Dipeptide Derivatives of Primaquine as Transmission-Blocking Antimalarials: Effect of Aliphatic Side-Chain Acylation on the Gametocytocidal Activity and on the Formation of Carboxyprimaquine in Rat Liver Homogenates

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Purpose. Dipeptide derivatives of primaquine (PQ) with reduced oxidative deamination to the inactive metabolite carboxyprimaquine were synthesized and evaluated as a novel class of transmission-blocking antimalarials.

Methods. Antimalarial activity was studied using a model consisting of mefloquine-resistant *Plasmodium berghei* ANKA 25R/10, Balb C mice, and *Anopheles stephensi* mosquitoes. Metabolic studies were performed with rat liver homogenates, and the incubates were analyzed by HPLC.

Results. All dipeptide derivatives and glycyl-PQ completely inhibited the appearance of oocysts in the midguts of the mosquitoes at 15 mg/kg, while *N*-acetylprimaquine was not active at this dose. However, none of the title compounds were able to block oocyst production at 3.75 mg/kg, in contrast with primaquine. Exception for sarc-gly-PQ, all remaining compounds prevented sporozoite formation in the salivary glands of mosquitoes at a dose of 3.75 mg/kg. Simultaneous hydrolysis to primaquine and gly-PQ ocurred with the following order of V_{max}/K_m: for primaquine formation, L-ala-gly-PQ > L-phe-gly-PQ > gly-gly-PQ; and for gly-PQ formation, L-phe-gly-PQ > L-ala-gly-PQ > gly-gly-PQ. In contrast, primaquine was not released from D-phe-gly-PQ, sarc-gly-PQ, and *N*-acetylprimaquine. Neither carboxyprimaquine nor 8-amino-6-methoxyquinoline were detected in any of the incubation mixtures.

Conclusions. The title compounds prevent the development of the sporogonic cycle of *Plasmodium berghei*. Gametocytocidal activity is independent of the rate and pathway of primaquine formation. Acylation of the aliphatic side-chain effectively prevents the formation of carboxyprimaquine, but the presence of a terminal amino group appears to be essential for the gametocytocidal activity.

KEY WORDS: primaquine; peptide derivatives; gametocytocidal activity; liver homogenates.

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INTRODUCTION

Malaria is the major public health problem in tropical areas, with an annual incidence of more than 110 million clinical cases and up to 1 million deaths (1). The rapid emergence and spread of chloroquine-resistant *Plasmodium falciparum* throughout the African continent is the major obstacle to present efforts to control the disease (1). Moreover, *P. falciparum* strains resistant to newer drugs such as mefloquine, have now been reported in South East Asia. Drug resistance results from the loss of drug activity on the asexual blood forms of the parasite, called blood schizonts, which are responsible for the clinical symptoms of the disease (2). The blood schizonts can differentiate into the sexual forms of the parasite, or gametocytes. These, once in the mosquito vector, can develop first into oocysts, and then into sporozoites, the infecting form of the parasite. Hence, a gametocytocidal drug could interrupt disease transmission from the host to the mosquito vector and could become an important strategy to achieve an effective control of malaria (3).

Currently, primaquine, **1**, is the only available transmission-blocking antimalarial displaying a marked activity against gametocytes from all species of parasite causing human malaria, including chloroquine-resistant *P. falciparum* (4). However, primaquine is rapidly metabolised in mammals to carboxyprimaquine **2**, which is devoid of significant antimalarial activity (5,6). After intravenous administration of primaquine to rats, monkeys, and humans it was found the plasma concentration of carboxyprimaquine rapidly exceeded that of the parent drug after 15—30 min (7–9). Although the pathway leading to the oxidative deamination at C-4′ of primaquine is not fully clarified, it seems to involve three enzymes in a two-step process: in the first step, monoamine oxidase (MAO) or cytochrome P-450 systems mediate the oxidation of primaquine to the aldehyde **3** (Fig. 1); in the second, the intermediate **3** is further oxidised to carboxyprimaquine **2** by aldehyde dehydrogenase (10). Interestingly, only one study specifically designed to search for new primaquine derivatives with potentially slower rates of oxidative deamination has been reported (10). Substitution of the hydrogen atoms with deuterium atoms at C-4′ of the aliphatic side-chain of primaquine was found to decrease the rate of oxidation to carboxyprimaquine 7-fold, while maintaining an *in vitro* activity against schizonts comparable to that of the parent drug (10).

Several peptide and amino acid derivatives of primaquine have been designed to reduce its toxicity, particularly its ability to induce oxidation of oxyhemoglobin to methemoglobin (11–13). Most of these derivatives, which contain L-leucine, L-alanine, or L-lysine directly linked to the C-4′ amino group (e.g. ala-leu-PQ or ala-leu-ala-lys-PQ), are rapidly hydrolysed to primaquine by aminopeptidases and endopeptidases (11,14). Therefore, it is of obvious interest to design new small peptide derivatives of primaquine, incorporating amino acids either that have the D-configuration or are *N*-alkylated, that could lead to peptidase-stable compounds with intrinsic antimalarial activity. Moreover, as secondary amides are known to be poor substrates for monoamino oxidase (15) and cytochrome P-450 systems (16), such compounds could completely block the formation of carboxyprimaquine.

The purpose of this research was: 1) to evaluate the gametocytocidal activity of dipeptide derivatives **4** of primaquine

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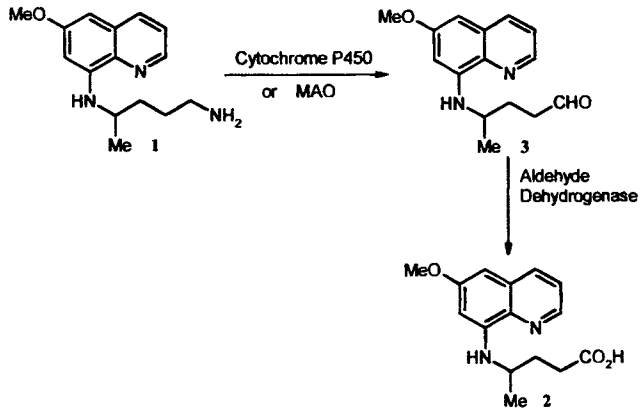


Fig. 1. Pathways for the oxidative deamination of primaquine.

(Fig. 2) and their potential as transmission-blocking antimalarials; 2) to compare the effect of side chains containing natural and non-natural aminoacids on the gametocytocidal activity; and 3) to assess the effect of the aliphatic side-chain acylation on the formation of carboxyprimaquine in rat liver homogenates. The 700 g supernatant fraction of rat liver homogenates was chosen as it includes all the enzymes most likely to metabolise both primaquine and its derivatives: cytochrome P450, monoamine oxidase, aldehyde dehydrogenase; and peptidases (10). Also included in this study were glycylprimaquine, **5**, which is a potential metabolic intermediate of **4**, and *N*-acetylprimaquine, **6**, to evaluate the importance of the terminal amino group to the gametocytocidal activity.

MATERIALS AND METHODS

General Procedures

Melting points were recorded on a Buchi 510 capillary melting-point apparatus and are uncorrected. The ^{1}H and ^{13}C-NMR spectra were recorded on a Jeol JNM-EX400 or Bruker MSX-300 spectrometer; coupling constants, *J*, are quoted in Hertz. The IR spectra were obtained with a Nicolet FTIR Impact 400 spectrophotometer. Microanalyses were obtained from MEDAC (U.K.) or ITQB laboratories (Portugal).

Chemicals

Primaquine (8-(4′-amino-1′-methylbutylamino)-6-methoxyquinoline), *N-tert*-butoxycarbonyl-L-phenylalanylglycine (Boc-L-phe-glyOH), *N-tert*-butoxycarbonyl-L-alanylglycine (Boc-L-ala-glyOH), sarcosine, *N-tert*-butoxycarbonyl-D-phenylalanine *N*-hydrosuccinimide ester (Boc-D-pheOSucc), and *N-tert*-butoxycarbonylglycine (Boc-glyOH) were purchased from Sigma (Sigma-Aldrich SA, Madrid, Spain). Glycine ethyl ester hydrochloride (glyOEt) and *tert*-butyl carbonic anhydride were purchased from Aldrich (Sigma-Aldrich SA, Madrid, Spain). *N-tert*-Butoxycarbonylglycylglycine (Boc-gly-glyOH) was from Bachem. Carboxyprimaquine was a kind gift of Prof. J. D. McChesney (University of Mississippi). All other chemicals and solvents were of reagent grade, except buffer substances and HPLC solvents which were analytical grade and LiChrosolve (Merck) grade, respectively. Column chromatography was performed using silica gel 60 mesh 70-230 (Merck).

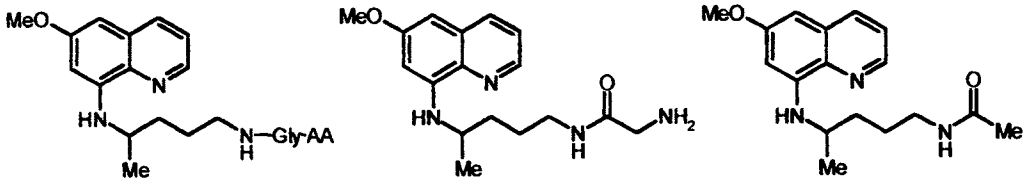
8-Amino-6-methoxyquinoline. A solution of 6-methoxy-8-nitroquinoline (0.41 g, 2 mmol) in 37% HCl (2 cm^3) was slowly added to a suspension of $SnCl_2$ (1.52 g, 8 mmol) and tin (0.057 g) in 37% HCl (2 cm^3) at 0°C. The reaction mixture was kept in ice for 1 h, after which a solution of 8M NaOH was added slowly, while maintaining the temperature bellow 10°C, until a brown oil was formed. This oil was extracted with dichloromethane (2 × 25 cm^3), the organic layers washed with water, dried with sodium sulfate and evaporated to yield pure 8-amino-6-methoxyquinoline (75%). ^{1}H-NMR ($CDCl_3$) δ: 3.83 (3H, s, CH_3O); 5.74 (2H, s, NH_2); 6.43 (1H, d, J = 2.4, C_7-H); 6.52 (1H, d, J = 2.4, C_5-H); 7.33 (1H, dd, J = 4.2, 8.4, C_3-H); 7.97 (1H, dd, J = 1.5, 8.4, C_4-H); 8.52 (1H, dd, J = 1.5, 4.2, C_2-H).

General Synthesis of Aminoacylglycyl Derivatives of Primaquine

The synthesis of derivatives **4a-c**, and **5** was achieved by coupling the appropriate BOC-protected aminoacid or dipeptide using the *N,N′*-diclohexylcarbodiimide (DCC) method. For compounds **4d-e**, it was necessary to prepare the corresponding dipeptide prior to the coupling reaction. The general procedure is exemplified for compound **4d**.

Step A

GlyOEt hydrochloride (0.349 g, 2.5 mmol) and dry triethylamine (0.25 g , 2.5 mmol) were added sequentially to a solution of Boc-D-pheOSucc (0.91 g, 2.5 mmol) in dry THF (12 cm^3) at 0°C. The mixture was stirred at room temperature for 24 h, then filtered. The filtrate was evaporated and the residue chromatographed on silica gel (ethyl acetate as eluent) to yield 0.83 g (94%) of *N-tert*-butoxycarbonyl-D-phenylalanylglycine ethyl ester (Boc-D-phe-glyOEt) as white crystals



4 **5** **6**

Fig. 2. Structures of investigated compounds. **a**, AA = gly; **b**, AA = L-phe; **c**, AA = L-ala; **d**, AA = D-phe; **e**, AA = sarcosine.

(mp. 92–94°C); IR: 3329, 1742, 1689, 1654 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.27 (3H, t,J = 7.1, CH_3CH_2); 1.39 (9H, s, $C(CH_3)_3$); 3.03 (1H, dd, J = 6.0, 12.2, CH_2Ph); 3.10 (1H, dd, J = 6.0, 12.2, CH_2Ph), 3.91 (1H, dd, J = 5.2, 18.2, CH_2gly); 4.03 (1H, dd, J = 5.2, 18.2, CH_2gly); 4.19 (2H, q, J = 7.1, CH_3CH_2); 4.42 (1H, m, CH); 5.03 (1H, brs, NH); 6.45 (1H, t, J = 5.2, NH); 7.20-7.31 (5H, m, Ph).

Step B

A solution of Boc-D-phe-glyOEt (0.544 g, 1.6 mmol) in THF (8 cm^3) and 1N NaOH (1.6 cm^3, 1 mol eq) was stirred at room temperature for 1 h. The solvent was evaporated and residue was dissolved in distilled water (1 cm^3). This solution was subjected to chromatography using an HCl-activated Amberlite IR120H ion-exchange column using ethanol:water (50:50) as eluent to give 0.41 g (82%) of Boc-D-phe-glyOH as a pale white solid (mp 160–161°C); IR: 3600 (br), 3377, 3361, 1683, 1665 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.41 (9H, s, $C(CH_3)_3$); 2.98 (1H, dd, J = 5.0, 14.0, CH_2Ph); 3.21 (1H, dd, J = 5.0, 14.0, CH_2Ph); 3.94 (1H, dd, J = 6.0, 16.0, CH_2gly); 4.02 (1H, dd, J = 6.0, 16.0, CH_2gly); 4.47 (1H, m, CH), 4.73 (1H, brs, NH), 6.74 (1H, brs, NH); 7.22-7.54 (5H, m, Ph).

Step C

Primaquine diphosphate (0.519 g, 1 mmol) and triethylamine (2 mol eq.) were added to a solution of Boc-D-phe-glyOH (0.367 g, 1 mol eq.) and DCC (1.1 mol eq.) in dichloromethane (6 cm^3) at 0°C. The mixture was stirred for 2 days. The urea that formed was separated by filtration, the filtrate evaporated and residue subjected to column chromatography on silica gel: ^{1}H-NMR δ ($CDCl_3$): 1.30 (3H, d, J = 6.3, C_1'-CH_3); 1.40 (9H, s, $(CH_3)_3$); 1.56–1.77 (4H, m, C_2'-H and C_3'-H); 2.97–3.30 (4H, m, CH_2Ph and C_4'-H); 3.63 (1H, m, C_1'-H);3.80 (1H, dd, J = 4.5, 16.5, CH_2gly); 3.88 (3H, s, CH_3O); 3.96 (1H, dd, J = 6.0, 16.5, CH_2gly); 4.20 (1H, m, $CHCH_2Ph$); 5.41 (1H, brs, NH); 5.98 (1H, dd, J = 4.5, 6.0, NH), 6.32 (1H, d, J = 2.5, C_7-H); 6.37 (1H, d, J = 2.5, C_5-H); 6.86 (1H, brs, NH); 7.17–7.34 (7H, m, C_3-H, Ph and NH); 7.94 (1H, dd, J = 1. 6, 8.2, C_4-H); 8.55 (1H, dd, J = 1.6; 4.2, C_2-H). This compound is a mixture of diastereoisomers and was used in the preparation of **4d** without further purification.

Step D

Boc-D-phe-gly-PQ (0.25 g, 0.45 mmol) was dissolved in a 1:1 solution of trifluoroacetic acid and dichloromethane (2 cm^3). After evaporation of the solvent, the yellow oil was treated with phosphoric acid to yield D-phenylalanylglycylprimaquine **4d** (74.5%): ^{1}H-NMR δ (d_6-DMSO): 1.30 (3H, d, J = 6.3, C_1'-CH_3); 1.62–1.75 (4H, m, C_2'-H and C_3'-H); 2.92 (1H, dd, J = 6.0, 11.4, CH_2Ph); 3.01–3.20 (3H, m, CH_2Ph, C_4'-Hs); 3.61 (1H, m, C_1'-H); 3.65 (1H, dd, J = 4.0, 14.0, CH_2gly); 3.75 (1H, dd, J = 5.0, 14.0, CH_2gly); 3.89 (3H, s, CH_3O); 3.95 (1H, m, $CHCH_2Ph$); 6.12 (1H, d, J = 9.0, C_8-NH); 6.29 (1H, d, J = 2.4, C_7-H); 6.44 (1H, d, J = 2.4, C_5-H); 7.19–7.30 (5H, m, Ph); 7.42 (1H, dd, J = 4.4, 8.0, C_3-H); 7.80 (1H, brs, NH), 8.04 (1H, dd, J = 1.2, 8.0, C_4-H); 8.55 (1H, dd, J = 1.2, 4.4, C_2-H), 8.70 (1H, brs, NH). ^{13}C-NMR δ (d_6-DMSO): 21.9 (C_1'-CH_3); 27.7 (C_3'); 35.1 (C_2'); 39.2 (C_4'); 40.3 (CH_2, phe); 43.8 (CH_2, gly); 48.7 (C_1'); 55.6 (CH, phe); 56.7 (CH_3O); 93.3 (C_7); 97.8 (C_5); 123.8 (C_3); 128.6 (*para*, phe); 130.1 (*meta*, phe); 131.1 (*ortho*, phe); 131.3 (C_{4a}); 136.2 (C_{8a}); 136.5 (C_4); 137.5 (*ipso*, phe); 146.0 (C_2); 146.4 (C_8); 160.7 (C_6); 169.6 (CO); 171.2 (CO). Calculated for $C_{26}H_{33}N_5O_3.2H_2PO_4$: C, 47.3; H, 5.9; N, 10.6; O, 26.7; P, 9.4. Found: C, 47.6; H, 6.1; N, 10.1; O, 27.1; P, 9.3. Compound **4d**, as well as **4b-c**, is a diastereomeric mixture which was used as such for subsequent experiments. Complete spectral assignments for these compounds were based on the corresponding COSY and HETCOR spectra. With the HPLC system described below it was not possible to separate the corresponding diastereoisomers.

Assay for Sporogonic Development of *Plasmodium berghei*

The mefloquine resistant *Plasmodium berghei* ANKA 25R/10 strain, Balb C mice, and *Anopheles stephensi* mosquitoes were used throughout the course of this study. The reason for using a mefloquine resistant strain *P. berghei* results from: a) the importance of evaluating the activity against resistant strains to commonly used antimalarial drugs, which are the major obstacle to control malaria, and b) the strain of *P. berghei* resistant to mefloquine was the only resistant strain available at the Centro da Malária (Portugal). 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 anesthetized (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.

In a parallel set of experiments, the development of sporozoites in mosquito salivary glands was examined. For these, an identical experimental protocol was adopted, except that disection of the mosquitoes and detection of sporozoites was performed twenty-three days after the blood meal.

Preparation of Liver Homogenates

Sprague-Dawley rats were injected intraperitoneally with 80 mg/kg/day of phenobarbital for 4 days, starved for 1 day, and euthanized by decapitation 24 hr after the last injection. The livers were removed, minced, and homogenized with pH 7.4 isotonic phosphate buffer (PBS), centrifuged at 700g for 10 min, and then pooled and kept at −70°C.

HPLC Analysis and Kinetics

The metabolism of compounds **4-6** in rat liver homogenates was monitored using HPLC following either the loss of the substrate or the formation of products. Incubations were carried out at 37°C in pH 7.4 PBS using 2mg 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 (1200 μl), the resulting mixture was centrifuged at 10000 rpm during 5 min and the supernatant analysed by HPLC. The following HPLC system was used: a Spectra-Physics Isochrom LC isochratic pump coupled to a Spectra-Physics SpectraChrom-100 variable wavelength detector and to a Merck-Hitachi 655A-40 autosampler injecting 20 μl/vial; a Merck LiChrospher® 100 RP-8 5 μm 250 × 4 mm column; mobile phase, acetonitrile-water containing sodium hexanesulfonate 10 mM, sodium acetate 2.5 mM and phosphoric acid 2.5 mM (40:60%) with a 1.0 cm^3/min flow rate. Retention times: primaquine, 11.6 min; **2**, 4.6 min; **4a**, 8.6 min; **4b**, 17.4 min; **4c**, 16.7 min; **4d**, 8.8 min; **4e**, 9.5 min; **5**, 12.8 min; **6**, 22 min. 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. Values of initial rate, V_i, were obtained from triplicate experiments and varied within ±5%.

RESULTS

Metabolism 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 (10), rat liver homogenates were specifically chosen to study the metabolism of derivatives **4**. Simultaneous formation of primaquine and of glycylprimaquie, **5**, was observed with compounds **4a-c** (Fig. 3). In contrast, derivatives **4d-e** were stable in the incubation mixtures, their concentration remaining constant for at least 3 h. Under the same conditions, N-acetylprimaquine, **6**, failed to regenerate primaquine.

Carboxyprimaquine was not observed in any of the incubation mixtures of compounds **4-6** over the timescale of the hydrolysis reactions (60 min). Similarly, 8-amino-6-methoxyquinoline, a minor metabolite arising from the oxidative deamination at C-1' (10,17), also was not detected in any of the reaction mixtures. Incubation of primaquine under the same conditions gave rise to carboxyprimaquine at a rate of 76.7 ng/h/mg. These results indicate that compounds **4** can undergo degradation by the pathways presented in Fig. 4, i.e., mediated by aminopeptidases (formation of **5**) and aminodipeptidases (direct release of primaquine).

The rates of formation of both products was found to be linear up to *ca.* 15 min. Plots of V_i versus [substrate] for each reaction produce the usual rectangular hyperbolae corresponding the Michaelis-Menten equation (data not shown). Interpolation of the data, using a non-linear least squares method, gives rise to the V_{max} and V_{max}/K_m values presented in Table I. Of course, the values determined for the formation of primaquine from **4a-c** include both the direct loss of the dipeptide unit together with the hydrolysis of **5** that is formed from the substrate. However, the rate of formation of primaquine from **5** (measured independently) is low compared to the corresponding values from **4a-c** (Table I). Therefore, the contribution to the formation of primaquine from compound **5** during the hydrolysis of compounds **4a-c** will be small and largely can be ignored. The data reveal some interesting features. First, at saturation, for any one compound the formation of primaquine and glycylprimaquine proceed at approximately similar rates. Second, the formation of both glycylprimaquine and primaquine is more rapid from **4b** and **4c** than it is from **4a**. This presumably reflects largely similar requirements of the exopeptidases (formation of **5**) and the endopeptidases (formation of **1**) for a lipophilic side chain in the terminal amino acid. Third, at sub-saturating concentrations the hydrolysis of **4b** to **5** is much higher than either for **4a** or **4c**. This must reflect the fact that the L-phenylalanyl side-chain is involved in binding interactions with enzyme active site. Overall, glycylglycylprimaquine, **4a**, is the least

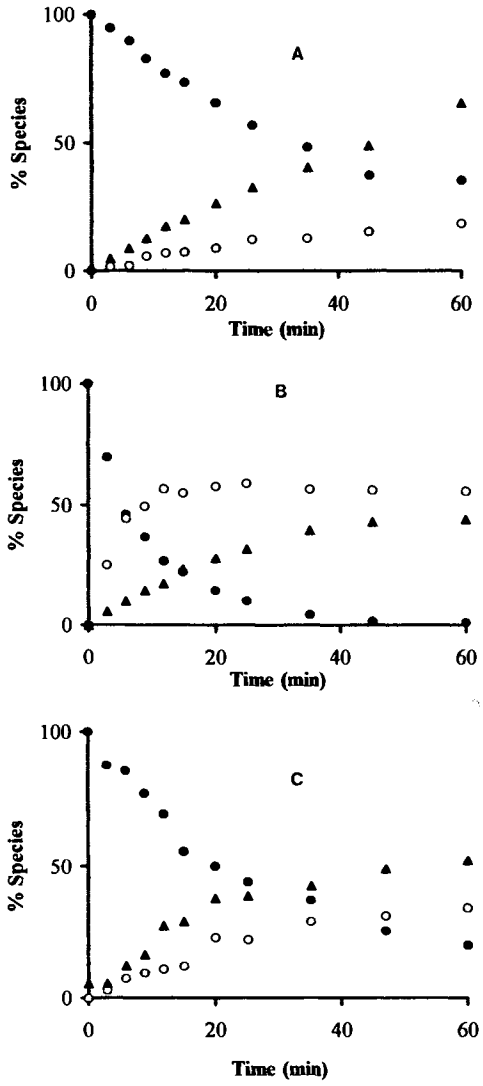


Fig. 3. Reaction profiles in liver homogenates for compounds **4a** (A), **4b** (B) and **4c** (C); •, substrate; ○, glycylprimaquine; ▲, primaquine.

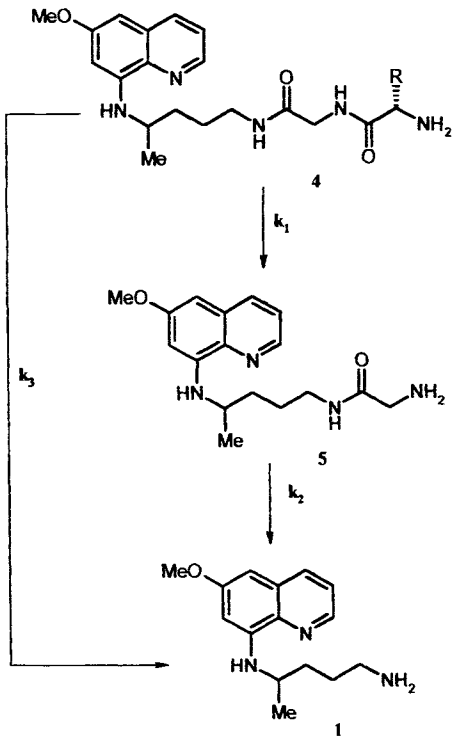


Fig. 4. Pathways for the hydrolysis of dipeptide derivatives **4** of primaquine in rat liver homogenates.

reactive compound, presenting the lowest values of V_{max} and V_{max}/K_m. The general order of reactivity of compounds **4** is in accordance with the hydrolytic activity of mammalian exopeptidases, being restricted to peptides containing an N-terminal residue with an α-unsubstituted amino group, the N-terminal and penultimate residues with the L-configuration and an hydrophobic side-chain in one of these residues (18).

Gametocytocidal Activity

The potential of compounds **4** to prevent the transmission of malaria was evaluated through their gametocytocidal activity against *P. berghei* using an model consisting of BalbC mice and *Anopheles stephensi* mosquitoes (19–21). The criteria used to assess the antimalarial activity of each compound were: (i) the minimal effective dose that prevents the appearance of oocysts in the midguts of mosquitoes, (ii) the percentage of mosquitoes with oocysts, (iii) the mean number of oocysts per infected mosquito, and (iv) the percentage of mosquitoes with sporozoites in their salivary glands (Table II).

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) (22). 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, except when the salivary glands infection was assayed (Table II).

Primaquine and all dipeptide derivatives **4** completely inhibited the production of oocysts at a dose of 15 mg/kg (Table II). Glycylprimaquine, **5**, was also very effective at this dose. Complete inhibition of oocyst production was still observed in mosquitoes fed on mice inoculated with 7.5 mg/kg of **4a**. However, only primaquine was able to completely block oocyst production at 3.75 mg/kg. The mean number of oocysts was also significantly ($P < 0.05$) affected by doses of 3.75 and 1.86 mg/kg of compounds **4a-c** and **5**. In contrast, at the lowest dose of 1.86 mg/kg, derivatives **4d-e** did not significantly ($P > 0.05$) reduce the oocyst production when compared with the control. *N*-Acetylprimaquine, **6**, which lacks a terminal basic amino group, was ineffective in this screen. A dose of 15 mg/kg of **6** did not significantly ($P > 0.05$) affect oocyst production when compared with the control.

The effect on the sporogonic development of *P. berghei* was also evaluated by checking the presence of sporozoites in the salivary glands of mosquitoes on day 23 post-feed. With the exception of compound **4e**, all remaining derivatives **4**, as well as primaquine, inhibited sporozoite production in the salivary glands at a dose of 3.75 mg/kg (Table II). However, only compounds **4a** and **4c** were able to inhibit salivary gland infections at dose of 1.86 mg/kg, being more effective than primaquine at this dose level.

DISCUSSION

Our aim has been to synthesize new dipeptide derivatives of primaquine that are less able to be transformed into the inactive carboxyprimaquine, yet which maintain gametocytocidal activity. Both mechanisms of cytochrome P-450 and MAO catalysed oxidation of amines appear to involve an initial single electron transfer to form a nitrogen-centred radical cation (15,23). Therefore, increasing the ionization potential through

Table I. Kinetic Parameters for the Formation of **1** and **5** from Primaquine Derivatives **4** and **5** by Rat Liver Homogenates at 37°C and pH 7.4

	Formation of 5			Formation of primaquine		
Compound	$10^6V_{max}/min^{-1}$	$10^4K_m/M$	$10^3(V_{max}/K_m)/M^{-1}min^{-1}$	$10^6V_{max}/min^{-1}$	$10^4K_m/M$	$10^3(V_{max}/K_m)/M^{-1}min^{-1}$
4a	11.0 ± 1.9	13.0 ± 4.6	8.50 ± 1.9	23.0 ± 2.5	9.40 ± 2.48	24.0
4b	35.0 ± 4.6	4.82 ± 1.39	73.0 ± 1.9	31.0 ± 9.4	8.80 ± 4.46	36.0
4c	38.0 ± 4.0	16.0 ± 3.7	24.0 ± 1.9	44.0 ± 4.9	9.00 ± 2.66	48.0
5	—	—	—	7.50 ± 4.51	8.50 ± 1.70	8.80

Table II. Effect of Compounds 4, 5, 6, and Primaquine, on the Sporogonic Development of Plasmodium berghei ANKA in *Anopheles stephensi* Mosquitoes

Compound	Dose (mg/kg)[a]	Oocysts[b]		Sporozoites[c,d]
		% of Infected mosquitoes	Mean n° ocysts per mosquito (± SE)	% of infected mosquitoes
1	Control	66	36.6 (11.1)	54
	15.0	0	0	—
	7.50	0	0	—
	3.75	0	0	0
	1.86	30	2.0 (1.0)	5
4a	Control	75	34.8 (6.8)	54
	15.0	0	0	—
	7.50	0	0	—
	3.75	26	1.6 (1.0)	0
	1.86	40	6.7 (3.2)	0
4b	Control	74	24.6 (5.4)	54
	15.0	13	0.2 (0.1)	—
	7.50	18	1.0 (0.5)	—
	3.75	19	1.3 (0.8)	0
	1.86	40	2.9 (1.1)	18
4c	Control	72	31.0 (5.5)	54
	15.0	10	0.3 (0.3)	ND
	7.50	20	1.2 (0.4)	ND
	3.75	47	2.5 (0.9)	0
	1.86	47	3.8 (2.6)	0
4d	Control	72	29.7 (5.5)	54
	15.0	0	0	ND
	7.50	14	1.1 (0.9)	ND
	3.75	50	7.5 (4.1)	0
	1.86	60	14.4 (5.3)*	26
4e	Control	72	33.4 (8.9)	54
	15.0	0	0	ND
	7.50	10	0.1 (0.1)	ND
	3.75	30	1.0 (0.5)	6
	1.86	53	12.4 (6.5)*	36
5	Control	90	45.8 (8.9)	ND
	15.0	0	0	ND
	7.50	3	0.03 (0.03)	ND
	3.75	33	2.9 (1.6)	ND
	1.86	33	3.7 (2.5)	ND
6	Control	85	31.0 (7.5)	ND
	15.0	75	14.1 (4.2)*	ND

[a] Doses are in milligrams of primaquine base/kilogram.
[b] counting of oocysts was carried out at day 10 post-feed.
[c] observation of sporozoites was carried out at day 23 post-feed.
[d] ND, not determined.
* $P > 0.05$ versus control, by Student's *t*-test.

nitrogen acylation is expected to reduce significantly the rate of oxidation. From the results reported herein, it is clear acylation of the aliphatic side-chain protects the terminal amino group against oxidative deamination, thereby preventing the formation of carboxyprimaquine.

All compounds **4** were able to prevent the development of the sporogonic cycle of *P. berghei* in *A. stephensi* mosquitoes at dose levels of 15 and 7.5 mg/kg. Significantly, the gametocytocidal activity of derivatives **4** is not related to their rate of peptidase-catalysed hydrolysis to primaquine. For example, compound **4a**, which has the lowest values of V_{max} and V_{max}/K_m for the formation of primaquine and glycylprimaquine, is more active than primaquine in the sporozoite assay, being able to prevent the infection of the salivary glands at a dose of 1.86 mg/kg. Furthermore, the primaquine derivatives **4d-e**, which are not hydrolyzed by peptidases, display intrinsic activity. Indeed, this is the first report of derivatives of primaquine containing non-natural amino acids, which are stable to peptidases. Philip *et al.* prepared D-valylleucyllysylprimaquine (D-val-leu-lys-PQ), L-valylleucyllysyl-primaquine (val-leu-lys-PQ), and D-alanylleucyllysyl-primaquine (D-ala-leu-lys-PQ) and found all three peptide derivatives showed radical curative activity against *P. cynomolgi* greater than that expected for the primaquine content of each compound (12). However, the possibility that D-val-leu-lys-PQ and D-ala-leu-lys-PQ act as prodrugs of primaquine cannot be ruled out. Indeed, the tripeptide derivative of primaquine containing ϵ-aminocaproic acid (EACA) as the *N*-terminal aminoacid (EACA-ala-leu-PQ)

releases primaquine, but not ala-leu-PQ or leu-PQ, in rat liver lysosomal preparations (14).

In contrast to derivatives **4d-e**, *N*-acetylprimaquine, **5**, which lacks a terminal basic amino group, was ineffective in this screen. Carboxyprimaquine, which itself lacks a terminal amino group, also is inactive (5), implying the presence of a terminal amino group is a major structural requirement for the gametocytocidal activity. This is consistent with the fact that the presence of a terminal amino group is also a major structural requirement for the tissue schizontocidal activity of 8-aminoquinolines (24,25).

In conclusion, a new, attractive approach to abolish effectively the oxidative deamination of primaquine is the acylation of the primary amino group at C-4′ with dipeptides containing non-natural amino-acids. Although the dipeptide derivatives **4** do not present an improved oocyst production inhibitory profile over primaquine, it was possible to achieve complete sporozoite production with two of these compounds at the lowest dose. This limited study suggests this approach may prove useful to the design of new antimalarials based on compounds that are rapidly metabolised by oxidative deamination in the aliphatic side-chain. Studies are in progress to evaluate the toxicity and the plasma stability of dipeptides derivatives of primaquine.

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