Repaglinide-gemfibrozil drug interaction: inhibition of repaglinide glucuronidation as a potential additional contributing mechanism

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Co-administration of gemfibrozil significantly increases the exposure of repaglinide.
- CYP3A4 and CYP2C8 are important in the metabolism of repaglinide.
- OATP1B1 polymorphism is an independent predictor of repaglinide pharmacokinetics.
- Gemfibrozil and its O-glucuronide are CYP2C8 and OATP1B1 inhibitors.

WHAT THIS STUDY ADDS

- Acyl glucuronidation is a major metabolic pathway of repaglinide *in vitro*.
- Gemfibrozil and its glucuronide inhibit the glucuronidation of repaglinide.
- UGT1A1 is a major enzyme responsible for the glucuronidation of repaglinide.

AIM

To further explore the mechanism underlying the interaction between repaglinide and gemfibrozil, alone or in combination with itraconazole.

METHODS

Repaglinide metabolism was assessed *in vitro* (human liver subcellular fractions, fresh human hepatocytes, and recombinant enzymes) and the resulting incubates were analyzed, by liquid chromatography-mass spectrometry (LC-MS) and radioactivity counting, to identify and quantify the different metabolites therein. Chemical inhibitors, in addition to a trapping agent, were also employed to elucidate the importance of each metabolic pathway. Finally, a panel of human liver microsomes (genotyped for UGT1A1*28 allele status) was used to determine the importance of UGT1A1 in the direct glucuronidation of repaglinide.

RESULTS

The results of the present study demonstrate that repaglinide can undergo direct glucuronidation, a pathway that can possibly contribute to the interaction with gemfibrozil. For example, [³H]-repaglinide formed glucuronide and oxidative metabolites (M2 and M4) when incubated with primary human hepatocytes. Gemfibrozil effectively inhibited (~78%) both glucuronide and M4 formation, but had a minor effect on M2 formation. Concomitantly, the overall turnover of repaglinide was also inhibited (~80%), and was completely abolished when gemfibrozil was co-incubated with itraconazole. These observations are in qualitative agreement with the *in vivo* findings. UGT1A1 plays a significant role in the glucuronidation of repaglinide. In addition, gemfibrozil and its glucuronide inhibit repaglinide glucuronidation and the inhibition by gemfibrozil glucuronide is time-dependent.

CONCLUSIONS

Inhibition of UGT enzymes, especially UGT1A1, by gemfibrozil and its glucuronide is an additional mechanism to consider when rationalizing the interaction between repaglinide and gemfibrozil.

Introduction

Repaglinide is an insulin secretagogue used in the treatment of type 2 diabetes and a drug interaction with gemfibrozil, with or without itraconazole, has been reported [1]. For example, gemfibrozil significantly increases repaglinide area under the concentration vs. time curve (AUC) and maximum plasma concentration (C_{max}) 8.1-fold and 2.4-fold, respectively. When co-dosed with itraconazole, the increase in repaglinide AUC is even greater (19.4-fold). The results of additional clinical studies implicate inhibition of cytochrome P450 (CYP) 3A4 in the case of itraconazole, and inhibition of CYP2C8 and organic anion transporting peptide 1B1 (OATP1B1, SLCO1B1) in the case of gemfibrozil, although the effect of the CYP2C8*3 allele remains controversial [1–5].

Although gemfibrozil is a weak inhibitor of CYP2C8 and OATP1B1 *in vitro*, its 1-O-glucuronide has been shown to be a potent mechanism-based inhibitor of CYP2C8 and a good inhibitor of OATP1B1 [6, 7]. *In vitro* studies have

confirmed also that CYP2C8 plays an important role in the oxidative metabolism (M4 formation) of repaglinide (Figure 1) [8, 9]. In agreement, co-administration of gemfibrozil significantly reduces the plasma exposure of M4 and the ratio of M4 : repaglinide [10, 11]. However, the results of all the *in vitro* studies so far suggest that CYP3A, rather than CYP2C8, is more important in the oxidative metabolism of repaglinide [8, 9]. This is inconsistent with the *in vivo* effect of co-administered CYP3A4 inhibitors (e.g. itraconazole, ketoconazole and ciclosporin A) on repaglinide exposure (~two-fold increase in AUC) [10, 12, 13]. This contradiction is yet to be explained.

The results of a clinical radiolabel study have demonstrated that the dicarboxylic acid metabolite (M2) is a major metabolite of repaglinide following an oral dose of 2 mg [14 C]-repaglinide (Figure 1). In these human volunteers, about 66% of administered repaglinide is excreted as M2 in the faeces and urine [14]. However, the predominance of M2 production cannot explain the observed interaction between repaglinide and gemfibrozil, because

Figure 1

Proposed biotransformation pathways for repaglinide with enzymes responsible for each pathway. The bold texts represent the major enzyme involved in the reaction

M2 formation is believed to be primarily CYP3A4-driven and gemfibrozil is not a significant inhibitor of the enzyme [6].

In the present study, an attempt was made to assess the metabolism of repaglinide in more complete systems, including liver 9000 *g* supernatant (S9) fraction and primary hepatocytes. Such systems contain the requisite microsomal and cytosolic drug-metabolizing enzymes and can enable a more integrated view of repaglinide metabolism *in vitro*. In turn, the data were used to rationalize further the mechanisms underlying the observed drug interaction with gemfibrozil, alone or in combination with itraconazole. During the course of the study, suspensions of primary human hepatocytes were shown to form a repaglinide glucuronide and an effort was made to characterize further the effect of gemfibrozil on its formation.

Methods

Materials

Human liver microsomes (HLM), human liver 9000 *g* supernatant (S9) fraction (HLS), human liver cytosol (HLC), recombinant human UDP-glucuronosyltransferases (UGT) and CYPs were purchased from BD Gentest (Woburn, MA). Repaglinide, *d*₅-repaglinide and gemfibrozil 1-*O*-glucuronide were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Rifamycin SV was purchased from MP Biomedicals (Solon, OH). Gemfibrozil, montelukast, ketoconazole, NADPH, UDPGA and alamethicin were obtained from Sigma-Aldrich (Milwaukee, WI). Primary human hepatocytes were obtained from CellZDirect (Durham, NC). All other reagents and solvents were analytical grade or better. All stock solutions were prepared in DMSO and then diluted in acetonitrile to appropriate concentrations to ensure less than 1% organic solvent concentration in the final incubations.

Synthesis of [³H]-repaglinide

[³H]-repaglinide was prepared by the Radiochemistry group at Bristol-Myers Squibb (Princeton, NJ). Briefly, an iodinated analogue of repaglinide was synthesized from repaglinide followed by de-iodination with carrier-free tritium gas. The resulting tritium label is on the aromatic ring of the benzoic acid moiety which is metabolically stable based on literature reports on repaglinide metabolism. [³H]-repaglinide was purified by HPLC to give a final product with a specific radioactivity of 20.3 Ci mmol⁻¹ and radiochemical purity of 99.73%.

Incubations with liver fractions in the presence of NADPH

In all incubations, the organic solvent concentrations were kept less than 1%. Various concentrations of repaglinide were incubated in 0.1 M potassium phosphate buffer (pH 7.4) with HLS (2 mg ml⁻¹), CYP3A4 (25 pmol ml⁻¹), CYP2C8

(25 pmol ml⁻¹), HLC (2 mg ml⁻¹) and the combinations of CYP3A4 or CYP2C8 with HLC in the presence or absence of NADPH (1 mM) for 45 min at 37°C (1 ml total incubation volume). Some incubation included 1 mM potassium cyanide (KCN) as trapping agent. In all cases, the incubation was stopped by addition of acetonitrile (1 ml) containing *d*₅-repaglinide as internal standard. The mixture was centrifuged (3000 *g*, 20 min) and an aliquot (10 µl) of the resulting supernatant was subjected to LC-MS analysis (see below).

To assess the kinetics of M2 formation, repaglinide (various concentrations) was mixed with HLC (2 mg ml⁻¹) and CYP3A4 or CYP2C8 (25 pmol ml⁻¹) in the presence of 1 mM NADPH and incubated for 5 min. As described above, the incubation was stopped by addition of acetonitrile (1 ml) containing *d*₅-repaglinide as internal standard. The mixture was centrifuged (3000 *g*, 20 min) and an aliquot (10 µl) of the resulting supernatant was subjected to LC-MS analysis (see below).

Incubations with HLM in the presence of UDPGA

Repaglinide (0.2 µM) was incubated in 0.1 M potassium phosphate buffer (pH 7.4) with HLM (0.5 mg ml⁻¹) or a panel of recombinant UGTs (0.25 mg ml⁻¹) in the presence of alamethicin (50 µg ml⁻¹) and UDPGA (5 mM) for 45 min at 37°C. The total incubation volume was 1 ml. The incubations were terminated and sample processed as described above.

For the assessment of time-dependent inhibition of RG formation by gemfibrozil 1-*O*-glucuronide, various concentrations (up to 300 µM) gemfibrozil 1-*O*-glucuronide were pre-incubated in 0.1 M potassium phosphate buffer (pH 7.4) with 0.5 mg ml⁻¹ HLM or 0.25 mg ml⁻¹ UGT1A1 in the presence of alamethicin (50 µg ml⁻¹) at 37°C for 25 min. The reaction was then started with the addition of 0.2 µM repaglinide and 5 mM UDPGA. Parallel incubations without the 25 min pre-incubation were conducted for comparison.

Incubations with primary human hepatocytes

Repaglinide (0.1 µM) was incubated with fresh human hepatocytes (1 million ml⁻¹) suspended in L-15 medium (Sigma, Milwaukee, WI) in the presence of solvent alone, 100 µM gemfibrozil, 3 µM itraconazole, a combination of gemfibrozil and itraconazole, or 75 µM rifamycin SV for 2 h at 37°C. The total incubation volume was 2 ml. To assess the effect of gemfibrozil pre-incubation, 100 µM gemfibrozil or solvent alone was pre-incubated with hepatocytes for 30 min before the addition of 0.1 µM repaglinide. The incubations were terminated and sample processed as described above.

Incubations with [³H]-repaglinide

[³H]-repaglinide (0.49 µM, 10 µCi) was incubated with HLS (2 mg ml⁻¹) and HLM (0.5 mg ml⁻¹) in the presence of NADPH (1 mM) in 0.1 M phosphate buffer (pH 7.4) or fresh

human hepatocytes (1 million cells ml^{-1}) in L-15 medium at 37°C. The total incubation volume was 1 ml for HLS and HLM incubations and 2 ml for hepatocyte incubation. After 45 min (HLS and HLM) or 2 h (hepatocytes), the metabolic reaction was stopped by the addition of 1 ml acetonitrile. Samples were centrifuged (3000 g , 20 min) and an aliquot of supernatant (50 μl) was subjected to analysis, 25% of the sample to LC-MS and 75% collected through a fraction collector into a 96-well Lumi-plate for TopCount radioanalysis.

Analytical methods

The LC/MS system consisted of an HPLC system and an Applied Biosystems/Sciex API-4000 Q-trap with an electrospray ionization source (Ontario, Canada). The HPLC system consisted of two Shimadzu LC-10ADvp pumps with a SCI-10ADvp controller (Columbia, MD) and Agilent 1100 well-plates autosampler (Agilent Technologies, Palo Alto, CA). Metabolites were separated on a Waters Atlantis C18 column (150 $\times$ 2.1 mm, 5 μm , Waters, Milford, MA) using 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.30 ml min^{-1} . The gradient started from 20% B to 60% B in 13 min, followed by 60% B to 90% B in 0.5 min and was maintained at 90% B for 2 min prior to column re-equilibration. Metabolites, parent drug and internal standard were monitored at MRM transition of m/z 451.3 > 228.3 (m/z 451 metabolites), m/z 469.3 > 246.2 (M4), m/z 485.3 > 262.2 (M2), m/z 453.3 > 230.3 (parent), and m/z 458.3 > 230.3 (IS) using positive ion electrospray ionization.

A Phenomenex Synergi hydro-RP column (250 $\times$ 4.6 mm, 4 μm , Torrance, CA) was used to separate the metabolites for radioanalysis. The gradient started from 20% B to 60% B in 13 min, followed by 60% B to 90% B in 7 min and was maintained at 90% B for 2 min prior to column re-equilibration. Fractions were collected into 96 wells and counted in a Perkin Elmer TopCount.

A shorter analytical method was used to monitor the formation of repaglinide glucuronide (RG). RG and internal standard were separated out on a Waters Atlantis C18 column (50 $\times$ 2.1 mm, 5 μm , Waters, Milford, MA) using 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.30 ml min^{-1} . The gradient started from 10% B to 90% B in 2.5 min and was maintained at 90% B for 0.5 min prior to column re-equilibration. Total run time was 5 min. RG and internal standard were monitored at MRM transitions of m/z 629.3 > 453.3 and m/z 458.3 > 230.3, respectively, using positive ion electrospray ionization.

Results

M2 formation

Incubations of [^3H]-repaglinide with NADPH-fortified HLM generated mainly M4 (m/z 469) and two additional peaks

with a mass of m/z 451. The latter represent oxidative dehydrogenation products of repaglinide (Figure 2A). The production of M2 (m/z 485) was not prominent. However, in the presence of HLS, M2 production was much higher at the expense of the m/z 451 metabolites (Figure 2B).

To assess the contribution of cytosolic enzymes in the production of M2, recombinant CYP3A4 and CYP2C8 were co-incubated with HLC in the presence of NADPH. For both recombinant CYP3A4 and CYP2C8, fortification with cytosol generated a major peak corresponding to M2 (Figure 2C). Incubation with NADPH-fortified cytosol alone produced a negligible amount of M2 (data not shown). Upon addition of cyanide, M2 production was completely inhibited, indicating that a cyanide-trappable intermediate was involved in the formation of M2 (Figure 2C).

To illustrate further the role of CYP3A4 and CYP2C8 in M2 formation, repaglinide was incubated with HLS at three different concentrations with or without ketoconazole and montelukast, typical inhibitors of CYP3A and CYP2C8, respectively [15, 16]. The addition of ketoconazole potentially inhibited the formation of M2 by ~70%, regardless of the initial repaglinide concentration. In contrast, the addition of montelukast only had minimal effect (<20% inhibition) on the formation of M2 (Figure 2D).

Finally, in order to explore possible kinetic differences, recombinant CYP3A4 and CYP2C8 (50 pmol ml^{-1} each) were incubated with HLC (2 mg ml^{-1}) over a wide repaglinide concentration range (0.09 μM to 200 μM). Under these conditions, the formation of M2 was linear with respect to both protein concentration and time. For both CYPs, M2 formation rate was not saturated up to a repaglinide concentration of 200 μM (Figure 3).

Hepatocyte studies

Incubation of [^3H]-repaglinide (0.5 μM) with human hepatocytes resulted in the formation of three major metabolites, M2, M4 and repaglinide glucuronide (RG), which accounted for 11, 13 and 12% of the total initial radioactivity, respectively (Figure 4A). The collision-induced dissociation spectrum of RG is shown in Figure 4B.

The addition of gemfibrozil, itraconazole and the combination of both inhibitors differentially inhibited the metabolism of repaglinide in human hepatocytes (Table 1). For example, gemfibrozil inhibited M4 (78%) and RG (79%) formation, and repaglinide turnover by 80%. Itraconazole inhibited repaglinide turnover by 52%. The combination of gemfibrozil and itraconazole completely abolished repaglinide turnover. Pre-incubation of gemfibrozil resulted in similar magnitude of inhibition as gemfibrozil without pre-incubation. The addition of an OATP1B1 inhibitor, rifamycin SV [17], inhibited the turnover of repaglinide by 26%.

Repaglinide glucuronide formation

When repaglinide was incubated with a panel of recombinant UGTs, several (UGT1A1, 1A3, 1A7, 1A8 and

Figure 2

M2 formation in the presence of CYP3A4, 2C8, HLM, HLS, and mixtures of cytosol and CYP3A4/2C8. Data represent the average of duplicate measurements. A) radiochromatograms of [^3H]-repaglinide (0.49 μM) incubated with HLM (0.5 mg mL^{-1}) in the presence of NADPH. B) radiochromatogram of [^3H]-repaglinide (0.49 μM) incubated with HLS (2 mg mL^{-1}) in the presence of NADPH. C) relative activities of M2 formation in different enzymatic systems in the absence or presence of cyanide. D) Inhibition of M2 formation in HLS by ketoconazole and montelukast (1 μM each). Control (■); montelukast (1 μM) (□); ketoconazole (1 μM) (□)

Figure 3

Kinetics of M2 formation in mixtures of HLC (2 mg mL^{-1}) with 25 pmol mL^{-1} of either CYP3A4 or CYP2C8. The units on the Y-axis are LC/MS peak area ratios of M2 vs. internal standard. CYP3A4 (25 pmol) + HLC (2 mg mL^{-1}) (■); CYP2C8 (25 pmol) + HLC (2 mg mL^{-1}) (▲)

1A9) were able to generate RG. The relative activity on the per mg protein basis was in the order of UGT1A3 > UGT1A1 > UGT1A8 > UGT1A9 > UGT1A7, with low levels of activity detected with UGT2B7 and UGT2B17 (Figure 5). Similar to M2 formation, under linear kinetic conditions, both UGT1A1- and UGT1A3-catalyzed RG formation was not saturated up to 200 μM repaglinide (data not shown).

In order to investigate further the importance of UGT1A1 in the glucuronidation of repaglinide, a panel of HLM genotyped for the *UGT1A1**28 allele was used. Namely, microsomes from 15 genotyped donors (five each carrying *UGT1A1**1*1, *UGT1A1**1*28, or *UGT1A1**28*28) were used in the incubations. As shown in Figure 6A, the rate of RG formation was significantly lower in microsomes from *28 homozygous subjects than the rates in microsomes from wild-type and *1*28 heterozygotes (46% and 62%, respectively). In summary, UGT1A1 plays a significant role in the glucuronidation of repaglinide.

Figure 4

Radiochromatogram of [³H]-repaglinide (0.49 μM) incubated with 1 million cells mL^{-1} fresh human primary hepatocytes in suspension for 2 h (A) and the MS2 spectra of RG (B)

Table 1

The inhibitory effects of gemfibrozil and itraconazole on the depletion of repaglinide (0.1 μM initial concentration) formation of M2, M4 and repaglinide glucuronide (RG) in fresh hepatocyte incubations (1 million cells mL^{-1})

Inhibitor	% Inhibition* Repaglinide turnover	RG	M4	M2
Gemfibrozil (100 μM)	80%	79%	78%	22%
Itraconazole (3 μM)	52%	19%	2%	35%
Rifamycin SV (75 μM)	26%	32%	-40%	-26%
Gemfibrozil + itraconazole	108%	78%	80%	85%
Gemfibrozil pre-incubation	71%	67%	86%	18%

*Data expressed as % of control (solvent alone) incubations. All experimental data are the average of duplicate measurements.

The formation of RG in HLM was inhibited by gemfibrozil with an IC_{50} value of 113 μM (Figure 7A). In incubations with recombinant UGT1A1, 1A3, 1A8 and 1A9, the addition of 100 μM gemfibrozil inhibited at least 50% of the

glucuronidation activity of repaglinide by all of these UGT isoforms (Figure 7B). This is consistent with earlier reports describing gemfibrozil inhibition of UGT1A1- and UGT1A3-catalyzed statin glucuronidation [18].

Lastly, the formation of RG in HLM and UGT1A1 was inhibited by gemfibrozil 1-O-glucuronide in a time-dependent manner (Figure 8). Without pre-incubation, gemfibrozil 1-O-glucuronide only marginally inhibited RG formation in both HLM and UGT1A1. However, pre-incubation for 25 min enhanced the inhibition potency with IC_{50} s of 130 and 69 μM for HLM and UGT1A1, respectively. LC/MS monitoring of gemfibrozil and gemfibrozil 1-O-glucuronide in the pre-incubated samples confirmed the presence of gemfibrozil 1-O-glucuronide and the absence of gemfibrozil, thus excluding the possibility of the potential breakdown of gemfibrozil 1-O-glucuronide to gemfibrozil leading to the observed inhibition of RG formation.

Discussion

The interaction between repaglinide and gemfibrozil has garnered considerable attention since the initial clinical report in 2003 [10], and numerous attempts have been made to rationalize it mechanistically [4–7, 9, 11, 19, 20]. At the present time, the interaction is attributed to inhibition of CYP2C8 and OATP1B1 by a combination of gemfibrozil and its 1-O-glucuronide. However, careful review of the existing literature reveals that the observed drug interaction cannot be explained using currently available *in vitro* data. This is further exacerbated by the fact that even though repaglinide is recommended by the US FDA as a preferred *in vivo* probe for the assessment of CYP2C8 inhibition [21], there has not been any convincing *in vitro* data to justify this recommendation.

In the presence of HLM, a relatively high concentration of repaglinide (20 μM) is known to be oxidized *in vitro* to multiple oxidative metabolites [8], such as M4, a carboxylic acid metabolite (M2) and a dealkylated primary aniline metabolite (M1). Formation of M2 and M1 is attributed to CYP3A4, whereas the formation of M4 is attributed to CYP2C8.

Therefore, the present study was conducted with an initial focus on the formation of M2, which is reported as the major metabolite in humans [14]. Because the formation of M2 from repaglinide is most likely a multi-step reaction, an attempt was made to dissect its formation pathway by using a combination of liver subcellular fractions in conjunction with a small molecule trapping agent. Because of the low dose and low plasma concentrations of repaglinide observed in the clinical studies, the lowest (sub- μM) concentrations of repaglinide allowable by the assay sensitivity were employed throughout the study. Based on the data presented herein, it is proposed that repaglinide undergoes CYP3A4-catalyzed metabolism to a

Figure 5

Repaglinide glucuronidation activities of different recombinant human UGT enzymes. Repaglinide ($0.2 \mu\text{M}$) was incubated with 0.25 mg ml^{-1} UGT for 45 min. Data represent the average of duplicate incubations. The units on the Y-axis are LC/MS peak area ratios of RG vs. internal standard

cyanide trappable intermediate (iminium or an aldehyde), which can be further oxidized by an unidentified cytosolic enzyme(s) to M2. In agreement with previous studies [8], the study employing chemical inhibitors demonstrated that CYP3A4 is the primary enzyme ($\sim 70\%$) to catalyze the initial reaction leading to M2 formation whereas CYP2C8 plays a relatively minor role (Figure 2D). So while M4 is catalyzed by CYP2C8 and is detectable in plasma [11], CYP3A4-catalyzed M2 is major *in vivo*.

Although the formation of repaglinide glucuronide has not been reported in detail, based on the results of the present study, its formation is proposed (Figure 4A). A review article by Hatorp [22] mentioned an acyl glucuronide as a major human metabolite after oral dosing of [^{14}C]-repaglinide. However, the cited article by van Heiningen *et al.* [14] did not describe the acyl glucuronide at all. Consistent with the review paper by Hatorp, the acyl glucuronide metabolite was listed as a major metabolite (M7) of repaglinide in the product label of Prandin® [23]. M7 was listed as one of the metabolites in the van Heiningen *et al.* article, but without description of its identity. Furthermore, other insulin secretagogues like nateglinide and mitoglinide have been shown to form acyl glucuronides [24, 25]. Most of the radioactivity was excreted in the faeces in the reported radiolabel study [14]. Given the potential instability of acyl glucuronide in the gut, it could be challenging to recover any acyl glucuronide excreted into the gastrointestinal tract. Therefore, RG is very likely formed and could represent a major metabolic pathway *in vivo*.

In this study, we found that repaglinide is a good substrate of UGT1A1 and UGT1A3 among other UGTs leading to the formation of a glucuronide metabolite, RG. Interestingly, gemfibrozil inhibited the formation of RG with an estimated IC_{50} of $113 \mu\text{M}$. The incubations with individual human isolated fresh hepatocytes clearly demonstrated the formation of RG. With the help of a ^3H tracer, it was found

that RG was formed in human hepatocytes in a substantial amount (1/3 of total metabolism). In human hepatocytes, the addition of $100 \mu\text{M}$ gemfibrozil inhibited both M4 and RG formation by 78 and 79%, and the overall turnover of repaglinide by 80% (Table 1). In contrast, the addition of itraconazole ($3 \mu\text{M}$) did not exhibit substantial inhibition of M4 and RG formation, but inhibited the overall turnover by 52%. This largely reflected the inhibition of M2 formation (35%) (Table 1), consistent with the role of CYP3A4 in the reaction. As would be expected, the combination of gemfibrozil and itraconazole abolished the metabolism of repaglinide in human hepatocytes. All of these are consistent with the magnitude of clinical interactions between repaglinide and gemfibrozil/itraconazole. The addition of the prototypical OATP1B1 inhibitor, rifamycin SV, to the hepatocyte incubations resulted in some inhibition of repaglinide turnover (26%). This magnitude of interaction is in qualitative agreement with clinical observations of the relationship between repaglinide exposure and OATP1B1 polymorphism. For example, in subjects with the SLCO1B1 521CC genotype, the $\text{AUC}(0,\infty)$ of repaglinide was 107% and 188% higher, respectively, than in subjects with the SLCO1B1 521TC or 521TT genotype [5].

The IC_{50} value of gemfibrozil in the inhibition of RG is comparable with the reported total maximum plasma concentration of gemfibrozil ($150 \mu\text{M}$) after repeated dosing of 600 mg twice daily [26]. However, the IC_{50} value is much higher than the free plasma gemfibrozil concentration given its high binding to plasma proteins. On the other hand, the liver concentration of gemfibrozil is unknown but could be much higher than the plasma concentration. Therefore, the inhibition of RG formation is likely to contribute to the observed clinical drug–drug interaction between gemfibrozil and repaglinide. In addition to the direct inhibition of RG formation by gemfibrozil, gemfibrozil-1-O-glucuronide inhibited RG formation in a

Figure 6

A) Repaglinide glucuronidation activities in a panel of individual HLM from 15 donors categorized into three groups based on *UGT1A1* allele status. *P* values shown are from two-tailed unpaired *t*-test. B) Scatter plot of RG activity vs. β -estradiol-3-O-glucuronidation activity in a panel of HLM. E3G: β -estradiol-3-O-glucuronide. E3G activity was determined previously in the same set of HLM by Zhang *et al.* [31]. The units on the Y-axis are LC/MS peak area ratios of RG vs. internal standard. *1* (○); *1*28 (■); *28* (△)

time-dependent fashion with apparent IC_{50} s of 130 and 69 μ M after 25 min of pre-incubation in HLM and UGT1A1, respectively. The exact mechanism of the time-dependent inhibition is unclear and further characterization of this phenomenon is underway. The total plasma concentration of gemfibrozil 1-O-glucuronide was reported to be 20 μ M, but the liver concentration is likely to be much higher with literature estimated free liver concentrations up to 83 μ M [6, 7]. The IC_{50} values reported here could, therefore, be relevant to clinical exposures. Nevertheless, the time-dependent inhibition of both CYP2C8 and UGT1A1 by gemfibrozil 1-O-glucuronide could explain the lasting effect of gemfibrozil on the pharmacokinetics of repaglinide [11, 27].

In this study, UGT1A3 showed slightly higher activity in RG formation than UGT1A1 on a per mg protein basis.

Figure 7

A) The inhibition of repaglinide (0.2 μ M) glucuronidation in HLM (0.5 mg ml⁻¹) by various concentrations of gemfibrozil. B) the inhibition of repaglinide glucuronidation in selected recombinant UGTs by gemfibrozil (100 μ M). Data represent the average of duplicate incubations

Earlier studies on liver UGT mRNA concentrations and limited data on protein expression levels in the liver have established that UGT1A1 is likely to be expressed in a much higher amount than UGT1A3. However, because the UGT2B family has high expression levels in the liver, their contribution to RG formation cannot be discounted, even though UGT2B7 and UGT2B17 showed much lower activity than UGT1A1 on a per mg protein basis [28–30]. In order to understand better the role of UGT1A1 in the glucuronidation of repaglinide, we used a panel of UGT1A1 genotyped HLMs including five each of *UGT1A1**1*1, *1*28 and *28*28 [31]. Although the difference in activity between UGT1A1 wild-type and *28 heterozygotes was not statistically significant, the glucuronidation activity of the *28 homozygotes was significantly lower (*P* = 0.008 vs. wild-type; *P* = 0.03 vs. heterozygotes). Furthermore, an excellent correlation was found between repaglinide glucuronidation and β -estradiol-3-O-glucuronidation (typical UGT1A1 marker activity) in this set of HLM (Figure 6B) [31]. Thus, UGT1A1 is an important contributor to the glucuronidation of repaglinide. It is concluded that UGT1A1 could play an

Figure 8

The inhibition of repaglinide (0.2 μM) glucuronidation in HLM (0.5 mg ml⁻¹) and UGT1A1 (0.25 mg ml⁻¹) at various concentrations of gemfibrozil 1-O-glucuronide with or without 25 min pre-incubation. Data represent mean and standard deviations of triplicate incubations. A) HLM with pre-incubation, B) HLM without pre-incubation, C) UGT1A1 with pre-incubation and D) UGT1A1 without pre-incubation

important role in the clearance of repaglinide in addition to CYP2C8 and CYP3A4.

As with any other *in vitro* studies, this study is not without limitations. Because of the assay sensitivity constraints, the repaglinide concentrations were at least 100 times more than what was observed in the clinic at relevant doses. Although linear kinetics was observed for both M2 and RG formation in this study, the supraphysiological concentration of repaglinide may provide misleading results. Furthermore, the inhibitory potency of both gemfibrozil and its glucuronide on RG formation is relatively weak in comparison with the CYP2C8 inhibitory potency of gemfibrozil 1-O-glucuronide [7]. Thus the clinical impact of the time-dependent inhibition on the RG formation is unknown but it can be investigated by monitoring the circulating RG concentrations in clinical samples.

In summary, we herein describe for the first time the glucuronidation of repaglinide *in vitro* as an

important clearance pathway that is inhibited by high concentrations of gemfibrozil and its 1-O-glucuronide in a time-dependent manner. The glucuronidation is mainly catalyzed by UGT1A1. Therefore, any attempt to use *in vitro* data to model the repaglinide interaction with gemfibrozil will have to take into account the inhibition of not just CYP2C8 and OATP [19], but also UGT1A1. At the same time, the differential effects of gemfibrozil and its glucuronide on all three components has to be taken into account. When combined with itraconazole, the effect of gemfibrozil on the AUC of repaglinide is marked (17-fold) [10]. Such an interaction likely reflects 'supra-proportional' inhibition, resulting from the combined effects of CYP3A (itraconazole), OATP, CYP2C8 (gemfibrozil and its 1-O-glucuronide) and UGT (gemfibrozil and its 1-O-glucuronide) inhibition. At the present time, the impact of UGT1A1 polymorphism on the magnitude of the interaction is unknown and warrants further study.

Competing interests

There are no competing interests to declare. The authors alone are responsible for the content and writing of the paper.

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