Annual Report
Comprehensive Research on Rice
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Project Title: Molinate: A Metabolic Explanation for Species Differences in Susceptibility to Male Reproductive Toxicity

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Level of 1997 Funding: $29,834

Objectives and Experiments Conducted to Accomplish Objectives:

This project has been funded for three 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. Although negative data is hard to interpret, 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 is to use this information to determine whether molinate toxicity is species specific and to assess more rationally the risk posed to man.

Broadly, the objectives of the research have been to:

I. Develop a single dose rat animal model for molinate-induced testicular toxicity
II. Determine whether molinate or a molinate metabolite was responsible for toxicity
III. Establish which metabolic pathways are toxic, nontoxic and detoxification pathways
IV. Compare the ability of the susceptible species (rat) and the test species (man) to form and detoxify the toxic metabolite of molinate
V. Investigate the biological events perturbed by the molinate metabolite and determine the likelihood of those events occurring in man.

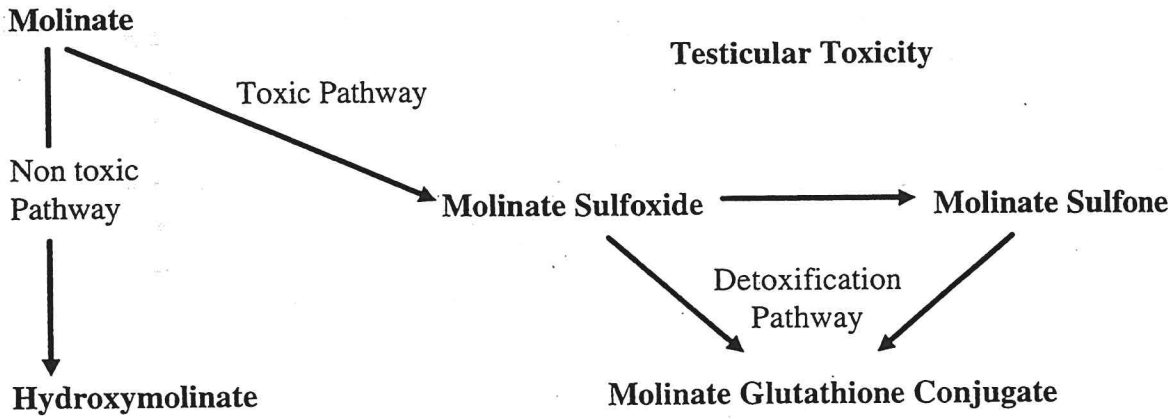
Within this framework our previous studies have implicated molinate sulfoxidation as the metabolic pathway associated with testicular damage. With the knowledge that metabolic activation is important in molinate induced testicular toxicity we have used *in vitro* methodology to compare the capacity of rat vs human liver to metabolize molinate via not only the toxic pathway but also nontoxic and detoxification pathways.

In the most recent year of support, the objectives were 1) to extend human vs. rat comparative metabolism studies using fresh liver slices, 2) to measure testicular levels of molinate sulfoxide thus further implicating molinate sulfoxidation as important in toxicity and 3) to identify the testicular protein to which molinate binds.

Objective I. Metabolism of molinate in rat and human liver slices.

Molinate undergoes oxidative metabolism to form either a nontoxic ring hydroxylated product or molinate sulfoxide which has been implicated as involved in testicular toxicity (Fig 1). Therefore if a species favored metabolic clearance via the nontoxic pathway it would be expected to be less susceptible to testicular damage. Similarly, the sulfoxide (or a sulfone product of sulfoxide oxidation) can be detoxified by conjugation with cellular glutathione. A species with high capacity to form the glutathione conjugate would also be predicted to have a lesser sensitivity to testicular damage. Liver metabolism would be important in governing susceptibility to toxicity since molinate sulfoxide formed in the liver circulates in the blood and can reach the testis by this route (see Objective 2 below).

Fig 1. Metabolism of Molinate via Oxidation and Glutathione Conjugation



In earlier *in vitro* metabolism studies, the capacity of human and rat liver microsomes to form the toxic sulfoxide vs nontoxic hydroxymolinate was compared. The data indicated that at low dose levels, humans would form less sulfoxide than rats and therefore would be less likely to be susceptible to testicular damage. However, as would be expected from a non-homogeneous population, the human liver preparations showed wide variability in their metabolic capabilities and results for some human livers overlapped with data obtained for the rat. With the liver slice studies carried out this year, the whole cell system allowed measurement of the detoxification pathway catalyzed by cytosolic glutathione transferase which is not present in microsomal preparations. Human liver slices formed a markedly greater (x 5) amount of molinate glutathione conjugate compared with rat slices(Fig 2A). In agreement with the microsomal study, human liver was also less capable than rat of forming the toxic sulfoxide metabolite(Fig 2B). Taken together, these data would predict that the human would be less susceptible than the rat to testicular toxicity after exposure to molinate.

Fig. 2 A Metabolism of Molinate in Human and Rat Liver Slices: Formation of Molinate Glutathione Conjugate (N =2 for both species)

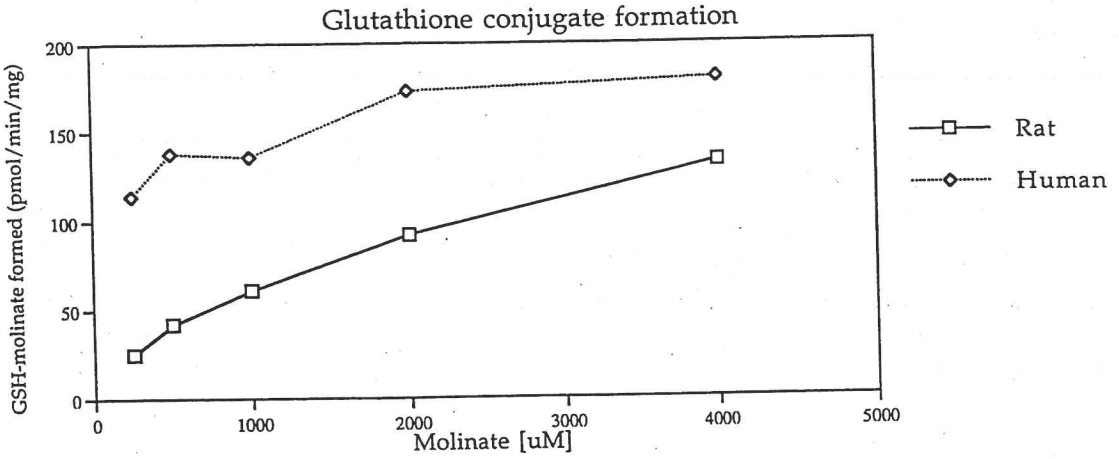
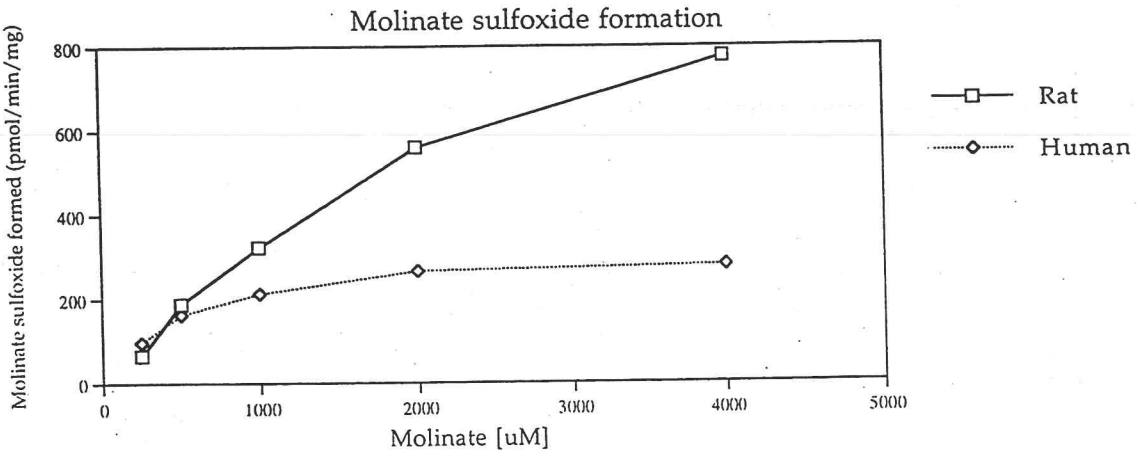


Fig. 2 B Metabolism of Molinate in Human and rat Liver Slices: Formation of Molinate Sulfoxide (N =2 for both species)



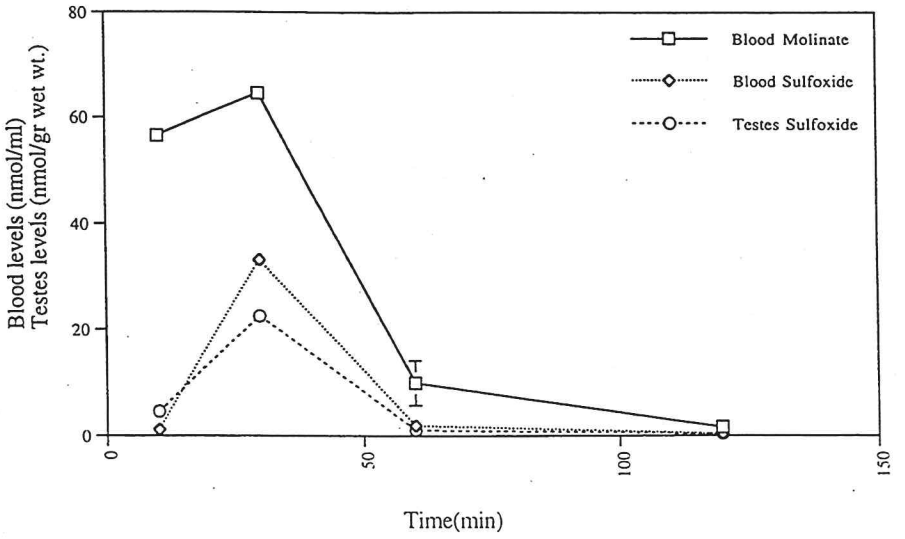
To date, molinate metabolism has only been carried out using two human liver slice preparations. We anticipate 2 more preparations through arrangement with Dr Carol Green at SRI although due to the difficulties of obtaining fresh human tissue, the exact time when these preparations is available is difficult to predict.

Objective 2. Relationship of Molinate Testicular Toxicity to Levels of Molinate Sulfoxide

Earlier in this project, data was obtained suggesting that whole rat seminiferous tubules with associated interstitial Leydig cells were unable to metabolize molinate. With this in mind Objective 2 proposed to inhibit cytochrome P450, the liver enzyme system we had shown to be responsible for sulfoxide generation. Using cytochrome P450 inhibitors, we proposed to decrease the amount of sulfoxide reaching the testis and therefore decrease testicular toxicity. The goal of this was to implicate further circulating sulfoxide formed by the liver as responsible for molinate-induced testicular toxicity.

Data obtained in the previous year of support has brought new considerations to this approach. Firstly, repeat studies with testis microsomes revealed that, similarly to liver microsomes, the testis preparation was capable of forming the sulfoxide metabolite. Moreover, all cytochrome P450 inhibitors tested inhibited both the nontoxic and toxic cytochrome P450-dependent pathways. Although the elegance of the original experimental design was not possible due to the lack of selectivity of the cytochrome P450 inhibitors, *in vivo* studies have measured testicular and blood levels of sulfoxide after molinate administration (Fig 3) and shown that similar levels are found in the testis and the blood suggesting that liver metabolism and transport of the sulfoxide in the blood contributes substantially to determining the amount of sulfoxide in the testis.

Fig. 3. Blood and Testis Levels of Molinate and Molinate Sulfoxide after Intraperitoneal Administration of Molinate (400 mg/kg)



Objective 3. Identification of a Carboxylesterase as the Major Protein Bound by Molinate Metabolites.

It is well established in toxicology that studies which incorporate both metabolic parameters and mechanistic understanding of the events leading to toxicity allow the best estimate of risk and extrapolation between species. Preliminary data indicated that incubation of liver microsomes with [^{14}C]-molinate, resulted in tight binding of radioactivity to only one protein suggesting that this protein could be involved in toxicity. Therefore studies were undertaken to identify the protein. Both the sulfoxide and sulfone metabolites of molinate are electrophilic and capable of covalent binding to cellular proteins therefore either or both metabolites could be responsible for the binding reaction. Protein binding of [^{14}C]molinate as well as [^{14}C]molinate sulfoxide and [^{14}C]molinate sulfone was investigated in liver and testis microsomal preparations. All three compounds in preparations from both tissues bound extensively and tightly to a single protein of approximately 60 KDa molecular weight. PMSF, a known inhibitor of serine esterases, prevented [^{14}C]molinate binding suggested that the molinate binding protein could be an esterase. Consistent

with its identity as an esterase was the protein's molecular weight under denaturing and non-denaturing conditions and a pI of 6.1. The protein was purified to homogeneity, subjected to MALDI-TOF mass spectral analysis and an N-terminal sequence for the first 17 amino acids was obtained. The N-terminal sequence showed 100% homology with a carboxyesterase, hydrolase A. Testicular esterase activity, localized to the Leydig cell by histochemical staining was markedly. inhibited by molinate. From this, the current hypothesis under investigation is that molinate-induced inhibition of esterase activity in the Leydig cell inhibits mobilization of cholesterol esters required for testosterone biosynthesis.

Summary of 1997 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. Within this framework, our previous studies have implicated molinate sulfoxidation as the metabolic pathway associated with testicular damage. *In vitro* methodology has been used to compare the capacity of rat vs human liver to metabolize molinate via not only the toxic sulfoxidation pathway but also nontoxic and detoxification pathways.

The major accomplishments in the previous year of support were:

Objective 1. Metabolism of molinate in rat and human liver slices.

In liver slice studies carried out this year, all molinate metabolic pathways including the detoxification pathway catalyzed by cytosolic glutathione transferase were measured. Human liver slices formed a markedly greater (x 5) amount of molinate glutathione conjugate compared with rat slices. In agreement with our earlier microsomal work, human liver was also less capable than rat of forming the toxic sulfoxide metabolite. Taken together, these data would predict that in the human, less sulfoxide would be available to cause toxicity.

Objective 2. Relationship of Molinate Testicular Toxicity to Levels of Molinate Sulfoxide

I*n vivo* studies have measured testicular and blood levels of sulfoxide after molinate administration and shown that similar levels are found in the testis and the blood suggesting that liver metabolism and transport of the sulfoxide in the blood contributes substantially to sulfoxide levels in the testis. It was demonstrated that testis microsomes, similarly to liver microsomes, were capable of metabolizing molinate to the toxic sulfoxide raising the possibility that both testis and liver metabolism could contribute to testicular toxicity.

Objective 3. Identification of a Carboxylesterase as the Major Protein Bound by Molinate Metabolites. One protein is extensively bound in both liver and testis microsomes incubated with radiolabelled molinate. This protein was identified as a carboxyesterase, hydrolase A and proposed to be involved in testosterone biosynthesis.

Publications

1. 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. Accepted subject to minor revision.
2. Jewell, W.T. and M.G. Miller. Identification of a carboxylesterase as the major protein bound by molinate. Toxicology and Applied Pharmacology. Accepted subject to minor revision.

Abstracts

1. Jewell W.T. and M.G. Miller. Molinate-induced testicular toxicity: metabolic activation in rat and human liver. *The Toxicologist*, 31: 53, 1997.
2. 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.

Awards

Best Graduate Student Platform Presentation at Joint Meeting of NorCal SETAC and NorCal SOT, Spring 1996. Awarded to William Jewell. "Role of Metabolism in Molinate-induced Testicular Toxicity".

Best Graduate Student Poster Presentation at Joint Meeting of GETA and NorCal SOT, Spring 1997. Awarded to William Jewell. " Molinate-induced testicular toxicity: metabolic activation in rat and human liver."

Concise General Summary of Current Years Results:

This project has been funded for three consecutive years by the California Rice Research Board. 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 at relevant environmental exposure levels.

To date our studies strongly implicate molinate sulfoxidation as the metabolic pathway associated with testicular damage. *In vivo* studies have found similar levels of sulfoxide in the testis and blood after molinate administration suggesting that liver metabolism and blood transport of the sulfoxide contributes substantially to sulfoxide levels in the testis. However, testis microsomes also metabolized molinate to the toxic sulfoxide raising the possibility that both testis and liver metabolism could contribute to testicular toxicity. *In vitro* studies have compared the capacity of rat vs human liver slices to metabolize molinate via not only the toxic pathway but also nontoxic and detoxification pathways has been measured. Compared with rat liver, human liver slices metabolized molinate via glutathione conjugation, the metabolic pathway responsible for sulfoxide detoxification, to a much greater extent (x 5). In agreement with our earlier microsomal work, human liver was also less capable than rat of forming the toxic sulfoxide metabolite. Taken together, these data would predict that in the human substantially less sulfoxide would be available to cause toxicity.

It is well established in toxicology that studies which incorporate both metabolic parameters and mechanistic understanding of the events leading to toxicity allow the best estimate of risk and extrapolation between species. With this in mind, a putative biological target molecule has been identified. One protein is extensively bound in both liver and testis microsomes incubated with radiolabelled molinate. This protein was identified as a carboxyesterase, hydrolase A, and proposed to be involved in testosterone biosynthesis. At low non toxic doses of molinate, the esterase is inhibited but rapidly recovers. Therefore it would be important to understand the biological threshold which is exceeded at higher dose levels.