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Cytotoxic effects of tebufenozide in vitro bioassays

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ABSTRACT

Tebufenozide is considered an environmentally friendly pesticide due to its specificity on target insects, but the effects on human are well studied. Studies on the toxicity of tebufenozide at molecular and cellular level is poorly understood. The present study reveals non-selective cytotoxic effects of tebufenozide, and the apoptotic mechanism induced by tebufenozide on HeLa and Tn5B1-4 cells. We demonstrate that the viability of HeLa and Tn5B1-4 cells is inhibited by tebufenozide in a time- and concentration-dependent manner. Intracellular biochemical assays showed that tebufenozide-induced apoptosis of two cell lines concurrent with a decrease in the mitochondrial membrane potential and an increase reactive oxygen species generation, the release of cytochrome-c into the cytosol and a marked activation of caspase-3. These results indicate that a mitochondrial-dependent intrinsic pathway contributes to tebufenozide induced apoptosis in HeLa and Tn5B1-4 cells and suggests potential threats to ecosystems and human health.

1. Introduction

Tebufenozide[N-tert-*butyl*-N′-(4-ethylbenzoyl)-3,5-dimethylbenzohydrazide](KOWLogP=4.25, Ph 7), a molt-inducing insecticide that mimics the action of ecdysone, is widely used in agriculture and forestry. It was developed by Rohm and Haas Company (Philadelphia, PA, USA) for Lepidoptera control. The first few toxicity tests on non-target vertebrates and invertebrates executed with formulations of tebufenozide required high substance concentrations to make a toxicological effect visible (Addison, 1996), although more and more examples in recent years have illustrated that chemical substances of anthropogenic origin potentially exert health of human through long-term accumulate (Bolognesi et al., 1994). In this paper, a study on cytotoxic and apoptotic effects of tebufenozide was carried out to evaluate its safety, and we found the tebufenozide has significant cytotoxicity.

As multicellular organisms, apoptosis is an essential biological process for human and insects. Apoptosis is a genetically programmed type of cell death mechanism (Elmore, 2007). It is a dynamic process that is essential to eliminate unwanted or abnormal cells and plays an important role in the stability of the internal environment and the balance of development in multicellular organisms (Kerr et al., 1972; Taylor et al., 2008). Despite a wide range of inducing signals, some common pathways exist in apoptosis (Nagata, 1997). For instance, in the intrinsic pathway, mitochondria irreversibly commit cells to apoptosis by releasing death factors into cytosol (Kroemer et al., 1995). Cytochrome-c, a death factor, can form a complex with Apaf-1 in the presence of dATP in the cytosol. This is followed by activation of caspase-9 and caspase-3 which results in the activation of a cascade of caspases (Petronilli et al., 1999). Finally, these activated caspases degrade key structural and nuclear proteins and irreversibly commit the cells to death (Kaufmann et al., 1993; Tang and Kidd, 1998). Meanwhile, the apoptotic signals not only direct to mitochondrial membrane potentials, but also disrupt electron transport, which results in an increased generation of reactive oxygen species (ROS) that causes oxidative stress to cells (Rhee et al., 2013). Breaching of cytochrome-c-cardiolipin interaction can be achieved by increase in intracellular ROS, which is one of the early events of apoptosis induced by a vast variety of agents (Orrenius and Zhivotovsky, 2005). If cells under the condition of the abnormal environment stress, they will undergo apoptosis, and when apoptosis continues to organism, the harm caused cannot be estimated any more. The research on cell apoptosis mechanism induced by tebufenozide provide a theoretical basis for understanding the manufacturing process pests.

The objective of current study on tebufenozide was to

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investigate the insecticidal activity and its mechanisms of toxicity in entomic level. Due to its specificity mainly towards lepidopteran insects it is regarded as a pesticide with high environmental safety (Hahn and Liess, 2001; Sundaram MPalli et al., 1998). However, the effects of tebufenozide on mammalian or insect cells have not been evaluated, especially whether tebufenozide could induce death by apoptosis in cells and what is its regulatory mechanisms. In our study, we used HeLa cells and Tn5B1-4 cells as representatives to work out the mechanism of toxicity of Tebufenozide in cellular level.

2. Materials and methods

2.1. Chemicals and regents

Tebufenozide [N-*tert*-butyl-N′-(4-ethylbenzoyl)-3,5-dimethylbenzohydrazide] was purchased from Abcam (Cambridge, MA, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), propidium iodide (PI), DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate) and rhodamine123 (Rh-123) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The Mitochondria/Cytosol Fractionation Kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The BCA Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). Most antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) except for horseradish peroxidase (HRP)-conjugated anti-rabbit IgG which was from Sangon Biotech Co., Ltd (Shanghai, China). Other reagents and chemicals used were of analytical grade and purchased locally.

2.2. Cell lines and culture conditions

Human cervical carcinoma HeLa cells (ATCC, CCL-2, laboratory stock), that was cultured in Dulbecco's modified Eagle's medium (DMEM)(Hyclone, Logan, Utah, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), streptomycin (100 μg/mL) and penicillin (100 U/mL) (Hyclone, Logan, Utah, USA), and incubated at 37 °C in a 5% CO_2 incubator. The medium was exchanged once per two days. After treatment, the HeLa cells were harvested by 0.25% Trypsin-EDTA (Gibco, Grand Island, NY, USA). *Trichoplusia ni* (Lepidoptera) BTI-Tn5B1-4 cells (Tn5B1-4 cells) was obtained from the academy of agricultural sciences (Beijing, China), that was maintained in TNM-FH medium (Hyclone, Logan, Utah, USA) supplemented with 10% heat-inactivated FBS, streptomycin (100 μg/mL) and penicillin (100 U/mL) and incubated at 28 °C in biochemical incubator.

2.3. Cell viability test

The cell viability was measured by MTT assay (Cetin and Bullerman, 2005; Stockert et al., 2012). The cells suspensions (2×10^5 cells/mL) were seeded onto the 96-well plates (150 μL/well). The cells were grown for 24 h until they reached 2–4 $\times 10^6$ cells/ml. 200 μL tebufenozide was changed to the final concentrations of 25, 50, 100, 200 and 400 μg/mL, and 0.1% DMSO was used as the control. After 24, 48 and 72 h of treatment, 20 μl of MTT (5 mg/mL) was added to each well. After 4 h incubation, 150 μL of DMSO was added to each well. Then, the absorbances were measured at 492 and 630 nm by a Synergy H1 microplate reader (BioTeck, Winooski, VT, USA). Each experiment was repeated at least three times in quadruplicate.

2.4. Alkaline comet assay

The alkaline comet assay was performed according to a previously reported method (Xiang et al., 2013) with slight modifications to evaluate total DNA Single Strand Breaks (SSBs). Cells at 1×10^5/mL were cultured in each well of 6-well plates. After the HeLa and Tn5B1-4 cells were treated by 50, 100 and 200 μg/mL tebufenozide for 24 h, the cells were collected, washed and suspended in PBS (pH 7.4). 30 μL cell samples (1×10^4 cells) were then used and suspended in 120 μL of 1% molten low-melting-point agarose at 37 °C. The mono-suspension was cast on a microscopic slide that had been covered with a layer of 0.8% regular-melting-point agarose. The agarose was gelled at 4 °C and the slides were then immersed in fresh lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% Triton X-100, 10% DMSO, pH 10) for 30 min at 4 °C. After lysis, the slides were washed in distilled water three times and immersed in fresh alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 10 min at 4 °C. An electric field was then applied at 20 V (1 V/cm) and 300 mA for 10 min. The slides were neutralized to pH 7.5 in 0.4 mM Tris buffer, stained with 40 μL of 20 μg/mL propidium iodide (PI) solution, and examined by fluorescence microscopy (Lecois, DM3000), and 200 stained cells from each treatment group were counted. The extent of DNA migration was determined by using an image analysis system (*Comet Assay Software Project*, CASP, www.casp.of.pl).

2.5. Apoptosis analysis by acridine orange (AO)/ethidium bromide (EB) assay

The dual staining of acridine orange (AO) and ethidium bromide (EB) was used to measure live cells from apoptotic and necrotic cells (Li et al., 2013). The cells were harvested and washed three times with PBS (pH 7.4) after being incubated with 50, 100 and 200 μg/mL tebufenozide for 24 h. Then the cells were stained with AO and EB (to a final concentration 100 μg/ml for both) and incubated at 37 °C for 15 min in the dark and washed three times with PBS (pH 7.4). The morphology of the treated cells was examined by fluorescence microscopy, and 200 stained cells from each treatment group were counted.

2.6. Mitochondrial membrane potential (MMP) analysis

The mitochondrial membrane potential (*MMP*) was measured using the Rh-123 (Scaduto and Grotyohann, 1999). Rh-123 is a positively charged molecule which accumulates in the energized mitochondria. A decrease in the fluorescence intensity of Rh-123 indicates a decline in *MMP*. After being treated with tebufenozide at concentrations of 50, 100 and 200 μg/mL for 24 h, HeLa and Tn5B1-4 cells were harvested, washed twice with PBS (pH 7.4) and stained with Rh-123 for 20 min at 37 °C in the dark. After incubation, the cells were washed twice by PBS (pH 7.4) to remove extracellular Rh-123. The fluorescence of the TE treated cells was examined by fluorescence microscopy. The fluorescence intensity was measured using *ImageJ* software, and 200 stained cells from each treatment group were counted. Each experiment was conducted three times, and the results were reported as the mean of the three experiments.

2.7. Intracellular ROS assay

The production of intracellular ROS was measured by oxidations of DCFH-DA (Pollard et al., 1992; Wang and Joseph, 1999). After treatment with 50, 100 and 200 μg/mL of tebufenozide for 24 h, the HeLa and Tn5B1-4 cells were harvested by centrifugation at 100*g* for 5 min and washed twice with PBS (pH 7.4). Briefly, cells