/. These data are not consistent with an effect of DMSO on GSH synthesis, and therefore implicate an effect on GSH utilization. A decreased rate of GSH conjugation of the bioactivated metabolite of paracetamol would have a saving effect on GSH levels, but would also be associated with an increase in toxicity. A decreased rate of cytochrome P-450-dependent bioactivation of paracetamol would cause a decreased utilization of GSH and decreased toxicity.

Although not the only isozyme capable of such a reaction, hepatic cytochromes P-450j and its ortholog P-450 LM3a have been implicated in the bioactivation of paracetamol /19,21/. Ethanol, known to induce these orthologs, aggravates paracetamol hepatotoxicity when administered chronically /22/, and inhibits toxicity when administered concomitantly with paracetamol /23/. Microsomal dimethylnitrosamine N-demethylase, at low substrate concentrations, is a cytochrome P-450j-dependent activity /18/. We found that DMSO competitively inhibited dimethylnitrosamine demethylase, with a Ki of 0.93 mM. We also found that at higher concentrations of dimethylnitrosamine, where demethylation is non-specific and dependent upon all of the isozymes of cytochrome P-450 present rather than any particular one, that DMSO was without effect on this activity. We interpret this to mean that DMSO interacts specifically with the ethanol-inducible isozyme of cytochrome P-450, not with any other isozymes of cytochrome P-450. DMSO has previously been reported to interact with the rabbit liver ortholog, LM3a /24/. Our finding could explain the lack of DMSO protection against pulmonary toxicity, since the ethanol-inducible isozyme is not

present in lung /25/, and therefore paracetamol would be bioactivated in the lung by an isozyme that was not inhibited by DMSO.

While the Ki for DMSO inhibition of metabolism does not appear to be small enough to ensure such strong inhibition in the whole animal, DMSO undergoes both reduction to the sulfide, and oxidation to the sulfone. We found that the sulfide is a competitive inhibitor of demethylase, with a far greater affinity for cytochrome P-450 than the sulfoxide, exhibiting a Ki of 0.0175 mM. Furthermore, inhibition by the sulfide was specific to the low Km demethylase activity, as we had found for DMSO itself. The sulfone was without significant effect (data not shown). The sulfide was not used in the whole animal studies due to its extreme volatile nature and consequent rapid loss from the lungs /26/.

We considered the possibility that DMSO is without direct effect on the demethylase, but that inhibition of demethylase is due to DMSO conversion to DMS, with subsequent inhibition by DMS. If this were the case, then microsomal DMSO inhibition would increase with incubation time as more DMSO was converted to DMS, but inhibition would not increase with increasing DMSO concentration above a maximal substrate concentration. Metabolism was linear with time (data not shown) and decreased as DMSO concentrations increased from 5 to 20 mM, a range far above normal substrate saturation for microsomal enzymes. We therefore conclude that DMSO has a direct inhibitory effect on demethylase, albeit a far smaller effect than DMS. Due to the differences in Ki, approximately 1% conversion of DMSO to DMS would cause approximately equal inhibition by DMSO and DMS. Therefore even if only a small amount cycles between reduced and oxidized in the whole animal, DMS can be expected to be responsible for a substantial portion of the inhibition.

Rat nasal epithelium is reported to contain the ethanol-inducible isozyme of cytochrome P-450 /25/. Therefore, if DMSO protects in the liver by its metabolite DMS inhibiting the ethanol-inducible isozyme, DMSO should protect the rat from nasal epithelial necrosis following paracetamol administration also. In contrast to this theory for the action of DMSO, we saw no protection /1/. One possible explanation for this finding is that the nasal epithelium lacks the enzyme required to reduce DMSO to the sulfide. We are presently studying this possibility.

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