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Diclofenac in *Arabidopsis* cells: Rapid formation of conjugates☆

Qiuguo Fu [a, b, *, 1], Qingfu Ye [b], Jianbo Zhang [c], Jaben Richards [a], Dan Borchardt [d], Jay Gan [a]

[a] Department of Environmental Sciences, University of California, Riverside, CA 92521, United States
[b] Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou 310029, China
[c] Department of Health Sciences and Technology, ETH Zürich, Schmelzbergstrasse 9, 8092 Zürich, Switzerland
[d] Chemistry Department, University of California, Riverside, CA 92521, United States

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ABSTRACT

Pharmaceutical and personal care products (PPCPs) are continuously introduced into the soil-plant system, through practices such as agronomic use of reclaimed water and biosolids containing these trace contaminants. Plants may accumulate PPCPs from soil, serving as a conduit for human exposure. Metabolism likely controls the final accumulation of PPCPs in plants, but is in general poorly understood for emerging contaminants. In this study, we used diclofenac as a model compound, and employed ^{14}C tracing, and time-of-flight (TOF) and triple quadruple (QqQ) mass spectrometers to unravel its metabolism pathways in *Arabidopsis thaliana* cells. We further validated the primary metabolites in *Arabidopsis* seedlings. Diclofenac was quickly taken up into *A. thaliana* cells. Phase I metabolism involved hydroxylation and successive oxidation and cyclization reactions. However, Phase I metabolites did not accumulate appreciably; they were instead rapidly conjugated with sulfate, glucose, and glutamic acid through Phase II metabolism. In particular, diclofenac parent was directly conjugated with glutamic acid, with acyl-glutamatyl-diclofenac accounting for >70% of the extractable metabolites after 120-h incubation. In addition, at the end of incubation, >40% of the spiked diclofenac was in the non-extractable form, suggesting extensive sequestration into cell matter. The rapid formation of non-extractable residue and dominance of diclofenac-glutamate conjugate uncover previously unknown metabolism pathways for diclofenac. In particular, the rapid conjugation of parent highlights the need to consider conjugates of emerging contaminants in higher plants, and their biological activity and human health implications.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are of significant environmental concern due to their large use volumes and continuous emission into the environment (Chen et al., 2014; Kidd et al., 2007; Kim et al., 2007; Mompelat et al., 2009; Popova et al., 2013; Tixier et al., 2003). The human use of PPCPs results in their collection at wastewater treatment plants (WWTPs). However, it is well known that many PPCPs are not completely removed at WWTPs, and are released to surface water when treated effluents are discharged, or biosolids are recycled, often as fertilizers to agricultural fields (Boxall et al., 2003; Jelic et al., 2011; Kim et al., 2007; Mompelat et al., 2009; Tixier et al., 2003; Wong, 2006). Treated wastewater is also increasingly used for agricultural irrigation, especially in arid and semi-arid regions (Dalkmann et al., 2014; U.S. Environmental Protection Agency, 2012; Wu et al., 2014). Moreover, use of some PPCPs for therapeutic and disease control purposes in animal production, often at much higher rates than human consumption, acts as another important route for their contamination of agricultural soil (Boxall et al., 2003).

When grown in contaminated soil, plants may accumulate PPCPs and transfer them to herbivores or carnivores along food chains, including humans, posing unintended risks (Fu et al., 2016; Malchi et al., 2014; Oaks et al., 2004; Paltiel et al., 2016; Wu et al., 2014). Like mammals, higher plants possess many enzymes to metabolize and hence detoxify xenobiotics (Bártíková et al., 2015; Sandermann Jr., 1994). Metabolism therefore plays a critical role in controlling the actual accumulation of PPCPs in higher plants (McCutcheon and Schnoor, 2004). Metabolism of xenobiotics in

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* Corresponding author. Department of Environmental Sciences, University of California, Riverside, CA 92521, USA.
E-mail addresses: fuqiuguo@zju.edu.cn, qiuguo.fu18@gmail.com (Q. Fu).
[1] Current address: Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland, Email: qiuguo.fu@eawag.ch.

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higher plants shares pathways somewhat similar to those in mammals, and is characterized by three phases of detoxification. In Phase I metabolism, oxidation (e.g., hydroxylation), hydrolysis, and dealkylation reactions, mainly catalyzed by cytochrome P-450 enzymes, may take place, resulting in intermediates with increased polarity. In Phase II metabolism, the parent and its Phase I metabolites may be conjugated with endogenous molecules (e.g., glucuronic acid, glucose, amino acids, glutathione, sulfate), contributing to further detoxification (Bartha et al., 2014; Farkas et al., 2007; Huber et al., 2012; McCutcheon and Schnoor, 2004). However, Phase III metabolism in higher plants differs from that in animals in that plants lack an excretory pathway. Phase III metabolism in plants involves deposition of conjugates from Phase II metabolism into plant vacuoles or incorporation into cell wall matter (e.g., lignin), forming phase III residues (McCutcheon and Schnoor, 2004).

Metabolism of PPCPs has been extensively studied in humans and rodents at the stage of discovery and development prior to commercialization (Todd and Sorkin, 1988). In comparison, relatively little is known about their metabolism in plants (Matamoros and Bayona, 2006; Shen et al., 1999; Stierlin et al., 1979). In limited studies to date, most researchers have stopped at identifying intermediates from Phase I metabolism (Bartha et al., 2014; Gröning et al., 2007; Huber et al., 2012; Wu et al., 2016). However, studies on pesticides show that conjugation and conversion to non-extractable residue are often dominant pathways for plant metabolism of xenobiotics (LeFevre et al., 2015a,b; McCutcheon and Schnoor, 2004). Therefore, simply monitoring for the free form of parent PPCPs may be a gross oversimplification, leading to incomplete knowledge of risks.

In this study, we used diclofenac as a model PPCP compound to characterize its Phase I, II and III metabolism in *Arabidopsis thaliana* cells. *Arabidopsis thaliana* is a model plant widely used in molecular and plant biology, with well-established and standardized cultivation protocols of cell lines and the whole plant. In addition, there is a wealth of knowledge about its genetic and enzymatic compositions, as well as mutated cell lines of *Arabidopsis*, which may potentially be valuable for understanding plant metabolism of PPCPs at the genetic and enzymatic levels. Diclofenac is a non-steroidal anti-inflammatory drug and widely used to relieve pain and inflammation, with around 940 tons of annual global consumption (Zhang et al., 2008). However, diclofenac is not efficiently removed at WWTPs and can be detected at the up to μg L^{-1} level in surface water (Zhang et al., 2008) and up to μg kg^{-1} dry soil in treated waste water irrigated soil (Kinney et al., 2006). The veterinary use of diclofenac was reported as a threat to several species of vultures (Cuthbert et al., 2014; Oaks et al., 2004). The distribution of extractable products from Phase I/II metabolism and non-extractable residues from Phase III metabolism was quantitatively evaluated using ^{14}C-diclofenac. Extractable metabolites, including conjugates, were identified using ultra-performance liquid chromatography-triple quadrupole tandem mass spectrometry (UPLC-QqQ-MS/MS) and liquid chromatography time-of-flight high-resolution mass spectrometry (LC-TOF-HRMS), and further validated in *Arabidopsis* whole plants.

2. Materials and methods

2.1. Chemicals

Carbon 14-labled diclofenac sodium (2-[(2,6-dichlorophenyl) amino] benzene acetic acid, monosodium salt) was purchased from American Radiolabeled Chemicals (Saint Louis, MO). The radiochemical and chemical purity was >99%, and the specific radioactivity was 55 mCi $mmol^{-1}$. Chemical structure and ^{14}C labeling position are shown in Fig. S1. Diclofenac-d_4 (Fig. S1) and non-labeled diclofenac were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and TCI America (Portland, OR), respectively. The analytical standard 2,6-dichlorobenzoic acid was obtained from Santa Cruz Biotechnology (Dallas, TX). Standards of 5-hydroxy-diclofenac and 4-hydroxy-diclofenac were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Standard of acyl-glutamatyl-diclofenac was purchased from Biosynthesis (Lewisville, Texas) through custom synthesis. All organic solvents (HPLC grade) used in this study were from Fisher (Fair Lawn, NJ). Ultrapure water was produced using a Barnstead E-Pure water purification system (Thermo Scientific, Dubuque, IA).

2.2. Arabidopsis thaliana cell incubation experiments

Arabidopsis thaliana PSB-D was obtained from Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH). The cells were cultured in suspension at 25 °C and 130 rpm in the dark. Detailed information about medium composition and preparation is available as Text S1 in the Supporting Information.

To explore metabolism of diclofenac in *A. thaliana* cells were inoculated into fresh culture and cultivated for 4 d at 25 °C and 130 rpm in the dark to produce the seed culture. Before use, 10% (v/v) of the seed culture containing actively growing cells was inoculated into fresh cell culture, with an estimated initial cell density at 3.3 g (dry weight) cell per liter. A stock solution of ^{14}C-diclofenac was prepared in methanol and the diclofenac stock solution contained non-labeled diclofenac at 1.08 g L^{-1} and ^{14}C-diclofenac at 1.8×10^7 dpm mL^{-1}. The addition of non-labeled diclofenac was to facilitate analysis on UPLC-QqQ-MS/MS and LC-TOF-HRMS.

A 100-μL aliquot of the stock solution was spiked into 30 mL of *A. thaliana* cell culture, resulting in a nominal initial concentration of diclofenac of 3.59 mg L^{-1} and a specific radioactivity of 6×10^4 dpm mL^{-1} (0.3% methanol). The elevated concentration was used to facilitate the identification of metabolites. In parallel, several different control treatments were included for quality control. A blank cell culture control with *A. thaliana* cells only was included to check for any potential contamination. The medium control with diclofenac but without cells was used to evaluate abiotic degradation of diclofenac under the experimental conditions. In addition, a carrier solvent control with *A. thaliana* cells and 100 μL methanol (without diclofenac) was used to check the potential effect of methanol on cell growth. Moreover, a control treatment with nonviable cells (prepared by autoclaving at 121 °C for 45 min) was similarly spiked with ^{14}C-diclofenac and used to quantify adsorption of ^{14}C-diclofenacto the cell materials. All treatments and controls contained three replicates.

2.3. Sampling and sample preparation

Sampling was performed immediately after the treatment, and after 6, 12, 24, 48, 96, and 120 h of incubation. The cells were immediately separated from the medium by centrifugation at 3000 rpm for 30 min. The harvested cells were washed with deionized water and then freeze-dried overnight in a freeze drier (Labconco, Kansas City, MO). The collected aqueous medium and cells were separately extracted and analyzed, as described below.

The medium (30 mL) was condensed and cleaned up with a 150-mg HLB cartridge (Waters, Milford, MA). The cartridge was preconditioned with 7 mL of methanol and then 7 mL of deionized water. After fortification of 50 μL of 10 mg L^{-1} diclofenac-d_4, the medium solution was loaded onto the HLB cartridge. Analytes were eluted using 20 mL methanol under gravity. The eluent was then dried using a gentle nitrogen stream and reconstituted in 1.0 mL of methanol before instrument analysis.

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