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Identity, Synthesis, and Cytotoxicity of Forchlorfenuron Metabolites in Kiwifruit

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ABSTRACT: Forchlorfenuron (CPPU) is a plant growth regulator widely used in kiwifruit production. Although research on the toxicological and environmental effects of CPPU is well-established, the nature and toxicological properties of its metabolites are much less well-known. Using high resolution mass spectrometry and nuclear magnetic resonance, the CPPU previously unidentified metabolites in Xuxiang and Jinyan kiwifruit were identified as *N*-(2-chloro-4-pyridinyl)-*N*′-(2-hydroxy-4-methoxyphenyl)-urea (metabolite **1**) and *N*-phenyl-*N*′-4-pyridinylurea (metabolite **2**, CAS: 1932-35-0). Their structures were confirmed by synthesis (metabolite **1**) and by comparison with a commercial standard (metabolite **2**). Quantitative studies demonstrate that CPPU and its metabolites are mainly retained in the kiwifruit peel, while the content is dependent on the nature of the peel surface, with the smoother peel of Jinyan kiwifruit retaining smaller amounts of the compound. Cell viability experiments in Caco2 and Lo2 cells show that the metabolites may have a lower cytotoxicity compared to the parent compound CPPU.

KEYWORDS: *forchlorfenuron, metabolite, kiwifruit, cytotoxicity, residue*

INTRODUCTION

Plant growth regulators are synthetic chemicals that mimic natural plant hormones used to regulate plant growth in agricultural and forestry production.[1] Forchlorfenuron (CPPU, *N*-(2-chloro-4-pyridinyl)-*N*′-phenylurea) belongs to the cytokinin group of plant growth regulators and is used in many countries worldwide. CPPU induces parthenocarpy and promotes cell expansion by acting synergistically with endogenous auxins;[2,3] it is also a popular agrochemical to accelerate growth in kiwifruit, grapes, and melons.[1] Although the maximum residue limit (MRL) of CPPU varies among countries or economic zones (e.g., the EU), limits for various foodstuffs vary between 0.05 to 0.1 mg/kg in China, with a limit of 0.05 mg/kg set for kiwifruit (CNS GB 2763-2019). The MRL set by EU in kiwifruit was 0.01 mg/kg.[4]

CPPU is classified as an unlikely endocrine disruptor and has low acute toxicity to mammals.[4] Despite the fact that legal limits have been set for the agricultural use of CPPU, which should therefore in theory protect consumers, the long-term health effects are still unclear and health conscious consumers are still reluctant to consume CPPU treated foodstuffs.[5,6] However, while the toxicological profile of CPPU is well-established, there remains much more ambiguity with regards to the nature of the metabolites formed after application in fruits, which could have a similar or even greater toxicity than CPPU itself. In fact, in recognition of the importance of agrochemical metabolites on health and the environment, in the EU, maximum residue limits include not only the parent chemical but also some of the major identified metabolites. For example, the MRL for prochloraz is defined as "the sum of prochloraz and its metabolites containing the 2,4,6-trichlorophenol moiety expressed as prochloraz".[7] Therefore, much research has been carried out to further investigate the potential toxicological and environmental hazards associated with agrochemical metabolites.[8−11]

There have been many residue studies on CPPU itself, among which Sharma et al. studied CPPU residues in grapes, as well as in environmental samples from soil and water.[12] Much progress has been made toward the quick and accurate quantification of CPPU in fruits and vegetables.[1,13−16] In addition, hyperspectral imaging technology and hand-held detector have been developed that can be used to non-destructively distinguish between CPPU treated and untreated kiwifruit.[5,17] Repeated-dose toxicity study of CPPU in rats indicate the danger that CPPU has potential adverse effects on the ovaries and steroid production.[18] In addition, animal studies in zebrafish indicate that CPPU adversely affects cardiac morphology and function.[19]

In comparison, the potential toxicity associated with CPPU metabolites has received scant attention. In 2005, the Australian Pesticides and Veterinary Medicines Authority reported an assessment of new activity of CPPU in produce a Sitofex 10 EC plant growth regulator, stating that the 3-hydroxyphenyl and 4-hydroxyphenyl metabolites of CPPU were present in kiwi vines, grape vines, and apple trees after treatment with a single dose of ^{14}C-forchlorfenuron (75 mg/L).[20] Additionally, Zhang et al. found that various metabolites formed after the hydroxylation and glycosylation of CPPU,[20] which are common biotransformation pathways in pesti-

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cides.[9,10,21,22] However, our knowledge of CPPU metabolites, including their biotransformation pathways and toxicity (both human and environmental), is still limited, warranting further research in this area.

Kiwifruit (*Actinidia*) is an edible fruit rich in vitamin C and nutrients such as dietary fiber, vitamin E, potassium, and folic acid present in woody vines originally native to central and eastern China. In addition, kiwifruit stands out among other fruits since it is also rich in various antioxidants and enzymes that are beneficial to human metabolism.[23] CPPU is widely used in kiwifruit to increase its fresh weight and yield,[24,25] promoting fruit expansion by increasing osmotically driven water absorption without being limited by the carbohydrate supply. During fruit development, CPPU increased the abundance of 16 proteins related to defense while CPPU changed the expression of 19 polar metabolites in the exocarp.[2] Despite its benefits to production, the large-scale and excessive use of CPPU has reduced consumer acceptance with a concomitant reduction in the price of Chinese kiwifruit.[6]

Herein, we aim to analyze and synthesize two previously unidentified metabolites of CPPU using high resolution mass spectrometry and nuclear magnetic resonance. Additionally, we quantified the content of CPPU and its metabolites in kiwifruit. We then used Caco2 and Lo2 cell lines to assess the cytotoxicity of CPPU and its metabolites. This work helps to clarify the nature of CPPU metabolites present in kiwifruit and should promote safer agricultural practices with regards to its application, while further determining the safety of CPPU grown fruit.

MATERIALS AND METHODS

Chemicals and Instrumentation. Commercially prepared CPPU (1 g active ingredient/L) was provided by Sichuan Lanyue Agrochemical Co., Ltd. (Sichuan, China). HPLC grade acetonitrile and methanol were purchased from Thermo Fisher Scientific (Shanghai, China). The CPPU standard was obtained from Sigma-Aldrich, while distilled water was obtained from Watsons. Metabolite **2** (*N*-phenyl-*N*′-4-pyridinylurea) was purchased from Beijing FYF Chemicals Co., Ltd. The ^{1}H and ^{13}C NMR spectra were obtained with a Bruker Avance 400 MHz instrument (Bruker, Germany) in deuterated dimethyl sulfoxide (DMSO-d_6). The coupling constants (J) are expressed in hertz, while peak multiplicities are abbreviated as singlet, s; doublet, d; triplet, t; multiplet, m.

Plant Material. Two kiwifruit varieties were used in this study, "Xuxiang" (green kiwifruit, *Actinidia deliciosa*) and "Jinyan" (gold kiwifruit, *Actinidia chinensis* × *Actinidia eriantha*), both of which were harvested in 2017 from two orchards located in Mei County (Shaanxi province) in central China, a geographical area representative of Chinese kiwifruit production.[26] In terms of temperature and precipitation, 2017 was a regular year, and neither orchard recorded abnormal climatic stress conditions. The two orchards, composed of adult vines, were managed by conventional agronomic practices. The commercial preparation of CPPU (1 g of active ingredient/L) was diluted with water to two concentrations, 10 mg/L (the standard concentration employed by farmers, low concentration treatment)[2,3] and 80 mg/L (a concentration higher than the recommended dosage, high concentration treatment).[4] The control plants were treated with water instead of CPPU. Each treatment contained six vines of each variety, for a total of 36 vines. Three aqueous solutions were used by soaking the kiwifruit for 3 s on the 25th day after full bloom of the fruit trees.

Sample Processing. Sampling was divided into two stages. The first stage was to collect kiwifruit for metabolite identification. Two hours after treatment, kiwifruit was collected randomly. In addition, kiwifruit was randomly collected every other day for the first 40 d after the treatment, and kiwifruit was also collected on the 60th, 80th, and 100th d after treatment. Representative samples were processed according to a previous method but with minor modifications.[27] First, the whole kiwifruit was directly chopped and homogenized in a blender (Philips, HR2096, Xi'an, China), and the fully homogenized sample (10 g) was added into a centrifuge tube, together with 15 mL of acetonitrile. This mixture was shaken for 1 min in a vortex blender and then centrifuged at 3000 rpm for 7 min. The supernatant (2 mL) was transferred to another centrifuge tube and centrifuged at 12 000 rpm for 10 min. The supernatant was passed through a 0.22 μm polytetrafluoroethylene (PTFE) filter (Anpel, Shanghai, China) and transferred to a vial for subsequent analysis.

In the second stage, kiwifruit was randomly harvested at commercial maturity in early October 2017 (123 d after treatment), after which the fruit was immediately transported to the laboratory and ripened at room temperature. The kiwifruit was peeled manually, and the pulp and peel were individually homogenized. To quantify CPPU and its metabolites in kiwifruit, the pulp (10 g) and peel (2 g) were mixed with acetonitrile (15 mL), and the subsequent processing operation was the same as described above.

HPLC-Q/Orbitrap-ESI/MS Analysis. CPPU and its metabolites were identified and quantified using a Thermo Scientific Dionex UltiMate 3000 HPLC and Thermo Scientific Q Exactive Focus Plus Orbitrap mass spectrometer coupled with a heated electrospray ionization (ESI) interface. The process was performed as described previously with some modifications.[28] Separations were conducted with a reverse-phase Hypersil Gold C18 column (3 μm, 2.1 mm × 150 mm) (Thermo Fisher Scientific) at 35 °C. Samples (injection volume: 5 μL) were eluted using a mixture of water (solution A) and methanol (solution B); the mobile phase had a continuous flow rate of 0.3 mL/min and the following gradient elution program: 0.0−4.0 min, 5% B; 4.0−25.0 min, from 5% to 95% B; 25.0−35.0 min, 95% B; and 35.0−40.0 min, 5% B.

The identification of metabolites with mass spectrometry was performed in both the electrospray positive ion and negative ion modes following the conditions described in a previous report.[29] Full MS data-dependent MS^2 mode was used. Full mass scan (full MS, 80−800 m/z) was used at a resolution of 70 000. The automatic gain control (AGC) target was set to 1×10^6 ions, and the maximum injection time (IT) was set to auto mode. In MS^2, the resolution was set to 17 500, with an isolation window of 4 m/z, an isolation offset of 1 m/z, and an AGC target of 5×10^4, using a stepped normalized collision energy of 30/40/50. The ESI source parameters were the following: spray voltage (+) 4000 V, spray voltage (−) 2800 V, capillary temperature 320 °C, sheath gas 35, aux gas 5, S-Lens RF level 50, and probe heater temperature 350 °C.

Quantification was carried out in switching mode. The lock mass option was set for ions at m/z = 248.05852 and 214.09749, corresponding to CPPU ($C_{12}H_{11}ClN_3O^+$) and metabolite **2** ($C_{12}H_{11}N_3O^+$), respectively, in positive ion mode, and m/z = 292.04944 corresponding to metabolite **1** ($C_{13}H_{12}ClN_3O_3^-$) in negative mode (Figure 1). The rest of the parameters were the same as those described in the above identification.

Data Analysis. Mass spectral data were analyzed with Compound Discoverer (CD) 2.1 (Thermo Fisher Scientific, Shanghai, China) to identify the metabolites of CPPU. An expected and unknown workflow with modifications was used. This workflow finds and identifies both expected and unknown metabolites with statistics and performs retention time alignment, detects expected compounds and biotransformation products with resolution aware isotope pattern matching, and detects unknown compounds and groups expected compounds and unknown compounds across all samples. The workflow predicts elemental compositions for all unknown compounds, fills gaps across all samples, hides chemical background (using blank samples), and flags unknown compounds that share common fragments by the "Compound Class Scoring" node.

A maximum shift of 0.5 min and mass tolerance of 5 ppm were performed for retention time alignment. For finding expected compounds, a minimum number of 2 isotopes, 5 ppm mass tolerance, and a minimum peak intensity of 100 000 were used. Compounds were grouped according to a retention time tolerance of 0.1 min. For

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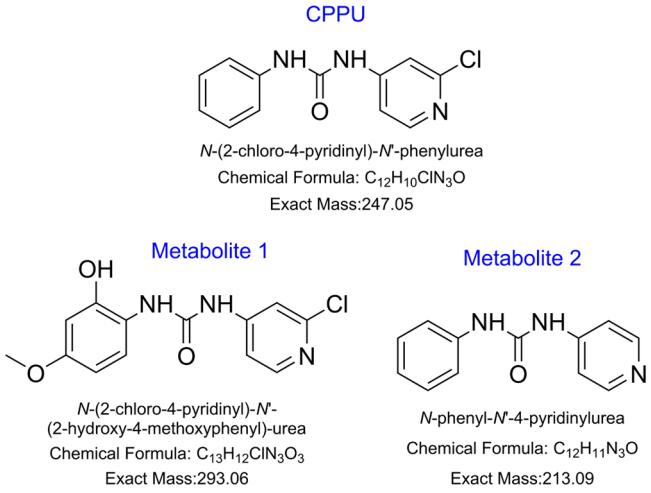

Figure 1. Chemical structures, formulas, and exact masses of CPPU and its metabolites.

the detection of unknown compounds, the peak intensity extracted in each set of data was not less than 100 000, and the signal-to-noise ratio (S/N) was larger than 3. Compounds were grouped according to a retention time tolerance of 0.2 min and a mass tolerance of 5 ppm.

The quantification of CPPU and its metabolites was performed in TraceFinder 3.3 software from Thermo Fisher Scientific with external standardization (0.1−800 μg/L).

Synthesis of Metabolite 1. 2-Chloro-4-pyridinamine (1 g, 7.78 mmol) was added in the dioxane (25 mL). Triphosgene (807.89 mg, 2.72 mmol) was added in the mixture. The mixture was heated to 120 °C and stirred for 1.5 h to obtain 2-chloro-4-isocyanatopyridine as a light-yellow oil. 2-Amino-5-methoxyphenol (1.8 g, 12.94 mmol) was added directly to the solution of 2-chloro-4-isocyanatopyridine (1.20 g, 7.78 mmol) at 20 °C and stirred for 1 h. The reaction was concentrated in a vacuum. The combined crude product was purified by prep-HPLC equipped with a Phenomenex Luna C18 column (15 μm, 70 mm × 250 mm). The mobile phase comprised (A) water containing 0.1% trifluoroacetic acid and (B) acetonitrile. The elution gradient ranged from 15% to 45% B for 20 min to obtain the metabolite **1** as a brown solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.88 (s, 1H), 8.23 (s, 1H), 8.17 (d, J = 5.7 Hz, 1H), 7.84 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 1.3 Hz, 1H), 7.27 (dd, J = 5.6, 1.5 Hz, 1H), 6.49 (d, J = 2.6 Hz, 1H), 6.39 (dd, J = 8.9, 2.6 Hz, 1H), 3.69 (s, 3H) (Figure S1 in the Supporting Information). ^{13}C NMR (126 MHz, DMSO-d_6): δ 155.84, 152.33, 151.27, 150.16, 149.99, 148.13, 120.98, 120.50, 112.04, 111.36, 104.07, 101.83, 55.47 (Figure S2 in the Supporting Information).

Cell Culture and CCK-8 Assay. Caco2 and Lo2 cells were purchased from ATCC and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (v/v). Cells were incubated at 37 °C under a 5% CO_2

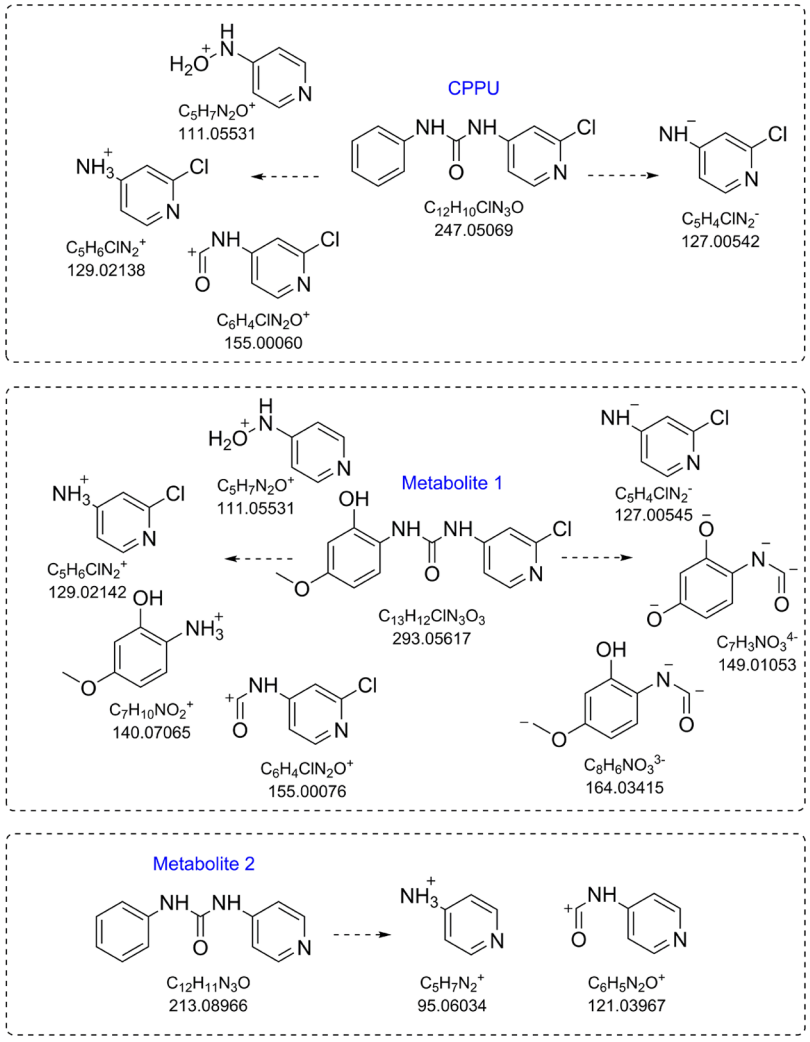

Figure 2. Fragmentation pattern of CPPU and its metabolites.

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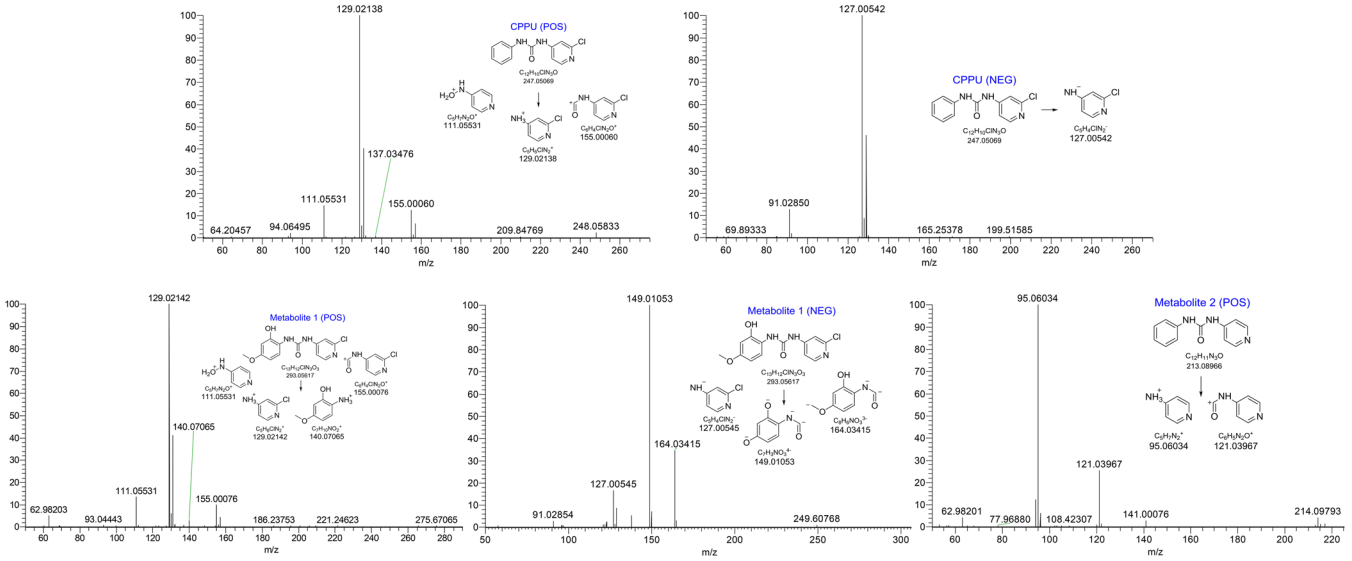

Figure 3. Mass spectra of CPPU and found metabolites in positive (POS) ion and negative (NEG) ion modes.

atmosphere. The samples were diluted with complete cell culture media to different compound concentrations. Caco2 cells (100 μL, 5 $\times$ 10^4 cells/mL) were seeded in 96-well plates with the wells in the outermost circle full of phosphate buffer saline (PBS, pH 7.4, Gibco). After 24 h, the old medium was removed, and each tested well was washed twice with 150 μL of PBS. Cells were treated with the compound at different concentrations (100 μL, 5−70 μM) for 24 h. The culture medium was subsequently discarded, and each well was washed. The cell viability was determined using the CCK-8 method described by Park et al. with slight modifications.[30] The WST-8 solution (CCK-8, Abmole Bioscience Inc., M4839) was added and incubated for 2 h. The absorbance was measured with a PerkinElmer Victor X3 microplate reader at 450 nm.

Statistical Analysis. Each experiment was performed in triplicate except for the cell experiment, which was repeated six times. Data were represented as the mean ± standard deviation (SD). All statistical analyses were carried out with SPSS 22.0 for Windows (SPSS Inc., Chicago, IL). Duncan's multiple-range test were assessed to test significant differences at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of CPPU Metabolites. The characteristic fragments of CPPU were detected in both positive and negative modes. In positive ion mode, characteristic fragment ions at m/z = 111.05531, 129.02138, and 155.00060 were detected, whereas in the negative mode, the fragment ion was at m/z = 127.00542 (Figures 2 and 3). In negative mode, metabolite **1**, protonated molecule ion $[M - H]^-$ at m/z 292.04944, was found. Metabolite **1** was further represented by three fragment ions at m/z = 127.00545, 149.01053, and 164.03415 with a retention time of 17.63 min (Figures 2 and 3). Using the modules "Elemental composition" in Xcalibur Qual Browser and "Composition Change" in CD, the chemical formula of metabolite **1** was determined to be $C_{13}H_{12}ClN_3O_3$ (Table 1), obtained by adding the CH_2O_2 molecular fragment to the chemical formula of CPPU ($C_{12}H_{10}ClN_3O$). The "Transformations" module of the CD software suggested that oxidation and methylation of the parent compound CPPU led to the formation of metabolite **1**.

To ascertain the location of chemical transformation in the original CPPU compound, the molecular formula of metabolite **1** was input into "Inclusion" in the method, and the sample was reinjected to obtain its secondary fragment in positive ion mode. In positive ion mode, the characteristic fragment ions of metabolite **1** were the same as in CPPU, at m/z = 111.05531, 129.02142, and 155.00076 (compare Figures 2 and 3). However, the minor fragment at m/z = 140.07065 for metabolite **1** (predicted as $C_7H_{10}NO_2$ with the "Elemental composition" module) indicated that oxidation and methylation took place in the benzene ring, which is consistent with previous reports.[4,20]

Table 1. Retention Time, Formula, and Fragments of CPPU and its Metabolites

			fragment (m/z)	
compound	RT (min)	formula	$[M + H]^+$	$[M - H]^-$
CPPU	18.49	$C_{12}H_{10}ClN_3O$	111.05531	127.00542
			129.02138	
			155.00060	
metabolite **1**	17.37	$C_{13}H_{12}ClN_3O_3$	111.05531	127.00545
			129.02142	149.01053
			155.00076	164.03415
metabolite **2**	18.48	$C_{12}H_{11}N_3O$	95.06034	
			121.03967	

The two characteristic fragment ions at m/z = 149.01053 and 164.03415 of metabolite **1** in negative mode (absent in CPPU) were further analyzed and determined to correspond to $C_7H_3NO_3$ and $C_8H_6NO_3$, respectively, as found with the "Elemental composition" module. The possible fragmentation pathways for metabolite **1** leading to the fragment ion peaks observed in the high resolution mass spectrometry are summarized in Figures 2 and 3. The structure of metabolite **1** was further confirmed by synthesis and subsequent nuclear magnetic resonance analysis (Figures S1 and S2). The retention time of metabolite **1** and the nature of the characteristic fragment ions all further established metabolite **1** as *N*-(2-chloro-4-pyridinyl)-*N*′-(2-hydroxy-4-methoxyphenyl)-urea (Figure 1).

An additional biotransformation product of the parent CPPU was discovered in positive ion mode (metabolite **2**), protonated molecular ion $[M + H]^+$ at m/z = 214.09749, with two main fragments at m/z = 95.06034 and 121.03967 and retention time at 18.48 min (Figures 2 and 3). The

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"Transformations" module of CD shows metabolite **2** was formed from reductive dechlorination, as evidenced from the loss of chlorine and the addition of hydrogen from the parent CPPU. The chemical formulas of the fragments at m/z = 95.06034 and 121.03967 were predicted by the "Elemental composition" module as $C_5H_7N_2$ and $C_6H_5N_2O$, respectively. On the basis of the original structure of CPPU, the predicted molecular structure of metabolite **2** is shown in Figure 1. Further confirmation of metabolite **2** as *N*-phenyl-*N*′-4-pyridinylurea (CAS: 1932-35-0) was obtained from the retention time and the characteristic fragment ions of the purchased standard.

According to the data in articles studying CPPU residues,[25,31] the content of CPPU decreased rapidly in the first 10 days after using CPPU. In addition, metabolites **1** and **2** can be detected in the first 10 days after CPPU use (data not published). We deduce that, in the early stage after using CPPU, CPPU is metabolized into other compounds with the rapid growth of kiwifruit.

Quantification of CPPU and Metabolites in Kiwifruit. While the careful use of plant growth regulators according to established guidelines can increase the economic profitability of kiwifruit, long-term excessive and large-scale use of CPPU can degrade kiwifruit quality and lead to environmental degradation.[32] Therefore, in order to detect final CPPU levels in the pulp and peel of Xuxiang and Jinyan kiwifruit, we used the synthesized metabolite **1** standard, the purchased metabolite **2**, and commercially obtained CPPU. At high concentration treatment conditions, the CPPU content was much higher in the peel of kiwifruit than in the pulp (Figure 4), with concentrations in the pulp of the Xuxiang and Jinyan varieties of 0.84 ± 0.09 and 1.28 ± 0.14 μg/kg, respectively. The residue in kiwifruit pulp exposed to the high concentration treatment still did not exceed the national guidelines set in China (CNS GB 2763-2019, 0.05 mg/kg). However, no CPPU was detected in the pulp of either variety at low concentrations.

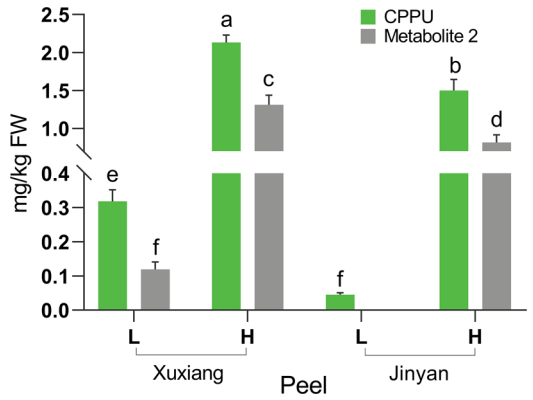

Figure 4. Residues of CPPU and identified metabolites on the peel of Xuxiang and Jinyan kiwifruit.

In both high and low concentration treatments, the Xuxiang peel had higher CPPU contents than the peel of the Jinyan variety ($p < 0.05$). We suggest that a prime factor for this difference in content is related to the characteristics of the peel surface of both varieties. The Xuxiang variety has a rough and hairy skin, whereas the Jinyan peel is smooth, hairless, and thin. Hence, agrochemicals are much more likely to be retained within the surface of a peel having irregular characteristics, such as in the Xuxiang variety that favor retention; the chemicals will more likely adhere to the peel surface. Conversely, as the Jinyan peel had a lower CPPU content, the concentration of the agrochemical in the pulp was simultaneously higher as the CPPU was able to penetrate the surface more easily. Therefore, we suggest that a rational approach for choosing the optimal concentration of plant growth regulators should consider the kiwifruit variety treated.

In the final commercial maturation stage, metabolite **1** was not detected in kiwifruit and metabolite **2** was not present in the pulp of the Xuxiang and Jinyan varieties. When treated at a low concentration, metabolite **2** was only detected in the peel of the Xuxiang variety, and it was present at a concentration of 0.12 ± 0.02 mg/kg (Figure 4). In the high concentration treatment, the metabolite **2** content of the Xuxiang peel was still higher compared to the Jinyan peel ($p < 0.05$). These results indicate that the kiwifruit peel is the main site for the retention of CPPU and its metabolites, which is consistent with other reports.[33] We point out that, in certain places, it is customary to eat kiwifruit directly without peeling the smooth peel, such as that found in the Jinyan variety. Therefore, our results indicate that this could be an unhealthy practice, and this investigation should bolster awareness efforts for the removal of kiwifruit peel prior to consumption.

Cytotoxicity. We used two cell lines, colon cancer Caco2 cells and human normal liver Lo2 cells, to compare the cytotoxicity of the parent compound CPPU and metabolites **1** and **2**, which were diluted to different concentrations (5−70 μM) with complete medium. The results showed that, over the entire dilution range, metabolites **1** and **2** decreased the cell viability of Caco2 and Lo2 cells to a smaller degree than CPPU (Figure 5), although the results were not significant ($p > 0.05$). On the basis of the prediction of the cytotoxicity, this may suggest that the toxicity of CPPU was reduced after

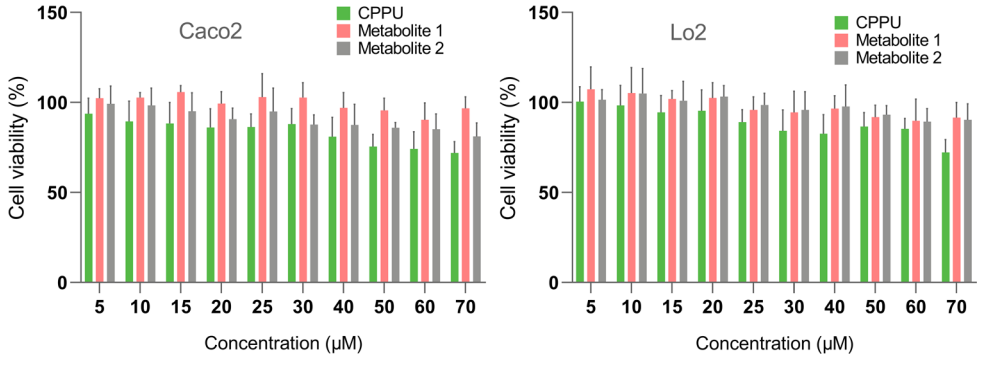

Figure 5. Effects of CPPU and its metabolites on Caco2 and Lo2 cell viability (% of control). Data are presented as mean ± SD (n = 6).

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metabolism, consistent with a previous study by Zhang et al., who reported that the metabolic biotransformation of forchlorfenuron on the basis of hydroxylation and glycosylation reduced the cytotoxicity of the parent compound.[20]

We believe that this study is a valuable contribution toward highlighting the importance of CPPU metabolites and their toxicological properties when applied to kiwifruit, furthering our understanding of the impact of agrochemical use. From our results, a applicable method of CPPU application on the basis of kiwifruit varieties is suggested to minimize toxicological concerns. That is, the concentration of plant growth regulators applied on the hairy and thick skin of kiwifruit varieties, such as Xuxiang, should be lower than that of hairless and thin skin, such as Jinyan.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c02492.

Figures of ^{1}H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CPPU, forchlorfenuron

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