# Toxicological evaluation on human colon carcinoma cell line (CaCo-2) of ionic liquids based on imidazolium, guanidinium, ammonium, phosphonium, pyridinium and pyrrolidinium cations†

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Toxicological evaluation of a new group of ionic liquids was performed on human colon cancerous cells—CaCo-2. They belong to different classes of cations: imidazolium (IM), dimethyl-guanidinium (dmg) and tetramethyl-guanidinium (tmg), methyl-pyrrolidinium (MPyr), 2-methyl-1-ethyl-pyridinium (2-MEPy), quaternary ammonium (benzyltriethyl-ammonium–BzTEA; phenyltrimethyl-ammonium–PhTMA; tri-n-octyl-methylammonium-Aliquat) and tri-*n*-hexyl-tetra-*n*-decylphosphonium (P6,6,6,14). The new results were compared with data obtained in previous reported studies performed in our lab, and we clearly saw that toxicity can vary significantly with the type of anion. Dicyanoamide-[DCA] and bis(trifluoromethanesulfonyl)-amide-[$NTf_2$] were seen to visibly change the impact of some cations. Some were considerably less harmful for CaCo-2 monolayer when the anion was [DCA] or [$NTf_2$], while others induced an abnormal increase of cellular metabolism when [$NTf_2$] was present and therefore, they were considered toxic. However, some cations induced similar responses in the presence of a broad number of anions as (1-butyl-3-methylimidazolium)-[$C_4$MIM] (with the exception of [$FeCl_4$]), (1-(2-hydroxyethyl)-3-methylimidazolium)-[$C_2$OHMIM] and [$C_4$MPyr] and did not cause toxicity. Consequently, they are considered promising cations for building human friendlier solvents. But, a reasonable number of other combinations involving different classes of cations were also seen to not significantly affect viability of the CaCo-2 monolayer.

## Introduction

Ionic liquids are claimed to be "green solvents" due to the fact they are not flammable and have very low vapour pressures, which opposes to conventional organic solvents. They can dissolve a wide range of materials and can be tuned by different combinations of cations and anions, resulting in solvents with very distinct properties. Some can be applied as thermal fluids, lubricants, plasticizers, dispensants and surfactants, antimicrobial agents, anticorrosion and electropolishing agents, amongst others. In addition, ionic liquids have been largely studied in biocatalysis, since they have been shown to improve catalyst efficiency.[1–10]

For application of ionic liquids in industry, it is convenient to study their impact on humans and environment and therefore, several studies have been performed on aquatic and terrestrial microorganisms and also in cell lines, including some from human origin. Methyl-imidazolium (MIM) toxicity was investigated in several aquatic models as the bacterium *Vibrio fischeri*, the freshwater crustaceous *Daphnia magna* and the algae *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* and it was seen to increase with the alkyl chain length.[11–13] But, for the green algae *Oocystis submarina*, toxicity followed the trend [butyl-MIM] < [benzyl-MIM] < [hexyl-MIM] < [ethyl-MIM].[14] Dependence on the anion was not obvious for the fish *Danio rerio* (zebrafish) since 96 hours exposure to different ionic liquids ([butyl-MIM], combined with hexafluorophosphate-[$PF_6$], bis(trifluoromethanesulfonyl)amide-[$NTf_2$], tetrafluoroborate-[$BF_4$] and dicyanoamide-[DCA]), lead to a $LC_{50}$ (concentration in water which kills 50% of the test batch of fish) higher than 100 mg/L in all cases.[15] In contrast, the algae *Selenastrum capricornutum* was affected differently by the type of anion and toxicity increased in the following order: hexafluoroantimonate ([$SbF_6$]) < [$PF_6$] < [$BF_4$] < trifluoromethanesulfonate ([$CF_3SO_3$]) < octylsulfate ([$C_8H_{17}OSO_3$]) < [Br] ~ [Cl].[16,17] Other examples are *Scenedesmus vacuolatus* and *Lemna minor* that were more sensitive to ionic liquids when the cation was combined with [$NTf_2$].[17] Introduction of

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hydroxyl ether and cyano groups in the imidazolium alkyl chain was seen to reduce aquatic toxicity and some cations such as morpholinium-[Morp], pyrrolidinium-[Pyr], the non-cyclic quaternary ammonium and [Choline] have been reported to have lower toxicity than pyridinium-[Py] and [MIM] cations.[11,18–20] Several studies also suggest that most of the studied ionic liquids are not less toxic to the aquatic environment than conventional solvents.[2,12,13,19,21] Toxicity assays have also been performed on bacteria and yeast strains more predominant in the terrestrial environment: *Escherichia coli*, *Pichia Pastoris* and *Bacillus cereus*, for instance. As for marine models, effect of the [MIM] cation on these microorganisms was dependent on the anion and the level of toxicity varied with the type of microorganism. *Bacillus cereus* and *Pichia Pastoris* were able to tolerate [1-butyl-3-MIM] [$PF_6$] up to a concentration of 1% (v/v) and 10% (v/v), respectively, whereas a concentration of 0.7% (v/v) was already toxic to *E. coli*. In contrast to, [1-butyl-3-MIM] [$BF_4$] at 1% (v/v) induced already a significant growth inhibition in all the microorganisms.[22] Thus, the anion [$PF_6$] seems to be inducing less toxicity than [$BF_4$]. Studies in *E. coli* and *Caenorhabditis elegans*, a well-studied free-living soil roundworm, showed that [MIM] toxicity, in the terrestrial environment, also increases with the alkyl chain length.[23,24] Imidazolium ionic liquids were demonstrated to be toxic to garden watercress *Lepidium sativum L.* and the longer the alkyl chain length the more absorbed was the ionic liquid, which consequently blocked nutrient uptake, leading to decreased root germination.[25] More recently, some studies have been performed on cell lines. The first experimental works date from 2004 and were done on leukemia–IPC-81 and glioma–$C_6$ rat cell lines and on human cancer cervical cells–HeLa.[13,26] Other human cell lines have followed: breast cancer cell line (MCF7) and colon cancer cell lines–HT-29 and CaCo-2.[27–30] Confluent CaCo-2 cells have the advantage to be able to differentiate in human erythrocytes upon reaching confluence and thus, they are a good model to study toxicity.[31] CaCo-2 monolayer and HT-29 cells in a low confluent state were both used in some experimental studies in order to assess toxicity and likely anti-proliferative activity against cancerous cells from the colon, respectively.[29,32] Cytotoxicities of Pyr and piperidinium (Pip) ionic liquids were estimated in MCF7 cells and they fall into the same range of toxicity as imidazolium ionic liquids and were considerably less toxic than their Py homologues.[27,28] Pyr, Pip and Py cations were seen to decrease as much cellular viability as longer their alkyl chains.[27,28,33] Concerning the assays carried out in colon cancer cells, any results were found regarding anti-proliferative activity; however some important conclusions were made in terms of toxicity. [$C_4$MIM], [$C_2$OHMIM], [$C_5$OHMIM] and [Choline] were the safest ionic liquids to CaCo-2 monolayer. [$C_8$MIM] and [$C_{10}$MIM] were highly toxic due to the length of the alkyl chains, but surprisingly, the introduction of a carboxylic group in the end of a $C_{10}$ alkyl chain reduced considerably toxicity, which did not occur in the presence of an ester.[29,32] In general, the cation tri-*n*-octyl-methylammonium (Aliquat) was very toxic, but dimethyl-guanidinium (dmg) toxicity looked to be more dependent on the anion and [$PF_6$] gave a worse result than either [DCA] or [$NTf_2$].[29,32]

Chemical and thermal stability of ionic liquids suggests they might potentially accumulate in the environment after disposal. Biodegradation studies have been undertaken according to the Organisation for Economic Cooperation and Development (OECD) Standard tests, which rates a compound as "readily biodegradable" if at least 60% mineralization occurs within the first 10 days during the 28-day test period. Results show that biodegradability seems to increase with the increase of the alkyl chain length and with the presence of ester functionality; and that it possibly depends on the type of anion, as well.[34–36] In opposition to [MIM] ionic liquids, some [Py]-based ILs can be completely degraded and therefore, they constitute attractive targets as green chemicals.[33,34]

In this study, we present new toxicological data for a large set of ionic liquids, some of them never studied before. Experimental work was performed in colon carcinoma CaCo-2 cells monolayer, which can differentiate in human erythrocytes.[31] Together with these results, we have compiled data of other ionic liquid toxicity assays that our laboratory have tested previously,[29,32] with the aim to establish rules for the designing of human friendly ionic liquids.

## Results and discussion

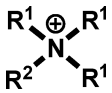
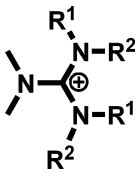
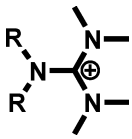
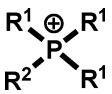
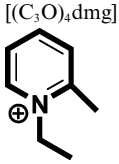
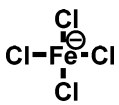
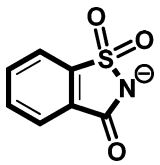
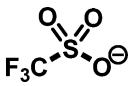
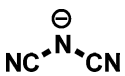
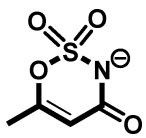
In this work, we have tested many ionic liquids (see ESI)† including dialkyl-dimethyl-guanidinium and tetramethyl-guanidinium with alkyl chains of variable length (Table 1). The obtained dose-dependent toxicity curves describe very different behaviours: some ionic liquids decreased drastically cellular viability while others induced decreases inferior to 50% or even changed it insignificantly. Consequently, many toxicity curves do not fit to standard dose–response curves like the ones suggested by Ranke *et al.*[13] In view of that and to make a clearer presentation of the data, we adjusted a function that best fitted to the experimental data and presented the toxicity trends. Each experimental point is an average of three repliques. This allowed us to compare the different ionic liquids and to establish general conclusions for the designing of non-toxic solvents, which was the aim of this work. In the ESI,† we have also joined data obtained in previous work performed in our lab.[29,32]

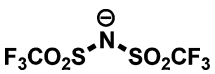
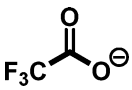
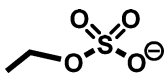
Accordingly with published reports, toxicity of guanidinium was also verified to be dependent on the length of the chain [12–14,23,24,32] (Fig. 1, Table 2). [(mh)$_2$dmg] [$BF_4$], [(di-o)$_2$dmg] [$BF_4$] and [(di-h)$_2$dmg] [Cl]/[SAC]/[$FeCL_4$] (Saccharine–SAC) were toxic (Fig. 1, Table 2 and ESI†).

Despite the presence of butyl-chains, [(di-b)$_2$dmg] [$BF_4$] lead to a great cell loss at the highest studied doses, whereas [(eb)(mb)dmg] [$BF_4$] and [(eb)$_2$dmg] [$BF_4$] did not have a significant impact on the CaCo-2 monolayer (Fig. 1). It appears that, [dmg] toxicity also increases with the number of longer chains, which explains the toxicity of [(di-b)$_2$dmg] [$BF_4$]. Consequently, [(eb)(mb)dmg] [Cl] did not affect cells viability whereas [(di-b)(eb)dmg] [Cl] induced a rapid decay of the viability (see ESI†). [(di-b)tmg] [I] was not toxic, but [(di-deca)tmg] [I] and [(di-hept)tmg] [I] were very toxic due to the effect of the chain length, as well (Table 2 and ESI†).

Results obtained in previous works showed that toxicity of guanidinium can depend considerably on the anion: [(di-h)$_2$dmg] [Cl] was very toxic but [(di-h)$_2$dmg] [$NTf_2$] and [(di-h)$_2$dmg] [DCA] were not (see ESI†).[32]

**Table 1** Schematic representation of the structures of all the studied cations and anions

| Cations | | | |
|---|---|---|---|
|  |  |  |  |
| $R^1$=$CH_3$, $R^2$=n-$C_4H_9$ [$C_4$MIM]<br>$R^1$=$CH_3$, $R^2$=n-$C_8H_{17}$ [$C_8$MIM]<br>$R^1$=$CH_3$, $R^2$=n-$C_{10}H_{22}$ [$C_{10}$MIM]<br>$R^1$=$CH_3$, $R^2$ =$CH_2Ph$ [BzMIM]<br>$R^1$=$CH_2Ph$, $R^2$=$CH_2Ph$ [BzIMBz] | $R^1$=$CH_3$, $R^2$=Ph [PhTMA]<br>$R^1$=n-$C_2H_5$, $R^2$=$CH_2Ph$ [BzTEA]<br>$R^1$=n-$C_8H_{17}$, $R^2$=$CH_3$ [Aliquat] | $R^1$=$R^2$=n-$C_6H_{13}$ [(di-h)$_2$dmg]<br>$R^1$ =$R^2$=n-$C_8H_{17}$ [(di-o)$_2$dmg]<br>$R^1$=$CH_3$, $R^2$=n-$C_4H_9$ [(mb)$_2$dmg]<br>$R^1$=$CH_3$, $R^2$=n-$C_6H_{13}$ [(mh)$_2$dmg]<br>$R^1$=n-$C_2H_5$, $R^2$=n-$C_4H_9$ [(eb)$_2$dmg]<br>$R^1$=n-$C_2H_5$,$CH_3$ $R^2$=n-$C_4H_9$ [(eb)(mb)dmg]<br>$R^1$=n-$C_2H_5$, n-$C_4H_9$ $R^2$=n-$C_4H_9$ [(di-b)(eb)dmg]<br>$R^1$=$R^2$=$CH_2CH_2OCH_3$ [$(C_3O)_4$dmg] | R=n-$C_4H_9$ [(di-b)tmg]<br>R=n-$C_7H_{15}$ [(di-hept)tmg]<br>R=n-$C_{12}H_{21}$[(di-dodec)tmg] |
|  |  |  | |
| $R^1$=n-$C_6H_{13}$, $R^2$=n-$C_{14}H_{29}$ [$P_{6,6,6,14}$] | R = n-$C_4H_9$ [$C_4$MPyr] | [2-MEPy] | |
| Anions | | | |
| [Cl] |  | [$FeCl_4$] |  |
| [I] | | | |
| [$BF_4$] | | | |
| [SAC] |  | [TfO] |  |
| [DCA] |  | [ACS] |  |

| | | | |
|---|---|---|---|
| [$NTf_2$] |  | | |
| [TFA] |  | | |
| [$EtOSO_3$] |  | | |

In this study, the existence of ether functionality of the dimethyl-guanidinium was also assessed and interestingly, toxicity was reduced when the ether was present. In this situation, [$(C_3O)_4$dmg] [Cl] is not toxic for CaCo-2 monolayer up to a concentration of 15000 µM (Fig. 1).

Functionalized chains had already been seen to contribute positively for non-toxic ionic liquids: the presence of hydroxyl groups in [MIM] alkyl chains was published to decrease toxicity[18,20,32] and, the addition of a COOH group was also shown by our group to dramatically reduce the toxicity of [$C_{10}$MIM].[29]

Data shows that [BzMIM] [Cl] and [BzIMBz] [Cl] are both not toxic. But, while [BzMIM] [DCA] is still not harmful, [BzIMBz] [DCA] decreased cellular viability in more than 50% at 15000 µM, which suggests the presence of benzyl group increases toxicity (Fig. 2, Table 3, and ESI†). Tetra-ethyl-ammonium seems a more toxic anion than methyl-imidazolium when they

**Table 2** Toxicity data of dialkyl-dimethyl-guanidinium and tetramethyl-guanidinium cations. CaCo-2 monolayer cells were exposed to the ionic liquids for a period of 4 hours. They were considered toxic when a 50% decrease of cellular viability was reached within the studied concentration range. $EC_{50}$ represents the concentration necessary to reduce 50% cell viability

| Cation | Anion | Toxicity[c] ($EC_{50}$ μM) |
|---|---|---|
| [(di-b)$_2$dmg] | [$BF_4$] | T (9200) |
| [(eb)$_2$dmg] | [$BF_4$] | NT |
| [(mb)$_2$dmg] | [$BF_4$] | T[b] |
| [(mh)$_2$dmg] | [$BF_4$] | T (404) |
| [(di-o)$_2$dmg] | [$BF_4$] | T[a] (5635) |
| [(eb)(mb)dmg] | [$BF_4$] | NT |
| | [Cl] | NT |
| [(di-b)(eb)dmg] | [Cl] | T (11350) |
| ($C_3O$)$_4$ [(di-h)$_2$dmg] | [Cl] | NT |
| [(di-h)$_2$dmg] | [Cl] | T[a] (< 750) |
| | [SAC] | T[a,b] |
| | [$FeCl_4$] | T[a] (< 750) |
| [(di-b) tmg] | [I] | NT |
| [(di-deca)tmg] | [I] | T[a] (955) |
| [(di-hept)tmg] | [I] | T (< 750) |

[a] Ionic liquid with lower solubilities in the aqueous cell culture medium.
[b] Cell viability reached values higher than the control cells (> 40%).
[c] Each experimental point represents the average of 3 repliques.

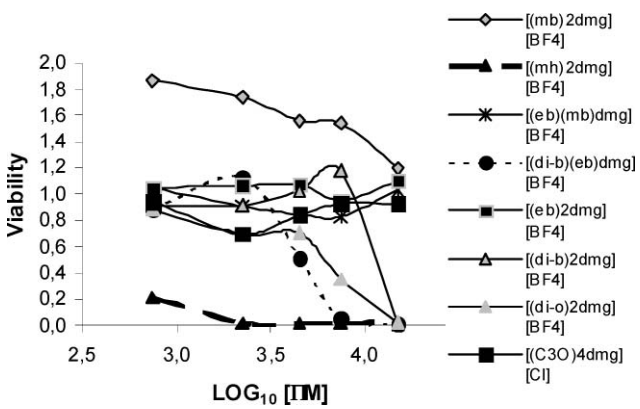



**Fig. 1** Effect of the alkyl chain length of the dimethyl-guanidinium cation for the same anion–[$BF_4$] and effect of the ether functionality in the toxicity of the same cation but linked to chloride. CaCo-2 cell monolayer was exposed to the ionic liquids for 4 hours and viability was assessed by the MTT colorimetric reagent.

are combined with chloride, because [BzTEA] [Cl] induced an abnormal increase of cellular metabolism, in about 50%. However, substitution of [Cl] with [DCA] cancelled this effect (Fig. 2, Table 3, and ESI†), showing that [DCA] can contribute to a lower toxicity. In addition, both [PhTMA] [Cl] and [PhTMA] [DCA] induced no harm on the CaCo-2 monolayer (Fig. 2, Table 3, ESI†). As shown above, combination of the previous cations with [DCA] obeys to the following trend of increasing toxicity: [BzTEA] ≈ [PhTMA] < [BzMIM] < [BzIMBz] (Fig. 2).

For some cations, the presence of [$NTf_2$] lead to an abnormal augment in cell viability compared to control cells (the same behaviour was obtained for $LiNTf_2$ as well—data not shown), and consequently, these were considered unsafe (Table 3 and ESI†). This effect was clearly demonstrated for the cations mentioned above: [BzIMBz], [BzMIM], [BzTEA] and [PhTMA] (ESI†). [$NTf_2$] was also reported to be toxic for some aquatic

**Table 3** Toxicity data of pyrrolidinium, pyridinium, imidazolium and tetra-alkyl-ammonium cations. CaCo-2 monolayer cells were exposed to the ionic liquids for a period of 4 hours. They were considered toxic when a 50% decrease of cellular viability was reached within the studied concentration range. $EC_{50}$ represents the concentration necessary to reduce 50% cell viability

| Cation | Anion | Toxicity[b] ($EC_{50}$ μM) | Cation | Anion | Toxicity[b] ($EC_{50}$ μM) |
|---|---|---|---|---|---|
| [$C_4$MPyr] | [DCA] | NT | [BzIMBz] | [Cl] | NT |
| | [ACS] | NT | | [DCA] | T (10823) |
| | [SAC] | NT | | [$NTf_2$] | T[a] |
| | [I] | NT | | [SAC] | T (14013) |
| [$C_8$MIM] | [DCA] | T (6076) | [PhTMA] | [Cl] | NT |
| | [SAC] | T (6076) | | [DCA] | NT |
| | [$NTf_2$] | NT | | [$NTf_2$] | T[a] |
| [$C_{10}$MIM] | [Cl] | T (1910) | [BzTEA] | [Cl] | T[a] |
| [BzMIM] | [Cl] | NT | | [DCA] | NT |
| | [DCA] | NT | | [$NTf_2$] | T[a] |
| | [$NTf_2$] | T[a] | | | |
| | [SAC] | NT | | | |

[a] Cell viability reached values higher than the control cells (> 40%).
[b] Each experimental point represents the average of 3 repliques.

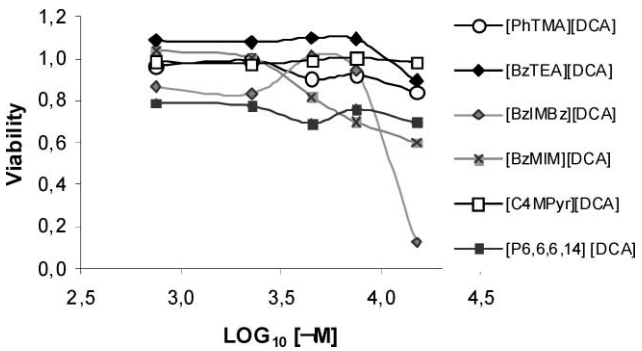



**Fig. 2** Effect of [PhTMA], [BzTEA], [BzMIM], [BzIMBz], [$C_4$MPyr] and [P6,6,6,14] on CaCo-2 monolayer, linked to the same anion- [DCA]. CaCo-2 cells monolayer was exposed to the ionic liquids for 4 hours and viability was assessed by the MTT colorimetric reagent.

microorganisms.[17] However, other results obtained in this work suggested that the type of cation can circumvent this effect and ionic liquid toxicity can be reduced. Cells treated with [$C_8$MIM] [$NTf_2$] had their metabolism increased in only about 20%, while [$C_4$MIM] [$NTf_2$] and [(di-h)$_2$dmg] [$NTf_2$] were seen in previous studies not to induce such effect (ESI†).[32] In a similar way, [Aliquat] [$NTf_2$] and [P6,6,6,14] [$NTf_2$] were also considered non-toxic (ESI†).[32] These are examples demonstrating that both ions can influence considerably toxicity.

Independently on the tested anion ([SAC], [ACS], [I] and [DCA]), impact of [$C_4$MPyr] on CaCo-2 cells monolayer was insignificant (Fig. 3). This agrees with published reports that demonstrated that [Pyr] can be less toxic than [MIM].[18–20] In this study, data also suggests that [Pyr] is possibly toxicologically more favourable than ammonium-based ionic liquids since their toxicity curves show a slight decay of viability at the highest applied doses, which did not occur for the pyrrolidinium ionic liquid (Fig. 2). Consequently, [$C_4$MPyr] shows potential for designing of human friendlier ionic liquids. In addition, [2-MEPy] [$EtSO_4$] also gave a very interesting result and might

**Table 4** Toxicity of aliquat and phosphonium cations on CaCo-2 cells monolayer. Cells were exposed to the ionic liquids for a period of 4 hours. They were considered toxic when a 50% decrease of cellular viability was reached within the studied concentration range. $EC_{50}$ represents the concentration necessary to reduce 50% cell viability

| Cation | Anion | Toxicity [a] ($EC_{50}$ μM) |
|---|---|---|
| [Aliquat] | [Cl] | T[a](<750) |
| | [$FeCl_4$] | T[a](<750) |
| | [TFA] | T[a](<750) |
| | [TfO] | T[a](<750) |
| [P6,6,6,14] | [$FeCl_4$] | T[a](1069) |
| | [DCA] | NT |
| | [Cl] | T (<750) |

[a] Ionic liquid showed a lower solubility in the aqueous cell culture medium.
[b] Each experimental point represents the average of 3 repliques.

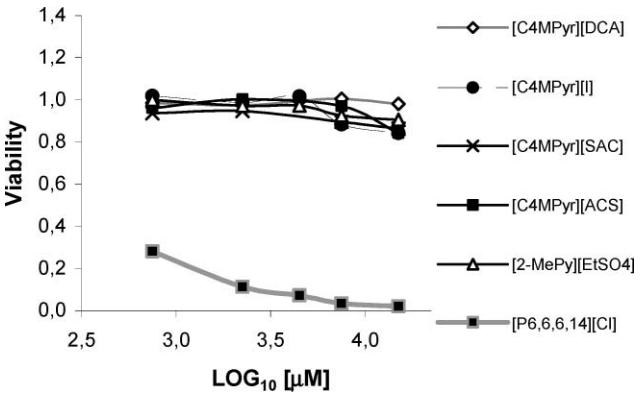



**Fig. 3** Effect of [$C_4$MPyr] [DCA]/[I]/[SAC]/[ACS], [2-MEPy] [EtSO4] and [P6,6,6,14] [Cl] ionic liquids on the CaCo-2 cells viability. Cells were exposed to ionic liquids for 4 hours and viability was assessed by the MTT assay.

present another good candidate, however studies with different anions need to be conducted (Fig. 3).

[P6,6,6,14] [Cl]/[$FeCl_4$] were very toxic, but combination with [DCA] reduced considerably toxicity (Table 4, Fig. 2, 3, ESI†). This contributes to strengthen the idea that this anion can lower toxicity. [Aliquat] [Cl]/[$FeCl_4$]/[TFA]/[TfO] had a very negative impact on this model and it is not a good candidate for building greener solvents (Table 4, ESI†). These results might be generated by the very long alkyl chains present in both [P6,6,6,14] and [Aliquat] cations.

## Conclusions

Our study focuses on the toxicological evaluation of over 80 ionic liquids from different classes of cations and anions. The work was performed on confluent human colon cancerous cells (CaCo-2), a suitable model for cytotoxicity studies.[31]

In general, the toxicity of the cation increases when longer alkyl chains are present. The presence of a benzyl group does not seem to contribute to non-toxic [MIM] based ionic liquids (Fig. 2), however, introduction of a COOH group was already seen previously by our group to lead to a great reduction of [$C_{10}$MIM] toxicity (ESI†).[29] In addition, introduction of ether functionality in the dimethyl-guanidinium lowered toxicity of this cation. (Fig. 1)

The type of anion can affect strongly toxicity of the ionic liquid, and some seem to have a bigger impact in the overall toxicity than others, as [$NTf_2$] and [DCA]. Comparison of all toxicological data produced in our lab allowed us to conclude that [$C_4$MIM], [$C_2$OHMIM] and [$C_4$MPyr] are potential good candidates for building human friendly ionic liquids, but some combinations with different cations exist that were very promising, as well: [$C_5O_2$MIM] [$PF_6$], [$C_{10}O_2$HMIM] [$PF_6$/DCA], [2-MEPy] [$EtSO_4$], [BzTEA] [DCA], [BzIMBz] [Cl], [BzMIM] [Cl], [PhTMA] [Cl/DCA], [(di-h)$_2$dmg] [DCA/$NTf_2$], [(eb)(mb)dmg] [Cl/$BF_4$], [(eb)$_2$dmg] [$BF_4$] and [$(C_3O)_4$dmg] [Cl] (ESI†).

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