Annual Report
Comprehensive Research on Rice
January 1, 1998 - December 31, 1998

Project Title: Molinate: A Metabolic Explanation for Species Differences in Susceptibility to Male Reproductive Toxicity.

Status of Proposal: ___/New   _X_/Continuing
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Objectives and Experiments Conducted to Accomplish Objectives:

This project has been funded for four consecutive years by the California Rice Research Board. The overall objective has been to investigate the role of metabolism and biological events underlying the male reproductive damage caused by the economically important rice herbicide molinate (Ordram). The majority of animal studies which implicate molinate as a male reproductive toxicant have been carried out in the rat. However, both human epidemiological and non-human primate studies have given no positive indication that molinate causes male reproductive toxicity in man. Therefore, this research was undertaken to understand the metabolic and mechanistic basis for the testicular damage seen after administration of molinate to the rat. The goal was to use this information to determine whether molinate toxicity is species specific and to assess more rationally the risk posed to man.

Our previous studies have implicated molinate sulfoxidation as the metabolic pathway associated with testicular damage (Jewell et al 1998). The molinate metabolite generated via sulfoxidation was found to bind a specific protein located in the Leydig cell of the testis (Jewell and Miller, 1998). This protein was identified as a carboxyesterase, hydrolase A, and proposed to be involved in testosterone biosynthesis. Molinate was hypothesized to inhibit this enzyme and hence decrease the availability of cholesterol derived from hydrolysis of cholesterol esters and required for testosterone biosynthesis. With the knowledge that metabolic activation is important in molinate induced testicular toxicity we have used *in vitro* methodology to compare the capacity of rat vs human liver to metabolize molinate via not only the toxic pathway but also nontoxic and detoxification pathways (Jewell and Miller, Accepted, 1999).

In the most recent year of support, the OBJECTIVES of the research were as follows:
1. To complete molinate metabolism studies in human vs. rat liver slices.
2. To determine whether molinate sulfoxide inhibits the esterase activity of hydrolase A using the purified protein and a cholesterol ester substrate.
3. To determine the time course and dose dependence of testicular esterase inhibition after molinate administration

Objective 1. To complete molinate metabolism studies in human vs. rat liver slices.

Liver slice and microsome studies have measured metabolites formed via the toxic (sulfoxide) and nontoxic(hydroxymolinate) microsomal pathways as well as the cytosolic detoxification(glutathione conjugation) pathway of molinate metabolism (Fig 1).

Fig 1. Metabolism of Molinate via Oxidation and Glutathione Conjugation
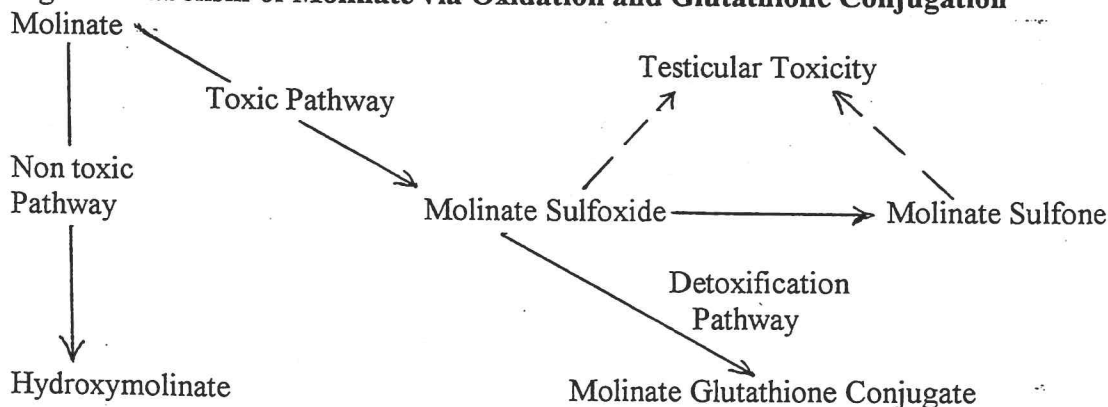
A metabolic profile for the *in vitro* metabolism of molinate in human and rat liver has been obtained. Km and Vmax values indicated that sulfoxidation would be the preferred high dose pathway while hydroxylation would predominate at low dose levels in both species (Table 1). Examination of the detoxifying conjugative pathways of molinate metabolism in liver slices showed that the human liver was more efficient that the rat liver at detoxifying molinate sulfoxide via glutathione conjugation (Fig. 2). Oxidative metabolism of molinate in both rats and humans was mediated by cytochrome P-450. Overall, at low doses molinate metabolism would occur via the nontoxic hydroxylation pathway in both species. At high doses, where sulfoxidation would predominate, the human is more capable than the rat of detoxification via glutathione conjugation. This data has been submitted and accepted for publication.

Table 1

	Vmax (pmol/min/mg)		Km (uM)	
	Rat	Human	Rat	Human
Sulfoxidation	1426 ± 139*	815 ± 140*	400 ±15.6**	264 ± 200**
Hydroxylation	35.4 ± 9.65†	58.6 ± 17.8†	138 ± 9.4	124 ± 48

Table #1 : Kinetic rate constants for molinate metabolism in liver microsomes.
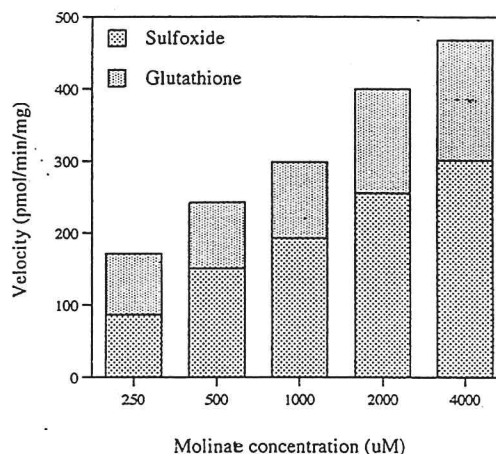(*,** $p < 0.05$, †$p < 0.11$; n=3 for rat microsomes and n=6 for human microsomes).

	Vmax (pmol/min/mg)		Km (uM)	
	Rat	Human	Rat	Human
Sulfoxidation and GSH conjugation	638± 241	510 ± 190	2837±827*	981±411†
Hydroxylation	8.33± 3.23	42.2± 21.3	587± 235*	303± 143†

Table #2 : Kinetic rate constants for molinate metabolism in liver slices.
(*† $p < 0.05$, n=3).

Figure 2
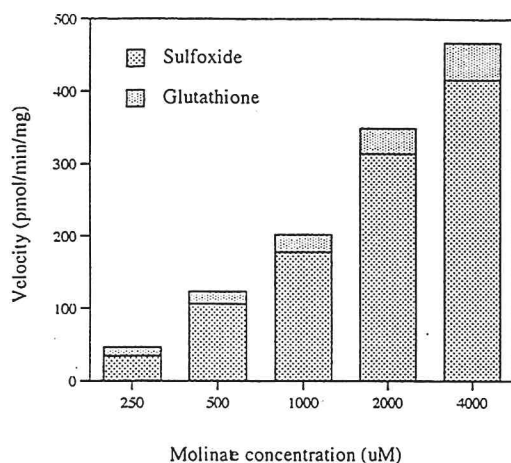
A. RAT   **B. HUMAN**

Objective 2. To determine whether molinate sulfoxide inhibits the esterase activity of hydrolase A using liver microsomes and a cholesterol ester substrate.

In the previous year of support, it was found that one protein was extensively bound in both liver and testis microsomes incubated with radiolabelled molinate. This protein was identified as a carboxyesterase, hydrolase A. Esterase activity in the testis is also inhibited by molinate. Since cholesterol is required for testosterone biosynthesis and cellular cholesterol is mobilized via hydrolysis of cholesterol esters, our hypothesis was that the esterase activity of hydrolase A is required for cholesterol mobilization in the testosterone-producing Leydig cell. To determine if hydrolase A was involved in cholesterol ester hydrolysis, the experimental design was to take rat liver microsomes and measure the hydrolysis of a radoiolabelled cholesterol ester, cholesterol [^{14}C]oleate in the presence and absence of molinate.

Cholesterol [^{14}C]oleate was incubated for 30 min at 37°C with liver microsomes which had been preincubated with molinate(400uM). The incubation mixture was extracted with chloroform and radioactive products were separated by thin layer chromatography. The band corresponding to the [^{14}C]- radiolabelled oleate hydrolysis product was scraped off the silica gel plate and the amount of radioactive material quantified by liquid scintillation counting. In the absence of molinate, 66.7 pmol of the oleate was hydrolysed by the microsomal preparation. When molinate was included in the incubation, the amount of oleate formed was markedly decreased (15.8 pmol) to approx. 25% of that found without molinate. This data supports the hypothesis that binding of a molinate metabolite to hydrolase A results in inhibition of cholesterol ester hydrolysis which could consequently decrease the ability of the Leydig cell to synthesis testosterone. These data are consistent with the a recently published study carried out by Zeneca Central Toxicology laboratory which demonstrated that intratesticular levels of testosterone were decreased after molinate administration to rats (Ellis et al., 1998).

Objective 3. To determine the time course and dose dependence of testicular esterase inhibition after molinate administration

The dose and time dependence of molinate induced inhibition of testicular esterase activity was measured with the goal of identifying dose levels where inhibition was minimal and establishing how rapidly esterase activity returned to control levels. In the recent Ellis et al., (1998) study, Leydig cell esterase activity was measured by histochemical staining at various time points after administration of molinate (40 mg/kg) and inhibition was found to be short lived with complete recovery 48 hrs after dosing. The present study measured esterase enzyme activity which represents the biochemical corollary of the histochemical stain.

Two substrates were used to measure esterase activity in testis microsomes: p-nitrophenol acetate(PNA) which is reported to be relatively specific for hydrolase A and alpha-naphthyl acetate to measure total non specific esterase(NSE) activity. At low dose levels (5 & 50 mg/kg) the onset of inhibition of PNA hydrolysis was rapid and recovery had not occurred 2 days later. However, 7 days after molinate administration, recovery was complete(Fig.3). Inhibition of NSE activity followed a similar pattern.

Figure 3

Effect of Molinate on p-Nitrophenyl Acetate Hydrolysis by Testis Microsomes
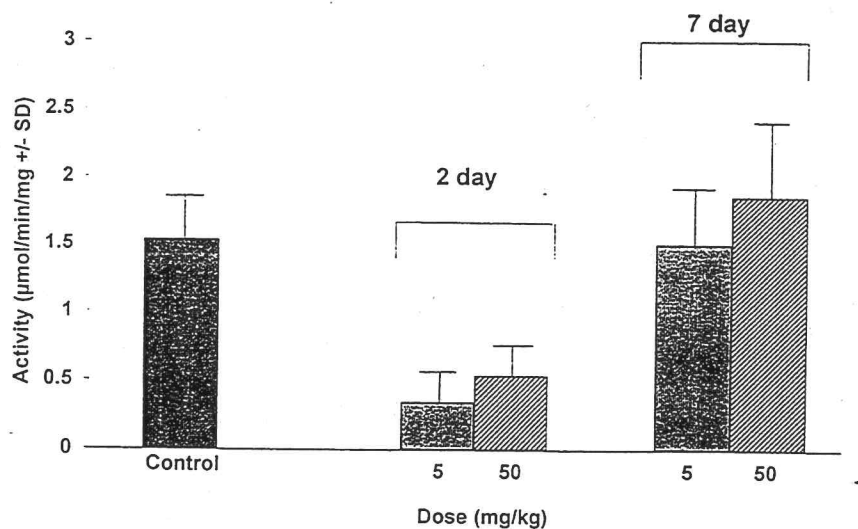
References

1.Ellis, MK, AG Richardson, JR Foster, FM Smith, PS Widdowson, MJ Farnworth, RB Moore, MR Pitts, and GA Wickramaratne. The reproductive toxicity of molinate and metabolites to the male rat:effects on testosterone and sperm morphology. Toxicol. Appl. Pharmacol. **151**, 22-32, (1998).

2. Jewell, WT, RA Hess, and MG Miller. Testicular toxicity of molinate in the rat: metabolic activation via sulfoxidation. Toxicol. Appl. Pharmacol. **149**, 159-166, (1998).

3. Jewell, WT, and MG Miller. Identification of a carboxylesterase as the major protein bound by molinate. Toxicol Appl.Pharmacol. **149**, 226-234, (1998)

4. Jewell, W.T. and M.G. Miller. Comparison of human and rat metabolism of molinate in liver microsomes and slices. Drug Metabolism and Disposition (accepted).

Summary of 1998 Research (Major Accomplishments) by Objective:

The overall objective of this research is to understand the metabolic and mechanistic basis for the testicular damage seen after administration of molinate to the rat. The goal is to use this information to determine the likelihood of those events occurring in man. Our previous studies implicated molinate sulfoxidation as the metabolic pathway associated with testicular damage. The present studies compared the metabolic capabilites of man vs rat tpo generate and detoxify sulfoxidation products. We also had previously identified a protein bound by the molinate metabolite as an esterase. Inhibition of this enzyme was proposed to decrease hydrolysis of cholesterol esters with a consequent reduction in the availability of cholesterol required for testosterone biosynthesis. The present studies have directly measured cholesterol ester hydrolysis in the presence of molinate and examined the reversibility of molinate induced esterase inhibition. The major accomplishments in the previous year of support were:

Objective 1. To complete molinate metabolism studies in human vs. rat liver slices.

Liver slice and microsome studies have measured metabolites formed via the toxic (sulfoxide) and nontoxic(hydroxymolinate) microsomal pathways as well as the cytosolic detoxification(glutathione conjugation) pathway of molinate A metabolic profile for the *in vitro* metabolism of molinate in human and rat liver has been obtained. At low doses molinate metabolism occurs primarily via the nontoxic hydroxylation pathway in both species. At high doses, where sulfoxidation would predominate, the human is more capable than the rat of detoxification via glutathione conjugation. Overall, the data predicts the human would be less suceptible than the rat. A paper comparing the *in vitro* metabolism of molinate in human and rat has been accepted for publication.

Objective 2. To determine whether molinate sulfoxide inhibits the esterase activity of hydrolase A using liver microsomes and a cholesterol ester substrate.

Molinate inhibited the hydrolysis of cholesterol [^{14}C]oleate to levels approx. 25% of control This data supports the hypothesis that binding of a molinate metabolite to hydrolase A results in inhibition of cholesterol ester hydrolysis which could consequently decrease the ability of the Leydig cell to synthesis testosterone. Diminished testicular testosterone would account for the testicular damage seen after high dose levels of molinate.

Objective 3. To determine the time course and dose dependence of testicular esterase inhibition after molinate administration

Low dose levels of molinate (5 & 50 mg/kg) rapidly inhibited testicular esterase activity. Recovery had not occurred 2 days after dosing but enzyme activity had returned to control levels 7 days from when molinate was administered. Since these dose levels of molinate cause no obvious testicular histological damage the data would suggest that esterase inhibition of short duration does not result in testicular damage.

Publications

Jewell, W.T., R.A. Hess, and M.G. Miller. Testicular toxicity of molinate in the rat: metabolic activation via sulfoxidation. Toxicology and Applied Pharmacology. **149**, 159-166, (1998).

Jewell, W.T. and M.G. Miller. Identification of a carboxylesterase as the major protein bound by molinate. Toxicology and Applied Pharmacology. **149**, 226-234, (1998)

Jewell, W.T. and M.G. Miller. Comparison of human and rat metabolism of molinate in liver microsomes and slices. Drug Metabolism and Disposition (accepted, subject to minor revision).

Abstracts

1. Jewell, W.T. and M.G. Miller. Identification of a carboxylesterase as the major testis protein bound by molinate: toxicological significance. Society of Toxicology Annual Meeting, Seattle WA, March 1998.

2. B.S. Winder, W.T. Jewell, and M.G. Miller. Esterase inhibition by molinate: role in toxicity. Society of Toxicology Annual Meeting, New Orleans, LA, March 1999.

Awards

Reproductive and Developmental Toxicology Speciality Section Society of Toxicology. Graduate Student Award to William Jewell. "Identification of a carboxylesterase as the major testis protein bound by molinate: toxicological significance"

Concise General Summary of Current Years Results:

The overall objective of this research is to understand the metabolic and mechanistic basis for the testicular damage seen after administration of molinate to the rat. The goal is to use this information to determine the likelihood of those events occurring in man. Our previous studies implicated molinate sulfoxidation as the metabolic pathway associated with testicular damage. The present studies compared the metabolic capabilities of man vs rat to generate and detoxify molinate sulfoxidation products. At low doses molinate metabolism would occur primarily via the nontoxic hydroxylation pathway in both species. At high doses, where sulfoxidation would predominate, the human is more capable than the rat of detoxification via glutathione conjugation. Overall, the data predicts the human would be less susceptible than the rat. Previous studies had also identified a protein bound by the molinate metabolite as an esterase. Inhibition of this enzyme was proposed to decrease hydrolysis of cholesterol esters with a consequent reduction in the availability of cholesterol required for testosterone biosynthesis. The present studies have directly measured cholesterol ester hydrolysis in the presence of molinate and examined the reversibility of molinate induced esterase inhibition. The data support the hypothesis that molinate inhibits the hydrolysis of cholesterol esters. Diminished testicular testosterone would account for the testicular damage seen after high dose levels of molinate. However low dose levels of molinate (5 & 50 mg/kg) rapidly inhibited testicular esterase activity but enzyme activity recovered within 7 days. Since these dose levels of molinate cause no testicular toxicity, the data would suggest that esterase inhibition of short duration does not result in testicular damage.