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Subacute oral toxicity assessment of benalaxyl in mice based on metabolomics methods

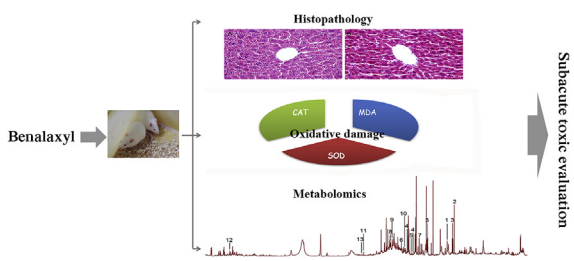
Xinru Wang [a, b], Dezhen Wang [b], Zhiqiang Zhou [b], Wentao Zhu [b, *]

[a] Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou 310008, China
[b] Beijing Advanced Innovation Center for Food Nutrition and Human Health, Department of Applied Chemistry, China Agricultural University, Yuanmingyuan West Road 2, Beijing 100193, China

HIGHLIGHTS

- Mice were exposed to benalaxyl for 30 consecutive days.
- Metabolic responses were assessed using untargeted and targeted metabolomics approaches.
- Significant oxidative damage was observed, without histopathological injury.
- Distribution of benalaxyl in brain was stereoselective.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the metabolic responses of mice after 30 days of exposure to benalaxyl were assessed using NMR-based untargeted metabolomics and LC-MS-based targeted profiling of 20 amino acids. Urinary ^{1}H NMR analyses revealed alterations in energy metabolism, lipid metabolism, vitamin B metabolism, the urea cycle and amino acid metabolism, and targeted analyses indicated that the serum levels of asparagine, histidine, lysine and aspartic acid were significantly altered. Additionally, significant oxidative stress was observed in the liver and kidney, although no apparent histopathological injury was observed. The tissue distribution indicated a significant stereoselectivity in the brain, where (−)-R-benalaxyl was enriched. These data provide a comprehensive picture of the subacute toxic effects of benalaxyl in mice. The results of this study suggested that, for a toxicity evaluation, metabolomics analysis is much more sensitive than traditional toxicological methods. The results also highlight the combined use of untargeted and targeted metabolomics approaches in evaluating the health risks of xenobiotics.

1. Introduction

As an important broad-spectrum acetanilide fungicide, benalaxyl (methyl-N-phenylacetyl-N-2, 6-xylyl alaninate, Fig. 1) is active against Oomycetes belonging to Peronosporaceae, a family responsible for many diseases in economically important crops such as grapes, lettuce, tomato, potato, soybean and tobacco(-Giraudi et al., 1999; Xu et al., 2009). Although benalaxyl is generally regarded as a pesticide of high efficiency and low toxicity, there is still a considerable risk for non-targeted organisms. Many efforts have been made to understand the cytotoxicity, acute toxicity and subchronic toxic effects of benalaxyl in earthworms, Chinese lizards and Scenedesmus obliquus (Xu et al., 2009; Huang et al., 2012; Al-Sarar et al., 2014; Wang et al., 2014). Very little information is

* Corresponding author.
E-mail address: wentaozhu@cau.edu.cn (W. Zhu).

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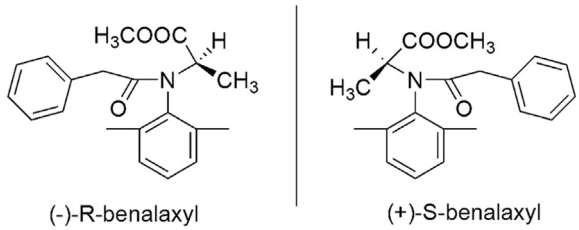

Fig. 1. Chemical structure of benalaxyl enantiomers.

available about the health risks of benalaxyl in mammals exposed at low levels for long periods of time.

Omics approaches, based on the analysis of gene transcription, protein expression and metabolic profiles, have offered an opportunity to unravel the complex pathways affected by chemical contaminants, and these approaches enhance our understanding of the corresponding health risk in organisms(Santos et al., 2010). The metabolomics approach in particular is widely used for understanding toxin-induced endogenous biochemical perturbations. While the other two omics technologies (transcriptomics and proteomics) only focus on the intermediate steps (mRNA and protein expression) (Bugrim et al., 2004), metabolomics reveals the end-point metabolite responses, which serve as direct signatures of biochemical perturbations and therefore provide insights into the mechanisms of toxicity(He et al., 2012). Thus, metabolomics is considered a useful tool in the field of toxicology research(Wang et al., 2009).

Metabolomics analyses can generally be separated into two groups: targeted and untargeted analyses. Targeted metabolomics is used when a defined set of metabolites is to be examined, typically focusing on one or more selected pathways of interest(Dudley et al., 2010). Untargeted metabolomics involves simultaneously measuring as many metabolites as possible without bias(Patti et al., 2012). In contrast to targeted metabolomics, untargeted metabolomics is global in scope and reveals the comprehensive metabolism of a whole organism. The most frequently used platforms in metabolomics are mass spectrometry (often combined with GC and LC) and Nuclear Magnetic Resonance (NMR) spectroscopy(Dunn et al., 2011). An NMR spectroscopy-based metabolomics approach coupled with pattern recognition analysis, such as partial least-squares- discriminant analysis (PLS-DA) and principle components analysis (PCA), allows analysis of biofluids to provide valuable information on biochemical perturbations and identify potential patterns for metabolite disturbances associated with toxic effects(Deng et al., 2014).

Oxidative stress is one mechanism of toxicity caused by xenobiotics. It has been reported that the toxicity mechanisms of many pesticides are associated with stimulation of free radical production, lipid peroxidation and alterations in antioxidant capability(Abdollahi et al., 2004). Benalaxyl has been reported to induce oxidative stress in *Scenedesmus obliquus* (Huang et al., 2012), *Eremias argus* (Wang et al., 2014) and Chinese hamster ovary cells (Al-Sarar et al., 2014). However, the effect of benalaxyl on mice is unclear.

The objective of the present study was to evaluate the subacute multi-toxic effects of benalaxyl on mice. Histopathological investigations were conducted, and oxidative stress and tissue distribution were studied after 30 days of exposure. Additionally, an NMR-based untargeted metabolomics strategy was used to evaluate the metabolic changes, and an LC-MS based targeted metabolomics strategy was used to assess the disorder of twenty amino acids in mice.

2. Experimental methods

2.1. Chemicals

Racemic benalaxyl standard (>99%) was obtained from Institute for Control of Agrochemicals, China Ministry of Agriculture. D_2O and 2,2′,3,3′-tetradeutero-3-(trimethylsilyl) propionic acid sodium salt (TSP-D4) were purchased from Sigma-Aldrich (St. Louis, MO). L-Alanine, L-Arginine, L-Asparagine, L-Aspartate, L-Cysteine, L-Glutamate, L-Glutamine, L-Glycine, L-Histidine, L-Isoleucine, L-Leucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine and L-Valine were obtained from Aladdin Reagent (Shanghai, China). Algal amino acid mixture-^{13}C, ^{15}N (98 atom % ^{13}C, 98 atom % ^{15}N), purchased from Sigma, was used for internal standards. All other chemicals were from commercial sources.

2.2. Animals and administration

Eight-week-old male CD-1 mice were obtained from Vital River Laboratory Animal Company (Beijing, China). They were individually housed in stainless steel metabolism cages with free access to water and food. All mice were acclimated for one week before the experiment. During the experiment, mice were maintained at 22 °C on a 12 h light/dark cycle. Animals were treated in accordance with the current Chinese legislation approved by the independent Animal Ethical committee at China Agricultural University.

Fifteen male mice were randomly assigned to 3 groups, including one control group and two treatment groups (low and high doses). According to the previous data, the acute oral half-lethal dose (LD_{50}) of benalaxyl on mice was 680 mg kg^{-1}. Thus, 6 and 60 mg kg^{-1} were used as the low and high doses of the treatment groups. *Rac*-benalaxyl was suspended in corn oil and administered to mice through oral gavage for 30 consecutive days. The control group was treated with an equivalent volume of corn oil.

2.3. Sample preparation

During the treatment, the body weight of each mouse was monitored daily. Individual urine samples were collected 24 h after the final gavage. Then after centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was collected and kept at −80 °C for further NMR spectroscopic analysis. Mice were sacrificed 24 h after the final dose. Blood was collected and centrifuged at 4000 rpm for 10 min at 4 °C to obtain serum samples. Tissues including liver, kidney, heart, lung, spleen and brain were also collected. All samples were stored at −80 °C.

2.4. Histopathology

For histopathological examination, liver and kidney samples fixed in 10% formalin were processed into 4 μm paraffin sections and stained with hematoxylin and eosin. This was carried out as a paid service by a qualified pathologist in the College of Veterinary Medicine, China Agricultural University.

2.5. Oxidative stress analysis

The activities of superoxide dismutase (SOD) and catalase (CAT) and the levels of the lipid peroxidation product malonaldehyde (MDA) in liver and kidney were analyzed to assess the oxidative stress caused by benalaxyl treatment. The livers and kidneys of the mice were homogenized on ice and after centrifugation, the supernatants were collected and used for measurements. The