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Report

Effect of Aliphatic Side-Chain Substituents on the Antimalarial Activity and on the Metabolism of Primaquine Studied Using Mitochondria and Microsome Preparations

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The substitution of two deuterium atoms on the α-carbon of the primaquine side chain was found to produce a sevenfold decrease in the rate of conversion of primaquine to carboxyprimaquine by enzymatic oxidative deamination, but the deuterium substitution was found to have no significant effect on the *in vitro* antimalarial activity or on *in vitro* hepatocyte toxicity. Placing a single methyl group on the α-carbon was found to produce only a slight decrease in the rate of oxidative deamination. Although metabolic attack occurred adjacent to either the aniline nitrogen or the aliphatic amine, metabolic attack occurred primarily adjacent to the more basic nitrogen at the 1′-position, even when this position bore a methyl substituent. Primaquine, the α-dideutero analogue, and the α-methyl analogue were all found to have about the same *in vitro* antimalarial activity as determined in the liver hepatocyte assay.

KEY WORDS: malaria; drug metabolism; deuterium isotope effect; cytotoxicity, quinocide.

INTRODUCTION

Primaquine diphosphate is widely used as an antimalarial for both its prophylactic and radical cure activities. In mammalian systems, less than 1% of the drug is excreted unchanged in the urine and it has been shown that the major metabolite found in the plasma results from the α-oxidation of the primary amine group. The metabolite arising from this oxidative deamination, carboxyprimaquine (Fig. 1), was first discovered as a microbial metabolite of primaquine (1), then later in rats (2), monkeys (3), and humans (3,4). The tranformation of primaquine to carboxyprimaquine is extremely rapid following i.v. administration of the drug. Using rats (2) or monkeys (3), it was found that within 15 min of administering the drug, the plasma concentration of carboxyprimaquine exceeded that of primaquine and 3.0 hr after administration, the concentration of carboxyprimaquine was more than 10 times greater than that of the parent drug.

The major objective of the present study was to discover structural variations that could be made in the basic structure of primaquine that might slow the rate of metabolic inactivation of the parent compound while maintaining antimalarial activity. One structural variation that was made consisted of placing two deuterium atoms on the carbon adjacent to the primary amine group (α,α-dideuteroprimaquine, Fig. 2), which could be expected to slow the rate of oxidative deamination by 5- to 10-fold. The other structural variation considered was to move the methyl group from the 4′-position adjacent to the aniline nitrogen to the 1′-position adjacent to the primary aliphatic amine (afforded by quinocide; Fig. 2).

Rather than use a whole animal model system, where the disposition of primaquine is strongly effected by both metabolic transformation and tissue compartmentalization, the present study utilized rat liver homogenates. One could anticipate that at least three enzyme systems [cytochrome P-450, monoamine oxidase (EC 1.4.3.4), and aldehyde dehydrogenase (EC 1.2.1.4)] might be involved in the transformation of primaquine to carboxyprimaquine. The initial α-oxidation adjacent to the nitrogens could be mediated either by a cytochrome P-450 system (located in the microsome 12,500*g* to 100,000*g* fraction) or by monoamine oxidase (located in the mitochondria 700*g* to 12,500*g* fraction), while the final oxidation of the intermediate aldehyde to the carboxylic acid would be mediated by aldehyde dehydrogenase (located in the 100,000*g* supernatant fraction). In the present study of the relative rates of the metabolic transformations of primaquine, α,α-dideuteroprimaquine, and quinocide; either a 700*g* supernatant fraction (containing monoamine oxidase, cytochrome P-450, and aldehyde dehydrogenase) or a 12,500*g* supernatant fraction (containing cytochrome P-450 and aldehyde dehydrogenase) was utilized to simplify the investigation.

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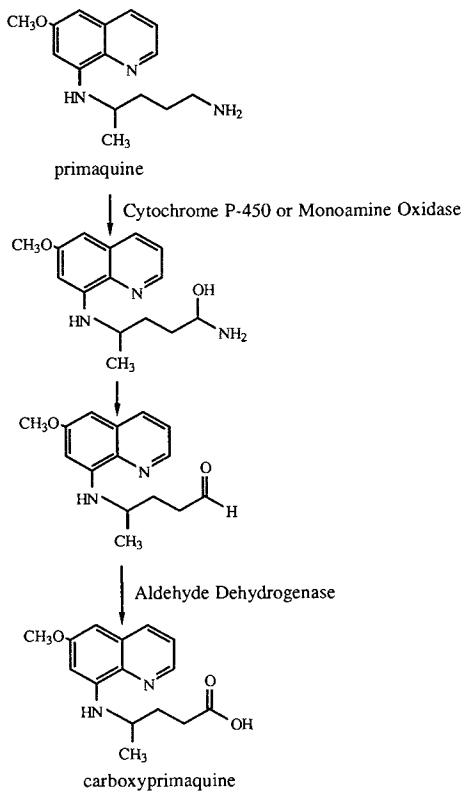

Fig. 1. Oxidative deamination pathway for primaquine.

MATERIALS AND METHODS

Liver Homogenate Incubations

The livers of male Wistar rats were homogenized in a Potter–Elvehjem tube with ice-cooled, pH 7.4, 0.05 *M* Tris buffer containing 0.15 *M* KCl (3.0 ml of buffer/g of liver). For the crude microsome preparation, the homogenates were centrifuged at 12,500*g* for 10 min at 4°C, and the supernatants were removed and used immediately or stored at −20°C for later use (within 15 hr). The crude mitochondria–microsome supernatant was prepared in an identical manner at the same time, except that the homogenates were centrifuged at 700*g* for 10 min.

Incubations were performed in triplicate in small open beakers containing a glass marble and shaken at 100 osc/min at 37°C under a 100% oxygen atmosphere. Each beaker contained 1.0 ml of the drug substrate [400 μg/ml stock solutions of either primaquine diphosphate (Aldrich Chemical Co.) or α,α-dideuteroprimaquine diphosphate (prepared by synthesis in these laboratories (5)) or quinocide diphosphate [obtained through Dr. David E. Davidson, Jr., Walter Reed Army Medical Institute of Research], 1.0 ml of either of the two liver homogenates, and 3.0 ml of cofactors (stock solution comprised of 0.64 mg/ml of NADP · 4.5 H_2O, 2.3 mg/ml of glucose-6-phosphate, 1.03 mg/ml of anhydrous $MgSO_4$ in 0.05 *M*, pH 7.4, Tris buffer). Control samples were prepared using the same quantities of primaquine substrate, liver homogenate, and cofactors, except that the liver homogenates were heated on a steam bath for 10 min. After the incubations, the samples were immediately placed on ice, then extracted with solvents for HPLC analysis as given below.

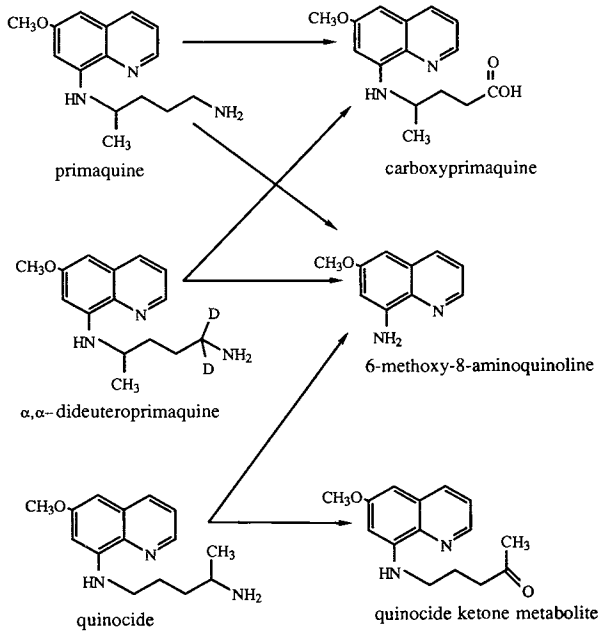

Fig. 2. Metabolic pathways for primaquine and quinocide.

Quantitation of Carboxyprimaquine

Carboxyprimaquine is difficult to extract from biological samples because of the amphoteric nature of the metabolite where the quinoline nitrogen has a pK_a of 3.20 and the carboxylic acid group has a pK_a of 3.1 (15). An HPLC procedure based on an optimized extraction at pH 3.10 using [4-(1-naphthylamino)butyric] acid as the internal standard has been reported (13) to give good absolute recoveries (90%) and good accuracy. The method was originally developed for the determination of carboxyprimaquine in urine samples and has been slightly modified as follows: after cooling the incubation mixture, 25 μl of the internal standard [4-(1-naphthylamino)butyric acid, 200 μg/ml stock solution] was added directly to the incubator beaker. The contents of the beaker were transferred to a centrifuge tube along with 2 ml of pH 3.10, 0.2 *M* phosphate buffer, then extracted with 2 × 5 ml of ethyl acetate. After centrifugation, the combined ethyl acetate extracts were taken to a volume of approximately 0.5 ml in a Brinkmann sample concentrator operated at 40°C under reduced pressure with a nitrogen stream. After washing down the walls of the concentrator tube with 1.0 ml of methanol, the sample was subjected to HPLC analysis.

The HPLC system consisted of a 25-cm Whatman ODS-3 column (5-μm particle size) with a Water's Associates Model 440 dual-wavelength (254-nm and 280-nm) detector used with a dual-pen recorder. The mobile phase was used at a flow rate of 1.0 ml/min and 1.0 liter of the mobile phase contained 1.0 g *N,N*-dimethyloctylamine, 1.65 g K_2HPO_4, 4.1 g KH_2PO_4, 300 ml acetonitrile, and 700 ml water.

Along with each series of incubation experiments, both "positive control" and "negative control" samples were run to guard against the possibility of artifacts. For the negative control, the drug substrate (primaquine in this case) was added to a liver homogenate that had been heat inactivated,

the cofactors were added, the sample was incubated alongside the test samples, the internal standard was added, then the incubation mixture was extracted and chromatographed as described above. Carboxyprimaquine was not detected in any of the negative control samples in the study. For a positive control sample, a 5-ml sample containing the metabolite (carboxyprimaquine) of known concentration was processed as above, then the peak heights of the carboxyprimaquine (retention time = 33 min) and the internal standard (retention time = 28 min) were measured to determine the response factor to be used for the assay of the incubation mixtures.

For a statistical analysis of the incubation experiments, the yields of the metabolite and the standard deviation were the result of the assay on incubations performed in triplicate, rather than multiple assays of a single incubation.

Quantitation of 6-Methoxy-8-aminoquinoline and the Quinocide Ketone Metabolite

After cooling the incubation mixture, 25 μl of the internal standard (250 μg/ml stock solution of *N*-ethylaniline) was added directly to the incubation beakers. The mixture was transferred to a centrifuge tube along with 500 μl of 10% Na_2CO_3, then extracted with 2 × 5 ml of ethyl acetate. After centrifugation, the combined ethyl acetate extracts were taken to a volume of approximately 0.5 ml in a Brinkmann sample concentrator operated at 40°C under reduced pressure with a nitrogen stream. After washing down the walls of the concentrator tube with 1.0 ml of methanol, the sample was subjected to HPLC analysis.

The HPLC column, flow rate, detector, and mobile phase composition were the same as used for the carboxyprimaquine assay, except that the acetonitrile content was increased to 35 from the 30% used in the former assay. The assay was calibrated by extracting and processing known quantities of 6-methoxy-8-aminoquinoline, the quinocide ketone metabolite, and *N*-ethylaniline in a 5-ml sample using the above procedure. Retention times for the HPLC system were as follows: 6-methoxy-8-aminoquinoline, 7.8 min; *N*-ethylaniline, 10 min; quinocide, 15 min; primaquine, 16 min; and the quinocide ketone metabolite, 19 min.

Negative control samples using heat-inactivated liver homogenates and quinocide were run alongside the test incubations to ensure that the observed levels of metabolite formation were not the result of a simple chemical decomposition.

Synthesis of the Quinocide Ketone Metabolite [8-(4′-Oxypentylamino)-6-Methoxyquinoline]

Using a generalization of a procedure previously reported (6), a mixture of 4,4-ethylenedioxypentanol [3.5 g, prepared from levulinic acid according to the procedure reported by Bulat *et al.* (7)] and iodotrimethyl silane (14.2 g) in chloroform (100 ml) was stirred at room temperature for 1 hr. The reaction mixture was poured into water (100 ml) and the organic layer was separated. The aqueous layer was extracted with chloroform (100 ml) and the combined organic layer was washed with sodium bisulfite, brine, dried over anhydrous sodium sulfate, and evaporated to give a yellow-brown oil. This was chromotographed over silica gel and elution with hexane:ethyl acetate (95:5) yielded 4-oxopentyliodide as pale yellow oil (2.8 g). This compound slowly decomposed on standing ans was used in the next reaction immediately: PMR ($CDCl_3$) 3.23 (2H,t,J = 6.6 Hz 1-CH_2), 2.59 (2H,t,J = 7 Hz, 3-CH_2), 2.12 (3H,s,5-CH_3), 2.07 (2H,t,J = 6.6 Hz, 3-CH_2); 13C NMR ($CDCl_3$): 207.0 (C-4), 43.8 (C-3), 30.1 (C-5), 27.1 (C-2), 6.5 (C-1). The PMR spectrum was essentially identical to that previously reported (14) for the product obtained by a different synthetic route.

A mixture of 6-methoxy-8-aminoquinoline [1.74 g; prepared by the method of Haskelberg (8)], 4-oxopentyliodide (2.7 g), and triethylamine (3 ml) was heated under a nitrogen atmosphere at 80–90°C for 12 hr. The residue was dissolved in chloroform (100 ml) and successively washed with sodium hydroxide (10%, 50 ml), brine, dried over anhydrous sodium sulfate, and evaporated. The residue was chromatographed over neutral alumina and elution with chloroform yielded 8-(4′-oxopentylamino)-6-methoxyquinoline, which was crystallized from ethanol:water to give brownish plates (600 mg): mp 56–57°C; IR (KBr) 3330 (NH), 1710 (C=O); PMR ($CDCl_3$ 8.53 (1H,dd,J = 5 and 1.5 Hz,2-C*H*), 7.91 (1H,dd,J = 9 and 1.5 Hz,4-C*H*), 7.29 (1H,dd,J = 9 and 5 Hz,3-C*H*), 6.35 (1H,d,J = 2.6 Hz,7-C*H*), 6.31 (1H,d,J = 2.6 Hz,5-C*H*), 6.13 (1H,t,J = 6 Hz,N*H*), 3.88 (3H,s,OCH_3), 3.29 (2H,9,J = 6 Hz, 1′-CH_2), 2.59 (2H,t,J = 8 Hz,3-CH_2), 2.14 (3H,s,5′-CH_3), 2.01 (2H,p,J = 8 Hz, 2′-CH_2); 13C-NMR ($CDCl_3$), 208.3 (C-4′), 159.4 (C-6), 145.8 (C-8), 144.4 (C-2), 135.3 (C-8a), 134.8 (C-4), 129.8 (C-4a), 121.9 (C-3), 96.7 (C-7), 92.2 (C-5), 55.2 (OCH_3), 42.5 (C-3′), 41.1 (C-1′), 30.0 (C-5′), 23.1 (2′); mass spectra (70 ev), m/z (relative intensity), 258 (M+, 12), 239 (6), 201 (52), 187 (100), 172 (13), 159 (37), 144 (85), 129 (17), 116 (23).

Table I. Comparison of Oxidative Deamination of Primaquine in Microsomes and in Mitochondria Liver Preparations

Preparation	Carboxyprimaquine formed (μg/ml)
A. Microsomes[a]	0.335 ± 0.012
B. Mitochondria and microsomes[b]	0.534 ± 0.018[c]

[a] Incubation using the 12,500*g* supernatant liver homogenate fraction.
[b] Incubation using the 700*g* supernatant obtained from the same liver homogenate as Preparation A.
[c] Standard deviation of the metabolite yield from multiple incubations.

In Vitro Antimalarial Activity and Liver Cell Cytotoxicity

The *in vitro* cytotoxicity and the antimalarial activity of

Table II. Effect of Deuterium Substitution on the Metabolism of the Primaquine Side Chain in a Microsome–Mitochondria Preparation

Substrate	Carboxyprimaquine formed (μg/ml)
Primaquine	1.35 ± 0.56
α,α-Dideuteroprimaquine	0.201 ± 0.007

Table III. Effect of Methyl Group Substitution on the Metabolism of the Primaquine Side Chain in a Microsome–Mitochondria Preparation

Substrate	Oxidation at carbon-4′ [a] (μg/ml)	Oxidation at carbon-1′ [b] (μg/ml)
Primaquine	0.023 ± 0.012	1.68 ± 0.08
Quinocide	0.064 ± 0.033	0.79 ± 0.49

[a] Formation of 6-methoxy-8-aminoquinoline.
[b] Formation of carboxyprimaquine or the quinocide ketone metabolite.

the three test compounds were measured using *Plasmodium yoelii*-infected liver tissue cultures using the method previously used for the evaluation of the activity of primaquine (9). The sporozoites of *Plasmodium yoelii*-265BY isolated from the salivary glands of *Anopheles stephensi* (25,000 to 40,000 sporozoites per disk) were used to treat *Thamnomys gazellae* hepatocytes (10,000 cells per disk). The test compounds were first dissolved in 100 μl of of dimethylsulfoxide, which was then diluted to the working concentration using minimal Eagle's medium supplemented with 2.2 g/liter sodium bicarbonate, 2.0 g/liter bovine albumin, 10 mg/liter bovine insulin, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3.5×10^{-5} *M* hydrocortisone. The test compound-culture medium mixture was added to the test system 2 hr following the addition of the sporozoites and again 24 hr following the first addition. After a total of 48 hr, the cultures were fixed with methanol and stained with 10% Giemsa, then the schizonts were counted by microscopic examination. The schizonticide LD50 values were calculated using a log dose–response probit analysis using the percentage dead schizonts vs drug concentration. Liver cell toxicity was measured by counting dead hepatocytes after vital staining with 0.25% trypan blue.

RESULTS AND DISCUSSION

In general, simple aliphatic primary amines undergo a two-step metabolic transformation leading to the ultimate formation of a carboxylic acid metabolite with an aldehyde (or its cabinolamine equivalent) as the intermediate (10). The conversion of primaquine to carboxyprimaquine (Fig. 1) can also be envisioned as a two-step process involving an initial oxidative step mediated either by a cytochrome P-450 system in the microsomes or by monoamine oxidase (EC 1.4.3.4) in the mitochondria followed by a second oxidative step mediated by aldehyde dehydrogenase (EC 1.2.1.4). From a comparison of the relative rates of transformation of primaquine to carboxyprimaquine by the microsome and the mitochondria fractions (Table I), it appeared that the cytochrome P-450 and the monoamine oxidase systems had nearly equal roles in the metabolic converstion of primaquine to carboxyprimaquine. Because of the importance of both of these systems in the metabolism of primaquine, all subsequent experiments were conducted using the 700*g* microsome-mitochondria preparation.

Replacement of the two hydrogens on the 1′-carbon adjacent to the aliphatic amine with two deuterium atoms had a pronounced effect on the conversion of primaquine to carboxyprimaquine (Table II). It was observed that the rate of metabolism of α,α-dideuteroprimaquine was 6.7 times slower than that for primaquine, which was similar to the kinetic isotope effects ranging from 2 to 12 that have been reported for similar metabolism studies (10).

Metabolic α-oxidation of primaquine at the 1′-position would give rise to carboxyprimaquine, while α-oxidation at the 4′-position would give rise to 6-methoxy-8-aminoquinoline (Fig. 2). In an early study of the metabolism of primaquine using an *in vivo* system, Baty reported (11) a very low yield of 6-methoxy-8-aminoquinoline, however, more recent *in vivo* studies have failed to find the presence of the metabolite (12,13). In the present *in vitro* study, it was found that the metabolic transformation of primaquine gave rise to only 0.023 μg/ml of 6-methoxy-8-aminoquinoline (Table III), which was 59 times lower than had been observed for the conversion of primaquine to carboxyprimaquine. For primaquine, it is clear that oxidation adjacent to the aliphatic nitrogen is more extensive than adjacent to the aniline nitrogen.

Considerations of the differences in the basicities of the two nitrogens aside, one might have expected the α-oxidation of the 1′-position to predominate over oxidation at the 4′-position, because of the steric effect of the 4′-methyl group which would inhibit the conversion of primaquine to 6-methoxy-8-aminoquinoline. However, the observed preference for attack at the 1′-position seen with primaquine (Table III) was probably not due primarily to steric effects because quinocide, which does not have a blocking methyl group at the 4′-position, formed 6-methoxy-

Table IV. *In Vitro* Antimalarial Activity and Hepatocyte Cytotoxicity of Primaquine and Its Side Chain Analogues

Compound	Schizonticide LD50 (μg/ml)	Hepatocyte cytotoxicity	
		At 0.1 μg/ml	At 1.0 μg/ml
Primaquine	0.020	0	+
α,α-Dideuteroprimaquine	0.020	0	+
Quinocide	0.025	0	+ + +

8-aminoquinoline at a rate that was only 2.8 times faster than primaquine. It would thus appear that the directing effect of the 4′-methyl group on the metabolism of primaquine is quite small compared to the large difference between oxidation adjacent to the two types of nitrogens in the side chain. In general, the placement of the methyl group at either the 1′ or the 4′ position slows the rate of metabolism by only two- to threefold, while the rate of attack adjacent to the more basic nitrogen is one to two orders of magnitude higher regardless of the placement of the methyl group.

It was found that the *in vitro* antischzonticidal activity of primaquine, α,α-dideuteroprimaquine, and quinocide was essentially the same (Table IV). Also, quinocide was slightly more toxic than either of the two other compounds. Although α,α-dideuteroprimaquine has the same intrinsic *in vitro* antimalarial activity and *in vitro* heptocyte toxicity as primaquine, the effect of the deuterium substitution on antimalarial activity and toxicity may be more pronounced when these activities are measured *in vivo*. The results of the present study show that the α,α-dideuterium substitution significantly slows the removal of the primary amine group [a group that is essential for targeting the drug to the parasite in the liver (2)], which could lead one to speculate that the antimalarial activity of α,α-dideuteroprimaquine could be higher than that for primaquine when studied *in vivo* even though the present study shows that the intrinsic antimalarial activity of α,α-dideuteroprimaquine and primaquine are the same. Presently an *in vivo* evaluation of the antimalarial activity of α,α-dideuteroprimaquine and other α,α-dideutero analogues is under way to determine if this potential therapeutic advantage can be realized.

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