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Characterization of ionic liquid cytotoxicity mechanisms in human keratinocytes compared with conventional biocides

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Keywords

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Abstract

The ability to chemically modify ionic liquids (ILs) has led to an expansion in interest in their use in a diversity of applications, not least as antimicrobials and biocides. Relatively little is known about cytotoxicity mechanisms of ILs in comparison to other biocides currently in widespread use, as well as their practical significance for the ecological environment and human health. Using NCTC 2544 and HaCat human keratinocyte cells, this study aimed to characterize cytotoxicity rates and mechanisms of a range of ILs. Using both lactate dehydrogenase (LDH) and 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity assays, it was confirmed that at biocide-relevant concentrations, ILs with longer alkyl chains exhibited greater biocidal activity than those with shorter alkyl chains, with comparable activity to the commonly used biocides chlorhexidine, benzalkonium chloride and cetylpyridinium chloride, at relevant in-use biocide concentrations. Mode of cell death, measured using fluorescence-activated cell sorting (FACS) and caspase 3/7 activity, determined necrosis to be the primary cytotoxic mechanism at higher concentrations of the biocides stated above, and with ILs [C_{14}MIM]Cl and [C_{14}quin]Br, with apoptosis observed at borderline necrotic concentrations. Perhaps most interestingly, modification of anion had a significant effect on cytotoxicity. The use of N[SO_2CF_3] as an anion to [C_{16}MIM] attenuated cytotoxicity 10-fold in comparison to other anions, suggesting cytotoxicity may also be a tuneable property when using ILs as biocides.

1. Introduction

The capacity to modify the chemical, physical and biological properties of ionic liquids (ILs), through independent modification of their constituent cations and anions, has led to an expanding interest in their use as task specific solvents in a diversity of applications, ranging from synthesis, catalysis and extraction (Berthod et al., 2018; de Melo et al., 2020; Ferlin et al., 2013), through to bioremediation, topical drug delivery and antimicrobial applications. Ionic liquids have been at the forefront in the emerging search for environmentally friendly chemical compounds to replace volatile organic compounds in conventional industrial processes. The ability to modify inherent structural components of the constituent ions permits almost limitless possibilities for tuning these property sets aimed towards improving existing use, or tailoring their use to new and emerging applications.

Unsurprisingly, given the potential range of ILs available, it is now widely accepted that it is no longer feasible to universally label them as 'green' solvents, given the biological toxicity attributable to ILs. Indeed, studies on the potential for collateral toxicity linked to their widespread application have centred around contamination of the aquatic environment, including toxicity to bacteria responsible for biodegradation and damage to cellular morphology of plants and wildlife, as well as bioaccumulation in marine trophic chains (Couling et al., 2006; Liu et al., 2015; Nędzi et al., 2013; Piotrowska et al., 2018; Pretti et al., 2006; Samorì et al., 2007; Sintra et al., 2017; Wells and Coombe, 2006). However, the ability of environmental bacteria to biodegrade certain ILs has been described (Sydow et al., 2018; Thamke et al., 2019), and may therefore mitigate the risks associated with ecotoxicity resulting from accidental environmental exposures. Furthermore, the literature has heralded the potential of ILs as antimicrobial agents (Pernak et al., 2004, 2003; Yu et al., 2016).

Other studies have highlighted the ability of ILs used in this study, namely 1-alkyl-3-methylimidazolium and 1-alkylquinolinium ILs, to effectively eradicate biofilms of clinically relevant pathogens (Busetti et al., 2010; Carson et al., 2009; Pendleton and Gilmore, 2015; Venkata Nancharaiah et al., 2012). Herein exists a division between alternate fields in IL research, one which attempts to ameliorate potential toxicity and another which seeks to harness toxicity as a desirable, tuneable property in the creation of a new designer biocidal compounds. The future potential of widespread consumer use of ILs as biocides (for example as disinfectants, preservatives, and antiseptics) raises the largely unaddressed issue of the potential for toxicity at 'in-use' concentrations to the user; with respiratory, digestive and skin related contact the most likely points of direct exposure. The inherent characteristic of low vapour pressure of ILs removes inhalation as a significant risk of exposure in day to day applications. This leaves topical (skin) exposure as the predominant concern were ILs to be expanded for consumer use.

The skin is a critical target organ in environmental exposure, with numerous studies utilising skin keratinocyte cell lines to identify toxicity, irritation and carcinogenesis. These include the effect of heavy metal ions (Holmes et al., 2020, 2016; Orlowski et al., 2016), surfactants and pharmaceuticals (Abruzzo et al., 2017; Choi et al., 2018; Kim et al., 2019; Sugita et al., 2017), as well as antimicrobial agents (Kim et al., 2008; Lam et al., 2020; Pulingam et al., 2020) and wound sensor materials (Bhushan et al., 2019). In addition, the human keratinocyte cells lines NCTC 2544 and HaCat have previously been used to evaluate ultra-short antimicrobial peptides (Pasupuleti et al., 2009). As keratinocytes represent the major cell type in the human epidermis this *in vitro* model has been identified as a useful screening tool (Wilhelm et al., 2001).

Alongside appropriate test systems is the need for appropriate comparative control compounds. The gold standards in the field of biocides for some time has been chlorhexidine (CHX) and the quaternary ammonium compound benzalkonium chloride (BC). The effect of CHX on epidermal necrosis has been studied (Faria et al., 2007), as well as its effect on the expression of apoptotic and cellular stress proteins HSP70, Bcl-2 and GRP78 (Faria et al., 2007), cytoskeletal organisation (Faria et al., 2009; Giannelli et al., 2008) and mitochondrial function (Giannelli et al., 2008; Negrelo Newton et al., 2004). Benzalkonium chloride has been investigated for the ability to induce death, either by apoptosis or necrosis (Brasnu et al., 2008), cause corneal toxicity (Dart, 2003), and has been found to cause a reduction in myosin light chain phosphorylation linked to ATP leakage (Geerling et al., 2001).

We have previously reported 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity assays (McLaughlin et al., 2011), and IL degradation by bacterial flora of the marine environment (Megaw et al., 2013). In this study we have expanded our characterization of imidazolium and quinolinium based ILs to cytotoxicity in NCTC 2544 and HaCat human keratinocytes at relevant biocidal concentrations, based on established antimicrobial activity of these compounds. Herein we describe a cytotoxic mode of death which is primarily necrotic, however, apoptosis is observed for ILs which are not rapidly cidal, indicating a mechanisms of cell death which is dependent on alkyl chain length and rate of kill. This study characterizes the cytotoxicity profile and mechanism of a number of ILs at relevant biocidal concentrations against human keratinocytes, an important step in the assessment the safety of ILs for use in biocidal applications. These data are compared to the rate and mechanisms of cytotoxicty of a range of biocides currently in widespread use.

2. Materials and Methods

2.1. Cell Culture Conditions

The human skin keratinocyte cell lines NCTC 2544 and HaCat were used in this study. NCTC 2544 cells were originally obtained from the Interlab Cell Line Collection (Genoa, Italy). Cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK), supplemented with 2 mM *L*-glutamine and 1 % penicillin/streptomycin (both from Invitrogen) and 10% foetal bovine serum from (Biosera, East Sussex, UK). HaCat cells were purchased from Cell Line Service (Eppelheim, Germany). Cells were cultured in DMEM containing 4.5 g/L *D*-glucose from Invitrogen, supplemented with 2 mM *L*-glutamine, foetal bovine serum and 1 % penicillin/streptomycin. Cells were grown at 37 ºC and 5% CO_2 with cells subcultured at 80-90% confluency. Cell monolayers were rinsed with PBS and treated with trypsin-EDTA (Invitrogen) to detach cells before re-suspension in fresh media.

2.2. Antimicrobial Compounds

The antimicrobial compounds BC, CHX and cetylpyridinium chloride (CPyr), and the anionic surfactant SDS were purchased from Sigma (Poole, UK). Ionic liquids were synthesised and characterized in house as previously described, for both 1-alkyl-3-methylimmidazolium chloride (Carson et al., 2009) and 1-alkylquinolinium bromide ionic liquids (Busetti et al., 2010).

For 1-alkyl-3-methylimmidazolium chloride ionic liquids, a mixture of distilled (vacuum distilled from CaH_2 suspension) 1-methylimidazole (1.00 mol) and either 1-chlorooctane or 1-chlorotetradecane (1.05 mol) (used as supplied from Sigma-

Aldrich) was heated with an oil bath at 100 °C in a one-neck round-bottom flask (500 cm^3) equipped with a stir bar and reflux condenser, under an inert dinitrogen atmosphere. The end of the reaction was confirmed by testing for the presence of unreacted 1-methylimidazole, by adding a few drops of the reaction mixture to a solution of copper(II) sulfate in water. The development of a blue coloration indicates the reaction is not complete and should be heated for longer. After three days and a negative 1-methylimidazole test, the reaction was connected to a high vacuum pump (at 1 mBar) and heated for 4 h at 100 °C (for $[C_8MIM]Cl$) to distil out unreacted 1-chloralkane, or recrystallised from boiling ethyl ethanoate (for $[C_{14}MIM]Cl$). For each compound, purity was found to be 98–99% by ^1H NMR spectroscopy. All salts were solid at room temperature, with melting points determined using DSC.

For 1-alkylquinolinium bromide ionic liquids, A mixture of quinoline (0.100 mol) and a slight excess (0.105 mol) of either 1-bromoctane or 1-brometetradecanewas heated for 3 d at 80 °C under dinitrogen. When the reaction was completed (verified by ^{1}HNMR spectroscopy), the solid was obtained and purified via recrystallisation from boiling toluene/ethyl ethanoate mixtures. This mixture was then cooled at -15 °C, filtered under vacuum, washed with cold toluene (ca. 50 cm^3) and dried under vacuum. This recrystallisation process was repeated twice. The compound was then dried in a vacuum oven overnight at 70 ◦C. For each compound in the series, purity was found to be 98–99% by ^1H NMR spectroscopy. ^1H and ^{13}C NMR data revealed no organic impurities. All salts were solid at room temperature, with melting points determined using DSC.

158 **2.3. MTT Cytotoxicity Assay**

159 MTT cytotoxicity assays were set up as described previously (McLaughlin et al.,
160 2011). NCTC 2544 and HaCat cells were plated in the absence of
161 penicillin/streptomycin in their respective growth medium as previously outlined.
162 After cell counting, cell lines were seeded at a concentration of 7.5 $x10^3$ cells per well
163 in 96-well plates and left to adhere and recover for 24 h. After 24 h, media was
164 removed and replaced with fresh media containing different concentrations of the
165 compounds outlined in Section 2.2. (a log10 dilution series in the range 1 mM to
166 $1x10^{-8}$ mM was used). All compounds were initially dissolved in dimethyl sulfoxide
167 (DMSO) with heating at 37 ºC to aid dissolution where required. These stocks were
168 diluted in culture media resulting in a final compound concentration of 1 mM with a
169 final DMSO concentration no higher that 0.5 %. Compound-free DMSO controls
170 were included to determine any toxicity related to solvent. Cells were exposed to each
171 concentration of each compound at time points 15 min and 24 h. After the allocated
172 exposure time had elapsed, media was removed and replaced with fresh media
173 containing 24 μM of MTT (Sigma, Poole, UK). Cells were incubated for 2 h at 37 ºC,
174 5% CO_2 after which culture media was removed and any insoluble formazan crystals
175 dissolved using 200 μL DMSO per well. Absorption values per well at 570 nm were
176 measured using a Biotek EL808 spectrophotometer (Labtech, East Sussex, UK).
177 Three independent replicates were carried out for each experiment and percentage cell
178 viability was calculated relative to untreated control.

179 **2.4. Lactate Dehydrogenase Assay**

180 Leakage of cytoplasmic contents was measured using the *in vitro* Toxicology Assay
181 Kit, Lactate Dehydrogenase (LDH) based (Sigma), as per manufacturer's instructions.

Briefly, HaCat cells were plated at a concentration of 12.5 $x10^3$ per well in 96-well plates. After 24 h cells were treated with different concentrations of biocides or ILs under study in the range 1 x 10^0 to 1 x 10^{-4} mM. Following incubation for either 15 min or 4 h, plates were centrifuged at 2000 rpm for 4 min and half the media was removed for LDH analysis. LDH assay mixture was added as recommended by the manufacturer and incubated in the dark for 20 min. Percentage LDH release was calculated by subtraction of background 690 nm measurement from 490 nm measurement. Untreated cells were used as a control for 0% compound-induced LDH release and triton X-100 (Sigma) as 100% release.

2.5. Flow Cytometry

Apoptosis and necrosis were discriminated by use of annexin V FITC coupled antibody (BD Pharmingen, Oxford, UK), and propidium iodide (Sigma). HaCat cells were plated in 6 well plates at $1x10^5$ cells per well and allowed to rest for 24 h. Cells were then exposed to increasing concentrations of each compound under study for 24 h. Culture media for each well was collected, cells were washed with PBS, and trypsin-EDTA was used to detach cells. Detached cells, PBS wash and culture supernatant were all combined upon collection. Apoptotic and necrotic cells were pelleted by centrifugation at 2000 rpm for 5 min after which the cell pellet was resuspended in binding buffer at a final concentration of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 18 mM $CaCl_2$ (pH 7.4). Annexin V and propidium iodide were mixed with the cell suspension and incubated at room temperature in the dark for 15 min. A minimum of 10^4 events were acquired using a FACS Canto II (BD Biosciences, Oxford, UK). Acquisition, gating and analysis were performed with FACSDiva software (BD Biosciences).

2.6. Caspase Activity

Activation of caspase 3 and 7 was assessed using the ApoTox-Glo apoptosis toxicity assay, as per the manufacturer's instructions (Promega, Southampton, UK). Cells were plated at a concentration of 10 $x10^3$ per well in opaque 96-well plates and allowed to rest for 24 h. Cells were treated with sub-necrotic concentrations of biocides or ILs. After a 24 h incubation, Caspase-Glo 3/7 reagent was added in a one-to-one ratio to media, mixed for 30 s on an orbital shaker and incubated for 30 min before luminescence measurement on a MicroLumat Plus (EG&G Berthold).

2.7. Western Blot

HaCat cells were plated at a concentration of 3.5 $x10^5$ cells per well in 6-well plates and left for 24 h. Media was removed, cells were washed in PBS and exposed to media containing different concentrations of biocides or ILs for 24 h. Cells were washed with PBS and lysed on ice in buffer containing 50 mM Tris-HCl, 20 mM EDTA and 1% nonidet P-40 for 15 minutes. Samples were centrifuged for 15 min at 14,500 rpm and the supernatant retained. Equal loading of protein samples on SDS-PAGE was determined by bicinchoninic acid (BCA) assay (Thermo Scientific Pierce, Leicestershire, UK). Whole cell lysates were separated on reduced 4-12% Bis-Tris NuPAGE gels using NuPAGE MOPS running buffer (Invitrogen). After blotting to PVDF membrane (GE Healthcare, Buckinghamshire, UK), blotted membranes were blocked with 5% non-fat dry milk in PBS for 1 h and probed for GRP78 with mouse α-KDEL (Stressgen, British Columbia, Canada), mouse α-γ-tubulin (Sigma) and HRP-conjugated goat α-mouse secondary (Jackson Immunoresearch, PA, USA). Antibody binding was detected using immobilon western substrate (Millipore, UK) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, using either two-tailed t-tests or one-way ANOVA followed by Tukey's multiple comparisons post-test.

3. Results

3.1. Cell viability measured by MTT assay

In order to assess the toxicity of 1-alkyl-3-methyimmidazolium chloride ([C_nMIM]Cl) and 1-alkylquinolinium bromide ([$C_n$quin]Br), a cytotoxicity screen was carried out in two human keratinocyte cell lines. NCTC 2544 and HaCat cell lines have been extensively utilised in the study of compounds pertinent to cutaneous toxicity. Alongside [C_nMIM]Cl and [$C_n$quin]Br, the commonly employed biocides CHX, BC and CPyr were studied for contextual comparison, along with the C_{12} alkyl chain containing anionic surfactant, sodium dedecylsulphate (SDS). Cytotoxicity values at a range of concentrations for both 15 min and 24 h are shown in **Figure 1**. Calculated half-maximal effective concentration (EC_{50}) values are shown in **Table 1**.

In NCTC 2544 cells at 15 min, EC_{50} values fell broadly into two groups. The least toxic compounds, with an EC_{50} value calculated to be greater than 1 mM, were the C_8-containing compounds [C_8MIM]Cl and [C_8quin]Br. The most toxic group of compounds, with an EC_{50} value of approximately 0.03-0.07 mM, were the common biocides CHX, BC and CPyr, as well as the C_{14} alkyl chain ILs [C_{14}MIM]Cl and [C_{14}quin]Br. This clustering was conserved at 24 h also. While [C_8MIM]Cl and [C_8quin]Br were still the least toxic of the biocides tested, there was a minor

253 difference, with [C_8MIM]Cl 3-fold less toxic than [C_8quin]Br at 0.255 and 0.086 mM
254 EC_{50} values respectively. CHX was the next most toxic at 0.012 mM, with BC, CPyr
255 and [C_{14}MIM]Cl and [C_{14}quin]Br approximately similar, with EC_{50} values falling
256 between 0.002 and 0.004 mM.

257 When the same 15 min and 24 time points were repeated in HaCat cells a number of
258 variations were observed. At 15 min, C_8 alkyl chain containing compounds were still
259 the least toxic with EC_{50} values of >1 mM. CHX was observed to be more toxic in
260 HaCat cells at 15 min, associating more closely with BC, CPyr and the C_{14} ILs with
261 EC_{50} values clustered between approximately 0.05 and 0.03 mM. The pattern of
262 toxicity at 24 h in HaCat cells was similar to that observed in NCTC 2544 cells. At
263 specific concentrations at 24 h in **Figure 1**, toxicity to HaCat cells was marginally
264 greater for [C_8MIM]Cl, [C_{14}quin]Br and [C14quin]Br over that observed in NCTC
265 2544 cells.

266 **3.2. Cytolysis and LDH Release**

267 We sought to identify the ability of ILs and biocides to induce necrosis using
268 cytolysis assays over very short contact time periods of 15 min when compared to 4 h
269 (**Figure 2**). Chlorhexidine induced the greatest LDH release, with 20% of total release
270 occurring at 15 min for 0.1 mM and 71% at 15 min with 1 mM. 4 h exposure resulted
271 in total LDH release at both 0.1 and 1 mM concentrations. BC, [C_{14}MIM]Cl and
272 [C_{14}quin]Br exhibited a similar pattern of LDH release, with negligible release at
273 15 min for 0.1 mM, rising to 70-90% of total release at 15 min exposure at 1 mM.
274 LDH release at 4 h was total at both 0.1 and 1 mM, the same as for CHX. [C_8MIM]Cl
275 and [C_8quin]Br exhibited no LDH release at 15 min at any tested concentration. Only

1 mM [C8quin]Br induced LDH release at 4 h with 35% of total release, no release for [C8MIM]Cl was observed at any tested concentration at 4 h.

3.3. Cytotoxicity of anion pairs with [C_{16}MIM] cation

Research in our group has identified [$C_{14\text{-}16}$MIM] as a lead structure of potent antibacterial and antibiofilm activity (Carson et al., 2009). In order to enhance the biocidal activity, a number of anions have been tested in order to assess their contribution to improving antimicrobial activity. This has led to the identification of $AgCl_2$ anion conferring increased broad spectrum antimicrobial activity against Gram negative bacteria such as *P. aeruginosa* PAO1 (Gilmore et al., 2013). To fully assess the effect on cytotoxity of various anions, $AgCl_2$ and other anions were tested in HaCat cells at 24 h (**Figure 3**) with corresponding EC_{50} values shown in **Table 2**. Inclusion of a cuprate anion ($CuCl_4$) or silver-containing anions ($AgCl_2$ or $AgBr_2$) resulted in a small increase in toxicity when compared to the chloride anion. The inclusion of cuprate anion as $CuBr_2$ had similar toxicity to the chloride anion at all concentrations. A significant decrease in toxicity over the chloride anion was observed for [C_{16}MIM] N[SO_2CF_3] at a number of concentrations, most notably at 0.01 mM (**Figure 3**) ($p < 0.0001$).

3.4. Analysis of apoptotic and necrotic cell death

Flow cytometry using propidium iodide and annexin V staining were carried out to identify apoptotic and necrotic cells following IL exposure. Loss of membrane integrity and entry of propidium iodide signified necrotic cells, whereas propidium iodide negative but annexin V positive cells indicated apoptosis. The ubiquitous antimicrobial compounds CHX and BC were tested as comparative controls. CHX and BC were observed to induce a large necrotic population of PI positive, annexin V

negative cells, at both 1 mM and 0.1 mM. Approximately 10% of the cell population were necrotic at 0.01 mM after 24 h exposure. Interestingly, the necrosis observed at 0.01 mM coincided with a small but statistically significant level of apoptosis. Treatment-free controls exhibited low levels of apoptosis at 1.4% of the total cell population, 0.01 mM CHX resulted in an increase in apoptotic cells to 4.0%, and 0.01 mM BC resulted in a rise to 4.4% after 24 h exposure.

Of the ILs tested, the results partitioned into two groups. As for **Figure 1**, the data in **Figure 4A** shows that toxicity is linked to alkyl chain length. The C_{14} alkyl chain bearing compounds [C_{14}MIM]Cl and [C_{14}quin]Br, exhibited a profile similar to CHX and BC over the concentration range tested. This was one of total necrosis at 0.1 and 1 mM, with lower levels of necrosis observed at 0.01 mM, which also coincided with increased apoptosis. This increase in apoptosis was 6.5 and 5.6% for [C_{14}MIM]Cl and [C_{14}quin]Br respectively, compared to 1.4 % in controls.

The C_8 alkyl chain group comprising [C_8MIM]Cl and [C_8quin]Br were toxic at 1 mM only, similar to the findings by MTT assay in **Figure 1**. [C_8MIM]Cl was marginally less toxic overall as measured by fluorescence-activated cell sorting (FACS), not bringing about complete toxicity at 1 mM. At this concentration, decreased necrosis and increased apoptosis (10.3%) was observed, similar to the trend observed at lower doses of the C_{14} compounds. This was not observed for [C_8quin]Br, which transitioned from little observable toxicity at 0.1 mM to total necrosis at 1 mM.

To confirm the increased apoptosis by an alternative method, activation of caspase 3/7 was measured at 0.001 and 0.01 mM for CHX, BC, [C_8MIM]Cl and [C_{14}MIM]Cl (**Figure 4B**). Statistically increased levels of apoptosis were observed in three conditions, 0.01 mM CHX, 0.01 mM BC and 0.01 mM [C_{14}MIM]Cl. Basal caspase

3/7 activity was recorded at 1100 Relative Luminescence Units (RLU), this increased to 1700 RLU with 0.01 mM CHX, 3700 RLU with 0.01 mM BC and 6400 RLU with 0.01 mM [C_{14}MIM]Cl. The pattern of increase in apoptosis closely corresponded to that observed for annexin V (**Figure 4A**) with induction shown at identical concentrations, the greatest induction observed for [C_{14}MIM]Cl and the least for CHX.

3.5. Endoplasmic Reticulum stress as measured by GRP78 expression

GRP78 is the major regulator and response chaperone of the endoplasmic reticulum (ER) related to the induction of ER stress and the unfolded protein response (Kim et al., 2006). It has previously been shown that CHX induces an upregulation of GRP78 in murine L929 fibroblast cells (Faria et al., 2009). We sought to investigate this possibility in HaCat cells for both biocides and 1-alkylmethylimmidazolium chloride ILs. In western blots (**Figure 5A**) with quantification of three independent experiments by densitometry, corrected for fluctuations in γ-tubulin levels (**Figure 5B**), no significant changes in GRP78 expression was observed in HaCat cells for any compound at 24 h.

4. Discussion

Ionic liquid toxicity studies published to date predominantly focus on contact/exposure times of 24 h or greater, a duration far in excess of that expected for biocide applications, where short contact times and rapid toxicity are necessary. The data presented focuses on short-term exposure, conditions which are more likely to correspond to small to medium scale biocidal applications, compared to large scale

347 industrial use. The biocides CHX (Giannelli et al., 2008) and BC (Brasnu et al., 2008)
348 have been investigated at 15 min time points, and right down to 1 min exposures.
349 Previous studies have used criteria based on EC_{50} values to judge the toxicity of ionic
350 liquids, based on the UFT Merck Ionic Liquids Biological Effects Database (Fatemi
351 and Izadiyan, 2011; Radošević et al., 2013). Based on the leukaemia cell line IPC-81,
352 ILs are classified as possessing either very high (EC_{50} <0.001 mM), high (0.001 <
353 EC_{50} <0.1 mM), moderate (0.1 < EC_{50} < 5 mM), or low (EC_{50} >5 mM) toxicity. Using
354 the EC_{50} values for HaCat cells following 15 min exposure, CHX, BC, CPyr,
355 [C_{14}Qn]Cl, and [C_{14}MIM]Cl would be considered to have high toxicity, with
356 moderate toxicities observed for SDS, [C_8Qn]Br, and [C_8MIM]Cl.

357 The toxicity profiles of CHX, CPyr and BC match closely those of both [C_{14}MIM]Cl
358 and [C_{14}quin]Br (**Figure 1**), and can justifiably be grouped as inducing a similar
359 cytotoxic impact. Comparison of [C_8MIM]Cl and [C_8quin]Br to SDS reveals both C_8
360 alkyl chain containing ILs exhibit a moderate toxicity, similar to anionic surfactants
361 present in numerous consumer products over 15 min exposures. The effect of alkyl
362 chain length on toxicity is well established (Cvjetko Bubalo et al., 2014; Diaz et al.,
363 2018; Li et al., 2012; Radošević et al., 2013), and this study shows how IL alkyl chain
364 length correlates to commonly used biocides and surfactants.

365 LDH release (**Figure 2**), a measure of cytoplasmic leakage, corresponded closely to
366 that measured by MTT. This exhibited a time delay where initial high LDH release
367 closely corresponded to downstream loss in cell viability in **Figure 1**. The degree of
368 correlation is indicative of necrotic release of cellular contents, including LDH, as the
369 major component of downstream cell death. It is also notable that treatment groups
370 showing significantly increased levels of death via apoptosis (**Figure 4**), exhibited

371 little or no LDH release, further indicating the correlation between LDH release and
372 necrotic cell death.

373 MTT cytotoxicity of varying anions is interesting in two respects. While silver has
374 been shown to possess enhanced antimicrobial properties against Gram negative
375 bacteria such as *P. aeruginosa* (Gilmore et al., 2013; Poulter et al., 2009; Yuan et al.,
376 2017), this does not translate into a comparable increase in cytotoxicity. Additionally,
377 use of $N[SO_2CF_3]$ actually attenuates the cytotoxicity of $[C_{16}MIM]$ ten-fold compared
378 to all other anions tested, with the exception of chloride (4-fold). This may indicate
379 the ability to "tune" for increased biocidal activity without a corresponding increase
380 in mammalian cell toxicity.

381 The identification of apoptosis and necrosis through FACS analysis of annexin V and
382 propidium iodide has previously been used in the study of CHX (Faria et al., 2009)
383 and BC (Brasnu et al., 2008). The results observed for HaCat cells (**Figure 4**)
384 correspond closely to that reported for CHX and BC in the literature with L929
385 fibroblasts and Chang/IOBA-NHC cells respectively. In this study and others for
386 CHX and BC, necrosis was observed at higher concentrations and apoptosis at
387 borderline necrotic concentrations, though HaCat cells appear less sensitive overall
388 than Chang or IOBA-NHC cells (Brasnu et al., 2008). This pattern was also observed
389 for both $[C_{14}MIM]Cl$ and $[C_{14}quin]Br$. $[C_8MIM]Cl$ conformed to the pattern of an
390 apoptotic population at sub-necrotic levels, however this was not observed with
391 $[C_8quin]Br$. Apoptosis, as measured by both FACS and caspase 3/7 activation,
392 corresponded closely. A number of recent studies have used a similar approach to
393 examine cell death mode of other cell types following treatment with imidazolium-
394 based ILs. Apoptosis was shown to contribute to cell death in both hepatocellular
395 carcinoma (HepG2) cells and human cervical carcinoma (Hela) cells, following

396 treatment with [C_{12}MIM]Cl and [C_{16}MIM]Cl (Wan et al., 2018; Wang et al., 2020;
397 Xia et al., 2018). In work by Li and co-workers, [C_8MIM]Br induced apoptosis in rat
398 phaeochromocytoma (PC12) cells, triggered by excessive reactive oxygen species
399 (ROS) and mediated by mitochondrial depolarization and permeability transition (Li
400 et al., 2012).

401 Mitochondrial permeability transition has been shown to be affected by CHX
402 (Negrelo Newton et al., 2004) as well as depletion of cellular ATP content (Hidalgo
403 and Dominguez, 2001). This is also the case for BC which induces ATP depletion in
404 epithelial cells, dephosphorylation of myosin light chain as well as cytoskeletal
405 thinning of cortical actin bundle and reduced cell adhesion (Guo et al., 2007).
406 Toxicity mechanisms of ionic liquids have been reviewed recently (Bubalo et al.,
407 2017). Whilst membrane damage remains a key mechanism of toxicity, enzyme
408 inhibition, alterations in cell morphology and oxidative damage were also highlighted
409 as contributing factors.

410 Analysis with CHX has previously shown upregulation of GRP78, BCL-2 and HSP70
411 when assayed by fluorescence microscopy (Faria et al., 2009, 2007). This led us to
412 postulate that ILs and BC may also perturb the endoplasmic reticulum and may
413 contribute through GRP78 induction to apoptosis via the unfolded protein response.
414 As shown in **Figure 5**, none of the biocides or ILs tested caused a significant
415 alteration to GRP78 expression after 24 h exposure. This was true even up to
416 0.01 mM, which in the case of CHX, BC and [C_{14}MIM]Cl are only 60, 20 and 40
417 percent viable, or in the case of 0.1 mM [C_8MIM]Cl, 40 percent viable. Upregulation
418 of GRP78 in L929 (Faria et al., 2009) may be a cell specific process not observed in
419 HaCat cells, however the apoptotic profile of L929 and HaCat cells is similar as

measured by annexin V and propidium iodide. This data suggests that GRP78 and the ER play no role in apoptotic cell death for the ILs and biocides tested.

5. Conclusion

At this point in their evolution, ILs lack substantive cellular mechanistic studies. This study describes a thorough characterization of cell death mechanisms as a result of IL exposure, finding necrosis as the major mechanism of death. While ILs have been shown to be capable of caspase activation and apoptosis, this is a distinct minority of the cell population in comparison to necrosis as signified by LDH leakage. Research on BC and CHX point to future areas of investigation, such as studies of reactive oxygen species generation, cytoskeletal changes and cellular metabolism, along with *in vivo* toxicity studies to assess teratogenicity in particular. Of particular interest is the ability to tune cytotoxicity through anion selection. This suggests unwanted cytotoxicity to the user may in fact be a tuneable property, which can be attenuated without affecting the ability of ILs to perform as biocides. Addressing gaps in knowledge in these areas is of profound importance for the onward development of ILs as consumer grade product constituents, as well as their continued development for safe use in industrial applications.

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