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A carbamate-based approach to primaquine prodrugs: Antimalarial activity, chemical stability and enzymatic activation

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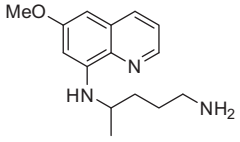
ABSTRACT

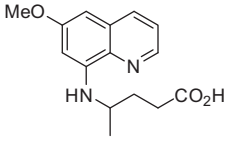
O-Alkyl and *O*-aryl carbamate derivatives of the antimalarial drug primaquine were synthesised as potential prodrugs that prevent oxidative deamination to the inactive metabolite carboxyprimaquine. Both *O*-alkyl and *O*-aryl carbamates undergo hydrolysis in alkaline and pH 7.4 phosphate buffers to the parent drug, with *O*-aryl carbamates being ca. 10^6–10^{10} more reactive than their *O*-alkyl counterparts. In human plasma *O*-alkyl carbamates were stable, whereas in contrast their *O*-aryl counterparts rapidly released the corresponding phenol product, with primaquine being released only slowly over longer incubation periods. Activation of the *O*-aryl carbamates in human plasma appears to be catalysed by butyrylcholinesterase (BuChE), which leads to carbamoylation of the catalytic serine of the enzyme followed by subsequent slow enzyme reactivation and release of parent drug. Most of the *O*-aryl and *O*-alkyl carbamates are activated in rat liver homogenates with half-lives ranging from 9 to 15 h, while the 4-nitrophenyl carbamate was hydrolysed too rapidly to determine an accurate rate constant. Antimalarial activity was studied using a model consisting of *Plasmodium berghei*, Balb C mice and *Anopheles stephensi* mosquitoes. When compared to controls, ethyl and *n*-hexyl carbamates were able to significantly reduce the percentage of infected mosquitos as well as the mean number of oocysts per infected mosquito, thus indicating that *O*-alkyl carbamates of primaquine have the potential to be developed as transmission-blocking antimalarial agents.

1. Introduction

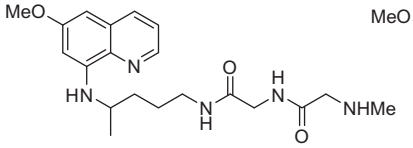
Malaria remains the world's top-priority tropical disease due to its high death burden, as well as to its economic and social impacts on the development of malaria-endemic countries.[1] The emergence and spread of multidrug-resistant *Plasmodium falciparum*, the causative agent of the most lethal form of human malaria, is still the major obstacle in achieving an effective control of the disease.[2] Most antimalarials in clinical use or under development are potent blood-schizontocides, that is they act rapidly against the parasitic forms that invade erythrocytes and cause the usual symptoms to effect a cure from malaria within a reasonable time (ideally 3 days or less).[3–5] However, the ultimate goal of global eradication of malaria parasites from the human population requires the radical cure of all life cycle stages of all malaria species infecting humans.[6]



1, Primaquine



2, Carboxyprimaquine



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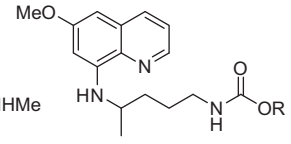

4, R = aryl, alkyl

Currently, primaquine, **1**, is the only available antimalarial that displays a marked activity against gametocytes from all species of parasite causing human malaria, including chloroquine-resistant *P. falciparum*; it is thus capable of interrupting disease transmission

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from the host to the mosquito vector.[7] Primaquine is also the only antimalarial effective against the latent exoerythrocytic forms (hypnozoites) of *Plasmodium vivax* responsible for relapsing malaria.[7,8] In addition, primaquine also displays blood schizontocidal activity, particularly against *P. vivax* and *P. falciparum*, but at doses that can induce side effects such as methaemoglobinemia.

However, primaquine is rapidly metabolised in mammals to carboxyprimaquine, **2**, which is devoid of significant antimalarial activity against the several forms of the parasite.[7] After intravenous administration of primaquine to rats, monkeys and humans it was found that the plasma concentration of carboxyprimaquine rapidly exceeded that of the parent drug after 15–30 min.[9–11] The oxidative deamination of the alkyl side chain of primaquine most likely involves three enzymes in a two-step process: first, monoamine oxidase (MAO) or cytochrome P450 systems mediate the oxidation of primaquine to the corresponding aldehyde; second, the aldehyde intermediate is further oxidised to carboxyprimaquine by aldehyde dehydrogenase.[7]

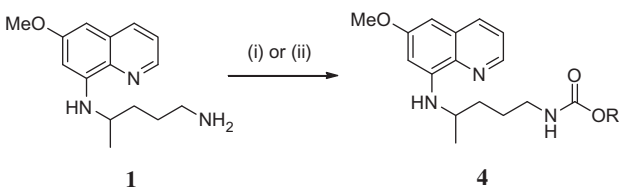
The synthesis of prodrugs is a widely accepted approach used to overcome metabolic deactivation and bioavailability problems commonly found in amine drugs.[12,13] In the case of primaquine, any prodrug should be activated at a rate adequate to maintain sustained levels of the parent drug while being unable to undergo oxidative deamination prior to the release of primaquine. Since both mechanisms of cytochrome P450- and MAO-catalyzed oxidation of amines appear to involve an initial single electron transfer to form a nitrogen-centred radical cation,[14] increasing the ionization potential through nitrogen acylation is expected to reduce significantly the rate of oxidation. For example, acylation of the terminal amino group of primaquine with dipeptides (e.g. **3**) blocked the formation of carboxyprimaquine in rat liver homogenates, without affecting the antimalarial activity.[15]

In the present work we assess carbamates **4** as potential prodrugs for primaquine. The carbamate prodrug approach requires the derivative to be enzymatically activated to a carbamic acid, which rapidly decomposes to the parent amine.[16] Carbamates can be activated by esterases or cytochrome P450, and their chemical and enzymatic reactivity can be modulated by appropriately choosing the alcohol carrier.[16,17] We have now synthesized a series of *O*-alkyl and *O*-aryl carbamate derivatives **4** of primaquine comprising different alcohol and phenol pro-moieties, in order to determine the impact of these leaving groups on (i) the chemical stability, (ii) rates of activation in human plasma and by rat liver homogenates and (iii) the potency of these derivatives. The target compounds were evaluated in vivo for their gametocytocidal activity.

2. Results and discussion

2.1. Synthesis

Primaquine carbamates were synthesised either by reaction of primaquine with the appropriate alkyl or aryl chloroformates or, alternatively, with an equimolar amount of carbonyl diimidazole and the appropriate alcohol (Scheme 1). Carboxyprimaquine, **2**, was prepared according to the procedure reported by McChesney et al.[18]



1 4

Scheme 1. Reaction conditions: (i) $ClCO_2R$, TEA; (ii) CDI, ROH.

2.2. Hydrolysis in aqueous buffers

The rates of hydrolysis of primaquine carbamates, 4, were investigated in aqueous buffers containing 20% (v/v) of acetonitrile. The observed pseudo-first-order rate constants, k_{obs}, were proportional to the hydroxide ion concentration (Eq. 1), up to pH 12.

$$k_{obs} = k_{OH^-}[OH^-] \quad (1)$$

In general, compounds **4** undergo alkaline hydrolysis to primaquine and the corresponding alcohol or phenol with a wide range of rates, as shown by the k_{OH^-} values listed in Table 1. Primaquine and the product phenols were readily observed by HPLC, their identities being confirmed by spiking with authentic samples. However, compound **4h** (R = CH_2CO_2Et) decomposes very rapidly to **4i** (R = CH_2CO_2H) via ester rather than carbamate hydrolysis.

It is possible to consider two different mechanisms that are consistent with both the products of hydrolysis and the observed kinetics. Carbamates **4** can undergo direct hydroxide attack at the carbonyl carbon atom (path A, Fig. 1). Alternatively, compounds **4** can undergo elimination by an E1cB mechanism, that is involving the formation of an isocyanate **6**, resulting from departure of the alcohol from the carbamate anion **5** (path B, Fig. 1). The latter then reacts rapidly with hydroxide ion to yield the conjugate base of carbamic acid, **7**. Eqs. (2) and (3) can be derived from Fig. 2 for the addition–elimination (path A) and elimination–addition (path B) pathways, respectively:

$$k_{obs} = \frac{k_1 K_w}{K_a + [H^+]} \quad (2)$$

$$k_{obs} = \frac{k_1 K_a}{K_a + [H^+]} \quad (3)$$

where K_w is the auto-protolysis constant for water. For both equations, if $[H^+] \gg K_a$ then k_{obs} varies linearly with $1/[H^+]$, which is exactly what is observed, as expressed by (Eq. 1).

Since both Eqs. (2) and (3) are kinetically equivalent they cannot be used to distinguish between the two possible mechanisms. However, the following strongly suggest that aryl carbamates, and carbamates derived from alcohols with pK_a <13, hydrolyse via the elimination–addition pathway (path B, Fig. 1), while alkyl carbamates derived from alcohols with $pK_a \geqslant 13$ hydrolyse via addition–elimination pathway (path A, Fig. 1). First, from the k_{OH^-} values for *O*-aryl carbamates **4d–g** presented in Table 1, it is clear that electron-withdrawing substituents in the phenol moiety enhance the reactivity. A plot of log k_{OH^-} versus σ^- values gives a Hammett ρ value of 3.0 (Eq. 4), which is identical to that reported for the alkaline hydrolysis of aryl *N*-phenylcarbamates.[19] These compounds are known to hydrolyse via a E1cB mechanism, with formation of isocyanate intermediates.

$$\log k_{OH^-} = (2.97 \pm 0.27)\sigma^- + (0.49 \pm 0.16) \quad (4)$$
$$(n = 4, r^2 = 0.98, SD = 0.31)$$

Second, *O*-aryl carbamates **4d–g** are ca. 10^6–10^{10} more reactive than their *O*-alkyl counterparts **4a**,**b**, reflecting the better nucleofugacity of phenols. The trifluoroethyl carbamate, **4c**, also hydrolyses ca. 10^4 times more rapidly than the other alkyl carbamates. A similar result was observed for aryl *N*-phenylcarbamates when compared to their alkyl counterparts, this difference of reactivity being ascribed to a change of mechanism from E1cB to direct attack of hydroxide ion at the carbamate carbonyl for those compounds containing poorer leaving groups.[18] Third, the Brønsted plot of log k_{OH^-} versus the pK_a of the leaving alcohol (Fig. 2) shows that only carbamates with good leaving groups (phenols and

Table 1
Second-order-rate constant for alkaline hydrolysis, k_{OH^-}, half-lives, $t_{1/2}$, for non-enzymatic hydrolysis at pH 7.4, first-order-rate constants and half-lives for activation in human plasma and rat liver homogenates at 37 °C, k_{plasma} and k_{liver}, respectively, and lipophilicity data for primaquine carbamates **4**

Compound	R	pK_a (ROH)	k_{OH^-} (M^{-1} s^{-1})	pH 7.4 buffer	Human plasma		Rat liver homogenate		$c\log P$ [h]	$\log D_{7.4}$
				$t_{1/2}$ (d)	k_{plasma} (s^{-1})	$t_{1/2}$ (min)	k_{liver} (h^{-1})	$t_{1/2}$ (h)		
4a	Et	15.9[a]	9.34×10^{-7}	NR[e]	NR	NR	4.56×10^{-2}	15.1	3.09	3.02
4b	$(CH_2)_5Me$	16.2[b]	3.49×10^{-7}	NR	NR	NR	6.63×10^{-2}	10.4	4.63	4.64
4c	CH_2CF_3	12.37[a]	3.95×10^{-3}	NR	NR	NR	5.04×10^{-2}	13.7	3.56	3.41
4d	C_6H_4–4-MeO	10.20[c]	9.10×10^{-1}	> 30	2.45×10^{-5}	467	6.70×10^{-2}	10.3	4.29	4.23
4e	C_6H_5	9.95[c]	2.64	12	5.34×10^{-5}	217	7.26×10^{-2}	9.5	4.26	4.27
4f	C_6H_4–4-Cl	9.38[c]	7.17	4	3.06×10^{-4}	38.3	5.85×10^{-2}	11.8	4.78	4.44
4g	C_6H_4–4-NO_2	7.14[c]	2.40×10^{4}	2.1 min	7.12×10^{-2}	0.16	ND[g]	ND[g]	4.28	4.23
4h	CH_2CO_2Et	12.9[b]	2.5[d]	10[d]	ND[f]	ND[f]	ND[f]	ND[f]	3.03	3.09

[a] Ref. 39.
[b] Ref. 40.
[c] Ref. 41.
[d] Ester hydrolysis.
[e] NR—no reaction detected.
[f] ND—not determined.
[g] Too fast to be determined accurately.
[h] Ref. 39.

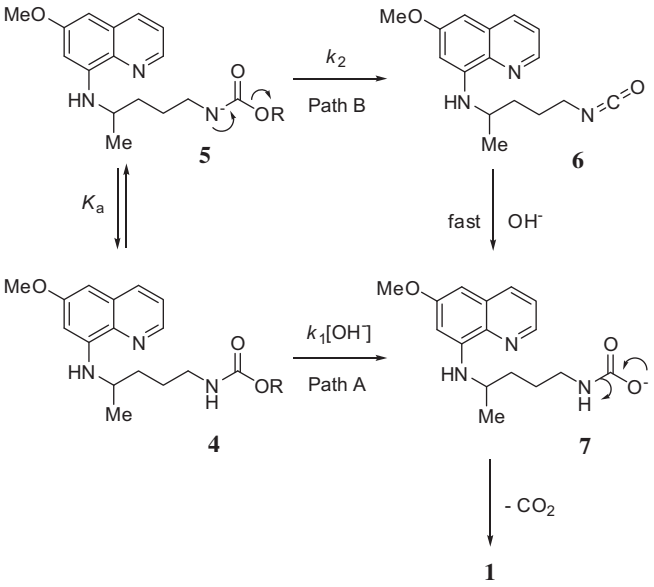


Figure 1. Mechanisms of hydrolysis for carbamates 4; path A refers to the addition–elimination pathway and path B referrers to the elimination–addition pathway (E1cB mechanism).

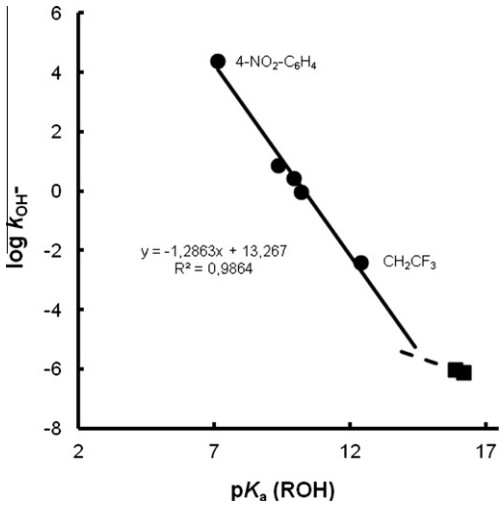


Figure 2. Brønsted plot of $\log k_{OH^-}$ versus the pK_a of the leaving alcohol, for the alkaline hydrolysis of carbamates 4 (●, aryl carbamates; ■, alkyl carbamates).

trifluoroethylethanol) show high sensitivity to leaving group variation, consistent with a with considerable acyl-oxygen bond cleavage as expected for the E1cB mechanism.[17,19]

The reactivity of carbamates **4** was also studied in pH 7.4 phosphate buffer, at 37 °C. As revealed by the half-live values, $t_{1/2}$, presented in Table 1, *O*-alkyl carbamates **4a**–**c** were stable at pH 7.4 (no measurable decomposition for two weeks), while the *O*-aryl carbamates **4d**–**f** hydrolysed very slowly to primaquine and the corresponding phenol product. In contrast, the 4-nitrophenol carbamate **4g** was rapidly hydrolysed, with a half-life of only 2 min. Overall, rates of hydrolysis at pH 7.4 largely reflect the nucleofugacity of the phenol pro-moiety.

2.3. Activation in human plasma

The alkyl carbamates **4a**–**c** proved to be stable in human plasma, their concentration in the incubation mixtures remaining constant for at least 3 h. In contrast, hydrolysis of *O*-aryl carbamates **4d**–**g** in 50% human plasma followed strict first-order kinetics for at least four half-lives, (r^2 >0.95 in all cases), reflecting their enhanced chemical reactivity. From the rate data presented in Table 1, it is clear that human plasma enzymes markedly accelerate the rate of hydrolysis of carbamates **4d**–**g**. To assess whether the rate-enhancement observed with human plasma, compared to hydrolysis in pH 7.4 buffer, could be ascribed to the action of plasma esterases, the effect of diisopropyl fluorophosphate (DIFP) on the rate of hydrolysis of **4f** was also studied. DIFP is a potent inhibitor of the serine hydrolase cholinesterase (E.C. 3.1.1.8, also called pseudocholinesterase, plasma cholinesterase or butyrylcholinesterase, BuChE).[20] Most significantly, a complete inhibition of the hydrolysis of carbamate **4f** was observed when the human plasma was pre-incubated with 20 mM DIFP, indicating that plasma activation of aryl carbamates is BuChE-catalysed.

Carbamates **4d**–**g** were hydrolysed in human plasma quantitatively to the corresponding phenol product, but none of the substrates released primaquine over the time scale of the reactions (ca. five half-lives). However, primaquine was detected when **4f** and **4g** were incubated for longer periods (ca. 10 half-lives), corresponding to 15% and 16% of prodrug conversion to the parent drug, respectively (Fig. 3). This suggests that *O*-aryl carbamates **4d**–**g**

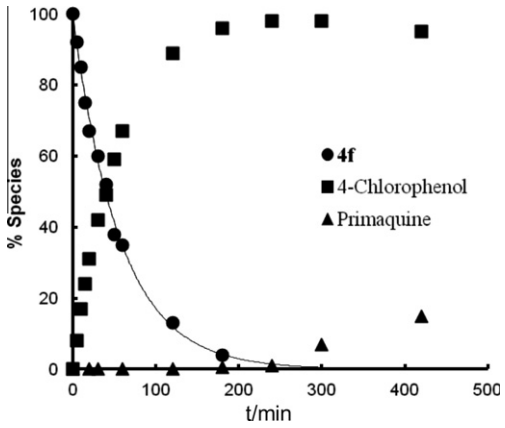


Figure 3. Reaction profile for the hydrolysis of carbamate **4f** in human plasma (●, **4f**; ■, 4-chlorophenol; ▲, primaquine).

react with the catalytic serine residue of BuChE to form a relatively stable carbamoylated enzyme, which is subsequently slowly hydrolysed to primaquine. This result is in line with reports showing that aryl carbamates can rapidly carbamoylate esterases, while the resulting modified enzyme hydrolyses at slower rates to regenerate the active enzyme and liberate the amine moiety.[21–23]

The general order of reactivity for the plasma-catalysed hydrolysis of **4d**–**g** is identical to that observed in the alkaline hydrolysis and a good correlation between $\log k_{OH^-}$ and $\log k_{plasma}$ is obtained, as shown in (Eq. 5):

$$\log k_{OH^-} = (0.77 \pm 0.07) \log k_{plasma} - (4.46 \pm 0.16) \quad (5)$$
$$(n = 4, r^2 = 0.98, SD = 0.24)$$

The $\log k_{plasma}$ values also correlate with Hammett σ^- values for the substituents in the phenol moiety to give a ρ value of 2.3 (r^2 = 0.99). Overall, these results suggest that both alkaline and plasma hydrolyses have similar structural requirements, that is reactivity in human plasma appears to be dependent on the alcohol leaving group ability rather than on lipophilicity of the prodrug, and that plasma hydrolysis has a substantial contribution from the E1cB process. A similar correlation between alkaline and plasma hydrolyses has also been reported for the plasma activation of acyloxymethyl-type prodrugs of sulfonamides.[24] These results suggest that the k_{OH^-} values can be used as a crude indicator for the metabolic susceptibility of a prodrug series towards serine-dependent enzymes such as BuChE and carboxylesterases.[25,26]

2.4. Activation in rat liver homogenates

Based on previous studies reporting the nearly equal roles of cytochrome P450, MAO and aldehyde dehydrogenase in the conversion of primaquine into carboxyprimaquine,[27] rat liver homogenates were specifically chosen to study the metabolism of derivatives 4. Both aryl and alkyl carbamates were slowly hydrolysed in rat liver homogenates, with half-lives ranging from 9 to 15 h, while 4-nitrophenyl carbamate, 4 g hydrolysed too rapidly to be monitored by HPLC. No clear correlation emerged between the rate data in Table 1 and the pK_a of the alcohol/phenol leaving group, $\log P$ or $\log D_{7.4}$ values for compounds **4a**–**g**.

HPLC analysis of the incubation mixtures revealed that only *O*-aryl carbamates released primaquine, corresponding to 12–15% of prodrug conversion to the parent drug. Surprisingly, primaquine was not detected when *O*-alkyl carbamates **4a**–**c** were activated in liver homogenates, contrasting with the observation that secondary and tertiary alkyl carbamates readily liberate the corresponding primary and secondary amines via oxidative dealkylation, as reported for ritonavir, amprenavir and loratidine.[28–30] Importantly, neither carboxyprimaquine or 8-amino-6-methoxyquinoline, a minor metabolite arising from the oxidative deamination at C-1′,[31,32] were detected in any of the incubation mixtures over the timescale of the hydrolysis reactions. The reason for the incomplete release of primaquine from the corresponding carbamates in rat liver homogenates is not yet fully understood, but one might speculate that for *O*-aryl carbamates **4d**–**g** might form a stable carbamoyl enzyme resulting from the reaction with liver carboxylesterase (CE). It has been reported that rat liver contains CE concentrations high enough to contribute significantly to the hydrolysis of carmabates,[33,34] and thus this enzyme is likely to be involved in the hydrolysis of derivatives **4d**–**g** in rat liver homogenates.

2.5. In vivo studies: gametocytocidal activity

The potential of compounds **4** to prevent the transmission of malaria was evaluated through their gametocytocidal activity against *Plasmodium berghei* using a model consisting of Balb C mice and *Anopheles stephensi* mosquitoes.[35,36] Carbamates **4f**–**g** were excluded from this assay due to their chemical reactivity. For each compound, the effect of the single dose of 15 mg/kg on the appearance of oocysts in the midguts of mosquitoes was studied. The criteria to assess the activity were: (i) the percentage of mosquitoes with oocysts and (ii) the mean number of oocysts per infected mosquito. Sporogonic studies using laboratory-raised vectors generates data with significant inherent variation which might have several origins, such as a genetically heterogeneous mosquito population or individual variation in vector feeding duration (therefore affecting the number of ingested gametocytes).[37] In order to assure that this inherent variation would not affect the interpretation of the experimental data, the largest possible number of mosquitoes was used for each compound and dose. As the result of the high number of mosquitoes necessary to perform all experiments it was decided to use separate controls for each compound (Table 2).

Inspection of the activity data presented in Table 2 shows that *O*-alkyl carbamates **4a**,**b** reduced the percentage of infected mosquitoes and the production of oocysts when compared to controls. In contrast, carbamates **4c**–**e** did not significantly affect the percentage of mosquitoes with oocysts and the mean number of oocysts per infected mosquito when compared to controls. Compounds **4a**,**b** were less active than primaquine, which almost completely inhibited the production of oocysts at a dose of 15 mg/kg. These results suggest that the gametocytocidal activity displayed by compounds **4a**–**e** is not dependent on the release of the parent drug and that *O*-alkyl carbamates **4a**,**b**, the most potent compounds in this series, are active per se.

Table 2
Effect of carbamates **4a**–**e** and primaquine on the sporogonic development of *P. berghei* in *A. stephensi* mosquitoes

Compound	% of infected mosquitoes	Mean No. oocysts per mosquito (±SE)[a]
Controls	91	58.8 (8.9)
4a	19	14.8 (6.8)
4b	24	24.6 (5.4)
4c	72	41.0 (5.5)[b]
Controls	80	46.1 (9.1)
4d	74	39.7 (5.5)[b]
4e	65	33.4 (8.9)
Primaquine	3.9	1.6 (1.3)

[a] Counting of oocysts was carried out at day 10 post-feed.
[b] P >0.05 versus control, by Student's t-test.

3. Conclusions and relevance to prodrug design

The use of carbamates as prodrugs of basic primary amine drugs is still open to discussion mainly due to the lack of in vitro/in vivo prodrug activation data.[16] The results presented herein show that primaquine carbamates derived from phenols are readily hydrolysed both in alkaline solutions and human plasma, with chemical and enzymatic reactivities being dependent on the electronic effects on the alcohol moiety. Activation of *O*-aryl carbamates in human plasma appears to be catalysed by BuChE, leading to carbamoylation of the catalytic serine and subsequent slow enzyme reactivation and release of parent drug. Both *O*-aryl and *O*-alkyl carbamates are slowly activated in rat liver homogenates, but only *O*-aryl derivatives released primaquine at a measurable rate. When assayed for gametocytocidal activity, ethyl, **4a**, and *n*-hexyl, **4b**, derivatives were able to significantly reduce the percentage of mosquitoes with oocysts and mean number of oocysts per infected mosquito when compared to controls, thus indicating that carbamates of primaquine have the potential to be developed as transmission-blocking antimalarial agents.

4. Material and methods

4.1. Chemistry

4.1.1. General

Melting points were recorded using a Buchi 510 capillary melting-point apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Bruker MSX-300 spectrometer. The ^{1}H NMR data are reported as follows: chemical shifts, expressed in ppm, using internal TMS as reference; number of protons; multiplicity (s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; br, broad); and coupling constants, *J*, quoted in Hertz. The IR spectra were recorded using a Nicolet FTIR Impact 400 spectrophotometer either as thin films or KBr pellets. Mass spectra (FAB-MS) were obtained on a VG Mass Lab 20–250 using glycerol as matrix. Microanalyses were obtained from MEDAC (UK) or ITN laboratories (Portugal). All kinetic measurements were made using either a Shimadzu UV-2100 spectrophotometer equipped with a Shimadzu CPS-260 temperature controller or an HPLC system comprising a Merck-Hitachi L-6000 pump, a Merck-Hitachi diode array detector and a 20 μL Rheodyne injector. Primaquine, (8-(4-amino-1-methylbutylamino)-6-methoxyquinoline), the alkyl and aryl chloroformates and carbonyl diimidazole (CDI) were purchased from Aldrich.

4.1.2. 8-(4-Ethoxycarbonylamino-1-methylbutylamino)-6-methoxyquinoline (4a)

Ethyl chloroformate (0.19 mL, 2 mmol) was added to a suspension containing primaquine diphosphate (0.911 g, 2 mmol) and triethylamine (3 mol equiv) in tetrahydrofuran (5 mL) at −10 °C. The mixture was stirred at −10 °C for 30 min and then left at room temperature for 90 min. The reaction mixture was filtered and the filtrate evaporated. The residue was re-dissolved in dichloromethane and the organic layers were washed (satd $NaHCO_3$, water), dried (anhydrous $MgSO_4$) and evaporated to dryness to yield an oily residue that was subjected to column chromatography on silica gel (ether/light petroleum, 7:3) to give 4a as a brown oil in 53% yield. ^{1}H NMR ($CDCl_3$): δ 1.15 (3H, t, J = 7.1), 1.23 (3H, d, J = 6.4), 1.51–1.61 (4H, m), 3.11–3.20 (2H, m), 3.52–3.58 (1H, m), 3.81 (3H, s), 4.02 (2H, q, J = 7.1), 4.61 (1H, br s), 5.93 (1H, d, J = 8.2), 6.20 (1H, d, J = 2.5), 6.26 (1H, d, J = 2.5), 7.23 (1H, dd, J = 8.2, 4.2), 7.85 (1H, dd, J = 8.2, 1.6), 8.45 (1H, dd, J = 4.2, 1.6); ^{13}C NMR ($CDCl_3$): δ 14.7, 20.9, 26.9, 34.0, 41.0, 47.9, 55.3, 60.7, 91.7, 96.8, 121.9, 130.0, 134.9, 135.4, 144.4, 145.0, 156.8, 159.5; FAB-MS, *m/z* (%) 332 (75, MH^+), 201 (100), 175 (37). Anal. Calcd for $C_{18}H_{25}N_3O_3$: C, 65.23; H, 7.60; N, 12.68. Found: C, 65.17; H, 7.91; N, 12.51.

4.1.3. 8-[4-(1-Hexyloxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4b)

The procedure described for **4a** was used with *n*-hexyl chloroformate (0.49 mL, 3 mmol) and primaquine diphosphate (0.911 g, 2 mmol) to afford **4b** as a brown oil (42%). ^{1}H NMR ($CDCl_3$): δ 0.86 (3H, t, J = 6.8), 1.14–1.21 (4H, m), 1.25 (3H, d, J = 6.4), 1.44–1.67 (8H, m), 3.12–3.21 (2H, m), 3.47–3.52 (1H, m), 3.77 (3H, s), 4.01 (2H, t, J = 7.0), 4.55 (1H, br s), 5.89 (1H, d, J = 8.4), 6.16 (1H, d, J = 2.6), 6.22 (1H, d, J = 2.6), 7.19 (1H, dd, J = 8.2, 4.2), 7.80 (1H, dd, J = 8.2, 1.6), 8.41 (1H, dd, J = 4.2, 1.6); ^{13}C NMR ($CDCl_3$): δ 14.4, 21.0, 22.9, 25.9, 27.2, 29.4, 31.9, 34.3, 41.4, 48.2, 55.6, 65.3, 92.1, 97.1, 122.2, 130.3, 135.2, 135.9, 144.7, 145.3, 157.2, 159.8; FAB-MS, *m/z* (%) 388 (47, MH^+), 201 (100), 175 (64). Anal. Calcd for $C_{22}H_{33}N_3O_3$: C, 68.19; H, 8.58; N, 10.84. Found: C, 68.25; H, 8.53; N, 10.86.

4.1.4. 8-[4-(2,2,2-Trifluoroethoxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4c)

Trifluoroethanol (0.07 mL, 1 mmol) in dried tetrahydrofuran (5 mL) was slowly added to a solution of carbonyl diimidazole (0.162 g, 1 mmol) in dried tetrahydrofuran (5 mL). After stirring for 1 h at room temperature, a solution of primaquine free base (0.263 g, 1 mmol; obtained by treating an aqueous solution of primaquine diphosphate with an excess of Na_2CO_3 and then extracting with dichloromethane, followed by drying and evaporation of the solvent) in dried tetrahydrofuran (5 mL) was added to reaction mixture. After overnight at room temperature the reaction mixture was filtered and the solvent was evaporated under reduce pressure. The residue was re-dissolved in dichloromethane (50 mL) and the organic phase was washed (satd $NaHCO_3$, water), dried (anhydrous $MgSO_4$) and evaporated to dryness. The residue was further purified by dry column chromatography (silica gel; ether/light petroleum, 7:3) to give **4c** as brown oil in 43% yield. IR (cm^{-1}) ν_{max} 3360, 1737; ^{1}H NMR ($CDCl_3$): δ 1.23 (3H, d, J = 6.4), 1.58–1.68 (4H, m), 3.15–3.23 (2H, m), 3.51–3.60 (1H, m), 3.81 (3H, s), 4.36 (2H, q, J = 9.0), 4.89 (1H, br s), 5.92 (1H, d, J = 8.4), 6.20 (1H, d, J = 2.5), 6.27 (1H, d, J = 2.5), 7.23 (1H, dd, J = 8.2, 4.2), 7.85 (1H, dd, J = 8.2, 1.5), 8.45 (1H, dd, J = 4.2, 1.5); ^{13}C NMR ($CDCl_3$): δ 20.7, 26.6, 33.8, 41.4, 47.8, 55.3, 74.5, 91.8, 96.9, 121.9, 124.9, 130.0, 134.9, 135.6, 144.4, 144.9, 154.6, 159.5; FAB-MS, *m/z* (%) 386 (48, MH^+), 201 (100), 175 (60). Anal. Calcd for $C_{18}H_{22}F_3N_3O_3$: C, 56.10; H, 5.75; N, 10.90. Found: C, 56.36; H, 5.59; N, 10.77.

4.1.5. 8-[4-(4-Methoxyphenoxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4d)

The procedure described for **4a** was used with 4-methoxyphenyl chloroformate (0.49 mL, 4 mmol) and primaquine diphosphate (1.82 g, 4 mmol) to afford **4d** as a yellowish solid (49%). Mp: 96–98 °C; IR (cm^{-1}) ν_{max} 3330, 1703; ^{1}H NMR ($CDCl_3$): δ 1.32 (3H, d, J = 6.4), 1.67–1.80 (4H, m), 3.57–3.69 (2H, m), 3.72–3.79 (1H, m), 3.79 (3H, s), 3.89 (3H, s), 5.02 (1H, br s), 6.02 (1H, d, J = 8.4), 6.29 (1H, d, J = 2.4), 6.34 (1H, d, J = 2.4), 6.85 (2H, d, J = 9.0), 7.01 (2H, d, J = 9.0), 7.31 (1H, dd, J = 8.2, 4.2), 7.92 (1H, dd, J = 8.2, 1.6), 8.53 (1H, dd, J = 4.2, 1.6); ^{13}C NMR ($CDCl_3$): δ 20.8, 26.9, 33.8, 41.2, 47.8, 55.2, 55.6, 91.7, 97.8, 114.3, 121.8, 122.4, 129.9, 134.8, 135.3, 144.3, 144.7, 144.9, 154.9, 156.8, 159.4; FAB-MS, *m/z* (%) 410 (51, MH^+), 286 (93), 201 (100), 175 (67). Anal. Calcd for $C_{23}H_{27}N_3O_4$: C, 67.45; H, 6.65; N, 10.26. Found: C, 67.40; H, 6.53; N, 10.24.

4.1.6. 8-(4-Phenoxycarbonylamino-1-methylbutylamino)-6-methoxyquinoline (4e)

The procedure described for **4a** was used with phenyl chloroformate (0.50 mL, 4 mmol) and primaquine diphosphate (1.82 g, 4 mmol) to afford **4e** as a light brown gum (42%). IR (cm^{-1}) ν_{max} 3367, 1734; ^1H NMR ($CDCl_3$): δ 1.33 (3H, d, J = 6.4), 1.67–1.80 (4H, m), 3.29–3.34 (2H, m), 3.64–3.68 (1H, m), 3.89 (3H, s), 5.05 (1H, br s), 6.02 (1H, d, J = 8.4), 6.30 (1H, d, J = 2.4), 6.34 (1H, d, J = 2.6), 7.11 (2H, d, J = 7.5), 7.15–7.37 (4H, m), 7.93 (1H, dd, J = 8.2, 1.6), 8.54 (1H, dd, J = 4.2, 1.6); ^{13}C NMR ($CDCl_3$): δ 20.6, 26.6, 33.8, 41.2, 47.8, 55.2, 91.7, 96.7, 121.6, 121.8, 125.2, 129.2, 129.9, 134.8, 135.5, 144.3, 145.1, 151.0, 154.7, 159.5; FAB-MS, *m/z* (%) 380 (45, MH^+), 201 (100), 175 (44). Anal. Calcd for $C_{22}H_{25}N_3O_3$: C, 69.64; H, 6.64; N, 11.07. Found: C, 69.43; H, 6.67; N, 11.19.

4.1.7. 8-[4-(4-Chlorophenoxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4f)

The procedure described for **4a** was used with 4-chlorophenyl chloroformate (0.56 mL, 4 mmol) and primaquine diphosphate (1.82 g, 4 mmol) to afford **4f** as a yellowish solid (36%). Mp: 106–108 °C; IR (cm^{-1}) ν_{max} 3354, 1736; ^1H NMR ($CDCl_3$): δ 1.34 (3H, d, J = 6.1), 1.68–1.80 (4H, m), 3.28–3.30 (2H, m), 3.64–3.69 (1H, m), 3.89 (3H, s), 5.05 (1H, br s), 6.02 (1H, d, J = 8.2), 6.29 (1H, d, J = 2.5), 6.34 (1H, d, J = 2.4), 7.05 (2H, d, J = 8.7), 7.30 (2H, d, J = 8.7), 7.32 (1H, dd, J = 8.2, 4.5), 7.93 (1H, dd, J = 8.2; 1.6), 8.50 (1H, dd, J = 4.5, 1.6); ^{13}C NMR ($CDCl_3$): δ 20.6, 26.5, 33.8, 41.2, 47.9, 55.2, 91.7, 96.8, 121.9, 122.9, 129.2, 129.9, 130.0, 134.8, 135.5, 144.4, 145.2, 150.3, 154.0, 159.2; FAB-MS, *m/z* (%) 416 (11, MH^++2), 414 (33, MH^+), 201 (100), 175 (42). Anal. Calcd for $C_{22}H_{24}ClN_3O_3$: C, 63.84; H, 5.84; N, 10.15. Found: C, 64.01; H, 5.79; N, 9.99.

4.1.8. 8-[4-(4-Nitrophenoxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4g)

The procedure described for **4a** was used with 4-nitrophenyl chloroformate (1.01 g, 5 mmol) and primaquine diphosphate (2.28 g, 5 mmol) to afford **4g** as a brown oil (10%). IR (cm^{-1}) ν_{max} 3332, 1705; ^1H NMR δ ($CDCl_3$): 1.33 (3H, d, J = 6.2), 1.65–1.74 (4H, m), 3.31–3.35 (2H, m), 3.70 (1H, m), 3.89 (3H, s), 5.22 (1H, br s), 6.01 (1H, d, J = 8.4), 6.30 (1H, d, J = 2.4 Hz), 6.35 (1H, d, J = 2.4), 7.30 (1H, dd, J = 8.2, 4.0), 7.30 (2H, d, J = 8.9), 7.93 (1H, dd, J = 8.2, 1.1), 8.20 (2H, d, J = 9.0), 8.53 (1H, dd, J = 4.0, 1.5); ^{13}C NMR ($CDCl_3$): δ 21.1, 26.8, 34.2, 41.8, 48.2, 55.6, 92.2, 97.3, 116.2, 122.3, 125.5, 126.6, 130.3, 135.3, 144.7, 144.8, 145.2, 153.1, 156.1, 159.8; FAB-MS, *m/z* (%) 425 (33, MH^+), 201 (100), 175 (41). Anal. Calcd for $C_{22}H_{24}N_4O_5$: C, 62.25; H, 5.70; N, 13.20. Found: C, 62.27; H, 5.69; N, 13.15.

4.1.9. 8-[4-(2-Ethoxycarbonylmethoxycarbonyl)amino-1-methylbutylamino]-6-methoxyquinoline (4h)

The procedure described for **4c** was used with ethyl glycolate (0.09 mL, 1 mmol) and primaquine free base (0.262 g, 1 mmol) to afford **4h** as a brown oil (27%). IR (cm^{-1}) ν_{max} 3365, 1740, 1732; ^1H NMR ($CDCl_3$): δ 1.26 (3H, d, J = 7.1), 1.29 (3H, t, J = 7.0), 1.62–1.76 (4H, m), 3.20–3.30 (2H, m), 3.57–3.65 (1H, m), 3.89 (3H, s), 4.19 (2H, q, J = 7.1), 4.65 (2H, s), 4.93 (1H, br s), 6.02 (1H, d, J = 8.2), 6.28 (1H, d, J = 2.5), 6.34 (1H, d, J = 2.5), 7.30 (1H, dd, J = 8.2, 4.2), 7.92 (1H, dd, J = 8.2, 1.6), 8.53 (1H, dd, J = 4.2, 1.6); ^{13}C NMR ($CDCl_3$): δ 14.1, 20.6, 26.6, 33.8, 41.1, 47.8, 55.2, 61.1, 61.6, 91.7, 96.8, 121.8, 129.9, 134.8, 135.2, 144.2, 144.8, 155.5, 159.4, 168.7. Anal. Calcd for $C_{20}H_{27}N_3O_5$: C, 61.68; H, 6.99; N, 10.79. Found: C, 60.82; H, 6.73; N, 10.64.

4.1.10. ({4-[(6-Methoxyquinolin-8-yl)amino]pentyl}carbamoyloxy) acetic acid (4i)

A solution of **4h** (72.2 mg, 0.2 mmol) in aqueous 0.1 N NaOH in dioxane (3 mL) was stirred for 2 h. The reaction mixture was treated with 1 N HCl and extracted with dichloromethane (10 mL). The resulting organic phase was dried (anhydrous $MgSO_4$) and evaporated to dryness to afford **4i** as a brown oil (36%). IR (cm^{-1}) ν_{max} 3480 (br), 3320, 1730, 1712; ^1H NMR ($CDCl_3$): δ 1.23 (3H, d, J = 6.4), 1.50–1.84 (4H, m), 3.46–3.58 (2H, m), 3.60–3.81 (1H, m), 3.81 (3H, s, OCH_3), 4.62 (2H, s), 4.82 (1H, br s), 5.92 (1H, d, J = 8.4), 6.20 (1H, d, J = 2.4), 6.26 (1H, d, J = 2.5), 7.23 (1H, dd, J = 8.2, 4.2), 7.84 (1H, dd, J = 8.2, 1.6), 8.45 (1H, dd, J = 4.2, 1.6), 11.51 (1H, br s); ^{13}C NMR ($CDCl_3$): δ 20.5, 24.3, 33.5, 40.1, 47.6, 55.2, 67.7, 91.8, 96.8, 121.8, 129.8, 134.7, 135.3, 144.3, 144.8, 155.8, 159.4, 170.4. Anal. Calcd for $C_{18}H_{23}N_3O_5$: C, 59.82; H, 6.41; N, 11.63. Found: C, 60.11; H, 6.33; N, 11.44.

4.1.11. Carboxyprimaquine (3)

Carboxyprimaquine, **3**, was synthesized according to the procedure reported by McChesney et al.[18] Anal. Calcd for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.71; H, 6.55; N, 10.14.

4.2. In vitro studies

4.2.1. Hydrolysis in aqueous solutions

All kinetic experiments were carried out in aqueous borate and NaOH buffers, containing 20% (v/v) acetonitrile to aid substrate solubility, at an ionic strength of 0.5 M ($NaClO_4$). Kinetic studies on the aryl carbamates were carried out by UV spectrophotometry at fixed wavelength by recording the decrease of substrate absorbance in the appropriate buffer solutions at 37 °C. In a typical run, reaction was initiated by adding a 15 μL aliquot of a 10^{-2} M stock solution of substrate in acetonitrile to a cuvette containing 3 ml of the pre-equilibrated buffer solution at 37 °C. The pseudo-first-order rate constants, k_{obs}, were obtained from the slopes of plots of $\ln(A_t - A_\infty)$ versus time, where A_t and A_∞ represent the absorbance at time t and at infinity, respectively. For alkyl carbamates **4a**–**c** and **4h**, the kinetic studies were carried out by HPLC following the loss of the substrate and formation of primaquine. In this method a 15 μL aliquot of 10^{-2} M stock solution of substrate was added to a reaction flask containing 3 ml of the buffer solution. At regular intervals, 50 μL samples of the reaction mixture were neutralised with HCl solutions and analysed using a Lichrosorb RP-8 column (5 μm particles, 4 × 250 mm; Merck) and an eluant consisting of acetonitrile/water/KH_2PO_4 1 M (55:40:5) at a flow rate of 1 mL/min. The hydrolysis of **4a**–**f** and **4h** in pH 7.4 buffer were also studied by HPLC.

4.2.2. Hydrolysis in human plasma

Human plasma was obtained from the heparinised blood of healthy donors, and was pooled and frozen at −70 °C before use. Typically, a 35 μL aliquot of 10^{-2} M stock solution of compound **4** was added to 350 μL of the human plasma diluted to 50% (v/v) with pH 7.4 isotonic phosphate buffer, at 37 °C. At appropriate intervals, 75 μL aliquots were added to acetonitrile (350 μL) to quench the reaction and precipitate plasma proteins. These samples were centrifuged (10,000 rpm for 10 min) and the clear supernatant analysed by HPLC for the presence of both substrate and primaquine, using a Lichrosorb RP-8 column (5 μm particles, 4 × 250 mm; Merck) and an eluant consisting of acetonitrile/buffer (40:60%) at a 1.0 mL/min flow rate. The buffer contained 10 mM sodium hexanesulfonate, 2.5 mM sodium acetate and 2.5 mM phosphoric acid. For compounds **4d**–**g**, the corresponding phenol products were also analysed. Quantitation of the substrate and corresponding products was achieved from measurement of peak areas relative to those of corresponding standards subjected to the same chromatographic conditions. The first-order-rate constants, k_{plasma}, were determined in triplicate.

4.2.3. Hydrolysis in rat liver homogenates

The metabolism of primaquine carbamates **4** in rat liver homogenates[15] was monitored using HPLC following either the loss of the

substrate and the formation of primaquine. Incubations were carried out at 37 °C in pH 7.4 phosphate buffer saline (PBS) using 2 mg protein/mL of liver homogenates and a NADPH generating system consisting of glucose-6-phosphate 6.25 mM, NADP 1.25 mM, $MgCl_2$ 6 mM and 2.5 U/mL of glucose-6-phosphate dehydrogenase. The substrates, dissolved in pH 7.4 PBS, were added to give initial concentrations of 0.25–5.0 mM. At regular intervals, 25–100 μL samples of the liver incubates were withdrawn and added to acetonitrile (1.2 mL), the resulting mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was analysed using the same HPLC described for studies in human plasma. The first-order-rate constants, k_{liver}, were determined in triplicate.

4.2.4. Apparent partition coefficients

The apparent partition coefficients between 1-octanol and pH 7.4 phosphate buffer, $D_{7.4}$, were determined at 22 °C. Both the aqueous and octanol phases were mutually saturated before the experiment. The volumes of each phase were chosen so that solute concentrations in the aqueous phase after distribution were readily measured. The compounds were dissolved in 1-octanol and the octanol–phosphate buffer mixtures were shaken for 30 min to reach an equilibrium distribution. Each phase was analysed separately by HPLC. Partition coefficients were calculated from the ratio of the peak area in 1-octanol to the peak area in buffer and the reported values are the average of three experiments. Partition coefficients for compounds **4** were calculated using the ALOGPS 2.1 program (available at http://146.107.217.178/lab/alogps/).[38]

4.3. In vivo studies

4.3.1. Sporogonic development of *Plasmodium berghei*

P. berghei ANKA 25R/10 strain, Balb C mice and *A. stephensi* mosquitoes were used throughout the course of this study. Mice were infected by intraperitoneal inoculations of 10^7 erythrocytes parasitised with *P. berghei* ANKA. After four days, when the presence of parasites in the blood (namely gametocytes) was observed by microscopic observation of Giemsa stained blood films, mice were randomly separated into five different groups of six animals. Each group was treated by intraperitoneal administration with one single dose of each compound (1.86, 3.75, 7.50 and 15.0 mg/kg body weight in inoculation volumes of 0.1–0.2 mL; controls consisted of mice given a saline solution). Two hours after administration, mice were anesthetised (diethyl ether) and placed on top of individual cages containing ca. 50 glucose-starved mosquitoes. These were allowed to feed for 2 h. After the blood meal, unfed females were removed from each cage. Engorged mosquitoes were maintained in the insectarium at 21 ± 1 °C and 60% relative humidity, receiving a solution containing 10% glucose, 0.05% PABA and 0.001% gentamicine (all v/v). Ten days after the blood meal, 10 mosquitoes of each cage were randomly collected and dissected for microscopic detection (100× and 400×) of oocysts in midguts. Dissection was performed, using a stereomicroscope, in a drop of saline solution.

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