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SHORT COMMUNICATIONS

Primaquine metabolism by human liver microsomes: effect of other antimalarial drugs

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Abstract—A number of drugs have been studied for their effect on the metabolism of the antimalarial drug primaquine by human liver microsomes (N = 4) *in vitro*. The only metabolite generated was identified as carboxyprimaquine by co-chromatography with the authentic standard. Ketoconazole, a known inhibitor of cytochrome P450 isozymes, caused marked inhibition of carboxyprimaquine formation with IC_{50} and K_i values of 15 and 6.7 μM, respectively. This finding and the dependency of metabolite formation on NADPH indicates that cytochrome P450 isozyme(s) catalysed metabolite production. Of compounds actually or likely to be coadministered with primaquine to malaria patients, only mefloquine produced any inhibition (K_i = 52.5 μM). Quinine, artemether, artesunate, halofantrine and chloroquine did not significantly inhibit metabolite formation. It seems unlikely that the concurrent administration of mefloquine, or other antimalarials, with primaquine will lead to appreciably altered disposition.

The 8-aminoquinoline derivative, primaquine [6-methoxy-8(4-amino-1-methylbutylamino)quinoline] is an important antimalarial drug because of its activity against several life-cycle stages of all human malaria [1]. It is the only drug that can be used for curative treatment in vivax and ovale malaria and the only drug that kills gametocytes of *Plasmodium falciparum*. Despite considerable experience in the clinical use of primaquine and well documented toxicity profiles (e.g. haemolytic anaemia, particularly in individuals with glucose-6-phosphate dehydrogenase deficiency), there is limited information on aspects of biochemical and clinical pharmacology. Primaquine undergoes rapid and extensive metabolism with a carboxylic acid formed by oxidative deamination identified as the major plasma metabolite in man [2], monkeys [3] and rats [4]. Carboxyprimaquine formation has been shown to take place in rat liver [5], human bone marrow cells [6] and microbes [7]. To date there has been no direct evidence for the involvement of cytochrome P450 isozyme(s) in the formation of carboxyprimaquine. However, it has been well established that primaquine inhibits hepatic microsomal cytochrome P450-dependent enzymes, both *in vitro* and *in vivo* [8–15]. We have recently demonstrated that primaquine inhibits the metabolism of mefloquine to carboxymefloquine in human liver microsomes with a K_i value of 8.6 μM [16].

Since primaquine is used with other antimalarial drugs and other therapeutic agents, there is the potential for pharmacokinetic/metabolic drug interactions. In the present work we have screened a number of antimalarial drugs for their propensity to inhibit the metabolism of primaquine by human liver microsomes *in vitro*. The drugs studied were mefloquine [racemic, (+), (−)], quinine, artemether, artesunate, halofantrine and chloroquine. For comparative purposes, the antifungal drug, ketoconazole, which is a well established inhibitor of cytochrome P450 enzymes was also studied.

Materials and methods

Drugs and chemicals. Primaquine-diphosphate and quinine hydrochloride were obtained from the Aldrich Chemical Co. (Gillingham, U.K.). The carboxylic acid metabolite of primaquine was a gift from Professor J. D. McChesney, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, MI, U.S.A. [^{14}C]Primaquine (sp. act. 155 μCi/μL; radiochemical purity > 96%) was obtained from New England Nuclear (Boston, MA, U.S.A.). Halofantrine hydrochloride was a gift from SmithKline and Beecham Research (Welwyn Garden City, U.K.). Mefloquine hydrochloride was a gift from the Walter Reed Army Medical Research Center (Washington D.C., U.S.A.) Mefloquine (+) and (−) enantiomers were kindly supplied by Roche (Basle, Switzerland). Artemether and artesunate were from the World Health Organization (Geneva, Switzerland). Ketoconazole was a gift from Janssen (Belgium). Chloroquine diphosphate and NADPH were from the Sigma Chemical Co. (Poole, U.K.). Solvents were of HPLC-grade and obtained from Fisons plc (Loughborough, U.K.). All other reagents were of analytical grade and supplied by BDH (Poole, U.K.).

Human liver samples. Histologically normal livers were obtained from kidney transplant donors soon after clinical death (three males, one female; aged 8–49 years). Ethical approval for the study was granted and consent to removal of the liver samples was obtained from the donors' relatives. Livers were stored as 10–20-g portions at −80° until required.

Preparation of liver microsomes. Washed microsomes were prepared by the classical differential centrifugation technique of Purba *et al.* [17]. Microsomal protein was determined by the method of Lowry *et al.* [18] and cytochrome P450 by the method of Omura and Sato [19].

Kinetic and inhibition studies. Incubations containing [^{14}C]primaquine (0.1 μCi), primaquine (100 μM), microsomal protein (2 mg) and 0.067 M phosphate buffer (pH 7.4) to a final volume of 2.5 mL were performed in Sovirel tubes (15 mL capacity) at 37° with agitation. Microsomal reactions were started by the addition of 1 mM NADPH. Primaquine and the putative inhibitors were dissolved in methanol which was evaporated to dryness before addition of other reaction constituents. The range of inhibitor concentrations used for calculation of IC_{50} values (the concentration producing a 50% reduction in enzyme activity) was 25–500 μM. The reaction was terminated after 1 hr by the addition of 2 mL 0.05 M octane-sulfonic acid in glycine buffer (pH 2.5).

Carboxyprimaquine was the only metabolite of primaquine formed under the incubation conditions described. Radiolabelled unmetabolized primaquine and carboxyprimaquine were extracted into 6 mL of ethylacetate by mechanical tumbling for 20 min. The organic layer was

then separated by centrifugation (2500 *g* for 10 min) and evaporated to dryness in a Buchler vortex evaporator. Extraction of total radioactivity was greater than 95%. The residue was reconstituted in methanol (50 μL) and an aliquot (20 μL) was analysed by radiometric HPLC. Carboxyprimaquine was identified according to the retention time (R_t) of the authentic standard (R_t = 4.5 min; R_t of primaquine = 6.5 min). Separation was performed on a Techopak 10 C-18 (30 cm × 0.39 cm) column protected by an inline guard column. The system was operated at room temperature in an isocratic mode with the mobile phase of 65% methanol and 35% distilled water containing 0.1 M octanesulfonic acid (adjusted to pH 3.6 with phosphoric acid) at a flow rate of 1.5 mL/min. This procedure was modified from that described previously [5]. Coefficients of variation for the assay procedure ranged from 4 to 6%.

Initial velocity conditions for enzyme activity were established employing a primaquine concentration of 100 μM which was linear over 6 hr and up to 2 mg microsomal protein. The coefficient of variation of the rate of metabolite formation between different incubations (N = 5) of the same liver was <10%.

The kinetic parameters (V_{max} and K_m) of formation of carboxyprimaquine at primaquine concentrations of 25–500 μM were determined in the absence and presence of some of the inhibitors using an iterative programme (ENZPACK) based on non-linear least-squares regression analysis to fit the experimental data to the Michaelis–Menten equation. The apparent K_i was determined by substituting K_m and V_{max} values in the appropriate equation [20] and by the graphical method of Dixon [21].

Results and Discussion

Microsomal protein and cytochrome P450 content was 15.4 ± 3.0 mg of liver and 0.36 ± 0.05 nmol/mg protein, respectively (mean ± SD, N = 4). The enzyme activity for carboxyprimaquine formation at a substrate concentration of 100 μM was 18.67 ± 3.68 nmol/hr/mg protein. No metabolite was present in the absence of NADPH.

Mean IC_{50} values and enzyme kinetic parameters determined in the absence and presence of putative inhibitors are shown in Table 1. The most potent inhibition was evident with ketoconazole (IC_{50} = 6.7 μM). The inhibitory effects of racemic, (+) and (−) mefloquine were compared. (+) Mefloquine had slightly greater inhibitory potential (IC_{50} = 167 μM) than the other two forms. Quinine, artemether, artesunate, halofantrine and chloroquine were essentially non-inhibitory (IC_{50} values > 500 μM).

The K_m value for carboxyprimaquine formation determined by regression analysis from Lineweaver–Burk plots with mean data from four livers was 181.8 μM and the V_{max} value was 45.5 nmol/hr/mg microsomal protein. Ketoconazole was the most potent inhibitor studied as judged by the K_i value of 6.7 μM (non-competitive inhibition), while mefloquine produced competitive inhibition with a K_i value of 52.5 μM (Fig. 1).

We recently demonstrated [16] that the metabolisn of mefloquine to carboxymefloquine by human liver microsomes was NADPH dependent and inhibited by ketoconazole (K_i = 11.2 μM). These findings were consistent with the involvement of cytochrome P450 isozyme(s) in metabolite formation. Similar findings in the present study indicate that carboxyprimaquine formation is also cytochrome P450 dependent.

The inhibition of primaquine metabolism by mefloquine (K_i = 52.5 μM) is apparently less marked than the inhibition of mefloquine metabolism by primaquine (K_i = 8.6 μM; [16]). This finding is consistent with previous data indicating that primaquine is a more potent inhibitor of cytochrome P450-mediated metabolism than mefloquine [10, 14, 15]. However, in the clinical setting with a dose differential (750 mg mefloquine, 45 mg primaquine) and considerable differences in blood levels and pharmacokinetics, the question arises: will mefloquine inhibit primaquine metabolism? An *in vivo* interaction is very much dependent on blood (or more importantly, hepatic) concentration of drug. After oral administration of 45 mg primaquine, peak plasma levels of 150–200 ng/mL (0.4 μM) are reached within 2 to 3 hr [22]. After oral administration of 750 mg mefloquine peak plasma concentrations are approximately 1500–2000 ng/mL (5 μM) at 4–18 hr [23]. Using the equation proposed by Resetar *et al.* [24] of $i = 100[I]/(K_i(1 + [S]/K_m) + [I])$ it can be calculated that mefloquine will produce 10% inhibition (i = per cent inhibition) of primaquine metabolism. In contrast to mefloquine, at a peak concentration of 6 μM ketoconazole [25], the calculated *in vivo* inhibition will be 50%.

Although quinine inhibited mefloquine metabolism (K_i = 28.5 μM; [16]), there was no inhibition of carboxyprimaquine formation. None of the other antimalarials studied inhibited primaquine metabolism.

Table 1. Characteristics of inhibition of carboxyprimaquine formation by various drugs

Inhibitors	K_m (μM)	V_{max} (nmol/hr mg protein)	IC_{50} (μM)	K_i (μM)	Nature of inhibition
Control	181.8	45.5	—	—	—
Ketoconazole	200.0*	28.6*	15	6.7	Non-competitive
Mefloquine (+, −)	400.7†	44.4†	220	52.5	Competitive
Mefloquine (+)	—	—	167.5	—	—
Mefloquine (−)	—	—	250	—	—
Quinine	—	—	>500	—	—
Artemether	—	—	>500	—	—
Artesunate	—	—	>500	—	—
Halofantrine	—	—	>500	—	—
Chloroquine	—	—	>500	—	—

Results are expressed as the mean data for four livers.
* At the inhibitor concentration of 10 μM.
† At the inhibitor concentration of 50 μM.

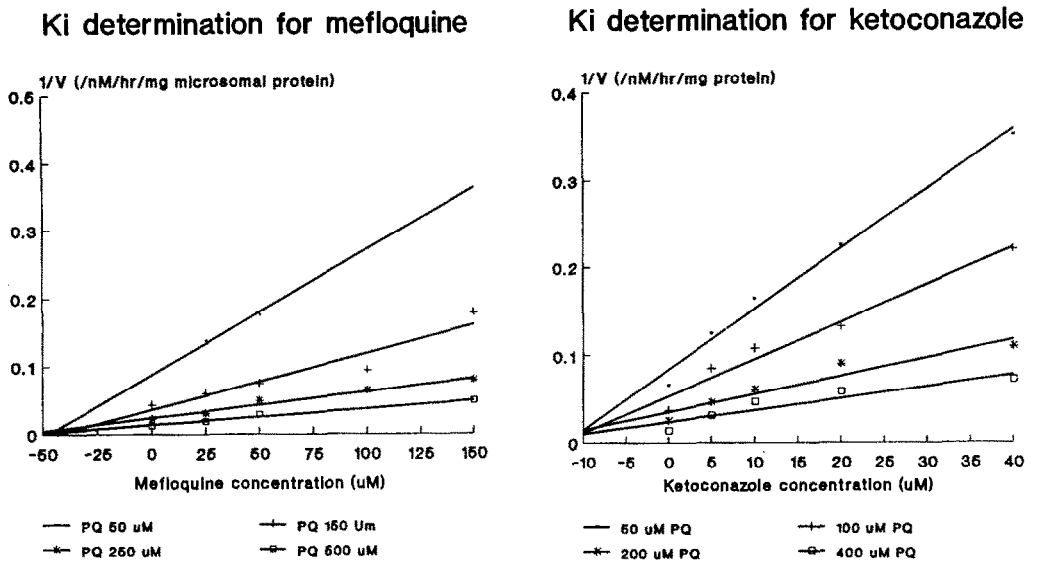

Fig. 1. Dixon plots (reciprocal of velocity, $1/v$, versus inhibitor concentration) showing inhibition of primaquine metabolism by ketoconazole and mefloquine.

In conclusion, the present study has shown carboxyprimaquine to be the only metabolite of primaquine formed in human liver *in vitro*. The affinity of primaquine as a substrate for cytochrome P450 isozyme(s) (K_m = 182 μM) appears to be very much less than as an inhibitor of mefloquine metabolism (K_i = 8.6 μM; [16]). We need to clarify which isozyme is responsible for primaquine metabolism. It seems unlikely that the concurrent administration of mefloquine (or any other antimalarial) with primaquine can lead to appreciably altered primaquine disposition, although some caution is necessary with ketoconazole and possibly other antimycotics. Any inhibition of metabolism *in vivo* could have implications for both efficacy and toxicity, although the importance of metabolites in the tissue parasiticidal actions, and toxicity of primaquine is still not completely known.

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Inhibitory potency of some isatin analogues on human monoamine oxidase A and B

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Abstract—Isatin is an endogenous compound which acts as a selective inhibitor of monoamine oxidase (MAO) B. In this study a range of isatin analogues were tested for their *in vitro* inhibition of human MAO A and B. Most of the analogues were less potent than isatin. Hydroxylation of the aromatic ring changed the inhibitory potency in favour of MAO A, with 5-hydroxyisatin being a potent and selective MAO A inhibitor (IC_{50} 8 μM). Isatinic acid, which is formed reversibly from isatin at alkaline pH, showed no inhibition.

Isatin has long been known as a pharmacologically active agent which exerts a number of *in vitro* and *in vivo* effects (for review, see Ref. 1). In particular, it is anxiogenic at low doses in rodents [1, 2]. It has recently emerged as a major constituent of tribulin, a naturally occurring, low molecular mass inhibitor of monoamine oxidase (MAO) and benzodiazepine binding [3–5], the output of which is increased in various states of stress or anxiety (e.g. Refs 6, 7). However, isatin does not account for all the activity ascribed to tribulin [8]. Isatin is a more potent inhibitor of MAO B than MAO A [5], whereas inhibition by tribulin is equipotent [4]. Isatin is also a relatively weak inhibitor of benzodiazepine receptor binding compared with tribulin [9].

Such discrepancies between the inhibitory properties of isatin and tribulin point to the presence of as yet unidentified components of tribulin, which can selectively inhibit MAO A and also inhibit benzodiazepine binding more potently. In the present study, we have examined the MAO inhibitory effect of hydroxylated and other analogues of isatin, including a *spiro*-tetrahydroisoquinoline condensation product of dopamine and isatin (dopamine–isatin), *N*-methylisatin, indole and oxindole, to try to understand how chemical modification of isatin affects inhibitory activity. We have also investigated the effect of isatinic acid, formed reversibly from isatin at alkaline pH.

Materials and Methods

The inhibitory effect of isatin and its analogues was tested on human placental MAO A and human platelet MAO B, using 170 μM [^{14}C]5-hydroxytryptamine (sp. act. 2.5 μCi/μmol) and 5 μM [^{14}C]phenylethylamine (sp. act. 12.5 μCi/μmol), respectively, as described previously [10]. These concentrations, which are close to the K_m value, allow competitive inhibition to be detected. IC_{50} values for the compounds tested were calculated from their inhibition curves, using a range of concentrations from 10^{-8} to 10^{-4} M for each compound. The results represent means of at least six independent experiments, with a standard error of less than 5%. Radiolabelled 5-hydroxytryptamine and