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Assessment of the cytotoxicity of ionic liquids on *Spodoptera frugiperda* 9 (*Sf-9*) cell lines *via in vitro* assays

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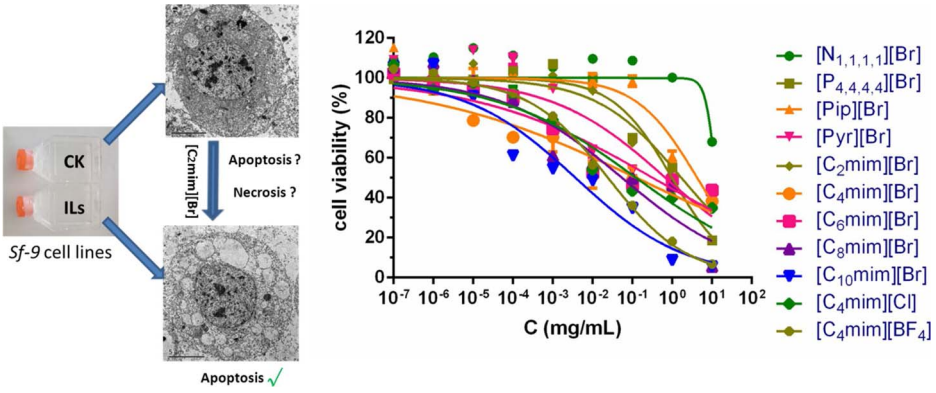
GRAPHICAL ABSTRACT



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ABSTRACT

Cytotoxicity studies are important tools for the assessment of the toxicity of ionic liquids (ILs). In the present study, the cytotoxicity of eleven ILs against *Spodoptera frugiperda* 9 (*Sf-9*) cell lines were evaluated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The effect on cellular morphology, ultrastructural morphology, and nuclear morphology induced by 1-ethyl-3-methylimidazolium bromide ([C_2mim][Br]) was studied via inverted light microscopy observation, acridine orange staining, and transmission electron microscope (TEM) analysis, respectively. The effect on cell DNA fragmentation, cell apoptosis and cell cycle induced by [C_2mim][Br] was also investigated *via* DNA agarose gel electrophoresis and flow cytometry analysis, respectively. The results showed that the cytotoxic effect of ILs on *Sf-9* cells was related to the IL structures, concentrations, and length of exposure. The morphological features of apoptosis induced by [C_2mim][Br] such as cell shrinkage and convolution, apoptotic bodies, pyknosis, and karyorrhesis were observed. All these phenomena confirmed that *Sf-9* cells exposed to [C_2mim][Br] died *via* apoptosis. This study complements the current knowledge about the cytotoxic properties of ILs on insect cells and highlights the mechanism by which ILs kill these cells. Furthermore, it provides a basis for further studies on the future applications of ILs as insecticides.

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1. Introduction

Ionic liquids (ILs) are defined as molten salts with melting temperatures around or below 100 °C, which are usually constructed from organic cations and organic or inorganic anions [1]. Regarding the structures of ILs, cations are composed of large head groups, such as ammonium, imidazolium, pyridinium, piperidinium, pyrrolidinium, morpholinium or cholinium, and alkyl side chains that can vary in length, number or position; the anions type include halides, acetate, fluorine, and cyano derivatives [2]. Since the first synthesis of the air- and water- stable ILs containing 1-ethyl-3-methylimidazolium cations [3], ILs have been praised by researchers and production personnel due to their unique advantages, including low volatility, non-flammability, high ionic conductivity, high thermal stability, excellent dissolving capacity, and wide electrochemical window [4–7]. Owing to these various properties, ILs have become versatile solvents and are used in different fields, such as extraction and separation [8,9], electrochemistry [10], analytical chemistry [11], synthesis and catalysis [12], and material science [13]. Most importantly, ILs are tunable, meaning that their cations and anions can be tailored to obtain various task-specific ILs with different physical and chemical properties [14,15].

With the wide use of ILs in the chemical industry and in academic research, it is unavoidable that ILs would be released into the living environment. However, not enough attention has been paid to the effect of ILs on the environment. Therefore, extensive efforts are required to obtain data on the ecotoxicity of ILs. To our knowledge, ILs are less volatile than organic solvents, and are unlikely to enter the atmosphere or cause atmospheric toxicity. However, most ILs are water soluble and enter the water and soil easily [16]. At present, the toxicity of ILs toward microorganisms (bacteria, fungi, yeast) [17,18], algae [19,20], plants [21,22], vertebrates [23], invertebrates [24], and cells [25,26] had been studied. These toxicity studies generally focused on external data, such as minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC), inhibitory concentration resulting in 50% of inhibition of the activity of biological or biochemical systems by tested compound (IC_{50}), and effective concentration of tested compound causing 50% of reduction on processes, such as growth or reproductive activity (EC_{50}), and median lethal dose (LD_{50}). These data were mainly obtained *via* in *vivo* studies. Although *in vivo* studies provide useful data about the toxicity of ILs toward organisms, sometimes they are time consuming and laborious. *In vitro* assays have many advantages, including speed, quantitative results, reproducibility, and low cost. Therefore *in vitro* assays have been widely used to assess the toxic effect of ILs. Moreover, *in vitro* cytotoxicity studies can clarify the effect of ILs on internal cellular structures and microstructures, and the cellular mechanisms that underlie toxicity.

In the past, increasing amounts of cytotoxic data were obtained through the study of ILs toxicity against rat [27,28], human [29–31], and fish [26,32] cell lines, in studies that they mainly used IC_{50} values to assess IL cytotoxicity. Studies regarding IL toxicity on insect cell lines and the effect of IL on internal cell structures have been rarely reported. In recent years, in order to clarify the mechanisms of the effect of ILs on organism, ILs toxicity toward selected cell lines has been studied *via* DNA fragmentation assays, flow cytometry analysis, and TEM analysis. These studies have indicated that ILs cause cell death *via* necrosis or apoptosis. For example, Li et al [28] investigated the cytotoxicity mechanism of imidazolium based ILs towards rat pheochromocytoma (PC12) cells *via* various methods, such as reactive oxygen species (ROS) levels detection, DNA fragmentation and lactate dehydrogenase release (LDH) analysis, and caspase-3 activity assay. The results showed that IL $[C_8mim][Br]$ may induce the apoptosis of PC12 cell lines. Radošević et al [26] assessed the cytotoxicity of imidazolium-based ILs towards Channel Catfish Ovary (CCO) cell lines *via* fluorescent microscopy observation and flow cytometry analysis, the results suggested that ILs with longer side chain length may cause the CCO cell lines necrosis. The work investigating toxic effect mechanism of ILs is necessary to their risk evaluation, however, the toxicity mechanism is not yet clear and still requires further investigation. Thus, it is of great importance to further realize the exact mechanisms underlying the toxic effect of ILs toward cell lines.

Insects are the most diverse groups of animals on our planet, which include a million species, thus they were usually used as models assessing the toxicity of toxins [33]. In the present study, the cytotoxic effect of ILs toward *Sf-9* insect cells was assessed. The *Sf*-9 cell lines were originally established from immature ovaries of the lepidopteran *S. frugiperda* pupae, otherwise known as the fall armyworm [34], which had previously been used as reliable tool for testing toxins and drug toxicity [33,35,36]. Larvae of *S. frugiperda* pupae live in the soil, and since a recent study showed that ILs were strongly absorbed and retained in soil for a period of time [37]. Thus, *Sf-9* cells were chosen as a model to assess ILs toxicity on soil-dwelling invertebrate organisms in this work.

Commonly used ILs containing assorted cations and anions were chosen, and their cytotoxicity toward *Sf-9* cells was investigated *via* MTT assay. To our knowledge, the application of imidazolium-based ILs was the most for industry and academic research among the all known ILs. Moreover, This IL $[C_2mim][Br]$ is the least cytotoxic among the imidazolium ILs in this study. So, $[C_2mim][Br]$ was chosen for investigating its toxicity mechanism toward *Sf-9* cell lines *via* various methods, such as acridine orange (AO) staining, DNA fragmentation assays, flow cytometry analysis, and transmission electron microscope (TEM) analysis. Information obtained from this study can complement the currently insufficient knowledge about IL cytotoxicity on insect cells by clarifying the mechanism of IL cytotoxicity. Moreover, it can provide some ideas and preliminary data for the future applications of ILs as insecticides.

2. Materials and methods

2.1. Materials and reagents

The ILs tetramethylammonium bromide $[N_{1,1,1,1}][Br]$, tetrabutylphosphonium bromide $[P_{4,4,4,4}][Br]$, 1-methyl-ethylpiperidinium bromide [MEPip][Br], 1-methyl-1-ethylpyrrolidinium bromide [MEPyrro][Br], 1-ethyl-3-methylimidazolium bromide $[C_2mim][Br]$, 1-butyl-3-methylimidazolium bromide $[C_4mim][Br]$, 1-hexyl-3-methylimidazolium bromide $[C_6mim][Br]$, 1-octyl-3-methylimidazolium bromide $[C_8mim][Br]$, 1-decyl-3-methylimidazolium bromide $[C_{10}mim][Br]$, 1-butyl-3-methylimidazolium tetrafluoroborate $[C_4mim][BF_4]$, 1-butyl-3-methylimidazolium hexyfluoroborate $[C_4mim][BF_6]$, and 1-butyl-3-methylimidazolium chloride $[C_4mim][Cl]$ were purchased from Shanghai Cheng Jie Chemical Co., LTD. (Shanghai, China), each with purity > 99%. The chemical structures of these ILs are shown in Table 1. *Sf-9* cells were obtained from the China Center for Type Culture Collection (CCTCC). 3-(4,5-Dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) was purchased from Amresco (USA). Acridine orange (AO) was purchased from Solarbio. Annexin V-EGFP Apoptosis and Cell Cycle Detection Kits were purchased from Jiangsu KGI Biotechnology Co., LTD. (Jiangsu, China). Fetal bovine serum (FBS) and gentamycin were purchased from Beijing North Carolina Souren Biotechnology Research Institute (Beijing, China). Insect DNA Kit (200) was obtained from OMEGA Bio-TEK (Doraville, GA, USA). Other reagents obtained from commercial sources were of analytical grade and were used without further treatment.

2.2. Cell culture

Sf-9 cells were cultured in 25 cm^2 T-flasks (Corning, USA) containing Trichoplusia ni medium-formulation Hink (TNM-FH) supplemented with 10% (v/v) fetal bovine serum (FBS) and 10 μg/mL gentamycin and maintained at 25 °C. To ensure the presence of sufficient nutrients, the medium was replaced every 2 – 3 days.