Drug-Derived Surface-Active Ionic Liquids: A Cost-Effective Way To Expressively Increase the Blood-Stage Antimalarial Activity of Primaquine


In the previous disclosure of room-temperature ionic liquids derived from primaquine and cinnamic acids, which displayed slightly enhanced blood-stage activity compared to the parent drug, we have now combined this emblematic antimalarial with natural fatty acids. This affords surface-active ionic liquids whose liver-stage antimalarial activity is either retained or slightly enhanced, while revealing blood-stage antimalarial activity at least one order of magnitude higher than that of the parent compound. These findings open new perspectives towards the cost-effective recycling of classical drugs that are either shelved or in decline, and which is not limited to antimalarial agents.

Introduction

Most available active pharmaceutical ingredients (API), including antimalarials, are available as salts, e.g., chloroquine phosphate, mepacrine hydrochloride, sodium artesunate, or primaquine phosphate. Although the preparation of saline pharmacokinetic forms of API is indeed a key step in drug development and formulation, most so-called conventional salts are solids frequently associated with undesirable features, such as polymorphic conversion or low bioavailability, among others. In order to avoid these limitations, ionic liquids (IL) derived from API (API-IL) have recently attracted much attention, since IL are organic salts with low melting points, often below 100 °C, and usually below room temperature (room temperature ionic liquids – RTIL). Formulating API as IL may not only minimize bioavailability and polymorphism-related issues, but also offer the possibility to fine-tune the biological and physicochemical properties of the API-IL, through convenient combination of selected organic cations and anions. As such, development of API-IL emerges as an attractive tool for cost-effective rescuing of drugs with limited bioavailability, possibly also improving their therapeutic effects.[1] In view of this, and in line with our long term research focused on the rescuing of classical antimalarial drugs,[2] we have previously developed IL derived from primaquine (PQ) that were active against liver- and blood-stage forms of Plasmodium parasites.[3] Biophysical studies using model membranes suggested that the observed enhancement of the blood-stage activity of these IL, as compared to the parent drug, might arise from a better interaction of the IL with the membranes of Plasmodium-infected erythrocytes.[4] Hence, we hypothesized that PQ's blood-stage activity might be further enhanced upon its acid-base pairing with amphiphilic carboxylic acids, e.g., natural fatty acids, likely able to efficiently interact with membranes of Plasmodium-infected red blood cells (PIRBC).

Results and Discussion

In the view of the above, we first converted PQ phosphate into the compound's deprotonated form, PQ (1), which was next reacted with natural fatty acids 2a–g to afford a small set of PQ-derived organic salts 3a–g (Scheme 1). These salts were obtained in near-quantitative yields, with 3a–d and 3g being isolated as yellow-orange RTIL, and 3e–f as orange IL. Spectroscopic data
undegraded up to temperatures as high as 82°C, stable than the commercial PQ phosphate salt, they remain
fatty acid hydrocarbon chain. Relevantly, data in Table 1 show
the treatment of diseases that mainly affect tropical and sub-
tropical regions of the world, all compounds were first submit-
ted to the assessment of the effect of adding each of
mixing with surfactant cetyltrimethylammonium bromide
(CTAB) improves the solubility of IL
values displayed by IL 3 do not merely reflect the activity of
their parent compounds, i.e., PQ and fatty acids 2, either alone
or in a 1:1 mixture, as inferred from comparison of PQ laurate
salt 3c with PQ or lauric acid (2c) alone or in an equimolar mixture (Table 1). Indeed, the clearly distinct behaviour of 3c as
compared to the 1:1 mixture of PQ with lauric acid shows that
these IL have their own identity as chemical entities, not being
the simple sum of their parts.

These in vitro data support our initial assumption that
formulation of PQ as a fatty acid-derived organic salt might
enhance its blood-stage antimalarial activity. This expect-
ation was based on the amphiphatic nature of fatty acids, which
suggested that IL 3 might behave as surface-active ionic liquids
(SAIL),10 with enhanced ability to internalize PiRBC. Accordingly,
we carried out surface tension studies (cf. SI for experimental
details) on IL 3 to gain further insight into the effect of the fatty
acid chain’s length on surface activity. Although the test IL 3
exhibit low solubility in pure water, their surface activity is
demonstrated by saturated aqueous solutions of 3b, 3c and
3e. The surface tension value measured at 25.0°C for 3b, 3c
and 3e was 37.3 mN m⁻¹, 28.9 mN m⁻¹ and 26.0 mN m⁻¹,
respectively. These values are much lower than that the surface
tension of ultra-pure water (72.0 mN m⁻¹ at 25°C), indicating
that these IL have a strong adsorption at the air-liquid interface.
Mixing with surfactant cetyltrimethylammonium bromide
(CTAB) improves the solubility of IL 3b-e and 3g. This enabled
the assessment of the effect of adding each of 3b-e and 3g on
the critical micelle concentration (cmc) and on the surface
tension at the cmc (γcmc) of CTAB solutions at a constant SAIL
molar fraction, defined as xSAIL = nSAIL/(nSAIL + nCTAB), of 0.10. All
tested salts 3 are SAIL, as they form mixed micelles with CTAB

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield [%]</th>
<th>Td [°C][a]</th>
<th>IC₅₀ [nM] [b]</th>
<th>IC₅₀ [nM] [b]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3D7 strain</td>
<td>Dd2 strain</td>
</tr>
<tr>
<td>3a</td>
<td>87</td>
<td>81.8; 189.6</td>
<td>432 ± 162</td>
<td>204 ± 37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(92–757)</td>
<td>(117–304)</td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>99</td>
<td>138.5; 203.4</td>
<td>225 ± 142</td>
<td>225 ± 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(125–325)</td>
<td>(87–341)</td>
<td></td>
</tr>
<tr>
<td>3c</td>
<td>99</td>
<td>161.7; 297.0</td>
<td>510 ± 142</td>
<td>356 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(249–657)</td>
<td>(323–389)</td>
<td></td>
</tr>
<tr>
<td>3d</td>
<td>36</td>
<td>–</td>
<td>164 ± 64</td>
<td>127 ± 39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(117–212)</td>
<td>(93–162)</td>
<td></td>
</tr>
<tr>
<td>3e</td>
<td>99</td>
<td>–</td>
<td>247 ± 118</td>
<td>127 ± 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(158–336)</td>
<td>(90–163)</td>
<td></td>
</tr>
<tr>
<td>3f</td>
<td>95</td>
<td>186.9; 328.9</td>
<td>n.d.[c]</td>
<td>n.d.[c]</td>
</tr>
</tbody>
</table>

[a] Temperature of observed degradation events. [b] Half-maximal inhibitory concentration ± SD (95% confidence intervals). [c] Compound insoluble under assay conditions. [d] Phosphate salt (commercial formulation of this API).

**Scheme 1.** Synthesis route to PQ-derived organic salts 3a-g. (i) 1 (1 molar equivalent, eq), 2a-g (1 eq), methanol (MeOH), room temperature (RT), 30 min.

supplied in the Supporting Information (SI) were in agreement
with the expected structures, and complete transfer of the
carboxylic acid proton to the 8-aminoquinoline drug was
confirmed by proton nuclear magnetic resonance (¹H-NMR), as
shown for 3b in Figure S1 (SI).

As thermal stability is an important issue for all API used in
the treatment of diseases that mainly affect tropical and sub-
tropical regions of the world, all compounds were first submit-
ted to simultaneous thermal analysis (STA). The IL present
at least two major thermal decomposition events (Table 1), at
temperature values that increase along with the size of the
fatty acid hydrocarbon chain. Relevantly, data in Table 1 show
that, despite the IL 3a-c and 3f are somewhat less thermally
stable than the commercial PQ phosphate salt, they remain
undegraded up to temperatures as high as 82°C (3a) or even
higher (3b-c and 3f).

The compounds were screened in vitro for their activity
against blood-stage forms of the chloroquine-sensitive 3D7,
and the chloroquine-resistant Dd2 strains of P. falciparum. The
antiplasmodial activity of IL 3 was ca. one order of magnitude
higher than that of the parent drug against both strains
(Table 1).

Despite the fact that there are many well-known antimal-
alorials with more potent blood-stage activity[8] than that
displayed by these compounds, this is an important outcome
for PQ-based compounds, which typically act as tissue
shizontocidals and gametocytocidals.[9,10] Moreover, the IC₅₀

Table 1. Synthesis yields, thermal degradation data, and in vitro activity of IL 3a-f.

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and cause a significant decrease in the mixture cmc as compared to neat CTAB (Table 2 and Figure 1A). Moreover, our data suggest that there may be an unusual relationship between cmc and the length of the fatty acid chain, highlighting the dodecanoate (laurate) salt 3c as the SAIL with strongest surface activity (Table 2). Indeed, the cmc of the CTAB + 3c mixture is ca. 13 times smaller than that of neat CTAB and the γcmc for the mixture with 3c is quite low, reaching 24.4 mN m⁻¹, consistent with a strong surface adsorption. We further assessed the dependence of the mixture cmc on the molar fraction of SAIL (cf. Figure S3, in the SI), and it was observed that the cmc decreases with increasing molar fraction of 3c (Figure 1B).

In summary, surface tension studies confirm the behavior of 3 as SAIL, which may be associated to the significant improvement of in vitro activity against blood-stage malaria parasites. Despite the relatively low number of compounds 3 studied thus far, and the fact that solubility issues limit the size of the fatty acids that can be used, these findings highlight a new path for antimalarial drug development that is worthy of further exploration. Indeed, other biocompatible amphiphilic acids may be useful to produce novel PQ-derived SAIL with promising blood-stage antimalarial activity. Nevertheless, this will be worthless if such SAIL are toxic to human cells or devoid of the valuable liver-stage antimalarial activity that characterizes PQ-based compounds. Consequently, we further screened SAIL 3a–f in vitro for their activity against liver forms of P. berghei and for their toxicity to human Huh-7 cells. Our results show that all six SAIL, 3a–f display dose-response dependence (Figure 2 – bars) and 3b–f are more active than the parent drug, both in free form 1a and in bisphosphate form 1b, with 3c being the most active compound evaluated. SAIL 3c also displays higher activity than the original fatty acid 2c and than the mixture 1b + 2c. Additionally, none of the compounds is cytotoxic up to 10 μM, as shown by the cell confluency data displayed (Figure 2 – dots). To the best of our knowledge, this

### Table 2. Critical micellar concentration (cmc) and surface tension at the cmc (γcmc) for CTAB and different CTAB/SAIL mixtures with a molar fraction of SAIL, xSAIL, equal to 0.10. Typical uncertainties are: cmc, ±5%, and γcmc, ±2%.

<table>
<thead>
<tr>
<th>System</th>
<th>cmc/mmol·kg⁻¹</th>
<th>γcmc/mN·m⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAB</td>
<td>0.84</td>
<td>33.0</td>
</tr>
<tr>
<td>CTAB + 3b</td>
<td>0.41</td>
<td>31.8</td>
</tr>
<tr>
<td>CTAB + 3c</td>
<td>0.061</td>
<td>24.4</td>
</tr>
<tr>
<td>CTAB + 3d</td>
<td>0.67</td>
<td>30.0</td>
</tr>
<tr>
<td>CTAB + 3e</td>
<td>0.60</td>
<td>25.4</td>
</tr>
<tr>
<td>CTAB + 3g</td>
<td>0.12</td>
<td>27.2</td>
</tr>
</tbody>
</table>

Figure 1. Surface tension plots and cmc determination, at 25°C, of aqueous CTAB/SAIL mixtures: (A) surface tension vs. the logarithm of total CTAB + SAIL concentration, expressed in molality; the cmc values are obtained from the intersection points of the linear fit in each system; (B) cmc vs. molar fraction of 3c in mixtures with CTAB, showing the marked effect of 3c in reducing cmc.

Figure 2. In vitro screening of the effect of SAIL 3a–f, at 1 and 10 μM, on growth of liver forms of P. berghei parasites (bars), and confluency of their host cells, Huh-7 hepatocytes (dots).
works constitutes the first report of PQ-derived SAIL acting as dual-stage antiplasmodial hits.

**Conclusion**

In conclusion, we advance PQ-derived SAIL as a new dual-stage antiplasmodial chemotype. We are aware that in vitro activity of these SAIL against blood-stage *P. falciparum* is not as high as those of first-line antimalarials like artemisinin or artesunate, but the considerable activity increase relative to that of the parent drug suggests that a similar cost-effective approach can be explored for both antimalarial and other API. Likewise, the preliminary assessment of the hepatic-stage antiplasmodial activity of these SAIL indicates that, although they are not as potent as reference drug atovaquone, their potency is higher than that of the parent PQ, which is a reference drug for all liver forms of *Plasmodium*, including hypnozoites, against which atovaquone is inactive.

Further studies in these systems and fully test our hypothesis, the first-time disclosure of PQ-derived SAIL that are active against both blood- and liver-stage *Plasmodium* parasites, while having the ability to co-assemble into colloidal nanostructures (micelles), is of undeniable impact in the medicinal chemistry field. Indeed, drug-derived SAIL enclose the remarkable potential of being exploitable, in the near future, as new bioactive chemotypes able to act both as drug and drug delivery systems. Ongoing studies in our lab, using similar SAIL derived from other antimalarial and anti-infective agents, will hopefully contribute to the establishment of API-derived SAIL as a cost-effective and simple approach towards the rescuing of classical drugs that are either shelved or in decline.

**Experimental Section**

**Chemistry**

*Conversion of primaquine phosphate into 1a:* Conversion of commercial primaquine phosphate into its free base form 1a was performed as previously described by Ferraz et al. Briefly, triethylamine was added to a suspension of chloroquine biphosphate in dichloromethane (DCM), and the mixture was stirred for 30 minutes. The organic layer was washed with water (x3), dried over anhydrous Na$_2$SO$_4$, and evaporated to dryness under reduced pressure, to afford 1 with correct $^1$H-NMR spectral data.

*Synthesis of ionic liquids 3:* 1 molar equivalent (1 eq) of primaquine (1a) was dissolved in methanol. In parallel, 1 eq of fatty acid was dissolved in methanol. The methanolic solution of the drug was placed under magnetic stirring and the methanolic fatty acid solution was added dropwise. Upon addition of the acid, the reaction mixture was kept under stirring for 30 min, at room temperature. The solvent was removed by evaporation under reduced pressure in the rotary evaporator, and finally dried at high vacuum. The residue obtained was analyzed by $^1$H-NMR and $^{13}$C-NMR, allowing to verify the identity of the desired salt, with an anion/cation stoichiometry of 1:1, according to the structural data presented in the Supporting Information.

*Simultaneous thermogravimetric analysis:* The compounds under study were subjected to heating from room temperature to 500°C at a speed of 5°C/min, obtaining the thermograms shown in the Supporting Information. For a better visualization of the degradative events, the derivatives of the thermogravimetric curves are also present in the thermograms. The thermal stability of the compounds was evaluated in a simultaneous thermal analyzer (STA) from Scancsi, model 7200RVR.

*Surface tension measurements:* A DCA T11 tensiometer from Dataphysics GmbH with a Pt–Y alloy Wilhelmy plate was used and all measurements were performed at 25.0 ± 0.5°C. The measurements for cmc determination of the CTAB/SAIL solutions were performed by adding aliquots from a stock mixed solution to the solution in the measuring vessel. No dynamic surface tension effects were observed.

*In vitro assays*  

*Plasmodium liver stages:* The inhibition of infection in the hepatic stage by the compounds was assessed by measuring the luminescence intensity of lysates of Huh-7 cells infected with a firefly luciferase-expressing *P. berghei* line, as previously described by Plomen et al.

Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% v/v fetal calf serum, 1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine and 10 mM 4-(2-hydroxyethyl)trimethylammoniumchloride (HEPES), pH 7, and maintained at 37°C with 5% CO$_2$. For infection assays, Huh-7 cells (1.2 × 10$^5$ per well) were seeded in 96-well plates the day before drug treatment and infection. Medium in the cells was replaced by medium containing the appropriate concentration of each compound approximately 1 h prior to infection with sporozoites freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes. Sporozoite addition was followed by centrifugation at 1700 g for 5 min.

The evaluation of the parasitic load was evaluated at 48 h infection by luminescence measurement of cell lysates, following addition of the luciferin substrate.

The effect of the compounds on the viability of Huh-7 cells was assessed by the AlamarBlue assay (Invitrogen, UK), using the manufacturer’s protocol.

**Blood stage**

*Parasite cultivation:* Laboratory-adapted *P. falciparum* 3D7 (chloroquine- and mefloquine-sensitive), Dd2 (chloroquine-resistant, mefloquine-resistant) were continuously cultured and sorbitol synchronized, as previously described by Nogueira et al.

**Determination of IC$_{50}$ values:** Staging and parasitaemia activity was determined by light microscopy of Giemsa-stained thin blood smears. Antimalarial activity was determined using the SYBR Green I assay, as performed by Machado et al. Briefly, early ring stage parasites (>80% of rings) were challenged with a 1:3 serial dilution of each compound, in concentrations ranging from 10,000–0.169 nM. Fluorescence intensity was measured with a multi-mode microplate reader (Triad, Dynex Technologies), with excitation and emission wavelengths of 485 and 535 nm, respectively, and analysed by nonlinear regression using GraphPad Prism to determine IC$_{50}$ values.

The Supporting Information contains structural characterization (NMR, MS spectra) and additional surface tension data.
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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Antimalarial · blood-stage · fatty acid · ionic liquid · liver-stage · Plasmodium · SAIL · surface activity


