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Cytotoxic activity of salicylic acid-containing drug models with ionic and covalent binding

Ksenia S. Egorova[1*], Marina M. Seitkalieva[1], Alexandra V. Posvyatenko[2,3], Victor N. Khrustalev [4,5] and Valentine P. Ananikov[1,6*]

[1]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky prospect 47, Moscow, 119991 Russia

[2]Institute of Gene Biology, Russian Academy of Sciences, Vavilova str. 34/5, Moscow, 119334 Russia

[3]D. Rogachev Federal Scientific Clinical Centre of Pediatric Hematology, Oncology and Immunology, Ministry of Health of Russian Federation, Samory Mashela str., Moscow, 117198 Russia

[4]Peoples' Friendship University of Russia, 6 Miklukho-Maklay Street, Moscow 117198, Russian Federation

[5]A.N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, 28 Vavilov Street, Moscow 119991, Russian Federation

[6]Saint Petersburg State University, Universitetsky Prospekt 26, Stary Petergof, 198504, Russia

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ABSTRACT: Three different types of drug delivery platforms based on imidazolium ionic liquids (ILs) were synthesized in high preparative yields, namely the models involving: i) ionic binding of drug and IL; ii) covalent binding of drug and IL; and iii) dual binding using both ionic and covalent approaches. Seven ionic liquids containing salicylic acid (SA-ILs) in the cation or/and in the anion were prepared, and their cytotoxicity towards the human cell lines CaCo-2 (colorectal adenocarcinoma) and 3215 LS (normal fibroblasts) was evaluated. Cytotoxicity of SA-ILs was significantly higher than that of conventional imidazolium-based ILs and was comparable to the pure salicylic acid. It is important to note, that the obtained SA-ILs dissolved in water more readily than salicylic acid, suggesting benefits of possible usage of traditional non-soluble active pharmaceutical ingredients in an ionic liquid form.

Most of active pharmaceutical ingredients (API) currently used in modern medicine are solid substances suffering from several significant disadvantages. Being crystalline compounds, API may exist as various polymorphs or pseudopolymorphs with different solubility and bioavalability, and the determination of a particular form of an API in the drug composition is a difficult and important task.[1,2] One of the most common strategies for overcoming this issue is the conversion of neutral API into salts with sodium, chloride, phosphate, citrate and other counter-ions. Ionic-core micelles were also proposed to be used for targeted drug delivery.[3-5] Recently, it has been suggested that ionic liquids, or liquid salts, also can be used as ionizing agents in pharmaceutics.[2,6]

Ionic liquids (ILs) comprise an outstanding class of chemicals, which during the last several decades have found application in such diverse scientific and industrial fields as organic synthesis,[7] nanoparticle research,[8] catalysis,[9] biomass conversion,[10] electrochemistry,[11] enzymatic reactions,[12] extraction,[13] and medicine.[14,15] Initially ILs, being non-volatile and non-flammable substances, were considered environmentally benign, but results of intense studies proved otherwise.[14] As biological activity of ILs has become apparent, their potential application in medicine has attracted much attention. First, ILs were explored as alternative reaction media for synthesis of various drugs.[16] Then it became evident that ILs might be introduced into the drug formula to relieve administration of insoluble preparations into the organism in the form of active pharmaceutical ingredient ionic liquids, or API-ILs.[15-18] Being unique tunable chemicals, ILs are ideal candidates for incorporating various functional groups,[19] including biologically active substances.[20] One of the latest areas of IL application in pharmaceutics involves using them as functionalization agents for non-ionizable active substances.[6,16,20] Figure 1 shows most remarkable structural features, which make ILs valuable potential candidates for new-age drug delivery platforms.

Presently, there are two main strategies of combining API and ILs: (a) using API as an anion or a cation (*ionic transport*, Fig. 1A) and (b) covalently linking API to IL (*covalent transport*, Fig. 1B). Moreover, the cationic-anionic structure of IL allows combining API with different properties within one molecule (both cation an anion may bear different or same API, Fig. 1C).[21] Poorly soluble API may be transformed into readily soluble API-ILs.[2,22] Synthesis and activity of several drugs, such as ampicillin,[23,24] aspirin[25] and salicylic acid,[26] in the IL form have been investigated. Particularly, ampicillin ILs demonstrated dramatically higher antibiotic activity than conventional sodium ampicillin.[23]

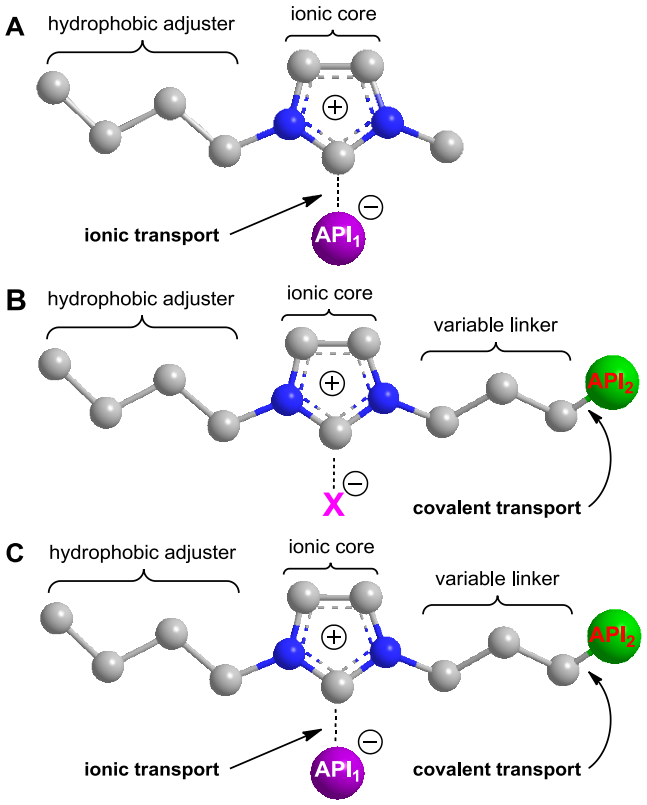

Figure 1.**Three types of drug development platforms based on ILs:** API-IL containing ionic API as its anion (A), covalently linked API within its cation (B), or both binding options (C).

In this work, we used salicylic acid (SA) as a model drug. SA is a relatively simple molecule, and its biological activity has been studied thoroughly.[27] We synthesized seven imidazolium-based ionic liquids (SA-ILs), which corresponded to the three API-IL classes stated in Figure 1: ILs bearing SA in their anion ([EMIM][Sal], [BMIM][Sal], [HMIM][Sal]); ILs with SA covalently linked to the cation ([EMIM-OSal][Cl], [PrMIM-OSal][Cl], [EMIM-OSal][BF_4]), and an IL bearing SA both in the anion and cation ([EMIM-OSal][Sal]). Formulae of SA-ILs and conventional ILs used in the study are shown in Figure 2.

Synthesis of [EMIM][Sal][26,28] and [BMIM][Sal][28,29] has been described earlier, whereas preparation of [HMIM][Sal], [EMIM-OSal][Cl], [PrMIM-OSal][Cl], [EMIM-OSal][BF_4], and [EMIM-OSal][Sal] have, to our knowledge, not been published before. As a representative example, the scheme of synthesis of [EMIM-OSal][Sal] is shown in Figure 3. The preparation procedure involves three stages: (1) synthesis of chloroalkyl ethers of salicylic acid; (2) incorporation of imidazole core; and (3) anion exchange reaction with sodium salicylate. Structures and purity of the synthesized ILs were confirmed using ^{1}H, ^{13}C NMR spectroscopy, and mass spectrometry; the melting temperature was measured for solid substances. The corresponding experimental details and characterization data for all synthesized compounds are provided in the Supporting information.

The molecular structure of [PrMIM-OSal][Cl] was established by X-ray analysis (Figure 4). The geometry of the imidazolium cation in this salt is comparable to that observed in other reported analogous compounds. The imidazolium ring is connected with the salicylic fragment via the alkane bridge, which adopts the t-g$^{(+)}$-t-g$^{(-)}$ (t – *trans*, g – *gauche*) conformation. It is well-known that, in ionic liquids, a 2-position imidazolyl [NC(H)N] proton possesses the highly acidic nature and is able to compete with active hydrogen atoms for the formation of hydrogen bonding interactions with halogenide anions.[30] In the case of [PrMIM-OSal][Cl], however, the hydrogen atom of the hydroxyl group in the sidearm functionalized with salicylic acid forms a strong O3—H3…Cl1 hydrogen bond with a chloride anion [O…Cl 2.969(2), H…Cl 2.14(4) Å, ∠O—H…Cl 172(3)°], whereas the acidic imidazolyl proton links two O1 and O3 oxygen atoms of a neighboring cation by the bifurcate hydrogen bond (see the Supporting information). Thus, cations and anions of [PrMIM-OSal][Cl] are bound by the above hydrogen bonds into *zigzag* chains towards [010] (Figure S19, see the Supporting information). The chains are packed in stacks along the ***a*** axis.

Salicylic acid is a known cytotoxin with anti-cancer effects.[31,32] Therefore, to investigate whether SA retains its activity upon incorporation into an IL, we used the MTT assay in two human cell lines, CaCo-2 (colorectal adenocarcinoma) and 3215 LS (normal fibroblasts). The resultant data on cytotoxicity of the SA-ILs, as well as conventional imidazolium-based ILs and pure salicylic acid, are shown in Table 1 (expressed as 24-h IC_{50}, half maximal inhibitory concentration after 24-hour exposure). Details on experimental procedures and statistical analysis are provided in the Supporting information.

Our first observation was that the studied ILs, both conventional and newly synthesized ones, demonstrated similar toxicity in the cancer and normal cell lines. Several ILs, such as [BMIM][BF_4], [Ala-OMe][BF_4], [EMIM][Sal], and [BMIM][Sal], exhibited significantly higher toxicity towards normal fibroblasts, as compared with cells of colorectal adenocarcinoma (Table 1, entries 4-7). Salicylic acid also did not distinguish between the two cell lines and was even less toxic towards the cancerous one; thus, we could not determine IC_{50} in CaCo-2, because the saturated solution of SA did not kill 100% cells (Table 1, entry 13).

Introduction of salicylic acid into imidazolium-based ILs dramatically increased their cytotoxicity. The replacement of the chloride anion with salicylate led to a significant decrease of 24-h IC_{50} (Table 1, entries 1-3 and 6-8). [BMIM][Sal] was even more cytotoxic than [BMIM][BF_4], for which rather high toxicity had been demonstrated in this and other[33] studies (Table 1, entries 4 and 7). Introduction of salicylic acid into the cation resulted in a similar increase of cytotoxicity (Table 1, entries 9-11).

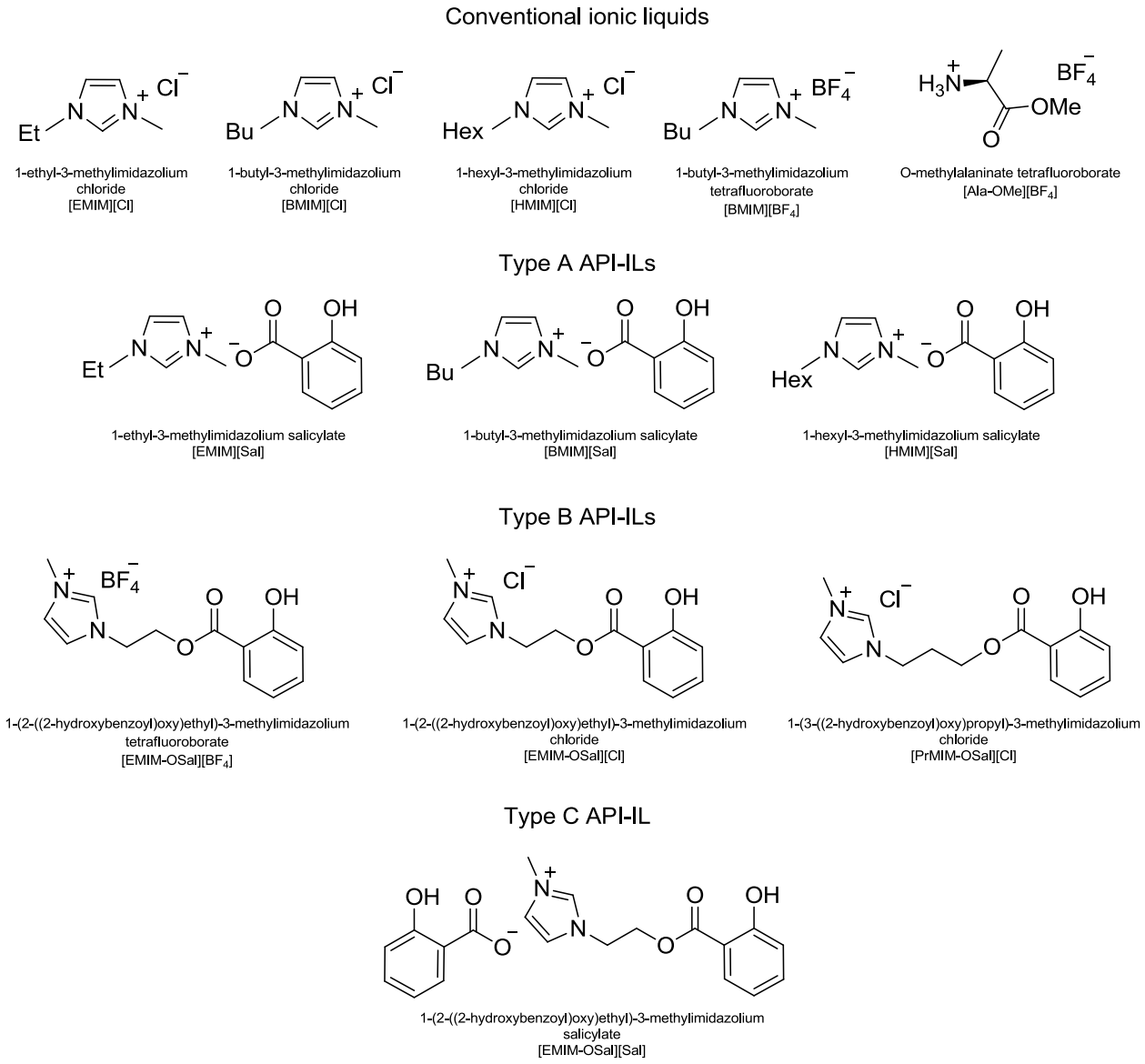

Figure 2. **Ionic liquids with various combinations of anions and cations utilized in the present study.** (See Figure 1 for types A-C.)

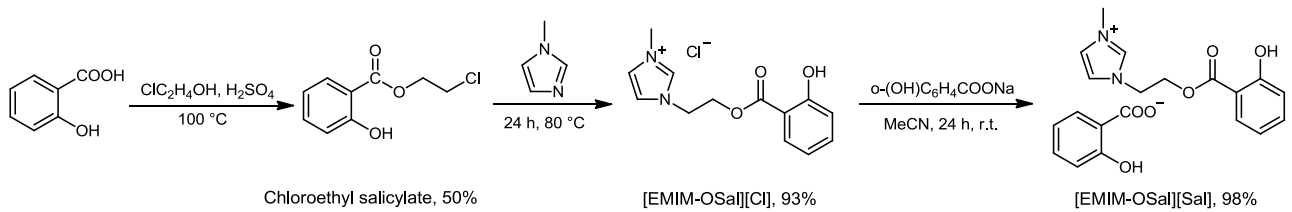

Figure 3. **Synthetic procedure for the preparation of [EMIM-OSal][Sal].**

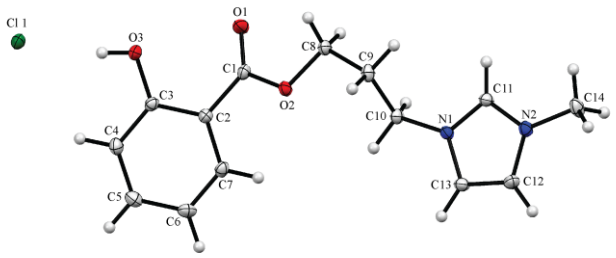

Figure 4. **Molecular structure of [PrMIM-OSal][Cl] determined by X-ray analysis.** (N1—C11 1.329(3) Å, N1—C13 1.377(3) Å, N2—C11 1.330(3) Å, N2—C12 1.372(3) Å, C12—C13 1.357(4) Å, N1—C11—N2 108.2(2)°, C1—O2—C8—C9 -179.5(2)°, O2—C8—C9—C10 64.0(3)°, C8—C9—C10—N1 166.7(2)°, C9—C10—N1—C11 -55.8(4)°.)

Of note, cytotoxicity of the ILs with SA in the cation was comparable to that of the amino acid-based IL [Ala-OMe][BF_4] (Table 1, entries 5 and 9-11). In our previous work, we demonstrated that the presence of an amino acid led to unexpectedly high cytotoxicity of ILs with the tetrafluoroborate anion, possibly due to specific interactions between the amino acid and transporter proteins on the surface of the cell.[33] In the case of salicylic acid, the anion had insignificant influence on the cytotoxicity. In contrast to the conventional [BMIM][Cl] and [BMIM][BF_4], [EMIM-OSal][BF_4] and [EMIM-OSal][Cl] possessed similar toxicity (Table 1, entries 2 and 4, 9 and 10), suggesting the inherent toxicity of the anion to be overwhelmed by that of the cation.

We also studied the cytotoxicity of [EMIM-OSal][Sal], which contained salicylic acid both in its cation (covalent linkage) and anion (ionic interaction) (Table 1, entry 12). Its IC_{50} was 0.71 mM and did not differ significantly from the SA-ILs bearing SA in the anion only (Table 1, entries 6-8). This observation suggests that in the case of imidazolium-based ILs, one part of salicylic acid per an IL molecule is enough for reaching the maximum possible cytotoxicity, and that increasing the SA content does not lead to higher biological activity. In general, IC_{50} of SA-ILs belonging to the three structural API-ILs types did not differ significantly, though SA-IL bearing covalently linked salicylic acid within their cation tended to demonstrate lower toxicity (IC_{50} 2.85-4.12 mM), than SA-ILs with salicylate anions (IC_{50} 0.64-2.00 mM). This observation may be possibly explained by known allosteric activity of SA.[34] The covalent linkage between SA and IL may hinder binding of the former to its cell targets; on the other hand, the ionic linkage allows for more flexible interactions.

Interestingly, in most cases the cytotoxicity of SA-ILs did not differ significantly from that of the pure salicylic acid (Table 1, entries 6-13). We were unable to determine the exact 24-h IC_{50} for SA in CaCo-2 cells (at maximum concentration, SA killed about 95% cells) and estimated it as 5-7 mM; in 3215 LS, 24-h IC_{50} for SA was 1.14 mM. Therefore, the introduction of SA into an ionic liquid did not impede its cytotoxic action, but significantly increased its solubility. For [EMIM-OSal][Cl], solubility in water was 267 g/L (r.t.), as compared to 2.20 g/L (r.t.)[35] for pure salicylic acid. All SA-ILs were readily dissolved in water in the concentration range used for the testing (see Table S1 in the Supporting information).

Table 1. Cytotoxicity of studied ILs towards cell lines 3215 LS and CaCo-2

	IL	**3215 LS** (24-h IC_{50}, mM)*	**CaCo-2** (24-h IC_{50}, mM)*
1	[EMIM][Cl]	46.15 (45.70-46.59)	32.10[33]
2	[BMIM][Cl]	30.08 (19.47-40.70)	18.85[33]
3	[HMIM][Cl]	7.54 (2.55-12.54)	6.21[33]
4	[BMIM][BF_4]	4.88 (4.27-5.50)	11.19[33]
5	[Ala-OMe][BF_4]	2.63 (2.32-2.94)	6.24 (4.32-8.17)
6	[EMIM][Sal]	1.39 (0.64-2.14)	5.97 (4.51-7.91)
7	[BMIM][Sal]	0.64 (0.19-1.09)	4.34 (2.48-7.61)
8	[HMIM][Sal]	2.00 (0.69-3.31)	1.96 (0.79-3.14)
9	[EMIM-O][Sal] BF_4	4.12 (2.51-5.73)	4.77 (3.27-6.27)
10	[EMIM-OSal][Cl]	2.85 (1.30-4.40)	3.82 (3.34-4.31)
11	[PrMIM-OSal][Cl]	3.19 (0.84-5.54)	2.55 (2.08-3.13)
12	[EMIM-OSal][Sal]	0.71 (0.58-0.84)	-**
13	Salicylic acid	1.14 (0.67-1.94)	~5-7***

* 95% confidence interval is shown in parentheses.

** No data available.

*** Approximate value (see the text).

Salicylic acid is known to cause apoptosis *in vitro* and *in vivo*,[31,32,36] but the exact mechanisms of its action just begin to emerge. SA was shown to impact mitochondrial activity[37] and to inhibit protective catalase in malignant cells directly, therefore activating the signaling mediated by reactive oxygen species (ROS), which triggers apoptosis in the cell.[32] SA decreased glucose consumption by breast cancer cells MCF-7[38] and increased caspase activity in CaCo-2 cells when applied at such low concentration as 10 μM, the latter effect being dependent on the oxygen availability.[31]

The fact that SA retains its ability to cause cell death upon covalent or ionic linking to an IL suggests that its mechanism of action remains unperturbed and does not include metabolic modifications.

In summary, we investigated cytotoxicity of salicylic acid-containing imidazolium-based ILs (SA-ILs) belonging to three currently available structural classes of API-ILs: API-ILs with ionic API as their anion (type A); API-ILs with covalently linked API within their cation (type B); and API-ILs bearing API both in the anion and cation (type C). Cytotoxicity of the studied SA-ILs was significantly higher than that of conventional imidazolium-based ILs and was comparable to that of the pure salicylic acid. In contrast to salicylic acid, SA-ILs possessed relatively high water solubility. According to our results, API-ILs of types A and C demonstrated similar biological activity, which was higher than that of type B.

Nevertheless, all three API-IL types proposed in this work are promising candidates for future studies. The obtained results confirm the possibility of using IL advantages, such as ionic linkage and improved solubility, in pharmaceutics.

ASSOCIATED CONTENT

Supporting Information: materials, experimental procedures, details on X-ray, MS, and NMR studies.

AUTHOR INFORMATION

Corresponding Author

E-mail: egorova-ks@ioc.ac.ru, val@ioc.ac.ru.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

IL, ionic liquid; API-IL, active pharmaceutical ingredient ionic liquid; SA-IL, salicylic acid-containing ionic liquid; IC_{50}, half maximal inhibitory concentration.

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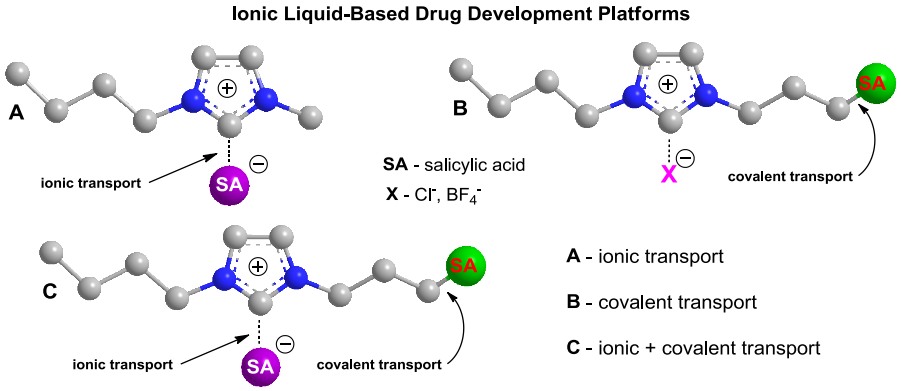

Lay Summary: Three different drug development platforms involving ionic binding of the drug components, covalent binding of the drug components and dual ionic/covalent binding were explored in model imidazolium ionic liquids. Tunable nature of ionic liquids and readily accessible synthetic procedures provide excellent opportunities for diverse applications.