RSC Advances

This article can be cited before page numbers have been issued, to do this please use: M. Vraneš, A. Tot, S. Jovanovic-Santa, M. Karaman, S. Dozic, K. Tesanovic, V. Koji and S. Gadzuric, *RSC Adv.*, 2016, DOI: 10.1039/C6RA16182K.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

View Article Online
DOI: 10.1039/C6RA16182K

Journal Name

ARTICLE

Toxicity reduction of imidazolium-based ionic liquids by the oxygenation of the alkyl substituent

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

M. Vraneš,[a] A. Tot,[a] S. Jovanović-Šanta,[a] M. Karaman,[b] S. Dožić,[a] K. Tešanović,[b] V. Kojić [c] and S. Gadžurić[a]

In this work five different salicylate based ionic liquids are prepared in order to study their toxicity, namely: 1-butyl-3-methylimidazolium salicylate, [bmim][Sal], 1-(4-hydroxy-2-oxybutyl)-3-methylimidazolium salicylate, [OHC_2OC_2mim][Sal], 1-(3-hydroxypropyl)-3-methylimidazolium salicylate, [OHC_3mim][Sal], 1-etoxyethyl-3-methylimidazolium salicylate, [C_2OC_2mim][Sal] and imidazolium salicylate [Im][Sal]. For that purpose aquatic organisms (*Artemia salina*) and human non-tumor cell line (normal fetal lung fibroblasts, MRC-5) are used. Introduction of the polar groups (in the form of hydroxyde and/or ether group) in the alkyl side chain of the imidazolium cation and their influence on the reduction of the ionic liquid's toxicity is also demonstrated. The results indicate that both, toxicity against *A. salina* and cytotoxicity against healthy cell line of lipophobic ionic liquids are significantly lower comparing with non-functionalized analogues and the same order of magnitude as the reference standard sodium salicylate. These facts open the possibility of designing new non-toxic ionic liquids that can be used as active pharmaceutical ingredients in the liquid form, adjusting only lipophilicity of the cations introducing polar oxygen groups in the side alkyl chain of the cation.

1. Introduction

Pharmaceutical companies currently rely on crystalline solid forms for the delivery of active pharmaceutical ingredients (APIs) for the reasons of their high purity, thermal stability, synthetic methods and easy handling.[1-3] Nowadays, many therapeutics are used as the adequate salts, as well as pharmaceutical formulations in the preclinical phase of development, due to their better physical properties, better solubility, bioavailability, permeability and drug delivery potential.[2-6] Pharmacokinetic features of the APIs in the form of the salts directly depend on their absorption mechanism and lipophilicity. The main problem when salts are applied as APIs is the polimorphism or pseudopolimorphism, since the each form shows different solubility and bioavailability.[1,2,5,6] To exceed this problems, the liquid forms of the drugs are proposed to be used. Thus, ionic liquids (ILs) as the salts with the melting temperatures below 100 ^{o}C with the biologicaly active components (cation or/and anion) were recently studied as the potential APIs.[6-12,13]

The ionic liquids are known as the salts with the tunable properties and the high thermal stability, which gives the new perspective in the pharmaceutical industry. There are several strategies how the ionic liquids can be used as the APIs:[8, 13-18]

1. API-ILs as pharmaceuticals prepared as single or dual-active liquid salts of APIs by liquefaction using the oligomeric ions or by formation of liquid co-crystals;
2. Synthesis of new API-ILs starting from existing API-IL
3. ILs for solubilization of poorly soluble drugs including: i) APIs solubilized within micelles acting as reservoirs for controlled release, ii) IL-assisted non-aqueous microemulsions stabilized by an addition of external surfactant or iii) ILs designed as hydrophilic-lipophilic balanced (HLB) hydrotropes that keep the APIs dissolved once added to water.

Application of the ionic liquids as potential drugs is still restricted due to the lack of the data concerning their toxicity and biodegradability.[8,19,20] It was shown that many commercial ILs are toxic. Thus, synthesis of the new ILs with environmentally friendly cations and biologically active anions such as aminoacids is proposed. For that purpose, many choline-based ILs were synthesized, but most of them show thermal, hydrolytical and electrochemical unstability.[21-24] At the other hand, commercially available imidazolium ILs are more stable, but more toxic, which greatly reduce their applications. One of the strategies in preparing low-toxic ILs is reducing toxicity of the imidazolium ion applying fine tuning of the essential properties by the variation of the alkyl substituents in the position N1 or N3 of the imidazolium ring.[25] Introducing the oxygen atom in the side alkyl chain decreases

[a] University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Trg D. Obradovića 3, 21000 Novi Sad (Serbia)
[b] University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Trg Dositeja Obradovića 3, 21000 Novi Sad (Novi Sad)
[c] Institute of Oncology Sremska Kamenica, Institutski Put 4, 21204 Sremska Kamenica (Serbia).
tel:(+381) 21 4852744
fax:(+381) 21 454 065
email: milan.vranes@dh.uns.ac.rs

†Electronic Supplementary Information (ESI) available: NMR and IR spectra of newly synthesized ionic liquids. See DOI: 10.1039/x0xx00000x

View Article Online
DOI: 10.1039/C6RA16182K

lipophilicity due to a presence of the polar groups, decreasing in the same time toxicity of the ILs.[25-27] Also, the presence of the ether and/or hydroxide functions increases solubility of the ILs in water and the solubility of the salts in ILs, promoting them to the excellent candidates for API-ILs synthesis.[26,28]

Second strategy is to obtain biologically active ILs by selection of the adequate anion. One of the best studied anions is the salicylate which in the form of acetyl-salicylic acid (Aspirin) is one of the most commonly used medicaments in the world.[5,7,29,30] It is well known that the aspirin is only partialy soluble in water (0.33 g in 100 ml of water) and in the acidic stomach enviroment, which results in undissolved particles adhering to the gastrointestinal mucosea causing irritation and gastric distress.[29-31] It is obvious that better drug delivery can be achieved applying the salicylate in the liquid form. Salicylic acid is also known as a cytotoxin with a prominent anticancer effect. Also, some salicylate based ionic liquids show a luminescence properties,[32] which facilitates its tracking and marking in the body.

Taking into account all above mentioned facts, in this paper we synthesized a series of ionic liquids with imidazolium cations functionalized with ether and/or hydroxide groups and salicylate anion, in order to study their toxicity and compare obtained results with the unsubstituted analogues of 1-butyl-3-methylimidazolium salicylate. In such way, the influence of the oxygen functions in the substituent on the imidazole ring on the ILs toxicity will be discussed.

2. Experimental section

All chemicals for ILs synthesis were used without purification as purchased from the manufacturer: 1-methylimidazole (Sigma Aldrich, CAS number: 616-47-7, ω ≥ 0.99), 2-chloroethyl ether (Sigma Aldrich, CAS number: 628-34-2, ω ≥ 0.99), 3-chloro-1-propanol (Sigma Aldrich, CAS number: 627-30-5, ω ≥ 0.98), 2-(2-chloroethoxy)ethanol (Sigma Aldrich, CAS number: 628-89-7, ω ≥ 0.99), ethyl acetate (Sigma Aldrich, CAS number: 141-78-6, ω ≥ 0.998), sodium salicylate (Reanal, CAS number: 54-21-7; ω ≥ 0.995), imidazole (Sigma Aldrich, CAS number: 288-32-4, ω ≥ 0.99), N-methylimidazole (Sigma Aldrich, CAS number: 616-47-7, ω ≥ 0.99), hydrochloric acid (Sigma Aldrich, CAS number:7647-01-0), acetone (Lachner, 67-64-1)

Five different salicylate based ionic liquids are synthesized. Synthesis of 1-butyl-3-methylimidazolium salicyalte ionic liquid, [bmim][Sal], is described in our previous paper.[33] Other ILs, namely: $[OHC_2OC_2mim][Sal]$, $[OHC_3mim][Sal]$, $[C_2OC_2mim][Sal]$ and [Im][Sal] were prepared starting from the corresponding chloride salts: $[OHC_2OC_2mim][Cl]$, $[C_3OHmim][Cl]$, $[C_2OC_2mim][Cl]$ and [Im][Cl] according to the synthetic path presented in Figure 1.

3-chloro-1-propanol (or 2-(2-chloroetoxy)ethanol or 2-chloroethyl ether) and 1-methylimidazole were added to a round-bottom flask. Ethyl acetate was used as a solvent, and 3-chloro-1-propanol (or 2-(2-chloroetoxy)ethanol or 2-chloroethyl ether) was added in 10% excess. The mixture was kept under the reflux for 48 h at 70 °C with stirring, until two phases were formed. The top phase, containing unreacted starting material was removed. The bottom phase was washed four times with new portion of ethyl acetate. The products were obtained in the liquid state, and additionally stored under vacuum with P_2O_5 for the next 72 h.

Non-substitued imidazolium chloride, [Im][Cl], was synthesized by the potentiometric acid-base titration. The reaction was conducted by slow addition of HCl (0.1053 $mol \cdot dm^{-3}$) to a water solution of imidazole with constant stirring, until achieving adequate pH. Additionaly, IL was dried under vacuum in order to remove water for the next 24 h and obtained solid [Im][Cl] was stored under P_2O_5.

Obtained chloride ionic liquids were transfered into salicylates by the addition of the equimolar amount of sodium salicylate using acetone as a solvent. Resulting solution was stirred and refluxed for 12 h. After that, the white precipitate (NaCl) was removed and the acetone solution of the ionic liquid was obtained. Acetone is removed by the evaporation under the vacuum at 70 °C for 1 h achieving the constant mass. Water content was determined by the Karl-Fischer titration and chloride content by the ion chromatography. It was found that water content was less than 200 ppm and chloride content less than 8.3 ppm in all synthesized ionic liquids.

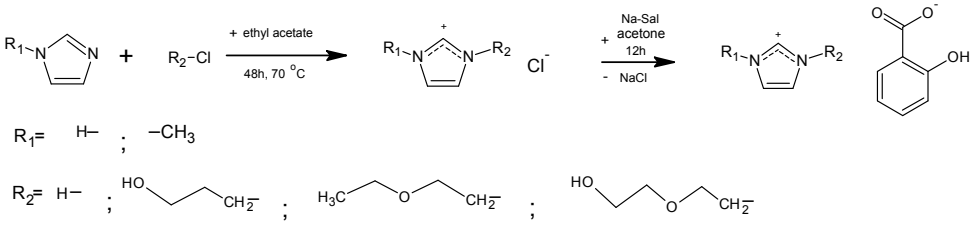


Figure 1. Synthetic paths for: $[OHC_2OC_2mim][Sal]$, $[OHC_3mim][Sal]$; $[C_2OC_2mim][Sal]$ and [Im][Sal]

For additional characterization the IR and NMR spectra (Figures S1 and S2 in Supporting information) of the newly synthesized ionic liquids were recorded. NMR spectra were recorded in D_2O at 25 °C on a Bruker Advance III 400 MHz spectrometer. Tetramethylsilane was used as accepted internal standard for calibrating chemical shift for ^1H and ^{13}C. ^1H homodecoupling and 2D COSY method were used routinely for the assignation of the obtained NMR spectra. ^{13}C spectra were assigned by selective decoupling technique.

View Article Online
DOI: 10.1039/C6RA16182K

[OHC$_2$OC$_2$mim][Sal]

^{1}H NMR (D_2O): 3.45 (*m*, 2H, $NCH_2CH_2OC\boldsymbol{H}_2CH_2OH$); 3.58 (*m*, 2H, $NCH_2CH_2OCH_2C\boldsymbol{H}_2OH$); 3.67 (*s*, 3H, $C\boldsymbol{H}_3$); 3.67 (*t*, 2H, $NCH_2C\boldsymbol{H}_2OCH_2CH_2OH$); 4.13 (*t*, 2H, *J* = 4.7, $NC\boldsymbol{H}_2CH_2OCH_2CH_2OH$,); 6.77 (*d*, 1H, $J_{3',4'}$ = 8.2 Hz, H-3'); 6.10 (*t*, 1H, $J_{3',4'}$ = 7.5 Hz, H-5'); 7.17 and 7.25 (2*xs*, 2H, H-4 and H-5); 7.29 (*m*, 1H, H-4'); 7.67 (*dd*, 1H, $J_{4',6'}$ = 1.3 Hz, $J_{5',6'}$ = 7.8 Hz, H-6'); 8.44 (*s*, 1H, H-2)

^{13}C NMR (D_2O): 38,63 (NCH_3); 51.77 ($N\boldsymbol{C}H_2CH_2OCH_2CH_2OH$); 63.08 ($NCH_2CH_2OCH_2\boldsymbol{C}H_2OH$); 70.99 ($NCH_2\boldsymbol{C}H_2OCH_2CH_2OH$); 74.51 ($NCH_2CH_2O\boldsymbol{C}H_2CH_2OH$); 118.97 (C-3'); 120.75 (C-1'); 122.03 (C-5'); 125.17 and 126.07 (C-5 and C-4); 133.18 (C-6'); 136.66 (C-4'); 138.83 (C-2); 162.47 (C-2'); 177.80 (C=O).

[C$_2$OC$_2$mim][Sal]

^{1}H NMR (D_2O): 1.05 (*t*, 3H, J = 7.0 $NCH_2CH_2OCH_2C\boldsymbol{H}_3$); 3.43 (*q*, 2H, *J* = 7.1, $NCH_2CH_2OC\boldsymbol{H}_2CH_3$); 3,66 (*t*, 2H, $NCH_2C\boldsymbol{H}_2OCH_2CH_3$); 3.73 (*s*, 3H, $NC\boldsymbol{H}_3$); 4.14 (*t*, 2H, $NC\boldsymbol{H}_2CH_2OCH_2CH_3$); 6.80 (*d*, 1H, $J_{3',4'}$ = 8.3 Hz, H-3'); 6.84 (*t*, 1H, $J_{3',4'}$ = 7.6 Hz, H-5'); 7.23 and 7.27 (2*xs*, 2H, H-4 and H-5); 7.31 (*m*, 1H, H-4'); 7.72 (*bd*, 1H, $J_{5',6'}$ = 7.8 Hz, H-6'); 8.47 (*s*, 1H, H-2)

^{13}C NMR (D_2O): 16.83 ($NCH_2CH_2OCH_2CH_3$); 38,39 (NCH_3); 51.76 ($N\boldsymbol{C}H_2CH_2OCH_2CH_3$); 69.39 ($NCH_2CH_2O\boldsymbol{C}H_2CH_3$); 70.38 ($NCH_2\boldsymbol{C}H_2OCH_2CH_3$); 74.51 ($NCH_2CH_2O\boldsymbol{C}H_2CH_3$); 118.94 (C-3'); 120.88 (C-1'); 121.99 (C-5'); 125.12 and 126.19 (C-5 and C-4); 133.19 (C-6'); 136.59 (C-4'); 138.70 (C-2); 162.54 (C-2'); 177.71 (C=O).

[OHC$_3$mim][Sal]

^{1}H NMR (D_2O): 2.00 (*m*, 2H, $NCH_2C\boldsymbol{H}_2CH_2OH$,); 3.55 (*t*, 2H, *J* = 6.1, $NCH_2CH_2C\boldsymbol{H}_2OH$); 3.78 (*s*, 3H, $NC\boldsymbol{H}_3$); 4.15 (*t*, 2H, *J* = 7.2, $NC\boldsymbol{H}_2CH_2CH_2OH$); 6.84-6.94 (*m*, 2H, H-3' and H-5'); 7.29 and 7.34 (2*xs*, 2H, H-4 and H-5); 7.40 (*m*, 1H, H-4'); 7.75 (*d*, 1H, $J_{5',6'}$ = 8.1 Hz, H-6'); 8.54 (*s*, 1H, H-2)

^{13}C NMR (D_2O): 34.35 ($NCH_2\boldsymbol{C}H_2CH_2OH$); 38,39 ($NCH_3$); 49.19 ($N\boldsymbol{C}H_2CH_2CH_2OH$); 60.64 ($NCH_2CH_2\boldsymbol{C}H_2OH$); 119.04 (C-3'); 120.74 (C-1'); 122.14 (C-5'); 124.99 and 126.33 (C-5 and C-4); 133.24 (C-6'); 136.77 (C-4'); 138.63 (C-2); 162.44 (C-2'); 178.08 (C=O).

[Im][Sal]

^{1}H NMR (D_2O): 6.35-6.45 (*m*, 2H, H-3' and H-5'); 6.83-6.93 (*m*, 3H, H-4, H-5 and H-4'); 7.34 (*dd*, 1H, $J_{5',6'}$ = 7.8 Hz, $J_{4',6'}$ = 0.9 Hz, H-6'); 8.10 (*s*, 1H, H-2)

^{13}C NMR (D_2O): 118.62 (C-3'); 120.34 (C-1'); 121.21 (C-5'); 121.62 (C-5 and C-4); 132.84 (C-6'); 135.52 (C-2); 136.26 (C-4'); 162.14 (C-2'); 177.81 (C=O).

Infrared spectra were recorded as neat samples from (4000-650) cm^{-1} on a Thermo-Nicolet Nexus 670 spectrometer fitted with a Universal ATR Sampling Accessory.

[OHC$_2$OC$_2$mim][Sal]

3500-3300 (H-bond OH); 3145 (sym. stretching *v* HC((4)C(5)H)); 3094 (sym. stretching *v* HC(2)); 2867 (sym. stretching νCH_3); 1584 (in-plane vibrations of imidazolium ring); 1484 (stretching ν CC); 1380 (stretching ν C-O); 1323 (in-plane bending mode δ O-H); 1125 and 1067 (stretching ν C-O from ether group); 807 (in-plane bending mode δ CC)

[C$_2$OC$_2$mim][Sal]

3142 (sym. stretching *v* HC((4)C(5)H)); 3077 (sym. stretching *v* HC(2)); 2974 (asym. stretching *v* CH_3); 2871 (sym. stretching *v* CH_3) 1585 (in-plane vibrations of imidazolium ring); 1483 and 1284 (stretching ν CC); 1376 (stretching ν C-O); 1166 (stretching ν C-O); 1144 and 1026 (stretching ν C-O from ether group); 806 (in-plane bending mode δ CC);

[OHC$_3$mim][Sal]

3260 (stretching OH); 2954 (asym. stretching *v* CH_3); 2880 (sym. stretching *v* CH_3); 3081 (sym. stretching *v* HC(2); 3144 (sym. stretching *v* HC((4)C(5)H); 1585 (in-plane vibrations of imidazolium ring); 1483 and 1284(stretching ν CC); 1376 (stretching ν C-O); 1166 (stretching ν C-O); 806 (in-plane bending mode δ CC); 702 (out-of-plane deformation γ CC)

[Im][Sal]

3159 (sym. stretching *v* HC((4)C(5)H)); 3047 (sym. stretching *v* HC(2); 2978 (asym. stretching *v* HC(2)); 1602 (in-plane vibrations of imidazolium ring); 1488 (stretching ν CC) 1383 (stretching ν C-O); 1463 and 1346 (stretching ν C-N); 809 (in-plane bending mode δ CC); 667 (out-of-plane deformation γ CC)

2.1 Toxicity assays

2.1.1 Toxicity on aquatic microcrustaocean *A. salina*

Mean lethal concentration (LC_{50}) was determined by a modification of the in vitro test described by Parra et al.[34] Culture of *Artemia salina* (Great Salt Lake Artemia Cysts, Ogden UT, USA) was incubated in the artificial sea water (ASW) at temperature of 30 °C under the constant illumination and aeration for 30 h. After incubation, 10-20 larvae of *A. salina* were transferred to each test tube and another 200 µL of ASW and 20 µL of the proper ionic liquids. Test was carried out in five replicates for each concentration of each IL, with the negative control of 220 µL of ASW without the addition of ionic liquids. The control solvent was distilled water (20 µL). Then, microplates were incubated at 30 °C under the constant illumination and the mortality of the larvae was monitored after 24 and 48 h.

2.1.1 In vitro antiproliferative assay

Cell lines and cell culture. Human non-tumor cell line (normal fetal lung fibroblasts MRC-5, ATCC CCL 171) was used in this study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5% of glucose. Media was supplemented with 10% of fetal calf serum (FCS, Sigma) and antibiotics: 105 IU mL^{-1} of penicillin and 100 $\mu g \cdot mL^{-1}$ of streptomycin (ICN Galenika). Cell line was cultured in flask (Costar, 25 cm^2) at 37 °C in a 100% humidity atmosphere containing 5% of CO_2. Only viable cells were used in the assays. Cell viability was determined by the trypan blue dye exclusion assay.

Antiproliferative activity. Antiproliferative activity of the tested salicylate derivatives was evaluated by tetrazolium colorimetric MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay.[35] Cells were exposed to the test compounds during 72 h in concentrations ranged from 10^{-8} to 10^{-4} $mol \cdot L^{-1}$. Reference compound used in the MTT assay was sodium salicylate. Exponentially growing cells were

View Article Online
DOI: 10.1039/C6RA16182K

harvested, counted by trypan blue exclusion test, seeded onto 96-well plates at a density of 5000 cells/well and allowed to stand overnight. Solutions of the tested compounds in medium (10 μmol·L^{-1}/well) were added so final concentrations ranging from 10^{-8} to 10^{-4} mol·L^{-1}. After 72 h tretament period, cells viability was determined by the addition of 10 μL of sterile MTT solution (5 mg·mL^{-1}). The precipitated formazan crystals were solubilized with acidifed 2-propanol (100 μL of 0.04 mol·L^{-1} HCl in 2-propanol) and the absorbance was recorded (Multiscan MCC340, Labsystems) at 540 and 690 nm after a few minutes incubation at room temperature. Wells containing cells without tested compounds were used as control. Wells without cells, containing only complete medium and MTT were used as blank. Cytotoxicity (CI) was calculated according to the formula:

$$CI(\%) = 1 - \frac{A_{sample}}{A_{control}} \cdot 100 \quad (1)$$

Data analysis. Two independent experiments were conducted in quadruplicate for each concentration of tested compound. Mean values and standard deviations (σ) were calculated for each concentration. Antiproliferative activity was expressed as IC_{50} value, defined as the dose of compound that inhibits cell growth by 50%. The IC_{50} of each tested compound was determined by median effect analysis.[36]

3. Results and discussion

Described tests on aquatic microorganisms (Artemia salina) and on human non-tumor cell line (normal fetal lung fibroblasts, MRC-5) were performed in order to investigate influence of the alkyl chain substituent on the ILs toxicity. Assay based on toxicity against *Artemia salina* is considered as rapid, convenient, low cost and one of the most reliable methods for the preliminary detection of mycotoxines, heavy metals and pesticides toxicity.[34,37,38] Also, this test is often used for testing of general toxicity of compounds before their commercial use .[37,39] In this work, it was used for the lethal concentration (LC_{50}) determination.

In order to investigate toxicity against human healthy cells, MRC-5 cell line was used, which is commonly applied in vaccine development,[40] as a transfection host in virology research,[41] and for *in vitro* cytotoxicity testing.[42] In order to evaluate the cytotoxicity of the synthesized salicylate ionic liquids, obtained IC_{50} values were compared with the results obtained for non-funcionalized ionic liquid [bmim][Sal] and for the sodium salicylate. First reference compound is highly toxic, while second one is commonly used in medicine as an analgetic, antipyretic, and non-steroidal anti-inflammatory drug which antitumor potential due to inducing necrosis and/or apoptosis in cancer cell.[43-46]

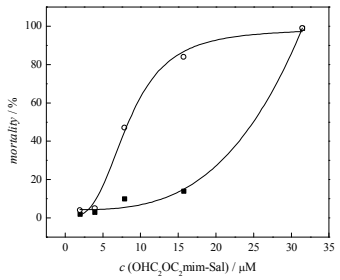
Obtained toxicity results are presented in Table 1 and in the Figures 2 and 3.

Table 1. Toxicity of the studied salicylate salts

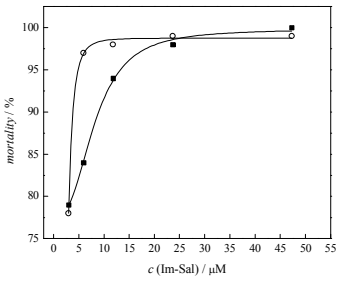
	Salt	LC_{50} (*A. salina*)/μM	IC_{50} (MRC-5)/μM
1	Sodium salicylate	8.87	**
2	[bmim][Sal]	*	27.54
3	[OHC_2OC_2mim][Sal]	8.41	**
4	[C_2OC_2mim][Sal]	8.18	**
5	[OHC_3mim][Sal]	10.18	**
6	[Im][Sal]	*	**

** Very high toxicity at low concentrations, LC_{50} could not be determined.*
*** IC_{50} is not detected in the investigated concentration range meaning that these compounds can be consider as non-toxic.*

Ionic liquids [bmim][Sal] and [Im][Sal] showed high toxicity even at the lowest ILs concentrations (3 μM) on *A. salina* larvae within the first 24 h, thus the corresponding values of LC_{50} were not detected.



a)



b)

View Article Online
DOI: 10.1039/C6RA16182K

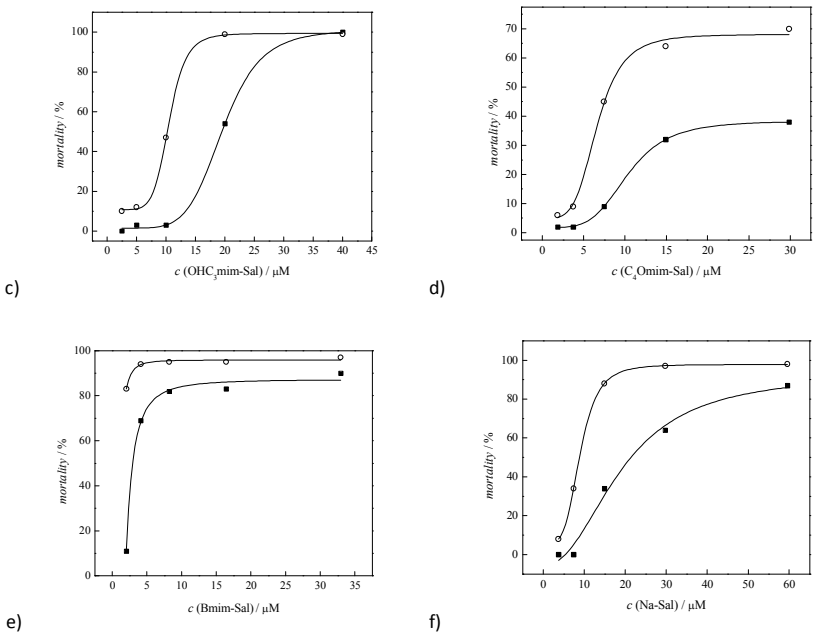
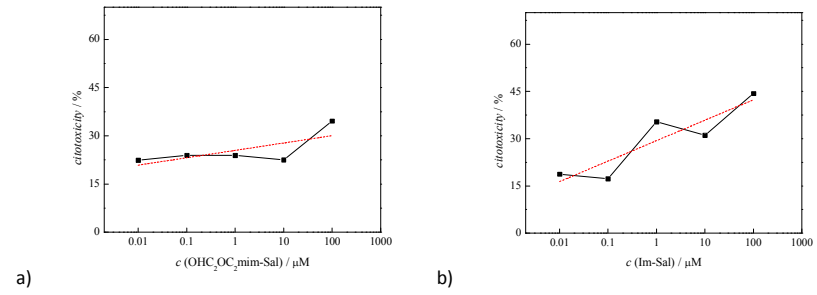


Figure 2. *A. salina mortality in function of concentration of: a) [OHC$_2$OC$_2$mim][Sal], b) [Im][Sal], c) [OHC$_3$mim][Sal], d) [C$_4$Omim][Sal],e) [bmim][Sal], f) Na-Sal (■, after 24 hours; ○, after 48 hours)*

The introduction of the oxygen in the form of a hydroxy and/or ether group in the side alkyl chain of the imidazolium ion leads to a significant reduction of ILs toxicity on *A. salina*, wherein the LC_{50} values of these newly synthesized ILs after 48 h were found to be similar as the LC_{50} obtained for the reference sodium salicylate. A significant reduction of toxicity can be observed comparing the results obtained for [bmim][Sal] and ILs with the oxygen in the alkyl substituent (Table 1, Figure 2). It can be seen that introduction of the oxygen in the form of the hydroxide group in [OHC$_3$mim][Sal], had a greater impact on reducing toxicity then the presence of oxygen in the form of ether group in the side alkyl chain of [C$_2$OC$_2$mim][Sal]. This observation is in the agreement with those reported by Stolte et al.[27,47] In the case of [OHC$_2$OC$_2$mim][Sal] the introduction of OH group in the alkyl chain with the existing ether oxygen had a lower impact on reducing toxicity, comparing introduction of OH group in the non-functionalized alkyl chain.

In the case of healthy lung cells, MRC-5, it was observed that only [bmim][Sal] expressed significant toxicity, its IC_{50} was 27.54 μM (Table 1). Otherwise, oxygen-modified ILs were not toxic against healthy cell line MRC-5. The citotoxicity tests were performed in the ionic liquid concentration range from (0.01 to 100) μmol·mL^{-1} and the results are presented graphically in Figure 3.



View Article Online
DOI: 10.1039/C6RA16182K

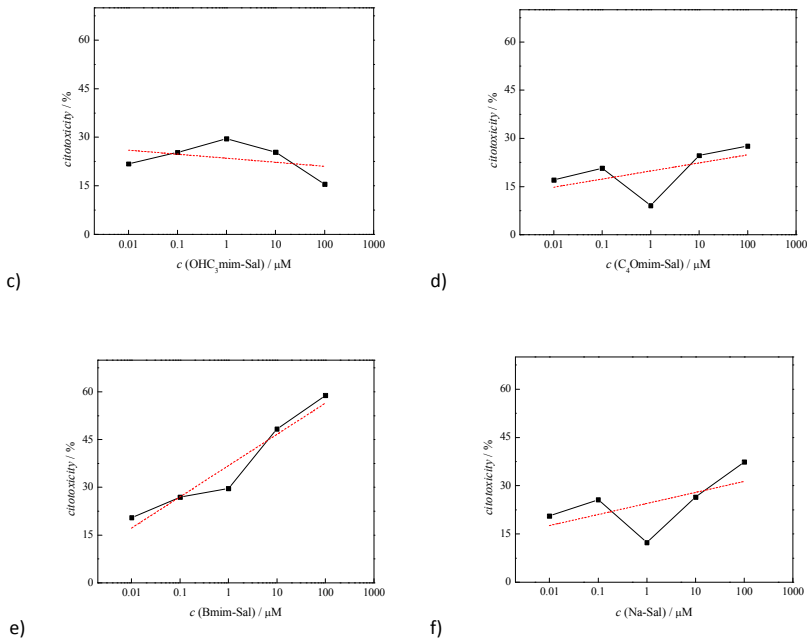


Figure 3. *MRC-5 citotoxicity in function of concentration of: a) [OHC_2OC_2mim][Sal], b) [Im][Sal], c) [OHC_3mim][Sal], d) [C_4Omim][Sal],e) [bmim][Sal], f) Na-Sal (symbol+lines - experimental values; dashed red lines - calculated values)*

It is known that structure of the cation has the greater impact on the ILs toxicity.[25,27,47-49] From the results presented in this manuscript it is obvious that alkyl side chain is the primary factor that affects the toxicity of imidazolium based ionic liquids, namely change of its polarity. This phenomenon can be explained by the assumption that the lipophilic cations are adsorbed or intercalated in the cell membrane, causing "perturbations" in the membrane (expansion or swelling, incerase in fluidity, lowering of the phase transition temperature and alteration of the ion permeability of the membrane).[25,49-52] The presented results indicate that the presence of a lipophilic butyl group leads to proportional destabilization of the lipid double layer membrane, which results in a linear increase of the toxicity with increasing IL concentration (Figure 3e). Introduction of the oxygen in the alkyl substituent increases polarity of the ion, while lipophilicity decreases. This weakens the interactions between lipid cell membrane and ionic liquid which significantly reduces the toxicity (Figures 3a, 3c and 3d). Lower toxicity of the ionic liquid which in the side chain contains hydroxide group in relation to the corresponding ether group, leads to the conclusion that the toxicity is largely affected by the type of functional group of the cation. The terminal group of the alkyl chain is the most accessible for the interactions with cellular membrane and its reactivity, namely hydrophilicity/ hydrophobicity, is mainly responsible for the toxicity of the whole ionic liquid. Ionic liquid [C_2OC_2mim][Sal] at the end of the alkyl chain has a more hydrophobic methyl group compared to [OHC_3mim][Sal] where side chain terminates with distinctly polar OH group, explaining thus the higher toxicity of [C_2OC_2mim][Sal]. Also, it can be observed that additional oxygenation of the alkyl chain does not decrease toxicity of the ionic liquids, which is in agreement with the results reported by Samori et al.[53]

Ionic liquids examined in this work, contains biological active anion – salicylate, and cation as a drug carrier. Due to dissociation of the ionic compounds, cation and anion can be delivered separately to the cells. Thus, decreasing the lipophilicity of the cation, its toxicity and bioavailability can be reduced without affecting bioavailability of salycilate anion.

Conclusions

In this paper the toxicity of newly synthesized salicylate based ionic liquids has been investigated using aquatic organisms (*Artemia salina*) and human non-tumor cell line (normal fetal lung fibroblasts, MRC-5). Also, the influence of the oxygenation (in the form of hydroxy and/or ether groups) of the alkyl side chain on the toxicity reduction was studied. The results indicate that both, toxicity against *A. salina* and cytotoxicity against healthy cell line of lipophobic ionic liquids are significantly lower then non-functionalized analogues and the same order of magnitude as the reference standard sodium salicylate. The most significant impact on the reduction of the toxicity shows the introduction of a hydroxide function at a terminal position of the alkyl substituent of the imidazolium cation, while the introduction of a larger number of oxygen does not contribute to reduced toxicity. Bearing in mind the obtained results, it opens the possibility of designing new non-toxic ILs adjusting only lipophilicity of the cations introducing polar oxygen groups in the side alkyl chain.

View Article Online
DOI: 10.1039/C6RA16182K

Acknowledgements

This work was financially supported by the Ministry of Education, Science and Technological Development of Republic of Serbia under project contract ON172012.

References

1. S.M. Berge, L.D. Bighley and D.C. Monkhouse, *J. Pharm. Sci.,* 1977, **66**, 1.
2. A.T.M. Serajuddin, *Adv. Drug Deliv. Rev.,* 2007, **59**, 603.
3. R. Banerjee, R.M. Bhatt, N.V. Ravindra and G.R. Desiraju, *Cryst. Growth Des.,* 2005, **5**, 2299.
4. I. Pasquali, R. Bettini and F. Giordano, *Adv. Drug Delivery Rev.,* 2008, **60**, 399.
5. P.C.A.G. Pinto, D.M.G.P. Ribeiro, A.M.O. Azevedo, V.D. Justina, E. Cunha, K. Bica, M. Vasiloiu, S. Reisa and M.L.M.F.S. Saraiva, *New J. Chem.,* 2013, **37**, 4095.
6. I.M. Marrucho, L.C. Branco and L.P.N. Rebelo, *Annu. Rev. Chem. Biomol. Eng.,* 2014, **5**, 527.
7. K.S. Egorova, M.M. Seitkalieva, A.V. Posvyatenko, V.N. Khrustalev and V.P. Ananikov, *ACS Med. Chem. Lett.,* 2015, **6**, 1099.
8. J.L. Shamshina, P.S. Barber and R.D. Rogers, *Expert Opin. Drug Delivery,* 2013, **10**, 1367.
9. S. Bontha, A.V. Kabanov and T.K. Bronich, *J. Control. Release,* 2006, **114**, 163.
10. J.O. Kim, G. Sahay, A.V. Kabanov and T.K. Bronich, *Biomacromolecules,* 2010, **11**, 919.
11. M. Kamimura, J.O. Kim, A.V. Kabanov, T.K. Bronich and Y. Nagasaki, *J. Control. Release,* 2012, **160**, 486.
12. O.A. Cojocaru, K. Bica, G. Gurau, A. Narita, P.D. McCrary, J.L. Shamshina, P.S. Barber and R.D. Rogers, *Med. Chem. Comm.,* 2013, **4**, 559.
13. J.L. Shamshina, S.P. Kelley, G. Gurau and R.D. Rogers, *Nature*, 2015, **528**, 188.
14. H.D. Williams, Y. Sahbaz, L. Ford, T.H. Nguyen, P.J. Scammells and C.J. Porter, *Chem. Commun.,* 2014, **50**, 1688–90
15. M. Omar, *UK. J. Pharm. Biosci.* 2016, **4**, 41.
16. D. Dobler, T. Schmidts, I. Klingenhofer and F. Runkel, *Int. J. Pharm.,* 2013, **441**, 620.
17. S.S. Kumar, M. Surianarayanan, R. Vijayaraghavan, A.B. Mandal and D.R. MacFarlane, *Eur. J. Pharm. Sci.,* 2014, **51**, 34–44
18. P.D. McCrary, P.A. Beasley, G. Gurau, A. Narita, P.S. Barber, O.A. Cojocaru and R.D. Rogers, *New J. Chem.,* 2013, **37**, 2196.
19. K.S. Egorova and V.P. Ananikov, *ChemSusChem,* 2014, **7**, 336.
20. T.P. Thuy Pham, C.W. Cho and Y.S. Yun, *Water Res.*, 2010**, 44**, 352.
21. X.D. Hou, Q.P. Liu, T.J. Smith, N. Li and M.H. Zong, *PLoS ONE*, 2013, **8**, 59145.
22. M. Petkovic, J.L. Ferguson, H.Q. Nimal Gunaratne, R. Ferreira, M.C. Leitão, K.R. Seddon, L.P.N. Rebelo and C.S. Pereira, *Green Chem.*, 2010, **12**, 643.
23. P. Nockemann, B. Thijs, K. Driesen, C.R. Janssen, K. Van Hecke, L. Van Meervelt, S. Kossmann, B. Kirchner and K. Binnemans, *J. Phys. Chem. B*, 2007, **111**, 5254.
24. K.D. Weaver, H.J. Kim, J. Sun, D.R. MacFarlane and G.D. Elliott, *Green Chem.,* 2010, **12**, 507.
25. S. Stolte, M. Matzke, J. Arning, A. Böschen, W.R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem.,* 2007, **9**, 1170.
26. S. Tanga, G.A. Bakerb and H. Zhao, *Chem. Soc. Rev.,* 2012, **41**, 4030.
27. J. Arning, S. Stolte, A. Böschen, F. Stock, W.R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem.,* 2008, **10**, 47.
28. M. Vraneš, A. Tot, S. Armaković, S.J. Armaković and S. Gadžurić, *J. Chem. Thermodyn.,* 2016, **95**, 174.
29. K. Bica, C. Rijksen, M. Nieuwenhuyzena and R.D. Rogers, *Phys. Chem. Chem. Phys.*, 2010, **12**, 2011.
30. L. Alfonso, G. Ai, R. Spitale and G.J. Bhat, *Br. J. Cancer,* 2014, **111**, 61.
31. A. Shiotani, T. Kamada and K. Haruma, *J. Gastroenterol.,* 2008, **43**, 581.
32. P.S. Campbell, M. Yang, D. Pitz, J. Cybinska and A.V. Mudring, *Chem. Eur. J.,* 2014, **20**, 4704.
33. M. Vraneš, S. Armaković, A. Tot, S. Papović, N. Zec, S.J. Armaković, N. Banić and B. Abramović, *J. Chem. Thermodyn.,* 2016, **93**, 164.
34. A.L. Para, R.S. Yhebra, I.G. Sardińas and L.I. Buela, *Phytomedicine*, 2001, **8**, 395.
35. T. Mossman, *J. Immunol. Methods*, 1983, **65**, 55.
36. A. Sotto, V. Foulongne, D. Sirot, R. Labia and J. Jourdan, *Int. J. Antimicrob. Agents*, 2002, **19**, 75.
37. M. Hartl and H.U. Humpf, *Food Chem. Toxicol.,* 2000, **38**, 1097.
38. W. Gouveia, T.F. Jorge, S. Martins, M. Meireles, M. Carolino, C. Cruz, T.V. Almeida and M.E.M. Araújo, *Chemosphere,* 2014, **104**, 51.
39. A. Manilal, S. Sujith, S. Kiran, J. Selvin and C. Shakir, *Glob. J. Pharmacol.,* 2009, **3**, 90.
40. A.B. Johnson and A. Lewis, Molecular Biology of the Cell, 4th edition, New York, Garland Science, 2002
41. A. Stary, P. Kannouche, A.R. Lehman and A. Sarasin, *J. Bio. Chem.,* 2003, **278**, 18767.
42. D.J.S. Patinha, L.C. Tome, C. Florindo, H.R. Soares, A.S. Coroadinha and I.M. Marrucho, *ASC Sustainable Chem. Eng.,* 2016, **4**, 2670.
43. L.K. Sztriha, K. Sas and L. Vecsei, *J. Neurol. Sci.,* 2004, **229/230**, 163.
44. N. Gong, M. Zhang , X.B. Zhang, L. Chen, G.C. Sun and T.L. Xu, *Neuropharmacol.,* 2008, **54**, 454.
45. Y. Sakaeda, M. Hiroi , T. Shimojima, M. Iguchi , H. Kanegae and Y. Ohmori, *Biochem. Biophys. Res. Comm*., 2006, **350**, 339.
46. T.P. McDade, R.A. Perugini, F.J. Vittimberga Jr., R.C. Carrigan and M.P. Callery, *J. Surg. Res.,* 1999, **83**, 56.
47. S. Stolte, J. Arning, U. Bottin-Weber, A. Müller, W.R. Pitner, U. Welz-Biermann, B. Jastorff and J. Ranke, *Green Chem*., 2007, **9**, 760.
48. H. Konemann, *Toxicology,* 1981, **19**, 209.
49. M.T.D. Cronin and T.W. Schultz, *Sci. Total Environ.,* 1997, **204**, 75.
50. A.P. van Wezel and A. Opperhuizen, *Crit. Rev. Toxicol.,* 1995, **25**, 255.
51. J. Ranke, S. Stolte, R. Stormann, J. Arning and B. Jastorff , *Chem. Rev.,* 2007, **107**, 2183.
52. J. Ranke, A. Müller, U. Bottin-Weber, F. Stock, S. Stolte, J. Arning, R. Stormann and B. Jastorff, *Ecotoxicol. Environ. Saf.,* 2007, **67**, 430.
53. C. Samorí, D. Malferrari, P. Valbonesi, A. Montecavalli, F. Moretti, P. Galleti, G. Sartor, E. Tagliavini, E. Fabbri and A. Pasteris, *Ecotoxicol. Environ. Saf.,* 2010, **73**, 1456.