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# "Seasoning" antimalarial drugs' action: chloroquine bile salts as novel triple-stage antiplasmodial hits

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Malaria is one of the "Big Three" global infectious diseases, having caused above two hundred million cases and over half a million deaths only in 2020. The continuous demand for new treatment options prioritizes the cost-effective development of new chemical entities with multi-stage antiplasmodial activity, for higher efficacy and lower propensity to elicit drug-resistant parasite strains. Following up on our long-term research towards the rescue of classical antimalarial aminoquinolines like chloroquine and primaquine, we have developed new organic salts by acid-base pairing of those drugs with natural bile acids. These antimalarial drug-derived bile salts were screened *in vitro* against the hepatic, blood and gametocyte stages of *Plasmodium* parasites, unveiling chloroquine bile salts as unprecedented triple-stage antiplasmodial hits. These findings pave a new pathway for drug rescuing, even beyond anti-malarial and other anti-infective drugs.





## Introduction

Malaria is an infection caused by *Plasmodium* spp. parasites that mainly affects low-income countries within tropical and subtropical regions of the globe, representing a huge global health and socio-economic burden in these areas. Alongside AIDS and tuberculosis, malaria belongs to the so-called "Big Three" global infectious diseases[1] carved as a top priority in the target 3.3 of the Goal 3 of the United Nation's Sustainable Development Agenda for 2030.[2]

Infections by *Plasmodium* parasites have been responsible for about 241 million cases of malaria and 627,000 deaths attributable to this disease in 2020 alone.[3] Despite the great advancements made during the first 15 years of this millennium, allowing effective reduction of malaria-related illness and fatalities, no significant progress in malaria control occurred in the last five years, eluding the attainment of the milestones set for 2030.[4] Malaria incidence is particularly high in Africa, where about 95% of the global malaria-related fatalities occur; this burden is aggravated in remote regions where sanitation and healthcare systems are deficient or even absent, and by the concerning rise of parasite resistance to artemisinins.[5-7] Collectively, these factors have contributed to the estimated rise in the number of malaria cases observed from 2015 to 2020.[8] Therefore, and despite several valuable drug candidates entering the pipeline in the past two decades, there is an enduring quest for new therapeutic options to tackle this major infectious disease. These options should, (i) concomitantly target multiple stages of parasite development, to increase therapeutic efficiency while reducing the chances of development of parasite resistance, and (ii) be cost-effective and chemically/thermally stable to be a real benefit for the settings where they are most needed.

Our group has long been interested in the rescuing of classical antimalarial drugs, as this is a valuable strategy to decrease the overall cost of the drug development process.[9] Specifically, we have been working towards improvement of classical antimalarial drugs like chloroquine (CQ) and primaquine (PQ), through simple and cost-effective chemical modifications.[10-12] Lately, our attention was drawn to conversion of active pharmaceutical ingredients (API) into ionic liquids (IL), which have been emerging as a promising and cost-effective strategy to work around some common limitations of API, such as polymorphism, low solubility, and poor bioavailability.[13] The prospective benefits of IL for the pharmaceutical industry are remarkable, not only for potentially improving the physico-chemical and pharmacokinetic properties of existing API, but also for facilitating drug formulation and delivery, especially in the case of surface-active ionic liquids (SAIL).[14] IL-based formulations are particularly interesting for the rescuing of ionizable API used to treat conditions that, like malaria, hit low-income countries the hardest, since such API can be easily paired with a suitable counter-ion via a simple acid-base neutralization reaction to produce a API-IL.[15] As such, we have recently pioneered a strategy to combine basic antimalarials like CQ and PQ with natural fatty acids, as a means to deliver new API-IL antiplasmodials exhibiting the properties of a SAIL; in our view, this might lead to more soluble and cell-permeable structures that could be able to bypass parasite resistance pathways. We thus produced fatty salts of CQ[16] and PQ[17] as new API-IL, showing clear improvements over the parent drugs. CQ-derived IL displayed higher activity than CQ against blood-stage parasites,[16] whereas PQ-derived IL displayed activity against both the parasite's hepatic and blood stages,[17] which constitutes a significant outcome, as PQ itself is not particularly effective against blood-stage parasites.[18] Additionally, we confirmed that the new API-IL had surface activity and self-aggregation properties, i.e., behaved like SAIL.[16,17] Motivated by these findings, we have now investigated whether any improvement could be obtained by replacing fatty with bile acids, given the well-known ability of the latter to emulsify and

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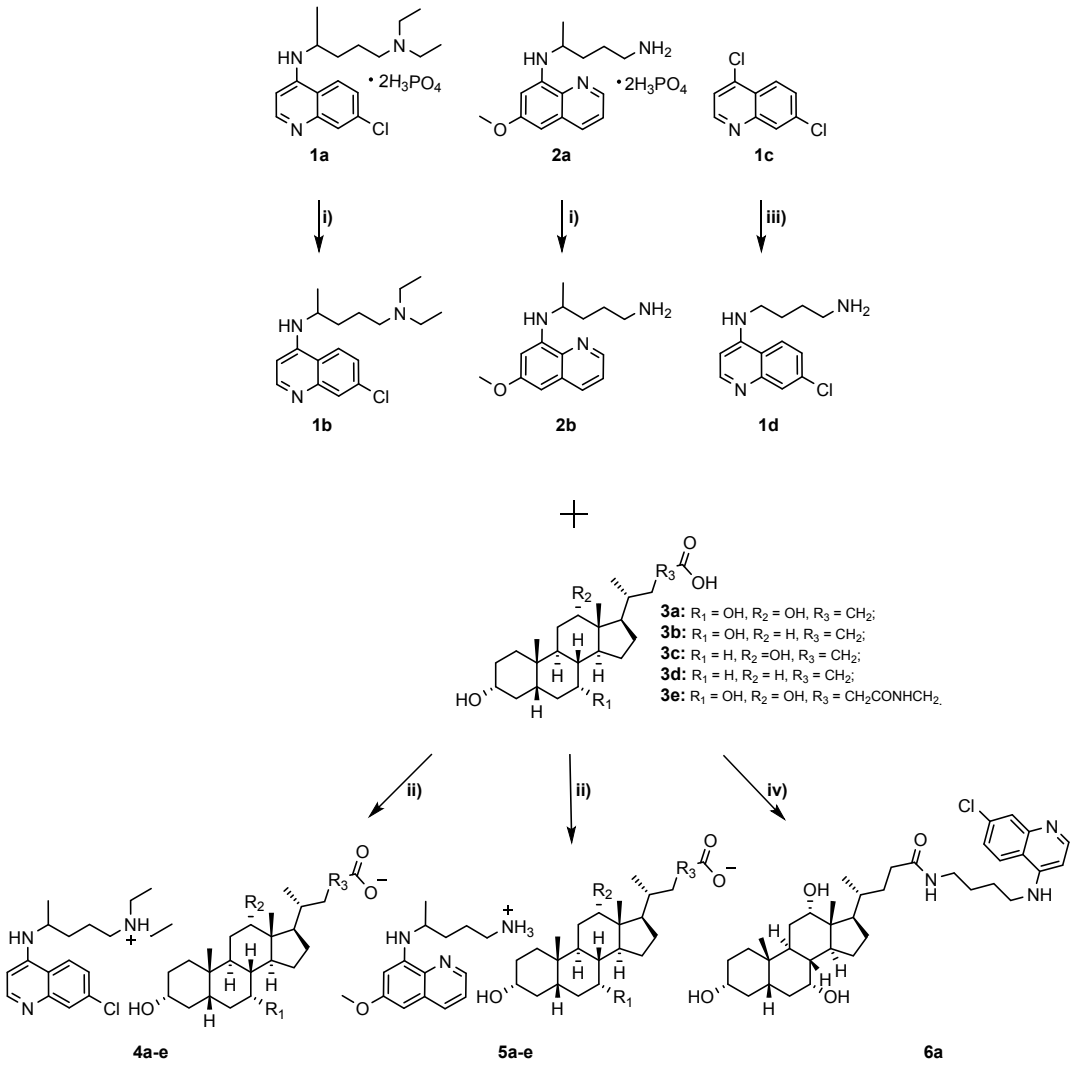
transport hydrophobic moieties within the body, and hence their potential as mediators in oral drug delivery.[19] Moreover, many natural steroids, and derivatives or analogues thereof, have been reported to possess interesting biological properties, including antimalarial activity.[20-25]

## Results and Discussion

**Synthesis and thermal properties of the drug-derived bile salts.**

The synthesis route towards the target organic salts is shown in **Scheme 1**. Briefly, after converting the commercial phosphate salts of chloroquine (CQ, **1a**) and primaquine (PQ, **2a**) into the respective free amines **1b** and **2b**, these were reacted with each one of the bile acids **3a-e** (by this order: cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and glycocholic acid) to produce the corresponding bile salts derived from either chloroquine (**4a-e**) or primaquine (**5a-e**) in quantitative or nearly quantitative yields (**Table 1**). The structures of the salts were confirmed by the standard spectroscopic analyses, and all relevant spectra are provided in the Electronic Supplementary Information (ESI). Importantly, the transfer of the carboxylic proton from the bile acids to the basic aminoquinolines was confirmed by proton nuclear magnetic resonance ($^1$H-NMR), as that proton could be observed in the spectra of the parent bile acids but not in those of the final bile salts. Also, an anion/cation 1:1 stoichiometry was confirmed by peak integration. The target bile salts were obtained as sugar-like white (**4a-e**) or brown (**5a-e**) solids.




**Scheme 1.** Synthesis route to bile salts **4a-e** and **5a-e**, and to amide **6a**: (i) **1a** or **2a**, 5% aqueous (aq.) $Na_2CO_3$, dichloromethane (DCM), room temperature (RT), 30 min; (ii) **1b** or **2b** (1 molar equivalent, eq.), bile acid **3** (1 eq), methanol (MeOH), RT, 30 min; (iii) **1c** (1 eq.), 1,4-diaminobutane (10 eq), 120 °C, 3 h; (iv) **1d** (1 eq.), **3a** (1.1 eq.), *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU, 1.1 eq.), *N,N*-diisopropylethylamine (DIEA, 2 eq.), dimethylformamide (DMF), RT, 24 h.

**Table 1.** Synthesis yields and thermal properties of the bile salts derived from CQ (**4a-e**) and from PQ (**5a-e**), of amide **6a** (covalent analogue of bile salt **4a**), and of the parent bile acids **3a-e**, as determined by combined DSC/LM (to determine $T_m$) and by TGA (to determine $T_d$).

| Compound | Synthesis yield/% | $T_m$/°C | $T_d$/°C |
|---|---|---|---|
| **4a** | 100 | 90 - 93 | 253 |
| **4b** | 94 | 75 - 76 | 255 |
| **4c** | 99 | 123 - 125 | 242 |
| **4d** | 98 | 101 - 106 | 367 |
| **4e** | 100 | 94 - 99 | 361 |
| **5a** | 65 | 97 - 98 | 359 |
| **5b** | 99 | 82 - 83 | 366 |
| **5c** | 100 | 111 - 127 | 348 |
| **5d** | 100 | 90 - 93 | 253 |
| **5e** | 100 | 75 - 76 | 255 |
| **6a** | 39 | ND[a] | 250 |
| **3a** | - | 203-204 | 360 |
| **3b** | - | 158-160 | 357 |
| **3c** | - | 177-178 | 357 |
| **3d** | - | 190-191 | 355 |
| **3e** | - | 134-135 | 328 |

[a] ND, not determined (decomposes prior to melting).

Of note, since the organic salts were produced as solids, their atomic structure using powder X-ray diffraction should be investigated in due course, in order to confirm if they can be regarded as protic ionic liquids, as herein assumed, or as co-crystals instead, as recently highlighted by Cherukuvada *et al*..[26]
The drug-derived bile salts were next characterized by combined differential scanning calorimetry (DSC) and light microscopy (LM) to determine their melting temperature, $T_m$, and by thermogravimetric analysis (TGA) to determine their degradation temperature, $T_d$ (specified here as the temperature at which the first degradation events are observed). Malaria's endemicity mainly in tropical and subtropical regions of the globe implies the need to ensure the thermal stability of compounds with potential antimalarial action and therefore this type of studies is warranted. **Table 1** shows the $T_m$ and $T_d$ value for the CQ-derived bile salts, **4a-e**, and the PQ-derived bile salts **5a-e**, and for the amide **6a**, alongside those of the parent bile acids, **3a-e**, for comparison. As can be seen, all the test compounds degrade at temperatures well above 200 °C, melting at a temperature significantly lower than $T_d$ (with exception of **6a**, which degrades prior to melting). These results demonstrate the high thermal stability of the target bile salts, **4a-e** and **5a-e**; indeed, the CQ-derived salts, **4a-e**, degrade in the range 242-255 °C, whereas the PQ-derived, **5a-e**, degrade in the range 348-367 °C, which is very similar to that of the parent bile acids, 328-360 °C.
Focusing now on the melting behavior of the compounds, a few interesting features arise. First, the drug-derived bile salts globally have melting points (m.p.) within the range 75-125 °C, which encompasses the upper limit that has been conventionally considered for IL (m.p. ≤ 100 °C). Second, DSC and LM data show that the melting of these bile salts is a gradual and complex process, not a sharp one; thus, the $T_m$ values in **Table 1** represent the temperature interval at which full melting (disappearance of any crystals) of these compounds is observed, considering the combined data from DSC and LM. In contrast, the parent bile acids have a sharp melting. Third, if we sort CQ-derived bile salts **4a-e** by increasing $T_m$, the order is **4d** < **4b** ≈ **4c** < **4a** < **4e**, and the same order is observed for the family of PQ-derived bile salts, **5d** < **5b** ≈ **5c** < **5a** < **5e**. This common trend strongly suggests that the drug-derived counter ions have a well-defined influence on the melting behavior of their bile salts. Moreover, it is tempting to interpret this trend based on the molecular structure of the compounds - specifically, in terms of the presence of one, two or three OH groups in the cholic ring, and on the head group nature - and the role of intermolecular forces (in particular, H-bonding) in the cohesion of the solid state. Thus, lithocholic acid-derived compounds **4d** and **5d** have the lowest $T_m$ in their respective families as intermolecular H-bonding is comparatively weaker in their case, since they possess a single OH group in the steroid ring. Compounds derived from chenodeoxycholic/deoxycholic acids, i.e., **4b**/**4c**, and similarly **5b**/**5c**, follow as they both have two OH groups in the steroid ring (the relative positions of the groups does not seem to have any important effect in $T_m$). Next are the derivatives of cholic acid, **4a** and **5a**, which have three OH groups in the steroid ring. Finally, glycocholic acid-derived compounds **4e** and **5e** have the highest m.p. not only because they have three OH groups on the steroid ring, but also the glycine amino acid head group, which can participate in additional H-bonding due to its amide bond.
Noteworthy, if we make a similar analysis on the parent bile acids (**Table 1**), a completely different order is observed: **3e** (glycocholic) < **3b** (chenodeoxycholic) < **3c** (deoxycholic) < **3d** (lithocholic) < **3a** (cholic); this order is somewhat surprising and non-trivial to interpret (particularly striking is the position of glycocholic acid **3e**, with the lowest m.p.). We did not find in the literature any systematic analysis of the melting behavior of bile acids, but nonetheless we note the fact here. While refraining from speculating further on these results, it is clear is that the type of interactions and packing in the solid state seems to be very different on the novel ionic compounds (where ions are present and, thus, ionic interactions come into play) as compared to the bile acids (without any charged entities).
Altogether, thermally stable antimalarial drug-derived bile salts were produced in very high yield and purity by a simple, cheap, and fast acid-base neutralization with no need for purification.

**Antimalarial activity of the drug-derived bile salts *in vitro*.**

The *in vitro* activity of bile salts derived from chloroquine (**4a-e**) and from primaquine (**5a-e**) against liver-, blood- and gametocyte forms of *Plasmodium* parasites was assessed alongside the commercial salts of the respective parent antimalarials, namely, chloroquine bisphospate (**1a**) and primaquine bisphosphate (**2a**), as well as of bile acids **3a-e**. Initially, blood-stage parasites of the *P. falciparum* CQ-sensitive 3D7 strain were exposed to all the test compounds for 72 h at 10 μM, to assess their antiplasmodial activity under these experimental conditions. The compounds' hemolytic activity

was evaluated in parallel, showing that none of them caused hemolysis at the same test concentration of 10 μM. All the bile salts **4a-e** and **5a-e** inhibited intraerythrocytic parasite growth in the 97-98% and 82-86% ranges, respectively, similarly to their corresponding parent antimalarials (98% for **1a** and 83% for **2a**); in turn, none of the parent bile acids **3a-e** inhibited the parasite's intraerythrocytic cycle under the same experimental conditions (data not shown). The half-inhibitory concentrations ($IC_{50}$) of the most active compounds, the CQ-derived bile salts **4a-e**, were then determined against both the CQ-sensitive (3D7) and the CQ-resistant (Dd2) strains of *P. falciparum* (**Table 2**). Relevantly, the bile salts **4a-e** displayed a ca. 3-fold higher activity than chloroquine bisphosphate, **1a**, against both strains. These organic salts (**4a-e**) are very similar to each other regarding activity, with **4a** and **4d** being the most active.

**Table 2.** *in vitro* activity of bile salts derived from CQ (**4a-e**) and from PQ (**5a-e**), of amide **6a** (covalent analogue of bile salt **4a**), of reference antimalarials chloroquine bisphosphate (**1a**) and primaquine bisphosphate (**1b**), and of parent bile acids **3a-e**, against blood and gametocyte stages of *P. falciparum.*

| Compound | $IC_{50}$ on *P. falciparum*/ nM (mean ± SD) | | *gametocyte inhibition at 10 μM (72 h) / %* |
|---|---|---|---|
| | 3D7 strain | Dd2 strain | |
| **4a** | 13 ± 1 | 216 ± 12 | 88 ± 4 |
| **4b** | 17 ± 1 | 242 ± 55 | 87 ± 15 |
| **4c** | 17 ± 2 | 205 ± 33 | 86 ± 13 |
| **4d** | 11 ± 1 | 207 ± 16 | 90 ± 5 |
| **4e** | 15 ± 1 | 212 ± 66 | 86 ± 8 |
| **5a** | 589 ± 116 | ND[a] | 91 ± 9 |
| **5b** | 380 ± 24 | ND[a] | 80 ± 4 |
| **5c** | 600 ± 59 | ND[a] | 86 ± 3 |
| **5d** | 424 ± 60 | ND[a] | 83 ± 3 |
| **5e** | 384 ± 91 | ND[a] | 81 ± 11 |
| **6a** | 108 ± 35 | 562 ± 46 | 91 ± 3 |
| **3a** | ND[a] | ND[a] | 39 ± 8 |
| **3b** | ND[a] | ND[a] | 62 ± 8 |
| **3c** | ND[a] | ND[a] | 42 ± 8 |
| **3d** | ND[a] | ND[a] | 65 ± 11 |
| **3e** | ND[a] | ND[a] | 41 ± 4 |
| **1a** | 33 ± 11 | 453 ± 68 | 72 ± 4 |
| **2a** | 415 ± 30 | 464 ± 100 | 93 ± 2 |

[a] ND, not determined.

In contrast, pairing PQ with the bile acids does not seem to improve the parent antimalarial drug's activity against blood-stage parasites, as PQ-derived bile salts **5a-e** had similar or lower activity than PQ itself against intraerythrocytic parasites. The gametocytocidal activity of these compounds was also evaluated (**Table 2**). Relevantly, although unconjugated bile acids **3a-e** displayed some gametocytocidal activity, this was significantly increased upon their combination with the antimalarial drugs. Relevantly, while the gametocytocidal activity of the PQ-derived bile salts **5a-e** was identical to that of primaquine bisphosphate (**2a**), in the case of the CQ-derived salts **4a-e**, gametocytocidal activity is clearly increased compared to that of chloroquine bisphosphate (**1a**). This is an interesting finding, as although PQ remains a reference gametocytocidal/transmission-blocking antimalarial drug,[27] more promising results were obtained with the CQ-derived bile salts than with those derived from PQ. The similarity between the activities of each compound precludes the extraction of any meaningful structure-activity relationships.

The drug-derived bile salts were further screened *in vitro* for their activity against hepatic forms of *P. berghei* parasites, and their toxicity to human hepatoma Huh-7 cells was concomitantly evaluated (**Figure 1**). While none of the new bile salts was toxic to this mammalian cell line, their activity against hepatic malaria parasites, at 10 μM, was significantly higher than that of their respective parent antimalarials and bile acids. Relevantly, the bile salts derived from CQ, **4a-e**, inhibited liver-stage *P. berghei* much more effectively than bile salts **5a-e**, derived from PQ, despite the latter being well-known for its activity against all hepatic forms of *Plasmodium* parasites.

The specific substitution pattern of the bile acid did not significantly modulate activity, which was similar for all compounds within each series. Nevertheless, particularly for PQ-derived salts **5**, it appears that conjugation with primary bile acids, i.e., in **5a-b**, leads to higher activity at 10 μM than conjugation with secondary bile acids, i.e., in **5c-d**.

Noteworthy, we have also synthesized the non-chiral analogue of bisdesethyl chloroquine, **1d**, and next coupled it to cholic acid (**3a**) to produce amide **6a** as a covalent analogue of the CQ-derived bile salt **4a** (Scheme 1). Amide **6a** was evaluated *in vitro*, for comparison, and found to display a much lower activity than its bile salt counterpart against all *Plasmodium* developmental stages (**Table 2** and **Figure 1**).

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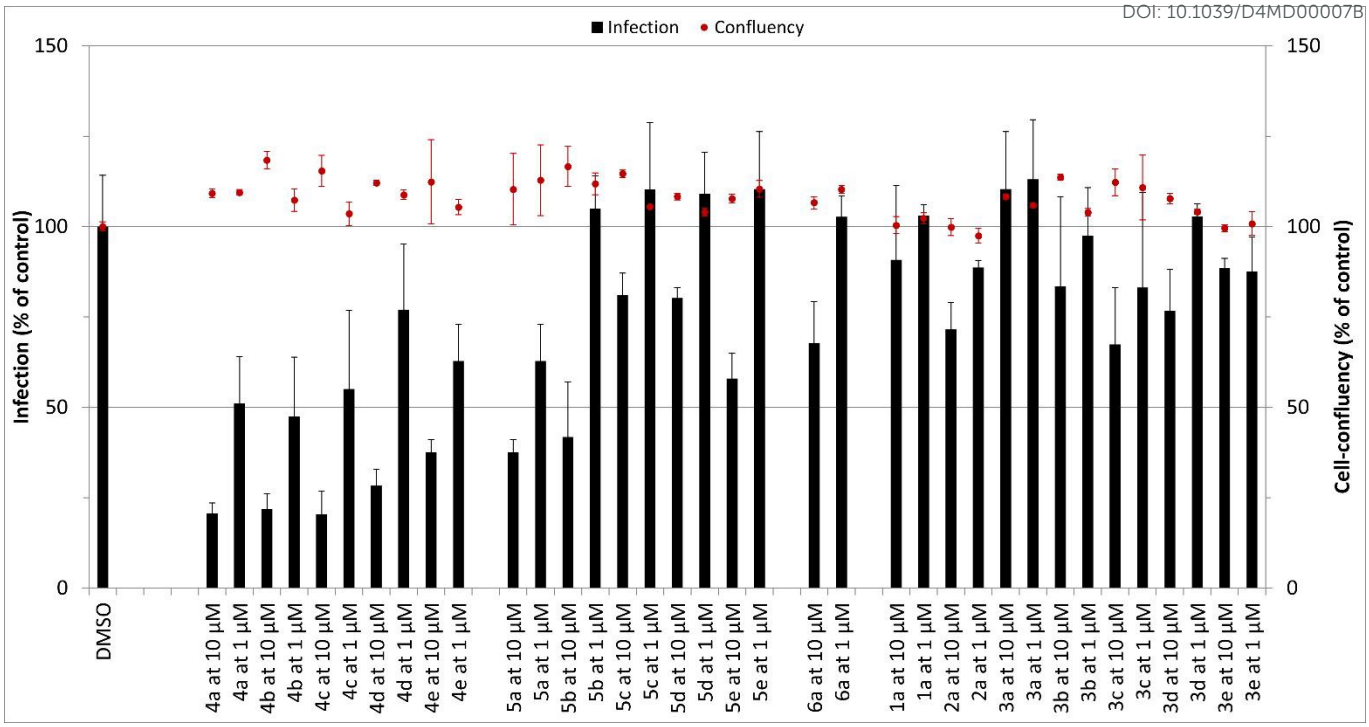


**Figure 2.** *in vitro* screening of the effect of bile salts derived from CQ (**4a-e**) and from PQ (**5a-e**), and amide **6a** (covalent analogue of bile salt **4a**), as well as parent compounds **1a**, **2a**, and **3a-e**, at 1 and 10 µM, on growth of liver forms of P. berghei parasites (bars), and confluency of their host cells, Huh-7 hepatocytes (dots).

## Conclusions

IL, classically defined as salts with melting points below 100 °C where a typically large organic cation is combined with an organic or inorganic anion, have unique physical, chemical, and biological properties, differing markedly from "conventional" salts.[28-30] The most remarkable feature of ILs is their tunability, as there are virtually endless cation/anion combinations that enable a fine tuning of the salts for the intended uses, warranting their growing interest in many fields of basic and applied research.[14,31] The boundaries of what can be defined as an IL have been pushed to include organic salts that have the distinctive features listed above, albeit with melting points higher than 100 °C, thus many authors prefer the broader term Group of Uniform Materials Based on Organic Salts (GUMBOs).[32-34] Regardless of terminology, the uniqueness of these salts has also been attracting the attention of Medicinal Chemists due to their potential interest in pharmaceutics, especially as a new way to formulate, deliver, and/or improve old/shelved drugs.[35-38] This is an attractive strategy to rescue obsolescent anti-infective agents, in an era where multi-drug resistance is escalating. This approach is particularly appealing to combat infectious diseases that mostly affect low-income regions, as formulating a known ionizable API by simple acid-base pairing with a suitable counter-ion may emerge as a convenient and affordable game changer. Although this strategy has been previously used,[35,39,40] we have pioneered its application to antimalarial aminoquinolines with promising results,[15-16] now consolidated by the findings herein reported. Indeed, we are disclosing a new family of CQ-derived bile salts that can inhibit *in vitro* all three developmental stages of malaria parasites. This is particularly relevant as CQ itself is not significantly active against either the liver or the gametocyte parasite stages. Moreover, by pairing the antimalarial drug with amphipathic natural bile acids as those used herein, we are producing novel antimalarial ILs whose likely surface-active properties will possibly contribute to improved pharmacokinetics. This aspect, which is currently under investigation, represents an unprecedented strategy in antimalarial drug development.

In summary, the activity of PQ-derived bile salts is preserved relative to the parent drug. Noteworthy, their CQ counterparts, while also retaining the blood-stage activity of the parent drug, showed increased hepatic-stage and gametocytocidal activities. This was not observed when attaching the CQ pharmacophore to a bile acid through a covalent bond, as in **6a** (resulting from the amide coupling of **1d** with **3a**), suggesting that – besides the much lower synthesis yield and the lack of easy tunability of covalent conjugates as compared to their ionic counterparts **4** – these exhibit more promising biological properties. Given the amphiphilic nature of bile salts, further studies are underway towards a detailed physico-chemical characterization of both families of bile salts **4a-e** and **5a-e** regarding their expected surface activity and self-assembling properties, as these will likely be a plus concerning absorption and distribution of the active drug. Overall, findings herein reported are paving a new pathway for the rescuing of drugs, far beyond anti-malarial and other anti-infective agents.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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