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Synthesis, characterization, and cellular localization of a fluorescent probe of the antimalarial 8-aminoquinoline primaquine

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ABSTRACT

Primaquine (PQ) is the only commercially available drug that clears dormant liver stages of malaria and blocks transmission to mosquito vectors. Although an old drug, much remains to be known about the mechanism(s) of action. Herein we develop a fluorescent tagged PQ to discover cellular localization in the human malaria parasite, *Plasmodium falciparum*. Successful synthesis and characterization of a primaquine-coumarin fluorescent probe (PQCP) demonstrated potency equivalent to the parent drug and the probe was not cytotoxic to HepG2 carcinoma cells. Cellular localization was found primarily in the cytosol of the asexual erythrocytic and gametocyte stages of parasite development.

Malaria is one of the world's most devastating tropical diseases, affecting 215 million people in 2015.[1] The disease is caused by multiple species with *P. falciparum* and *P. vivax* being the most important causes of morbidity and mortality. Despite the large number of drugs available to treat malaria, drug resistance has emerged, even against artemisinin combination therapy.[2] In addition, *P. vivax* and *P. ovale* infections lead to relapsing malaria due to dormant liver stages known as hypnozoites. For unknown reasons the hypnozoites arrest in development in hepatocytes and later become activated and lead to symptomatic blood stage infections weeks to years after the initial infection. The radical cure of hypnozoites is a major target for malaria elimination and the only commercially available drug that clears hypnozoites is primaquine (PQ), an 8-aminoqinoline drug (Fig. 1) that has been used for radical cure, post-exposure prophylaxis, and to block malaria transmission.[3] Currently the use of low dose PQ for malaria elimination is in debate due to potential toxicity with glucose 6-phosphate dehydrogenase (G6PD) deficiency, where hemolytic anemia occurs with varying severity.[4–6] Despite the limitations with PQ, the drug has important biological properties against that parasite that underscores a need to deepen our understanding of the mechanisms of action of PQ and other 8-AQ drugs.[3]

In this study we demonstrate the synthesis, the photophysical properties, and *in vitro* antiplasmodial activity of a novel primaquine-coumarin fluorescent probe (PQCP) (Fig. 1). The aim was to produce a probe that could be used to confirm the cellular localization of PQ to enhance studies on mechanism(s) of action so we can better understand how PQ works on the different parasite stages of development.

The synthesis of the primaquine-coumarin probe (PQCP) is shown in Fig. 2. Based on a previous report, Meldrum's acid was acylated with methyl 5-chloro-5-oxovalerate and subsequently treated with methanol to provide β-keto ester **1**.[7] Next, β-keto ester **1** was first reacted with resorcinol under acidic conditions and then hydrolyzed with lithium hydroxide to provide 4-(7-hydroxy-2-oxo-2*H*-chromen-4-yl) butanoic acid **2**.[8] Finally, primaquine and coumarin butanoic acid **2** was coupled under standard EDCI/DMAP coupling conditions to yield the probe PQCP.

The analysis of the photophysical properties of PQCP displayed extinction coefficients of 15,442 $M^{-1}\,cm^{-1}$ at $\varepsilon_{(264)}$, and 8806 $M^{-1}\,cm^{-1}$ at $\varepsilon_{(329)}$PQCP also has a Stokes shift (Δ) of 122 nm in MeOH (λ_{abs} 265 nm, λ_{em} 389 nm). When compared to PQ and coumarin, the Δ of PQCP is closer to the mean Δ value of 127 nm between PQ (194 nm) and coumarin (62 nm).

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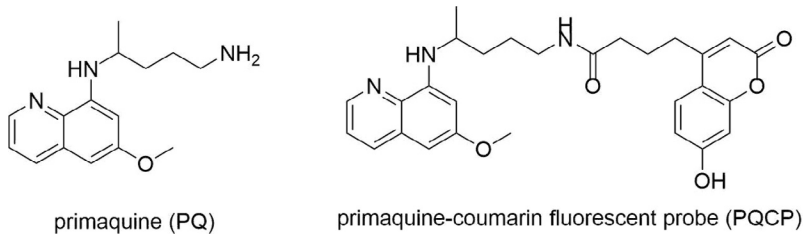

Fig. 1. Structures of antimalarial 8-aminoquinoline primaquine and primaquine-coumarin fluorescent probe.

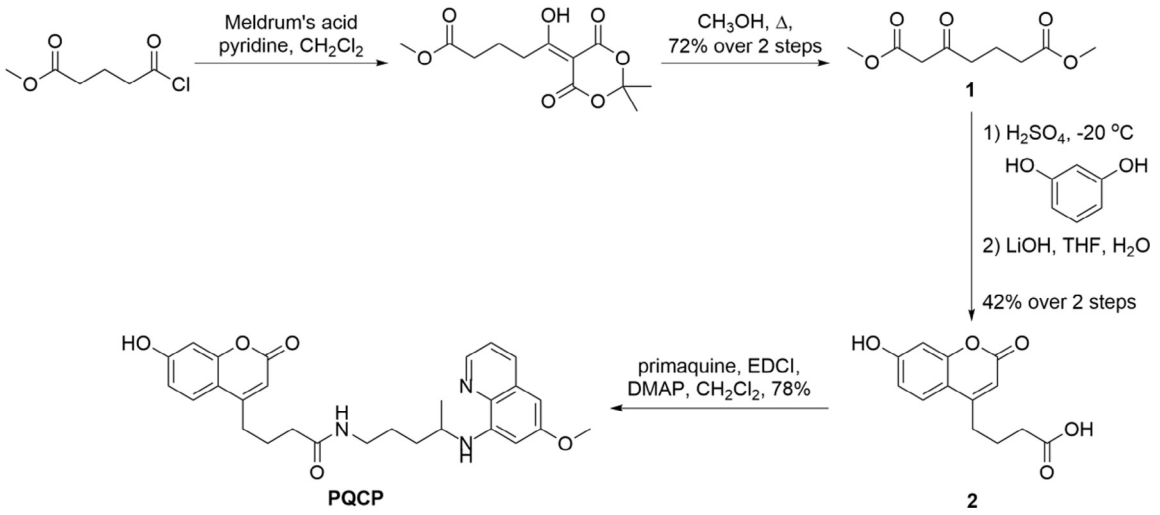

Fig. 2. Synthesis of the fluorescent PQCP probe.

The absorption spectra of PQCP with the parent PQ and coumarin are displayed in Fig. 3A. The spectra for PQ is dominated by the bands at ~265 nm and ~360 nm originating from a transition from the S_o to ^1L_B state and ^1L_A state, respectively. The ^1L_B state is a bond centered state with excess charge density on the quinolone ring while the ^1L_B state is an atom centered excess charge density state with the excess charge density localized on the heterocyclic nitrogen.[9] The spectrum of coumarin contains a single transition centered at 320 nm that arises from a strongly allowed π-π^* transition overlapping with a weaker n-π^* transition.[10] The absorption spectra of the PQCP complex are essentially the sum of the PQ and coumarin spectra, indicating there are no intramolecular interactions between the ring systems of the two chromophores within the complexes.

The corresponding steady state emission spectra of PQ, PQCP, and coumarin are displayed in Fig. 3B. Excitation of PQ at 320 nm results in emission spectra with maxima centered at 465 nm and is due to the radiative relaxation from the ^1L_A excited state while

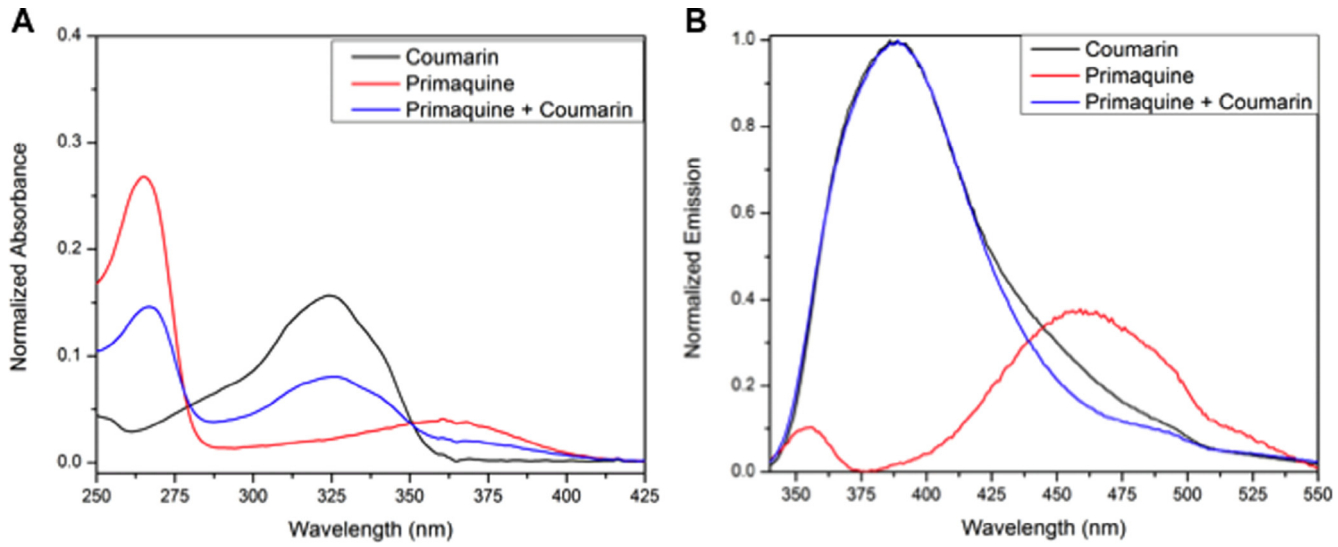

Fig. 3. A) Normalized optical absorption spectra of PQ, PQCP and coumarin; B) Normalized steady state emission spectra of PQ, PQCP and coumarin (excitation: 320 nm).

excitation of coumarin at 320 nm results in emission maxima centered at 380 nm arising from relaxation from the $^1S_1$ excited state. As the absorption spectrum of the PQCP complex is dominated by the coumarin chromophore in the region of 300 nm–350 nm, excitation of the complex at 320 nm results in an emission spectrum that is nearly identical to that of the isolated coumarin with only a small shoulder at 450 nm arising from the PQ chromophore.

Table 1
Potency of primaquine (PQ) and PQ-coumarin probe (PQCP) against chloroquine-susceptible (CQS) and -resistant (CQR) *Plasmodium falciparum* asexual blood stages and *P. berghei* liver stages *in vitro* (50% inhibitory concentrations, μM).

Parasite	Clone	PQ	PQCP
P. falciparum	3D7 (CQS)	5.77 ± 1.1	10.5 ± 1.1
	W2 (CQR)	2.35 ± 0.3	3.52 ± 0.4
	D6 (CQS)	5.16 ± 0.6	4.47 ± 0.6
P. berghei	1052 C11 (CQS)	17.2 ± 1.2	20.7 ± 1.1

Next the biological activity of PQ and PQCP was assessed. The potency against asexual blood stages of *P. falciparum* was found to be equivalent for the parent and the probe against three clones (Table 1 and Fig. 4A). Previous data suggests PQ is more potent against chloroquine-resistant (W2) than chloroquine-susceptible (3D7 and D6) *P. falciparum*;[11] a similar pattern of susceptibility was observed with PQCP being most active against W2, the chloroquine-resistant clone (Table 1). PQ also is active against liver stages of malaria therefore we used the model organism *P. berghei* (rodent malaria parasite) to determine the activity of the compounds against liver stages of development. In this *in vitro* model PQ and

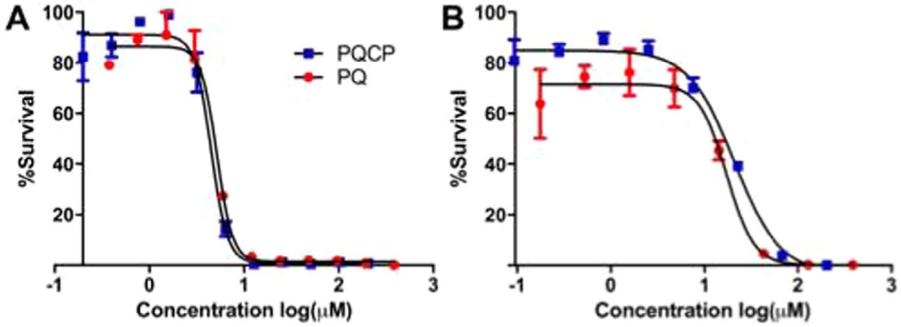

Fig. 4. A) Concentration response curves for PQ and PQCP against A) asexual blood stages of *P. falciparum* (D6 clone) and B) *P. berghei* liver stages *in vitro*.

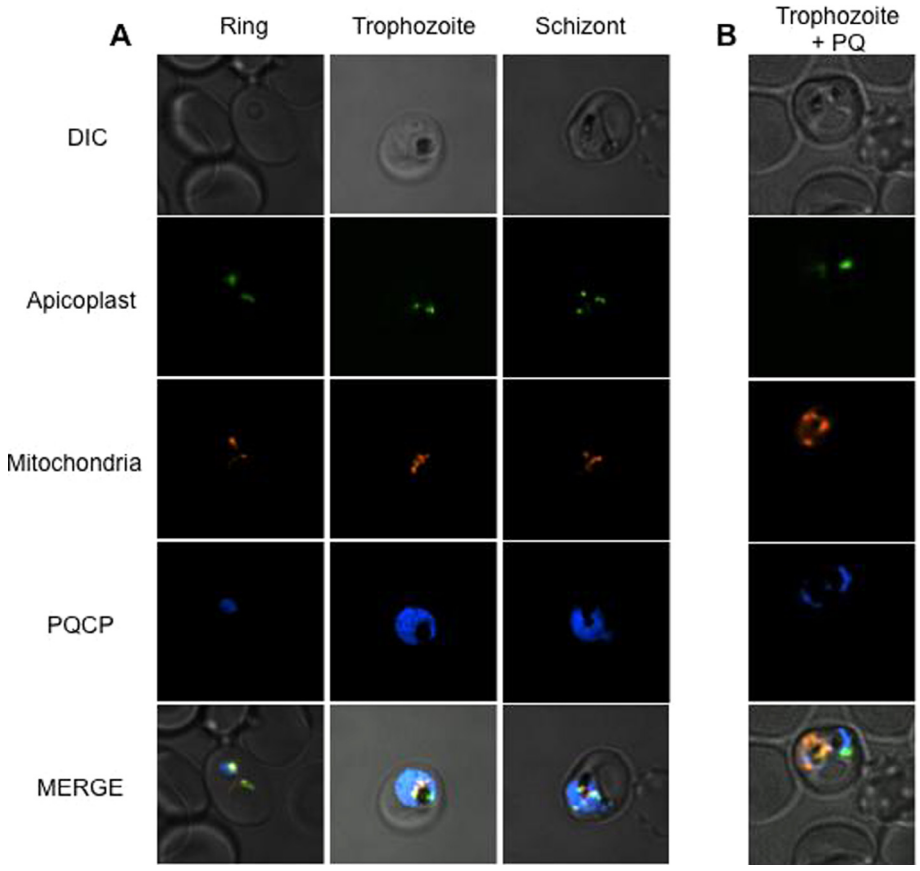

Fig. 5. A) Images of *P. falciparum* asexual blood stages showing localization of PQCP (blue) in the cytoplasm, mitochondria (red, stained with Mitotracker red), and apicoplast (green, GFP-tagged ACP leader). B) Images showing reduced concentration of PQCP in trophozoite stages pre-exposed to PQ (150 μM).

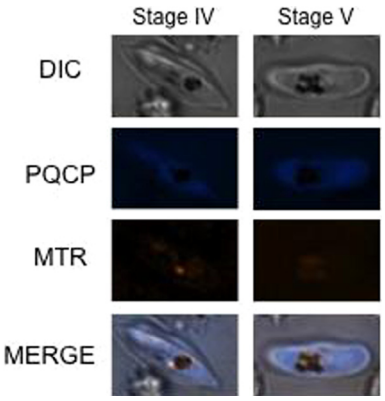

Fig. 6. Images of *P. falciparum* gametocytes (A, stage IV; B, stage V) showing localization of PQCP in the cytoplasm (blue) in the cytoplasm and the mitochondria (red, stained with Mitotracker red (MTR)).

PQCP were found to be equipotent with IC50s of 17.2 ± 1.2 and 20.7 ± 1.1 μM, respectively, against *P. berghei* infected hepatoma cells (Fig. 4B). Cytotoxicity against HepG2 was less for PQCP than PQ, with IC50s of 101 ± 0.93 μM and 44.5 ± 0.76 μM, respectively (Supplemental Fig. 1). These data collectively indicate that the addition of the fluorescent coumarin moiety does not adversely affect compound efficacy, especially against the parasites.

A goal of this study was to develop a probe to determine the cellular localization of PQ in malaria parasites at different stages of development. First we examined the intracellular localization of PQCP in asexual blood stages of *P. falciparum in vitro*. As shown in Fig. 5, PQCP localized throughout the cytoplasm of the asexual blood stages of development (ring, trophozoite and schizont) in red blood cells. There was no apparent localization in the apicoplast, the relict plastid of the parasite, the mitochondria, nuclei, or the food vacuole. In control experiments, neither PQCP nor coumarin found to accumulate in uninfected erythrocytes (data not shown).

The cellular localization of PQCP was next assessed in the most mature stages of gametocyte development (stages IV and V). Similar to the asexual stages, PQCP accumulated in the cytoplasm of gametocytes and was not concentrated in any organelle (Fig. 6). There was no evidence of PQCP accumulation in the food vacuole in gametocytes. As an additional control for the localization studies, PQ (150 μM) was preloaded into *P. falciparum* trophozoite-infected erythrocytes prior to exposure to PQCP (150 μM); these data showed saturation with much reduced accumulation of PQCP in the cytoplasm of the trophozoites (Fig. 5B).

These studies with PQCP suggest the major site of accumulation in the cytoplasm of the parasites at all stages of development that infect erythrocytes. It is possible that PQCP accumulates in the food vacuole or other organelles at lower concentrations not discernable by fluorescence microscopy. Unfortunately, localization of PQCP in liver stages of *P. berghei* infected hepatoma cells was not observed. This could be due to the higher concentrations of PQ and PQCP required for efficacy in this model (Table 1) or potentially the cleavage of the coumarin moiety by metabolically active hepatoma cells. Future studies with different fluorescent tags in metabolically active hepatocytes are warranted.

PQ is an important antimalarial drug and is the only compound commercially available that clears both hypnozoites and stage V gametocytes; both of these are critically important pharmacodynamics properties of drugs to support the elimination of malaria.[3] Another 8-AQ analog, tafenoquine, is in late stage development, yet like PQ, despite the known desirable properties against the parasite, the exact mechanisms of how the drugs act remain unknown.[3] The use of fluorescent probes or click chemistry to identify potential sites and targets of action in the parasite are tools to enhance our understanding of how these drugs act. Identifying the mechanism(s) of action also may reveal opportunities to identify chemical scaffolds without G6PD toxicity concerns to accelerate drug discovery for the malaria elimination efforts.

Herein we describe the synthesis and chemical and biological characterization of a fluorescent coumarin probe of PQ. The new synthesis was based upon established methods and resulted in compound PQCP that was characterized for photo-physical properties and antimalarial activity. The emission spectrum of PQCP is nearly identical to that of the isolated coumarin with a maximum centered at 380 nm with a small shoulder at 450 nm arising from the PQ chromophore. Testing for antimalarial activity suggests that PQ and PQCP are equipotent against asexual and late stage gametocyte stages of *P. falciparum* and that the drugs primarily accumulate in the cytoplasm of the parasite rather than any organelle. Given the importance of the spectrum of activity of PQ and the lack of knowledge of how the drug works, the PQCP probe or similar approaches can be used to better understand the drug and to identify potential targets for further drug discovery efforts.

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Conflict of interest

None.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.09.030.

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