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Fluoroquinolone-Based Organic Salts and Ionic Liquids as Highly Bioavailable Broad-Spectrum Antimicrobials †

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Abstract: The majority of antibiotics display low oral bioavailability due to reduced solubility in water and/or inefficient permeability across biological membranes. Their efficiency is further limited by the frequent presence of polymorphic structures with different pharmaceutical activities. In this communication, we present our latest results on the development of organic salts and ionic liquids from fluoroquinolones (FQ-OSILs) as highly efficient ionic formulations of this family of antimicrobials. Ciprofloxacin and norfloxacin were used as anions and as cations, by combination with biocompatible organic counter-ions. *In vitro* bioavailability studies showed that all prepared FQ-OSILs presented higher solubility in water than the original drugs. All compounds were found to be isomorphic and with tailorable antimicrobial activity according to the cation–anion combination against *Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumoniae* strains.

Keywords: active pharmaceutical ingredients as organic salts and ionic liquids (API–OSILs); antibiotics; ciprofloxacin; fluoroquinolones; ionic liquids; norfloxacin; polymorphism

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1. Introduction

The development of novel antibiotics did not present significant progress over the last years due to low returns, in particular regarding clinical trials. Norfloxacin [1] and ciprofloxacin [2] are the two pioneering antimicrobials from the fluoroquinolone family, which are primarily effective against gram-negative bacteria [3]. Typically, fluoroquinolones as well as many other active pharmaceutical ingredients (APIs) display spontaneous conversion into different polymorphs with different pharmaceutical profiles [4]. Crystal structures of neutral and zwitterionic forms of norfloxacin and ciprofloxacin have been described by Nikaido and Thanassi, with considerable differences in bioavailability [5]. Several different protolytic forms (anionic, neutral, zwitterionic, and cationic) presented by these drugs negatively influence their bioavailability in physiological media, which is highly dependent on their low solubility in water [6,7].

For more than a decade, active pharmaceutical ingredients as organic salts and ionic liquids (API-OSILs) have arisen in academia as an alternative formulation for low bioavailable drugs [8]. This third generation of ionic liquids consists of the combination of APIs as cations or anions, according to their functional groups, with organic counter-ions. This type of transformation has rendered distinctive physicochemical properties over the

original drugs and reduced toxicity to healthy cells, thus enhancing the pharmaceutical activity of the API [9–16]. Our works involving the preparation of API-OSILs from β-lactam [9–12] and fluoroquinolone [13,14] antibiotics, 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 therapeutic scope. Furthermore, the stability and solubility in physiological media of the API can be significantly altered, yielding novel formulations with different pharmacokinetics and potentially different delivery modes and applications [15,16]. In addition, the polymorphic tendency of an API can be considerably reduced, or even eliminated, hence tackling one of the most important issues in the pharmaceutical industry responsible for limitations in a drug's solubility and dosages.

The formation of organic salts based on cationic fluoroquinolones (mostly comprising ciprofloxacin (Cip), but also norfloxacin (Nor)) has been widely explored by the academic community by protonation of drugs' amine group(s). More precisely, these fluoroquinolones have been combined with several dicarboxylic acids [17–21], citric acid [17], as well as with other drugs [22–24] in order to assess bioavailability, crystallinity/amorphous profiles, and thermodynamic stability. Some works involving the combination of ciprofloxacin or enrofloxacin as cations and/or as anions, by combination with amino acids [25] and ionic polymers [26,27], have indeed presented, for the first time, enhanced antimicrobial activity of the salts in comparison with the parent drugs, in particular against gram-negative bacteria strains.

In this communication, we present our works on the synthesis and characterization of novel API-OSILs from Cip and Nor as cations [13] and as anions [14]. In the former, we combined both antimicrobials with methanesulfonate [Mes], gluconate [Glu], and glycolate [Gly] biocompatible anions; while in the latter, the combinations were performed with choline [Ch], cetylpyridinium [C_{16}Py] and *N*-methylimidazolium derivatives [xMIM]. The synthesized FQ-OSILs were characterized by NMR (^{1}H and ^{13}C) and FTIR spectroscopic techniques, elemental analysis, as well as differential scanning calorimetry (DSC). Water solubility at 25 and 37 °C was experimentally determined for the FQ-OSILs, with the exception of the ones containing the [C_{16}Py] cation. Finally, the antimicrobial activity of all FQ-OSILs on gram-negative (*Klebsiella pneumoniae*) and gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) bacteria was determined and presented here for the first time for the cationic salts.

2. Experiments

2.1. Materials and Methods

2.1.1. General Procedure for the Preparation of Cationic FQ-OSILs

A 1 M solution of the organic acid was added in stoichiometric amounts to a methanolic solution of ciprofloxacin or norfloxacin. After magnetically stirring for 24 h at room temperature, the solvent was evaporated using a rotavapor, and the desired FQ-OSIL was obtained in quantitative yield after drying in *vacuo* for 24 h [13].

2.1.2. General Procedure for the Preparation of Anionic FQ-OSILs

The halide salt was dissolved in methanol and passed through an ion-exchange column, Amberlite IRA-400-OH (5 eq., flux rate 0.133 mL/mL min). The methanolic solution of the corresponding hydroxide salt was added dropwise to ciprofloxacin or norfloxacin (1.2 eq) and completely dissolved in 2 M ammonia solution (50 mL/g). After stirring the mixture for 1 h, the solvent was evaporated in the rotavapor and the crude was recrystallized from a mixture of chloroform/methanol. Excess fluoroquinolone was filtered off, the solution was evaporated, and the desired FQ-OSIL was dried in *vacuo* for 24 h [14].

2.1.3. Determination of the Solubility in Water

An excess amount of compound was added to the corresponding solvent in 1.5 mL vials. Vials were kept under vigorous stirring at 1400 rpm and controlled temperatures (25 or 37 °C ± 0.1 °C) using a Thermomixer Comfort (eppendorf 1.5 mL) and collected at different time periods. The samples were centrifuged in a Biofuge 28 RS, Heraeus Sepatech, for 10 min at 14,000 rpm, in order to enhance physical separation of different phases. The quantification of the liquid phase was accomplished by UV-vis spectroscopy (model UV-1800—Pharma-Spec spectrophotometer from Shimadzu), with quartz cells at a wavelength of 273 (ciprofloxacin) and 275 nm (norfloxacin). Every experiment was conducted in triplicate, and a calibration curve was previously established for each compound (for all curves $R^2 > 0.998$).

2.1.4. Cytotoxicity of Compounds

Cytotoxic activities of FQ-OSILs were studied on 3T3 cells previously acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) biobank (Germany). Cells were cultured in Dulbecco's Modified Eagle's medium: Nutrient Mix F-12 (DMEM/F-12; Merck, Germany) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), GlutaMAX™ (Gibco, Grand Island, NY, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma, USA). For subculture, 3T3 cells were dissociated using trypsin-ethylenediamine tetraacetic acid (Sigma, USA), split in a 1:5 ratio, and seeded into Petri dishes with 25 cm^2 of growth area. Culture medium was replaced every 2 days until cells reached the total confluence after 3–5 days of initial seeding. Cells were maintained in a humidified atmosphere (95%), 5% CO_2, and 37 °C. Cytotoxicity was evaluated on 3T3 cells' viability after cells reached total confluence. Cells were treated with samples at 10 μM for 24 h. Samples effects were assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Germany), and the results were expressed in percentage of control (%).

2.1.5. Antimicrobial Activities

Antimicrobial activity of FQ-OSILs was evaluated against *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25,923) grown in Lysogeny broth (LB) and *Klebsiella pneumoniae* (ATCC 11,296) grown in Tryptic soy broth. All mediums were obtained from Merck (Darmstadt, Germany). The antimicrobial activity was accompanied by optical density at 600 nm, in order to verify the ability of compounds to inhibit the microorganisms' growth (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Vermont, USA). The IC_{50} was determined (0.001–10 nM) for the most potent samples (microorganism growth inhibition >50% at 10 μM). Results were expressed in percentage of growth inhibition relative to the control (growth medium with microorganism).

2.1.6. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relative to control treatment. Results are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at a level of 0.05 (p-value < 0.05). The determination of IC_{50} was performed by the analysis of non-linear regression by means of the equation:

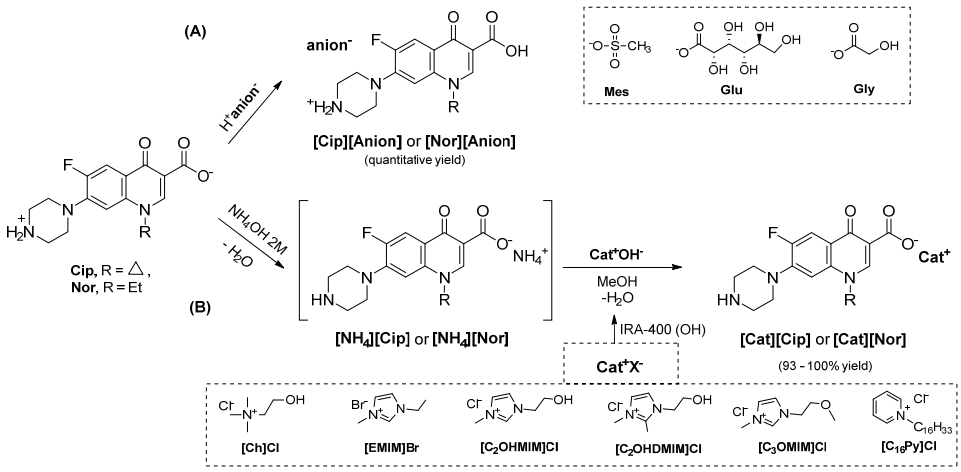
$$y = \frac{100}{[1+10(X-log(IC_{50}))]}$$

Calculations were performed using GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) software.

3. Results and Discussion

3.1. Synthesis

Ciprofloxacin and norfloxacin were combined as anions and as cations with biocompatible counter-ions by following well-established methodologies. In the case of the cationic FQ-OSILs, methanolic solutions of the organic acids (mesylic, gluconic, and glycolic) were added to a solution of the fluoroquinolones, yielding the corresponding FQ-OSILs in quantitative yields (Scheme 1A). As for the anionic FQ-OSILs, we followed an in-house developed methodology for the preparation of API-OSILs from zwitterionic drugs [12,15]. More precisely, we dissolved the drugs in ammonia buffered solution in order to prepare the corresponding ammonium salts *in situ*, which were subsequently exchanged with choline [Ch], 1-ethyl-3-methylimidazolium [EMIM], 1-hydroxy-ethyl-3-methylimidazolium [C_2OHMIM], 1-(2-hydroxyethyl)-2,3-dimethylimidazolium [C_2OHDMIM], 1-(2-methoxyethyl)-3-methylimidazolium [C_3OMIM], and cetylpyridinium [C_{16}Py] cations as hydroxide bases. These were freshly prepared from the corresponding halide cations by anion exchange using Amberlyst IRA-400 (OH^-) resin (Scheme 1B). The desired FQ-OSILs were obtained in 93–100% yields upon recrystallization.



Scheme 1. Synthetic methodology for the preparation of (**A**) cationic and (**B**) anionic Fluoroquinolone-based Organic Salts and Ionic Liquids (FQ-OSILs).

3.2. Spectroscopic Characterization

All products were characterized by ^{1}H and ^{13}C NMR and FTIR spectroscopic techniques, as well as elemental analysis.

In all cases, the ^{1}H NMR spectra showed that the cation/anion proportion was strictly 1.0:1.0, in agreement with the intended stoichiometry. In addition, only one set of signals was observed, meaning that the reactions were complete and only one product was formed.

Regarding the FTIR spectra of the cationic compounds, there were no clear signals consistent with the deprotonation of the organic acids due to superimposition with the drugs' amine signals at 1630 cm^{-1}. However, a signal at 1721 cm^{-1} was consistent with the presence of the fluoroquinolone's carboxylic acid group, which was absent in the FTIR spectra of the parent drugs. This observation was only achievable if the drugs' structure was no longer zwitterionic, and hence, it is protonated at the secondary nitrogen atom. In the case of the anionic FQ-OSILs, there were only slight changes in some vibrational frequencies, in particular from the free Cip and Nor carboxylate group (respectively 1589 and 1583 cm^{-1}) to 1581–1575 and 1583–1579 cm^{-1} in the respective final salts. In all cases, the 3600–3300 cm^{-1} area displayed strong signals in the spectra of the final compounds, as opposed to the initial ones, which may suggest the establishment of H-bonds between the cations and the anions.

3.3. Thermal Analysis

All prepared FQ-OSILs were studied by differential scanning calorimetry (DSC). Table 1 contains the obtained data, namely melting and glass transition temperatures, as well as the physical state at room temperature of the analysed compounds.

Table 1. Physical state at room temperature, melting (T_m) and glass transition (T_g) temperatures of ciprofloxacin, norfloxacin and corresponding OSILs.

Compound	Physical State	T_m/°C	T_g/°C
Cip	White solid	322.0	-
[Cip][Mes]	Pale yellow solid	145.5	125.2
[Cip][Glu]	Pale yellow solid	146 dec.	122.9
[Cip][Gly]	White solid	153.7	-
[Ch][Cip]	Pale yellow solid	111.2	-
[EMIM][Cip]	White solid	92.9	36.7
[C_2OHMIM][Cip]	White solid	111.6	49.5
[C_2OHDMIM][Cip]	White solid	197.5	-
[C_3OMIM][Cip]	White solid	112.4	46.4
[C_{16}Py][Cip]	Orange viscous liquid	-	-10.7
Nor	White solid	217.0	
[Nor][Mes]	Pale yellow solid	168.2	-
[Nor][Glu]	Yellow viscous liquid	-	1.8
[Nor][Gly]	White solid	115.3	91.6
[Ch][Nor]	Pale yellow solid	94.5	54.8
[EMIM][Nor]	Yellow solid	119.9	64.6
[C_2OHMIM][Nor]	Yellow viscous liquid	-	41.1
[C_2OHDMIM][Nor]	White solid	121.8	65.1
[C_3OMIM][Nor]	White viscous liquid	-	44.1
[C_{16}Py][Nor]	Yellow viscous liquid	-	5.9

All prepared salts displayed lower melting temperatures than the parent fluoroquinolones, as expected. In general, ciprofloxacin-based OSILs have a higher tendency to be solid at room temperature than norfloxacin ones. From the former, only [C_{16}Py][Cip] was found to be a room temperature ionic liquid (RTIL), while from the latter four were obtained as RTILs. All solid salts displayed no polymorphism in the studied conditions.

3.4. Water Solubility Studies

All FQ-OSILs presented higher solubility in water at 25 and 37 °C than the parent antibiotics (Figure 1).

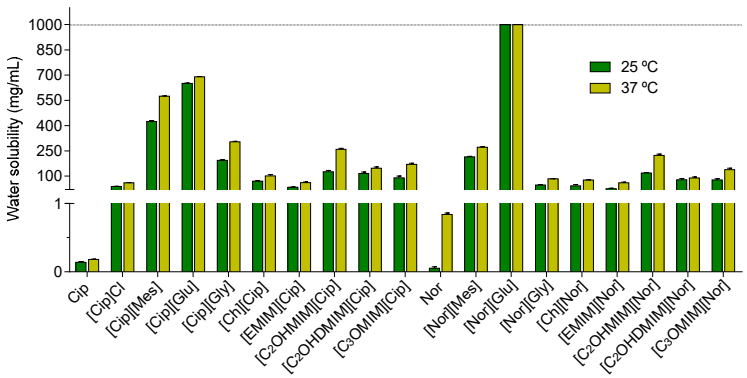


Figure 1. Water solubility at 25 °C (green) and 37 °C (yellow) for ciprofloxacin, norfloxacin, and corresponding OSILs. The dotted line represents the upper experimental threshold.

The general tendency points to less soluble anionic FQ-OSILs, in comparison with the cationic analogues. The most soluble salts based on both fluoroquinolones present the gluconate anion, which is consistent with the highly hydrophilic character of this carbohydrate. In fact, [Nor][Glu] is entirely soluble in water at both temperatures. As for the anionic FQ-OSILs, the observed general tendency is in agreement with the hydrophilic character of the studied cations.

3.5. Antimicrobial Activity Studies

The antimicrobial activity (IC_{50}) of the prepared FQ-OSILs and the corresponding starting materials were determined against gram-negative (*Klebsiella pneumoniae*) and gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria strains (Table 2).

Table 2. IC_{50} values (nM) for ciprofloxacin and corresponding OSILs (0.001–10 μM) against the tested microorganisms. The values in parentheses represent the confidence intervals for 95%.

Compounds	*K. pneumoniae*	$RDIC_{50}$	*S. aureus*	$RDIC_{50}$	*B. subtilis*	$RDIC_{50}$
Cip	196.50	-	29.16	-	3.84	-
[Cip][Mes]	**55.07**	3.6	31.96	0.91	**14.38**	0.3
[Cip][Glu]	**74.52**	2.6	29.81	1.0	**12.16**	0.3
[Cip][Gly]	**82.79**	2.4	24.06	1.2	**19.78**	0.2
[Ch][Cip]	**51.12**	3.8	181.40	0.2	**20.60**	0.2
[EMIM][Cip]	**64.80**	3.0	**24.24**	1.2	**16.17**	0.2
[C_2OHMIM][Cip]	**36.42**	5.4	61.95	0.5	**15.75**	0.2
[C_2OHDMIM][Cip]	**50.99**	3.9	**21.20**	1.4	**12.35**	0.3
[C_3OMIM][Cip]	**47.61**	4.1	82.05	0.4	**21.66**	0.2
[C_{16}Py][Cip]	**9.88**	19.9	1430	0.0	6.07	0.6
Nor	255.2	-	123.4		117.2	
[Nor][Mes]	**203.8**	1.3	230.3	0.5	65.12	1.8
[Nor][Glu]	225.2	1.1	176	0.7	98.67	1.2
[Nor][Gly]	242.4	1.1	118.2	1.0	67.49	1.7
[Ch][Nor]	**199.4**	1.3	127.1	1.0	95.49	1.2
[EMIM][Nor]	207.5	1.2	211.2	0.6	24.48	4.8
[C_2OHMIM][Nor]	201.0	1.3	91.70	1.3	88.61	1.3
[C_2OHDMIM][Nor]	**178.2**	1.4	**79.55**	1.6	**148.8**	0.8
[C_3OMIM][Nor]	224.5	1.1	106.7	1.1	68.12	1.7
[C_{16}Py][Nor]	**202.9**	1.3	**10.84**	11.4	87.29	1.3

In general, both anionic and cationic Cip-based OSILs were found to be more active against the pathogenic strains than the Nor analogues. The former were particularly active against *Klebsiella pneumoniae*, with relative decreases of IC_{50} ($RDIC_{50}$) between 2.4 ([Cip][Gly]) and 19.9 ([C_{16}Py][Cip]). [EMIM][Cip] and [C_2OHDMIM][Cip] also recorded 40% increased activity against *Staphylococcus aureus*. Moreover, decreased activity against commensal *Bacillus subtilis* was also desirably observable. The most promising Nor-based OSILs were [C_2OHDMIM][Nor], with a 40–60% increase in activity against the pathogenic strains and [C_{16}Py][Nor], with selective 11.4-fold increased activity against *S. aureus*.

4. Conclusions

In this work, we were able to show two simple and straightforward methods for the preparation of cationic and anionic non-polymorphic FQ-OSILs, which present very high water solubility and tailorable antimicrobial activities against sensitive pathogenic and commensal bacteria strains according to the established cation–anion combinations. These results may pave the way for the future development of personalized ionic formulations of antimicrobials with selective or broad-spectrum activities, according to patient need.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

API	Active Pharmaceutical Ingredient
API–OSILs	Active Pharmaceutical Ingredient Organic Salts and Ionic Liquids
C_{16}Py	cetylpyridinium
C_2OHDMIM	1-(2-hydroxyethyl)-2,3-dimethylimidazolium
C_2OHMIM	1-(2-hydroxyethyl)-3-methylimidazolium
C_3OMIM	1-(2-methoxyethyl)-3-methylimidazolium
Ch	choline
Cip	ciprofloxacin
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EMIM	1-ethyl-3-methylimidazolium
FQ	fluoroquinolone
FQ-OSILs	fluoroquinolone-based organic salts and ionic liquids
IC_{50}	half maximal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
Nor	norfloxacin
OSIL	Organic Salt and Ionic Liquid
$RDIC_{50}$	Relative Decrease in Inhibitory Concentration for 50% bacterial growth
RTILs	Room Temperature Ionic Liquids
T_g	glass transition temperature
T_m	melting temperature

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