Differentiating Toxicities of Conazole Fungicides Through Metabonomic Analyses of Multiple Tissues

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Abstract

The conazole fungicides represent a large group of compounds widely used agriculturally for the protection of crop plants (Hutson, 1998) and pharmaceutically in the treatment of topical and systemic infections (Sheehan, 1999). In 1999, the latest period for which agricultural usage estimates are available, 79 and 556 million pounds of fungicide active ingredient were used in the U.S. and world markets, respectively (Donaldson, 2002), creating concern over the impact these compounds may have through environmental exposure to humans and other organisms. In an attempt to better understand the toxicities of these compounds, an NMR (Nuclear Magnetic Resonance)-based metabonomics approach was used to determine differences in the toxicities of two conazole fungicides (myclobutanil and triadimefon) by analyses of metabolite changes occurring in blood serum, liver tissue, and testicular tissue of control and exposed rats. Metabonomics is the quantitative measurement of a broad spectrum of metabolic responses of living systems in response to disease onset or genetic modification. By monitoring changes in cellular metabolites in response to the introduction of a toxicant, the biochemical pathways affected can be determined and the specific toxic response characterized on a molecular level. Furthermore, metabonomic data can be used in conjunction with genomic and proteomic data to more fully characterize environmental effects. Through the combined efforts of the U.S. Environmental Protection Agency (U.S. EPA), the Procter and Gamble Company, and the Imperial College (London, England), distinct metabolite profiles produced by exposure to conazole fungicides were identified. These metabonomic profiles identify potential biological pathways responding to the exposures. One distinct change observed was induction of betaine levels in rats exposed to myclobutanil versus those exposed to triadimefon or control rats. This betaine effect indicates altered homocysteine metabolism. Homocysteine is an intermediate metabolite of the amino acid methionine, and altered homocysteine levels have been linked to a variety of health problems. These preliminary results support the case for metabonomics as part of the Computational Toxicology program in the ORD and suggest the potential of metabonomics for assessing the toxicity of compounds regulated by the U.S. EPA.

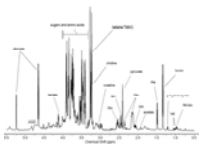
Introduction

Extensive use of conazole fungicides both agriculturally and pharmaceutically has created concern regarding the threat these compounds may pose as a result of environmental exposure to humans and other organisms. Toxicity studies conducted in rodents have shown the conazoles to target a variety of organs producing multiple effects. Among these are the production of liver tumors in mice, thyroid tumors in rats, and developmental and reproductive lesions (Zarn, 2003). Although grouped into a single class, the specific biological effects produced by individual members vary widely, presenting difficulty in fully assessing risk from conazoles. Therefore, the ability to rapidly differentiate the toxicities these compounds induce on a molecular scale will provide a route for more effectively setting risk assessments for individual members of this class of chemicals.

A recently developed approach involves the use of advanced analytical techniques such as NMR (Nuclear Magnetic Resonance) spectroscopy (figure 1) to analyze changes in the levels of cellular metabolites (e.g. sugars, carbohydrates, amino acids, etc.) that relate to the toxic mechanism(s) involved in responding to the presence of a given chemical. This approach, known as metabonomics (figure 2), has proven to be a powerful tool in the assessment of toxicity or other physiological alterations in a variety of different organisms (Bailey, 2003; Coen, 2003; Viant, 2003). We have employed metabonomics for the determination of changes in metabolite profiles in a variety of tissues measured using NMR spectroscopy for rats exposed to both toxic and non-toxic levels of conazole fungicides. For the present study, two conazoles producing different toxicities (triadimefon produces liver toxicity and myclobutanil is a testicular toxin) have been chosen to determine the feasibility of using this approach to differentiate responses.

Figure 1 - NMR spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique that allows one to observe the levels of metabolites in a variety of tissues and biofluids for metabonomic analyses.



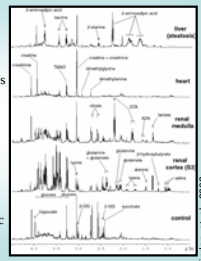
A portion of an NMR spectrum of a rat liver tissue extract with several metabolites labeled. Changes in the levels of these metabolites (e.g. sugars, carbohydrates, amino acids, etc.) can allow one to determine the nature of toxicity induced by exposure to a given compound.

Figure 2 - Metabonomics

Disease states or exposure to drugs or other biologically active compounds produces changes in the levels of metabolites present in tissues and biofluids (e.g. blood, urine, etc.). The identification of these effected metabolites yields valuable information for determining the nature of a disease or toxic response.

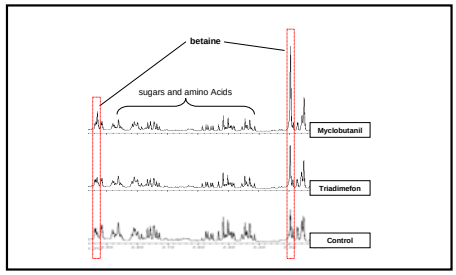
Using metabonomics, the presence of disease or toxicity can be determined rapidly and inexpensively in comparison to other approaches (e.g. genomics, proteomics, etc.).

Shown here are urine metabolite profiles in mice resulting from exposure to organ-specific toxins (i.e. liver, heart, and kidney)



Lindon, et. al., 2002

Figure 3 - Differential Responses to Specific Conazole Fungicides



Metabonomic analyses of liver tissues from both control rats and those dosed separately with two conazole fungicides with different toxicities revealed several changes in the levels of cellular metabolites. For example, a severe alteration in the level of betaine in those rats exposed to myclobutanil was observed. Betaine often serves in a protective capacity in the liver.

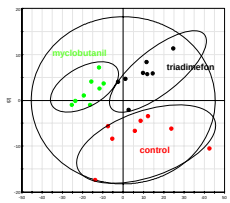
Figure 4 - Chemometric/Statistical Methods for Rapidly Determining Changes in Metabolites

The large number of metabolites changing in response to a given toxic insult can be overwhelming without the use of statistical tools that can quickly identify changes.

Various computational methods designed to simplify the analysis of large amounts of data (e.g., principal components analysis (PCA)) provide the ability to quickly determine metabolite changes as opposed to assessing changes simply by eye.

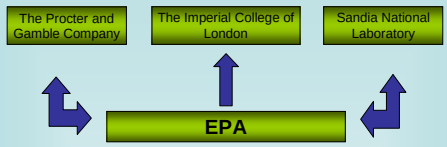
While these methods are effective, more- advanced methods are needed to observe very subtle changes that may carry information crucial to more accurately defining organism responses.

We are currently working with researchers at **Sandia National Laboratory (Albuquerque, NM)** to develop these methods (see poster titled: "Multivariate Curve Resolution of NMR Spectroscopy Metabonomic Data").



PCA plot of rat liver extracts from animals exposed to myclobutanil and triadimefon. Using plots like this, one can determine with relative ease the metabolites that are changing due to toxicity.

Figure 5 - Collaboration with Academia and Industry to Strengthen ORD's Metabonomics Program



- For the metabonomics program in ORD to succeed in the early stages of its development, support from researchers in both industry and academia was essential.
- Fortunately, a platform for collaboration with researchers at The Procter and Gamble Company (Cincinnati, OH) and those at The Imperial College of London (London, UK) was established. This collaboration has been successful not only in the pursuit of current projects (e.g. the work outlined here) but also in the establishment of potential future projects.

Results

While the concentration of a number of metabolites changed in response to exposure to both of the conazoles used separately in this study, the major goal was to determine if differences between the two compounds (myclobutanil and triadimefon) could be determined. Analyses of the effects of the conazoles on liver metabolite composition revealed distinct differences (figure 3). One of the most notable responses was a marked change in the levels of betaine (bē ' tān) that differed among treatments. While triadimefon only produced a slight change in this metabolite, myclobutanil produced a much larger change. As betaine serves a protective function in the liver, it is likely that this change reflects the liver's ability to cope with the toxic effects of myclobutanil. Rats exposed to triadimefon, a recognized liver toxin, do not show this large change potentially indicating the absence of this protective response. Both blood serum and testicular tissue were also analyzed using this approach and these displayed a number of metabolite changes in response to exposure (data not shown).

Conclusions

The potential for metabonomics to supply rapid and accurate information on the toxicity of compounds of interest to the EPA has been shown here through the differentiation of the effects of conazole fungicides in rats. In addition, the results of this work are being compared to genomic data derived from rats exposed to the same conazoles to determine correlations in gene expression changes and metabolite changes in order to integrate the two approaches. Furthermore, we are developing methods for advanced data analysis which will provide the means to more fully realize the potential of this new method for studying toxicity (figure 4). In addition to rodent studies, we have also begun working with environmental fish models (fathead minnow, zebra fish, and rainbow trout) in collaboration with other EPA researchers (NERL and NHEERL) and the University of Georgia to extend metabonomics into the ecotoxicology realm. As a result of Agency support and collaboratative efforts (figure 5) this initial study serves as a major step in the establishment of an integrated metabonomics program at EPA.

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