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A Novel Approach for Bisphosphonates: Ionic Liquids and Organic Salts from Zoledronic Acid

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Novel ionic liquids and organic salts based on mono- or dianionic zoledronate and protonated superbases, choline and *n*-alkylmethylimidazolium cations, were prepared and characterized by spectroscopic and thermal analyses. Most of the prepared salts display amorphous structures and very high solubility in water and saline solutions, especially the dianionic salts. Among the zoledronate-based ionic compounds, those containing choline [Ch] and methoxyethylmethylimidazolium [C_3OMIM] cations appear to have significant cytotoxicity against human osteosarcoma cells (MG63) and low toxicity toward healthy skin fibroblast cells. Because osteosarcoma is a bone pathology characterized by an increase in bone turnover rate, the results presented herein may be a promising starting point for the development of new ionic pharmaceutical drugs against osteosarcoma.

The discovery of novel effective drugs is currently one of the greatest challenges for pharmaceutical industry.[1] The convenient manipulation of solid active pharmaceutical ingredients (APIs) has been associated with several drawbacks such as polymorphic conversion, low bioavailability from crystalline solids, and spontaneous crystallization of amorphous forms.[2–5]

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In addition, it is recognized that the bioavailability of pharmaceutical drugs is significantly dependent on their water solubility as well as high permeability. In fact, many phase II trials of new APIs fail because of their decreased bioavailability, a consequence of poor solubility in water and biological fluids. The most common approach followed by the pharmaceutical industry to increase the bioavailability of an API is to transform it into a salt, usually by combining it with metallic cations such as sodium or potassium, or with chloride anions, according to the chemical structure of the API. Recently, the use of APIs as organic salts and ionic liquids (API-OSILs) as an alternative has been investigated by the academia.[6–9] The works involving the preparation of API-OSILs from ampicillin, fluoroquinolones, ibuprofen, ranitidine and lidocaine, and also acetylsalicylic and salicylic acids, among others, have shown that the combination of an API, either as a cation or as an anion, with suitable biocompatible counter-ions can increase the water solubility of the parent drug and even change its biological effect.[10–21] In face of these results, it is suggested that the oral bioavailability of the formed API-OSILs is particularly enhanced, or alternatively it may open new perspectives for their local administration/application. Therefore, the therapeutic dosage of the drug may be reduced, decreasing side effects.

Bisphosphonates (BPs) are considered the primary therapy for skeletal disorders due to their high affinity for bone tissue. BPs are stable analogues of the inorganic pyrophosphate, an endogenous regulator of bone mineralization, which can withstand hydrolysis in the gastrointestinal tract.[22–24] Their conformation allows targeting the bone because of their three-dimensional structure, which makes them primary agents against osteoclast-mediated bone loss. In addition, recently they have also been considered as potential antitumor agents due to their ability to induce tumor cell apoptosis, inhibition of cell adhesion, invasion and proliferation, modulation of the immune system to target and eliminate cancer cells as well as affect the angiogenic mechanisms. However, zoledronic acid, one of the most effective BPs, lacks absorption in the GI tract and is only administered intravenously. In addition, many debilitating side effects are described such as muscle, joint and bone pain, muscle spasms, numbness, among many others. In this context, there is a need to develop new ways to increase oral bioavailability of zoledronic acid, while the side effects are significantly reduced.

Potential formulations of zoledronic acid and other bisphosphonates for oral administration have been reported recently by pharmaceutical companies.[25–27] Such formulations comprise, among other possibilities, the use of BPs as organic

salts, in particular quaternary ammonium salts. However, no data on the synthesis, bioavailability and/or toxicity of such salts are presented.

Thus, in this line of thought we report the preparation of twelve OSILs from zoledronic acid (Zol-OSILs) in quantitative yields by following two distinct sustainable and straightforward methodologies, according to the type of cation. All prepared Zol-OSILs were characterized by spectroscopic and thermal analysis and their solubility in water and biological fluids was determined. An evaluation of the toxicity toward human skin fibroblasts and human lung and bone (osteosarcoma) cancer cells was performed. The zoledronic acid-based OSILs were prepared using the cations depicted in Figure 1, which were selected due to their low cytotoxicity.[20,21,28]

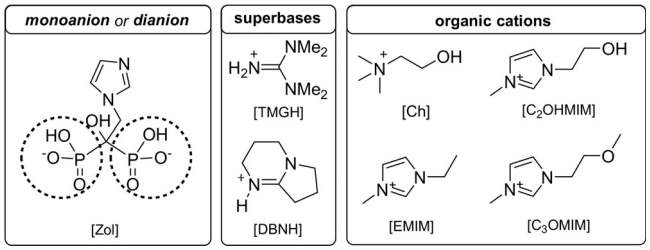
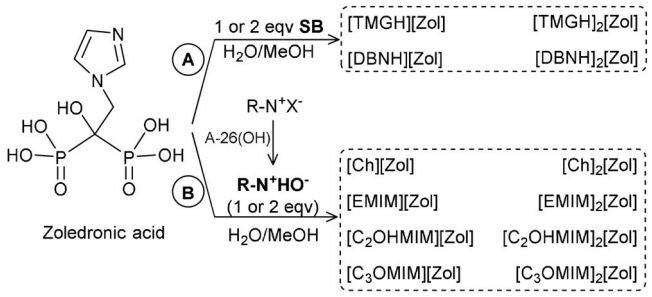


Figure 1. Structures of mono- and dianionic zoledronic acid, protonated superbases and organic cations.

Protic zoledronate-OSILs were prepared by adding 1 or 2 equivalents of organic superbases, more specifically 1,1,3,3-tetramethylguanidine (TMG) and 1,5-diazabicyclo(4.3.0)non-5-ene (DBN), to a solution of zoledronic acid, whose phosphonate group(s) become(s) deprotonated (general procedure A in Scheme 1).[15]



Scheme 1. Synthetic methodologies A and B for the synthesis of Zol-OSILs.

With this straightforward synthetic methodology two monoanionic and two dianionic zoledronic acid-based ionic salts were prepared. Room temperature ionic liquids (RTILs) were obtained whenever two units of superbase were added, while the monoionic compounds were obtained as protic organic solid salts.

For the deprotonation of zoledronic acid with cholinium and methylimidazolium salts, a different methodology was employed (general procedure B from Scheme 1), based on previous reports from our group on the preparation of other API-OSILs.[17,20] Briefly, the cations in the form of hydroxide salts are firstly prepared from the corresponding chloride or bromide salts by reaction with hydroxide exchange resins (e.g., Amberlyst A26-OH) in methanolic solution. The very basic solutions are then neutralized by addition to an aqueous solution of zoledronic acid yielding the desired Zol-OSILs in quantitative yields. By using one or two molar equivalents of cation salts we synthesized four monoionic organic salts and four dianionic Zol-based RTILs.

All products were characterized by NMR (^{1}H and ^{13}C) and FTIR spectroscopies, as well as elemental analysis. The thermal properties were evaluated by DSC and the solubility in water and saline solution was determined for all compounds. Additionally, the structure of [DBNH][Zol] was definitively established by single crystal X-ray diffraction analysis. This salt crystallized from an asymmetric unit composed of one zwitterionic Zol unit where both phosphonate groups are deprotonated and one nitrogen atom of the imidazolium moiety is protonated. The DBN unit is protonated at the nitrogen atom from the 6-membered ring (Figure 2). The crystal structure is stabilized by several intra and intermolecular hydrogen bonds (see packing in Figures S49 and S50 in the Supporting Information).

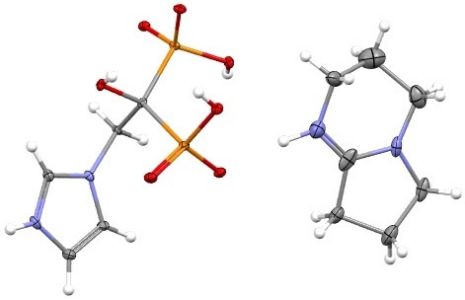


Figure 2. ORTEP-3 diagram of [DBNH][Zol] with thermal ellipsoids drawn at 30% probability level illustrating one cationic DBN unit and one zwitterionic Zol entity bearing two anionic phosphonate groups and one cationic imidazolium moiety. The hydrogen, carbon, oxygen, nitrogen and phosphorous atoms are shown in white, grey, red, blue, and orange, respectively.

All prepared Zol-OSILs were studied by DSC and the melting, crystallization and glass transition temperatures were determined (see Table 1) and compared with the data from the parent drug. Depending on the hydration level, zoledronic acid displays a melting point between 214 and 230 °C, to which decomposition rapidly follows.[29] Only two of the prepared Zol-OSILs, have a melting point in the same range, namely [TMGH][Zol] (225.3 °C) and [Ch][Zol] (220.4 °C). These two compounds, in addition to the remaining four solid compounds obtained, are not considered ionic liquids, but organic salts because their melting temperatures are higher than 100 °C.

[C_3OMIM][Zol] shows a first melt at 125.9 °C followed by a cold crystallization step at 139.8 °C, to which then follows a second melt that occurs at 185.0 °C. After this the compound remains in an amorphous state characterized by a glass transition temperature of 45.7 °C. [EMIM][Zol] also becomes amorphous after first melt (at 198.0 °C) with a glass transition temperature of 29.5 °C.

Table 1. Physical state, melting, crystallization and glass transition temperatures of the prepared Zol-OSILs.

Salt	Physical state	T_m [°C]	T_c [°C]	T_g [°C]
[TMGH][Zol]	White solid	225.3	–	–
$[TMGH]_2$[Zol]	Colorless paste	–	–	12.4
[DBNH][Zol]	White solid	208.7	–	45.7
$[DBNH]_2$[Zol]	Colorless paste	–	–	15.1
[Ch][Zol]	White solid	220.4	–	78.4
$[Ch]_2$[Zol]	White paste	18.6	13.2	–
[EMIM][Zol]	White solid	198.0	–	29.5
$[EMIM]_2$[Zol]	Colorless paste	–	–	31.7
$[C_2OHMIM]$[Zol]	White solid	143.8; 195.9	170.1[a]	57.3
$[C_2OHMIM]_2$[Zol]	Colorless paste	–	–	10.8
$[C_3OMIM]$[Zol]	White solid	125.9; 185.0	139.8[a]	45.7
$[C_3OMIM]_2$[Zol]	Colorless paste	–	–	3.4

[a] Cold crystallization.

Even though $[Ch]_2$[Zol] is a thick white paste at room temperature, it was possible to determine its melting temperature and the corresponding crystallization peak by DSC, which occur at 18.6 (*exo*) and 13.2 °C (*endo*), respectively. Except for this ionic liquid, all remaining Zol-RTILs are clearly amorphous compounds with defined glass transition temperatures.

As expected, all ionic liquids and salts were more soluble in water and saline solution at 37 °C than neutral zoledronic acid. Figure 3 summarizes the data obtained from the solubility studies. All dianionic zoledronate ionic liquids were more soluble in the tested media than the monoanionic siblings, with three of them ($[TMGH]_2$[Zol], $[DBNH]_2$[Zol] and $[C_3OMIM]_2$[Zol]) being completely soluble in water and saline solution. For unknown reasons, $[EMIM]_2$[Zol] was the only ionic compound which was less soluble in water than in saline solution (in which, in fact, it was entirely soluble). Moreover, the monoionic salts were found to be between 214 and 624-fold more soluble in water than the neutral parent drug. With the exception of $[C_2OHMIM]$[Zol], the monoionic compounds had solubility in saline solution lower than 25 mg mL^{-1} with a ratio between 1.5 and 7.0 times in comparison with zoledronic acid.

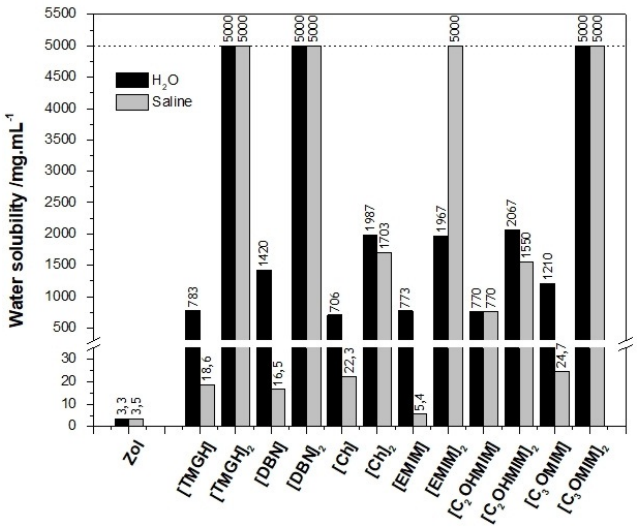


Figure 3. Solubility in water and saline solution at 37 °C of zoledronic acid and Zol-OSILs (detection limit is 5 g mL^{-1}, represented by the upper threshold).

The cytotoxicity (IC_{50}) data of the studies with the prepared zoledronic-containing ILs in different human cell lines and fibroblasts are presented in Table 2. In the tested concentration range, the activity of zoledronic acid toward normal and osteosarcoma cells only differed in one order of magnitude, while no activity was observed for breast cancer cells. The combination of the drug with [TMGH] yielded an inactive compound toward all types of cells tested. In general, all monoanionic salts present lower toxicity to skin fibroblasts than the dianionic ionic liquids, with the rare exception of those with [EMIM] cation. More specifically, the salts containing [TMGH], [DBNH], $[EMIM]_2$ and $[C_3OMIM]$ were found to be the least toxic from the tested set toward the non-neoplastic cells, with $IC_{50} \geq 2.5$ mM. These IC_{50} values are at least three orders of magnitude lower than the one found for Zol (2.43 μM). Regarding the effects on breast cancer cells (T47D), all tested salts, with the exception of [TMGH][Zol], showed cytotoxic activity whereas Zol was inactive. However, the profile was similar to the one found with skin fibroblasts, which means that they are not selective for any of these cells. In the case of the human osteosarcoma cell line MG63, [Ch][Zol] and $[C_3OMIM]_2$[Zol] showed IC_{50} values of 0.15 and 0.33 nM, respectively, which are three orders of magnitude lower than for zoledronic acid (57 mM). The selectivity ratio of [Ch][Zol] between normal and MG63 cells was found to be extremely high, in the order of 10^4, similar to the one found for $[EMIM]_2$[Zol], although its activity toward osteosarcoma cell line was lower ($IC_{50} = 0.5$ μM). Finally, [DBNH][Zol] and $[EMIM]_2$[Zol] also display very high selectivity ratios as they display very low toxicity toward fibroblasts.

Table 2. Cytotoxicity of Zol-OSILs on human skin fibroblasts (SF) and human breast cancer (T47D) and osteosarcoma (MG63) cell lines.

Compound	IC_{50} [mM]			IC_{50}(SF)/ IC_{50}(MG63)
	SF	T47D	MG63	
Zol	2.43×10^{-13}	ND[a]	5.74×10^{-4}	4.2
[TMGH][Zol]	ND[a]	ND[a]	ND[a]	–
$[TMGH]_2$[Zol]	1.02×10^{-3}	6.87×10^{-3}	6.97	1.5×10^{-4}
[DBNH][Zol]	2.50	1.98×10^{-1}	1.65×10^{-3}	1.5×10^{3}
$[DBNH]_2$[Zol]	5.96×10^{-7}	1.25×10^{-7}	ND[a]	–
[Ch][Zol]	3.98×10^{-3}	6.15×10^{-4}	1.51×10^{-7}	2.6×10^{4}
$[Ch]_2$[Zol]	1.46×10^{-7}	1.69×10^{-6}	2.10×10^{-2}	7.0×10^{-6}
[EMIM][Zol]	4.97×10^{-2}	2.11×10^{-3}	ND[a]	–
$[EMIM]_2$[Zol]	17.8	3.82×10^{-2}	5.01×10^{-4}	3.6×10^{4}
$[C_2OHMIM]$[Zol]	ND[a]	1.84×10^{-2}	5.60×10^{-4}	–
$[C_2OHMIM]_2$[Zol]	1.58×10^{-7}	1.48×10^{-7}	5.58×10^{-5}	2.8×10^{-3}
$[C_3OMIM]$[Zol]	14.7	2.01	12.3	1.2
$[C_3OMIM]_2$[Zol]	9.71×10^{-5}	8.02×10^{-3}	3.27×10^{-7}	3.0×10^{2}

[a] Not detected in the tested concentration range.

The data retrieved from this study may be particularly relevant, as osteosarcoma cells are known to induce disturbances

in bone metabolism, with an increase in bone turnover rate.[30–32] Thus, the synthesis of compounds with a simultaneous potential inhibitory effect on bone resorption and a selective cytotoxicity against osteosarcoma cells may represent an important strategy for the development of new drugs against osteosarcoma.

Experimental Section

General procedure (A) for the synthesis of Zol-OSILs with organic superbases as cations: To a dispersion of zoledronic acid (500 mg, 1.84 mmol) in MeOH/H_2O (15 mL, 1:1) a methanolic solution of 1 or 2 molar equivalents of organic superbase (15 mg mL^{-1}) was added dropwise under magnetic stirring. After reacting for 1 h the solvent was evaporated and the desired product was dried under vacuo for 24 h.

General procedure (B) for the preparation of Zol-OSILs with ammonium cations: The halide salts of the selected quaternary ammonium cations were dissolved in methanol and passed slowly through an anion-exchange column A-26(OH) (3 equivalents). The freshly formed methanolic solutions of the corresponding hydroxide salts (1 or 2 equivalents) were consequently added dropwise to zoledronic acid (500 mg, 1 equivalent) dispersed in H_2O under magnetic stirring. After 1 h, the solvent of the clear solution was evaporated and the desired product was dried under vacuo for 24 h.

The detailed experimental procedures of the solubility and cytotoxicity studies are described in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: API-OSIL · bioavailability · cytotoxicity · ionic liquids · zoledronic acid

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