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# SUPPLEMENTARY INFORMATION

# Cholinium-Based Ionic Liquids with Pharmaceutically Active Anions

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## Materials and Instruments

Nalidixic acid (≥98%) and niflumic acid (≥98%) were purchased from Sigma-Aldrich (St. Louis, Missouri USA). 4-Amino-salicylic acid (99%), pyrazinoic acid (pyrazinecarboxylic acid, 99%) and picolinic acid (2-picolinic acid, 99%) were purchased from Sigma-Aldrich (Sheinheim, Germany). The parent APIs were used without further purification. Cholinium chloride ((2-Hydroxyethyl)trimethylammonium chloride, ≥98%) was purchased from Sigma-Aldrich

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(St. Louis, Missouri USA), and was dried under reduced pressure at room temperature for at least 48h before use. The buffer solutions suitable for dissolution testing, simulated gastric fluid without enzymes–interchangeable with 0.1 N HCl (pH 1.0) and simulated intestinal fluid without enzymes–interchangeable with phosphate standard buffer pH 6.8 were purchased from Fluka (Seelze, Germany). Sodium chloride physiological solution (0.15M NaCl–isotonic ionic strength) was purchased from Fluka (Buchs, Switzerland). Milli-Q water (Milli-Q Integral Water Purification System) was used in all experiments throughout the work.

For the *in vitro* cytotoxicity assays, CellTiter 96®AQueous One Solution Cell Proliferation Assay was purchased from Promega (CA, USA); RPMI medium 1640, fetal bovine serum (FBS), L-Glutamine, penicillin-streptomycin solution, MEM medium, MEM nonessential amino acids (MEM-NEAA), sodium pyruvate (100x) and trypsin-EDTA solution were supplied by Gibco (Invitrogen Corporation, Paisley, UK). For cell culture, human colon carcinoma cell line Caco-2 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and human hepatocellular carcinoma cell line HepG2 was obtained from the European Collection of Cell Culture (ECACC, Wiltshire, UK).

The $^{1}H$ and $^{13}C$ NMR spectra of all samples were carried out on a Bruker AVANCE 400 spectrometer operated at room temperature with 16 and 500 scans, respectively. All spectra were acquired using $D_2O$ for field-frequency lock and NMR internal standard. Electrospray ionisation mass spectra (ESI-MS) were recorded on an API-Ion trap (PO03MS), and samples were measured in water solution. Elemental analysis were done on an element analyser (Vario EL Elemental Analyser). The water content was determined by Karl Fischer coulometric titration (Metrohm 831 KF coulometer). Halide impurities (chloride) were quantified by Chloride Ion Selective Electrode (Thermo Scientific. Orion 9617BNWP). The differential scanning calorimetry (DSC) measurements were done with a TA Instrument DSC Q200 Differential Scanning Calorimeter. The thermogravimetric analysis (TGA) were carried out with a TA Instrument Model TGA Q50. UV-vis spectra were measured using a Shimadzu Pharma-Spec UV-1800 spectrophotometer.

# Parent APIs

Nalidixic acid, the first analogue of a class of the most important and impressive antimicrobial drugs called quinolones, has been extensively used in the treatment of urinary tract infections.[1] The drug is effective against both Gram-positive and Gram-negative bacteria, and is the only FDA approved quinolone drug for pediatric formulation. The drug is insoluble in water at $< 0.1$ mg/ml solubility and its bioavailability is thus limited. High doses of the drug can occasionally trigger convulsions and hyperglycemia.[2]

Niflumic acid, member of the group of compounds denominated fenamates, is a potent nonsteroidal anti-inflammatory drug (NSAID).[3,4] Its action results in antipyretic, analgesic and anti-inflammatory effects.[5,6] Niflumic acid is widely used in rheumatic disorders[7] such as ankylosing spondylitis, osteoarthritis[8] and rheumatoid arthritis.[9]

4-Amino-salicylic acid is an antibiotic that has been used since the 1940s in the treatment of tuberculosis. Additionally, this FDA-approved anti-tuberculosis drug is safe and effective in the treatment of inflammatory bowel diseases, viz. Chron's disease[10] and distal ulcerative colitis,[11,12] and it has been also used successfully in the treatment of severe manganese (Mn)–induced Parkinsonism in humans.[13]

Pyrazinoic acid is the active metabolite of the anti-tuberculosis drug pyrazinamide,[14,15] a first line agent for the treatment of human tuberculosis.[16] The active form of the drug is not directly used to treat patients presumably due to poor absorption through the gastrointestinal tract.[14] Additionally, pyrazinoic acid is an important pharmaceutical intermediate in the synthesis of the medicament oltipraz, which is often used for treating schistosomiasis and preventing liver cancer.[17]

Picolinic acid is a metabolite of L-tryptophan detected in the human body[18,19] that has been reported to elicit a number of potential effects, particularly involving immune function and antimicrobial activity.[20] It is an approved food supplement[21] and an efficient metal ion chelating agent.[20] For example, chromium picolinate is used as a dietary supplement for obese people, since it has a beneficial effect on reducing diabetes risk[22] and zinc picolinate presents an antioxidant effect and may be used in patients with chronic obstructive pulmonary disease.[23] Picolinic acid is also a metabolite of fungi and is known as an inhibitor of phenolox-

idase.[24] Additionally, it exhibits antiviral activity against human HIV–1 and HSV–2 infected cells.[25] Furthermore, the study of Borawska and co-workers[26] demonstrate the potential of picolinic acid and it salts as food or cosmetic preservatives.

# Characterization of the Cholinium-based API-ILs

The prepared cholinium-based API-ILs were completely characterized by $^{1}$H and $^{13}$C NMR, electrospray ionisation mass spectrometry (ESI-MS) and CHN elemental analysis in order to check their expected structures and final purities. Additionally, the quantitative integration of their characteristic $^{1}$H NMR resonance peaks unfold the expected cation/anion correlations. Also, there were no peaks assigned to impurities in the $^{1}$H NMR spectra. The water content, determined by Karl Fischer titration, was less than 0.05 wt%. Halide impurities were quantified by Chloride Ion Selective Electrode (Thermo Scientific. Orion 9617BNWP) and the chloride content was found to be less than 0.005 $Cl^-$ wt%. The numbering system for the prepared cholinium-based API-ILs is depicted in Figure S1. The ESI-MS and elemental analysis data were provided by the Mass Spectrometry Laboratory, Analytical Services Unit, ITQB-UNL, Oeiras, Portugal.

**Cholinium Nalidixate ([$\mathbf{N_{1112OH}}$][Nalidixate])**

$\delta_H$(400 MHz; $D_2O$) 1.37 (3H, t, $J$ = 7.2 Hz, $n$), 2.62 (3H, s, $o$), 3.12 (9H, s, $a$), 3.48–3.38 (2H, m, $b$), 3.92–4.03 (2H, m, $c$), 4.46 (2H, q, $J$ = 7.2 Hz, $m$), 7.34 (1H, d, $J$ = 8.3 Hz, $k$), 8.45 (1H, d, $J$ = 8.3 Hz, $j$), 8.49 (1H, s, $h$); $\delta_C$(100 MHz; $D_2O$) 14.3 (s, $n$), 24.2 (s, $o$), 46.5 (s, $m$), 53.8 (t, $a$), 55.6 (s, $c$), 67.4 (t, $b$), 118.9 (s, $f$), 119.9 (s, $d$), 121.4 (s, $k$), 135.9 (s, $j$), 147.0 (s, $h$), 148.1 (s, $g$), 164.1 (s, $i$), 172.4 (s, $l$), 176.8 (s, $e$); ESI-MS: calc. for $C_{17}H_{25}N_3O_4$ $[M]^+$: m/z = 104.2, found 104.1; $[M]^-$: m/z = 231.2, found 230.8; Elemental analysis: calc. for $C_{17}H_{25}N_3O_4$: C, 60.9, H, 7.5, N, 12.5, found C, 61.0, H, 7.2, N, 12.8%.

**Cholinium Niflumate ([$\mathbf{N_{1112OH}}$][Niflumate])**

$\delta_H$(400 MHz; $D_2O$) 3.11 (9H, s, $a$), 3.42 (2H, dd, $J$ = 5.9, 4.1 Hz, $b$), 3.97 (2H, ddd, $J$ = 7.8,

5.3, 2.8 Hz, *c*), 6.82 (1H, dd, $J$ = 7.6, 5.0 Hz, *g*), 7.30 (1H, d, $J$ = 7.7 Hz, *l*), 7.43 (1H, t, $J$ = 7.9 Hz, *k*), 7.55 (1H, d, $J$ = 8.2 Hz, *j*), 7.84 (1H, s, *n*), 8.05 (1H, dd, $J$ = 5.0, 1.9 Hz, *h*), 8.12 (1H, dd, $J$ = 7.6, 1.9 Hz, *f*); $\delta_C$(100 MHz; $D_2O$) 53.8 (t, *a*), 55.5 (s, *c*), 67.4 (t, *b*), 114.7 (s, *g*), 115.8 (s, *d*), 116.8 (q, *n*), 118.9 (q, *j*), 122.8 (s, *p*), 124.0 (d, *l*), 125.4 (s, *p*), 129.6 (s, *k*), 130.3 (d, *i*), 140.3 (s, *m*), 140.9 (s, *h*), 149.2 (s, *f*), 155.0 (s, *e*), 173.5 (s, *o*); ESI-MS: calc. for $C_{18}H_{22}F_3N_3O_3$ $[M]^+$: m/z = 104.2, found 104.1; $[M]^-$: m/z = 281.2, found 280.7; Elemental analysis: calc. for $C_{18}H_{22}F_3N_3O_3$: C, 56.1, H, 5.75, N, 10.9, found C, 56.6, H, 5.5, N, 11.3%.

**Cholinium 4-Amino-salicylate ([$N_{1112OH}$][4-Amino-salicylate])**

$\delta_H$(400 MHz; $D_2O$) 3.09 (9H, s, *a*), 3.35–3.45 (2H, m, *b*), 3.89–4.00 (2H, m, *c*), 6.19 (1H, d, $J$ = 2.2 Hz, *f*), 6.28 (1H, dd, $J$ = 8.5, 2.2 Hz, *h*), 7.53 (1H, d, $J$ = 8.5 Hz, *i*); $\delta_C$(100 MHz; $D_2O$) 53.8 (t, *a*), 55.6 (s, *c*), 67.4 (t, *b*), 101.4 (s, *f*), 107.5 (s, *h*), 108.8 (s, *d*), 132.0 (s, *i*), 152.4 (s, *g*), 161.3 (s, *e*), 175.9 (s, *j*); ESI-MS: calc. for $C_{12}H_{20}N_2O_4$ $[M]^+$: m/z = 104.2, found 104.1; $[M]^-$: m/z = 152.1, found 151.8; Elemental analysis: calc. for $C_{12}H_{20}N_2O_4$: C, 56.2, H, 7.9, N, 10.9, found C, 56.6, H, 8.0, N, 10.8%.

**Cholinium Pyrazinate ([$N_{1112OH}$][Pyrazinate])**

$\delta_H$(400 MHz; $D_2O$) 3.12 (9H, s, *a*), 3.37–3.47 (2H, m, *b*), 3.98 (2H, ddd, $J$ = 6.9, 5.3, 2.8 Hz, *c*), 8.615 (1H, s, *g*), 8.62 (1H, d, $J$ = 2.5 Hz, *f*), 9.01 (1H, d, $J$ = 1.1 Hz, *e*); $\delta_C$(100 MHz; $D_2O$) 53.8 (t, *a*), 55.6 (s, *c*), 67.4 (t, *b*), 144.1 (s, *d*), 144.4 (s, *g*), 145.7 (s, *e*), 148.2 (s, *f*), 170.4 (s, *h*); ESI-MS: calc. for $C_{10}H_{17}N_3O_3$ $[M]^+$: m/z = 104.2, found 104.1; $[M]^-$: m/z = 123.1, found 122.7; Elemental analysis: calc. for $C_{10}H_{17}N_3O_3$: C, 52.85, H, 7.5, N, 18.5, found C, 52.3, H, 7.3, N, 18.2%.

**Cholinium Picolinate ([$N_{1112OH}$][Picolinate])**

$\delta_H$(400 MHz; $D_2O$) 3.09 (9H, s, *a*), 3.37–3.45 (2H, m, *b*), 3.87–4.00 (2H, m, *c*), 7.49 (1H, ddd, $J$ = 7.4, 5.0, 1.3 Hz, *e*), 7.85 (1H, d, $J$ = 7.8 Hz, *f*), 7.92 (1H, td, $J$ = 7.7, 1.7 Hz, *g*), 8.48 (1H, d, $J$ = 4.8 Hz, *h*); $\delta_C$(100 MHz; $D_2O$) 53.8 (t, *a*), 55.6 (s, *c*), 67.4 (t, *b*), 123.9 (s, *e*), 126.0 (s, *g*), 139.0 (s, *f*), 147.6 (s, *h*), 152.4 (s, *d*), 172.2 (s, *i*); ESI-MS: calc. for $C_{11}H_{18}N_2O_3$

$[M]^+$: m/z = 104.2, found 104.1; $[M]^-$: m/z = 122.1, found 122.2; Elemental analysis: calc. for $C_{11}H_{18}N_2O_3$: C, 58.4, H, 8.0, N, 12.4, found C, 58.9, H, 8.5, N, 12.1%.

# Thermal Characterization of the Cholinium-based API-ILs

Measurements of phase-transitions of the cholinium-based API-ILs were done with a TA Instrument DSC Q200 Differential Scanning Calorimeter. Cooling was accomplished by using a refrigerated cooling system capable of controlling the temperature down to -90 °C. The sample was continuously purged with 50 ml/min dry dinitrogen gas. About 5 to 10 mg of each API-IL was crimped in an aluminum standard sample pan. Indium ($T_m$ = 156.61 °C) was used as the standard compound for the calibration of the DSC. The cholinium-based API-IL samples was cooled to -90 °C and tempered (30 min) and finally heated to 100 °C (140 °C for $[N_{1112OH}]$[4-Amino-salicylate]). The cooling-heating cycles were repeated three times at different rates (10 °C/min, 5 °C/min and 1 °C/min). The transition temperatures obtained from the second and subsequent cycles at the same rate were reproducible. The thermal properties (melting points and glass transition temperatures) of the cholinium-based API-ILs prepared in this work are summarized in Table 1 (cf. Manuscript).

The decomposition temperatures of the cholinium-based API-ILs were measured with a TA Instrument Model TGA Q50, using nitrogen atmosphere at a flow rate of 60 ml/min. All samples were run in aluminium pans and heated to 600 °C at a rate of 1 °C/min until complete thermal degradation was achieved. Universal Analysis version 4.4A software was used to determined the onset decomposition temperature ($T_{onset}$), which is the intersection of the baseline weight with the tangent of the weight vs temperature curve during heating. The onset points of decomposition ($T_{onset}$) are listed in Table 1 (cf. Manuscript).

## Solubility

An excess of cholinium-based API-ILs and parent APIs were added to 0.5 ml of each solvent (Milli-Q water and buffer solutions suitable for dissolution testing, such as simulated gastric fluid without enzymes–interchangeable with 0.1 N HCl (pH 1.0), simulated intestinal fluid without enzymes–interchangeable with phosphate standard buffer pH 6.8, and 0.15M NaCl–isotonic ionic strength) in 1.5 ml safe-lock test tubes. These mixtures were placed in a Thermomixer Comfort (Eppendorf 1.5 ml) and kept under controlled temperature at 25 °C and 37 °C (±0.1 °C), whilst stirring at up to 1400 rpm. A study of the amount of cholinium-based API-ILs and parent APIs solubilized in the solvents over time ensured that the equilibrium was achieved. Before sampling, each solution was centrifuged for a short time (1 min), at isothermal conditions, to enhance the physical separation of the two phases. After this, the supernatant solutions were filtered (at isothermal conditions), to ensure that they were free of particulate matter. Concentrations were determined by measuring UV-vis spectroscopy using a Shimadzu Pharma-Spec spectrophotometer (model UV-1800), after appropriate dilution and interpolation from previously constructed calibration curves for cholinium-based API-ILs and parent APIs in Milli-Q water and buffer solutions. All the solubilities experiments were repeated at least three times.

## *In Vitro* Cytotoxicity Assays

*In vitro* cell toxicity test were performed using human colon adenocarcinoma Caco-2 cells and human hepatoma HepG2 cells. Caco-2 cells were routinely grown in RPMI 1640 supplemented with 10% of inactivated FBS, 2 mM of glutamine and 5000U of penicillin-streptomycin and human hepatocellular carcinoma cell line HepG2 was cultured in MEM with 10% of inactivated FBS, 2 mM Glutamine, 1% MEM-NEAA and 1% sodium piruvate. Stock of both cells were maintained as monolayers in 175 $cm^2$ culture flasks and incubated at 37 °C in a 5% CO2 humidified atmosphere. Briefly, Caco-2 and HepG2 cells were seeded at a density of 2 x $10^4$ cells/well and 6 x $10^4$ cells/well, respectively in 96-well plates and their media was replaced every 48 h. Caco-2 cells experiments were performed using cells after reaching

confluence (± 96 h after seeding) and HepG2 cells experiments were performed 24 h after seeding. Stock solutions of the API-ILs and parent APIs were prepared in Milli-Q water and all the API-ILs/parent APIs were homogenous in solution even when diluted in culture media containing 0.5% FBS. Caco-2 and HepG2 cells were incubated for 24 h with the API-ILs and parent APIs dilutions and control cells were performed using culture media (0.5% FBS and 2 mM glutamine or 5% FBS, 2 mM glutamine, 1% MEM-NEAA and 1% sodium piruvate for Caco-2 and HepG2 cells respectively) without supplementation with API-ILs or parent APIs.

After 24 hours of incubation, the samples were removed and 100 $\mu$L of a CellTiter 96®AQueous One Solution Cell Proliferation Assay reagent (containing MTS and PES) was added to each well and left to react for 4 h. This solution reagent contains a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and an electron-coupling reagent (phenazine ethosulfate, PES). PES has an enhanced chemical stability which allows it to be combined with MTS, leading to a stable solution. MTS is bio-reduced by cells into a coloured formazan product that is soluble in the tissue culture medium. Formazan was quantified spectrophotometrically at 490 nm in a BioTek FLx800 microplate reader (BioTek, USA). Each sample was incubated in three different wells and the obtained value was the average of three independent assays. Cell viability was determined by the ratio between the measured absorbance of the API-ILs/parent APIs-contacted cells and the measured absorbance of control. Results were expressed in terms of percentage of cellular viability relative to control (%).

# Figures

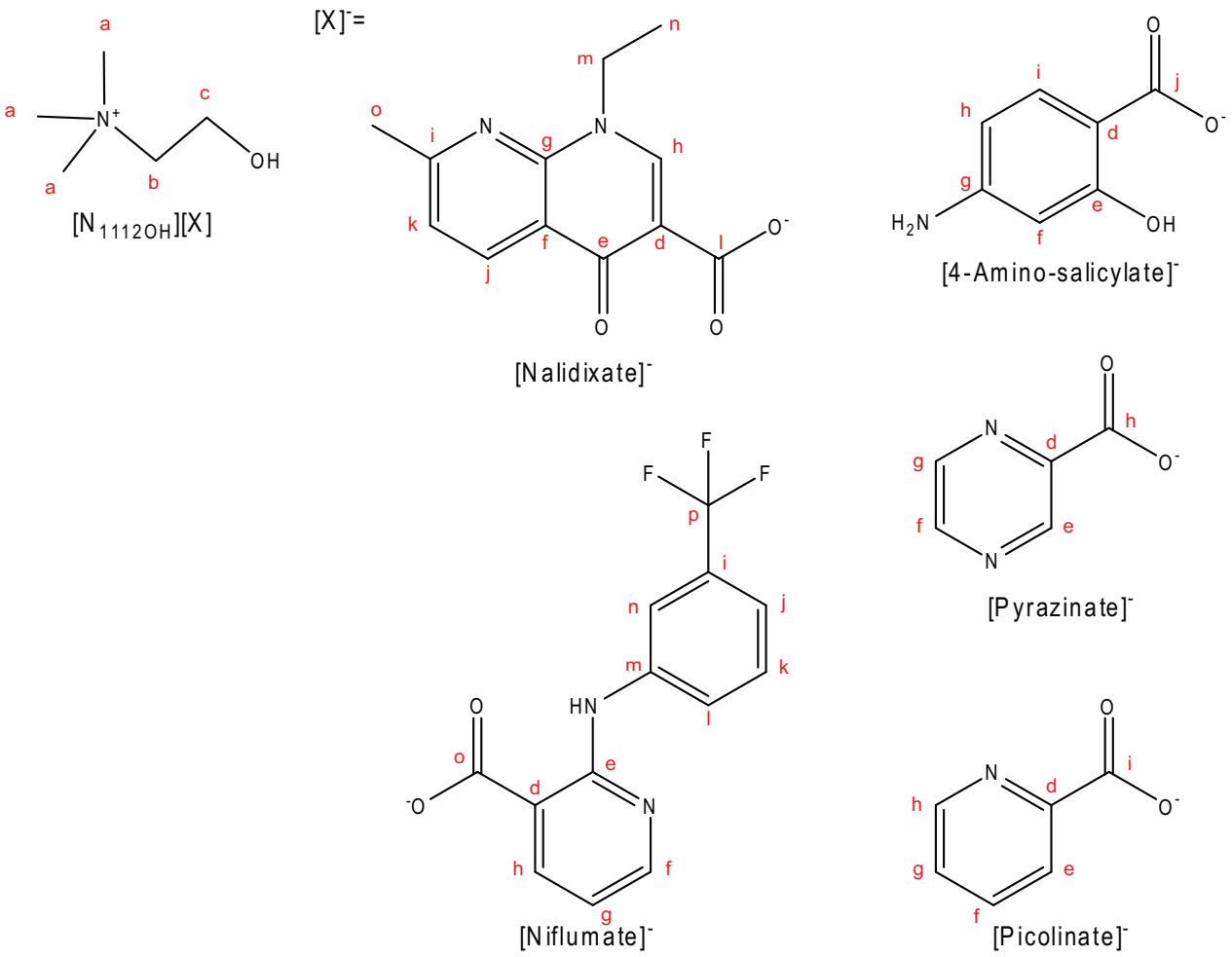


Figure S1: Prepared cholinium-based API-ILs with atom numbering.

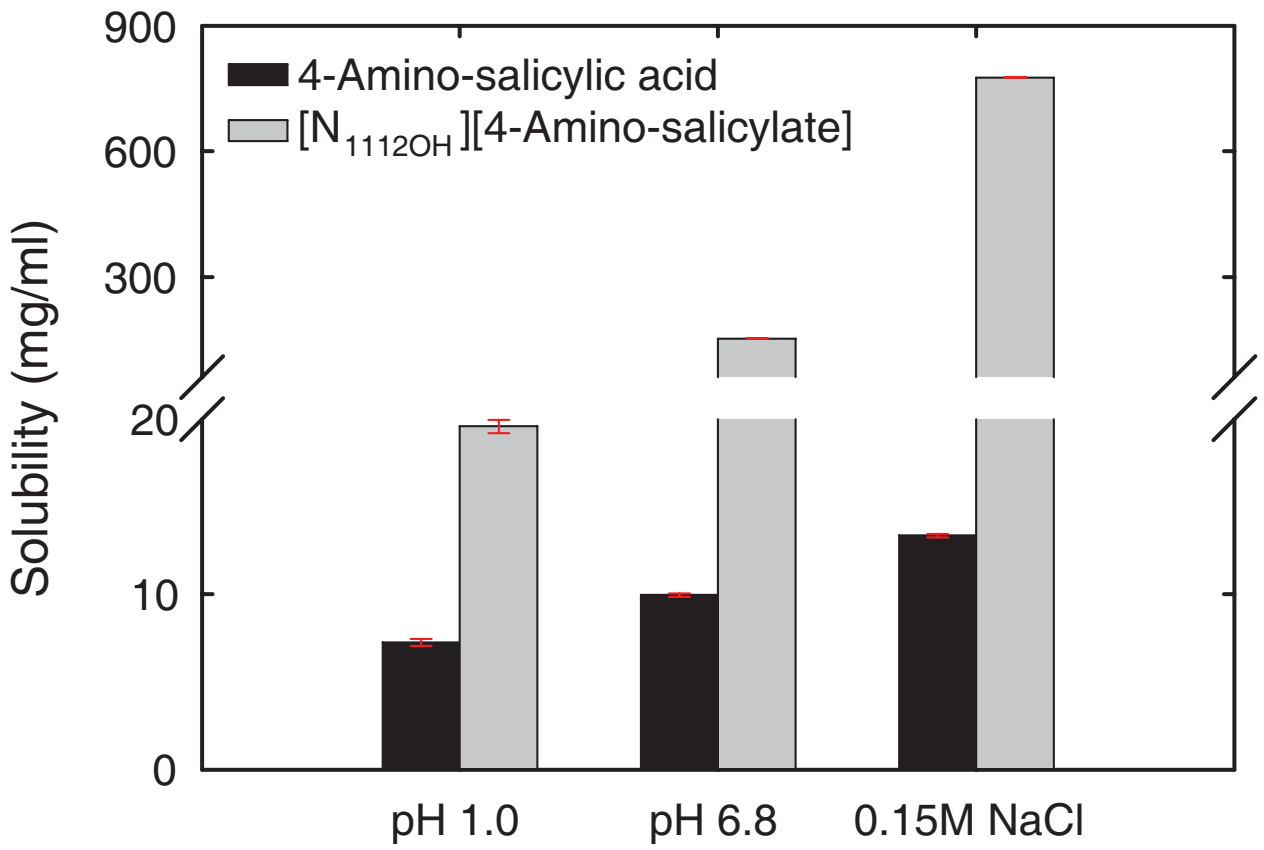


Figure S2: Solubility of $[N_{1112OH}]$[4-Amino-salicylate] and 4-Amino-salicylic acid (parent API) in buffer solutions suitable for dissolution testing, simulated gastric fluid (pH 1.0), simulated intestinal fluid (pH 6.8) and isotonic ionic strength aqueous solution (0.15M NaCl), at 37 °C.

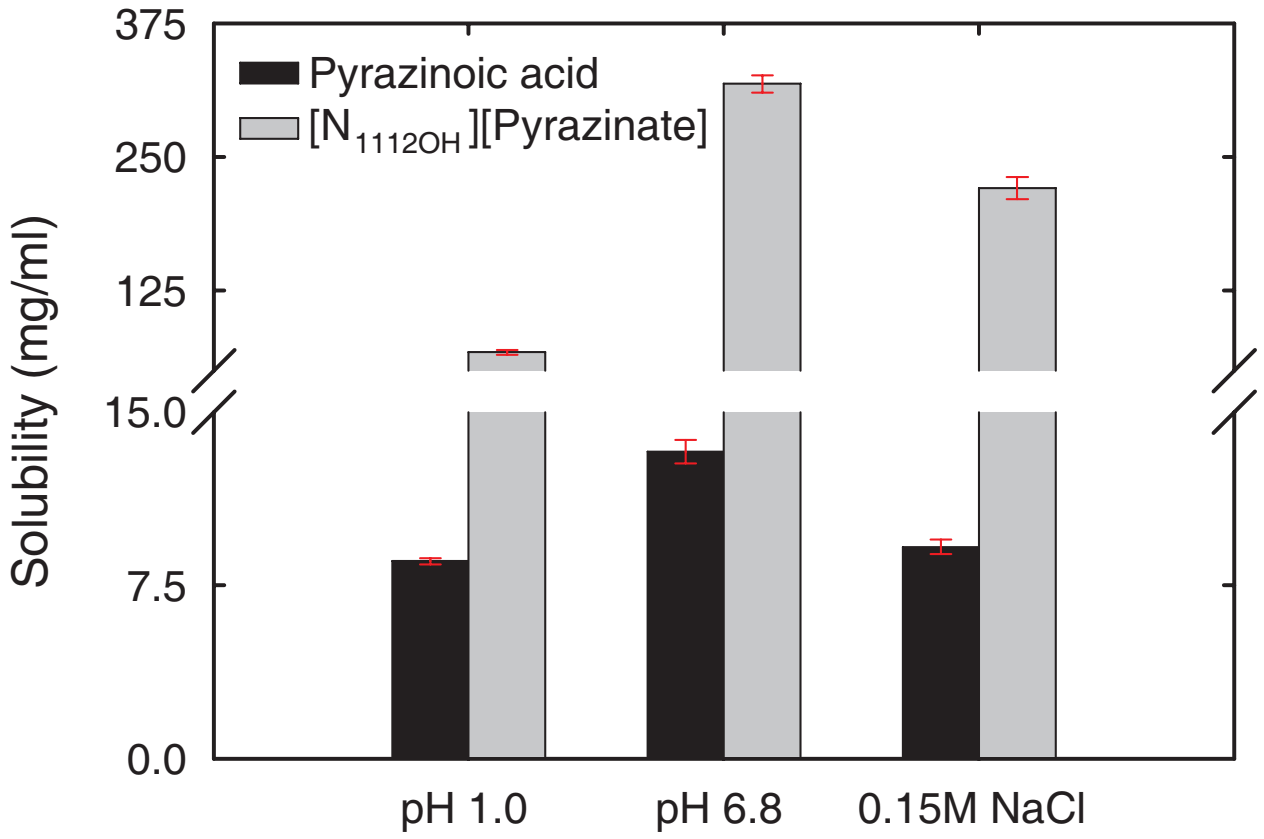


Figure S3: Solubility of $[N_{1112OH}][Pyrazinate]$ and pyrazinoic acid (parent API) in buffer solutions suitable for dissolution testing, simulated gastric fluid (pH 1.0), simulated intestinal fluid (pH 6.8) and isotonic ionic strength aqueous solution (0.15M NaCl), at 37 °C.

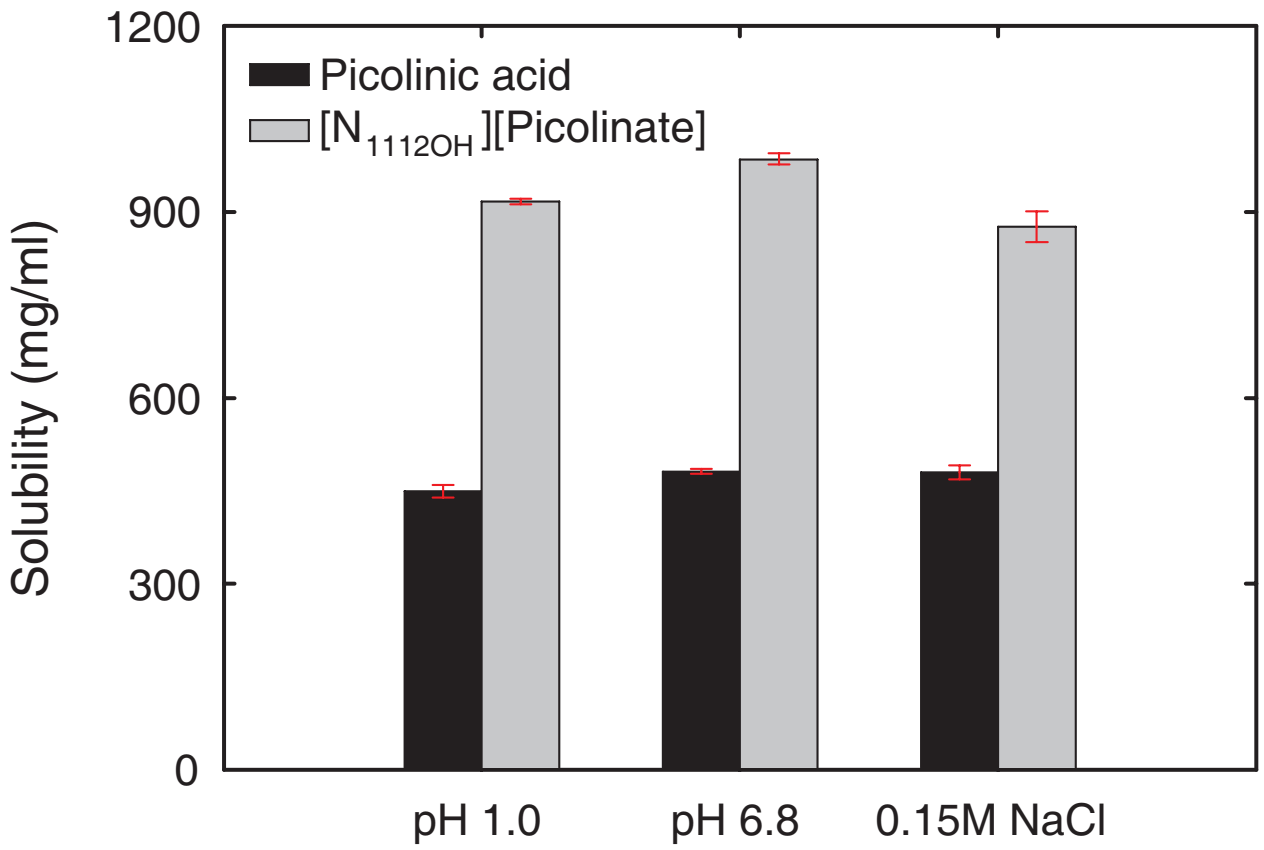


Figure S4: Solubility of $[N_{1112OH}][Picolinate]$ and picolinic acid (parent API) in buffer solutions suitable for dissolution testing, simulated gastric fluid (pH 1.0), simulated intestinal fluid (pH 6.8) and isotonic ionic strength aqueous solution (0.15M NaCl), at 37 °C.

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