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Halofantrine Metabolism in Microsomes in Man: Major Role of CYP 3A4 and CYP 3A5

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Abstract

We have clarified the contribution of the different enzymes involved in the N-debutylation of halofantrine in liver microsomes in man. The effect of ketoconazole and cytochrome P450 (CYP) 3A substrates on halofantrine metabolism has also been studied.

The antimalarial drug halofantrine is metabolized into one major metabolite, *N*-debutylhalofantrine. In microsomes from nine livers from man, N-debutylation of halofantrine was highly variable with apparent Michaelis–Menten constant V_{max} and K_m values of 215 ± 172 pmol min^{-1} mg^{-1} and 48 ± 26 μmol L^{-1}, respectively, (mean ± standard deviation). Formation of *N*-debutylhalofantrine was cytochrome P450 (CYP)-mediated. Studies using selective inhibitors of individual CYPs revealed the role of CYP 3As in the formation of *N*-debutylhalofantrine. α-Naphthoflavone, a CYP 3A activator, increased metabolite formation. In microsomes from 12 livers from man the rate of N-debutylation of halofantrine correlated strongly with CYP 3A4 relative levels ($P = 0{\cdot}002$) and less strongly, but significantly, with CYP 2C8 levels ($P = 0{\cdot}025$). To characterize CYP-mediated metabolism of halofantrine further, incubations were performed with yeast microsomes expressing specific CYP 3A4, CYP 3A5, CYP 2D6, CYP 2C8 and CYP 2C19 from man. The rate of formation of *N*-debutylhalofantrine was six- and twelvefold with CYP 3A4 than with CYP 3A5 and CYP 2C8, respectively. CYP 2D6 and CYP 2C19 did not mediate the N-debutylation of halofantrine, but, because in-vivo CYP 2C8 is present at lower concentrations than CYP 3A in the liver in man, the involvement of CYP 3As would be predominant. Diltiazem, erythromycin, nifedipine and cyclosporin (CYP 3A substrates) inhibited halofantrine metabolism. Similarly, ketoconazole inhibited, non-competitively, formation of *N*-debutylhalofantrine with an inhibition constant, K_i, of $0{\cdot}05$ μM. The theoretical percentage inhibition of halofantrine metabolism in-vivo by ketoconazole was estimated to be 99%.

These results indicate that both CYP 3A4 and CYP 3A5 metabolize halofantrine, with major involvement of CYP 3A4. In-vivo, the other CYPs have a minor role only. Moreover, strong inhibition, and consequently increased halofantrine cardiotoxicity, might occur with the association of ketoconazole or other CYP 3A4 substrates.

Halofantrine is a phenanthrene–methanol derivative (Figure 1) used in the treatment of chloroquine- and multi-drug-uncomplicated *Plasmodium falciparum* malaria (Cosgriff et al 1982).

In the seventies the halofantrine dosage regimen in both adults and children consisted of three oral doses of 8 mg kg^{-1} at 6-h intervals (24 mg kg^{-1} per cure; Boudreau et al (1988)). On the basis of a marked reduction in cure rates in south-east Asia, particularly Thailand (Nosten et al 1991), halofantrine dosages were subsequently increased to 500 mg three times daily for three days or to two treatments of 24 mg kg^{-1} day^{-1} at seven-day intervals (Ter Kuile et al 1993). Severe cardiotoxicity was subsequently reported, with significant prolongation of QTc intervals on the electrocardiogram (Nosten et al 1993).

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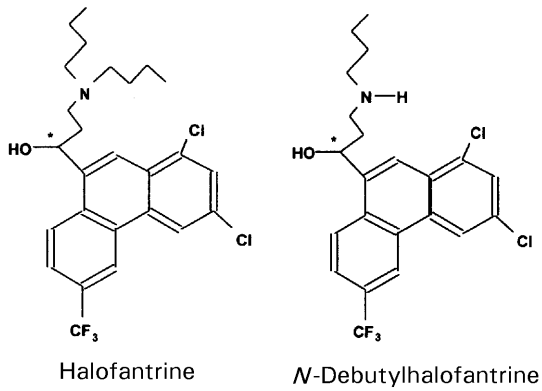

Figure 1. The chemical structures of halofantrine and its major metabolite *N*-debutylhalofantrine.

This cardiotoxicity was related to high halofantrine plasma concentrations; *N*-debutylhalofantrine (the major metabolite of halofantrine) was not implicated in cardiotoxicity (Karbwang & Na Bangchang 1994). Two hypotheses have been proposed to explain the dose-dependent cardiotoxic effects of halofantrine: halofantrine bioavailability is highly variable and peak concentrations might be increased when the drug is taken with fatty food; and metabolic inhibition because of concomitant medications.

Halliday et al (1995) have shown the major involvement of cytochrome P450 (CYP) 3A4 in hepatic metabolism of halofantrine in man and the minor in-vitro contribution of CYP 2D6 in the formation of *N*-debutylhalofantrine. The involvement of other isoenzymes was rapidly studied with chemical inhibitors. No correlation study between formation of *N*-debutylhalofantrine and other CYP levels was performed and the participation of CYP different from CYP 3A4 or CYP 2D6 was not studied in microsomes of a heterologous system expressing one CYP. The potential metabolic inhibition of halofantrine metabolism by other xenobiotics has not been evaluated.

To clarify the CYP enzymes involved in the oxidative metabolism of halofantrine in the liver in man we have studied the participation of CYP 1A2, CYP 2C8, CYP 2C9, CYP 2C19, CYP 2D6, CYP 2E1 and CYP 3A5.

The effects of ketoconazole, a selective inhibitor of CYP 3A, and any CYP 3A substrates on halofantrine metabolism were also examined to evaluate the risk of increased cardiotoxicity induced by inhibition of halofantrine metabolism during co-administration of these drugs.

Materials and Methods

Drugs and chemicals

Halofantrine and *N*-debutylhalofantrine were generous gifts from SmithKline Beecham (Paris, France). NADPH, α-naphthoflavone, coumarin, 7-hydroxycoumarin, diethyldithiocarbamate, cyclosporin, tolbutamide, *R*,*S*-mephenytoin, nifedipine, diltiazem and erythromycin were purchased from Sigma (Rueil Malmaison, France). Chlorzoxazone, 6-hydroxychlorzoxazone and hydroxytolbutamide were obtained from Bioblock Scientific (Illkirch, France). Mefloquine and sulphaphenazole were gifts from Dr Gasser of Hoffmann-La Roche (Basel, Switzerland). All other reagents were from commercial sources and were of the purest grade available.

Preparation of liver microsomes from man

Liver samples were obtained from sixteen liver-transplant donors. The protocol strictly followed French legal considerations and ethical committee approval was obtained before the study. All subjects were Caucasian. Liver fragments were immediately frozen and stored at −80°C. Microsome fractions were prepared as previously described by Dragacci et al (1987) and stored at −80°C until use. The protein content of microsomal preparations was determined by the method of Bradford (1972). The microsomal batches were denoted M6, M10, M11, M14, M14b, M17, M19, M22, M23, M24, M25b, M26N, M27b, M30, M31, M32 and M37.

Incubations with liver microsomes from man

The incubation mixture consisted of 50 μg microsomal proteins, NADPH (1 mM) in phosphate buffer (0·01 M, pH 7·4). Halofantrine was dissolved in phosphate buffer immediately before incubation. Incubations (100 μL final volume), in polypropylene test tubes, were performed in duplicate at 37°C in a shaking water-bath. After 4-min preincubation the reaction was started by addition of NADPH; it was stopped by the addition of hydrochloric acid (6 M, 50 μL). The rate of N-debutylation of halofantrine was linear over 60 min incubation for 0·1–1·5 mg mL^{-1} microsomal protein. The linearity of metabolite formation was verified at a halofantrine concentration of 50 μM, under initial velocity conditions. In the same way Halliday et al (1995), employing a substrate concentration of 10 μM, over 90 min and up to 2 mg mL^{-1} microsomal protein showed that N-debutylation of halofantrine was linear.

Analytical method

Halofantrine and its metabolite were quantified according to a previously described HPLC-UV detection method (Gimenez et al 1992). The

extraction procedure was slightly modified. After addition of 6 M hydrochloric acid (50 μL) and internal standard (40 mg L^{-1} mefloquine in methanol, 25 μL), the sample was gently mixed in methyl *t*-butyl ether (1 mL) for 5 min. After centrifugation (1000 *g*, 5 min), the organic layer was evaporated to dryness at 30°C under air. The residue was reconstituted in a mixture of mobile phase and methanol (50:50, v/v; 100 μL) and 40 μL was injected into the column. The extraction recovery of the analyte and of the internal standard was up to 64%. Separation was achieved on a Spherisorb C1 column (5 μm, 150 mm × 4·6 mm; Société Française de Chromato Colonne/Shandon, Eragny, France). The mobile phase was a 40:60 (v/v) mixture of acetonitrile and potassium phosphate buffer (50 mM, pH 5). The flow rate was 1·2 mL min^{-1} and the detector was set at 260 nm. The retention times of the internal standard, *N*-debutylhalofantrine and halofantrine were 3·9, 6·0 and 9·6 min, respectively.

Kinetics of formation of N-*debutylhalofantrine*

N-Debutylation of halofantrine was assessed in microsomal preparations obtained from nine livers from man (M10, M14b, M22, M23, M25b, M26N, M27b, M30 and M31) at halofantrine concentrations ranging from 2·5 to 200 μM (n = 9). The apparent Michaelis–Menten constants K_m and V_{max} were estimated by use of GRAFIT (Version 3.0, Erithacus Software, Staines, UK), a non-linear least-squares regression analysis software, by proportional weighting ($1/v^2$). The intrinsic clearance (CL_{int}) was calculated from the ratio of V_{max} to K_m. Data were analysed by linear transformation (Lineweaver–Burk plots), to determine whether N-debutylation of halofantrine conformed to single- or two-enzyme Michaelis–Menten kinetics. To evaluate the formation of a putative secondary metabolite, *N*-debutylhalofantrine (20 μM) was incubated under the same experimental conditions.

Inhibition or activation studies

All incubations were performed in duplicate with three different batches of microsomes (M6, M10, M19, M25b, M30 or M31). Incubations were performed with 50 μM halofantrine. α-Naphthoflavone at 0·5, 5 and 10 μM was tested as an activator. The inhibitors studied were: coumarin (10, 50 and 100 μM), sulphaphenazole (0·5 and 10 μM), quinidine (0·5 and 1 μM), diethyldithiocarbamate (2·5 and 100 μM), tolbutamide (500 and 1000 μM), *R*,*S*-mephenytoin (500 and 1000 μM) and ketoconazole (0·10, 0·20 and 0·30 μM). The inhibitor was added to the incubation mixture immediately before halofantrine. The extent of N-debutylation of halofantrine was expressed as percentage of the control result.

Determination of the relative CYP level in liver microsomes from man

For each batch of liver microsomes, proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) and electro-transferred on to nitrocellulose filters. CYP 1A2, CYP 2D6, CYP 3A4, CYP 2C8 and CYP 2C9/10 were detected by use of primary rabbit antibodies and peroxidase-conjugated secondary antibodies. They were then stained with 4-chloro-1-naphthol as previously described (Beaune et al 1985). Bands were quantified by densitometry (Hewlett-Packard Scan Jet II) and the results were expressed as arbitrary units ((mg microsomal protein)$^{-1}$). Linearity was checked by loading different amounts of microsomes. Twelve liver microsomes from man were characterized (M10, M11, M14, M17, M19, M22, M23, M24, M29, M30, M31 and M32).

Determination of CYP 2A6 and 2E1 activity

In the batch used to quantify CYP levels, coumarin 7-hydroxylation and chlorzoxazone 6-hydroxylation were used to probe CYP 2A6 (Yun et al 1991) and CYP 2E1 (Tassaneeyakul et al 1993) activity, respectively. The hydroxylation of chlorzoxazone was determined according to Tassaneeyakul et al (1993), with a substrate concentration of 250 μM. The hydroxylation of coumarin was determined as described by Yun et al (1991), with a substrate concentration of 20 μM. The analytical method was modified for quantification of 7-hydroxycoumarin in the incubation mixture. After addition of hydrochloric acid (6 M, 50 μL), the sample was gently mixed with dichloromethane (1 mL) for 5 min. After centrifugation (1000 *g* for 5 min), the organic layer (500 μL) was agitated with NaOH (0·01 M, 200 μL) for 5 min. After centrifugation (1000 *g* for 5 min) the aqueous layer (20 μL) was injected onto a Novapack C18 column (150 mm × 3·9 mm). The mobile phase was 20:80 (v/v) acetonitrile–acetate buffer (20 mM, pH 4·85) at a flow rate of 1·0 mL min^{-1} and eluates were detected by fluorimetry (λ_{ex} 358 nm, λ_{em} 458 nm).

Correlation studies

Correlations between the rate of N-debutylation of halofantrine and the activities of CYP 2E1 and

CYP 2A6 or with the relative CYP levels (CYP 1A2, CYP 2C8, CYP 2C9, CYP 2D6 and CYP 3A4) were investigated in microsomes from twelve livers from man. Correlation coefficients were calculated according to Spearman's rank test with the statistically significant level fixed at 0·05. Incubations were performed in duplicate with 200 μM halofantrine.

Halofantrine metabolism in yeast microsomes

Yeast microsomes were prepared according to Cullin & Pompon (1988). Incubation conditions were similar to those previously described for liver microsomes from man except for temperature (28°C) (Renaud et al 1990) and addition of rabbit liver NADPH-P450 reductase in equimolar ratio with CYP (Truan et al 1993). The kinetics of formation of *N*-debutylhalofantrine were assessed in microsomal preparations expressing specific CYP 2C8, CYP 2C19, CYP 2D6, CYP 3A4 and CYP 3A5 at halofantrine concentrations ranging from 2·5 to 180 μM. All experiments were performed in duplicate.

Effects of CYP 3A4 substrates on halofantrine metabolism

Inhibition experiments with CYP 3A4 substrates such as nifedipine (Guengerich et al 1986), diltiazem (Sutton et al 1997), cyclosporin (Prichard et al 1990) and erythromycin (Wrighton et al 1985) were performed using different concentrations of drug—1–50 μM except for cyclosporin (1–20 μM).

The incubation mixture consisted of 50 mg microsomal proteins (batches M22 and M30), NADPH (1 mM) and phosphate buffer (0·01 M, pH 7·4). Halofantrine was dissolved in phosphate buffer immediately before incubation. Incubations (100 μL final volume), in polypropylene test tubes were performed in duplicate at 37°C for 60 min in a shaking water-bath. The inhibitors were pre-incubated for 15 min with the microsomes before addition of halofantrine. IC50 values (concentrations resulting in 50% inhibition of the formation of *N*-debutylhalofantrine, compared with the control) were estimated by use of GRAFIT, non-linear least-squares regression analysis software, and are presented as the means of results from two experiments.

Inhibition of halofantrine metabolism by ketoconazole

Ketoconazole (0·025, 0·050 and 0·075 μM) was incubated with 20, 40, 60 or 90 μM halofantrine solutions in polypropylene test tubes at 37°C for 60 min in a shaking water-bath. The inhibitor was added at the same time as the substrate. The study was assessed, in duplicate, with microsomal preparations obtained from three livers (M29, M31 and M37).

The inhibition data were fitted using conventional relationships for competitive and non-competitive inhibition (Segel 1976). The choice of the type of inhibition was determined on the basis of several criteria which included the random distribution of the residuals, the size of the sum of squares of the residuals and the standard error of the parameter estimate.

The predicted in-vivo inhibition of the catalytic activities of the examined CYP were calculated for non-competitive inhibition from (Segel 1975):

$$\text{Inhibition percentage, i, } = \{[I]/([I] + K_i)\} \times 100 \quad (1)$$

where [I] is the concentration of inhibitor and K_i is the inhibition constant. Assuming this in-vitro relationship also applies in-vivo, the theoretical percentage inhibition of halofantrine clearance can be predicted by use of plasma ketoconazole concentrations found in man after oral administration.

Results

Kinetics of formation of N*-debutylhalofantrine*

The formation of *N*-debutylhalofantrine was CYP-mediated. *N*-Debutylhalofantrine was not produced in the absence of NADPH nor in the presence of liver microsomes heated for 60 min at 56°C. Under our incubation and analysis conditions no other metabolite could be detected in the incubation media.

Monophasic Michaelis–Menten kinetics were observed for the formation of *N*-debutylhalofantrine. The mean apparent V_{max} value was 215 ± 172 pmol min^{-1} (mg microsomal protein)$^{-1}$ and the mean K_m value was 48 ± 26 μM (Table 1). Very large (10-fold) interliver variability was observed for both V_{max} and K_m. The in-vitro CL_{int} was estimated at $4{\cdot}7 \pm 3{\cdot}3$ μL min^{-1} (mg microsomal protein)$^{-1}$.

Chemical inhibition or activation

The effects of selective substrates or inhibitors on the N-debutylation of halofantrine are summarized in Table 2. Ketoconazole, a CYP 3A inhibitor (Bourrié et al 1996), was inhibitory at 0·10 μM. In contrast, usual inhibitory concentrations of other selective CYP substrates or inhibitors did not inhibit formation of *N*-debutylhalofantrine.

Table 1. Michaelis–Menten kinetic parameters for N-debutylation of halofantrine in microsomes from nine livers from man.

Microsome	V_{max} (pmol min^{-1} mg^{-1})	K_m (μmol L^{-1})	CL_{int} (μL min^{-1} mg^{-1})
M10	135	42·5	3·2
M14b	145	45	3·2
M22	653	60	10·8
M23	175	65	2·7
M25b	103	20	5·0
M26N	210	95	2·2
M27b	83	67	1·2
M30	247	19	4·8
M31	182	19	9·7
Mean ± s.d.	215 ± 172	48 ± 26	4·7 ± 3·3

K_m and V_{max} are the apparent Michaelis–Menten constants and CL_{int} is the intrinsic clearance.

Table 2. Inhibition or activation of halofantrine (50 μM) metabolism by different CYP substrates or inhibitors, in liver microsomes obtained from three livers from man.

Inhibitor or activator	Concn (μM)	CYP	*N*-Debutylhalofantrine concn (mean ± s.d., %)
Control			100
α-Naphthoflavone	0·5		124 ± 29
	5·0	1A2	211 ± 98
	10·0		219 ± 118
Coumarin	10·0		108 ± 10
	50·0	2A6	157 ± 15
	100·0		178 ± 15
	0·5		94 ± 2
Sulphaphenazole	10	2C9/10	96 ± 0
Quinidine	0·5	2D6	106 ± 6
	1·0		94 ± 20
	2·5		104 ± 10
Diethyldithiocarbamate	100	2E1	92 ± 4
Ketoconazole	0·10		70 ± 16
	0·20	3A4	23 ± 3
	0·30		11 ± 8
Tolbutamide	500	2C8/9/10	91 ± 3
	1000		83 ± 0
R,*S*-Mephenytoin	500	2C19	65 ± 5
	1000		49 ± 6

α-Naphthoflavone, an activator of CYP 3A activity when present at concentrations greater than 1 μM (Kerlan et al 1992), increased the N-debutylation of halofantrine (10 μM of α-naphthoflavone caused the strongest activation).

Correlation experiments

The correlation between formation of *N*-debutylhalofantrine (v_i at 200 μM halofantrine) and CYP activity or relative selective CYP levels are shown in Table 3. Formation of *N*-debutylhalofantrine did not correlate either with CYP 1A2, CYP 2D6, CYP 2C9 levels or with CYP 2E1, CYP 2A6 activity. the strongest correlation was observed between the rates of N-debutylation of halofantrine and CYP 3A4 levels ($P = 0{\cdot}002$, n = 12) (Table 3). A weaker but still significant correlation was observed between N-debutylation of halofantrine and CYP 2C8 level ($P = 0{\cdot}025$, n = 12). Correlation was also found between CYP 3A and CYP 2C8 levels ($P = 0{\cdot}048$, n = 12).

Table 3. Correlation between N-debutylation of halofantrine and specific CYP isoform levels or CYP 2A6- and CYP 2E1-mediated reactions.

Correlation	Spearman coefficient r (n = 12)	*P*
N-Debutylation of halofantrine with:		
CYP 1A2 relative levels	0·531	0·078
CYP 2C8 relative levels	0·678	0·025
CYP 2C9 relative levels	0·517	0·086
CYP 2D6 relative levels	0·392	0·194
CYP 3A4 relative levels	0·925	0·002
N-Debutylation of halofantrine with:		
Coumarin oxidation (CYP 2A6)	0·413	0·172
Chlorzoxazone oxidation (CYP 2E1)	0·224	0·458
CYP 3A4 relative levels with:		
CYP 1A2 relative levels	0·404	0·180
CYP 2C8 relative levels	0·596	0·048
CYP 2C9 relative levels	0·397	0·188
CYP 2D6 relative levels	0·334	0·268

Halofantrine metabolism in yeast microsomes expressing specific CYP 2C8, CYP 2C19, CYP 2D6, CYP 3A4 or CYP 3A5 from man

The kinetic parameters of N-debutylation of halofantrine in yeast microsomes expressing CYP 2C8, CYP 3A4 or CYP 3A5 from man are shown in Table 4. Because transformation of substrate in yeast microsomes expressing CYP 2C19 or CYP 2D6 from man was weak, no kinetic parameters were calculated.

Effects of CYP 3A substrates on halofantrine metabolism

All CYP 3A substrates inhibited formation of *N*-debutylhalofantrine. IC50 values were 3·2, 13, 1·3

Table 4. Michaelis–Menten kinetic parameters for N-debutylation of halofantrine in microsomes of yeast expressing CYP 2C8, CYP 3A4 or CYP 3A5.

CYP expressing in yeast	V_{max} (pmol min^{-1} $(nmol\ P50)^{-1}$)	K_m (μmol L^{-1})	CL_{int} (μL min^{-1} $(nmol\ P50)^{-1}$)
CYP 2C8	39	156	0·25
CYP 3A4	299	96	3·10
CYP 3A5	69	130	0·53

K_m and V_{max} are the apparent Michaelis–Menten constants and CL_{int} is the intrinsic clearance.

Table 5. The nature of inhibition of halofantrine metabolism by ketoconazole in three different liver microsomes from man.

	Liver microsomes batch		
	M31	M37	M29
Fitting on non-competitive inhibition: reduce χ^2	0·0110	0·0150	0·0815
Fitting on competitive inhibition: reduce χ^2	0·0412	0·0452	0·1114
Ho thrue probability	0·002	0·0018	0·4401
Type of inhibition	Non-competitive	Non-competitive	Non-competitive

and 11·0 μM for cyclosporin, nifedipine, diltiazem and erythromycin, respectively.

Inhibition of halofantrine metabolism by ketoconazole

This inhibition was non-competitive (Table 5); the K_i value was 0·07, 0·03 and 0·05 μM, respectively, in the presence of the three microsomal preparations M31, M37 and M29.

After oral administration of ketoconazole, plasma concentrations ranged from 3·8–11·3 μM (Graybill et al 1980). Using these concentrations, the percentage of inhibition of formation of *N*-debutylhalofantrine was 99%.

Discussion

In liver microsomes from man halofantrine was metabolized to a single metabolite, *N*-debutylhalofantrine. *N*-Debutylhalofantrine was formed neither in the absence of NADPH nor in the presence of heated microsomes. Under our experimental conditions, *N*-debutylhalofantrine was the only metabolite identified and it was not transformed into a secondary metabolite.

The Lineweaver–Burk curve for formation of *N*-debutylhalofantrine was monophasic, indicating the involvement of a single isoenzyme or of several isoenzymes with similar apparent K_m values. The large variability in V_{max} and v_i is consistent with the large intersubject variability in liver CYP levels (Guengerich & Turvy 1991). This variability can contribute to the large intervariability in halofantrine pharmacokinetics which can be further exacerbated by low and erratic absorption.

In liver microsomes from man we showed the major involvement of CYP 3A4 and CYP 3A5 in halofantrine metabolism.

α-Naphthoflavone, an inhibitor of CYP 1A2 and an activator of CYP 3A, increased formation of *N*-debutylhalofantrine; ketoconazole (a CYP 3A inhibitor) at 0·30 μM inhibited more than 90% of halofantrine biotransformation. These findings and the lack of effect of quinidine, coumarin, sulphaphenazole, diethylthiocarbamate or mephenytoin suggest a major role for CYP 3As in the formation of *N*-debutylhalofantrine.

The nature of the inhibition of CYP 3A4 activity by ketoconazole is not well defined in the literature. Studies have found inhibition to be competitive (Von Moltke et al 1995), non-competitive (Von Moltke et al 1994) or mixed (Bourrié et al 1996). The different methods used to determine the nature of the inhibition (model, weighting procedure) might explain this variability. Further specific studies are necessary to demonstrate the exact nature of ketoconazole inhibition. Correlation studies between the rate of formation of *N*-debutylhalofantrine and CYP levels or activity further corroborated the involvement of CYP 3As in halofantrine metabolism. Only CYP 3A4 levels correlated highly with the rate of formation of *N*-debutylhalofantrine. The correlation observed between CYP 2C8 and CYP 3A4 levels might partly explain the significant correlation between CYP 2C8 level and the rate of formation of *N*-debutylhalofantrine. Incubation with microsomes of yeast expressing only this CYP 2C8 are necessary to prove such involvement. The lack of inhibition by high concentrations of tolbutamide further confirmed that CYP 2C8 was not involved in metabolite formation. No cross reaction between CYP 2C8 and CYP 3A4 antibodies has been reported (Transon et al 1996).

Halliday et al (1995) have shown that formation of *N*-debutylhalofantrine correlated with CYP 3A4 protein levels or with the rate of felodipine metabolism, and occurred in heterologous CYP 3A4 or CYP 206 expression systems (only these two expression systems were tested). However, comparison with our results was very difficult because no kinetic parameters (K_m, V_{max} or CL_{int}) were calculated. Halliday et al (1995) have reported that formation of *N*-debutylhalofantrine did not correlate with liver CYP 2D6 genotype or phenotype. Thus, in-vivo, CYP 2D6 is very unlikely to be responsible for much formation of *N*-debutylhalofantrine. In contrast with Halliday et al (1995), we

studied the participation of CYP 3A5 and of other major CYPs (CYP 1A2, CYP 2C8, CYP 2D6, CYP 2C19).

Formation of *N*-debutylhalofantrine in yeast microsomes was observed only in the presence of a system expressing CYP 2C8, CYP 3A4 or CYP 3A5. However, the intrinsic clearance of halofantrine in yeast microsomes expressing CYP 3A4 was six- and twelvefold than in the presence of yeast microsomes expressing CYP 3A5 or CYP 2C8, respectively. In addition, in liver from man CYP 3A4 was present in significantly higher concentrations than CYP 2C8 or CYP 3A5. Guengerich et al (1986) and De Waziers et al (1990) found that CYP 3A4 was present at a concentration of $250\,\text{pmol}\,\text{mg}^{-1}$ liver protein compared with only $20\,\text{pmol}\,\text{mg}^{-1}$ liver protein for CYP 2C8. Moreover, CYP 3A5 was present in only 20–30% of the liver from man and represented 15–30% of hepatic CYP 3A content (Wrighton et al 1990). Thus, in-vivo, the involvement of CYP 2C8 is expected to be minor relative to that of CYP 3A4. In man expressing CYP 3A5 in the liver, the bioavailability of halofantrine will be reduced and intersubject variability of halofantrine pharmacokinetics will be increased.

To investigate the consequences of CYP 3A involvement in halofantrine metabolism, the interaction between ketoconazole or CYP 3A substrates and formation of *N*-debutylhalofantrine were studied. All CYP 3A substrates inhibited halofantrine metabolism with IC50 values closely related to the higher range of their peak plasma concentrations. Hence, plasma concentrations of cyclosporin, diltiazem, erythromycin and nifedipine ranged from 0·2–0·7 μM (Erkko et al 1997), 0·1–0·4 μM (Koiwaya et al 1981), 1–4 μM (Washington & Wilson 1985) and 0·06–0·30 μM (Jakobsen et al 1979), respectively, whereas IC50 values were 3·2 μM, 1·3, 11 and 13 μM for cyclosporin, diltiazem, erythromycin and nifedipine, respectively. Irrespective of the uptake of the drug by the liver, therapeutic doses of diltiazem or erythromycin might be sufficient to inhibit the metabolism of concomitantly administered halofantrine. In the same way, inhibition by ketoconazole might be complete. Thus, halofantrine plasma concentrations might be increased and the risk of cardiotoxicity would be maximized. Indeed, arrythmias have been observed with terfenadine, a drug biotransformed via CYP 3As, when it was administered with ketoconazole (Honig et al 1993) or erythromycin (Honig et al 1992).

In summary, our experiments confirm that in-vivo, CYP 3A4 is the major isoform responsible for halofantrine metabolism. They further show that the contribution of CYP 3A5 to formation of *N*-debutylhalofantrine was less significant than CYP 3A4 involvement. The mediation of other CYPs, e.g. CYP 2D6 or CYP 2C8, in halofantrine metabolism seemed to be minor. We also showed that co-administration of halofantrine with CYP 3A4 substrates or potent inhibitors might dramatically increase the cardiotoxic risk by inhibition of metabolism.

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References

Beaune, P. H., Flinois, J. P., Kieffel, L., Kremers, P., Leroux, J. P. (1985) Purification of a new cytochrome P450 from human liver microsomes. Biochem. Biophys. Acta. 840: 364–370

Boudreau, E. F., Pang, L. W., Webster, H. K., Dixon, K. E., Pavanand, K. (1988) Malaria: treatment efficacy of halofantrine (Wr 171,669) in initial field trials in Thailand. Bull. WHO 66: 227–235

Bourrié, M., Meunier, V., Berger, Y., Fabre, G. (1996) Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalysed by human liver microsomes. J. Pharmacol. Exp. Ther. 277: 321–332

Bradford, M. M. (1972) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Cosgriff, T. M., Boudreau, E. F., Pamplin, C. L., Doberstyn, E. B., Desjardins, R. E. (1982) Evaluation of antimalarial activity of the phenanthrene methanol halofantrine. Am. J. Trop. Med. Hyg. 31: 1075–1079

Cullin, C., Pompon, D. (1988) Synthesis of functional mouse cytochrome P450 P1 and chemeric P40 P3-1 in the yeast *Saccharomyces cervisiae*. Gene 65: 203–217

De Waziers, I., Cugnenc, P., Yang, C. S., Leroux, J. P., Beaune, P. (1990) Cytochrome P450 isoenzymes, epoxide hydroxylase and glutathione transferase in rat and human hepatic and extrahepatic tissues. J. Pharmacol. Exp. Ther. 253: 387–394

Dragacci, S., Hamar-Hansen, C., Fournel-Gigleux, S., Lafaurie, C., Magdalou, J., Siest, G. (1987) Comparative study of clofibric acid and bilirubin glucuronidation in human liver microsomes. Biochem. Pharmacol. 36: 3923–3927

Erkko, P., Granlund, H., Nuutinen, M., Reitamo, S. (1997) Comparison of cyclosporine A pharmacokinetics of a new microemulsion formulation and standard oral preparation in patients with psoriasis. Br. J. Dermatol. 136: 82–88

Gimenez, F., Aubry, A.-F., Farinotti, R., Kirkland, K., Wainer, I. W. (1992) The determination of the enantiomers of halofantrine and monodesbutylhalofantrine in plasma and whole blood using sequential achiral/chiral high performance liquid chromatography. J. Pharm. Biomed. Anal. 10: 245–250

Graybill, J. R., Lundberg, D., Donovan, W., Levine, H. B., Rodriguez, M. D., Drutz, D. J. (1980) Treatment of cocci-

dioidomycosis with ketoconazole: clinical and laboratory studies of 18 patients. Rev. Infect. Dis. 2: 661–673

Guengerich, F. P., Turvy, C. G. (1991) Comparison of levels of several human microsomal cytochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. J. Pharmacol. Exp. Ther. 256: 1189–1194

Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T., Waxman, D. J. (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation drug metabolism. J. Biol. Chem. 261: 5051–5060

Halliday, R. C., Jones, B. C., Smith, D. A., Kitterincham, N. R., Park, B. K. (1995) An investigation of the interaction between halofantrine, CYP 2D6, CYP 3A4, studies with human liver microsomes and heterologous enzyme expression systems. Br. J. Clin. Pharmacol. 40: 369–378

Honig, P. K., Woosley, R. L., Zamani, K., Conner, D. P., Cantilena, L. R. (1992) Changes in the pharmacokinetics and electrocardiographic pharmacodynamics of terfenadine with concomitant administration of erythromycin. Clin. Pharmacol. Ther. 52: 231–238

Honig, P. K., Wortham, D. C., Zamani, K., Conner, D. P., Mullin, J. C., Cantilena, L. R. (1993) Terfenadine-ketoconazole interaction. Pharmacokinetic and electrocardiographic consequences. J. Am. Med. Assoc. 269: 1513–1518

Jakobsen, P., Lederballe, P. O., Mikkelsen, E. (1979) Gas chromatographic determination of nifedipine and one of its metabolites using electron-capture detection. J. Chromatogr. 162: 81–87

Karbwang, J., Na Bangchang, K. (1994) Clinical pharmacokinetics of halofantrine. Clin. Pharmacokinet. 27: 104–119

Kerlan, V., Dreano, Y., Bercovici, J. P., Beanne, P. H., Floch, H. H., Berthon, F. (1992) Nature of cytochromes P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. Biochem. Pharmacol. 44: 1745–1756

Koiwaya, Y., Matsuguchi, T., Nakamura, M., Etoh, A. (1981) Plasma concentrations of diltiazem after oral administration in coronary artery disease patients: a comparison with those in normal volunteers. Clin. Ther. 4: 127–132

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685

Nosten, F., Ter Kuile, F., Chongsuphajaisiddhi, T., Webster, H. K., Edstein, M., Phapun, L., Thew, K. L., White, N. J. (1991) Mefloquine-resistant falciparum malaria on the Thai-Burmese border. Lancet 337: 1140–1143

Nosten, F., Ter Kuile, F., Luxemburger, C., Woodrow, C., Kyle, D. E., Chongsuphajaisiddhi, T., White, N. J. (1993) Cardiac effect of antimalarial treatment with halofantrine. Lancet 341: 1054–1056

Prichard, L., Fabre, I., Fabre, G., Domergue, J., Saint Aubert, B., Mourad, G., Maurel, P. (1990) Cyclosporin A drug interactions, screening for inducers and inhibitors of cytochrome P450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and liver microsomes. Drug Metab. Dispos. 18: 595–606

Renaud, J. P., Cullin, C., Pompon, D., Beaune, P. H., Mansuy, D. (1990) Expression of human liver P450 3A4 in yeast. A functional model for the hepatic enzyme. Eur. J. Biochem. 194: 889–896

Segel, I. H. (1975) In: Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems, John Wiley and Sons, New York, pp 100–206

Sutton, D., Butler, A. M., Nadin, L., Murray, M. (1997) Role of CYP 3A4 in human hepatic diltiazem N-demethylation: inhibition of CYP 3A4 activity by oxidized diltiazem metabolites. J. Pharmacol. Exp. Ther. 282: 294–300

Tassaneeyakul, W., Veronese, M. E., Birkett, D. J., Gonzalez, F. J., Miners, J. O. (1993) Validation of 4-nitrophenol as an in-vitro substrate probe for human liver CYP 2E1 using cDNA expression and microsomal kinetic techniques. Biochem. Pharmacol. 46: 1975–1981

Ter Kuile, F. O., Dolan, G., Nosten, F., Edstein, M. D., Luxemburger, C., Phaipun, L., Chongsuphajaisiddhi, T., Webster, K., White, N. J. (1993) Halofantrine versus mefloquine in treatment of multidrug-resistant falciparum malaria. Lancet 341: 1044–1049

Transon, C., Lecoeur, S., Leemann, T., Beaune, P., Dayer, P. (1996) Interindividual variability in catalytic and immunoreactivity of three major human liver cytochrome P450 isozymes. Eur. J. Clin. Pharmacol. 51: 79–85

Truan, G., Cullin, C., Reisdorf, P., Urban, P., Pompon, D. (1993) Enhanced in vivo monoxygenase activities of mammalian P-450s in engineered yeast cells producing high level of NADPH P450 reductase and cytb5. Gene 125: 49–55

Von Moltke, L. L., Greenblatt, D. J., Cotreau-Bibbo, M. M., Harmatz, J. S., Shader, R. I. (1994) Inhibitors of alprazolam metabolism in vitro: effect of serotonin-reuptake-inhibitor antidepressants, ketoconazole and quinidine. Br. J. Clin. Pharmacol. 38: 23–31

Von Moltke, L. L., Greenblatt, D. J., Schmider, J., Harmatz, J. S., Shader, R. I. (1995) Metabolism of drugs by cytochrome P450 3A isoforms. Implications for drug interactions in psychopharmacology. Clin. Pharmacokinet. 29: 33–44

Washington, J. A., Wilson, W. R. (1985) A microbial and clinical perspective after 30 years of clinical use. Mayo Clin. Proc. 60: 189–203

Wrighton, S. A., Maurel, P., Schuetz, E. G., Watkins, P. B., Young, B., Guzelian, P. S. (1985) Identification of the cytochrome P450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. Biochemistry 25: 2171–2178

Wrighton, S. A., Brian, W. R., Sari, M.-A., Iwasaki, M., Guengerich, F. P., Raucy, J. L., Molowa, D. T., Vanderbranden, F. (1990) Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). Mol. Pharmacol. 38: 207–213

Yun, H. Y., Shimada, T., Guengerich, F. P. (1991) Purification and characterization of human liver microsomal cytochrome P-450 2A6. Mol. Pharmacol. 40: 679–685