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Potential Hepatic Toxicity of Buprofezin at Sublethal Concentrations: ROS-mediated Conversion of Energy Metabolism

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ABSTRACT

Buprofezin is known for its broad-spectrum action and environmental safety. The popularity of buprofezin has raised concerns about its potentially adverse effects on human health and risk to the environment. In this study, we first identified the liver as one of the major organs in which buprofezin accumulated, and we detected a severe oxidative stress response. Next, we demonstrated that sublethal concentrations of buprofezin promoted the conversion of energy metabolism from the aerobic tricarboxylic acid (TCA) cycle and oxidative phosphorylation to anaerobic glycolysis. Importantly, reactive oxygen species (ROS) generation partially accounted for the shunting of the energy metabolism through the buprofezin-mediated inhibition of cytochrome c oxidase activity. ROS directly perturbed the activities of several key TCA cycle enzymes, stimulated glycolysis, and indirectly disturbed the activity of the respiratory chain complex by altering mitochondrial DNA (mtDNA). These findings clarify the potential mechanisms of buprofezin toxicity and provide biomarkers for buprofezin-mediated hepatotoxicity at sublethal concentrations.

Key words: buprofezin; hepatic toxicity; energy metabolism; reactive oxygen species (ROS)

11. Introduction

Pesticides are widely used to control unwanted pests and disease vectors and to enhance food production in agriculture. Therefore, the potential adverse effects of environmental pesticide exposure on non-targeted organisms, particularly human beings, have become a major concern. According to a report from the World Health Organization (WHO) and the United Nations Environment Programme (UNEP), there are more than 26 million human pesticide poisonings worldwide per year, resulting in approximately 220,000 deaths [1]. In China, there are 0.5 million human pesticide poisonings per year, which are responsible for 0.1 million deaths [2]. Considering the environmental and health risks of pesticides, many traditional pesticides have been banned and replaced with lower-toxicity pesticides. Insect growth regulators such as chitin synthesis inhibitors appear to be promising because of their insect-specific mode of action and their lower toxicity in non-target organisms than conventional insecticides. However, chitin synthesis inhibitors at sublethal concentrations could have toxic effects on target and non-target organisms. Sublethal doses of hexaflumuron were shown to interfere with normal carbohydrate metabolism in certain instars of insects [3, 4]. Diflubenzuron and flucycloxuron were shown to affect both glutathione levels and glutathione S-transferase activity in the adult females of a larvivorous fish [5]. Buprofezin (2-tert-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one) is a typical inhibitor of chitin synthesis and molting. It acts specifically on immature developmental stages of

¹ Abbreviation: TCA cycle, tricarboxylic acid cycle; ROS , reactive oxygen species; mtDNA , mitochondrial DNA; SCO1, cytochrome c oxidase assembly protein; COX17, cytochrome c oxidase copper chaperone; CS, citrate synthase; OGDH, oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide); IDH2, isocitrate dehydrogenase 2 (NADP+); FH, fumarase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3; LDHB, lactate dehydrogenase B; MMP, mitochondrial membrane potential.

homopteran pests by inhibiting the incorporation of N-acetyl-[D-H3] glucosamine into chitin and interfering with cuticle formation, resulting nymphal mortality during molting [6, 7]. It also exhibits larvicidal activity against the brown rice planthopper, *Nilaparvata lugens*, and the greenhouse whitefly, *Trialeurodes vaporariorum* [8, 9]. According to the National Pesticide Toxicity Grading Standards Organization of China, buprofezin is graded as a low-toxicity pesticide [10]. However, current buprofezin production is approximately 4500 tons per annum, and due to its long half-life, buprofezin residues persist in plants and the environment [11]. According to the literature, the residues of buprofezin on oranges and lemons range from 0.05 to 0.69 mg/kg in Spain, Italy, Portugal, Australia and New Zealand, and the residues of buprofezin in rice samples range from 0.07 to 2.5 mg/kg in Korea, additionally, buprofezin residues are commonly detected in soil and water [12, 13]. Notably, and in contrast to expectations, recent experimental evidence indicates that buprofezin in the aquatic environment, even at low concentrations (16 μ M), causes detrimental effects to the early embryonic and larval development of African catfish [14]. Exposure to buprofezin at concentrations of 12.5–100 μ M was shown to significantly increase the frequency of micronuclei in cultured Syrian hamster embryo cells [15]. Therefore, these findings raise concerns about the potentially adverse effects of buprofezin on human health at sublethal and even nontoxic concentrations.

The liver is an important detoxification organ. It is a primary target organ of pesticides and is also the most sensitive organ to their effects [16]. Additionally, the liver exhibits high metabolic activity and energy demands and plays a central role in maintaining energy homeostasis. Energy metabolism disorders are important contributors to liver injury [17, 18]. Furthermore, mitochondrial dysfunction and the subsequent abnormalities in energy metabolism elicited in response to external stimuli have been implicated in the regulation of hepatic toxicity and even liver-related diseases [19-23]. It has been

reported that chlorpropham induces mitochondrial dysfunction in rat hepatocytes [24]; the organochlorine lindane impairs mitochondrial function and energy metabolism in HepG2 cells [25]; and atrazine leads to abdominal obesity and insulin resistance in rats by impairing mitochondrial function [26]. Although the liver has been identified as the main target of buprofezin toxicity [27], a limited number of studies have focused on buprofezin-mediated changes in cellular signaling, particularly in relation to mitochondrial function and energy metabolism processes.

The purpose of this study was to identify the target organ of buprofezin and elucidate the mechanism of buprofezin-induced energy metabolism disorder. Mice were treated with buprofezin, and substantial amounts of buprofezin accumulated in the mouse liver, resulting in an oxidative stress response. In addition, HepG2 cells were exposed to different concentrations of buprofezin, and ATP, pyruvate and lactate were measured, and the mRNA expression levels of critical genes in the TCA cycle and glycolysis were quantified by RT-PCR. Furthermore, indicators of oxidative phosphorylation, including the mitochondrial membrane potential (MMP) and mitochondrial respiratory chain (MRC) complex enzyme activities, were analyzed. Our research suggests possible mechanisms for evaluating, alleviating and preventing the adverse outcomes of chitin synthesis inhibitors from the perspective of energy metabolism.

2. Materials and methods

2.1. Oral exposure of animals

Male C57BL/6 mice (6–8 weeks of age) were obtained from Experimental Animal Center of the Academy of Military Medical Sciences of the Chinese PLA (Beijing, China). All mice were housed under standard conditions (24 ± 2 °C, $50 \pm 5\%$ humidity, with a 12:12 h light:dark cycle) and were acclimatized for 1 week before starting the treatments. The animals had free access to water and standard feed and

were randomly divided into four groups. For the control group, 0.9% sterile NaCl (200 μ L/mouse) was intragastrically administered every other day for a total of 9 administrations, and the other three exposure groups were given 0.0463, 0.139 or 0.417 g/kg (bw) buprofezin using the same protocol. The animals were sacrificed 18 h after the last treatment. The liver, brain, kidney, heart, spleen and stomach, were excised and quick frozen in liquid nitrogen, then stored at -80 °C.

2.2. Cell culture

The human hepatocellular carcinoma cell line (HepG2) was obtained from the Cell Bank of the Type Culture Collection (CAS, China). The cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL streptomycin (Gibco, USA) and 100 U/mL penicillin (Gibco, USA), in a humidified culture at 37 °C and 5% CO₂.

2.3. Determination of buprofezin contents

Gas chromatography–mass spectrometry (GC-MS) (TRACE-DSQ, Thermo Scientific, America) was utilized to analyze the buprofezin contents of the different tissues. The tissues were homogenized through sonication with CHCl₃ on ice for 10 min. Next, the homogenates were concentrated in a rotary evaporator for 5 min. Subsequently, 1 mL CHCl₃ was added to dissolve the extracts, which were then concentrated again with N₂. Finally, the extracts were dissolved in 1 mL of CHCl₃, and 1 μ L of the samples was used for the detection of buprofezin via GC-MS. The parameters were set as in our previous work [28].

2.4. Measurements of the methane dicarboxylic aldehyde (MDA) levels

Approximately 0.1 g of tissue was homogenized with 1 mL of 0.9% NaCl on ice. The homogenates were centrifuged for 30 min at 10,000 rpm at 4 °C. The supernatant was then transferred to a new tube for MDA determination according the manufacturer's instructions (Nanjing Jiancheng Bioengineering

Institute, China).

2.5. Cytotoxicity determination

The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay was applied to determine cytotoxicity before and after buprofezin exposure. Cells (1.0×10^4 /well) were plated in 96-well plates and treated with 0, 1, 3, 10, 30, 100, 300 or 1000 μ M buprofezin for 24 h. After culture at 37 °C for 24 h, MTT was added to the culture media at a final concentration of 0.5 mg/mL, and the cells were cultured for an additional 4 h. Thereafter, the supernatant was removed, and 100 μ L of dimethylsulfoxide (DMSO) was added to each well. The cultures were then shaken for 10 min in the dark, and the absorbance was detected at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Thermo Scientific Varioskan Flash reader (Thermo Fisher Scientific, USA).

2.6. Apoptosis detection

Approximately 5×10^5 cells/mL were seeded in 35-mm petri dishes and treated with 0, 3, 10 or 30 μ M buprofezin for 24 h. After exposure, the cells were collected and washed twice with cold PBS. Subsequently, the cells were resuspended in binding buffer and stained with 5 μ L of annexin V and 1 μ L of propidium iodide (PI) for 15 min according to the instructions of the apoptosis detection kit (Invitrogen, USA). Then, the cells were washed with cold PBS three times, and the proportions of apoptosis cells were determined with a BD Accuri™ C6 Flow Cytometer (BD, USA).

2.7. Western Blot analysis

Protein was extracted and quantified according to a previously reported method [29]. Protein samples of 30 μ g were then size-fractionated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Next, the membranes were blocked with 3% bovine serum albumin (BSA) and incubated overnight at 4°C with antibodies specific

for human caspase-3, cleaved caspase-3 and β -actin (Cell Signaling Technology, USA) that had been diluted with 3% BSA (1:1000). After exposure to a fluorescently labeled secondary antibody (1:2000) (IRDye 800 CW goat anti-rabbit IgG, LI-COR), the membranes were scanned using a LI-COR Odyssey Infrared Fluorescent System (LiCor Biosciences, USA).

2.8. Determination of the ATP, pyruvate and lactate level

After inoculation of the cells (1×10^6 cells/mL) into 35-mm petri dishes, the cells were treated with 0, 3, 10 or 30 μ M buprofezin for 24 h. Intracellular ATP levels were determined with a Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, USA) according to the manufacturer's instructions (Beyotime, China) [29]. Similarly, intracellular pyruvate and lactate levels were determined according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The protein contents of the samples were measured using Coomassie brilliant blue staining, and the protein concentrations were used to normalize each sample.

2.9. DNA/RNA isolation and real time quantitative PCR (q-PCR)

Approximately 5×10^5 cells/mL were seeded in 35-mm petri dishes and treated with 0, 3, 10 or 30 μ M buprofezin for 24 h. After exposure, the cells were washed twice with PBS, and genomic DNA was extracted with a Tissue DNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. Similarly, total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Qiagen, USA). Then, the extracted RNAs were reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (TaKaRa, China). qPCR was performed in a Real-Time PCR qTOWER 2.2 system (Analytik Jena AG, Jena, Germany). The primer sequences and reaction conditions for the RT-PCR are shown in Table 1.

2.10. 5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimidazolyl Carbocyanine Iodide (JC-1) assay

Approximately 5×10^5 cells/mL and 1×10^5 cells/mL were seeded in 35-mm petri dishes for flow cytometry analysis (C6, BD, USA) and microscopy analysis using a fluorescent microscope (Olympus, Japan). After exposure to 0, 3, 10 or 30 μ M buprofezin for 24 h, the cells were collected and washed twice with cold PBS. They were then cultured in fresh medium containing 2.0 μ M JC-1 (Nanjing Jiancheng Bioengineering Institute, China) at 37 °C for 20 min. Subsequently, the cells were washed three times, and their green and red fluorescence intensities were measured with a BD Accuri™ C6 Flow Cytometer (BD, USA). For microscopic observations, the cells were washed with cold PBS after exposure, cultured in fresh medium with JC-1 and then washed three times before observed under a fluorescence microscope (Olympus ix71, Japan).

2.11. Measurement of reactive oxygen species (ROS) production

After being seeded into 35-mm petri dishes (approximately 5×10^5 cells), the cells were exposed to 0, 3, 10 or 30 μ M buprofezin for 24 h. The cells were collected and incubated with 10 μ M 2', 7'-dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 30 min in the dark. Next, the cells were washed with cold PBS three times, and the DCFH-DA fluorescence was monitored at an emission wavelength of 530 nm and an excitation wavelength of 488 nm with BD Accuri™ C6 Flow Cytometer (BD, USA).

2.12. Isolation of mitochondria and measurement of MRC complex enzyme activities

Cells (approximately 1×10^6 cells) were seeded in 35-mm petri dishes and treated with 0, 3, 10 or 30 μ M buprofezin for 24 h. After exposure, the cells were washed and homogenized with cold extraction buffer (10 mM HEPES, pH 7.5, containing 1 M mannitol, 350 mM sucrose, and 5 mM EGTA) on ice [30]. The homogenate suspension was then centrifuged at 600 g for 5 min at 4 °C, after which the supernatant was transferred to a new tube and centrifuged at 11000 g for 10 min at 4 °C. The precipitate was resuspended in 0.25 mM sucrose. The isolated mitochondria were immediately used to

detect the activities of MRC complex enzymes (IV and V) according to the manufactures' instructions. The protein contents of the samples were measured using Coomassie brilliant blue staining and employed to normalize each sample.

2.13. Molecular docking

The structures of cytochrome c oxidase assembly protein (SCO1) (PDB: 1WP0) and cytochrome c oxidase copper chaperone (COX17) (PDB: 2RN9) were retrieved from a repository of experimentally elucidated biological macromolecules crystal structures at the Brookhaven Protein Data Bank (PDB, USA, <http://www.rcsb.org/pdb>). After preparing the target proteins according to previous studies, ligand docking analysis was performed using the LibDock program of Discovery Studio 2.5 [31]. Default settings were employed for all docking and subsequent scoring parameters. The numbers of conformers, polar and apolar Hot-Spots, and the LibDock score for the substrate molecules SCO1 and COX17 are provided in Table 2.

2.14. Statistical analysis

The results are expressed as the mean $\pm$ the standard error. The data were analyzed using one-way ANOVA followed by Tukey's post hoc test to identify significant differences between the groups treated with different concentrations, after checking normality (Kolmogorov–Smirnov) and homoscedasticity (Levene test) using the SPSS 17.0 statistical package. A Kolmogorov–Smirnov non-parametric test was performed when normality and homoscedasticity requirements were not satisfied after data transformation. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. Accumulation of buprofezin and the oxidative stress-response in the liver

As shown in Figure 1A and Figure 1B, the highest buprofezin concentrations were found in the liver

(35.84%) followed by the brain (23.58%), stomach (21.94%) and kidney (18.64%), while the levels of buprofezin in the mouse spleen and heart were below the limit of detection. As shown in Figure 1C, buprofezin tended to elevate the MDA level in all organs, and the most significant concentration-dependent increases were observed in the liver and brain.

3.2. Cell viability and apoptosis

As shown in Figure 2A, viability was significantly reduced by 100, 300 μ M and 1000 μ M buprofezin, to approximately 14, 44 and 45% of the vehicle control level, respectively. Conversely, at concentrations less than 30 μ M, buprofezin did not compromise cell viability, and 1-30 μ M concentrations were classified as sublethal. To confirm this concentration range, apoptotic characteristics were examined via flow cytometry. For apoptosis detection, we determined the effect of buprofezin at 1, 3, 10, 30 and 100 μ M. Because the apoptosis rate of cells treated with 1 and 3 μ M buprofezin was similar, and were relatively lower than that in other higher concentration groups, the figures were not given. As shown in Figure 2B, only marginal increases in apoptosis were observed following treatment with 30 μ M buprofezin for 24 h (vs. Annexin V⁺PI⁺ cells). Furthermore, the purpose of this study is to determine the energy metabolism disorder induced by buprofezin. Therefore, relatively higher concentration (3, 10 and 30 μ M) of buprofezin was chose for next testing. As shown in Figure 2C, 3, 10 and 30 μ M buprofezin treatments caused only slight activation of cleaved caspase-3.

3.3. Alteration of cellular energy metabolism and mitochondrial function

As shown in Figure 3A, ATP levels were attenuated in a concentration-dependent manner to 91.3, 87.9 and 67.2% of the levels in the vehicle control under treatment with 3, 10 and 30 μ M buprofezin, respectively. As shown in Figure 3B, the lactate levels were significantly increased to 1.14-, 3.17- and 3.51-fold the vehicle control level following treatment with 3, 10 and 30 μ M buprofezin, respectively. In

addition, the pyruvate content increased significantly under 30 μ M buprofezin treatment (Figure 3C). Taken together, the reduction of ATP combined with the elevation of lactate and pyruvate contents implied that energy metabolism shifted from the TCA cycle to glycolysis. To further support our hypothesis, the levels of key TCA cycle and glycolysis genes were analyzed. As shown in Figure 4A, buprofezin reduced the expression of pyruvate dehydrogenase (lipoamide) alpha 1 (PDHA1) and rate-limiting enzymes in the TCA cycle, including citrate synthase (CS), oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) (OGDH), isocitrate dehydrogenase 2 (NADP+) (IDH2) and fumarase (FH) in concentration-dependent manner, with the most significant attenuations being observed following 30 μ M treatment. Additionally, as shown in Figure 4B, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3) was increased slightly, and lactate dehydrogenase B (LDHB) levels were significantly increased after 30 μ M buprofezin treatment.

As shown in Figure 5A, the green fluorescence intensity was noticeably higher under increased concentrations of buprofezin, with the peak being observed at 30 μ M. In contrast to the green fluorescence, decreases in red fluorescence were observed in the buprofezin-treated groups, particularly under 30 μ M buprofezin treatment. In the merged graphs, the alterations in the buprofezin-treated groups indicated that the form of JC-1 changed from aggregates to monomers and that the MMP was reduced. A corresponding analysis was performed via flow cytometry to further validate the results. As shown in Figure 5B, the peaks were significantly moved to the right compared with the control (41.8% vs. 53.4%), which indicated an increase in green fluorescence following buprofezin treatment at 30 μ M. Additionally, we measured the ratio of the two color intensities and observed a slight increase after buprofezin exposure (Figure 5C). As shown in Figure 5D, the activity of Complex IV was significantly reduced to 82.2, 69.2 and 63.4% of the vehicle control levels following

buprofezin treatment at 3, 10 and 30 μ M, respectively. The activity of Complex V was also significantly decreased to 68% of the vehicle control level after exposure to 30 μ M buprofezin.

3.4. Mechanism for the conversion of energy metabolism

As shown in Figure 6A, intracellular ROS levels were significantly increased, reaching 1.28-, 1.59- and 1.91-fold the level in the vehicle control following exposure to 3, 10 and 30 μ M buprofezin, respectively. Moreover, the mtDNA contents were significantly reduced to 77.3%, 70.0% and 66.7% of the vehicle control level following exposure to concentration of 3, 10 and 30 μ M (Figure 6B). As shown in Figure 6C and 6D, buprofezin is predicted to bind to SCO1 and COX17 via hydrogen- and π -bonding interactions, which are involved in the assembly of mitochondrial cytochrome c oxidase. Furthermore, based on the DOCK_Scores, a state of equilibrium had been reached (Table 2). Subsequently, to obtain further evidence of the involvement of ROS in buprofezin-induced energy metabolism disorders resulting from the inhibition of cytochrome c oxidase activity, we measured ATP levels and activity of cytochrome c oxidase following 30 μ M buprofezin treatment in the absence and presence of N-acetyl-L-cysteine (NAC, Sigma, USA). After treatment with 30 μ M buprofezin, the ATP levels and cytochrome c oxidase activity in HepG2 cells pretreated with NAC recovered significantly (Figure 6E&F).

4. Discussion

Although buprofezin is extensively used as a pesticide against a wide range of hemipteran pests, few studies have focused on its tissue accumulation and tissue distribution in non-targeted organisms. Considering recent concerns about the safety of buprofezin, we determined its concentrations and distribution in various tissues in mice. These data, coupled with the MDA level in different organs, suggested that buprofezin accumulated in mammals in an organ-dependent manner following oral

exposure and that the liver is one of the major target organs. Previous studies have provided support for our findings that buprofezin induces an increase in liver weight and focal necrosis, and the liver has been identified as the main target of buprofezin toxicity in rats and dogs [27]. Similarly, studies have indicated that related insecticides have an adverse effect on the livers of target and non-target organisms. Exposure to 169.35 mg/kg cyromazine significantly reduced body weight and elevated liver weight in rats. In addition, cyromazine resulted in obvious oxidative damage [32]. Cyromazine was fed to Luhmann hens at a level of 0.15 ppm for 3 weeks, and hepatic lipids were increased [33]. Adult male rats were exposed (gavage) to 0, 2, 4, or 8 mg/kg of diflubenzuron for 28 days, but no significant alterations were observed in the liver and kidney histology [34]. Chlorfluazuron exposure resulted in 53% inhibition of the cytochrome P450 (CYP1A1/2) activities in human hepatic microsomes [35].

In addition to its role in detoxification, the liver is known to exhibit high metabolic activity and energy requirements and is responsible for maintaining both short- and long-term whole-body energy needs [36, 37]. Altered energy metabolism in the liver is related to numerous metabolic diseases, including insulin resistance, diabetes, and nonalcoholic fatty liver disease [38]. Therefore, alterations in energy metabolism are frequently involved in hepatic toxicity and are used as early markers of liver dysfunction [17, 18]. More importantly, it has been suggested that sublethal concentrations of pesticides can affect liver function by affecting energy metabolism [25]. Another study on related insecticides also identified ultrastructural changes in the mitochondria in last instar nymphs after treatment of the 1-day old penultimate instar nymphs of *S. gregaria* with 1000 ppm of Flufenoxuron or Lufenuron [39]. Therefore, it was necessary to study the biological effects of sublethal concentrations of buprofezin on energy metabolism modes in the liver to determine its potentially hepatotoxic effects. Here, the results of analyses of cell viability, apoptosis and cleaved caspase-3 together indicated that

buprofezin under 30 μM concentration did not induce significant cytotoxicity. Therefore, 3, 10 and 30 μM concentrations of the buprofezin were used in our experiments.

Mitochondria produce most ATP through aerobic respiration, and the reduction in ATP indicated that buprofezin might affect the aerobic respiration process. Because the majority of ATP is produced through the TCA cycle and oxidative phosphorylation, we speculated that buprofezin might inhibit the TCA cycle and oxidative phosphorylation and enhance the cells' glycolysis process to satisfy their energy demand. To assess the possible alterations in energy metabolism following exposure to sublethal buprofezin concentrations, the levels of the end-point metabolite for energy metabolism (ATP), the metabolic product of glycolysis (lactate) and a reactant in the first stage of the TCA cycle (pyruvate) were determined. The elevated levels of these indicators after buprofezin treatment confirmed our hypothesis. Both *in vivo* and *in vitro* experiments have also demonstrated that pesticides can reduce the ATP level in liver tissue and HepG2 cells [40, 41].

To obtain further support for our hypothesis, the levels of several key TCA cycle and glycolysis genes were analyzed. PDHA1 catalyzes the overall conversion of pyruvate to acetyl-CoA and CO_2 and provides the primary link between glycolysis and the TCA cycle. Moreover, CS, OGDH, IDH2 and FH are the rate-limiting enzymes in the TCA cycle [42, 43]. In addition, the levels of PFKFB3 and LDHB were examined. PFKFB3 is a key glycolytic enzyme, as its knockdown reduces glycolysis, and its overexpression increases glycolysis [44]. LDH is a tetrameric enzyme composed of combinations of two subunits (LDHA and LDHB), which catalyzes the conversion pyruvate to lactate in the glycolytic pathway [45]. The expression levels of these genes were consistent with the accumulation of ATP, lactate and pyruvate. Similar studies have also found that HepG2 cells exhibit enhanced glycolysis and reduced TCA cycle function following treatment with nAg [46]. Altogether, these data suggest that

buprofezin at sublethal concentrations promotes the conversion of energy metabolism from the TCA cycle to glycolysis.

Oxidative phosphorylation is the last stage of aerobic respiration, and its disruption results in a reduction in the membrane potential and subsequent disorders of the oxidation reduction system that are linked to energy metabolism. To investigate this stage of aerobic respiration, we examined alterations in the MMP [47]. A normal MMP is an essential prerequisite for maintaining normal mitochondrial oxidative phosphorylation, producing ATP and sustaining the normal physiological functions of cells. Dissipation of the MMP is the earliest sign of normal and cancer-related cell death [48, 49]. During the process of mitochondrial oxidative phosphorylation, the function of the electron transport chain is carried out by four enzyme complexes (I–IV) in the mitochondrial inner membrane, and ATP is synthesized by complex V [50, 51]. An overall decrease in the activity of the mitochondrial complexes suggests inhibition of the electron transport chain, which may lead to a loss of the MMP and the cellular stores of ATP. In the present study, the reduction of the MMP as well as Complex IV and Complex V confirmed that buprofezin disrupted oxidative phosphorylation.

Previous studies have indicated a relationship between energy metabolism and ROS generation. ROS may contribute to energy metabolism disorders by hampering the processes involved in aerobic metabolism (i.e., the TCA cycle and oxidative phosphorylation) and enhancing anaerobic glycolysis. Exposure to ROS-producing compounds perturbs the activities of several key TCA cycle enzymes, including OGDH, IDH2 and FH, in HepG2 cells, and the continuation of the TCA cycle in impaired mitochondria results in rapid production of ROS [44, 52-54]. ROS also impair mitochondrial membranes and damage mtDNA, which encodes the four complexes (I, III, IV and V), resulting in faulty complexes and oxidative phosphorylation, leaking more electrons to produce more ROS [55, 56]. Furthermore,

ROS are involved in upregulating glycolytic activity in hepatoma cells [57]. Bagchi and Stohs demonstrated that pesticides such as endrin, lindane, and 2,3,7,8 tetrachlorodibenzo-p-dioxin induce the production of ROS in rat hepatic mitochondria [58]. Based on these findings, we speculated that ROS might be one of major reasons that buprofezin reduced ATP levels and promoted altered energy metabolism. In support of our hypothesis, we observed an increase in ROS and a decrease in mtDNA levels. Although it is not clear whether mitochondrial dysfunction is the cause or consequence of ROS, the interaction of these factors may be closely interrelated and create a vicious circle [56]. To further explore the mechanism by which buprofezin induced the release of ROS and resulted in alteration of energy metabolism, Discovery Studio 2.5 software was used to determine the interactions between buprofezin and cellular proteins. In vitro and in vivo studies have indicated that some toxicants lead to the generation of ROS through the inhibition of cytochrome c oxidase (complex IV) and subsequently disrupt energy metabolism [59]. SCO1 family proteins are copper-binding proteins that are involved in the assembly of mitochondrial cytochrome c oxidase, and COX17 is a key mitochondrial copper chaperone that is responsible for supplying copper ions to cytochrome oxidase with the assistance of SCO1, SCO2, and COX11 [60, 61]. These two molecules play important roles in maintaining cytochrome c oxidase activity. We found that buprofezin effectively inhibited the activity of cytochrome c oxidase by binding to the active pockets of SCO1 and COX17, which elevated the level of ROS. In addition, treatment with an ROS inhibitor (NAC) reversed the reduced level of ATP and cytochrome c oxidase activity, which also indicated the involvement of ROS in buprofezin-induced conversion of energy metabolism.

5. Conclusions

In the present study, we demonstrated that the liver might be one of the major organs where

buprofezin accumulates and could represent the origin of an oxidative stress response following oral exposure to buprofezin. During this process, buprofezin at sublethal concentrations inhibited ATP production by promoting the conversion of energy metabolism from the aerobic TCA cycle and oxidative phosphorylation to anaerobic glycolysis (Figure 7). Importantly, we revealed that ROS generation at least partially accounted for the alteration of energy metabolism due to inhibition of the activity of cytochrome c oxidase by buprofezin, directly perturbed the activities of several key TCA cycle enzymes, stimulated glycolysis, and indirectly disturbed respiratory chain complex activity by altering mtDNA.

Acknowledgements

This study was supported by National Science Foundation of China (NSFC, No. 91543203, 21477070, 21377076, 21222701), Specialized Research Fund for the Doctoral Program of Higher Education of China (SRFDP, No. 20121401110003, 20131401110005), and Research Project Supported by Shanxi Scholarship Council of China (No. 2015-006).

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Figure captions

Fig. 1. The accumulation of buprofezin and the oxidative damage elicited by buprofezin in different tissues. (A) Determination of buprofezin concentrations and (B) the distribution of buprofezin accumulation in different tissues by GC-MS ($n = 8$). (C) Methane dicarboxylic aldehyde (MDA) levels in different mice tissues following exposure to various concentrations of buprofezin relative to the levels in untreated mice ($n = 8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, as determined using ANOVA and Tukey's post hoc test for the data from the stomach, kidney, spleen and heart (log transformation for stomach data; sin transformation for spleen data) and the Kolmogorov–Smirnov non-parametric test was employed for the data from the liver and brain.

Fig. 2. Effects of buprofezin on HepG2 cell viability and apoptosis. (A) MTT-based cell viability assay for HepG2 cells treated with various concentrations of buprofezin ($n = 6$). (B) Representative FACS scatter plots of apoptosis in cells exposed to various concentrations of buprofezin or 0.1% dimethylsulfoxide (DMSO) ($n = 3$). (C) Western Blot analysis of cleaved caspase-3, caspase-3 and β -actin in HepG2 cells following exposure to various concentrations of buprofezin or 0.1% DMSO for 24 h ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, as determined through ANOVA and Tukey's post hoc test (cleaved caspase-3) and the Kolmogorov–Smirnov non-parametric test (MTT).

Fig. 3. Cellular energy metabolism in HepG2 cells in response to buprofezin treatment. (A) Relative cellular ATP levels in the cells following buprofezin treatment for 24 h ($n = 3$). (B) Levels of lactate and (C) pyruvate in HepG2 cells treated with buprofezin for 24 h ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, as determined through ANOVA and Tukey's post hoc test.

Fig. 4. Changes in the expression of genes involved in the tricarboxylic acid cycle (TCA) cycle and glycolysis in HepG2 cells exposed to buprofezin for 24 h. (A) Pyruvate dehydrogenase (lipoamide)

alpha 1 (PDHA1) and citrate synthase (CS), oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) (OGDH), isocitrate dehydrogenase 2 (NADP+) (IDH2), fumarase (FH) and (B) 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3) and lactate dehydrogenase B (LDHB) mRNA expressions in cells following buprofezin treatment for 24 h, as determined via qRT-PCR analyses ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, as determined by ANOVA and Tukey's post hoc test (OGDH, IDH2, PDHA1, LDHB and sin transformation of FH) and Kolmogorov–Smirnov non-parametric test (CS and PFKFB3).

Fig. 5. The process of oxidative phosphorylation in HepG2 cells in response to buprofezin treatment. (A) Representative images of two colors (green and red) and the merged colors in cells with JC-1 staining captured via fluorescence microscope ($\times 200$) ($n = 3$). (B) Flow cytometry analysis of the green color after JC-1 staining of cells treated with buprofezin for 24 h ($n = 3$). (C) Ratios of the two colors (green/red) in the cells treated with buprofezin for 24 h, quantified by flow cytometry ($n = 3$). (D) Complex IV and complex V activities were assessed in the HepG2 cells following exposure to buprofezin for 24 h ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group, as determined by ANOVA and Tukey's a post hoc test (JC-1, Complex IV and log transformation of complex V data).

Fig. 6. The mechanism of buprofezin-induced conversion of energy metabolism. (A) Relative intracellular ROS production and (B) relative mtDNA contents in cells treated with buprofezin for 24 h ($n = 3$). (C-D) Proposed modes of buprofezin binding to cytochrome c oxidase assembly protein (SCO1) (C) and cytochrome c oxidase copper chaperone (COX17) (D) based on the molecular docking and molecular simulation study. (E) Activity of cytochrome c oxidase and (F) ATP levels in cells treated with buprofezin (30 μ M) for 24 h in the absence or presence of 3 mM NAC ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the 30 μ M concentration

group, as determined by ANOVA and Tukey's post hoc test (ROS, mtDNA, cytochrome c oxidase) and the Kolmogorov–Smirnov non-parametric test (ATP).

Fig. 7. Schematic diagram illustrating the molecular basis for the disruption of energy metabolism in HepG2 cells by exposure to sublethal buprofezin concentrations.

Table 1. Primers sequences used for real-time PCR

Gene	Sequence (5'→3')	annealing temperature (°C)
OGDH Sense	ACTGTGGCGACACTGAAGG	52.0
OGDH Anti-Sense	GGCTCTGGGTTATGTTGGT	
FH Sense	GGAGGCACTGCTGTTGGTA	52.0
FH Anti-Sense	CTGCTTCCTGGTTCATTTT	
CS Sense	GAATGCCAGAACTGCTAC	49.0
CS Anti-Sense	GTCTTCATAAATCAACTCCC	
IDH2 Sense	CGTATTATCTGGCAGTTCATC	53.5
IDH2 Anti-Sense	CATCAGTCTGGTCACGGTTT	
LDHB Sense	AGGAGTCCGTCAGCAAGAA	53.5
LDHB Anti-Sense	CAGCCATAAGGTAGCGAAA	
PFKFB3 Sense	CCTCACTCGCAGCCACTTC	59.8
PFKFB3 Anti-Sense	TCCACTCTTCCGACCTTCC	
PDHA1 Sense	CCGAATGGAGTTGAAAGCA	50.0
PDHA1 Anti-Sense	GTGAAAGTAAAGCCGTGAG	
Cytb Sense	CCTGAAACATCGGCATTA	45.0
Cytb Anti-Sense	GGGTGGGACTGTCTACTG	
18S rRNA Sense	AGTTGGTGGAGCGATTTGT	52.6
18S rRNA Anti-Sense	TGAGCCAGTCAGTGTAGCG	
GAPDH Sense	CTGATTTGGTCGTATTGGG	50.0
GAPDH Anti-Sense	TGGAAGATGGTGATGGGAT	

Table 2. Number of conformers, polar and apolar HotSpots, and the LibDock score for the substrate molecules 2RN9 and 1WP0.

Ligand	Conformers	HotSpots		LibDock score
		Polar	Apolar	
2RN9	6	100	84	71.840
1WP0	6	100	100	101.889

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7