

CYTOTOXIC EFFECTS OF IMIDAZOLIUM IONIC LIQUIDS ON FISH AND HUMAN CELL LINES

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Ionic liquids bring a promise of a wide range of “green” applications that could replace conventional volatile solvents. However, before these applications become large-scale, their toxicity needs to be investigated in order to predict the impact on human health and environment. In this study we assessed the cytotoxicity of imidazolium ionic liquids (in the concentrations between 0.1 mmol L⁻¹ and 10 mmol L⁻¹) in the ovarian fish cell line CCO and the human tumour cell line HeLa using the MTT cell viability assay. Our results showed that the most cytotoxic ionic liquid was 1-*n*-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide, [BMIM][Tf₂N], followed by 1-*n*-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF₄], 1-*n*-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF₆], and 1,3-dimethylimidazolium hexafluorophosphate [MMIM][PF₆]. Generally, the effects were concentration-dependent. They also depended on the type of anion and the *n*-alkyl chain length. The comparison between the fish CCO and human HeLa cell lines suggests that CCO cells provide a good biological system for initial toxicity testing of ionic liquids that could replace *in vivo* bioassays.

KEY WORDS: CCO cells, HeLa cells, imidazolium ionic liquids, MTT assay

Ionic liquids are organic salts in liquid state at room temperatures that have recently attracted considerable attention as potential “green” substitutes for conventional volatile solvents, as they do not evaporate and are not flammable (1-2). In addition, ionic liquids are stable thermally, chemically and electrochemically, and can dissolve a wide range of compounds. These desirable properties and almost limitless structural possibilities provide a tremendous potential for application in various fields, such as organic synthesis, biocatalysis, and extraction and separation of biologically important compounds.

However, before their production and use reaches the industrial scale, ionic liquids should be assessed for the impact on human health and environment. One of the useful assessment tools is *in vitro* testing on cell

lines, which can elucidate intracellular, molecular, or physiological mechanisms induced by chemical substances (3). Mammalian cell lines are routinely used for basal cytotoxicity studies (4), while continuous fish cell lines are used in the ecotoxicological assessment of chemicals and environmental samples (5). It is suggested that cytotoxicity tests using dedifferentiated cancer cells provide a quick and convenient first information about the toxic potential of chemical substances (6). The effects of ionic liquids on cell viability have already been reported in well-characterised tumour mammalian cell lines such as HeLa (7), CaCo-2 (8), MCF7 (9), and PC12 (10). Investigations of the effects of ionic liquids on the aquatic environment have mostly been based on the evaluation of the growth and survival of different

aquatic organisms (11-12). Even though fish cell lines are becoming the most important *in vitro* tool in aquatic ecotoxicology (13), investigations of ionic liquid cytotoxicity in continuous fish cell lines have not been reported yet.

The aim of our study was to assess the cytotoxicity of imidazolium ionic liquids in the ovarian fish cell line CCO and the human tumour cell line HeLa using the MTT cell viability assay.

MATERIALS AND METHODS

Ionic liquids

The ionic liquids 1-*n*-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF₄], 1-*n*-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF₆], and 1-*n*-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide, [BMIM][Tf₂N] used in the experiments were purchased from Acros Organics, USA. 1,3-dimethylimidazolium hexafluorophosphate [MMIM][PF₆] was synthesised by M. Cvjetko in the Laboratory of Cell Culture Technology and Biotransformations (Zagreb, Croatia). The structures of the tested ionic liquids are shown in Table 1.

Materials

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Trypan blue were purchased from Sigma, St. Louis, MO, USA. Heat-inactivated foetal bovine serum (FBS) was purchased from GIBCO, Paisley, Scotland, UK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemie, Steinheim, Germany. DMSO was purchased from Kemika, Zagreb, Croatia.

Cell lines

The CCO cell line, derived from the ovaries of Channel catfish (*Ictalurus punctatus*, Rafinesque, 1818), was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA; ATCC® number CRL-2772). The HeLa cell line, derived from the human cervical carcinoma, was obtained from the Ruđer Bošković Institute, Zagreb, Croatia. Both cell lines were cultured in 75 mL flasks in DMEM supplemented with 10 % inactivated FBS and

maintained at 5 % CO₂. CCO cells were incubated at 30 °C and HeLa cells were incubated at 37 °C.

Cell treatment

Samples of CCO and HeLa cells were taken at the exponential growth phase and counted using the Trypan blue. Cells were then seeded in 24-well plates at a density of 5x10⁴ cells mL⁻¹ in 1 mL of media. Stock solutions of the ionic liquids (1 mol L⁻¹) were prepared in the culture medium or DMSO. After 24 h of cell growth, the medium was replaced with fresh medium containing different concentrations of ionic liquids. Both cell types were exposed to ionic liquids in the concentration range from 0.1 mmol L⁻¹ to 10 mmol L⁻¹; CCO cells for 72 h and HeLa cells for 48 h. The final DMSO concentration in the medium was 0.1 % for each sample.

Cytotoxicity assay

The cytotoxicity of ionic liquids was measured using the MTT assay as described by Mosmann (14). The absorbance of purple formazan was measured at 570 nm using a spectrophotometer (Helios, Thermo Electro Corporation). The results are given as percentages of the control absorbance.

Statistical analysis

The obtained data are expressed as the mean±SEM of three independent experiments performed in triplicate. We used the one-way analysis of variance (ANOVA) with Dunnett's test and set the probability level of p<0.05 as statistically significant. The half maximal effective concentration (EC₅₀), defined as the concentration of ionic liquid that resulted in 50 % growth inhibition, was calculated from the dose-response curves using equations of related trend lines for the MTT assay.

RESULTS

Cytotoxicity of ionic liquids in CCO and HeLa cells

Figure 1a shows the effects of different [BMIM][BF₄] concentrations on CCO and HeLa cells, expressed as percentages of viability. The proliferation of CCO cells was not affected by [BMIM][BF₄] at the concentrations of 0.1 mmol L⁻¹ and 0.5 mmol L⁻¹, but in HeLa cells these caused a slight cytotoxic effect.

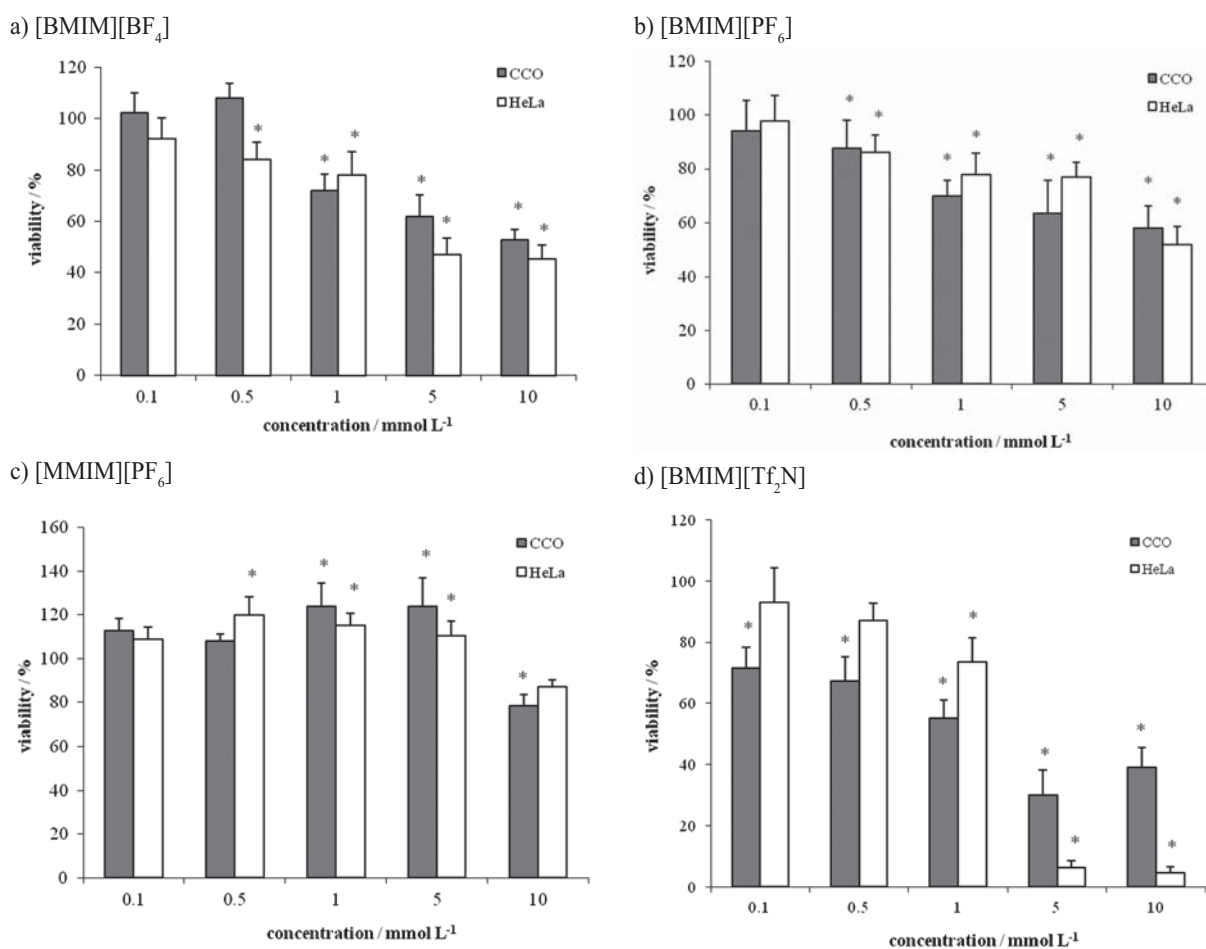


Figure 1 Effects of [BMIM][BF₄], [BMIM][PF₆], [MMIM][PF₆], and [BMIM][Tf₂N] at different concentrations on CCO and HeLa cell viability. Data expressed as a percentage of unexposed control cells ± SEM of three replicates for each exposure concentration. * denotes significant difference from control (p < 0.05).

Table 1 Ionic liquids used in the study

Systematic name	Trade name	Molecular weight	Chemical formula
1- <i>n</i> -butyl-3-methylimidazolium tetrafluoroborate	[BMIM][BF ₄]	226.02	
1- <i>n</i> -butyl-3-methylimidazolium hexafluorophosphate	[BMIM][PF ₆]	284.18	
1,3-dimethylimidazolium hexafluorophosphate	[MMIM][PF ₆]	242.11	
1- <i>n</i> -butyl-3-methylimidazolium bis (trifluoromethylsulphonyl) imide	[BMIM][Tf ₂ N]	419.36	

Table 2 EC₅₀ (mmol L⁻¹) of the selected imidazolium ionic liquids in CCO and HeLa cells

Ionic liquid	CCO cells	HeLa cells
[BMIM][Tf ₂ N]	3.26 ± 0.18	3.11 ± 0.11
[BMIM][BF ₄]	5.01 ± 0.32	4.42 ± 0.18
[BMIM][PF ₆]	10.32 ± 0.28	11.2 ± 0.15
[MMIM][PF ₆]	-	-

Higher concentrations of [BMIM][BF₄] (1 mmol L⁻¹ to 10 mmol L⁻¹) induced a significant cytotoxic effect in both cell lines. Survival rates decreased as [BMIM][BF₄] concentrations increased.

Figure 1b shows viability of CCO and HeLa cells exposed to different [BMIM][PF₆] concentrations (0.1 mmol L⁻¹ to 10 mmol L⁻¹). At 0.1 mmol L⁻¹, [BMIM][PF₆] did not cause significant cytotoxic effects in either cell line. Significant cytotoxic effects in both CCO and HeLa cells started at 0.5 mmol L⁻¹ and increased in the dose-dependent manner.

Exposure to [MMIM][PF₆] (Figure 1c) led to significant cytotoxic effects only in CCO cells at 10 mmol L⁻¹. At lower concentrations, [MMIM][PF₆] in fact significantly improved cell viability in both cell lines; in HeLa cells at doses up to and including 5 mmol L⁻¹ and in CCO cells at 1 mmol L⁻¹ and 5 mmol L⁻¹.

Exposure to [BMIM][Tf₂N] produced significant cytotoxic effects in CCO cells at all tested concentrations (Figure 1d). In contrast, significant cytotoxicity in HeLa cells was observed only at higher concentrations (1 mmol L⁻¹ to 10 mmol L⁻¹). In addition, at 5 mmol L⁻¹ and 10 mmol L⁻¹ this effect was much stronger in HeLa cells (95 %) than in CCO cells (65 %).

Comparison of cytotoxicity in CCO and HeLa cells

Table 2 shows the half maximal effective concentrations (EC₅₀) for selected ionic liquids in CCO and HeLa cells, that were calculated from cell viability data. The exception is [MMIM][PF₆] which could not decrease cell viability below 80 % in CCO cells and 85 % in HeLa cells, even at the highest tested concentration. The lowest EC₅₀ was calculated for [BMIM][Tf₂N] in both cell lines, followed by [BMIM][BF₄] and [BMIM][PF₆].

DISCUSSION

Even though ionic liquids are originally considered “green solvents”, recent studies on their ecotoxicity and degradability has shown that some are not as environmentally friendly as others (15). This calls for a careful and critical assessment that should be able to predict their effects on human health and environment.

Our study compared the cytotoxic effects and EC₅₀ of four imidazolium ionic liquids in fish CCO and

human tumour HeLa cells. [BMIM][BF₄] had about half the EC₅₀ [(5.01±0.32) mmol L⁻¹ in CCO cells and (4.42±0.18) mmol L⁻¹ in HeLa cells] of [BMIM][PF₆] [(10.32±0.28) mmol L⁻¹ for CCO cells and (11.2±0.15) mmol L⁻¹ for HeLa cells]. Similar results for HeLa cells have also been reported by Stepnowski et al. (7) and Wang et al. (16).

EC₅₀ did not differ much between CCO and HeLa cells, and since there is no available data on the toxicity of ionic liquids in fish cells, this finding may be relevant for the comparison of cytotoxicity data between fish and mammalian cell lines.

We also observed a significant stimulatory effect on CCO and HeLa cell viability by [MMIM][PF₆] at the concentrations of 0.5 mmol L⁻¹ to 5 mmol L⁻¹. This phenomenon is known as chemical hormesis, which is characterised by low-dose stimulation and high-dose inhibition (17). A similar hormetic effect of 1-*n*-octyl-methylimidazolium tetrafluoroborate [C₈MIM][BF₄] was observed in IPC-81 leukaemia cells (18) and of 1-*n*-butyl-3-ethylimidazolium tetrafluoroborate [BEIM][BF₄] in HeLa cells (7).

It seems that *n*-alkyl chain length correlates with toxicity, as [BMIM][PF₆] was more toxic in both cell lines than [MMIM][PF₆], which is consistent with the results reported by Ranke et al. (18).

It also seems that cytotoxicity may be related to the anion; the lowest EC₅₀ in both cell lines was found for [Tf₂N]. Kumar et al. (9) showed that the [Tf₂N] anion was more toxic in MCF7 cells than the bromide anion, and this toxicity could be associated with hydrolytic cleavage that resulted in the formation of free fluoride ions (19).

HeLa cells showed higher sensitivity when exposed to 5 mmol L⁻¹ and 10 mmol L⁻¹ of [BMIM][Tf₂N] than the CCO cells. This might point to HeLa-specific mechanisms of [BMIM][Tf₂N] action. Similarly specific mechanisms of action have been observed for other cell lines and imidazolium liquids (18).

Castaño et al. (20) compared the sensitivity of *in vitro* basal cytotoxicity tests (MTT assay) in fish and mammalian cell lines. These cells showed similar sensitivity when exposed to a range of chemicals. Although our results with CCO and HeLa cells are in agreement with this study, future *in vitro* cytotoxicity studies should include more cell lines to provide a more comprehensive information about the effects of ionic liquids in specific mammalian and fish cell lines.

In conclusion, our results obtained with CCO and HeLa cell lines show that the toxicity of the selected ionic liquids depends on the dose, anion type, and *n*-alkyl chain length. This has been the first study to assess the cytotoxicity of ionic liquids in fish CCO cells and it suggests that fish cell lines could be a good biological system for initial toxicity testing of ionic liquids that could replace *in vivo* fish bioassays. However, future studies on other fish cell lines should answer if differences between fish species and tissues/organs could affect toxicity data and their interpretation.

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Sažetak**CITOTOKSIČNI UČINCI IONSKIH TEKUĆINA NA STANIČNIM LINIJAMA RIBA I LJUDI**

S obzirom na širok raspon primjena ionskih tekućina, prethodno je potrebno ispitati njihovu toksičnost i mogući utjecaj na zdravlje ljudi i okoliš. U ovom radu ispitana je citotoksičnost odabranih imidazolijevih ionskih tekućina na stanicama ovarija riba CCO i ljudskoj tumorskoj staničnoj liniji HeLa primjenom MTT-metode. Izlaganje stanica različitim koncentracijama ionskih tekućina [(0,1 do 10) mmol L⁻¹] rezultiralo je uglavnom citotoksičnim učincima ovisnima o koncentracijama ionske tekućine. Na temelju dobivenih vrijednosti EC₅₀ najtoksičnija ionska tekućina je 1-*n*-butil-3-metilimidazolijev bis(trifluorometilsulfonil)imid [BMIM][Tf₂N], zatim 1-*n*-butil-3-metilimidazolijev tetrafluoroborat [BMIM][BF₄], 1-*n*-butil-3-metilimidazolijev heksafluorofosfat [BMIM][PF₆] i 1,3-dimetilimidazolijev heksafluorofosfat [MMIM][PF₆]. Općenito, toksičnost je bila ovisna o koncentraciji, tipu aniona i duljini *n*-alkilnog lanca. Nakon usporedbe rezultata toksičnosti na CCO i HeLa-stanicama smatramo da CCO-stanice mogu biti dobar biološki sustav za početna ispitivanja toksičnosti ionskih tekućina u cilju zamjene *in vivo* testova na ribama.

KLJUČNE RIJEČI: CCO-stanice, HeLa-stanice, imidazolijeve ionske tekućine, MTT-metoda

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