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Antiproliferative Organic Salts Derived from Betulinic Acid: Disclosure of an Ionic Liquid Selective Against Lung and Liver Cancer Cells

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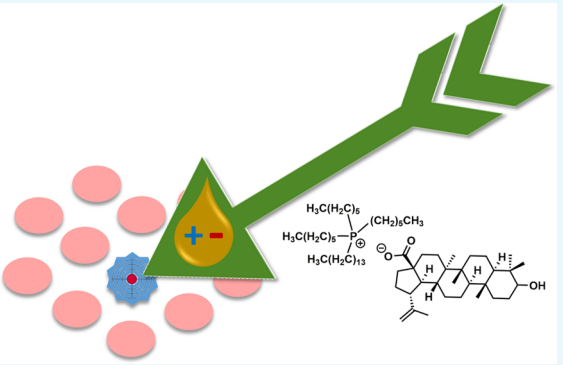
Supporting Information

ABSTRACT: In the last few years, we have been witnessing an increasing interest in ionic liquids (ILs) and organic salts, given their potential applications in biological and pharmaceutical sciences. We report the synthesis and in vitro evaluation of novel organic salts combining betulinate, known for its anticancer properties, with antimalarial drugs, primaquine, chloroquine, and mepacrine, and also with the trihexyltetradecylphosphonium ([$P_{6,6,6,14}$]) cation. The salts were screened for their in vitro activity against tumor lines HepG2 (liver), MG63 (osteosarcoma), T47D (breast), A459 (lung), and RKO (colon), and also on normal human fibroblasts. All betulinates prepared displayed antiproliferative properties, with the trihexyltetradecylphosphonium betulinate standing out for its higher selectivity. This unprecedented disclosure of a betulinic acid (BA)-derived IL with selective antitumor activity constitutes a relevant first step toward development of novel anticancer therapies based on BA-derived IL.



1. INTRODUCTION

Ionic Liquids (ILs) derived from active pharmaceutical ingredients (API) may open new perspectives toward low-cost rescuing or repurposing of classical drugs, given that most available API are found in the cationic or anionic form.[1−3] Therefore, the use of ILs has great potential to help the pharmaceutical industry to overcome two of its most concerning issues: solubility and polymorphism. In medicines, API may be single- or multicomponent, such as, for example, solvates (active + solvent), salts (active + counterion), or co-crystals (active + crystal co-forming component), among others.[4,5] In the last two cases, as in any crystalline solid, polymorphism may arise; and, even in solvated API pseudo-polymorphism may occur. This means that distinct polymorphs may exhibit different properties that may influence both the bioactivity and bioavailability of the drug. As such, different therapeutic windows may be associated with distinct polymorphic phases, which requires dose adjustment; moreover, there is a risk of toxicity arising from the appearance of a different undetected polymorphic phase that can cause harmful effects to patients. This situation has led to product failures and lengthy patent litigation.[2,5−7] Whenever this kind of problem appears, and because most drugs have at least one ionizable functional group, the most common strategy is formation of a salt of that drug, that is, conjugation of the drug with a counterion as this represents, in most cases, an improvement over the neutral drug. Thus, today, about 50% of the drugs are administered as salts.[2,8−11]

The formation of organic salts, including ILs, derived from API provides a simple tool to tune physical properties such as thermal stability, crystallinity, hygroscopicity, water solubility, and bioavailability. Thus, conversion of a neutral drug into a convenient organic salt often results in improved physical, chemical, and biological characteristics.[5,9,12,13] One example is that of antimalarial drugs, most of which are administered as salts (e.g., primaquine (PQ) bisphosphate, chloroquine (CQ)

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bisphosphate, mepacrine (MP) hydrochloride, proguanil hydrochloride, and sodium artesunate, among others).[14,15] As such, antimalarial API can be combined with either an inert counterion or a counterion displaying additional biological properties, eventually producing novel drug-derived ILs (API-IL) with therapeutic interest. In this connection, and following our long-term research focus in rescuing and repurposing antimalarial drugs such as PQ, (**1**),[16−22] CQ, (**2**),[23,24] or MP, (**3**)[25−27] (Figure 1) we have recently developed PQ-derived ILs with improved biological activity compared to the parent compounds.[28]

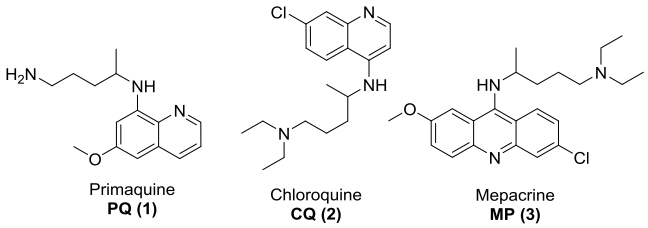

Figure 1. Primaquine (PQ, **1**), chloroquine (CQ, **2**), and mepacrine (MP, **3**).

Previous reports, both by us and by others, have highlighted the potential of repurposing antimalarials for cancer.[26,29−32] Some of the works recently conducted in this topic suggest that, more than the intrinsic antiproliferative properties of the antimalarials, it is their role as tumor cell sensitizers, able to potentiate the therapeutic action of available antitumor drugs, that makes them valuable tools to fight cancer.[30,31] On the basis of these reports, we reasoned that a combination of classical antimalarials such as PQ, CQ, or MP with the antitumoral compound betulinic acid (BA, **4** in Figure 2) might produce organic salts with improved water solubility as well as enhanced antiproliferative activity because of a possible sensitization of cancer cells to BA.

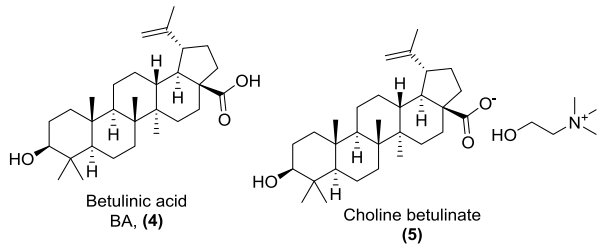

Figure 2. Structures of BA (**4**) and choline betulinate ([choline][BA], **5**).

The choice of BA, a triterpenoid that can be obtained from renewable sources and displays a variety of biological properties, namely, anti-inflammatory,[38,39] anti-HIV,[40,41] antimalarial,[42,43] immunomodulatory,[44,45] antiangiogenic,[46,47] antifibrotic,[48,49] and hepatoprotective,[50] was based on its reported cytotoxicity to tumor cell lines such as lung, ovary, neuroblastoma, and glioblastoma. Moreover, BA has quite low water solubility, so suitable formulations are needed to enable its future valorization as an API; previous reports suggest that BA-based organic cations and ILs may help to meet this goal, as choline betulinate (**5**, Figure 2) was found be at least 100 times more soluble in water than free BA.[50−53]

In view of the above, we pursued the chemical synthesis and in vitro evaluation of the antiproliferative properties of PQ, CQ, and MP betulinates. Trihexyltetradecylphosphonium ($[P_{6,6,6,14}]$) betulinate was also produced, based on the long-known anticancer potential of phosphonium salts.[33−37] Hopefully, a combination of the betulinate anion with organic cations, as the protonated forms of the aforementioned antimalarial drugs or the trihexyltetradecylphosphonium cation, might deliver organic salts with improved solubility, and a synergetic effect against tumor cell lines.[54] The target organic salts were synthesized and evaluated in vitro for their activity on one normal and five tumoral cell lines. The most interesting compound was further tested for its effects on important signaling pathways in cancer: the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK, or simply MEK) pathway, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, and the c-Jun N-terminal kinases (JNK) pathway.[55−60]

2. RESULTS AND DISCUSSION

2.1. Chemical Synthesis. There are two main methods for the chemical synthesis of ILs and organic salts, namely, metathesis and acid-base neutralization. While metathesis has some issues regarding yield and purity of the target products, acid-base neutralization is the "most intuitive" method, as it rests on a basic principle of chemistry, which is the direct neutralization of an acid with a base.[1,55−58] Moreover, the neutralization method is more advantageous for industrial applications, as it can be easily implemented on a large scale.[1,59,60] As such, we have been successfully employing this method for the synthesis of ILs and organic salts derived from different API.[1,28]

The target organic salts derived from BA and the antimalarial drugs [PQ][BA], [CQ][BA], and [MP][BA] (Figure 3a) were thus synthesized by the neutralization method, as previously reported,[28] whereby each of the basic antimalarials was reacted with an equimolar amount of BA.[1,28] For the chemical synthesis of $[P_{6,6,6,14}][BA]$ (Figure 3b), the commercially available chloride salt $[P_{6,6,6,14}][Cl]$ was converted into the hydroxide, using an Amberlyst anion exchange resin (A26-OH), prior to the neutralization reaction with BA. These procedures delivered the target ionic compounds in quantitative yields (Table 1) and free of detectable impurities, as confirmed by high-resolution mass spectrometry (HRMS) and by ^1H and ^{13}C NMR (see the Supporting Information).

An ^1H NMR analysis confirmed the complete proton transfer from BA to the basic building block, as the singlet at 12 ppm corresponding to the carboxylic proton in BA was not observed for any of the salts prepared, as illustrated by Figure 4, where the ^1H NMR spectra of AB, CQ, and [CQ][AB] in the 12.5−4.0 ppm region are overlapped; this superposition further shows an upfield shift of the vinylic protons in BA as a result of the neutralization process. Moreover, the expected cation/anion 1:1 ratio could be confirmed through a quantitative integration of characteristic ^1H resonance peaks from nonionizable protons, namely, either of the vinylic protons in betulinate (peaks at ca. 4.6 and 4.5 ppm, see Figure 4 and the Supporting Information) and either of the aromatic protons (peaks in the 8.5−6.0 ppm interval, see Figure 4 and the Supporting Information) in protonated PQ, CQ, or MP, or the methylic protons (at ca. 0.9 ppm, integrating to 12 protons) from the trihexyltetradecylphosphonium cation (see the Supporting Information).

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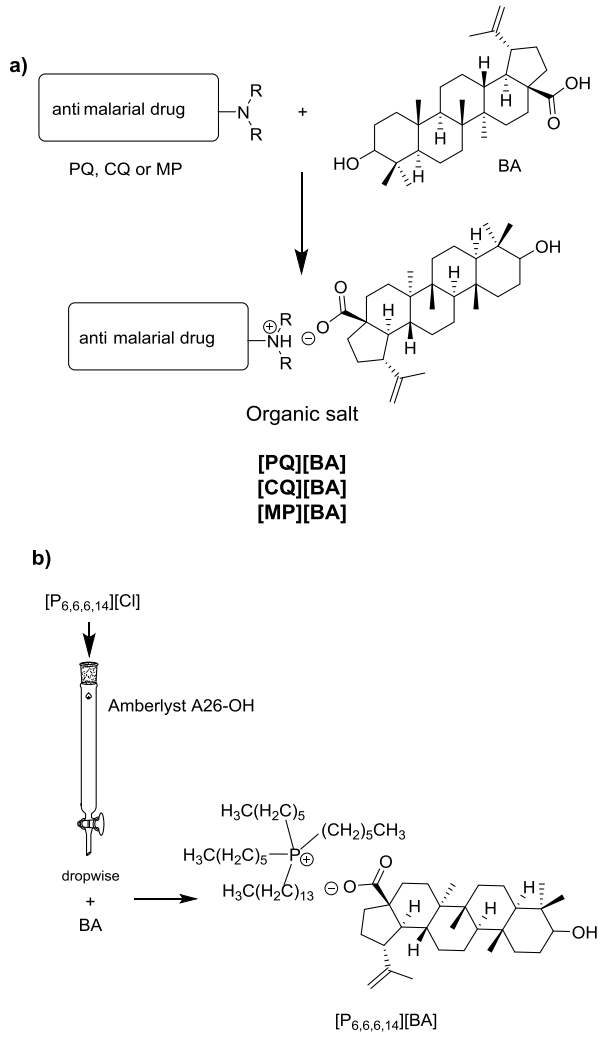

Figure 3. Synthetic route toward BA-derived organic salts, by combination with (a) antimalarials and (b) $[P_{6,6,6,14}]$.

Table 1. Yields and Aspect of the Target Organic Salts

compound	yield (%)	physical state	m.p.(dec)[a]/°C
[PQ][AB]	96.9	orange solid	170−175
[CQ][AB]	99.8	white solid	220−225
[MP][AB]	99.9	yellow solid	130−134
$[P_{6,6,6,14}]$[AB]	99.7	colorless liquid	

[a]Melting point (with observable decomposition).

Notably, as shown in Table 1, all but $[P_{6,6,6,14}][BA]$ were solid at room temperature, evidencing relatively high melting points, which were apparently accompanied by decomposition (drastic change in compound color at the melting temperature). In turn, $[P_{6,6,6,14}][BA]$ was a room-temperature IL.

2.2. Antiproliferative Activity in Vitro. Five tumor cell lines (RKO, T47D, MG63, A549, and HepG2) were used to evaluate the effects of the BA-derived salts on cell viability in vitro. The compounds were tested at 0.5, 5, 50, and 500 μM, and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method at 24 and 72 h. Human fibroblasts (HFF-1) were also used to assess compounds' selectivity, and BA was included for reference. All compounds displayed cytotoxic effects after 24 h, which were retained after 72 h in most cases (see Table S1, Supporting Information). The IL $[P_{6,6,6,14}][AB]$ had the most interesting behavior after 72 h (Table 2), as it was the least toxic to HFF-1 cells, while being cytotoxic to the cancer cell lines, particularly in the case of A549 (lung) and HepG2 (liver) cells. This holds great promise, as salts of $[P_{6,6,6,14}]$ have been reported as generally cytotoxic, in a nonselective fashion.[61−63]

2.3. Interference with Cellular Signaling Pathways. Given the interesting behavior of the IL $[P_{6,6,6,14}][BA]$, its effects on the modulation of three relevant intracellular signaling pathways, MEK, NF-kB, and JNK, were further investigated. The election of these particular signaling pathways was based on their reported relevance in tumorigenesis:[61−66] the MEK pathway is fundamental for neoplastic formation and development, and preclinical and laboratory studies support it as a viable target for the therapy of some types of cancer;[61,62] NF-kB regulates the expression of genes involved in many processes that appear to be critical for the development and progression of cancer, such as proliferation, migration, and apoptosis;[63,64] moreover, previous works suggest that BA might interfere with this pathway,[44] and the closely related Nrf2 pathway was recently found as relevant for the response of A549 and HepG2 cells, on which $[P_{6,6,6,14}][BA]$ was most active, to antitumoral compounds;[67] finally, the JNK signaling pathway, by being a regulator of proinflammatory genes, tissue remodeling, and apoptosis, constitutes an attractive target for new therapies in different pathological contexts.[65,66]

The ability of $[P_{6,6,6,14}][BA]$ to modulate the aforementioned signaling pathways was tested in vitro after 24 and 72 h, using suitable inhibitors of each of the pathways: U0126 for MEK, PDTC for NF-kB, and SP600125 for JNK, and the MTT method to assess cell viability (see Supporting

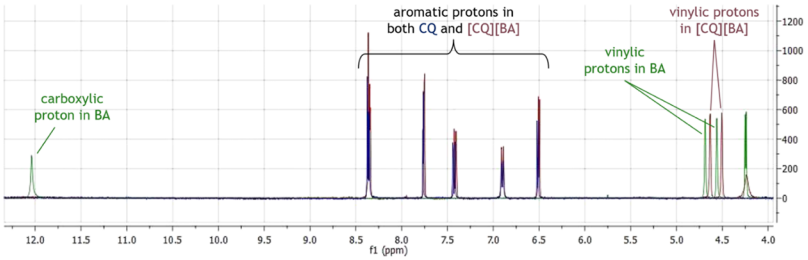

Figure 4. Superimposed ^{1}H NMR spectra of BA (green), CQ (blue), and [CQ][BA] (brown). The carboxylic proton peak at ca. 12 ppm exclusively observed for unmodified BA is missing in the spectrum of its [CQ][BA] salt, thus confirming the transfer of this proton to the basic building block. Peaks associated to the BA's vinylic protons further confirm changes because of the neutralization reaction, by being shifted upfield in the salt, as compared to unmodified BA.

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Table 2. IC_{50} Values (Mean ± SD, in μM) at 72 h, from Three Replicates in Three Independent Assays

compound	HFF-1	RKO	T47D	MG63	A549	HepG2
BA	24.4 ± 0.8	17.7 ± 0.9	11.7 ± 0.8	11.7 ± 0.8	6.2 ± 0.8	16.1 ± 0.9
[CQ][BA]	12 ± 1	15 ± 1	15 ± 1	nd	10.1 ± 0.9	12.3 ± 0.9
[PQ][BA]	11 ± 1	13 ± 1	11.6 ± 0.9	6 ± 1	10 ± 1	13.0 ± 0.9
[MP][BA]	nd[a]	nd	11 ± 1	5.3 ± 0.9	nd	4.1 ± 0.1
$[P_{6,6,6,14}][BA]$	101.2 ± 0.2	60.5 ± 0.5	68.1 ± 0.4	nd	19 ± 1	16.8 ± 0.9

[a]Consistent IC_{50} values could not be determined.

Table 3. Viability (Mean ± SD, as %) at 72 h of Cells Supplemented with Signaling Pathway Inhibitors, in the Absence (Control) or Presence of $[P_{6,6,6,14}][AB]$ at 50 μM

compound	inhibitor	HFF-1	RKO	T47D	MG63	A549	HepG2
control	U0126	99 ± 2	88 ± 3	101.6 ± 0.8	116.6 ± 0.8	94.5 ± 0.8	98 ± 2
	PDTC	58 ± 2	96 ± 3	94 ± 1	118.5 ± 0.9	88 ± 3	92 ± 1
	SP600125	92 ± 3	57 ± 4	84.4 ± 0.7	92.1 ± 0.5	101 ± 2	72.8 ± 0.6
$[P_{66614}][AB]$	U0126	97.2 ± 0.3	115 ± 1	105.9 ± 0.4	99.1 ± 0.5	98.0 ± 0.1	83.2 ± 0.7
	PDTC	80.4 ± 0.8	98.5 ± 0.9	80.2 ± 0.7	118.6 ± 0.4	131.0 ± 0.8	87.5 ± 0.6
	SP600125	83 ± 1	87 ± 2	80.9 ± 0.3	166.4 ± 0.3	109.0 ± 0.4	69.4 ± 0.3

Information, Table S2). Results obtained at 72 h are shown in Table 3, and suggest that:

- in the absence of $[P_{6,6,6,14}][BA]$, fibroblasts seem particularly dependent on the NF-kB pathway;
- addition of $[P_{6,6,6,14}][BA]$ to fibroblast cultures down-regulates NF-kB and upregulates JNK;
- JNK appears to be an important pathway for RKO, T47D, and HepG2 cells;
- upon addition of $[P_{6,6,6,14}][BA]$ to cancer cell cultures, RKO and HepG2 cell lines appeared to become less dependent on the JNK pathway, whereas T47D and HepG2 exhibited an upregulated NF-kB pathway;
- the three pathways seem affected in T47D cells upon addition of $[P_{6,6,6,14}][BA]$, but similar effects in the other cancer cell lines are not evident.

Overall, this differential behavior of cell signaling responses in fibroblasts as compared to, for example, HepG2, when treated with $[P_{6,6,6,14}][AB]$, may underlie the cancer versus normal cells' selectivity presented by this IL. Still, this preliminary assessment requires further investigation in order to draw conclusive interpretations.

3. CONCLUDING REMARKS

The physico-chemical properties, and particularly the tunability of organic salts and ILs, are of great interest for drug development.[9,68] The pharmaceutical industry first looked at ILs as "greener" alternates to solvents used in the synthesis of API[69] and, soon after, as potential co-adjuvants in drug formulations.[70−72] Upon discovery of bioactive organic salts, including API-ILs, many new ILs were developed as potential antineoplastic agents,[36,73−78] and several known ones were screened for their cytotoxicity to both normal and cancer cell lines from different species[13,53,79−85] Still, lack of sufficient knowledge on the possible mechanisms of antitumor action of ILs remains a major shortcoming toward rational design of novel anticancer ILs.[13] The present work represents initial, but promising steps toward the understanding of the antineoplastic potential of ILs and organic salts derived from a natural antitumor compound. Remarkably, all organic salts synthesized displayed antiproliferative activity in vitro, amongst which IL $[P_{6,6,6,14}][AB]$ stood out for its selectivity, compared with all the other organic salts and with the parent BA. A preliminary investigation on cellular signaling pathways eventually modulated by this IL did not show any marked trends; still, it suggested a differential response of human fibroblasts to $[P_{6,6,6,14}][AB]$, as compared to the other cell lines, which might be related to the considerably lower toxicity of this IL to normal cells, as compared to cancer ones.

We are aware that the antiproliferative activities displayed by the BA-derived organic salts herein reported are not extraordinary. However, the panel of tumor cells assayed is still a small one, and subsequent studies shall include a larger set of cancer cell lines, including drug-resistant ones. The same applies to the signaling pathways potentially affected by the test compounds, as a clearer picture on where these are interfering will guide the future choice of alternative cationic drugs to combine with BA in order to obtain more potent antiproliferative activity. On the other hand, one cannot forget that a major drawback in most anticancer therapies is the lack of selectivity that characterizes most of the potent chemotherapeutic agents currently used in the clinics, which makes the $[P_{6,6,6,14}][BA]$ IL herein reported a particularly relevant therapeutic hit. Moreover, conversion of any therapeutic agent that, like BA, is sparingly soluble in aqueous media, into bioactive organic salts with significantly improved water solubility, is by itself an important advance toward orally bioavailable drugs. Hence, taken together, these findings represent an important landmark toward future design and development of new anticancer agents, based on low-cost production of organic salts and ILs derived from antitumor compounds.

Overall, these are encouraging and unprecedented results disclosing the potential of BA-derived organic salts and ILs as a new type of antitumor agents, which deserve further development in the near future. Additional studies in this direction are under way, and will be timely reported.

4. EXPERIMENTAL SECTION

4.1. General Materials and Methods. PQ biphosphate, CQ phosphate, MP hydrochloride, BA, and tri-hexyltetradecylphosphonhium chloride were acquired from Sigma-Aldrich. Amberlyst A26-OH anion exchange resin was from Acros Organics. NMR analyses were carried out on a Brucker

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AVANCE III 400 MHz spectrometer, and samples were prepared in $(CD_3)_2SO$ with tetramethylsilane as an internal reference, from Euriso-Top. Chemical shifts are reported downfield in parts per million (ppm). Mass spectra were obtained on LTQ Orbitrap XL/LTQ Tune Plus 2.5.5/2.1.0 Xcalibur (Thermo Scientific) from methanolic (LC-MS grade, from VWR international) solutions of the ILs, using electrospray ionization and ion trap detection (ESI−IT MS).

4.2. General Procedure for the Synthesis of the Target Ionic Compounds. *4.2.1. Neutralization of the Commercial Salts of the Antimalarial Drugs.* The commercial salt forms of PQ (bisphosphate), CQ (bisphosphate), and MP (hydrochloride) were first converted into the respective free amine bases. To this end, 30% aqueous sodium hydrogen carbonate was added to the commercial antimalarial salt, and the solution stirred for 15 min at room temperature, in the dark. Formation of an oily layer was observed, and extracted with dichloromethane (3 × 10 mL); the resulting organic layer was then dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness under reduced pressure, to obtain the free amine.

4.2.2. Formation of the Target Antimalarial-BA Salts by Direct Acid-Base Neutralization. The antimalarial drug [as free amine base, obtained according to section 4.2.1] was dissolved in methanol; likewise, a methanolic solution of an equimolar amount of BA was prepared in parallel. This solution was then added dropwise to the methanolic solution of the antimalarial drug, at room temperature and under magnetic stirring. After 30 min, the solvent was evaporated, and the residue dried under vacuum until constant weight. This solid residue obtained was then analyzed by ^{1}H- and ^{13}C NMR, and HRMS, and spectral data obtained were consistent with the expected structures (see the Supporting Information).

4.2.3. Synthesis of [P6,6,6,14][BA]. Trihexyltetradecylphosphonium chloride was dissolved in methanol and passed through an anion exchange column Amberlyst A-26(OH), (5.5 molar equivalents), and the resulting trihexyltetradecylphosphonium hydroxide solution was slowly added dropwise to a methanolic solution of BA. The resulting mixture was stirred at room temperature for 1 h, after which the solvent was eliminated by evaporation under reduced pressure. The resulting liquid residue was dried until constant weight, and next analyzed by ^{1}H- and ^{13}C-NMR, and by HRMS, and spectral data were consistent with the expected structure (see the Supporting Information).

4.3. General Procedures for in Vitro Assays. The organic salts synthesized were evaluated for their effect on human cell viability. Different tumor cell lines were used, namely, RKO, T47D, MG63, A549, and HepG2. In parallel, HFF-1 human fibroblasts were used as a non-neoplastic control, in order to assess the selectivity of the test ionic compounds.

Cells were maintained in α-minimal essential medium containing 10% fetal bovine serum, 100 IU/mL penicillin, 2.5 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 50 μg/mL ascorbic acid. Cells were seeded at 10^4 cells/cm^2. After 24 h of cell attachment, the culture medium was renewed and supplemented with different concentrations (0.5, 5, 50, and 500 μM) of the test compounds. Cell cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C. Cellular viability/proliferation was assessed after 24 and 72 h, by the MTT assay. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. Half-maximal inhibitory concentration (IC_{50}) values were obtained by nonlinear regression analysis of concentration−effect curves, using GraphPad Prism software (version 2012).

In order to gather some insights about the intracellular mechanisms affected by $[P_{6,6,6,14}][BA]$, cell cultures were treated with this IL at 50 μM, and supplemented with different commercial signaling pathway inhibitors, namely, U0126 (1 μM, MEK inhibitor), PDTC (10 μM, NF-kB inhibitor), and SP600125 (10 μM, JNK inhibitor). Cell viability was assessed by the MTT method, and the results were presented as a percentage of the corresponding control (absence of the test IL).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03691.

Spectral data and traces of the organic salts prepared, proton NMR data on BA, PQ, CQ and MP, as well as comparison of in vitro data at 24 and 72 h available in [link to ESI] (PDF)

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Notes

The authors declare no competing financial interest.

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