1	In vitro cytotoxicity assessment of monocationic and dicationic pyridinium-based ionic
2	liquids on HeLa, MCF-7, BGM and EA.hy926 cell lines
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9	Abstract
10	Dicationic ionic liquids (ILs) generally possess higher thermal and electrochemical stability than
11	the analogous monocationic ILs, which makes them more suitable for high-temperature
12	applications as solvents for organic reactions, lubricants or stationary phase in gas
13	chromatography. However, knowledge on dicationic IL cytotoxicity is still scarce. Here we
14	explore the cytotoxicity of twelve mono- and dicationic pyridinium-based ILs on HeLa, MCF-7,
15	BGM and EA.hy926 cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
16	(MTT) assay, cell cycle arrest assays, apoptosis experiments and orange staining were carried out.
17	The results showed that dicationic ILs are generally less cytotoxic than their monocationic
18	counterparts. In monocationic ILs, cytotoxicity was stronger when they contain long alkyl chains,
19	because of their higher lipophilicity. However, the full effect of the length of the linkage alkyl
20	chain of dicationic ILs on cytotoxicity is not clear probably because the chain is "trapped"
21	between both cationic moieties. IL cytotoxicity is highly dependent on the cell type, and HeLa
22	cells exposed to [C ₁₂ Pyr]Br die via apoptosis. The present study increases our knowledge of IL
23	cytotoxicity on human and monkey cells and clarifies the cell death mechanism. The results
24	suggest that dicationic ILs offer the potential to replace some monocationic ILs because of their
25	lower cytotoxicity.

26 Keywords: Dicationic; ionic liquid; pyridinium; MTT assay; apoptosis.

27 **1. Introduction**

28 Ionic liquids (ILs) are molten salts at low temperature (around or below 100 °C). Low melting points are possible because the asymmetry of their ionic structure and the dispersion of their 29 charges result in reduced inter-ion interaction energies and decreased lattice enthalpy (ΔH_L) 30 31 (Ranke et al., 2004). ILs are typically composed of an organic cation with a bulky charge-carrying 32 head group (imidazolium, pyrrolidinium, pyridinium, piperidinium, etc.) and one or more alkyl 33 side chains, and a counterion which may be organic or inorganic, for example Cl⁻, Br⁻, AcO⁻ or $[Tf_2N]^-$ (Wu et al., 2018). ILs have been widely studied in research activities and even 34 35 incorporated into several industrial processes because of their exclusive combination of properties, which include negligible vapor pressure, non-flammability, wide liquid state 36 37 temperature range, high thermal and chemical stability and high ionic conductivity (Alvarez-38 Guerra and Irabien, 2011; Diaz et al., 2018; Lee and Lin, 2014). On a fundamental level, the most remarkable feature of ILs is the possibility of modulating their physicochemical properties, 39 40 including their hydrophobicity, viscosity, density or solubility and their biodegradability or 41 toxicity, by means of the appropriate selection of the cationic and anionic constituents (Montalbán 42 et al., 2015; Ventura et al., 2012), which means that they can be designed for a specific application (Viswanathan et al., 2006). Initially ILs were proposed as more benign solvents and candidates 43 44 to replace conventional organic solvents (Earle and Seddon, 2002), but they have also found 45 applications in other areas, where they can be used as reaction media (de Los Ríos et al., 2007), 46 catalysts (Welton, 2004), extraction agents (Montalbán et al., 2018a, 2016a) or supported liquid 47 membranes (Hernández-Fernández et al., 2008, 2007), and in lithium ion batteries (Balducci, 48 2017), dye-sensitized solar cells (Denizalti et al., 2018) or the synthesis of biopolymeric 49 nanoparticles (Lozano-Pérez et al., 2015).

50 Dicationic ILs are an interesting sub-class of materials that can be easily prepared by the 51 functionalization of a central linker fragment with two discrete cationic charge carriers 52 (Montalbán et al., 2018b; Steudte et al., 2014). Compared to monocationic ILs, dicationic ILs 53 may possess even higher thermal and moderately increased electrochemical stability, making

them more suitable for use in high-temperature applications as solvents for reactions (Han and 54 55 Armstrong, 2005), lubricants (Jin et al., 2006) or as stationary phases in gas chromatography (Qi 56 and Armstrong, 2007). Because ILs are widely used in academic research and have potential use 57 in industry, more studies related to their toxicity are required since they may enter to the living 58 environment and the risks associated with them are still far from being known in any depth (Wu 59 et al., 2018), especially in the case of the more recently discovered dicationic ILs. As mentioned 60 above, the release of ILs into the atmosphere is difficult because they are not volatile (Gómez-61 Herrero et al., 2018). However, a great number of ILs (all those which are hydrophilic), are 62 notably soluble in water and hence bioavailable so they can easily enter water streams or soils 63 (Diaz et al., 2018; Montalbán et al., 2016b) with serious effects on human health and the 64 environment. By contrast, ILs which contain a hydrophobic anion (PF_6^- , Tf_2N^- , among others) are practically non-soluble in water and have poor bioavailability. ILs with halogen anions (chloride, 65 bromide or iodide), such as the ILs studied in this work, are highly hydrophilic and extremely 66 water soluble, with a high degree of bioavailability (Huddleston et al., 2001). Given the wide 67 68 potential use of mono- and dicationic ILs in industrial processes, especially as an alternative to 69 conventional solvents, and their possible release into the environment by accidental spills or as residues, analysis of their toxicological fate is imperative. In addition, some ILs have recently 70 71 been seen to be of interest in the biological and pharmaceutical field as platforms to overcome 72 problems associated with new drugs, such as their low solubility in water and poor bioavailability 73 (Egorova et al., 2017; Vieira et al., 2019). To this end, several studies have reported that ILs are 74 able to increase the water solubility of poorly soluble drugs because of their capacity for 75 dissolving pharmaceutical ingredients and for acting as drug reservoirs in controlled release 76 devices (Mahkam et al., 2016; Miwa et al., 2016; Mizuuchi et al., 2008; Moniruzzaman et al., 77 2010). New dicationic imidazolium-based ILs have recently been synthesized and their potential 78 use as antimicrobial materials has been demonstrated (Gindri et al., 2014). These recent 79 applications of ILs make the study of their biocompatibility and cytotoxicity even more necessary.

Studies related to the toxicity of dicationic ILs are very scarce in the literature. e Silva et al. (2014) 80 studied the (eco)toxicity of dicationic cholinum-based ILs in Vibrio fischeri on the basis of the 81 82 luminescence inhibition of this bacterium. Steudte et al. (2014) used several toxicity tests of different levels of biological complexity to study a group of dicationic imidazolium and 83 pyrrolidinium-based ILs. They used enzyme inhibition tests (acetylcholinesterase), algae 84 (Scenedesmus vacuolatus), fish (Daphnia magna) and cell lines (IPC-81) to carry out the 85 86 experiments. Gindri et al. (2014) screened the cytotoxicity of a set of dicationic imidazolium-87 based ILs on MC3T3-E1 pre-osteoblast cells.

In this study, we evaluate the cytotoxicity of 10 pyridinium-based ILs (5 dicationic ILs and their 88 89 monocationic counterparts) on four cell lines: Human cervical cancer cells (HeLa), Human breast cancer cells (MCF-7), green monkey kidney epithelial cells (BGM) and human umbilical 90 91 immortalized cells (EA.hy926). A comparison between the cytotoxicity of dicationic and 92 monocationic ILs is carried out in order to study the influence of the dicationic moiety structure, 93 while the effect of the alkyl chain length and cell line on cytotoxicity in both groups of ILs is also analyzed. To the best of our knowledge, this is the first time that the cytotoxicity of these 94 dicationic ILs has been measured. 95

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2. Material and Methods

2.1. Ionic liquids

99 The full name and chemical structure of the ILs used are presented in Table 1. The monocationic 100 ILs $[C_2Pyr]Br$ (purity>99%), $[C_4Pyr]Br$ (purity>99%), $[C_6Pyr]Br$ (purity>99%), $[C_8Pyr]Br$ 101 (purity>99%) and $[C_{12}Pyr]Br$ (purity>99%) were purchased from IoLiTec (Germany). The 102 dicationic ILs used in this study ($[C_2(Pyr)_2]Br_2$, $[C_4(Pyr)_2]Br_2$, $[C_6(Pyr)_2]Br_2$, $[C_8(Pyr)_2]Br_2$ and 103 $[C_{12}(Pyr)_2]Br_2$) were synthesized and characterized as described in previous papers (Montalbán 104 et al., 2018b; Montalbán et al., 2018c).

Table 1. Abbreviations, full names and chemical structures of the studied ILs.

IL abbreviation	IL full name	Cation	Anion
[C ₂ Pyr]Br	1-ethylpyridinium bromide	N ⁺	Br-
[C ₄ Pyr]Br	1-butylpyridinium bromide		Br⁻
[C ₆ Pyr]Br	1-hexylpyridinium bromide		Br⁻
[C ₈ Pyr]Br	1-octylpyridinium bromide	N ⁺	Br-
[C ₁₂ Pyr]Br	1-dodecylpyridinium bromide		Br⁻
$[C_2(Pyr)_2]Br_2$	1,2-bis(pyridinium-1-yl)ethane dibromide	$\frac{1}{2}$	2Br-
[C ₄ (Pyr) ₂]Br ₂	1,4-bis(pyridinium-1-yl)butane dibromide		2Br⁻
$[C_6(Pyr)_2]Br_2$	1,6-bis(pyridinium-1-yl)hexane dibromide		2Br⁻
$[C_8(Pyr)_2]Br_2$	1,8-bis(pyridinium-1-yl)octane dibromide		2Br ⁻
$[C_{12}(Pyr)_2]Br_2$	1,12-bis(pyridinium-1-yl)dodecane dibromide	* (), +	2Br ⁻

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2.2. Cell lines and culture media

Human cervical cancer cells (HeLa), Human breast cancer cells (MCF-7), green monkey kidney 108 epithelial cells (BGM) and human umbilical immortalized cells (EA.hy926) were acquired from 109 110 the American Type Tissue Culture Collection (ATCC, USA). Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with a low glucose content (1 g/L) supplemented 111 with 10% (v/v) fetal bovine serum (FBS), 1 mM glutamax, 1 % antibiotics (penicillin-112 streptomycin) and 1 mM pyruvate. In all cases, cells were maintained at 37°C in 5% CO2 with 113 114 95% humidity atmosphere. Cells were sub-cultured and the medium was changed once a week. 115 In all cases, 0.25% trypsine-0.25 mM ethylenediaminetetraacetic acid (EDTA) were used. Before

and after the experiments, all cell lines were seen to be mycoplasma-free, as determined by theHoechts DNA stain method (Chen, 1976).

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2.3. Cytotoxicity assay

A total of 5×10^3 cells/well (150 µL of culture medium as described above) were seeded in a 96-121 well plate and incubated at 37 °C in a 5% CO₂ and 95% humidity atmosphere for 24 h. The IL 122 123 solutions were diluted in complete medium at 100 mM concentration for dicationic ILs and 250 μ M for the monocationic ILs. The resulting solution was then diluted successively (1:1 dilutions) 124 to obtain a total of 16 solutions of diminishing concentration. These dilutions differed depending 125 126 on the IL in question and their cytotoxic effect on each cell line. The dilutions were added to each 127 well at a final volume of 150 µL. Cells were incubated at 37 °C for 48 h. The medium was then 128 removed from the wells and 200 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-129 diphenyltetrazolium bromide, 1 mg/mL final concentration) was added. After 4 h incubation in 130 identical conditions, MTT was removed and 100 μ L of dimethyl sulfoxide (DMSO) was added. The absorbance at 560 nm was then recorded on a Fluostar Omega spectrophotometer. 131

132 The absorbance at each compound concentration was translated into inhibition percentage, *I*%,133 according to the following equation,

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$$I\% = \left[1 - \frac{A_T}{A_C}\right] x100$$
 [1]

135 where $A_{\rm T}$ and $A_{\rm C}$ are the absorbances of treated and control cells, respectively.

IC₅₀ values were obtained from a three parameter fitting of the semi-logarithmic curves (*I*% as a
function of the logarithm of the IL concentration) according to equation 2.

138
$$I\% = \frac{Imax}{1 + \left(\frac{IC_{50}}{C}\right)^n}$$
 [2]

139 where I_{max} is the maximum inhibition observed, IC₅₀ is the IL concentration at which 50% of the 140 cell population is dead, *C* is the IL concentration at which the inhibition *I*% is observed and *n* is the slope of the curve at the IC₅₀ value. The fitting was performed using GraphPad Prism v.6
software. All compounds were tested in three independent sets with triplicate points. The *in vitro*studies were performed in the SACE service with Level 2 Biosecurity (Support Service for
Experimental Sciences, University of Murcia, Murcia, Spain).

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2.4. Cell Cycle Arrest Assays

146 A total of 7.5×10^4 HeLa cells were seeded in 12-well plates and left at 37°C in a 5% CO₂ and 95% 147 humidity atmosphere for 24 h. Monocationic or dicationic ILs with C12 chain used at the 148 approximate IC₅₀ concentration were then added to each well. Cells were incubated for 24 h and 149 then removed from the wells with trypsin-EDTA, collected and centrifuged (200g, 5 min). The supernatant was removed and the cells were washed with phosphate-buffered saline (PBS) and 150 151 centrifuged (200g, 5 min). The supernatant was also removed, and the cells were treated with 152 ethanol (EtOH) at 70% (PBS, 30%) for 45 min at 4°C. Finally, EtOH was removed by 153 centrifugation. Cells were resuspended in 400 μ L of PBS, and 50 μ L RNase solution and 50 μ L of propidium iodide (PI) were added at a final concentration of 0.1 mg/mL and 40 mg/mL, 154 155 respectively. Cells were incubated for 30 min in the same conditions as for culture. The PI 156 fluorescence was measured for each cell in a Becton-Dickinson FACScalibur flow cytometer. In 157 each case 30000 events were acquired.

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2.5. Apoptosis Experiments

159 To explore the cell death mechanism provoked by ILs, apoptosis experiments were performed. 160 First, 7.5×10^4 of HeLa cells were typically seeded in a 12-well plate and maintained under normal 161 culture conditions. After 24 h, the cells were treated with monocationic and dicationic ILs with 162 C₁₂ chain at the approximate IC₅₀ concentration and incubated for a further 24 h. An apoptosis 163 positive control with 8 µM camptotecin was used. Following incubation, media and cells were 164 collected and centrifuged, the supernatant was discarded and cells were washed twice with PBS 165 as described above (without the PBS/EtOH mix in this case). After removing the PBS, 40 µL of 166 a solution containing Annexin V and PI (Annexin-V-Fluos from Roche) and 160 µL of incubation

167 buffer (Roche apoptosis kit) were added to the cell pellet. Cells were resuspended in this solution 168 and left at room temperature in the dark for 15 min. 200 μ L of PBS was added prior to determining 169 the emission of PI and Annexin V at 620 and 525 nm, respectively. These measurements were 170 carried out in a Becton-Dickinson FACScalibur flow cytometer. In each case, 10000 events were 171 acquired.

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2.6. Nuclei fluorescent imaging

173 Typically, 1.5×10^4 HeLa cells were cultured in the same conditions as described above (DMEM 174 supplemented, 37°C, 5% CO₂ and 95-100% humidity) in a 4-well chamber slide system and left 175 for 24 h. $[C_{12}Pyr]Br$ was then added at 8 μ M and the cells in each individual well were fixed every 176 30 minutes with 70% alcohol and left in PBS buffer. Finally, a solution of 1ng/ml of acridine 177 orange was added to each well, and the resulting cultures were incubated for 10 min. The resulting 178 slide was observed under an inverted microscope (NIKON mod. Eclipse TE 2000U) for phase 179 contrast and using a FITC filter for fluorescent images. Imaging cells were analyzed with ImageJ 180 software. The statistical analysis was made with GraphPad Prims 6 software.

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3. Results and Discussion

3.1. Cytotoxicity of mono- and dicationic pyridinium-based ILs

184 Carrying out *in vitro* cytotoxicity tests is important in the case of ILs and, especially when they 185 are going to be used in biological and pharmaceutical applications and in other areas in which 186 they could come into contact with humans, animals or the environment. The test is cost-effective, convenient and time saving (Vieira et al., 2019). IL cytotoxicity is greatly influenced by the 187 chosen cell line and by IL structural features such as the cationic moiety, the length of the alkyl 188 189 chain/s and the anion type (Wu et al., 2018). After exposing HeLa, MCF-7, BGM and EA.hy926 190 cells to a series of mono- and dicationic pyridinium-based ILs for 48 hours, IC₅₀ values were 191 calculated using a dose-response model, which was obtained from a sigmoidal fitting of dose-192 response curves as stated in Section 2.3. The calculated IC_{50} values are shown in Table 2. The 193 corresponding dose-response curves of monocationic and dicationic ILs are represented in Figures 1 and 2, respectively. It can be observed that the cytotoxicity of the ILs was dosedependent. The IC₅₀ of $[C_{12}Pyr]Br$ on EA.hy926 cells was the lowest, with a value of 0.1 μ M. By contrast, the IC₅₀ of $[C_4(Pyr)_2]Br_2$ on MCF-7 cells was the highest at 33.17 mM. From a preliminary study of the results, we can infer that the IL cytotoxicity greatly depends on the IL structure and on the cell line in question. As a general rule, the IC₅₀ values obtained in this work were high, reflecting the low cytotoxicity of the studied ILs and the probability that, at their environmental concentration, they have no strong cytotoxic effect.

Table 2. IC₅₀ values for HeLa, MCF-7, BGM and EA.hy926 cells exposed to mono- and
dicationic pyridinum-based ILs for 48 hours.

ц	IC ₅₀ (mM)			
IL	HeLa	MCF-7	BGM	EA.hy926
[C ₂ Pyr]Br	5.560±0.4307	26.300 ± 5.8194	4.743±0.9068	6.511±0.4474
[C4Pyr]Br	1.725 ±0.1771	6.439 ± 0.8727	3.756 ±0.7244	1.998 ± 0.2059
[C ₆ Pyr]Br	0.568 ± 0.0707	3.262 ± 0.5238	0.690 ± 0.1487	0.228 ± 0.03627
[C ₈ Pyr]Br	0.0159 ± 0.0018	0.054 ± 0.0047	0.211±0.0208	0.0007 ± 0.0001
[C12Pyr]Br	0.0046±0.0002	0.002±0.0002	0.018±0.0037	0.0001 ± 0.00002
$[C_2(Pyr)_2]Br_2$	18.360 ± 0.529	6.553 ± 2.797	13.220 ± 0.529	22.100 ± 1.971
$[C_4(Pyr)_2]Br_2$	13.760 ± 1.218	33.170 ± 2.696	22.140 ± 0.336	19.560 ± 1.381
$[C_6(Pyr)_2]Br_2$	18.250 ± 0.641	17.410 ± 1.458	24.000 ± 1.033	8.428 ± 2.659
$[C_8(Pyr)_2]Br_2$	1.078 ± 0.141	6.979 ± 0.716	26.260 ± 0.249	0.408 ± 0.037
[C ₁₂ (Pyr) ₂]Br ₂	0.160 ± 0.030	0.529 ± 0.043	0.751 ± 0.066	0.008 ± 0.001



Figure 1. Dose-response curves of HeLa, MCF-7, BGM and EA.hy926 cells treated with five
monocationic pyridinium-based ILs for 48 hours.



Figure 2. Dose-response curves of HeLa, MCF-7, BGM and EA.hy926 cells treated with five
dicationic pyridinium-based ILs for 48 hours.

214 3.1.1. Monocationic vs dicationic ILs





Figure 3. IC₅₀ values for HeLa, MCF-7, BGM and EA.hy926 cells exposed to monocationic and dicationic pyridinium-based ILs with different alkyl chain lengths (the number of carbon atoms *n* ranged from C_2 to C_{12}) for 48 hours.

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3.1.2. Influence of alkyl chain length

238 The cytotoxic effect of monocationic and dicationic pyridinium-based ILs with different alkyl 239 chain lengths (number of carbon atoms n = 2, 4, 6, 8 and 12) on HeLa, MCF-7, BGM and 240 EA.hy926 cells was studied. The results are shown in Figure 3. In the case of the monocationic 241 ILs, their cytotoxicity on all the cell lines increased with the alkyl chain length as their IC_{50} values 242 decreased. The link between increasing alkyl chain length and increasing cytotoxicity in 243 monocationic ILs has been established in numerous studies (Li et al., 2012; McLaughlin et al., 244 2011; Steudte et al., 2014; Vieira et al., 2019; Wu et al., 2018). Lengthening of the alkyl chain of 245 the cation is closely related to an increase in IL toxicity due to the increase of lipophilicity (Gal 246 et al., 2012; Montalbán et al., 2016b). This increase in the lipophilic nature and the presence of 247 delocalized charges would promote the disruption of the cell membrane and increase the internal 248 acidity, with negative effects on cellular viability because of enzyme denaturation and oxidative 249 stress (Gal et al., 2012; Vieira et al., 2019). According to Wu et al. (2018), the stronger the 250 lipophilicity, the greater the possibility of contact with the lipid bilayer and hydrophobic proteins 251 of the membrane, altering its normal physiological function and even leading to cell death.

252 As regards the dicationic ILs (Figure 3), a similar trend was found, but not so clear. With up to 253 six carbon atoms in the alkyl chain, the IC₅₀ of [C₂(Pyr)₂]Br₂, [C₄(Pyr)₂]Br₂ and [C₆(Pyr)₂]Br₂ on 254 EA.hy926 and HeLa cells showed values of the same order of magnitude and, from $[C_8(Pyr)_2]Br_2$, cytotoxicity greatly increased. In BGM and MCF-7 cell lines, the IL cytotoxicity did not start to 255 256 significantly increase until $[C_{12}(Pyr)_2]Br_2$. On the one hand, our results agree with other authors 257 such as Gindri et al. (2014), who found that imidazolium-based dicationic ILs with different 258 anions and cationic alkyl chains of 10 carbons were more toxic towards MC3T3-E1 pre-osteoblast 259 cells than those with 8 carbons. On the other hand, the trend observed in this work was also found 260 by Steudte et al. (2014) who reported that they did not observe the effect of greater toxicity on

promyelocytic leukemia rat cells (IPC-81) with increasing spacer length from 2 to 6 atoms of carbons in the studied dicationic ILs. However, they did find an effect when the length of the terminal side chains (not the linkage alkyl chain) increased from 1 to 5 atoms of carbons. The reason might be that, as stated before, the alkyl chain is "trapped" between the two cationic moieties and, although the hydrophobicity of the cation increases with the length of the linkage alkyl chain, the interaction of the alkyl chain with the cellular membrane is weaker and, consequently, cellular death is reduced.

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3.1.3. Influence of cell type

269 In this study, the cell lines were selected due to their features and origin. Three of them (HeLa, 270 MCF-7 and EA.hy926) were human cell lines and one of them (BGM) from monkey. HeLa cells 271 are derived from cervical carcinoma and represent prototypical cells of the human epithelium. 272 Epithelial cells were chosen because toxic compounds generally enter an organism through this 273 site of first contact so they are a suitable model for studying IL cytotoxicity on human cells due 274 to the direct contact (Stepnowski et al., 2004; Xia et al., 2018). MCF-7 cells are human breast 275 cancer cells, which have been widely used in IL cytotoxicity studies because they are a well-276 characterized cell line (Kumar et al., 2008; Salminen et al., 2007). EA.hy926 cells coat the inside 277 of blood vessels and are one of the most used and best characterized human vascular endothelial 278 cell lines (Vieira et al., 2019). When ILs are used in pharmacological approaches with intravenous 279 administration, the determination of IL cytotoxicity on endothelial vein cells such as EA.hy926 280 cells is of great value. BGM is a cell line of renal origin. It is a non-tumorigenic epithelial cell 281 line which was chosen because it is a good reference to assess the toxicological effects of different 282 compounds (Romero et al., 2004).

As can be seen in Figure 4 and Table 1, the IL cytotoxic effect is greatly influenced by the cell line, but the mechanism of IL cytotoxicity has not been fully elucidated yet. A similar conclusion was reached by other authors (Vieira et al., 2019). According to our results, it may be inferred that EA.hy926 cells are more sensitive to mono- or dicationic ILs with longer alkyl chains (C_{6^-} C_{12}), *i.e.* the most toxic of each series, while the cytotoxicity exhibited toward normal and cancer human cells is significantly different. For mono- or dicationic ILs with shorter alkyl chains (C₂-C₄), the cytotoxicity values are of the same order on all the cell lines and no trend can be established. The differences in the cellular viability of the different cell lines after exposure to the same IL could derive from the specific features and/or properties of the cells.



Figure 4. IC₅₀ values for HeLa, MCF-7, BGM and EA.hy926 cells exposed to monocationic (left)
and dicationic (right) pyridinium-based ILs for 48 hours.

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296 **3.2.** Flow cytometry analysis of mono- and dicationic pyridinium-based ILs

For the flow cytometry analysis, HeLa cells were chosen as a model for their well-characterized cell cycle. As comparison between mono- and dicationic ILs, the assays were carried out with $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$ since they are the most toxic ILs on HeLa cells in each series. The IC₅₀ concentration of both ILs was chosen for the experiments.

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3.2.1. Rate of apoptosis

To analyze the nature of cell death due to the effect of the ILs on HeLa cells, Annexin-V-Fluos Apoptosis Detection Kit was used to determine the rate of apoptosis provoked by 24 hours of treatment at the IC₅₀ concentration of $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$. The Annexin-V-Fluos stained graph consists of four quadrants (Q) where Q1 represents necrotic cells, Q2 and Q3 show late and early apoptotic cells, respectively and Q4 are the live cells. As negative control live HeLa cells which had not been treated with any IL were used, and the positive control, as mentioned in 308 Section 2.5, was HeLa cells treated with camptotecin, a chemical compound which induces 309 cellular apoptosis. The positive control is normally used as comparison and, if similar results are 310 found in the treated cells, it can be assumed that the cells have suffered apoptosis. As shown in 311 Figure 5, the apoptotic effect in HeLa cells was dependent on the type of IL and its concentration. 312 The apoptosis rate (Q2 + Q3) of the negative and positive controls was 19.82% and 56.9%, respectively. The apoptosis rate of the HeLa cells exposed to $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$ at 313 314 their IC₅₀ concentration was 24.92% and 30.3%, respectively. As expected, these values are intermediate between both controls, indicating that the mechanism acting during cell death was 315 mainly apoptotic. In addition, it could be inferred that early apoptosis and late apoptosis were the 316 main cell death mechanisms for the monocationic and the dicationic IL, respectively. The results 317 318 reveal that, to reach a similar rate of apoptosis, a considerably higher dicationic IL concentration 319 was necessary, showing again its less cytotoxic behavior compared to its monocationic analogous.





Figure 5. Flow cytometry analysis of apoptosis rate in HeLa cells after 24 hours of treatment with different concentrations of $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$. HeLa cells not treated with IL were used as negative control and HeLa cells treated with camptotecin were used as positive control.

326 3.2.2. Influence of IL treatment on the cell cycle

327 The observed induction of apoptosis due to pyridinium-based IL treatment on HeLa cells further 328 prompted us to study the inhibition mechanism of cell growth through cell cycle arrest by flow 329 cytometric analysis. The cell cycle of HeLa cells exposed to $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$ at the 330 IC₅₀ concentration for 24 hours was evaluated. According to Figure 6, the number of G1 phase 331 cells slightly increased with respect to the control (unexposed cells) from 49.1% to 54.6 % and 53.5% when cells were exposed to $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$, respectively. The number of 332 333 cells in the S and G2/M phases decreased in both cases. The S cell number decreased from 27.7% 334 to 23.4% and 24.5%, and the G2/M cell number from 16.6% to 13.1% and 13.3% with $[C_{12}Pyr]Br$ 335 and $[C_{12}(Pyr)_2]Br_2$, respectively. Our results are in good agreement with previous IL cytotoxicity studies. Thamke et al. (2019) reported that another pyridinium-based monocationic IL, 1-butyl-336 337 4-methylpyridinium chloride, arrested animal cells (MDA-MB-231) in S and G2/M phases of the 338 cell cycle. It can be concluded that the dicationic IL, $[C_{12}(Pyr)_2]Br_2$, is much less cytotoxic than 339 the homologous monocationic IL, [C₁₂Pyr]Br, because a 32-fold concentration is necessary to 340 have the same effect on the cell cycle of HeLa cells.



Figure 6. The cell cycle of HeLa cells exposed to different concentrations of $[C_{12}Pyr]Br$ and $[C_{12}(Pyr)_2]Br_2$ for 24 hours. HeLa cells not treated with IL were used as control.

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346 **3.3. Nuclear morphology in HeLa cells after being exposed to [C₁₂Pyr]Br**

347 Observing cellular morphology is useful for distinguishing between apoptosis and necrosis during 348 cell death. The nucleus fluorescence of HeLa cells after different exposure times (0, 30, 60 and 349 90 min) at a concentration of 8 μ M of [C₁₂Pyr]Br, the most toxic IL in this work, can be found in 350 Figure 7. The quantitative analysis showed that the fluorescence remained constant up to 60 min 351 of exposure. Only at 90 min was an increase in nucleus fluorescence observed, due to the 352 alteration in nucleus morphology occurred. Cell nuclei increased in size, perhaps due to the 353 significant G1 phase arrest of cell cycle progression. This provides an opportunity for cells to 354 either undergo repair mechanisms or follow the apoptotic pathway (Murad et al., 2016). 355 According to Huber and Gerace (2007) and Jorgensen et al. (2007), a critical nucleus size is 356 required for progression between the G1 and S phases in eukaryotic cells. However, in this case, cell nuclei did not reach a critical size to progress to the S phase as shown in Figures 5 and 6. 357 358 Therefore, we infer that $[C_{12}Pyr]Br$ at a concentration of 8 μ M after 90 min of treatment induces 359 apoptosis in HeLa cells. These results agree with our MTT and flow cytometry results.



Figure 7. Nuclear morphology in HeLa cells induced by $[C_{12}Pyr]Br$ at a concentration of 8 μ M over 0, 30, 60 and 90 min using acridine orange staining. HeLa images correspond to the initial time and 90 min of treatment.

364

365 4. Conclusions

Some trends can be identified for the twelve monocationic and dicationic pyridinium-based ILs 366 367 studied in this work. These are related to both their structural features and the cell line chosen to 368 test their cytotoxicity. The results suggest that the cytotoxicity of the studied ILs on the four cell 369 lines is highly dependent on their structure. The development of dicationic ILs, with a broad range 370 of potential applications, but with lower cytotoxicity than their monocationic counterparts, can be 371 considered in the sustainable design of new ILs for specific applications. The present results 372 confirm that the mechanism of cell death followed by the most toxic IL used in this study, 373 $[C_{12}Pyr]Br$, on HeLa cells was apoptotic. In addition, this work increases our knowledge of the 374 cytotoxicity of pyridinium-based ILs, helping to amend the gap in the literature in this respect.

- 375
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- 377

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