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A primaquine–chloroquine hybrid with dual activity against *Plasmodium* liver and blood stages

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ABSTRACT

We present a new class of hybrid molecules consisting of the established antiplasmodial drugs primaquine and chloroquine. No drug is known to date that acts comparably against all stages of *Plasmodium* in its life cycle. Starting from available precursors, we designed and synthesized a new-generation compound consisting of both primaquine and chloroquine components, with the intent to produce agents that exhibit bioactivity against different stages of the parasite's life cycle. In vitro, the hybrid molecule **3** displays activity against both asexual and sexual *P. falciparum* blood stages as well as *P. berghei* sporozoites and liver stages. In vivo, the hybrid elicits activity against *P. berghei* liver and blood stages. Our results successfully validate the concept of utilizing one compound to combine different modes of action that attack *different Plasmodium* stages in the mammalian host. It is our hope that the novel design of such compounds will outwit the pathogen in the spread of drug resistance. Based on the optimized synthetic pathway, the compound is accessible in a smooth and versatile way and open for potential further molecular modification.

Introduction

Malaria remains a devastating disease with an estimated 250 million clinical cases annually and a mortality of 1 million individuals (Murray et al., 2012). The spread of multidrug-resistant parasites impels the need for the development of new chemotherapeutic agents. No drug is known that acts comparably against all stages and against all *Plasmodium* species. Some 90% of current research programs are focussed on the development of drugs against asexual stages of *P. falciparum* (Warhurst et al., 2002), though this constitutes a small component of the overall complex, multi-faceted life cycle. Chloroquine (**2**) (Fig. 1) is a classical blood stage 4-aminoquinoline antimalarial agent active against blood schizonts: it is well tolerated and has been administered as a standard therapy for decades (Solomon and Lee, 2009). The use of chloroquine has rapidly declined since the early 1990s due to the widespread development of resistance. At the same time, official malaria treatment policy changed to combination therapy applying a mixture of two drugs. However, there is some evidence that withdrawal of chloroquine (**2**) from the market resulted in a decline of chloroquine resistant *Plasmodium* species due to their lower fitness compared to wild-type parasites (Ndiaye et al., 2012; Sinclair et al., 2009). These data suggest potential for the reintroduction of chloroquine (**2**) in malarial combination drug therapy. Two other antimalarial drugs – 8-aminoquinolines pamaquine and primaquine (**1**) (Fig. 1), have been neglected over the past years due to their possible side effects: methemoglobinemia and haemolytic anemia in glucose-6-phosphate dehydrogenase-deficient patients (Coleman and Coleman, 1996). This is unfortunate, as the 8-aminoquinoline primaquine is active against liver-stage schizonts and, as the only substance that eradicates hypnozoites and thus

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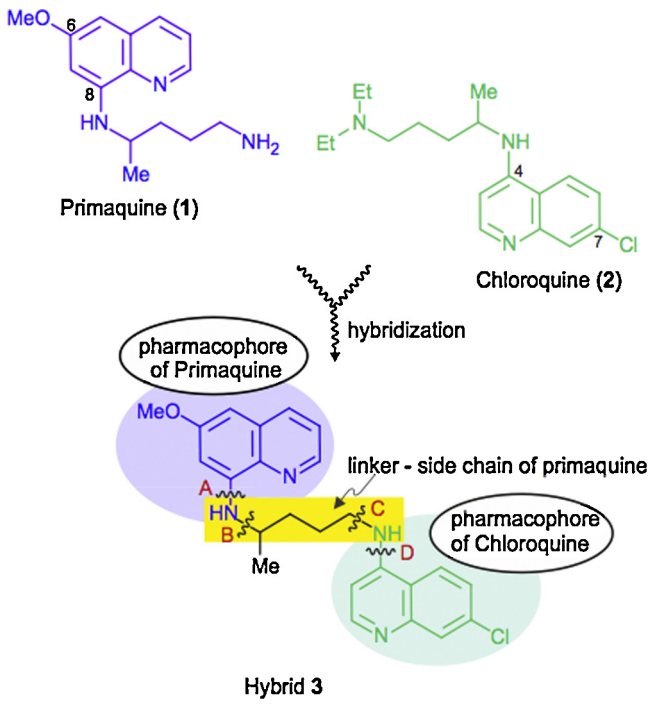

Fig. 1. Design of the target hybrid **3**: the concept of a "Siamese primaquine–chloroquine twin" jointed by the side chain of primaquine (**1**). A and D indicate synthetic routes toward hybrid **3** by the coupling of the terminal amino function of the side chain to the core moiety of either primaquine (**1**) or chloroquine (**2**). Reductive amination (B) or nucleophilic substitution (C) will connect the primaquine or chloroquine core to the side chain of the other pharmacophore.

avoids relapse, has been used since the 1950s as a prophylaxis against recurrent *P. vivax* infections (Tekwani and Walker, 2006). A drug active against the clinically silent liver stage, such as primaquine (**1**), would halt disease before clinical symptoms emerge (Baird et al., 2003). Blood-stage inhibiting compounds, however, act during the clinical manifestation of the disease, at which point the patient presents with pathology. An ideal remedy to this would be a low-toxicity, well-tolerated compound active against all stages of the *Plasmodium* life cycle, including the critical liver and blood stages, serving to prophylactically eliminate the pathogen from the liver and the parasite from the blood during antimalarial therapy. To this aim, we designed a new hybrid compound **3** (Fig. 1) composed of pharmacophores of two approved antimalarial drugs. The utilized concept combines the advantages of a hybrid drug (Dechy-Cabaret et al., 2000; Meunier, 2008; Peters, 2012) with dual activity toward both liver and blood stage *Plasmodium*. The use of the primaquine side chain as a linker allows us to connect the aminoquinoline moieties without introducing any new functional groups. The choice of the hybrid compound was also governed by the aim to improve pharmacodynamic and pharmacokinetic profiles of the parent compounds: Primaquine (**1**) as a medical drug that has to be administered daily due to its rapid metabolism to several harmful phenolic species that are can induce haemolytic anemia (Bowman et al., 2004), like quinoneimine and 5,6-quinone (formed via 5-hydroxyprimaquine and 5,6-dihydroxyquinoline respectively), and to the inactive metabolite carboxyprimaquine (Burchard, 2006).

Chloroquine (**2**), in contrast to primaquine (**1**), has a very long terminal half-life within 30–60 days (Frisk-Holmberg et al., 1984). It is gradually metabolized to N-deethylchloroquine and bisdeethylchloroquine in liver, while about 40–70% of the administered drug is excreted uncharged. This led us to expect metabolic stability of the 4-amino-carbon bond of chloroquine (**2**) and 8-aminomethine moiety of primaquine (**1**). Hence we designed our covalent bound conjugate with a 'two-fold authentic', linkage, i.e. from both drugs (like a 'Siamese twin') (Fig. 1). Furthermore, the structure **3** was investigated for its drug-like characteristics. The hybrid obeys the 'Lipinsky's rule of five' and lies in the chemical space defined for the well-absorbed compounds. The higher lipophilicity of the conjugate compared to those of its parent compounds was expected to assure sufficient membrane permeability. Furthermore, the designed hybrid structure possessed nucleophilic nitrogens that would be important for the protonation of the compound and its accumulation in digestive vacuoles. Moreover, we expected that structure **3** does not serve as a substrate of *P. falciparum* chloroquine-resistant transporter (PfCRT), which is responsible for the declined sensitivity of *Plasmodium* toward chloroquine-like compounds (Bhattacharjee et al., 2002; Fidock et al., 2000). These factors supported our hypothesis of hybrid **3** being a good drug candidate.

The newly synthesized hybrid **3** was investigated for its activity against all stages of *Plasmodium* in the mammalian host using in vitro assays and in vivo tests in rodents. The compound showed significant inhibitory effects against *Plasmodium* liver and blood-stage parasites both in vitro and in vivo.

Materials and methods

Chemical synthesis

All solvents were distilled before use. Commercially available material was purchased from Sigma Aldrich and used without further purification. Thin-layer chromatography was carried out using silica gel 60 F_{254} or aluminum oxide with a fluorescent indicator. Detection of the compounds was achieved by fluorescence quenching at 254 nm, fluorescence at 356 nm, or staining with iodine. Flash chromatography was performed using silica gel (20–63 mesh). NMR spectra were obtained on a Bruker DMX 600 and are reported in ppm relative to the internal solvent signal with coupling constants (*J*) in Hertz (Hz). Spectra were obtained at 25 °C. EI mass spectrometry was carried out on a Finnigan MAT 8200; ESI-HRMS was measured on a Bruker Daltonik micrOTOF-focus.

General method for extraction of primaquine

Primaquine (**1**) was obtained from a commercially available primaquine bisphosphate by extraction of the compound as a free base from an aqueous solution of sodium bicarbonate ($NaHCO_3$) by dichloromethane as follows. Primaquine bisphosphate (700 mg, 1.54 mmol) was dissolved in an aqueous $NaHCO_3$ (30 mL) and extracted with dichloromethane (3× 100 mL). The organic phases were combined, dried over sodium sulfate (Na_2SO_4), filtered, and concentrated under reduced pressure. The free primaquine base (**1**) was obtained as yellow oil (380 mg, 1.47 mmol).

Synthesis of hybrid compound 3 (N^1-(7-chloroquinolin-4-yl)-N^4-(6-methoxyquinolin-8-yl)pentane-1,4-diamine) by nucleophilic substitution under neat conditions

The free base of primaquine (**1**, 425.2 mg, 1.64 mmol) and 4,7-dichloroquinoline (**4**, 162.3 mg, 0.82 mmol) were heated at 120 °C for 6.5 h. The reaction mixture was allowed to cool down to room temperature before it was added an aqueous $NaHCO_3$ (10 mL), followed by extraction with dichloromethane (3× 40 mL). The combined organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on deactivated (7.5% NH_3) silica gel (petroleum ether–ethyl acetate, 1:1) to give the hybrid compound **3** as a beige solid (283.9 mg, 0.67 mmol, 82%): mp 65 °C (petroleum ether–ethyl acetate); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.31 (d, $^3J_{H\text{-}H}$ = 6.36 Hz, 3 H, Me), 1.80–1.83 (m, 2 H, 3-CH_2), 1.89–1.94 (m, 2