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THE EFFECTS OF PRIMAQUINE STEREOISOMERS AND METABOLITES ON DRUG METABOLISM IN THE ISOLATED PERFUSED RAT LIVER AND *IN VITRO* RAT LIVER MICROSOMES

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Abstract—The effect of the antimalarial drug primaquine, its stereoisomers and its proposed metabolites, on the metabolism of substrates for mixed function oxidase, has been studied in isolated perfused rat livers (IPRL) and/or *in vitro* microsomal suspension. Following acute administration to an IPRL preparation, racemic primaquine produced a dose related reduction in the hepatic clearance of antipyrine which at the highest dose of primaquine (5.0 mg) represented a decrease to 46% of control values. Antipyrine clearance was reduced to a comparable extent by the (+) and (−) isomers and the racemic mixture (each at a dose of 2.5 mg) with mean reductions of 45, 49 and 47%, respectively. These changes in clearance were reflected by significant increases in half-life relative to control. The apparent volume of distribution of antipyrine was unchanged in all experiments. Racemic primaquine and its (+) and (−) isomers were equipotent in inhibiting aminopyrine N-demethylase activity, producing reductions of 56, 59 and 55%, respectively, relative to control values. These three compounds also produced corresponding reductions of 73, 58 and 73% in ethoxyresorufin O-deethylase activity. The N-acetyl and 5-hydroxy derivative of primaquine produced inhibitory effects comparable to that seen for the parent drug. In contrast the carboxylic acid metabolite of primaquine, 6-desmethylprimaquine and 5-hydroxy-6-desmethyl primaquine did *not* influence aminopyrine N-demethylase activity. These results indicate that the propensity to inhibit drug metabolism by these primaquine related substances, is influenced by functional group substitution rather than the optical activity of the parent drug.

The antimalarial agent primaquine has been shown to inhibit drug metabolism both *in vivo* and *in vitro* in rats [1] and *in vivo* in man [2]. These studies utilized racemic primaquine, which comprises equal proportions of the (+) and (−) isomers. As the toxicity of these two isomers has been shown to differ markedly [3], the present study has examined the potency of the (+) and (−) isomers in inhibiting drug metabolism when compared to increasing doses of racemic primaquine.

We have used the clearance of antipyrine from the isolated perfused rat liver as an index of mixed function oxidase activity. The results obtained from this experimental model, when applied to the study of hepatic drug–drug interactions for the H_2-receptor antagonists [4], have agreed with findings from human studies [5]. In addition, this model allows the direct evaluation of *hepatic* drug–drug metabolism interactions in a whole organ system.

In further studies we have examined the effects of the (+) and (−) isomers, racemic primaquine, the carboxylic acid metabolite of primaquine and several putative metabolites of primaquine on *in vitro* microsomal mixed function oxidase activity.

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MATERIALS AND METHODS

Drugs and chemicals. Racemic (±) primaquine diphosphate was obtained from Aldrich Chemical Co. (Gillingham, U.K.). (+) Primaquine diphosphate, (−) primaquine diphosphate, 5-hydroxyprimaquine trihydrobromide, 6-desmethylprimaquine sulphate and 5-hydroxy-6-desmethylprimaquine trihydrobromide were gifts from the Walter Reed Army Medical Research Centre (Washington, DC). Samples of N-acetylprimaquine and the carboxylic acid metabolite of primaquine were gifts from Professor J. McChesney (Department of Pharmacognosy, University of Mississippi, MI).

Antipyrine, aminopyrine, β-naphthoflavone, Phenacetin, semicarbazide and NADPH were obtained from Sigma Chemical Co. (Poole, U.K.), ethoxyresorufin (ERR) from Pierce Chemicals Ltd. (Chester, U.K.) and resorufin (RR) from Eastman Kodak Co. (Rochester, NY). Norantipyrine and 4-hydroxyantipyrine were gifts from Dr. McKillip, ICI (Alderley Edge, U.K.) and 3-hydroxymethylantipyrine a gift from Professor D. Breimer (Leiden, Netherlands).

Solvents were of HPLC grade and obtained from Fisons Pharmaceutical (Loughborough, U.K.), all other reagents were of analytical grade and supplied by British Drug Houses (Poole, U.K.).

Animals. Male Wistar rats (200–250 g) were

housed in well ventilated cages and kept at a temperature of approximately 24°. They were allowed to feed *ad libitum* on pelleted food (Oxoid Breeding Diet, Oxoid Ltd., London, U.K.) and tap water.

Isolated perfused rat livers (IPRL). Rats were anesthetized with sodium pentobarbitone (60 mg/ml; 60 mg/kg i.p.) and their livers were isolated using standard techniques and then perfused in a constant flow (15 ml/min) recirculating system at 37° as previously described [4]. The principal indices of liver viability were steady oxygen consumption (1.5–2.0 μmoles/g liver/min), sustained bile production (0.4–0.6 ml/hr), constant perfusion pressure (6–8 cm H_2O), reproducible liver function test results and normal appearance.

Antipyrine disposition studies in the IPRL. The elimination of a 2.5 mg bolus dose of antipyrine (250 μl of a 10 mg/ml aqueous solution added to the perfusate reservoir), was studied over four hours in the following groups: (a) control (without addition of a putative inhibitor; N = 7); (b) (±) primaquine (after bolus doses of 0.5, 2.5 or 5.0 mg; N = 4 in each set); (c) (+) primaquine (after a bolus dose of 2.5 mg); (d) (−) primaquine (after a bolus dose of 2.5 mg). All drug solutions were added as aqueous solutions of the diphosphate salt of (+), (−) or (±) primaquine (10 mg/ml), into the perfusate reservoir, thereby simulating systemic dosage. Prior to use, the optical rotation of the (+) and (−) isomers was found to correspond to that previously reported [6].

Samples (1.5 ml) were taken from the perfusate reservoir for antipyrine estimations, predose and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. After centrifugation the separated perfusate plasma was removed and frozen at −20° until assayed. Additional samples were taken hourly to measure primaquine concentrations. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. The total amount of antipyrine lost through sampling was less than 5% of the dose. Bile was collected into preweighed vials. Perfusate blood gases were measured and liver function tests were performed before and after each experiment to ascertain the viability of the liver.

Drug assays.

Assay of antipyrine. Perfusate antipyrine levels were determined by the method of Shargel *et al.* [7], except that changes to chromatography conditions were made in order to avoid interference from the metabolites of antipyrine which have been previously described by Danhof *et al.* [8]. To perfusate plasma (200 μl) was added phenacetin as the internal standard (100 μg/ml, 20 μl), in a 1.5 ml capacity microfuge tube. Perfusate proteins were then precipitated by the addition of 100 μl each of $ZnSO_4$ (20%): methanol (50:50) and saturated $Ba(OH)_2$ solution. After vortex mixing (30 sec) and centrifugation (1000 *g*, 60 sec), 5 to 10 μl of the supernatant was injected on to the chromatograph. Separation was effected on a μBondapak C_{18} Rad Pak column (Waters Associates, Hartford, U.K.) housed in a Z-module (Waters Assoc.) and fitted with a CN-guard column (Waters Assoc.). The mobile phase was CH_3OH: Na_2HPO_4 (50 mM, pH 6.8) [45:55] flowing at 3 ml/min. Under these conditions 3-hydroxymethylantipyrine, norantipyrine, antipyrine, 4-hydroxyantipyrine and phenacetin were all resolved to baseline and eluted with retention times of 1.9, 2.4, 3.2, 3.8 and 5.0 min, respectively.

Assay of primaquine. Perfusate primaquine levels were determined by a selective and sensitive HPLC method as previously described by us [9].

In vitro *studies.* The potential of racemic primaquine to inhibit aminopyrine N-demethylation and ethoxyresorufin (ERR) deethylation, was compared with the degree of inhibition produced by the (+) and (−) isomers of this drug. As very limited quantities (≤5 mg) were available of the carboxylic acid metabolite of primaquine [9] and the proposed metabolites; N-acetylprimaquine, 5-hydroxyprimaquine, 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine [10], these compounds were only tested for their potential to influence aminopyrine N-demethylation.

Rats were killed by cervical dislocation, the livers rapidly removed and homogenized in ice-cold 0.67 M phosphate buffer (pH 7.5) containing 0.15 M KCl using a Teflon in glass homogenizer. The 25% homogenate was centrifuged at 13,000 *g* for 20 min at 4°. The resulting supernatant was decanted without disturbing the pellet and centrifuged at 105,000 *g* for 60 min at 4°. The microsomal pellet was resuspended in 0.2 ml phosphate buffer and microsomal protein was determined by the method of Lowry *et al.* [11]. The N-demethylation of aminopyrine was carried out with the following reaction mixture: aminopyrine (2.5 mM), semicarbazide (9.37 mM), test compound (0.10–0.18 mM), microsomes (0.5 ml of 4 mg/ml suspension) (and NADPH (0.6 mM). The hydroxylated metabolites of primaquine were stabilized by the addition of ascorbic acid (1 mM) to the reaction mixture. Formaldehyde production was measured with the Nash reagent [12] and absorbance determined at 415 nm.

ERR O-deethylase activity was determined by the method of Burke and Meyer [13]. Rats were pretreated for 3 days with β-naphthoflavone (i.p.: 75 mg/kg/day). The incubation mixture contained ERR (250 nM), test compounds (1 μM), microsomal protein (0.01 mg/ml) and NADPH (0.25 mM) in 0.067 M phosphate buffer (pH 7.5). The reaction was allowed to proceed in a cuvette for 3 min at 30°, during which time the formation of RR was monitored by the appearance of fluorescence (λ_{exc} = 510 nm; λ_{emm} = 586 nm).

Pharmacokinetic calculations and statistical analysis. Antipyrine pharmacokinetic parameters were calculated using standard model-independent pharmacokinetic formulae [14]. Statistical comparisons of antipyrine pharmacokinetic parameters were made by one way analysis of variance and of % inhibition of substrate metabolism by comparison of sample means with unknown variance using the *t*-statistic [15], accepting $P \leq 0.05$ as significant. Data are presented as mean ± S.D.

RESULTS

The disappearance of antipyrine from the perfusate, in the presence or absence of primaquine in the medium is shown in Figs. 1 and 2. Antipyrine

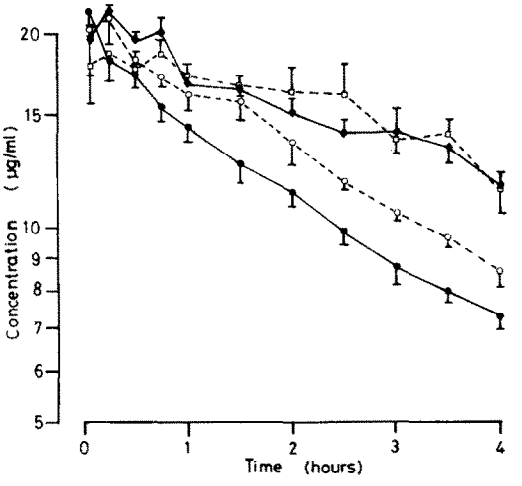

Fig. 1. Semilogarithmic plot of antipyrine elimination from perfusate in control livers (without addition of putative inhibitor); (●–●) and after primaquine in doses of 0.5 mg (○–○), 2.5 mg (◆–◆) and 5.0 mg (□–□). Antipyrine was eliminated more slowly with increasing doses of primaquine.

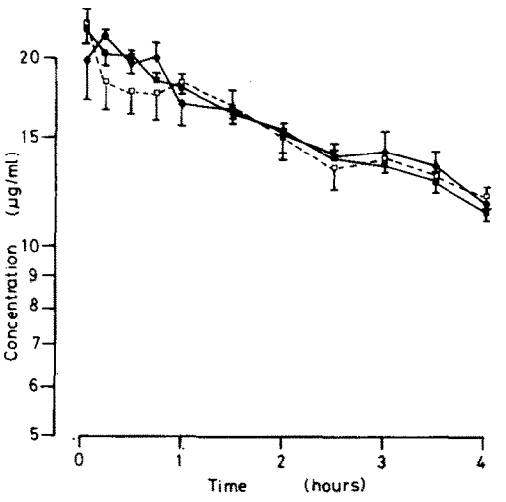

Fig. 2. Semilogarithmic plot of antipyrine elimination from perfusate after 2.5 mg doses of (±) primaquine (◆–◆), (+) primaquine (■–■) and (−) primaquine (□–□). Antipyrine elimination was comparable for all these substances.

concentrations in perfusate declined monoexponentially in all experiments. Increasing doses of (±) primaquine were associated with slower rates of fall of antipyrine levels (Fig. 1). However at doses of 2.5 mg, the (+), (−) and (±) isomers of primaquine resulted in no appreciable differences in the antipyrine profile (Fig. 2).

The resultant mean pharmacokinetic parameters for antipyrine are summarized in Table 1. Racemic primaquine produced a dose and plasma level related reduction in antipyrine clearance (Tables 1 and 2) which at the highest dose of primaquine represented a decrease to 46% of the control values. The dose of (±) primaquine that produced 50% inhibition of antipyrine clearance (ID_{50}) was 7.0 μmole (3.2 mg primaquine diphosphate, Fig. 3). Using the same experimental model, the ID_{50} of antipyrine clearance for cimetidine [4] was 2.8 μmole (0.7 mg cimetidine), indicating that primaquine is two to three fold *less* potent than cimetidine in inhibiting drug metabolism in the IPRL.

Antipyrine clearance was reduced to a comparable extent by the (+) and (−) isomers, and by the racemic mixture of primaquine (each at a dose of 2.5 mg) with mean reductions of 45, 49 and 47%, respectively. These changes in clearance were reflected by significant increases in elimination half-life compared to control (Table 1). The volume of distribution remained the same in all experiments and was comparable to the actual volume of the perfusion circuit (Table 1).

The mean perfusate concentrations of primaquine throughout the course of each set of experiments are listed in Table 2, showing that primaquine underwent hepatic elimination in all groups.

Racemic primaquine and its (+) and (−) isomers were equipotent in inhibiting aminopyrine N-demethylase activity producing reductions of 56, 59 and 55%, respectively (Table 3). These three substances produced corresponding reductions of 73, 58 and 73% in ERR deethylase activity. Both the N-acetyl and 5-hydroxy-derivatives of primaquine produced inhibitory effects comparable to that seen for the parent drug. By contrast the carboxylic acid metabolite of primaquine, 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine did not appreciably influence aminopyrine N-demethylase activity compared to control.

Table 1. Antipyrine pharmacokinetic parameters in the absence of any putative inhibitor (control) and following dosage with primaquine

Treatment group	Clearance (ml/min)	Half-life (min)	Volume of distribution (ml)	N
Control	0.550 ± 0.053	165 ± 17	130 ± 16	7
(±) PQ 0.5 mg	0.446 ± 0.044*	194 ± 20*	124 ± 5	4
(±) PQ 2.5 mg	0.292 ± 0.074*	300 ± 121*	118 ± 10	4
(±) PQ 5.0 mg	0.253 ± 0.083*	404 ± 143*	116 ± 28	4
(+) PQ 2.5 mg	0.302 ± 0.057*	277 ± 51*	118 ± 11	5
(−) PQ 2.5 mg	0.279 ± 0.035*	303 ± 45*	121 ± 9	5

* $P \leq 0.05$.

Table 2. Mean perfusate primaquine concentrations (ng/ml, mean ± S.D.) at various intervals post-dose during isolated perfused rat liver experiments

Drug	Dose (mg)	Time (min) 60	120	180	240
(±) Primaquine	0.5	136 ± 19	99 ± 9	68 ± 19	46 ± 18
(±) Primaquine	2.5	682 ± 237	710 ± 183	624 ± 191	481 ± 199
(±) Primaquine	5.0	1985 ± 819	1337 ± 415	1028 ± 151	1287 ± 413
(+) Primaquine	2.5	995 ± 121	859 ± 62	767 ± 94	673 ± 209
(−) Primaquine	2.5	819 ± 181	564 ± 116	455 ± 74	383 ± 68

DISCUSSION

The disposition of antipyrine from the IPRL is a useful experimental model for the study of hepatic drug–drug metabolism interactions [4, 16]. In the case of the H_2-receptor antagonists cimetidine and ranitidine, and in the present study primaquine, the qualitative findings from this experimental model have correlated with the conclusions reached from human studies [2, 5]. In addition, as a relatively simple and versatile experimental system, studies in the IPRL can readily examine the contribution of factors such as dose size, isomer form and drug metabolites to the hepatic component of drug metabolism interactions.

In the present study we have seen a dose and plasma level dependent inhibition of drug metabolism. This was shown by the progressive decrease in antipyrine clearance with increasing doses and associated higher plasma levels of (±) primaquine (Tables 1 and 2). This effect was virtually immediate, suggesting a direct interaction between the inhibiting agent and drug metabolizing enzymes, leading to a decrease in their activity. These changes in antipyrine clearance were reflected in prolonged half-life values. However, the values for volume of distribution were not different among any of the treatment groups and were comparable in size to the actual volume of the perfusion circuit (100 ml reservoir plus volume of perfused liver), suggesting there was no perturbation of antipyrine distribution by primaquine.

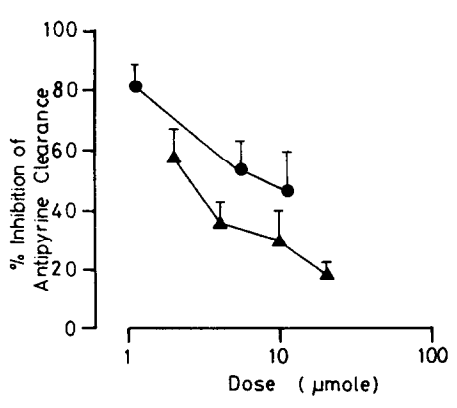

Fig. 3. Dose dependent inhibition of antipyrine clearance by primaquine (●–●), compared with cimetidine (▲–▲)— data derived from ref. 4. Cimetidine was two to three fold more potent than primaquine in inhibiting drug metabolism; ID_{50} for cimetidine = 2.8 μmole; ID_{50} for primaquine = 7.0 μmole.

We have previously examined the effects of various H_2-receptor antagonists on antipyrine clearance from the same experimental IPRL model [4] and found cimetidine (ID_{50} = 2.8 μmole) to be two to three fold more potent an inhibitor of drug metabolism than primaquine (ID_{50} = 7.0 μmole—Fig. 3). By contrast, in a recent study *in vivo* of the inhibition of the plasma disappearance of tolbutamide, primaquine was shown to be two to three fold more potent than cimetidine [16]. This reversal of the order of potency may be due either to dissimilarities in the experimental models used or to differences in the capacities of these inhibitor compounds to influence different substrate reactions.

In animal studies, the (+), (−) and racemic forms of primaquine have different therapeutic index values (i.e. ratios of toxic to effective doses). This disparity does not arise from differences in antimalarial activity, but is due to variations in the toxicity (primarily hepatotoxicity) between the three substances [3]. In fact, the potency to produce acute lethal toxicity in rodents by the (+) isomer is 2 to 3 fold greater than the racemic drug, which in turn is 2 to 3 fold more potent in this effect than the (−) isomer [3]. These differences in toxicity may reflect differences in the cytochrome P_{450} metabolism of the three substances. It is interesting to note that in the present study after equivalent 2.5 mg doses of the drugs the perfusate disappearance rates of the (+) isomer was slower than the racemate which in turn was slower than the (−) isomer (Table 2). In spite of this contrast in toxicity, and stereoselectivity in metabolism, the (+), (−) and racemic forms of primaquine were equipotent in their capacity to decrease antipyrine clearance in the IPRL (Fig. 2, Table 1). These findings were further supported by the *in vitro* microsomal studies, in which aminopyrine N-demethylase activity was influenced by primaquine isomers and the racemate to a comparable extent (Table 3).

In the case of ERR-O-deethylase activity, a similar pattern of inhibition was evident for the three substances, however the (+) isomer appeared marginally less potent than the (−) isomer or racemate (Table 3). In the present study, the results of the *in vitro* substrate inhibition experiments using racemic primaquine were in agreement with earlier reports [1].

In all experiments primaquine underwent hepatic elimination, with drug levels falling approximately

Table 3. Percentage inhibition (mean ± S.D.) of drug metabolism activity *in vitro* in liver microsomes—control (no putative metabolite added) = 100% activity

Substance (concentration)	Aminopyrine N-demethylase activity		Ethoxyresorufin O-deethylase activity	
Control	100	(N = 8)	100	(N = 5)
(±) Primaquine (0.11 mM)	56 ± 11*	(N = 8)	27 ± 4*	(N = 5)
(+) Primaquine (0.11 mM)	59 ± 11*	(N = 5)	42 ± 6*	(N = 5)
(−) Primaquine (0.11 mM)	55 ± 13*	(N = 5)	27 ± 3*	(N = 5)
N-Acetylprimaquine (0.17 mM)	55 ± 12*	(N = 4)		
Carboxylic acid metabolite of primaquine (0.18 mM)	103 ± 7	(N = 3)		
5-hydroxyprimaquine (0.10 mM)	55 ± 7	(N = 4)		
6-desmethylprimaquine (0.15 mM)	95 ± 7	(N = 4)		
5-hydroxy-6-desmethyl primaquine (0.10 mM)	91 ± 7	(N = 4)		

* Level of significance compared to control, $P \leq 0.005$).

30–60% between the first and fourth hours of the experiments (Table 2). Ideally, drug metabolism inhibition studies should be carried out in the presence of steady state levels of the putative inhibitor. Nonetheless in every case of the present study, drug concentrations persisted at levels equal to or greater than those encountered in human studies, where therapeutic doses of primaquine had been given [9].

Using the limited quantities of identified and 'postulated' metabolites of primaquine available, it was possible to evaluate the effects of these substances on microsomal aminopyrine N-demethylase activity (Table 3). Only the carboxylic acid derivative of primaquine has been identified as a metabolite of this drug in man [9]; however, it did not affect aminopyrine N-demethylation. By contrast, N-acetylprimaquine, which has been detected as a primaquine metabolite in microbial culture studies [18], but not in human plasma or urine [9], was as potent as racemic primaquine in inhibiting aminopyrine demethylation. As for the three hydroxylated derivatives [10], only 5-hydroxyprimaquine inhibited drug metabolism, whereas 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine did not produce any appreciable inhibition (Table 3).

The differences in potency of inhibition of drug metabolism between these primaquine derivatives implies that subtle changes in functional group substitution of the parent molecule, but not optical activity, may have considerable bearing on the propensity of these compounds to influence drug metabolism. In this vein, it appears that the methylene group of the 6-methoxy position is necessary for inhibition of microsomal drug metabolism. This 6-methoxy substitution is present in all the compounds studied except 6-desmethylprimaquine and 5-hydroxy-6-desmethylprimaquine, neither of which produced any inhibitory effect. Although the carboxylic acid metabolite of primaquine does retain this methylene group, it failed to inhibit drug metabolism. This implies that the retention of the terminal amine group on the side chain may also be an essential molecular component for this inhibitory phenomenon.

The present study has shown that in the IPRL, the inhibition of drug metabolism by racemic primaquine was rapid in onset and both dose and plasma level dependent. The results of further studies in the IPRL together with *in vitro* experiments in liver microsomes have shown the (+) and (−) isomers to be equipotent to racemic primaquine in inhibiting drug metabolism. Separate *in vitro* studies have also shown that the carboxylic acid metabolite of primaquine, which represents the major plasma metabolite in man, to have no influence on drug metabolism. In addition, although drug metabolism was inhibited by the N-acetyl and 5-hydroxy derivatives of primaquine, the importance of these observations awaits the complete elucidation of primaquine's metabolic fate.

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