CYP2C8 and CYP3A4 are the principal enzymes involved in the human *in vitro* biotransformation of the insulin secretagogue repaglinide

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Aims To identify the principal human cytochrome P450 (CYP) enzyme(s) responsible for the human *in vitro* biotransformation of repaglinide. Previous experiments have identified CYP3A4 as being mainly responsible for the *in vitro* metabolism of repaglinide, but the results of clinical investigations have suggested that more than one enzyme may be involved in repaglinide biotransformation.

Methods [^{14}C]-Repaglinide was incubated with recombinant CYP and with human liver microsomes (HLM) from individual donors in the presence of inhibitory antibodies specific for individual CYP enzymes. Metabolites, measured by high-performance liquid chromatography (HPLC) with on-line radiochemical detection, were identified by liquid chromatography-mass spectrophotometry (LC-MS) and LC-MS coupled on-line to a nuclear magnetic resonance spectrometer (LC-MS-NMR).

Results CYP3A4 and CYP2C8 were found to be responsible for the conversion of repaglinide into its two primary metabolites, M4 (resulting from hydroxylation on the piperidine ring system) and M1 (an aromatic amine). Specific inhibitory monoclonal antibodies against CYP3A4 and CYP2C8 significantly inhibited (> 71%) formation of M4 and M1 in HLM. In a panel of HLM from 12 individual donors formation of M4 and M1 varied from approximately 160–880 pmol min^{-1} mg^{-1} protein and from 100–1110 pmol min^{-1} mg^{-1} protein, respectively. The major metabolite generated by CYP2C8 was found to be M4. The rate of formation of this metabolite in HLM correlated significantly with paclitaxel 6α-hydroxylation ($r_s = 0.80$; $P = 0.0029$). Two other minor metabolites were also detected. One of them was M1 and the other was repaglinide hydroxylated on the isopropyl moiety (M0-OH). The rate of formation of M4 in CYP2C8 Supersomes^{TM} was 2.5 pmol min^{-1} pmol^{-1} CYP enzyme and only about 0.1 pmol min^{-1} pmol^{-1} CYP enzyme in CYP3A4 Supersomes^{TM}. The major metabolite generated by CYP3A4 was M1. The rate of formation of this metabolite in HLM correlated significantly with testosterone 6β-hydroxylation ($r_s = 0.90$; $P = 0.0002$). Three other metabolites were identified, namely, M0-OH, M2 (a dicarboxylic acid formed by oxidative opening of the piperidine ring) and M5. The rate of M1 formation in CYP3A4 Supersomes^{TM} was 1.6 pmol min^{-1} pmol^{-1} CYP enzyme but in CYP2C8 Supersomes^{TM} it was only approximately 0.4 pmol min^{-1} pmol^{-1} CYP enzyme.

Conclusions The results confirm an important role for both CYP3A4 and CYP2C8 in the human *in vitro* biotransformation of repaglinide. This dual CYP biotransformation may have consequences for the clinical pharmacokinetics and drug-drug interactions involving repaglinide if one CYP pathway has sufficient capacity to compensate if the other is inhibited.

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Introduction

Repaglinide (see Figure 2) is an insulin secretagogue used in the treatment of type 2 diabetes. This disease occurs as a result of inadequate insulin secretion, decreased peripheral tissue insulin sensitivity, or both, leading to impaired glucose utilization and hyperglycaemia [1].

Repaglinide is a carbamoylmethyl benzoic acid derivative and is the first of a new class of short acting oral insulin secretagogues. It stimulates the release of insulin from the pancreatic beta cells by binding to and closing ATP-dependent potassium channels. This depolarizes the plasma membrane leading to opening of voltage-dependent calcium channels. Influx of calcium ions, giving increased intracellular Ca^{2+}, triggers exocytosis of insulin [2]. The repaglinide binding site on the potassium channels is distinct and different from the sulphonylurea binding site [3, 4], and this may explain some of the differences observed in their actions.

As repaglinide is both rapidly absorbed (t_{max} of 0.5–1.0 h) and eliminated, with a half-life of approximately 1–2 h, the drug is suitable for administration preprandially, giving good overall glycaemic control without the risk of hypoglycaemia [5, 6].

Repaglinide is extensively metabolized by the hepatic cytochrome P450 enzyme system, with less than 2% of an oral dose being excreted unchanged in humans [7, 8]. Metabolites are primarily excreted via the bile into the faeces and none of the metabolites has been found to exert clinically relevant hypoglycaemic activity.

CYP3A4 has been identified as an important enzyme in the *in vitro* metabolism of repaglinide [9]. However clinical drug-drug interaction studies involving substrates, inhibitors and inducers of CYP3A4 have shown only marginal effect on repaglinide pharmacokinetics [10], except for induction by rifampicin [11] and inhibition by clarithromycin [12]. Repaglinide does not have a significant effect on the pharmacokinetics of the CYP3A4 substrates simvastatin, nifedipine and oral contraceptive steroids [10]. Thus other forms of human cytochrome P450s may be involved in the metabolism of repaglinide. Of particular interest are the CYP2C enzymes because of their known substrate overlap with CYP3A4 [14–18] and because many CYP2C substrates are carboxylic acids like repaglinide [13].

Of the four human members of the CYP2C family, both CYP2C18 and CYP2C8 have previously been assigned a very limited role in the metabolism of drugs and xenobiotics.

From previous *in vitro* studies with repaglinide (unpublished data on file at Novo Nordisk), the involvement of either CYP2C9 or CYP2C19 has been excluded.

Recently, CYP2C8 was shown to be involved in the metabolism of several structurally and functionally different drugs, such as carbamazepine [15], paclitaxel [14], amiodarone [19], cerivastatin [16] and rosiglitazone [20]. CYP3A4 also contributes to the metabolism of the first four of these drugs. Because of this substrate overlap between CYP2C8 and CYP3A4 and because both enzymes exhibit wide interindividual variability in their metabolic activity, their dual contribution to biotransformation pathways may lead to drug-drug interactions. Furthermore, it was recently shown that CYP3A4 substrates such as quinidine and amiodarone inhibit CYP2C8 activity and that ketoconazole, which is a potent and selective inhibitor of CYP3A4 activity at concentrations up to about 2 μM, almost abolished CYP2C8 activity *in vitro* at a higher concentration of 10 μM [21].

The aim of the present study was to define the roles of CYP2C8 and CYP3A4 in the metabolism of repaglinide in HLM.

Methods

Chemicals

[^{14}C]-Repaglinide (radiochemical purity > 98%, specific activity 63.2 μCi mg^{-1} dissolved in 99.9% ethanol) was synthesized in the laboratories of Novo Nordisk A/S. β-Nicotinamide adenine dinucleotide phosphate tetra sodium salt, reduced form (β-NADPH), was purchased from Aldrich Chemie, Germany. Formic acid 98–100%, acetonitrile, isocratic grade and sodium dihydrogen phosphate monohydrate were purchased from Merck, Germany.

[^{14}C]-Repaglinide biotransformation in human liver microsomes

A mixed donor pool of HLM from 15 histologically normal human liver samples was obtained from In Vitro Technologies, Inc. (Baltimore, MD, USA) and HLM from individual donors (coded HG3, HG23, HK23, HK34, HG42, HG43, HG56, HG66, HG70, HG89, HG93, HG112) were obtained from Gentest Corporation (Woburn, MA, USA). The company states that the human materials were prepared from human donor tissue that was obtained with informed consent in accordance with the Uniform Anatomical Gift Act (USA). The

informed consent specified that the tissue could be used for research if it was unsuitable for transplantation. These individual liver microsomal samples had previously been assessed for CYP activity and content by the supplier. CYP activities were determined using probe substrates selective for each enzyme. Testosterone 6β-hydroxylation and paclitaxel 6α-hydroxylation were used for CYP3A4 and CYP2C8 activity, respectively.

[^{14}C]-Repaglinide (22 μM) and pooled HLM (0.075 mg protein), were pre-incubated for 2 min at 37 °C in 50 mM phosphate buffer at pH 7.4. Previous studies demonstrated that the formation of metabolites exhibited linear kinetics up to a substrate concentration of 22 μM. β-NADPH, at a final concentration of 1 mM, was used to initiate the reaction, which was then allowed to proceed for 15 min at 37 °C in a total volume of 250 μl. Biotransformation was stopped by the addition of 250 μl cold acetonitrile and by placing the sample on ice for 5 min Insoluble material was removed by centrifugation at 9503 *g* for 10 min and 100 μl of the supernatant was analysed by HPLC.

Chromatography

A Prodigy C18/ODS 3.5 μm high performance liquid chromatography column (HPLC) (250 × 4.6 mm) and a C18/ODS SecurityGuard™ Cartridge (4 × 3.0 mm) were purchased from Phenomenex (Torrance, CA, USA). Chromatography was performed at ambient temperature. Eluent was initially passed through a UV detector set at 242 nm and radioactive metabolites were then detected using a Packard, Radiomatic FLO-ONE®*Beta* 150 TR Flow Scintillation Analyser, after mixing the eluent with a scintillation cocktail (Ultima-Flo™ M, Packard), delivered at a flow-rate of 3.0 ml min^{-1}. All other HPLC components were from Merck Hitachi LaChrom and consisted of an interface D × 7000 pump L-7100, an auto sampler L-7200 and a UV detector L-7400. The flow rate of mobile phase gradient was 1.0 ml min^{-1}. Eluent A was acetonitrile : water : formic acid (10 : 90 : 1, v/v), and Eluent B was acetonitrile : water : formic acid (90 : 10 : 1, v/v). The elution conditions were a linear gradient of 0% Eluent B increasing to 60% Eluent B over 30 min, followed by an increase to 100% Eluent B over 3 min and return to 0% B over 3 min. Results were analysed on Merck Hitachi LaChrom Software.

The formation rates of repaglinide metabolites were calculated using a standard curve based on [^{14}C]-repaglinide over the concentration range of 0.11–22 μM. The coefficient of variation of the slope of three replicate graphs was 25.47%. The minimum determinable rate of metabolite formation was 0.05 pmol min^{-1} pmol^{-1} CYP enzyme.

[^{14}C]-Repaglinide biotransformation by recombinant human P450 enzymes

Supersomes™ from insect cells expressing human CYP1A2, CYP2C8, CYP2C9*1 (Arg144, Ile359), CYP2C19, CYP2D6*1 & CYP3A4 (all coexpressed with P450 reductase) were obtained from Gentest Corporation (Woburn, MA, USA). Incubations and analysis were performed as described above, using a total of 50 pmol CYP in each incubation.

Inhibition experiments

Specific inhibitory antipeptide antibodies against CYP2C9, CYP2C19 [22], CYP2D6 [23] and CYP3A4 [24] were raised in the Department of Drug Metabolism, Novo Nordisk A/S. Specific inhibitory monoclonal antibodies against CYP1A2 (IH-MAb-1A2), CYP3A4 (IH-MAb-3A4) and CYP2C8 (IH-MAb-2C8) were obtained from Gentest Corporation. These antibodies are validated by the manufacturer against CYP1A1/A2, CYP1B1, CYP2A6/B6/C8/C9/C19/D6/E1 and CYP3A4 using appropriate catalytic assays. Pre-immune IgG was used as a control for nonspecific inhibition.

HLM (0.075 mg protein) were pre-incubated for 30 min at room temperature with varying amounts of purified inhibitory IgG (2–8 mg IgG mg^{-1} protein) and 50 mM NaH_2PO_4, pH 7.4. The amounts of IgG used were in accordance with earlier studies which produced the greatest degree of inhibition of enzyme activity. Incubations were conducted at 37 °C for 2 min and [^{14}C]-repaglinide was added at a final concentration of 22 μM. The reaction was stopped and samples were analyzed as described above.

In experiments using inhibitory monoclonal antibodies, HLM (0.075 mg protein) were pre-incubated for 20 min on ice with 15 μl IH-MAb-1A2 (2 mg MAb/mg total protein) and 10 μl 25 mM Tris buffer, 5 μl IH-MAb-2C8 (0.67 mg MAb mg^{-1} total protein) and 20 μl 25 mM Tris buffer or 10 μl IH-MAb-3A4 (1.33 mg MAb mg^{-1} total protein) and 15 μl 25 mM Tris buffer. Incubations were continued at 37 °C for 2 min before the addition of [^{14}C]-repaglinide (22 μM). β-NADPH (1 mM) was used to initiate metabolism. Further incubation and analysis was performed as described above. The amounts of IH-MAb used were as recommended by the manufacturer.

Identification of major [^{14}C]-repaglinide metabolites formed in vitro

[^{14}C]-Repaglinide (44 μM) was pre-incubated for 2 min at 37 °C with a total of 50 pmol of recombinant human CYP2C8 or CYP3A4 in 50 mM sodium phosphate

buffer, pH 7.4. β-NADPH (1 mM) was used to start the reaction, which was allowed to proceed for 120 min at 37 °C in a total volume of 250 µl. Biotransformation was stopped by the addition of 250 µl cold acetonitrile and placing the sample on ice for 5 min Insoluble material was removed by centrifugation at 9503 *g* for 10 min and the volume of the supernatant fraction was reduced by evaporation at 40 °C under a stream of nitrogen. Repaglinide concentrations, the amount of CYP enzyme and incubation time were increased to produce sufficient metabolites for identification using LC-MS and LC-MS-NMR.

LC-MS analysis

Structural information on the metabolites was obtained by LC-MS and MS^n analysis. The LC equipment consisted of a HP 1100 chromatographic system equipped with a quaternary pump, a UV detector set at 242 nm, a degasser and an autosampler (Agilent Technology, Palo Alto, CA, USA). The separation was performed using a Prodigy C18/ODS 3.5 µm column (250 × 4.6 mm) equipped with a C18/ODS SecurityGuard™ Cartridge (4 × 3.0 mm) using the same gradient as used for the analysis of metabolites in the HLM experiments. A 100–400 µl aliquot was injected onto the column. The flow-rate was 1 ml min^{-1}, and the eluant was split 1 : 3 between the MS and a fraction collector.

MS detection was performed using a Bruker Esquire LC ion trap (Bremen, Germany) equipped with an electro spray ionization (ESI) interface. The MS was used in positive ion mode and over the scan range 100–800 m z^{-1}. Fractions collected during LC-MS analysis were analysed directly by MS^n (product ion scan). The samples were introduced to the mass spectrometer by continuous infusion at a flow-rate of 3 µl min^{-1} by a syringe pump (Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA). The mass spectrometer was scanned in the range m/z 50–600 (or to 500, depending upon mass of the precursor ion) using unit mass resolution.

Directly coupled HPLC-MS-NMR analysis

The HPLC-NMR data were acquired using a Bruker DRX-600 MHz spectrometer equipped with a ^1H flow probe (cell of 3 mm i.d., with a volume of 120 µl). ^1H NMR spectra were obtained in the MS detected capillary stop-flow mode at 600.14 MHz. The MS settings were as described above. In order to suppress the solvent signals (residual water and acetonitrile), the 1D ^1H NMR spectra were collected using a NOESYPRESAT pulse sequence. Free induction decays (FIDs) were collected into 32K computer data points with a spectral width of 16025.64 Hz, and 90° pulses were used with an acquisition time of 1.36 s. Prior to Fourier transformation, an exponential apodization function was applied to the FID, corresponding to a line broadening of 1.0 Hz. Five thousand one hundred and twenty scans were accumulated in order to obtain an appropriate signal-to-noise ratio.

Analysis of results

Graphical analysis was performed using GraphPad Prism™ v 3.0 (GraphPad Software, Inc., San Diego, USA).

Inhibition of enzyme activities by antibodies was also expressed as percentage of pre-immune control values (mean ± SD), where appropriate. Correlations were performed using Spearman's rank correlation.

Results

Identification of the principal enzymes responsible for [^{14}C]-repaglinide biotransformation

Following incubation of [^{14}C]-repaglinide with a mixed pool of HLM and NADPH, one major and two minor metabolites were identified. No metabolites were detected in the absence of NADPH.

Inhibitory antipeptide antibodies against CYP2C9, CYP2C19 and CYP3A4 inhibited the formation of the major metabolite, later identified by LC-MS and LC-MS-NMR as M4 [25], by 15%, 35% and 25%, respectively (Figure 1). The M4 metabolite is formed by β-hydroxylation of repaglinide in the piperidine ring system (Figure 2 and Table 1). Inhibitory antipeptide antibodies against CYP2D6 and inhibitory monoclonal antibodies against CYP1A2 had no effect on this biotransformation pathway.

In Supersomes™ from insect cells expressing human CYP1A2, CYP2C8, CYP2C9*1, CYP2C19, CYP2D6*1 and CYP3A4,[^{14}C]-repaglinide biotransformation was mediated only by CYP2C8 and CYP3A4

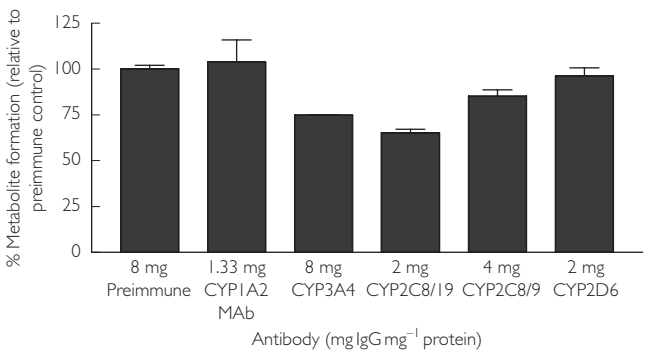


Figure 1 Effect of antipeptide antibodies or monoclonal antibodies (MAb) to different CYPs on metabolite M4 formation in a mixed donor pool of HLM (mean ± SD, $n = 3$ incubations).

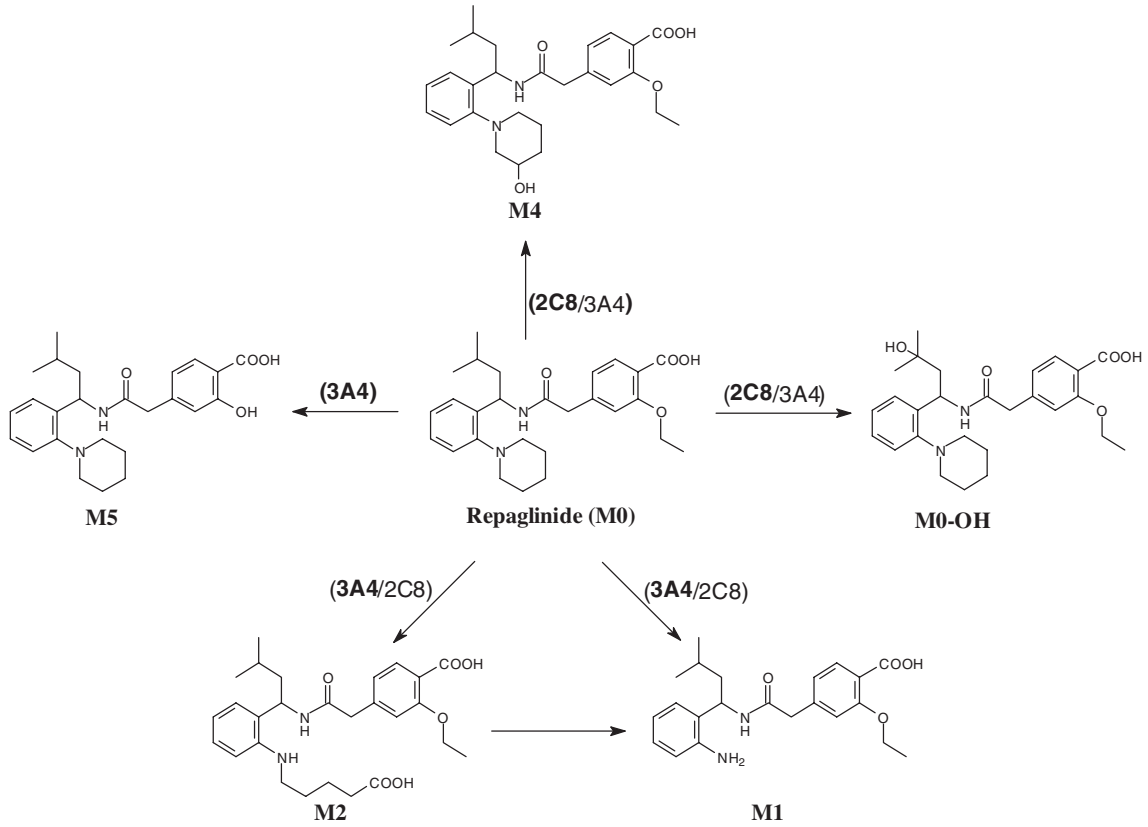


Figure 2 Biotransformation pathways of repaglinide *in vitro*. The principal enzyme responsible is highlighted in bold.

(Figure 3). As in the mixed pool of HLMs, three metabolites were formed by both expression systems using HPLC with on-line radiochemical detection. The rate of formation of the major metabolite, M4, in CYP2C8 Supersomes™ was 2.5 pmol min^{-1} $pmol^{-1}$ CYP enzyme and only about 0.1 pmol min^{-1} $pmol^{-1}$ CYP enzyme in CYP3A4 Supersomes™. The major metabolite in the CYP3A4 expression system was identified by LC-MS-NMR to be the previously designated M1 metabolite [25], which is an aromatic amine (Figure 2). The rate of M1 formation in CYP3A4 Supersomes™ was 1.6 pmol min^{-1} $pmol^{-1}$ CYP enzyme and in CYP2C8 Supersomes™ 0.4 pmol min^{-1} $pmol^{-1}$ CYP enzyme. A metabolite, previously undetected *in vitro*, was observed following incubation with both CYP2C8 and CYP3A4 Supersomes™ at rates of formation of 0.7 and 0.2 pmol min^{-1} $pmol^{-1}$ CYP enzyme, respectively. This metabolite was identified by LC-MS and LC-MS-NMR (Table 1) to be repaglinide hydroxylated on the isopropyl moiety (Figure 2), and is designated as M0-OH. A previously detected metabolite M5 [7] was also identified here by both LC-MS and LC-MS-NMR (results not shown) after incubation of drug with CYP3A4 Supersomes™ (Figure 2). This metabolite was not detected radiochemically as M5 co-eluted with repaglinide.

No repaglinide biotransformation was detected in CYP2C9 and CYP2C19 Supersomes™. The antipeptide antibodies against CYP2C9 and CYP2C19 showed approximately 20% inhibition of the formation of M4 in CYP2C8 Supersomes™ (results not shown).

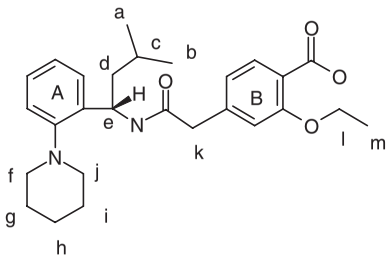
[^{14}C]-Repaglinide biotransformation in a panel of human liver microsomes

In a panel of HLM from 12 individual donors M0-OH, M1, M2 and M4 were formed at different rates (Figure 4). The formation of M0-OH varied from 0–195 pmol min^{-1} mg^{-1} protein; that of M4 varied from 160–880 pmol min^{-1} mg^{-1} protein; that of M1 formation varied from 100–1110 pmol min^{-1} mg^{-1} protein; and that of M2 varied from 0–190 pmol min^{-1} mg^{-1} protein. M4 was the major metabolite formed in 9 of the 12 HLM samples and M1 was the major metabolite in the other three samples. M2 was detected in 11 of the 12 HLM samples, but never as the major metabolite (Figure 4).

The formation of M4 was significantly correlated with CYP2C8 activity ($r_s = 0.80$; $P = 0.0029$) based on paclitaxel 6α-hydroxylation but not with CYP3A4 activity ($r_s = 0.077$; $P = 0.82$) based on testosterone 6β-hydroxylation. The formation of M1 showed a significant

Table 1 1H NMR assignments for repaglinide and its main metabolites formed by CYP2C8.

Compound	T_R *(min)*	*a,b*	*c*	*d*	*e*	*f,j* *Chemical shift (ppm, multiplicity)*	*g,h,i*	*k*	*l*	*m*	*A-ring*	*B-ring*
Repaglinide	30.8	0.81 (dd)	1.25 (m)	1.25 (m)	4.68 (dd)	Under acetonitrile	1.55 (m)	3.50 (s)	3.99 (q)	1.25 (t)	7.43-7.58 (m)	6.76-6.83 (d,s) 7.66 (d)
M4	26.5	0.82 (dd)	1.35 (m)	1.20 (m)	4.85 (dd)	3.58 (d) Under acetonitrile	4.60 (m) 1.40 (m) 2.25 (m)	3.50 (s)	3.99 (q)	1.25 (t)	7.43-7.58 (m)	6.80-6.83 (d,s) 7.68 (d)
M0-OH	20.2	1.11 (s) 1.01 (s)	-	2.25 (d)	4.95 (dd)	Under acetonitrile	1.42 (m)	3.50 (s)	3.92 (q)	1.22 (t)	7.40-7.51 (m)	6.76-6.82 (d,s) 7.56 (d)



1D 1H spectra were acquired by directly coupled HPLC-NMR in D2O : acetonitrile according to the relative amounts at the retention time in the chromatographic run. T_R is the chromatographic retention time in minutes. Spectra were referenced to the acetonitrile signal (δ = 2 ppm). dd = double doublet, s = singlet, d = doublet, t = triplet, q = quartet.

correlation with CYP3A4 activity ($r_s = 0.90$; $P = 0.0002$) and a significant but low correlation with CYP2C8 activity ($r_s = 0.60$; $P = 0.043$). M2 formation was significantly but weakly correlated with both CYP3A4 and CYP2C8 activity ($r_s = 0.66$; $P = 0.024$ and $r_s = 0.75$; $P = 0.0062$, respectively) and M0-OH showed a significant correlation with CYP2C8 activity ($r_s = 0.84$; $P = 0.0011$) but not with CYP3A4 activity ($r_s = 0.47$; $P = 0.13$).

The effect of specific inhibitory monoclonal antibodies against CYP3A4 (IH-MAb-3A4) and CYP2C8 (IH-MAb-2C8) on the formation of each repaglinide metabolite is illustrated in Figure 5. IH-MAb-2C8 inhibited the formation of M0-OH by 30% to 100% in the 12 HLM samples (mean 90%). IH-MAb-3A4 had no inhibitory effect on the formation of this metabolite in most samples.

IH-MAb-2C8 had a potent inhibitory effect on the formation of M4, which was greater than 73% in all HLM samples (mean 85%), whereas IH-MAb-3A4 did not inhibit this metabolic pathway.

Formation of M1 was only inhibited by IH-MAb-3A4 (71–100% inhibition, mean 80%), and both antibodies inhibited the formation of M2 to varying degrees, although IH-MAb-3A4 had the greatest inhibitory effect on M2 formation in all HLM samples (53–100%, mean 58%, Figure 5).

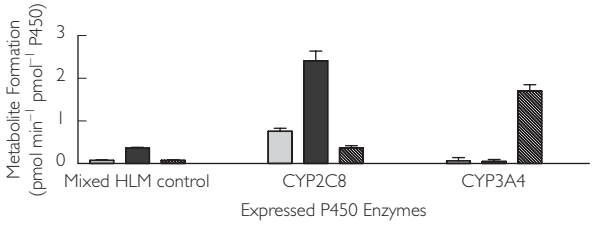


Figure 3 Repaglinide metabolite formation in Supersomes™ from insect cells transfected with individual human CYP enzymes (50 pmol) and CYP reductase (mean ± SD, $n = 3$ incubations). M0-OH (▭); M4 (▬); and M1 (▩).

Discussion

This study confirmed that the *in vitro* biotransformation of repaglinide is NADPH dependent and that the enzymes CYP3A4 and CYP2C8 mediate to varying degrees the formation of five of its metabolites, M0-OH, M4, M1, M2 and M5. M1, M2 and M5 are predominantly formed by CYP3A4 and M0-OH and M4 by CYP2C8. In Supersomes™ from insect cells transfected with individual human P450 enzymes, only CYP3A4 & CYP2C8 showed the capability to mediate repaglinide biotransformation. Under the incubation conditions used (15 min incubation) only three metabolites (M0-OH, M4 and M1) were formed, but if incubations were allowed to proceed for 120 min, metabolite M2 was formed in sufficient quantities to be also detected by HPLC with on-line radiochemical detection. M5 was detected after incubation of repaglinide with CYP3A4 Supersomes™ and analysis by LC-MS and LC-MS-NMR. The results of repaglinide biotransformation in Supersomes™ revealed M4 resulting from hydroxylation on the piperidine ring to be the major *in vitro* metabolite formed by CYP2C8 and M1 (an aromatic amine) to be the major *in vitro* metabolite formed by CYP3A4. This is confirmed by the correlation of M4 formation with paclitaxel 6α-hydroxylation, which is catalyzed by CYP2C8 [14], and the correlation of M1 with testosterone 6β-hydroxylation, which is catalyzed by CYP3A4.

M0-OH has not previously been identified *in vitro* or *in vivo*. Its formation results from hydroxylation on the isopropyl moiety. CYP2C8 is the principal enzyme responsible for the formation of M0-OH, but CYP3A4 also contributed. Similarly for the other repaglinide metabolites CYP2C8 or CYP3A4 is the main contributor to biotransformation, with the other enzyme having a minor role.

The inhibition experiments confirm M1 to be a CYP3A4 metabolite as the inhibitory monoclonal antibodies against CYP3A4 (IH-MAB-3A4) almost completely inhibited the formation of M1 in all the

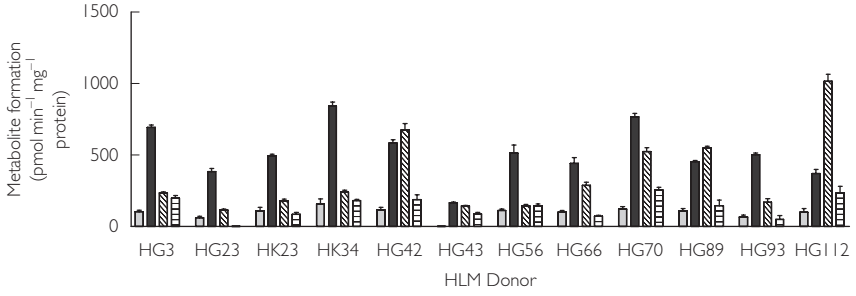


Figure 4 Repaglinide metabolism in a panel of 12 individual HLM samples (mean ± SD, $n = 3$ incubations). M0-OH (▭); M4 (▬); M1 (▩); and M2 (▤).

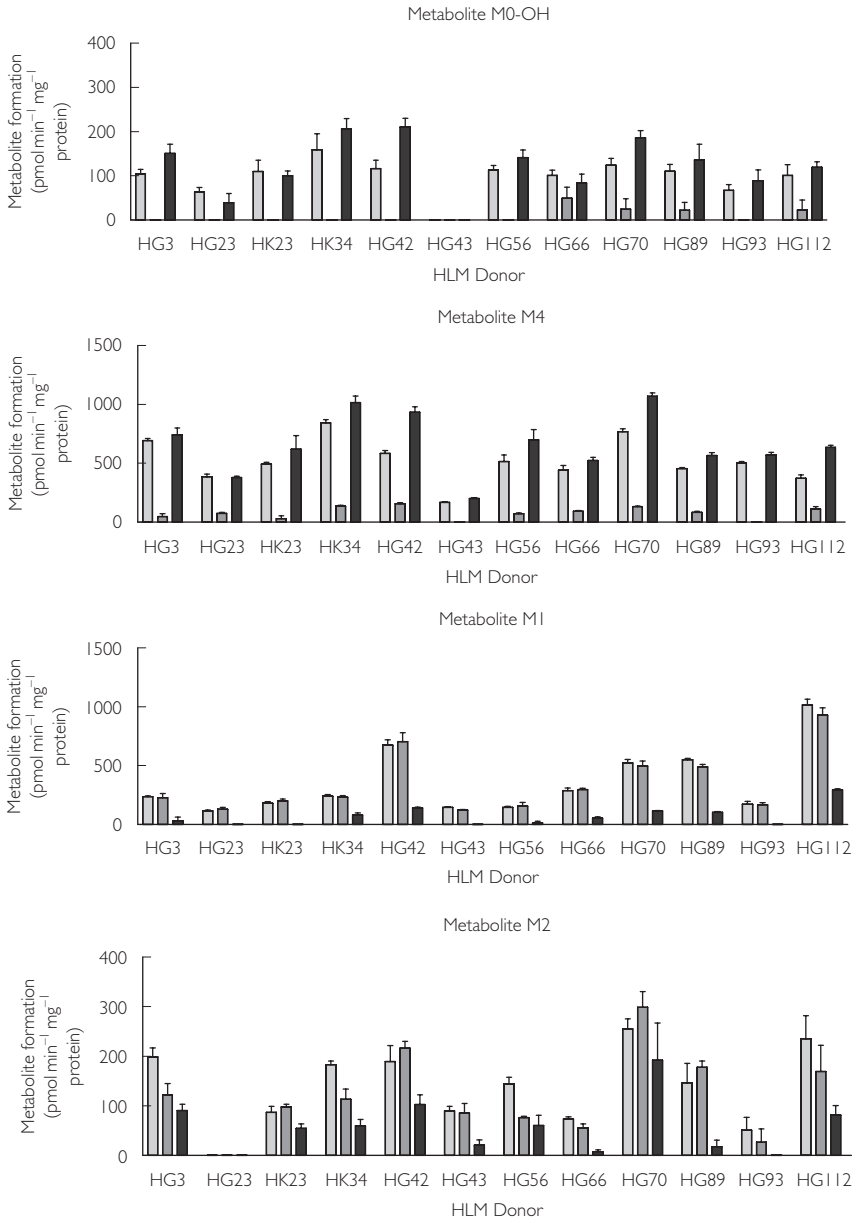


Figure 5 The effect of specific inhibitory monoclonal antibodies against CYP3A4 (IH-MAb-3A4) and CYP2C8 (IH-MAb-2C8) on the formation of repaglinide metabolites. Values are shown as metabolite formation in pmol min^{-1} mg^{-1} protein (mean ± SD, $n = 3$) for each individual HLM sample ($n = 12$). Preimmune IgG (▭); IH-MAb-2C8 (▨); and IH-MAb-3A4 (▬).

individual HLM samples. In previous studies M2 has been identified as the major human metabolite excreted in faeces, whereas M1 and M2 together with unidentified polar compounds are the major human metabolites excreted in urine [7]. In the present *in vitro* study M2 was formed but not as the major metabolite in any of the 12 HLM samples. This could be explained by the formation of the aromatic amine M1 by N-dealkylation of M2 [25] to a greater extent *in vitro*.

The metabolism of repaglinide by both CYP2C8 and CYP3A4 may explain why selective inhibition and possibly induction of CYP3A4 activity in humans *in vivo* has a less than predicted effect on repaglinide pharmacokinetics and effect.

Ketoconazole at low concentrations is used as a selective CYP3A4 inhibitor but it also inhibits CYP2C8, although at higher concentrations [21]. *In vivo* studies have investigated the effect of multiple dosing of ketoconazole on repaglinide pharmacokinetics and pharmacodynamics. No clinically relevant influence of ketoconazole administration on the blood glucose concentration or on the adverse event profile of repaglinide

was detected [10]. This can perhaps be explained by the possibility that ketoconazole, even after multiple dosing, does not reach the concentrations (approximately 10 μM) in the human liver necessary to inhibit CYP2C8 effectively [21]. However, clarithromycin, a mechanism-based inhibitor of CYP3A4 [27] increases the plasma concentration of repaglinide after 4 days of clarithromycin administration [12]. Therefore predictions of *in vivo* drug-drug interactions may require a consideration of their mechanism of inhibition.

A recent study showed some decrease in the plasma concentration of repaglinide after rifampicin administration for 5 days and repaglinide administration on day 6 [11]. However, another study showed no effect of rifampicin administration for 7 days on the pharmacokinetic or pharmacodynamic effects of repaglinide [28].

In the present study we showed that repaglinide is metabolized mainly by CYP2C8 and CYP3A4. Variation in the activities of these enzymes may lead to a better understanding of the difference in AUC and C_{max} observed within patient groups treated with repaglinide [26]. Metabolism by both these enzymes may also explain why selective inhibition and possibly induction of CYP3A4 in humans has a less than expected effect on repaglinide pharmacokinetics and effect.

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