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Metabolism of sulfamethoxazole in *Arabidopsis thaliana* cells and cucumber seedlings☆

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ABSTRACT

Reclaimed water is a historically underutilized resource. However, with increased population growth and global climate change, reclaimed water is evolving into an economical and sustainable water resource to meet the needs of citizens, industries, and agriculture. The use of recycled water for agricultural irrigation comes with the potential risk of environmental and food contamination by pharmaceuticals and personal care products (PPCPs). The levels of PPCPs in plants will depend on translocation and metabolism in plant tissues. However, relatively little is known about the metabolism of PPCPs in plants. In this study, the metabolism of the antibiotic sulfamethoxazole was investigated in *Arabidopsis thaliana* cells as well as cucumber seedlings grown under hydroponic conditions. Using high-resolution mass spectrometry and ^{14}C tracing allowed for sulfamethoxazole metabolism to be comprehensively characterized through all metabolic phases. Six phase I and II metabolites were identified in *A. thaliana* cell cultures and cucumber seedlings. Sulfamethoxazole metabolism followed oxidation and then rapid conjugation with glutathione and leucine. Direct conjugation with the parent compound was also observed via acetylation and glucosylation. At the end of 96 and 168 h incubation, N4-acetylsulfamethoxazole was the major metabolite and >50% of the radiolabeled sulfamethoxazole became non-extractable in both *A. thaliana* cells and cucumber seedlings suggesting extensive phase III metabolism and detoxification. The study findings provided information for a better understanding of the uptake and metabolism of sulfamethoxazole in higher plants, highlighting the need to consider metabolic intermediates and terminal fate when assessing the risk of PPCPs in the soil-plant continuum.

1. Introduction

Over the past two decades, pharmaceuticals and personal care products (PPCPs) have emerged as contaminants of environmental concern due to their extensive use and continuous emission into the environment (Daughton and Terns, 1999; Pedersen et al., 2005; Boxall et al., 2012). PPCPs are released into the environment primarily through the disposal of treated wastewater and biosolids from wastewater treatment plants (WWTPs) (Carballa et al., 2004). As climate change and population growth places an increasing stress on freshwater resources, especially in arid and semi-arid regions, communities have turned to utilizing municipal treated water for agricultural irrigation, which may result in soil contamination by PPCPs (Barnett et al., 2005; Tal, 2006; NRC, 2012). Furthermore, the heavy use of some pharmaceuticals, particularly antibiotics, for disease control and growth promotion in intensive animal farming also contributes to contamination of agricultural fields when animal wastes are used for fertilization (Hu et al., 2010).

The presence of PPCPs in irrigation water and soil can lead to contamination of food crops if plants can substantially accumulate these compounds. Various studies over the last decade have sought to quantify plant uptake of PPCPs, and in general, only low levels of PPCPs have been found in edible tissues (ng/kg) (e.g., Wu et al., 2013, 2014). The majority of studies to date have only targeted the parent form of PPCPs for analysis. However, plants have a cascade of enzymes that may extensively transform xenobiotics such as PPCPs after uptake (Celiz et al., 2009; Fu et al., 2016). Recently several published studies have explored the metabolism of pharmaceuticals in plants (e.g. Huber et al., 2009, 2012; Fu et al.,

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2016; LeFevre et al., 2016; Marsik et al., 2017). Therefore, consideration of metabolism and biologically active metabolites is much needed for a better understanding of the fate and risks of PPCPs in the soil-plant system.

Higher plants have many detoxification enzymes similar to those in animals. These enzymes function in plants as a 'green liver' (Sandermann, 1994). In general, metabolism of xenobiotics includes three phases. Phase I involves modification reactions such as oxidation, hydrolysis, and dealkylation reactions introducing reactive sites to the molecule. Phase II is characterized by conjugation with large polar biomolecules, such as sugars and amino acids, to further increase the polarity of the xenobiotic. Phase III is typified by sequestration, resulting in the formation of bound residues (Sandermann, 1992; Sandermann, 1994, Miller et al., 2016). As shown for many xenobiotics in mammals and plants metabolites from phases I and II often retain biological activity (Osborne et al., 1990; Pichersky and Gang, 2000; Miller et al., 2016), and therefore should not be discounted.

In this study, sulfamethoxazole was selected as the compound of interest because of its prevalence in WWTP effluents and increasing concerns over the propagation of antibiotic resistance (Yao et al., 2012; WHO, 2016). Since its introduction in 1961 sulfamethoxazole has been widely prescribed due to its potency against both gram-positive and gram-negative bacteria (Brunton et al., 2011). Currently, sulfamethoxazole's has been detected from ng L^{-1} to μg L^{-1} in surface and effluent waters and μg kg^{-1} to mg kg^{-1} in soils and manure (Hu et al., 2010; Shelver et al., 2010; Brausch et al., 2012). Recent long-term studies of waste-water application under realistic field conditions have highlighted the potential for sulfamethoxazole to be taken up and translocated in crop plants, including to the fruit (Christou et al., 2017).

The structures of sulfamethoxazole metabolites, including conjugates from Phase II metabolism, were identified using high-performance liquid chromatography coupled with time-of-flight high-resolution mass spectrometry (HPLC-TOF-HRMS) and further quantified using ultra-high performance liquid chromatography in tandem with a triple quadrupole mass spectrometry (UPLC-TQD-MS/MS). Furthermore, Phase III terminal products in the form of bound residues were quantified using ^{14}C labeling.

Arabidopsis thaliana cells were selected as the experimental organism due to their extensive use in the literature, commercial availability, and their membership in the commonly consumed Brassica family (e.g., cabbage, broccoli, kale). Further, *Arabidopsis thaliana* plants are found worldwide under several common names (e.g., Wall cress, mouse-ear cress, shiro-inu-nazuna) and are consumed by a wide variety of animals as well as humans (van Poecke and Dicke, 2004, TAIR institute, 2018). Cucumber (*Cucumis sativus*) was selected in the hydroponic experiment due to the fact that it is often consumed raw, rapid growth, and amiability to soilless culture (Texas A&M, AgriLife, 2018).

2. Materials and methods

2.1. Chemicals and solvents

Non-labeled sulfamethoxazole was purchased from MP Biomedicals (Solon, OH). Sulfamethoxazole-d_4 was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and ^{14}C-labeled sulfamethoxazole was obtained from American Radiolabeled Chemicals (Saint Louis, MO). Stock solutions of ^{14}C-sulfamethoxazole and non-labeled sulfamethoxazole were prepared in methanol to reach a specific radioactivity of 1.2×10^3 dpm μL^{-1} and a chemical concentration of 1.0 mg mL^{-1}, respectively. HPLC grade acetonitrile and methanol were used for extraction along with ultra pure water. Mobile phases were prepared using Optima™ LC/MS grade methanol and deionized water. Standards were prepared in HPLC grade methanol and stored in the dark at −20 °C. All solvents used in this study were purchased from Fisher (Fair Lawn, NJ).

2.2. Arabidopsis thaliana cell incubation experiment

PSB-D *A. thaliana* cell line (CL84840) was purchased from the Arabidopsis Biological Resource Center (ARBC) at the Ohio State University (Columbus, OH). The cells were maintained in liquid suspension culture at 25 °C and rotated at 130 rpm in the dark according to the ARBC protocol (2018). To explore metabolism of sulfamethoxazole in *A. thaliana* cells, 7 mL of cell culture was inoculated in 43 mL fresh culture and cultivated for 96 h at 25 °C and 130 rpm in the dark to produce the seed culture. A 30 μL aliquot of the non-labeled stock solution and 10 μL aliquot of ^{14}C-sulfamethoxazole were spiked into 30 mL of *A. thaliana* cell culture, resulting in a nominal initial concentration of sulfamethoxazole of 1 μg mL^{-1} and a specific radioactivity of 1.2×10^3 dpm mL^{-1} (0.3% methanol). Simultaneously, control treatments were prepared by autoclaving cell suspensions before chemical spiking (non-viable cell control), flasks containing sulfamethoxazole without cells (medium control), and flasks containing live cells but no sulfamethoxazole (background control). These control treatments were used to determine adsorption, abiotic degradation, and potential toxicity to cells. The incubation lasted for 96 h, and triplicate containers were sacrificed at 0, 3, 6, 12, 24, 48 and 96 h.

At each sampling interval, the entire culture was transferred to a 50 mL polypropylene centrifuge tube and centrifuged at 10,000 rpm for 15 min. The supernatant was collected and stored at −20 °C until further analysis and the plant cells were placed at −80 °C before freeze-drying for 72 h. After drying, cells were fortified with 50 μL of 10 mg L^{-1} sulfamethoxazole-d_4 as a recovery surrogate. Cells were extracted using a modified method previously established in Wu et al. (2012). Briefly, cells are sonicated in a Fisher Scientific FS110H sonication bath (50/60 Hz, Pittsburgh, PA) for 20 min with 30 mL acidified DI water (pH 4) followed by centrifugation at 10,000 rpm for 15 min. The supernatant was decanted into a new 50 mL centrifuge tubes. The cell matter was further extracted using 20 mL methyl tert-butyl ether (MTBE), followed by 20 mL acetonitrile. The MTBE and acetonitrile supernatants were combined, dried under nitrogen at 35 °C, and re-constituted in 1.0 mL methanol. The extract was then combined with the above water extract. The combined sample extract was loaded onto a preconditioned 150-mg Oasis© HLB solid phase extraction (SPE) cartridge and eluted with 20 mL methanol. The cleaned extract was dried under nitrogen and further recovered in 1.5 mL 50:50 MeOH:H_2O (v/v). The growth media was acidified to pH 3 and similarly extracted and cleaned as described above. Extraction recovery for the sample preparation protocol of the cell extract was 46% ± 13 and for the growth media was 57% ± 10.

Prior to instrument analysis, both cell and media extracts were transferred to micro-centrifuge tubes and centrifuged at 120,000 rpm in a bench-top SciLogex d2012 centrifuge (Rocky Kill, CT) and further filtered through a 0.22-μm polytetrafluoroethylene (PTFE) membrane (Millipore, Carrigtwohill, Cork, Ireland) into 2 mL glass vials. All final extracts in 2 mL glass vials were stored at −20 °C if not immediately analyzed.

At each time interval, 100 μL of the cell material extract or concentrated growth media was added to 6 mL Ultima Gold™ liquid scintillation cocktail (Waltham, MA) to measure the extractable ^{14}C-radioactivity on a Beckman LS 5000TD Liquid Scintillation Counter (LSC, Beckman, Fullerton, CA). Additionally, the extracted cell matter was air dried, and a 10 mg aliquot was combusted on an OX-500 Biological Oxidizer (R. J. Harvey Instruments, Hillsdale, NJ). The evolved $^{14}CO_2$ was captured in 15 mL

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