J. Pharm. Pharmacol. 1996, 48: 1049–1036
Received November 20, 1995
Accepted March 8, 1996

In-vitro Metabolism of YM17E, an Inhibitor of Acyl Coenzyme A:Cholesterol Acyltransferase, by Liver Microsomes in Man

TAISUKE UCHIDA, TAKASHI WATANABE, *EWOUD J. VAN HOOGDALEM AND SABURO HIGUCHI

*Drug Metabolism Department, Clinical Pharmacology Research Laboratories, Yamanouchi Pharmaceutical Co. Ltd, 1-8, Azusawa 1-chome, Itabashi-ku, Tokyo 174, Japan and *Biopharmaceutical Department, Yamanouchi Europe B.V., P.O. Box 108, 2350 AC Leiderdorp, The Netherlands*

Abstract

Because YM17E (1,3-bis[[1-cycloheptyl-3-(*p*-dimethylaminophenyl)ureido]methyl]benzene dihydrochloride) inhibits acyl coenzyme A:cholesterol acyltransferase (ACAT) it has potential application in the treatment of hypercholesterolaemia. In man and animals YM17E is extensively metabolized, via *N*-demethylation, to five active metabolites (M1, M2-a, M2-b, M3 and M4). The main objectives of this study were to examine inhibition of YM17E metabolism by the products and identify the cytochrome P450 isoforms in liver microsomes which catalyse in-vitro YM17E metabolism in man.

In microsomes in man, *N*-demethylation of YM17E to M1 occurred enzymatically; for up to 45 s the rate was linearly proportional to the microsomal protein concentration. This reaction was inhibited by metabolites M2-a, M2-b, M3 and M4. Further, *N*-demethylation of [^{14}C]-YM17E was also inhibited by its product, M1. These results showed that primary metabolism of YM17E was inhibited by its products, and supported the finding that the non-linear increase in plasma concentration of the parent drug and metabolites observed in an in-vivo study was due to inhibition by these products. Metabolic activity in microsomes from ten individual human livers demonstrated that YM17E *N*-demethylase activity correlated closely with testosterone 6β-hydroxylase activity. When cytochrome P450 isozyme-specific substrates and chemical inhibitors were used to inhibit YM17E *N*-demethylase activity, CYP3A-specific substrate and inhibitors such as nifedipine, ketoconazole and triacetyloleandomycin strongly inhibited this activity, whereas CYP1A-specific substrate or inhibitor, ethoxyresorufin and α-naphthoflavone, inhibited weakly. Other CYP inhibitors, in contrast, had few or no effects. An inhibition study using anti-rat CYP1A1, CYP2B1, CYP2C11, CYP2E1 and CYP3A2 antibodies demonstrated that only anti-rat CYP3A2 antibody inhibited YM17E metabolism, to 40% of control level, with no other antibodies showing an inhibitory effect. Of seven cDNA-expressed P450 isoforms in man (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1 and CYP3A4), CYP3A4, CYP2D6 and CYP1A2 isozyme exhibited substantial catalytic activity of *N*-demethylation of YM17E.

These results indicate the predominant role of CYP3A4 in liver metabolism of YM17E in man.

Acyl CoA:cholesterol acyltransferase (ACAT) catalyses intracellular cholesterol esterification with fatty acid to make cholesterol ester. As esterification of cholesterol is thought to be the rate-limiting step in the absorption of food- and bile-derived cholesterol from the gastrointestinal tract inhibition of ACAT is expected to be beneficial in therapeutic treatment of hypercholesterolaemia (Heider et al 1983; Largis et al 1989). A number of ACAT inhibitors has been reported to have inhibitory activity on ACAT, to diminish the absorption of cholesterol from the gastrointestinal tract and to reduce serum cholesterol levels in experimental animals (Matsuda 1994). In most cases the sole mechanism of hypocholesterolaemic effect is thought to be the inhibition of cholesterol absorption. Recently, however, in addition to inhibition of intestinal ACAT, a second mechanism has been proposed for the reduction of serum cholesterol levels, namely inhibition of hepatic ACAT. It was reported that the hypolipidaemic activity of the ACAT inhibitor CI-976 in rats was a result not only of inhibition of intestinal absorption of cholesterol but also of hepatic ACAT inhibition (Krause et al 1993). In addition, ACAT inhibitors at high plasma concentrations are also expected to suppress the progress of arteriosclerosis by acting directly on the artery wall (Bell 1986). Although many ACAT inhibitors are under clinical evaluation, none has shown the same hypocholesterolaemic efficacy in man as they have in animals (Harris et al 1990; Hainer et al 1994; Peck et al 1995). The reason for this is not clear but it is possible that the pharmacokinetic profile, for example low absorption and insufficient concentrations in the liver, is unsuitable for reducing serum cholesterol levels in man. Pharmacokinetic and metabolism study is, therefore, thought to be important for evaluation of the efficacy of ACAT inhibitors in man.

YM17E (1,3-bis[[1-cycloheptyl-3-(*p*-dimethylaminophenyl)-ureido]methyl]benzene dihydrochloride; Fig. 1) is a serum-cholesterol regulator that inhibits ACAT activity in rabbit liver microsomes with a 50% inhibition concentration (IC50) of 4.4×10^{-8} M. This compound also shows antihypercholesterolaemic activity in cholesterol-fed animal models (Iizumi et al 1991; Ito et al 1991). YM17E also induced a reduction in serum cholesterol levels in a clinical study (Uchida et al 1996).

YM17E is metabolized by *N*-demethylation, the major metabolic pathway in animals and man, to form five metabo-

Correspondence: Taisuke Uchida, Drug Metabolism Department, Clinical Pharmacology Research Laboratories, Yamanouchi Pharmaceutical Co. Ltd, 1-8, Azusawa 1-chome, Itabashi-ku, Tokyo 174, Japan.

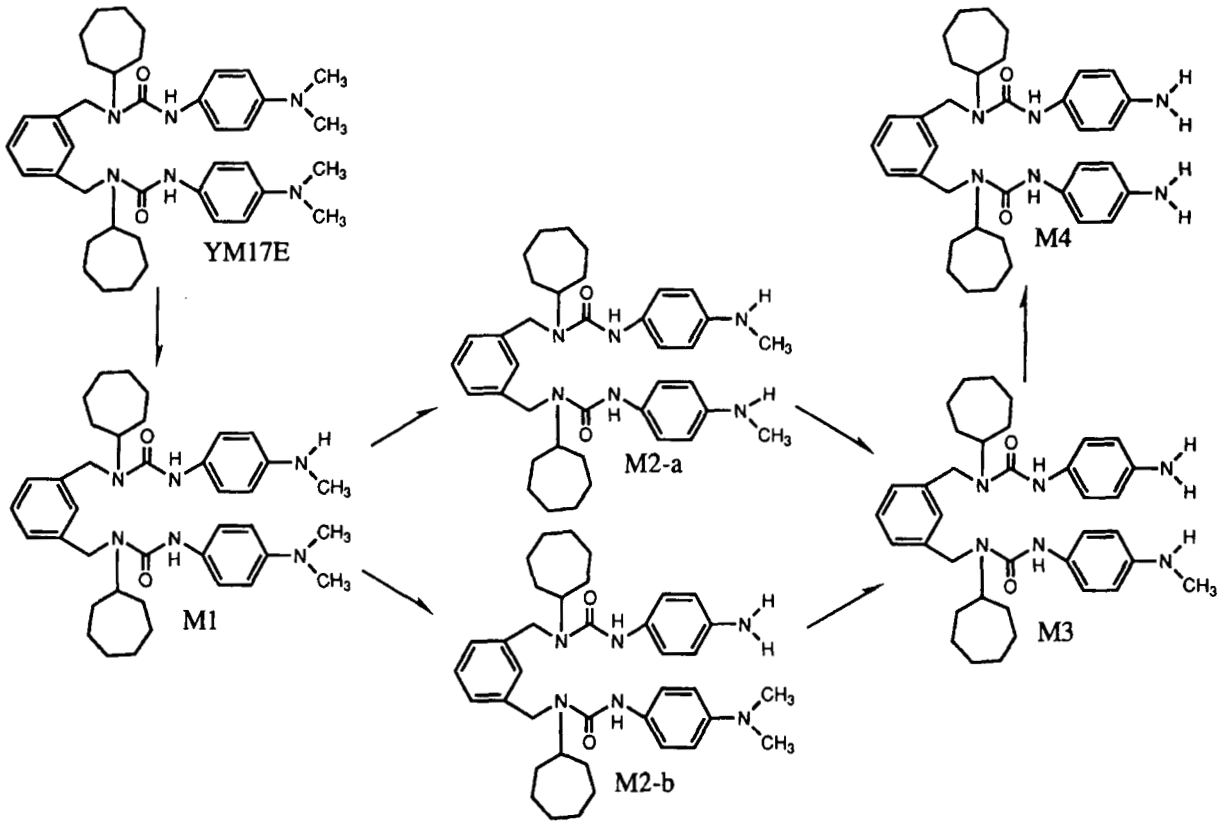

FIG. 1. Major metabolic pathways of YM17E.

lites, namely M1, M2-a, M2-b, M3 and M4, both in-vitro and in-vivo (Fig. 1; Uchida et al 1993a, 1993b, 1994). All five metabolites also have an inhibitory effect on ACAT (unpublished results), suggesting that not only the parent drug but also these five metabolites play a role in the hypocholesterolaemic effect in-vivo. In a study in man, plasma concentrations both of parent drug and of active metabolites increased more than in proportion to the increase in dose. If only parent drug increased non-linearly more than in proportion, saturation of the metabolism of parent drug would be a possibility. In this case, not only parent drug but also M1 increased more than in proportion to the increase in the dose. An explanation of this phenomenon could be either that parent drug and metabolites inhibited the metabolism of each other (Bast et al 1982; Suzuki et al 1984) or that rate of absorption was changed by increasing the dose. In this study we have investigated the first possibility by in-vitro use of the microsomal fraction of liver in man.

In general, the bioavailability of ACAT inhibitors is low because they are poorly absorbable and their plasma concentration is relatively low. YM17E is an absorbable inhibitor and reaches high plasma concentrations together with active metabolites (Uchida et al 1994). As high plasma concentrations of YM17E and active metabolites might explain why this inhibitor reduced serum cholesterol levels in a clinical study whereas other ACAT inhibitors did not, examination of its metabolism is considered to be important. To clarify whether YM17E metabolism is inhibited by its metabolites in the liver, and to identify those enzymes catalysing the *N*-demethylation of YM17E, we investigated the in-vitro metabolism of YM17E in human liver microsomal fraction.

Materials and Methods

Chemicals and reagents

YM17E and metabolites were obtained from Tsukuba Research Center, Yamanouchi Pharmaceutical (Tsukuba, Japan). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were purchased from Boehringer Mannheim (Mannheim, Germany). α-Naphthoflavone and quinidine were from Nakalai Tesque (Kyoto, Japan), triacetyloleandomycin, tolbutamide, metyrapone and nifedipine from Sigma (St Louis, MO, USA), 7-methoxycoumarin and coumarin from Extrasyntèse (Genay, France), and phenytoin, SKF-525A and debrisoquin from Research Biochemicals International (Natick, MA, USA). 7-Ethoxyresorufin was from Molecular Probes, (Eugene, OR, USA), hexobarbital from Tokyo Kasei Kougyou (Tokyo, Japan), ketoconazole from Biomol Research Laboratories (PA, USA), and propafenone from Yamanouchi Pharmaceutical. Anti-Rat cytochrome P450 antibodies were purchased from Daiichi Pure Chemical (Tokyo, Japan). Other reagents were obtained commercially and were of analytical grade.

Human microsomes

A hepato screen test kit with ten individual human liver microsomes (HBI-2, HBI-3, HBI-5, HBI-6, HBI-7, HBI-9, HBI-10, HBI-11, HBI-12 and HBI-13) and hepatosomes with pooled microsomes from two (HBI-6 and HBI-7) or seven (HBI-14, HBI-15, HBI-16, HBI-17, HBI-19, HBI-20 and HBI-21) different human livers were obtained from Human Biologics (Phoenix, AZ, USA). These microsomes were character-

ized by the metabolic activities of cytochrome P450 (CYP) isoforms, namely 7-ethoxyresorufin *O*-deethylation (CYP1A), caffeine 3-demethylation (CYP1A), coumarin 7-hydroxylation (CYP2A), tolbutamide methyl-hydroxylation (CYP2C), *S*-mephenytoin 4′-hydroxylation (CYP2C19), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), testosterone 6β-hydroxylation (CYP3A), lauric acid 11- (CYP2E) and 12-hydroxylation (CYP4A) and benzfetamine *N*-hydroxylation. CYP (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, CYP3A4) cDNA-expressing microsomes of lymphoblast cells were purchased from Gentest (Woburn, MA, USA).

Assay for M1

Reaction mixtures contained 0·5 mg protein mL^{-1} human liver microsomes, 100 mM sodium potassium phosphate buffer (pH 7·4), 6 mM $MgCl_2$, 1 U mL^{-1} glucose 6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, 1 mM NADP, 0·1 mM EDTA and an appropriate amount of YM17E or inhibitor, or both. The total volume of the reaction mixture was 0·5 mL. The reaction was started by adding the NADPH-generating system and maintained at 37°C for 45 s. Incubation was stopped by adding ice-cold methanol and then immediately placing the tube on ice. The tube was centrifuged at 1200 *g* for 15 min and the supernatant obtained was passed through a 0·22-μm filter (MilexGV; Nippon Millipore, Tokyo, Japan). A 100-μL portion was injected into the HPLC system. HPLC conditions were: column, Cosmosil 5PE 4·6 mm (i.d.) × 250 mm (Nakalai Tesque); temperature, 50°C; mobile phase, 20 mM Na_2HPO_4/10 mM citric acid buffer (pH 5) acetonitrile (4 : 6, v/v); flow rate, 1 mL min^{-1}. Amperometric detection was performed with an electrochemical detector Coulochem 5000A (ESA; Bedford, MA, USA) comprising a type 5022 guard cell and a type 5011 analytical cell. The guard cell was placed in line in front of the autosample processor 712 WISP (Waters, Milford, MA, USA), to remove interference in the mobile phase by electrolysis, and set at 0·3 V. The dual D1 and D2 detectors of the analytical cell were set at 0·03 and 0·23 V, respectively. The signal from D2 was monitored and the peak area of M1 was calculated by use of a CR4AX integrator (Shimazu, Tokyo, Japan. Calibration curves were obtained by plotting the peak area of M1 against the amount of M1 in the reaction tube and regression was performed with the weight of 1/y (y = peak area). The standard curves were linear over the range of 0·5 to 500 ng with a correlation coefficient of more than 0·99.

The microsomal protein concentration in inhibition studies using anti-rat P450 antiserum was 100 μg mL^{-1} in 0·5 mL of reaction mixture. Anti-serum or pre-immunized serum (15 μL) was incubated with human liver microsomes (25 μL) at room temperature for 2 h before reaction. The incubation time of experiments using cDNA-expressed microsomes was 10 min, which was set according to the linearity of the production rate with incubation time in a preliminary study. Other conditions were the same as those mentioned above. Inhibitory constant (IC50) values were calculated by means of the NONLIN84 program (Statistical Consultants, KY, USA) using unweighted data from the equation:

$$v/V_{max} = 1/(1 + ([I]/IC50)^s)$$

where V_{max}, v, [I] and s are uninhibited velocity, observed velocity, inhibitor (metabolites) concentration added and slope factor, respectively.

The inhibitory effect of M1 on *N*-demethylation of YM17E was confirmed by using [^{14}C]YM17E as substrate. The microsomal protein concentration in the reaction mixture was set at 1 mg mL^{-1} and the mixture was incubated for 10 min. [^{14}C]YM17E was added to the reaction mixture at 15 μM, and M1 was added at 5, 15 and 60 μM. Other conditions were as described above. Detection was performed by liquid scintillation counting after fractionation of the [^{14}C]M1 peak by HPLC.

Results

Reaction conditions and HPLC

Typical HPLC chromatograms of the reaction mixture used for quantitation of M1 are shown in Fig. 2. Although nothing in the human liver microsomal fraction interfered with the M1 peak and its quantitation (Fig. 2a), the HPLC chromatogram obtained before incubation showed a peak which had the same retention time as that of M1 and interfered with the M1 peak (Fig. 2b). The following results (*N*-demethylation of YM17E) are, therefore, calculated from the difference between the peak areas before and after incubation. *N*-demethylation activity of YM17E was proportional to the microsomal protein concentration in the reaction mixture in the range 50 to 1000 μg mL^{-1}. The amount of M1 increased linearly up to 45 s after the beginning of the reaction (Fig. 3) but tended to plateau thereafter, because M1 was further metabolized to M2-a or M2-b. The velocity of *N*-demethylation of YM17E in the subsequent experiments was therefore determined with a protein concentration of 500 μg mL^{-1} and an incubation time of 45 s. V_{max} and K_m of YM17E *N*-demethylation, determined using pooled microsomes from seven individual livers, were

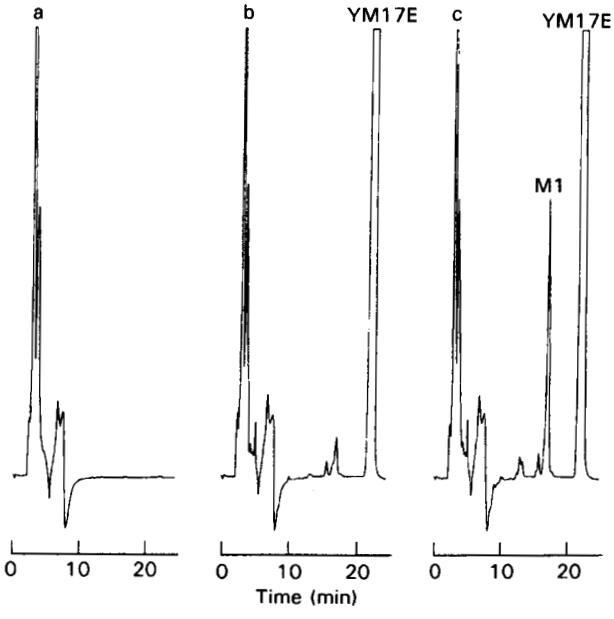

FIG. 2. HPLC chromatograms of microsomal reaction mixture: a. blank human liver microsomes; b. microsomes at 0 s on incubation with YM17E as substrate; c. microsomes after 45 s incubation with YM17E.

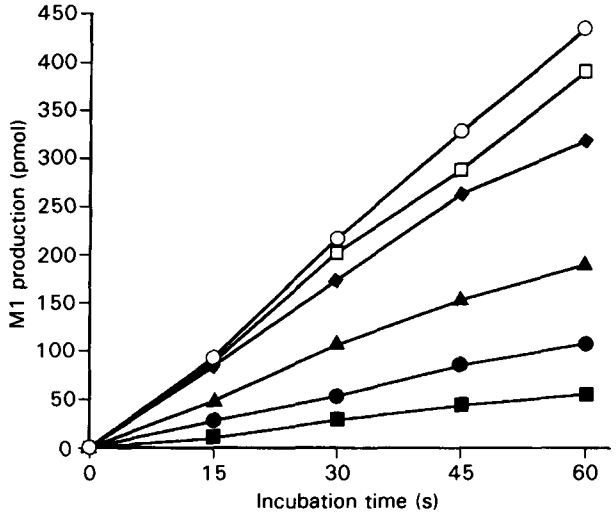

FIG. 3. Relationship between incubation time and M1 production for different substrate concentrations. YM17E concentrations were set at ■ 0·5 μM, ● 1 μM, ▲ 2 μM, ◆ 4 μM, □ 10 μM, ○ 20 μM. Pooled microsomes from two human livers (HBI-6 and HBI-7) were used.

1449 ± 294 pmol min^{-1} (mg protein)$^{-1}$ and $6{\cdot}7 \pm 2{\cdot}2$ μM, respectively (mean ± s.e. of three determinations; Fig. 4).

Inhibition of N-*demethylation of YM17E by metabolites*

Pooled microsomes from seven different human livers (HBI-14, HBI-15, HBI-16, HBI-17, HBI-19, HBI-20 and HBI-21) were used in this experiment. When M2-a, M2-b, M3 and M4 were added at various concentrations to the reaction mixture containing YM17E at a concentration of 4 μM, which is close to the K_m value, *N*-demethylation of YM17E was inhibited in a concentration-dependent manner. IC50 values of M2a, M2b, M3 and M4 were 3·8, 2·0, 2·4 and 5·0 μM, respectively (Table 1), showing that the inhibitory effect of these metabolites was similar, with M2b being somewhat more potent.

The inhibitory effect of M1 on *N*-demethylation of YM17E was confirmed by using [^{14}C]YM17E as a substrate. When M1 was added to the reaction mixture at 5, 15 and 60 μM, the activity decreased to 60·7%, 47·3% and 8·5% of control activity, respectively.

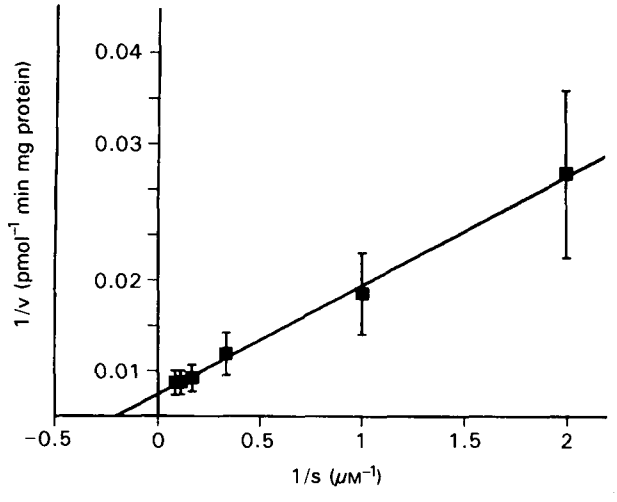

FIG. 4. Double reciprocal plot of *N*-demethylation of YM17E in pooled microsomes from seven human livers. The values are mean ± s.e. of three experiments.

Table 1. IC50 values of M2a, M2b, M3 and M4 for *N*-demethylation of YM17E in human liver microsomes.

Metabolite	IC50 (μM)
M2-a	3·8
M2-b	2·0
M3	2·4
M4	5·0

Pooled microsomes from seven different human livers (HBI-14, HBI-15, HBI-16, HBI-17, HBI-19, HBI-20 and HBI-21) was used in this experiment.

Individual variation in N-*demethylation of YM17E*

Table 2 shows the velocity of *N*-demethylation of YM17E at a concentration of 20 μM in ten individual hepatic microsomes containing different amounts of cytochrome P450 isoforms with different specific activities for substrates. A greater than tenfold difference was observed in the velocity of YM17E metabolism among these microsomes. The correlation coefficient between the *N*-demethylation activity of YM17E and those of various metabolic activities specific for cytochrome P450 isoforms was estimated (Table 3); only testosterone 6β-hydroxylase activity, for which CYP3A is specific (Waxman et al 1988, 1991), was correlated to that of *N*-demethylation of YM17E (correlation coefficient > 0·97). The activities of 7-ethoxyresorufin *O*-deethylation (CYP1A, Bourdi et al 1990; Lee et al 1991), caffeine 3-demethylation (CYP1A), coumarin 7-hydroxylation (CYP2A; Yamano et al 1990), tolbutamide methylhydroxylation (CYP2C9/10; Veronese et al 1993), *S*-mephenytoin 4′-hydroxylation (CYP2C19; Goldstein et al 1994), dextromethorphan *O*-demethylation (CYP2D6; Kupfer et al 1986; Kronbach et al 1987), chlorzoxazone 6-hydroxylation (CYP2E1; Peter et al 1990), lauric acid 11-hydroxylation (CYP2E1; Clarke et al 1994) and lauric acid 12-hydroxylation (CYP4A; Clarke et al 1994) were not correlated with that of *N*-demethylation of YM17E.

Inhibition by CYP inhibitors and substrates

Pooled microsomes from HBI-6 and HBI-7 were used in this experiment. *N*-demethylation of YM17E was inhibited by

Table 2. *N*-demethylase activity of YM17E in ten individual human liver microsomal fractions.

Microsomes	*N*-demethylation activity (pmol min^{-1} (mg protein)$^{-1}$)
HBI-2	1203·7
HBI-3	Not detected
HBI-5	9·6
HBI-6	1629·9
HBI-7	145·1
HBI-9	152·0
HBI-10	25·1
HBI-11	893·9
HBI-12	40·0
HBI-13	511·5

YM17E was added to the microsomal incubation mixture at a concentration of 20 μM and incubated for 45 s at 37°C. The values are means from duplicate assays.

Table 3. Correlation coefficient between the *N*-demethylation activity of YM17E and the specific activity of different CYP isozymes..

Specific activity for each CYP isozyme	Correlation coefficent
7-Ethyoxyresorufin *O*-deethylation (CYP1A)	0·046
Caffeine 3-demethylation (CP1A)	0·067
Coumarin 7-hydroxylation (CYP2A)	0·131
Tolbutamide methylhydroxylation (CYP2C9/19)	0·484
S-Mephenytoin 4′-hydroxylation (CYP2C19)	0·003
Dextromethorphan *O*-demethylation (CYP2D6)	0·000
Lauric acid 11-hydroxylation (CYP2E1)	0·177
Testosterone 6β-hydroxylation (CP3A)	0·979
Lauric acid 12-hydroxylation (CYP4A)	0·370

Table 4. Inhibition of *N*-demethylation of YM17E by various CYP inhibitors.

	Control activity (%) for inhibitor concentrations of:		
	0·2 μM	2 μM	20 μM
α-Naphthoflavone (CYP1A)	99·5	100·3	58·6
Quinidine (CYP2D)	93·7	95·5	86·7
Ketoconazole (CYP3A)	38·4	7·7	Not detected
Triacetyloleandomycin (CYP3A)	100·0	94·5	52·7
SKF525A (non-specific)	94·7	91·8	73·6
Metyrapone (non-specific)	90·4	72·4	23·6

YM17E was added at a concentration of 4 μM to pooled microsomes from two human livers (HBI-6 and HBI-7). The values are means from duplicate assays.

ketoconazole and triacetyloleandomycin, which are potent inhibitors of the CYP3A subfamily (Watkins et al 1985; Maurice et al 1992), and by non-specific CYP inhibitors such as SKF-525A and metyrapone (Table 4). When these inhibitors were used at 20 μM, activity decreased to 0%, 50%, 70% and 25% of control levels, respectively (Table 4). Addition of α-naphthoflavone 20 μM also inhibited this reaction to 60% of the control level. Quinidine, which is a specific inhibitor of CYP2D6 (Otton et al 1988), had only a slight effect on this reaction. When CYP-specific substrates were used, *N*-demethylase activity was reduced only by ethoxyresorufin and nifedipine, which are substrates of CYP1A and CYP3A4, respectively (Guengerich et al 1986; Bourdi et al 1990; Lee et al 1991), with activity decreasing to 80% and 50% of control levels when 2 and 20 μM was added, respectively (Table 5). Coumarin, tolbutamide, phenytoin, hexobarbital, debrisoquin and propafenone, which are specific substrates for CYP2A, CYP2C9/10, CYP9/19 and CYP2D6 (Siddoway et al 1987; Knodell et al 1988; Yamano et al 1990; Kato et al 1992; Veronese et al 1993; Goldstein et al 1994) had essentially no effect on this reaction. These results suggested that *N*-demethylation of YM17E was mainly catalysed by the CYP3A subfamily, while the CYP1A subfamily made only a minor contribution and the other isozymes no contribution to this reaction.

Metabolism by cDNA-expressed microsomes

Substrate concentrations were set at 2 and 20 μM, the approximate K_m and saturation concentrations, respectively. Because the metabolic velocities of these microsomes were low, with insufficient production of M1 for analytical sensitivity after 45 s incubation, incubation was performed for 10 min. As M1 production rate was confirmed to be linear for up to 10 min in these microsome experiments (data not

Table 5. Inhibition of *N*-demethylation of YM17E by known CYP substrates.

	Control activity (%) for inhibitor concentrations of:		
	0·2 μM	2 μM	20 μM
Ethoxyresorufin (CYP1A)	104·8	80·5	Not tested
Methoxycoumarin (CYP1A)	98·5	92·3	101·2
Coumarin (CYP2A)	95·0	92·5	89·4
Tolbutamide (CYP2C)	98·6	107·7	96·7
Phenytoin (CYP2C)	102·1	98·3	99·8
Hexobarbital (CYP2C)	104·8	105·0	102·5
Debrisoquin (CYP2D)	102·7	102·9	100·8
Propafenone (CYP2D)	105·6	109·7	92·5
Nifedipine (CYP3A)	99·8	93·0	53·1

YM17E was added at a concentration of 2 μM to pooled microsomes from two human livers (HBI-6 and HBI-7). Inhibition with 20 μM ethoxyresorufin was not conducted because of the poor solubility. The values are means from duplicate assays.

Table 6. Metabolism of YM17E by cDNA-expressed CYP isoforms.

Microsome used	YM17E *N*-demethylase activity for substrate concentrations:			
	pmol min^{-1}	(mg protein)$^{-1}$	pmol min^{-1}	(nmol P450)$^{-1}$
	2 μM	20 μM	2 μM	20 μM
Unexpressed microsomes	0·8	0·4	Not calculated	Not calculated
CYP1A1	0·2	0·9	10·0	37·8
CYP1A2	2·6	6·2	78·3	183·5
CYP2A6	Not detected	1·0	Not detected	22·8
CYP2B6	2·0	1·6	37·1	29·6
CYP2D6	3·4	15·8	13·3	61·9
CYP2E1	0·3	0·9	8·0	21·6
CYP3A4	10·7	21·2	357·3	707·9

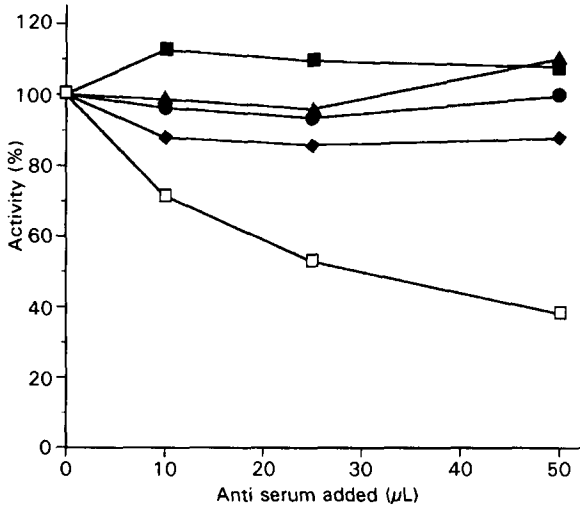

FIG. 5. Inhibition of *N*-demethylation of YM17E by rat anti-CYP antisera: 2 h before incubation; ■ anti-rat CYP1A1, ● anti-rat CYP2B1, ▲ anti-rat CYP2C11, ◆ anti-rat CYP2E1 and □ anti-rat CYP3A2 antisera were added to 50 μg protein from pooled microsomes from two human livers (HBI-6 and HBI-7).

shown), this condition was considered to be adequate for quantitative analysis. cDNA-expressed microsomes of each CYP metabolized YM17E at a different rate (Table 6). CYP3A4 isozyme showed the highest metabolic velocity at both 2 and 20 μM, followed by CYP2D6 and CYP1A2. Expression of the velocity of these microsomes in the units pmol min^{-1} (nmol P450)$^{-1}$ also showed that CYP3A4 had the highest activity for *N*-demethylation of YM17E (Table 6).

Inhibition of N-demethylation of YM17E by rat CYP antiserum

Pooled microsomes from HBI-6 and HBI-7 were used in this experiment. On addition of several anti-rat CYP antisera to the reaction mixture, anti-rat CYP3A2 antiserum inhibited *N*-demethylation of YM17E by 60% (Fig. 5). In contrast, other antisera had slight effect on this activity. As activity was not completely inhibited under the above conditions, even with CYP3A antiserum, an additional inhibition study was performed at the lower microsomal protein concentration of 10 μg mL^{-1} (data not shown). The same results were obtained, however, suggesting either that this antiserum has a limited effect on hepatic CYP in man or that other enzymes contribute to this reaction.

Discussion

On oral administration of YM17E to healthy volunteers, plasma concentrations of YM17E and its metabolites (M1, M2-a, M2-b) increased more than in proportion to the increase in dose (Uchida et al 1996) suggesting either that metabolites inhibited YM17E metabolism or that the absorption rate of YM17E from the gastrointestinal tract was affected by dose levels. In this study we have examined the possibility that YM17E metabolism is inhibited by its metabolites. The results show that M2-a, M2-b, M3 and M4 inhibited *N*-demethylation of YM17E in a concentration-dependent manner, with IC50 values of 3·8, 2·0, 2·4 and 5·0 μM, respectively (Table 1). Thus, inhibitory potency was closely similar, with that of M2-b being somewhat greater.

Because inhibition of *N*-demethylation of YM17E by M1 could not be determined by HPLC with electrochemical detection, this inhibition was examined using [^{14}C]YM17E and measurement of radiolabelled metabolite (M1) by liquid scintillation counting. Unfortunately, because the specific activity of [^{14}C]YM17E was not sufficient to enable precise determination of product ([^{14}C]M1), IC50 or K_i values were not estimated; the results showed, however, that M1 inhibited *N*-demethylation in an M1-concentration-dependent manner, indicating that M1 itself did inhibit M1 formation. These results indicate that in human liver microsomes *N*-demethylation of YM17E is inhibited by at least five metabolites, namely M1, M2-a, M2-b, M3 and M4. In man the concentrations of YM17E and these metabolites in liver after oral administration are not known, thus we cannot conclude that non-linear kinetics were a consequence of inhibition by the metabolic products. In a previous study (Uchida et al 1994), however, the combined concentration of YM17E and its active metabolites in rat liver after oral administration of [^{14}C]YM17E at 10 mg kg^{-1} were estimated to be approximately 20 μM, more than the IC50 value obtained in the present study. In addition, the highest values of the range of plasma concentrations obtained in studies on man were beyond those measured in the rat (Uchida et al 1996), suggesting the possibility that the concentration of these metabolites in the

human liver was high enough to cause the non-linear plasma concentrations of parent drug and M1.

We previously reported that the *N*-demethylation of YM17E in rat liver microsomes was at least partially catalysed by cytochrome P450 (Uchida et al 1993a). In human hepatic microsomes this reaction was also inhibited by cytochrome P450 inhibitors, namely SKF525A and metyrapone, indicating that cytochrome P450 is involved in this reaction. Many specific inhibitors or specific substrates of CYP isozymes have been reported and used for identification of CYP isozymes involving unknown metabolism. Among isozyme-specific inhibitors and substrates, ketoconazole showed the most potent inhibition, namely 90% of control at a concentration of 2 μM, whereas no metabolites were detected at 20 μM. Nifedipine also inhibited this *N*-demethylation in a concentration-dependent manner, suggesting that YM17E and nifedipine are metabolized by the same enzyme, namely CYP3A4. Inhibition by triacetyloleandomycin (Watkins et al 1985) also supports the involvement of CYP3A isozyme. The weak inhibition by 7-ethoxyresorufin and α-naphthoflavone suggested that the CYP1A subfamily plays a small role, but that other CYP isozymes are not involved in this reaction. When ten individual microsomes containing different amounts of each CYP isozyme were used, the activity of *N*-demethylation of YM17E correlated with that of testosterone 6β-hydroxylation, which is specific for the CYP3A subfamily (Waxman et al 1988, 1991). Other metabolic activity showed a lower correlation coefficient. The correlation coefficient for testosterone 6β-hydroxylation was greater than 0·97, suggesting the major contribution of CYP3A to this reaction in hepatic metabolism in man. Taken together, these results show that CYP3A4 is the major metabolizing enzyme of YM17E in microsomes and that CYP1A2 partially contributes to this *N*-demethylation.

The conclusion is further supported by metabolism in cDNA-expressed microsomes. These microsomes demonstrated that not only CYP3A4 and CYP1A2 but also CYP2D6 catalyse YM17E metabolism. We used YM17E at concentrations of 2 and 20 μM, i.e. approximately the K_m value and saturation state, respectively. The ratio of the reaction velocity of CYP2D6 to that of CYP3A4 is higher at a substrate concentration of 20 μM than at 2 μM, suggesting that the V_{max}/K_m values of CYP3A4 and 2D6 are different. The unit of these results is the velocity of product formation (mg protein)$^{-1}$, but the cDNA-expressed microsomes each contains a different amount of cytochrome P450. For example, CYP2D6-expressed microsomes contain about ten times more cytochrome P450 than do CYP3A4-expressed microsomes. Correcting the velocity for the amount of P450 shows more clearly the predominant role of CYP3A4 isozyme (Table 6). Shimada et al (1994) reported that two members of the P450 family, the CYP2C and CYP3A subfamilies, are the major P450 enzymes in the liver in man; these represent 18·2% and 28·8%, respectively, of total P450 whereas the CYP2D6 content is only 1·5%. Even though CYP2D6 does participate in catalysing this reaction in-vivo, its contribution is not significant.

As mentioned in the introduction, despite showing potent efficacy in several animal models, the hypocholesterolaemic activity of ACAT inhibitors in man is not clear. In general, plasma concentrations of ACAT inhibitors are low (Tse & Jaffe 1988; Hainer et al 1994), and ACAT inhibitors with low bioavailability and present at low concentrations in plasma had no effects on regulation of plasma lipids in man (Peck et al 1995). In a clinical study, a reduction in serum cholesterol levels was induced by a high plasma concentration of YM17E together with active metabolites. If ACAT-catalysis of esterification in the gastrointestinal tract were not the rate-limiting step in absorption of cholesterol from the gastrointestinal tract in man, or if the supply of cholesterol from liver were sufficient to maintain the serum cholesterol levels in man, poorly absorbable ACAT inhibitors might not be effective. YM17E was administered orally so that the drug reached the gastrointestinal mucosa to inhibit intestinal ACAT. It was then absorbed and metabolized to five metabolites, which also have an inhibitory effect on ACAT. The metabolism of YM17E and of these metabolites was inhibited by themselves, with the result that the plasma concentration required for the hypocholesterolaemic effect was maintained for longer. This characteristic of YM17E seems to be advantageous for reducing serum cholesterol levels because oral administration of YM17E is thought to act at two sites at least, the intestine and liver. In addition, a high plasma concentration of parent drug and active metabolites might also enable the development of anti-atherosclerotic activity on the arterial wall, but further investigation is required to determine whether this inhibitor shows anti-atherosclerotic activity in man.

References

Bast, A., Scheefhalts, L. W. C., Noordhoek, J. (1982) Dose-dependent kinetics of aminopyrine metabolism in the rat caused by product inhibition and determined by capillary GLC. (1982) Pharmacology 25: 130–137

Bell, F. P. (1986) Arterial cholesterol esterification by acylcoA-cholesterol acyltransferase: its possible significance in atherogenesis and its inhibition by drug. In: Fears, R. (ed.) Pharmacological Control of Hyperlipidaemia. J. R. Prous Science Publishers, Barcelona, pp 409–422

Bourdi, M., Larrey, D., Nataf, J., Bernuau, J., Pessayre, D., Iwasaki, M., Guengerich, F. P., Beaune, P. H. (1990) Anti-liver endoplasmic reticulum autoantibodies are directed against human cytochrome P450IA2. J. Clin. Invest. 85: 1967–1973

Clarke, S. E., Baldwin, S. J., Bloomer, J. C., Ayrton, A. D., Sozio, R. S., Chenery, R. J. (1994) Lauric acid as a model substrate for the simultaneous determination of cytochrome P4502E1 and 4A in hepatic microsomes. Chem. Res. Toxicol. 7: 836–842

Goldstein, J. A., Faletto, M. B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M., Ghanayem, B. I. (1994) Evidence that CYP2C19 is the major (*S*)-mephenytoin 4′-hydroxylase in humans. Biochemistry 33: 1743–1752

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, a prototype for genetic polymorphism in oxidative drug metabolism. J. Biol. Chem. 261: 5051–5060

Hainer, J. W., Terry, J. G., Connell, J. M., Zyruk, H., Jenkins, R. M., Shand, D. L., Gillies, P. J., Livak, K. J., Hunt, T. L., Crouse, J. R. (1994) Effect of the acyl-CoA:cholesterol acyltransferase inhibitor Dup 128 on cholesterol absorption and serum cholesterol in humans. Clin. Pharmacol. Ther. 56: 65–74

Harris, W. S., Dujovne, C. A., Bergmann, K. V., Neal, J., Akester, J., Windsor, S. L, Greene, D., Look, Z. (1990) Effects of the ACAT inhibitor CL277,082 on cholesterol metabolism in humans. Clin. Pharmacol. Ther. 48: 189–94

Heider, J. G., Pickens, C. E., Kelly, L. A. (1983) Role of acyl CoA: cholesterol acyltransferase in cholesterol absorption and its inhibition by 57-118 in the rabbit. J. Lipid Res. 24: 1127–1134

Iizumi, Y., Yanagita, Y., Masuyama, Y., Ohtsuka, H., Kakuta, H., Fujimari, N., Itoh, N., Matsuda, K., Ohata, I. (1991) Lipid-lowering

and anti-atherosclerotic activity of YM17E, an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) in animal models. 9th Int. Symp. Atherosclerosis, Rosemont, IL, USA, Abstract 96

Ito, N., Matsuda, K., Yasunaga, T., Araki, T., Iwaoka, K., Iizumi, Y., Ohhata, I. (1991) Synthesis and hypocholesterolemic activity of phenylene and pyridylene diurea derivatives as ACAT inhibitors. 202nd ACS National Meeting, New York, NY, USA, Abstract 109

Kato, R., Yamazoe, Y., Yasumori, T. (1992) Polymorphism in stereoselective hydroxylation of mephenytoin and hexobarbital by Japanese liver samples in relation to cytochrome P450 human-2 (CYPIIC9). Xenobiotica 22: 1083–1092

Knodell, R. G., Dubey, R. K., Wilkinson, G. R., Guengerich, F. P. (1988) Oxidative metabolism of hexobarbital in human liver: relationship to polymorphic *S*-mephenytoin 4-hydroxylation. J. Pharmacol. Exp. Ther. 245: 845–849

Krause, B. R., Anderson, M., Bisgaier, C. L., Bocan, T., Bousley, R., DeHart, P., Essenburg, A., Hamelehle, K., Homan, R., Kieft, K., McNally, W., Stanfield, R., Newton, R. S. (1993) In vivo evidence that the lipid-regulating activity of the ACAT inhibitor CI-976 in rats is due to inhibition of both intestinal and liver ACAT. J. Lipid Res. 34: 279–294

Kronbach, T., Mathys, D., Gut, J., Catin, T., Meyer, U. R. (1987) High-performance liquid chromatographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan *O*-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. Anal. Biochem. 162: 24–32

Kupfer, A., Schmid, B., Pfaff, G. (1986) Pharmacokinetics of dextromethorphan *O*-demethylation in man. Xenobiotica 16: 421–433

Largis, E. E., Wang, C. H., DeVries, V. G., Schaffer, S. A. (1989) CL 277,082: a novel inhibitor of ACAT-catalyzed cholesterol esterification and cholesterol absorption. J. Lipid Res. 30: 681–690

Lee, Q. P., Fantel, A. G., Juchau, M. R. (1991) Human embryonic cytochrome P450s: phenoxazone ethers as probes for expression of functional isoforms during organogenesis. Biochem. Pharmacol. 42: 2377–2385

Matsuda, K. (1994) ACAT inhibition as antiatherosclerotic agents: compounds and mechanisms. Med. Res. Rev. 14: 271–305

Maurice, M., Pichard, L., Daujat, M., Fabre, I., Joyeux, H., Domergue, J., Maurel, P. (1992) Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. FASEB J. 6: 752–758

Otton, S. V., Crewe, H. K., Lennard, M. S., Tucker, G. T., Woods, H. F. (1988) Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes. J. Pharmacol. Exp. Ther. 247: 242–247

Peck, R. W., Wiggs, R., Posner, J. (1995) The tolerability, pharmacokinetics and lack of effect on plasma cholesterol of 447C88, an acylCoA:cholesterol acyl transferase (ACAT) inhibitor with low bioavailability, in healthy volunteers. Eur. J. Clin. Pharmacol. 49: 243–249

Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P., Yang, C. S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. Chem. Res. Toxicol. 3: 566–573

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., Guengerich, F. P. (1994) Interindividual variations in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J. Pharmacol. Exp. Ther. 270: 414–423

Siddoway, L. A., Thompson, K. A., McAllister, C. B., Wang, T., Wilkinson, G. R., Roden, D. M., Woosley, R. L. (1987) Polymorphism of propafenone metabolism and disposition in man: clinical and pharmacokinetic consequences. Circulation 75: 785–791

Suzuki, T., Fujita, S., Kawai, R. (1984) Precursor-metabolite interaction in the metabolism of lidocaine. J. Pharm. Sci. 73: 136–138

Tse, F. L. S., Jaffe, J. M. (1988) Influence of high-fat meal on the absorption of a silicon-containing amide, an inhibitor of acyl-CoA:cholesterol acyltransferase, in man. Biopharm. Drug Dispos. 9: 201–210

Uchida, T., Usui, T., Teramura, T., Watanabe, T., Higuchi, S. (1993a) Metabolic *N*-demethylation of 1,3-bis[[1-cycloheptyl-3-(*p*-dimethylaminophenyl)ureido]methyl]benzene dihydrochloride, a novel acyl-coenzyme A:cholesterol acyltransferase inhibitor. Drug. Metab. Dispos. 21: 524–529

Uchida, T., Usui, T., Watanabe, T., Higuchi, S. (1993b) Simultaneous determination of a new inhibitor of acyl CoA:cholesterol acyltransferase, YM17E, and five metabolites using high-performance liquid chromatography with electrochemical detection. J. Chromatogr. Biomed. Appl. 613: 179–183

Uchida, T., Nakamura, E., Usui, T., Imasaki, H., Kawakami, R., Watanabe, T., Higuchi, S. (1994) Disposition and metabolism of a novel diurea inhibitor of acyl CoA:cholesterol acyltransferase (YM17E) in the rat and dog. Xenobiotica 24: 1223–1236

Uchida, T., Usui, T., watanabe, T., Higuchi, S., Nakata, M., Maezawa, K., Kikawa, Y., tsunoo, M., Nakaya, N., Goto, Y. (1996) Pharmacohinetic properties of YM17E, an inhibitor of acyl CoA:cholesterol acyl transferase, and serum cholesterol levels in healthy volunteers. eur. J. clin. Pharmacol. In press

Veronese, M. E., Doecke, C. J., Mackenzie, P. I., McManus, M. E., Miners, J. O., Rees, D. L. P., Gasser, R., Meyer, U. A., Birkett, D. J. (1993) Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. Biochem. J. 289: 533–538

Watkins, P. B., Erighton, S. A., Maurel, P., Schuetz, E. G., Mendez-Picon, G., Parker, G. A., Guzelian, P. S. (1985) Identification of an inducible form of cytochrome P-450 in human liver. Proc. Natl. Acad. Sci. 82: 6310–6314

Waxman, D. J., Attisano, C., Guengerich, F. P., Lapenson, D. P. (1988) Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6β-hydroxylase cytochrome P-450 enzyme. Arch. Biochem. Biophys. 263: 424–436

Waxman, D. J., Lapenson, D. P., Aoyama, T., Gelboin, H. V., Gonzalez, F. J., Korzekwa, K. (1991) Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. Arch. Biochem. Biophys. 290: 160–166

Yamano, S., Tatsuno, J., Gonzalez, F. J. (1990) The CYP2A3 gene product catalyses coumarin 7-hydroxylation in human liver microsomes. Biochemistry 29: 1322–1329