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An investigation into the formation of tebufenozide's toxic aromatic amine metabolites in human *in vitro* hepatic microsomes

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ABSTRACT

Tebufenozide is a nonsteroid ecdysone agonist that causes premature and incomplete molting in Lepidoptera. Studies conducted so far have shown the low toxicity of tebufenozide in mammals, birds and invertebrates. Tebufenozide potential metabolites such as aromatic amines are known to induce methemoglobinemia disorder in humans, most likely by the formation of *N*-hydroxy metabolites; therefore, the aim of this research is to investigate the formation of the potential toxic *N*-hydroxy derivatives in pooled human hepatic microsomal fractions. Analyses of metabolites by high performance liquid chromatography equipped by a time-of-flight detector (HPLC/TOF) indicated the formation of a hydroxylated metabolite (exact mass = 369; retention time: 6.65 min) and two de-dimethylethyl metabolites (exact masses = 313; retention times: 5.76 and 6.22 min). Hydroxylated tebufenozide metabolite resulted from hydroxylation at either the 3 or 5 position of the dimethylbenzoic acid moiety to form either 3-hydroxymethyl-5-methylbenzoic acid 1-(1,1-dimethylethyl)-2-(4-ethylbenzoyl) or 3-methyl-5-hydroxymethylbenzoic acid 1-(1,1-dimethylethyl)-2-(4-ethylbenzoyl), respectively. The two de-dimethylethyl-tebufenozide derivatives were 3,5-dimethylbenzoic acid-2-(4-hydroxyethylbenzoyl) and 3-hydroxymethyl-5-methylbenzoic acid-2-(4-ethylbenzoyl) or 3-methyl-5-hydroxymethylbenzoic acid-2-(4-ethylbenzoyl). Generally the metabolite formation rates increased with incubation time. The rate of hydroxylation of the dimethylbenzoic acid moiety was approximately 12 times higher than the hydroxylation of the ethylbenzoyl moiety. Tebufenozide does not appear to produce the toxic aromatic amine metabolites in human *in vitro* hepatic microsomes. This suggests that the fate of tebufenozide in humans is a process of detoxification rather than activation.

1. Introduction

Pesticides are an important group of compounds extensively used in agriculture, sanitation, and pest control. Humans are exposed to pesticides from various sources, such as residues in food and water, house use, and from occupational exposure [1,2], and exposure is dependent on the concentration of the contaminant and frequency and duration of the contact [3]. Tebufenozide, developed by Rohm and Haas Company (Philadelphia, PA, USA) for Lepidoptera control in agriculture and forestry, is one of the pest management alternatives designed to lessen the use of broad-spectrum synthetic pesticides [4]. The insecticide tebufenozide (*N*-*tert*-butyl-*N*′-[4-ethyl-benzoyl]-3,5-dimethylbenzohydrazide) belongs to a group of insect growth regulators, the benzoyl hydrazines. These substances have been found to act as agonists of ecdysteroidal molting hormones at the molecular level [5,6].

Upon the implementation of REACH legislation and the European Chemicals Agency, there is a need for robust and quick methods to characterize the metabolic fate and metabolic interactions of selected chemicals. One of the important groups is pesticides or in general, agrochemicals. A scheme for metabolic studies has originally been developed for the screening of potential drugs at the preclinical screening phase and this scheme has been applied for the study of pesticide metabolism in humans [7–10]. Pesticides are metabolized by many enzymes, including the cytochrome P450 enzymes, which play a key role in the metabolism of a wide variety of xenobiotics (Phase I).

Studies conducted so far on tebufenozide have shown that its acute toxicity in mammals, birds, non-target terrestrial invertebrates, and most aquatic organisms is low [11]. Degradation of tebufenozide occurs mainly *via* microbial processes and photolysis in natural aquatic systems [12]. Tebufenozide did not show a negative endocrine disruptor impact on the estuarine crustacean, the opposum shrimp *Neomysis integer*, *in vivo* [13]. Tebufenozide was found to activate human pregnane X receptor (PXR) receptor in HepG2 cells, but not human constitutive androstane receptor (CAR) in C3A hepatoma cells. CYP3A11 and CYP2B10 mRNA levels were induced in mouse primary hepatocytes treated with tebufenozide for 24 h. Human CYP3A4 mRNA was induced by 10 μM tebufenozide to 15-fold in the metabolically competent, human-derived HepaRG cell line [14].

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While tebufenozide is known as a substance with high environmental safety due to its mode of action mainly towards lepidopteran insects, its fate in humans needs to be better understood. The potential toxicity of tebufenozide metabolites has been assumed to be low since all of the *in vivo* toxicology studies on tebufenozide involve the generation of detoxified metabolites. Tebufenozide major metabolites illustrate low acute oral toxicity (LD_{50} values > 5000 mg/kg). In addition, its metabolites are inactive in bacterial mutagenicity assays [15]. On the other hand, methemoglobinemia disorder has been reported in connection with tebufenozide toxicity in humans [15]. This disorder, also known as "blue skin people", is caused by the abnormally high level of methemoglobin in the blood. Methemoglobin is actually a form of hemoglobin but attracts higher levels of oxygen, which means that less oxygen is released into tissues. Tebufenozide metabolites, such as aromatic amines, are known to induce methemoglobinemia, most likely by the formation of *N*-hydroxy metabolites [16]. Therefore, the objective of this study was to investigate the fate of tebufenozide in human hepatic preparations *in vitro*, since a human study could not be performed *in vivo* except under special circumstances and occupational exposures.

2. Materials and methods

2.1. Chemicals

High-performance liquid chromatography HPLC grade solvents were obtained from Rathburn (Walkerburn, UK). All other chemicals used were from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available. Water was in-house freshly prepared with Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP grade (ultra pure, 18.2 MΩ).

2.2. Human liver homogenates and microsomes

Human liver samples used in this study were donated by the University Hospital of Oulu as surplus from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. The livers were transferred to ice immediately after the surgical excision and cut into pieces, snap-frozen in liquid nitrogen and stored at −80 °C. The homogenate was prepared from livers of ten individuals by homogenizing liver tissue in four volumes of 0.1 M phosphate buffer (pH 7.4), *i.e.* the homogenate contained 200 mg of liver tissues/ml. The microsomes were prepared by standard differential ultracentrifugation [17]. The tissues were typically homogenized in 100 mM phosphate buffer and centrifuged at 10,000*g* for 20 min. The resulting supernatant, referred to as S9 fraction, was centrifuged at 100,000*g* for 60 min to yield the microsomal pellets and a cytosolic supernatant. The pellet was typically re-suspended in a volume of phosphate buffer and stored at −70 °C. The final microsomal pellet was suspended in 100 mM phosphate buffer, pH 7.4. Protein content was determined by the Bradford method [18].

2.3. In vitro assay of tebufenozide metabolites

The standard incubation mixture contained serial concentration of tebufenozide (final concentration 5, 25 and 100 μM), 0.1 mg pooled human liver microsomal proteins and 10 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). Tebufenozide was dissolved in dimethylsulfoxide (final concentration in the reaction medium 1.0%). After 2 min incubation at 37 °C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) the reaction was started by adding NADPH. The mixture was incubated at 37 °C for 0, 20, 40, 60 min and the reaction stopped with 200 μl of ice-cold acetonitrile. All incubations were carried out in duplicate samples. After centrifugation at 10,000 ×*g* for 15 min the supernatant was kept at −20 °C and analyzed by LC–MS.

For measuring the metabolites in liver homogenates incubation mixtures, which contained the same final tebufenozide concentrations as microsome incubations, were prepared. Other components in mixture were 40 μl human liver homogenates (contains 0.14 mg microsomal protein), 50 mM uridine 5′-diphosphoglucuronic acid, 10 mM glutathione, 12 mM adenosine-3′phosphate-5′-phosphosulfate and 10 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). Incubation method was similar as in microsome incubations.

2.4. LC–MS analysis

Before analysis samples were centrifuged for 10 min at 13,400 ×*g* with Eppendorf MiniSpin centrifuge (Eppendorf AG, Hamburg, Germany). Chromatographic separation was carried out with Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA, USA). Column used was a Waters XTerra MS C18 (2.1 mm × 50 mm, particle size of 3.5 μm) together with Phenomenex C18 2.0 mm × 4.0 mm precolumn (Phenomenex, Torrance, CA, USA). Temperature of column oven was 30 °C. The flow used was 0.4 ml/min. The eluents used were ultrapure-grade water containing 0.1% acetic acid (A) and acetonitrile (B). Linear gradient elution from 10% B to 35% B in 8 min followed by 35% B to 95% B in 2 min was applied. Solvent B was thus maintained at 95% for 1 min before reequilibration (5 min). Total analysis time was 16 min.

MS data was measured with a Micromass LCT (Micromass Ltd, Manchester, England) time of flight (TOF) mass spectrometer equipped with Z-Spray ionization source. A generic positive electrospray ionization method was used for all substrates and metabolites. Capillary voltage used was 3500 V, cone voltage 15 V and desolvation and source temperatures 300 and 150 °C, respectively. Nitrogen was used as desolvation and cone gas with flow rates of 700 and 150 l/h. The mass spectrometer and HPLC system were operated under Micromass MassLynx 3.4 software. For exact mass measurements the lock mass was leucine encephalin ($[M + H]^+$ at *m/z* 556,2771) and it was delivered into ionization source through tee union using a syringe pump (Harvard Apparatus, Holliston, USA). Molecular fragments were generated in-source with cone voltages of 25 or 35 V.

3. Results and discussion

High performance liquid chromatography equipped by time-of-flight mass spectrometry (HPLC-TOF) was used for the characterization of potential tebufenozide metabolites formed in human hepatic microsomes and homogenate incubation mixtures. Three metabolites were detected from the extracted mass chromatograms formed by 60 minute incubation with human liver microsomes at a tebufenozide concentration of 100 μM as shown in Fig. 1. One hydroxylated tebufenozide and two de-dimethylethyl-tebufenozide derivatives were detected. Hydroxylated tebufenozide metabolite (exact mass = 369; retention time: 6.65 min) resulted from hydroxylation at either the 3 or 5 position of the dimethylbenzoic acid moiety to form either 3-hydroxymethyl-5-methylbenzoic acid 1-(1,1-dimethylethyl)-2-(4-ethylbenzoyl) or 3-methyl-5-hydroxymethylbenzoic acid 1-(1,1-dimethylethyl)-2-(4-ethylbenzoyl), respectively. The two de-dimethylethyl-tebufenozide derivatives (exact masses = 313; retention times: 5.76 and 6.22 min) were 3,5-dimethylbenzoic acid-2-(4-hydroxyethylbenzoyl) and 3-hydroxymethyl-5-methylbenzoic acid-2-(4-ethylbenzoyl) or 3-methyl-5-hydroxymethylbenzoic acid-2-(4-ethylbenzoyl). Conjugated metabolites were not detected in human liver microsomes fortified with the appropriate cofactors for Phase II reactions.

The trend of metabolite formation as a function of incubation time at different tebufenozide concentrations was studied. The two de-dimethylethyl-tebufenozide derivatives were the major metabolites, while lower levels of hydroxylated tebufenozide were detected. The percentage of all metabolites increased with increased

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