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Evaluation of drug uptake and deactivation in plant: Fate of albendazole in ribwort plantain (*Plantago laceolata*) cells and regenerants

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ABSTRACT

Albendazole (ABZ) is a benzimidazole anthelmintic widely used especially in veterinary medicine. Along with other drugs, anthelmintics have become one of a new class of micro-pollutants that disturb the environment but the information about their fate in plants remains limited. The present study was designed to test the uptake and biotransformation of ABZ in the ribwort plantain (*Plantago lancelota*), a common meadow plant, which can come into contact with this anthelmintic through the excrements of treated animals in pastures. Two model systems were used and compared: cell suspensions and whole plant regenerants. In addition, time-dependent changes in occurrence of ABZ and its metabolites in roots, basal parts of the leaves and tops of the leaves were followed up. Ultrahigh-performance liquid chromatography coupled with high mass accuracy tandem mass spectrometry (UHPLC-MS/MS) led to the identification of 18 metabolites of ABZ formed in the ribwort. In both model systems, the same types of ABZ biotransformation reactions were found, but the spectrum and abundance of the ABZ metabolites detected in cell suspensions and regenerants differed significantly. Cell suspensions seem to be suitable only for qualitative estimations of drug biotransformation reactions while regenerants were shown to represent an adequate model for the qualitative as well as quantitative evaluation of drug uptake and metabolism in plants.

1. Introduction

The benzimidazole drug albendazole (ABZ) is a broad-spectrum anthelmintic widely used in veterinary and also in human medicine for the treatment of intestinal parasites as well as for the treatment of systemic worm infections (Danaher et al., 2006). The usefulness of this drug is uncontested, yet at the same time its massive use represents a clear risk to the environment. ABZ and its metabolites enter the environment via different pathways, with the most important route being the excretion of these substances in the urine and faeces of livestock animals (Boxall et al., 2003). ABZ and its metabolites excreted to the environment may persist there and impact non-target organisms. Whereas the humans and/or animals for which the drugs have been developed are exposed for a defined period of treatment, smaller organisms and plants in these environments are exposed chronically (Weiss and Porzelt, 2008; Fisher, 2008).

For this reason ABZ as well as other anthelmintics have been classified as emerging environmental contaminants (Wagil et al., 2015). The negative environmental effects of benzimidazole drugs have been described many times. Comprehensive studies, for example, have revealed the toxicity of benzimidazoles to different aquatic organisms (Wagil et al., 2015) (Bunduschuh et al., 2016). ABZ can also severely affect earthworms living in soil (Wang et al., 2009). Moreover, the exposure of lower development stages of parasitic helminths to non-lethal concentrations of anthelmintics in manure and soil may also promote the development of drug-resistant strains of helminths (Wolstenholme et al., 2004).

In addition to animals, plants also rank among the non-target organisms which may be affected by anthelmintics in the environment (pastures with treated animals, fields fertilized with dung from treated animals, aquatic ecosystems). But in contrast to animals, the information about the impact and fate of veterinary drugs including anthelmintics in plants remains limited (Bártíková and Skálová, 2016). Plants are able to uptake and transform drugs to non- or less-toxic compounds and store them in vacuoles and cell walls (Bártíková et al., 2015). However, it is important to keep in mind that some drug metabolites

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can be similar or even more toxic than their parent compound (Halling-Sorensen and Tjornelund, 2002). For this reason, more detailed information about the metabolic pathways of each drug is necessary for a complex evaluation of eco-toxicological risks.

The biotransformation of drugs and other xenobiotics occurs through two phases. Oxidation along with reduction or hydrolysis of the xenobiotic represents phase I; in this step, reactive and hydrophilic groups are inserted or uncovered in the structures of xenobiotics. In phase II, xenobiotics or their phase I metabolites can undergo conjugation reactions with endogenous compounds. Glutathione and saccharides represent the main conjugation agents in plants. Plants have evolved an extremely sophisticated battery of xenobiotic-metabolizing enzymes, some of them similar to those in humans and animals, but several others are plant-specific (Bártíková et al., 2015).

In our previous studies, the uptake and metabolism of ABZ was tested in the reed (Podlipna et al., 2013) and harebell (Stuchlikova et al., 2016). Reed and harebell cells were able to uptake and biotransform ABZ into several metabolites. Most of the ABZ metabolites can be considered as deactivation products, but some of them remain biologically active. When the metabolic pathways of ABZ in the harebell and reed were compared, marked inter-species differences were revealed. Nevertheless, all known data have been obtained using *in vitro* suspension cultures, thus no information has been found about ABZ uptake and metabolism in the whole plant. For this reason, the present study was designed to test and compare the ABZ uptake and biotransformation in cell suspension and in whole plant regenerants. In addition, time-dependent changes in occurrence of ABZ and its metabolites in roots, basal parts of the leaves and tops of the leaves were followed up. The ribwort plantain (*Plantago lanceolata*), a perennial herb, was chosen, as the contact of these plants with anthelmintics via the dung of treated animals is widespread. The aim of this study was to increase information about ABZ fate in plants and to compare and evaluate the predictive values and limitations of the two model systems commonly used in biotransformation studies.

2. Material and methods

2.1. Chemical and reagents

ABZ and all other chemicals (UHPLC, MS, or analytical grade) were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Preparation and cultivation of whole plant regenerants

The regenerants were achieved from sterilized seeds (in 70% ethanol for 1 min and sterilized by 1% sodium hypochlorite supplemented by 0.02% detergent TWEEN 20 for a period of 10 min). The seeds germinated on the basal MS medium (Murashige and Skoog, 1962) solidified with agar (0.8%), at 25 °C, with a16-h photoperiod at 72 μmol of photons/m^2/s using a fluorescent lamp Philips TL-D-840 (21% blue light 400–490 nm a 38% Red light 600–680 nm). The regenerants were cultivated in Magenta boxes under the same conditions and regularly subcultivated every 4 weeks.

2.3. Cultivation of plant cell suspensions

The cell culture was initiated from the leaves of regenerants by setting the cuts on solid MS medium supplemented by synthetic phytohormones (0.225 mg/ml 2,4-dichlorophenoxyacetic acid, and 0.215 mg/ml kinetin). The callus cultures were cultivated in the dark, at 25 °C and regularly subcultivated every 3 weeks. The cell culture was then transferred into the liquid MS medium and cultivated in Erlenmeyer flasks on a horizontal shaker in the dark at 25 °C to obtain the suspension cultures.

2.4. Incubation of suspension cultures and regenerants with anthelmintics

The suspension cultures and *in vitro* regenerants were incubated with ABZ in 10 μM for various periods (the suspensions for 1 week and the regenerants for 1, 3 and 6 week). The samples of cell suspensions, roots, tops of leaves (approx. 1 cm from the leaf top) and basal parts of leaves (approx. 1 cm from the bottom of rosette) were collected and frozen (−80 °C) before analysis. All samples were prepared in triplicate.

2.5. Sample preparation and extraction for analysis

All the samples were homogenized using the FastPrep-24 homogenizer (Santa Ana, CA, USA). The homogenized samples were subjected to liquid-liquid extraction (LLE) according to the method described previously by Vokřál et al. (2012) and Podlipna et al. (2013). The obtained supernatants were evaporated to dryness using the concentrator Eppendorf (30 °C). The dry samples were quantitatively reconstituted in a mixture of water/acetonitrile (70/30, v/v) by sonication and filtrated through syringe filters with PTFE membrane. One microliter of the samples were injected into a UHPLC-MS system.

2.6. UHPLC-MS/MS conditions

UHPLC-MS/MS chromatograms of the samples were measured using electrospray ionization (ESI) on a triple quadrupole mass analyzer (Shimadzu, Japan). UHPLC was performed on a Nexera liquid chromatograph (Shimadzu, Japan) using a Zorbax RRHD Eclipse Plus C18 column 150 × 2.1 mm, 1.8 μm (Agilent Technologies, Waldbronn, Germeny), temperature 40 °C, flow rate 0.4 ml/min, injection volume 1 μl. The mobile phase consisted of water (A) and acetonitrile (B), both with the addition of 0.1% formic acid. The linear gradient was as follows: 0 min – 15% B, 8 min – 40% B, 10 min – 95% B followed by 1 min of isocratic elution. MS was used with the following setting of tuning parameters: capillary voltage 4.5 kV, heat block temperature 400 °C, DL line temperature 250 °C, the flow rates of nitrogen were 12 l/min (drying gas) and nebulizing gas were 2.5 L/min, respectively. The isolation width $\Delta m/z$ 2 and the collision energy 20 eV (found as the optimal energy for the fragmentation of the studied metabolite ions) using argon as the collision gas were employed for the MS/MS experiments. The detected metabolites were identified based on the presence of protonated molecules $[M+H^+]$ and the interpretation of their product ion spectra. All measurements were carried out in the positive ion ESI mode, as this mode provides a better sensitivity for the studied metabolites. The relative peaks areas of the metabolites were integrated using mebendazole (MBZ) as an internal standard. All data are presented as arithmetic mean ± SD (n = 3).

3. Results

The biotransformation of anthelmintic drug ABZ was studied in the ribwort plantain (*Plantago lanceolata*) *in vitro* in cell suspension and *in vivo* in whole plant regenerants. The suspensions were incubated in culture medium with 10 μM ABZ for 1 week. The regenerants were cultivated in soil extract agar supplemented with 10 μM ABZ for 1, 3 and 6 weeks.

3.1. Identification of ABZ metabolites

The identification of the chemical structures of the metabolites revealed that enzymatic systems of the ribwort plantain (*Plantago lanceolata*) were able to transform ABZ via several reactions of phase I and II of drug metabolism. The fragmentation patterns, retention times and theoretical *m/z* values of elemental composition of all ABZ metabolites formed in the ribwort during phase I and II biotransformation are summarized in Table 1. The schemes of the proposed metabolic