INHIBITION OF TOLBUTAMIDE METABOLISM BY ANTIMALARIAL DRUGS

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INTRODUCTION

There is increasing evidence that some antimalarial drugs have the propensity to inhibit hepatic microsomal drug oxidation. Murray (1984) studied the effects of quinoline derivatives on cytochrome P-450, using aminopyrine as substrate *in vitro* and found that primaquine, amodiaquine and quinine were relatively potent inhibitors of the N-demethylase activity, but that quinidine and chloroquine were essentially non-inhibitory. Similar results were obtained by Back *et al.*, (1983), the data indicating that primaquine inhibited aminopyrine N-demethylation at very much lower concentrations than chloroquine. Riviere and Back (1985) have also shown that mefloquine (MQ) inhibits hepatic microsomal enzymes both *in vitro* and *in vivo* and produces comparable inhibition *in vitro* to that seen with primaquine (PQ). More recently, Riviere and Back (1986) provided more *in vitro* evidence of the inhibitory potential of quinoline derivatives; primaquine, mefloquine and to a lesser extent quinine (Q) produced the most marked inhibitory effects on tolbutamide and ethinyloestradiol metabolism; quinidine (QD) and amodiaquine were of intermediate potency and chloroquine was essentially non-inhibitory.

Since MQ is being used as a combination product, it is important to ascertain whether the components of mefloquine/sulfadoxine/pyrimethamine (MSP) and the combination (MSP) itself have any inhibitory effects on microsomal drug metabolism. In addition, since quinine and quinidine are widely used in the treatment of falciparum malaria, particularly in cerebral malaria where intravenous administration is required, the inhibitory effects of these two quinolines were also studied and are reported herein.

It has been suggested that tolbutamide is a good marker drug for assessing changes in hepatic enzyme activity (Back *et al.*, 1984). Tolbutamide is converted in the liver to hydroxytolbutamide by NADPH linked microsomal enzymes. Hydroxytolbutamide is then further metabolised *in vivo* to carboxytolbutamide, via tolbutamide aldehyde, by enzymes in the cytoplasmic fraction (Hansen and Christensen, 1977). Oxidation of tolbutamide to hydroxytolbutamide is the rate limiting step in elimination of the drug and its metabolite (Rowland and Matin, 1973; Hansen and Christensen, 1977).

The present study was designed to investigate the relative inhibitory potential of

MQ, sulfadoxine (S), pyrimethamine (P), Q, Qd and MSP *in vitro* using human liver microsomes and tolbutamide as substrate.

MATERIALS AND METHODS

Chemicals: Drugs and chemicals used were: mefloquine HCl, pyrimethamine and sulfadoxine were gifts from Hoffmann-La Roche, Basle, Switzerland. NADPH, quinine HCl and quinidine HCl were obtained from Sigma. Tolbutamide and hydroxytolbutamide were obtained from Hoechst. All other reagents were obtained from B.D.H. (Poole, U.K.).

Human liver: Samples of 50-750 gm of histologically normal livers were obtained from kidney transplant donors (4 male, 2 female; Table 1). Ethical approval for the study was granted and consent to removal of the liver samples was obtained from the donors' relatives. Each sample was cooled to 0°C, divided into 3-20 gm portion and frozen in liquid nitrogen within one hour of removal and stored at -70°C until used. Washed microsomes were prepared using the classical differential sedimentation method as described by Purba *et al.*, (1986). Cytochrome P-450 was assayed by the method of Omura and Sato (1964). Microsomal protein was determined by the method of Lowry *et al.*, (1951).

The hydroxylation of tolbutamide was carried out in 15 ml screw cap glass tubes at 37°C with vigorous agitation. Incubations contained tolbutamide 150 μM, the alleged inhibitors (MQ, P, Q and Qd 50-500 μM, S 10-200 μM, MSP (based on MQ concentration 5-45 μM with a fixed ratio of MQ:S:P = 1:2:10), $MgCl_2$ (5 mM), EDTA (1mM), KCl (1mM), NADPH (1mM), microsomal protein 2 mg and 1/15 M phosphate buffer pH 7.5 (total volume 2.5 ml). Tolbutamide and inhibitors were dissolved in methanol which was evaporated to dryness before the addition of other reaction constituents, ie. 1/15 M phosphate buffer, mixture (contained KCl 1mM, EDTA 1mM, $MgCl_2$ 5mM, in phosphate buffer), 2 mg of microsomal protein, followed by NADPH (1mM). The reaction was stopped after 8 min by

Table 1

Tolbutamide 4-hydroxylase activity of human liver microsomes.

Liver (age, sex)	Cyt P-450 (nmole/mg protein)	Tolbutamide hydroxylase activity (nmole/min/mg protein)	Protein (mg/gm liver)
L1 (4 yr, M)	0.31	0.43	18.5
L3 (35 yr, M)	0.13	0.07	18.4
L4 (66 yr, F)	0.84	0.18	17.4
L5 (60 yr, M)	0.32	0.15	12.7
L6 (21 yr, M)	0.24	0.13	12.5
C2 (60 yr, F)	0.36	0.23	14.5
Mean	0.35	0.20	15.7
± S.D.	0.25	0.13	2.8

adding 100 µl of 6M HCl.

The effect of a fixed concentration of the various inhibitors on 4-hydroxy tolbutamide formation was investigated, using tolbutamide (at various concentrations, 50–250 µM) as the substrate; this enabled the determination of the nature of the inhibition from Lineweaver-Burk plots.

4-hydroxytolbutamide was quantified by HPLC as previously described (Back *et al.*, 1984) using a reversed-phase column (ODS-2) and a mobile phase of 0.05% phosphoric acid and methanol (50:50).

Data analysis: The IC_{50} values for MQ, S, P, Q, Qd and MSP on tolbutamide hydroxylation were determined by plotting the enzyme activity as a percentage of the control against the inhibitor concentration.

Km and Vmax values were determined from Lineweaver-Burk plots and calculated by linear regression.

Ki values for non-competitive inhibition were calculated as follows:

$$Ki = \frac{[I]}{\frac{Vmax\ C}{Vmax\ I} - 1}$$

Vmax C = Vmax control
Vmax I = Vmax in the presence of inhibitor
[I] = concentration of inhibitor

Ki values for competitive inhibition were calculated as follows:

$$Ki = \frac{[I]}{\frac{Km\ I}{Km\ C} - 1}$$

Km C = Km control
Km I = Km in the presence of inhibitor
[I] = concentration of inhibitor

Statistical analysis was by Student's paired t-test.

RESULTS

Microsomal protein content was 15.7 ± 2.8 mg/g of liver (mean ± S.D.), cytochrome P-450 content was 0.35 ± 0.25 nmole/mg microsomal protein, tolbutamide 4-hydroxylase activity was 0.20 ± 0.13 nmole/min/mg microsomal protein at a substrate concentration of 150 µM (Table 1).

There was no significant correlation between the cytochrome P-450 content and the tolbutamide 4-hydroxylase activity when all livers were included (r = 0.13).

MSP, MQ, S, Q, Qd and P significantly inhibited tolbutamide metabolism at the highest concentration studied, as shown by the reduction in enzyme activity (Tables 2 and 3 and Fig. 1). MSP showed significant inhibition of tolbutamide metabolism at 15, 25, 35 and 45 µM. S significantly inhibited tolbutamide metabolism at 20, 50, 100 and

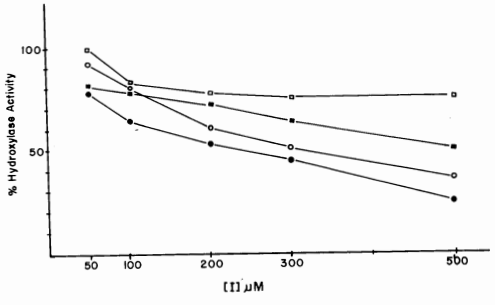


Fig. 1–The effect of different inhibitors on Tolbutamide hydroxylase activity. Each point is the mean of four experiments. □–□ = pyrimethamine, ○–○ = quinine ■–■ = quinidine, ●–● = mefloquine.

Table 2

Inhibitory effect of MQ, Q, Qd and P on 4-hydroxytolbutamide formation. Values are enzyme activity, nmole/min/mg protein. Results are mean ± S.D. of 4 experiments in each group.

Concentration of inhibitors (μM)	Enzyme activity in the presence of			
	Mefloquine	Quinine	Quinidine	Pyrimethamine
Control	0.17 ± 0.04	0.17 ± 0.04	0.25 ± 0.13	0.16 ± 0.07
50	0.13 ± 0.05[b]	0.16 ± 0.06	0.21 ± 0.12	0.15 ± 0.07
100	0.11 ± 0.05[b]	0.14 ± 0.05[a]	0.20 ± 0.12[a]	0.13 ± 0.07
200	0.09 ± 0.05[b]	0.11 ± 0.04[a]	0.18 ± 0.10[a]	0.12 ± 0.05
300	0.08 ± 0.04[b]	0.09 ± 0.04[b]	0.17 ± 0.10[a]	0.10 ± 0.05[a]
500	0.05 ± 0.03[b]	0.06 ± 0.02[b]	0.13 ± 0.06[a]	0.12 ± 0.05[a]

a = $p \leq 0.05$, significantly different from controls.
b = $p \leq 0.005$, significantly different from controls.

Table 3

Inhibitory effect of S and MSP on 4-hydroxytolbutamide formation. Values are enzyme activity. Results are mean ± S.D. of 4 experiments in each group.

Concentration (S)	4-OH tol (nmole/min/mg)	Concentration (MSP)	4-OH tol (nmole/min/mg)
Control	0.25 ± 0.13	Control	0.16 ± 0.07
10	0.24 ± 0.15	5	0.13 ± 0.05
20	0.22 ± 0.14[a]	15	0.09 ± 0.05[a]
50	0.19 ± 0.13[b]	25	0.06 ± 0.04[a]
100	0.15 ± 0.12[b]	35	0.04 ± 0.02[a]
200	0.10 ± 0.09[b]	45	0.03 ± 0.02[a]

a + p $\leq$ 0.05, significantly different from control.
b + p $\leq$ 0.01, significantly different from control.

200 μM. MQ showed significant inhibition at all four concentrations studied (50 to 500 μM), whereas Q and Qd showed significant inhibition at 100, 200, 300 and 500 μM, and P only showed significant inhibition at 300 and 500 μM.

The Km values for tolbutamide 4-hydroxylase determined by weighted regression analysis from the Lineweaver-Burk plots with mean data from 4 livers was 112 μM and the Vmax value was 0.34 nmol/min/mg microsomal protein. Table 4 shows the enzyme kinetic parameters in the presence of inhibitors ie. MSP, M, S, Q and Qd.

Table 4

Inhibition of Tolbutamide 4-hydroxylation in human liver microsomes (n = 4 livers)

	Ic 50 (μM)	Km (μM)	Ki (μM)	Vmax (nmole/min/mg)	Type of inhibition
Control	–	112	–	0.34	
Mefloquine	260	119	260	0.18	non-competitive
Quinine	353	84	617	0.24	un-competitive
Quinidine	512	115	1,156	0.28	non-competitive
Pyrimethamine	1,038	–	–	–	–
Sulfadoxine	76	222	76	0.37	competitive
MSP	20	1,190	2	0.47	competitive

The IC_{50} values were determined graphically (Fig. 1). The Ki values were determined by substituting Km and Vmax values in the appropriate equation (Webb, 1963). MQ and Qd were non-competitive inhibitors, S and MSP were competitive inhibitors, Q was an uncompetitive inhibitor (Table 4).

DISCUSSION

The present work provides more evidence of the potential of some antimalarial drugs to inhibit hepatic microsomal enzyme activity. Using tolbutamide as a substrate it is clear that S, MSP and to a lesser extent MQ and Q, produce the most marked inhibitory effects, Qd is of intermediate potency and P is the least potent inhibitor.

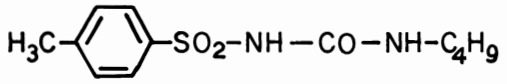
These results are substantially in agreement with the study by Riviere and Back (1986) on the inhibitory effect of antimalarials on tolbutamide and ethinyloestradiol metabolism in rat liver, which indicated an order of potency PQ > MQ > Q > Qd > AQ > CQ. It also concurs reasonably well the study by Murray (1984) on the effect of five of the quinoline drugs on aminopyrine N-demethylase activity, which indicated an order of potency on aminopyrine N-demethylase activity Q > PQ > AQ > Qd > CQ.

S was found to be a very potent competitive inhibitor of tolbutamide metabolism. A recent study by Tjia (personal communication) on the inhibitory effects of a number of sulphonamide drugs *in vitro* using human liver and tolbutamide as substrate showed the order of inhibitory potency to be sulphenazole > sulfadoxine > sulphamethizole > dapsone >> sulphamethoxazole.

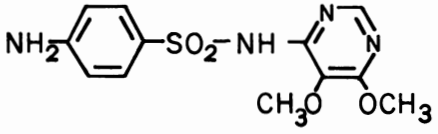
Since S has a similar structure to tolbutamide (Fig. 2), it is possible that S and tolbutamide are metabolized by the same form of cytochrome P-450, and thus compete for the same binding site; hence the competitive inhibition of tolbutamide metabolism (Fig. 3).

The finding that S is a potent microsomal enzyme inhibitor *in vitro* could also explain the longer $T\frac{1}{2}$ and MRT when it is coadministered with MQ (as MSP) in healthy volunteers (Karbwang *et al.*, 1987). It is possible that S inhibits the metabolism of MQ in *vivo* as well as inhibiting drug metabolising enzymes *in vitro*.

However, some caution should be exercised because the fact that a compond shows



Tolbutamide



Sulfadoxine

Fig. 2–The structures of (1) Tolbutamede, (2) Sulfadoxine.

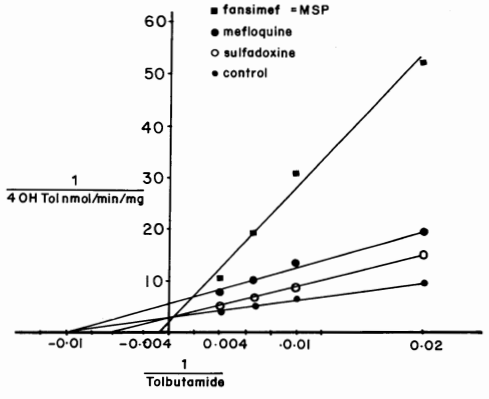


Fig. 3–Lineweaver-Burk plot showing competitive inhibition of Tolbutamide hydroxylation by Fansimef and sulfadoxine and non-competitive inhibition by mefloquine. Each point is a mean of four livers.

enzyme inhibition *in vitro* does not mean that it will automatically lead to clinically important pharmacokinetic interactions when co-administered with other drugs in man. Riviere *et al.*, (1985) have demonstrated that in normal therapeutic doses, PQ but not MQ inhibits antipyrine metabolism in man, despite the latter being a potent inhibitor *in vitro*. This may reflect the different pharmacokinetics and hepatic accumulation of the antimalarials. Since enzyme inhibition *in vivo* is a reflection not only of binding to the enzyme but also of actual drug concentration present in the liver, particularly in the vicinity of the enzyme it is possible that hepatic concentrations of MQ are very much less than PQ. Hepatic extraction of MQ may be low as there is evidence of the avid binding of MQ to both plasma proteins and red blood cells. Another explanation for these findings is that the concentration of MQ was insufficient to inhibit antipyrine metabolism. The peak plasma concentration of MQ was attained at approximately 24 hr after dosing, by which time the metabolism of antipyrine was already completed.

Further studies in man should be attempted in order to understand the clinical relevance of the inhibitory potential of the antimalarial drugs. The inhibitory effect of MQ on antipyrine should be re-evaluated in man at the proper timing of antipyrine administration ie. 12 to 24 hr after MQ administration.

SUMMARY

The effects of mefloquine (MQ), the combination of MQ with sulfadoxine-pyrimethamine (MSP), sulfadoxine (S), pyrimethamine (P) quinine (Q) and quinidine (Qd) on *in vitro* hepatic metabolism has been studied using tolbutamide as a substrate.

The hydroxylation of tolbutamide was determined in the presence of variable concentrations of each compound. Tolbutamide hydroxylase activity in control microsomes was 0.20 ± 0.13 nmole/min/mg microsomal protein at a substrate concentration of 150 μM. All compounds studied inhibited tolbutamide metabolism as shown by a decrease in 4-hydroxytolbutamide formation. The order of potency of the inhibitors was MSP > S > MQ > Q > Qd > P.

MQ, MSP, S, Q, and Qd were examined in detail for the type of inhibition. MQ and Qd

were non-competitive inhibitors, whereas MSP and S were competitive inhibitors and Q was an uncompetitive inhibitor of tolbutamide 4-hydroxylation.

These data provide more information on the inhibitory potential of some antimalarial drugs on microsomal enzymes in human liver. S has been shown to be a potent inhibitor *in vitro* and this finding possibly explains the longer $T\frac{1}{2}$ and MRT of MQ when co-administered with S in healthy volunteers. Further studies in man should be attempted in order to understand the clinical relevance of the inhibitory potential of the antimalarial drugs.

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