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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 1996 Funding: $29,772

Objectives and Experiments Conducted to Accomplish Objectives:

This research project is investigating the possibility that species differences in metabolism play a key role in determining species sensitivity to molinate induced male reproductive toxicity. Our previous studies in rats strongly implicated a molinate metabolite generated via sulfoxidation as the chemical species responsible for molinate-induced testicular toxicity. Recently, it was demonstrated that this metabolite could not be generated by testis preparations therefore, in order to exert its toxic action, it must be formed by liver metabolizing enzymes and then travel in the blood to the testis. To assess the likelihood that the toxic sulfoxidation pathway would be important in the human metabolism of molinate, *in vitro* metabolism studies were carried out using liver microsomal preparations from human and rats. The goal was to determine whether humans readily formed the toxic metabolite. 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 nonhomogenous 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.

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, preliminary data has shown that when liver microsomes are incubated with [^{14}C]-molinate radioactivity is found associated with only one protein. Our tentative identification of that protein is that it is an esterase. In the testis, hydrolase A is an esterase located in the testosterone-producing Leydig cell. Esterases are important in the testis as they hydrolyze cholesterol esters which are the precursors in the synthesis of testosterone. We propose that the sulfoxide may be binding to and inhibiting an esterase which could be important for testosterone synthesis.

Summary of 1996 Research by Objective

1. To determine which molinate metabolite is responsible for testicular toxicity and to quantify its formation and detoxification by conducting *in vivo* metabolism and toxicity studies.
2. To compare molinate metabolism in human and rat liver slices and, based on the amount of toxic metabolite formed, predict species differences in susceptibility to toxicity.
3. To explore the feasibility of developing a physiologically based pharmacokinetic model (PBPK) for molinate metabolism in rats and humans to improve interspecies extrapolation.

OBJECTIVE 1. Identity, Formation and Detoxification of the Molinate Toxic Metabolite in the Rat. A single dose animal model for molinate male reproductive toxicity was developed in the rat (for details, see Annual Report 1995). Our studies have indicated that molinate is not the chemical species responsible for testicular damage and the best candidate for a testicular toxicant is either molinate sulfoxide or a metabolite generated via the sulfoxide pathway. In the rat, molinate is oxidatively metabolised to form either molinate sulfoxide or hydroxy molinate[1,2] (Fig.1). Formation of hydroxymolinate can be viewed as a nontoxic metabolic pathway. The sulfoxide can be further metabolized to a second chemically reactive species, a sulfone. Both the sulfoxide and sulfone would be detoxified by conjugation with glutathione ultimately giving rise to the same conjugate.

Fig. 1 Molinate Metabolism

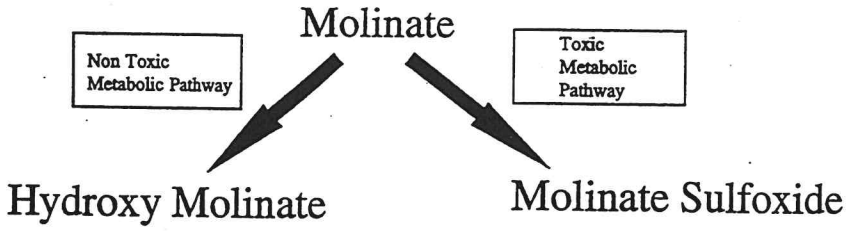


Figure 1. Proposed Toxic and Nontoxic Pathways of Molinate Metabolism

Our initial candidate for a toxic metabolite was molinate sulfoxide. However, intratesticular injection of the sulfoxide gave no indication of damage, whereas the sulfone was so reactive it caused indiscriminant effects on testicular tissue uncharacteristic of the molinate lesion. The lack of toxicity after intratesticular injection of the sulfoxide is in contrast to the pronouced lesion found after intraperitoneal administration of this metabolite. However, for reasonably stable compounds, levels achieved after intra-testicular administration fall very rapidly and cannot be equated with testis levels after intraperitoneal administration. Thus, the sulfoxide, which our studies have shown to be of sufficient stability to circulate in the blood, remains the top candidate metabolite responsible for testicular toxicity. The site of molinate sulfoxidation and the enzyme responsible for metabolism have been investigated. Whole seminiferous tubules with associated interstitial Leydig cells were prepared from rat testis and were found to be incapable of metabolizing molinate further indicating the importance of the liver as the organ responsible for metabolic activation of molinate. In liver microsomes, the cytochrome

P450 enzyme system and not flavin monoxygenase, was shown to be responsble for sulfoxide formation. This is important in our proposal for 1997 where, since cytochrome P450 activity can be manipulated using specific inhibitors, the goal will be to modulate sulfoxidation and monitor changes in testicular toxicity thus providing strong data that circulating sulfoxide is responsible for the molinate-induced testicular toxicity.

OBJECTIVE 2. Molinate Metabolism in Rat and Human Liver Preparations. Previously, preliminary *in vitro* studies compared the metabolic capabilities of rat vs. human liver microsomal preparations, with the goal of establishing whether humans metabolize molinate via the toxic sulfoxidation pathway or via the nontoxic hydroxy molinate pathway. The data predicted that sulfoxidation capacity in the rat was greater than in the human. However, the data had been obtained only in one rat and one human preparation. These microsomal studies have been completed for 3 rat preparations and 7 human preparations.The major molinate metabolites detected were hydroxymolinate and molinate sulfoxide. No sulfone could be found in the incubation media. Overall, with the increase in the number of samples the rat data changed little but the human data demonstrated a wide variability.

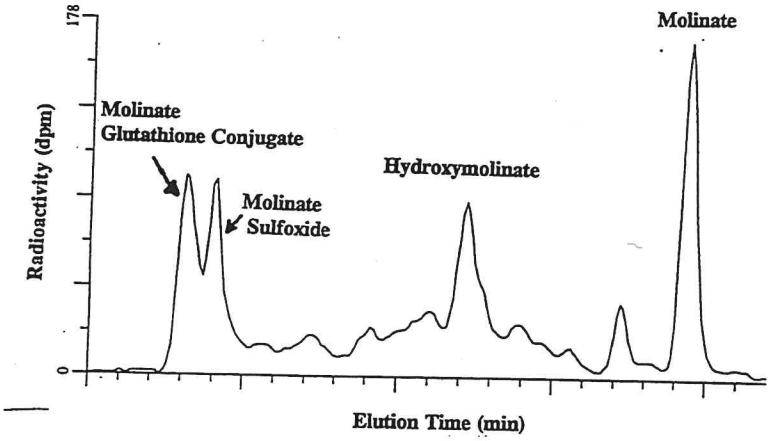
Enzyme kinetic data are presented in Table 1. The K_m represents the affinity of the enzyme for molinate and is defined as the substrate concentration where the velocity of the reaction proceeds at a half maximal rate. The lower the K_m value the more readily the substrate is metabolized at low concentrations. The Vmax value indicates the maximal capacity of the enzyme to form a particular metabolite. In the rat, the sulfoxidation pathway has lower affinity and higher capacity than the hydroxylation pathway. This means that at low dose levels(substrate concentration) the primary route of metabolism would be hydroxylation. However, as the capacity of this pathway is readily exceeded, higher dose levels would result in a shift of metabolism to the toxic sulfoxide metabolite. In the human, a similar situation exists. However, the numbers indicate that hydroxylation in humans has a higher capacity therefore the shift to sulfoxidation would occur at higher dose levels. As more human liver preparations were analyzed, it was apparent that the metabolic capability of liver preparations obtained from individual humans was highly variable. Nevertheless, it still can be concluded that at low doses the preferred pathway is the nontoxic hydroxylation pathway.

Table 1

	Sulfoxidation		Hydroxylation	
Rat #	Km (uM)	Vmax	Km (uM)	Vmax
1	422	1230	142	23.3
2	388	1524	147	46.9
3	390	1524	125	36.1
Mean	400+/-15.6	1426+/-139	138+/-9.4	35.4+/-9.65
Human #				
1	450	632	114	74.1
2	609	1045	100	21.3
3	203	2500	286	81.9
4	74	746	63	68.5
5	116	820	191	67.1
6	92	952	189	67.1
7	248	690	92	53.5
Mean	256 +/- 187	1055 +/- 605	148 +/- 72	61.9 +/- 18.4

Since human variability has blurred the species differences in metabolism via the toxic and nontoxic pathways, it becomes increasingly important to define glutathione transferase-catalyzed detoxification in both species. Since conjugation with glutathione occurs in the soluble cytosol and not the particulate microsomal fraction of the liver, these studies are being carried out in liver slices. Preliminary data has been obtained with rat liver slices. Molinate is rapidly metabolized and HPLC separation conditions are being optimized to separate metabolites. The radioactive HPLC trace shown below(Fig. 2) indicates extensive metabolism of the radiolabelled parent compound to form primarily molinate sulfoxide and polar metabolites as well as hydroxymolinate. The polar metabolites coelute with the synthesized glutathione conjugate. However, it is also possible that a component of these polar compounds are glucuronide and/or sulfate conjugates which can be determined after glusalase hydrolysis to cleave the conjugate and regenerate hydroxymolinate.

Fig. 2



The importance of liver glutathione in the detoxifciation of the sulfoxide has been demonstrated by the rapid depletion of liver glutathione levels (approx. 50% depletion) after exposure to molinate. Glutathione levels return to control values after 24 hr after molinate administration when the majority of molinate would have been cleared from the animal.

OBJECTIVE 3. Exploration of a PBPK Model for Molinate.

The above two objectives represent the majority of the work carried out in the 1996 year of funding. Development of a physiologically based pharmacokinetic (PBPK) model for molinate metabolism in rats and humans still represents an important goal. However, additional primary information is still required before such a model can be realistically implemented. Classically Km/Vmax relationships are viewed as the hardest to obtain and this is an ongoing effort.

Additional Mechanistic Studies

In addition to pharmacokinetic analyses which predict the likelihood of formation of a toxic metabolite, understanding the mechanism by which a chemical causes toxicity can be very

important in determining whether that chemical is likely to be a significant risk to exposed humans. With this in mind preliminary mechanistic studies have found that when rat liver microsomes were incubated with [^{14}C]-molinate and microsomal proteins were separated by gel electrophoresis, only one band retained radioactive material. This indicated that molinate was being metabolized to a reactive species which preferentially bound to only one protein. It has also been observed that non-specific esterase activity is inhibited in the testis of molinate-treated animals. The bound protein is at a molecular weight (57-60 Kda) consistent with the esterase, hydrolase A, which is also found in the Leydig cell of the testis. If this enzyme (which, if inhibited, could decreases testosterone synthesis) is the target for the action of molinate sulfoxide, exposure levels which do not result in significant inhibition could be considered sub threshold and presenting minimal risk.

Summary of 1996 Research (Major Accomplishments) by Objective:

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The **Objectives** were:

1. To determine which molinate metabolite is responsible for testicular toxicity and to quantify its formation and detoxification by condcting *in vivo* metabolism and toxicity studies.

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Additional Data

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References

1. DeBaun, J.R., D.L.Bova, C.K.Tseng, and J.J.Menn. Metabolism of ^{14}C-Ordram (Molinate) in the rat. 2.Urinary metabolite identification. J. Agric. Food Chem., (26)5, 1096-1104, 1978.
2. Madan, A., A.Parkinson, and M.F.Faiman. Identification of the human and rat P450 enzymes responsible for the sulfoxidation of S-methyl N,N-diethylthiolcarbamate (DETC-ME). Drug Metab. Dispos. 23, 1153-1162, 1995.

Publications or Reports

1. W.T. Jewell and M.G. Miller. Testicular toxicity in rats after administration of molinate: Role of metabolism in toxicity. International Congress of Toxicology-VII, Seattle, WA. 1995.
2. W.T. Jewell and M.G. Miller. Role of metabolism in testicular toxicity of molinate (Ordram) in male rats. *The Toxicologist,* **30,** 275, 1996.
3. W.T. Jewell and M.G. Miller. Molinate-induced testicular toxicity: Metabolic activation in rat and human. Society of Toxicology Annual Meeting, Cincinnati, OH, March 9-13, 1997.

(Abstracts 2 and 3 are currently papers in preparation)

Concise General Summary of Current Years Results:

This research project is investigating the possibility that species differences in metabolism play a key role in determining species sensitivity to molinate induced male reproductive toxicity. Molinate-induced testicular toxicity is caused by a toxic metabolite formed in the sulfoxidation pathway of molinate metabolism. Our recent studies have demonstrated that this metabolite could not be generated by testis preparations and therefore, in order to exert its toxic action, it must be formed by liver metabolizing enzymes and then travel in the blood to the testis. Since liver metabolism will play an important role in the delivery of the toxic metabolite to the testis, the ability of liver enzymes to metabolically activate molinate has been studied in the rat and human in order to predict species differences in susceptibility to toxicity. The data indicated that at low dose levels humans would form less sulfoxide than rats therefore would be less likely to be susceptible to testicular damage. However, human liver preparations showed wide variability in their metabolic capabilities and species differences in metabolism were blurred by the wide range of values obtained for the human data. Additional data has been obtained which indicates that a molinate metabolite (putatively the sulfoxide) binds strongly to one protein and that this protein may have esterase activity and be involved in testosterone synthesis.