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Abstract

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The role of human CYP2C8 and CYP2C9 variants in pioglitazone metabolism in vitro

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Key words: pioglitazone, CYP2C8, CYP2C9, pharmacogenetics
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Introduction The cytochrome P450 enzyme CYP2C8 appears to have a major role in pioglitazone metabolism. The present study was conducted to further clarify the role of individual CYPs and of the CYP2C8/9 polymorphisms in the primary metabolism of pioglitazone in vitro.

Methods Pioglitazone (2-400 µM) was incubated with isolated cytochrome P450 enzymes or human liver microsomes, some of them carrying either the CYP2C8*3/*3 genotype (and also the CYP2C9*2/*2 genotype) or the CYP2C8*1/*1 genotype (5 samples each). The formation of the primary pioglitazone metabolite M-IV was monitored by HPLC. Enzyme kinetics were estimated assuming a single binding site.

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Conclusion CYP2C8, CYP1A2 and CYP2D6 are major CYPs forming M-IV in vitro. The higher activity of CYP2C8*3/CYP2C9*2 microsomes may result from a contribution of CYP2C9*2, or from differences in CYP2C8 expression. Substrate specific inhibitory effects of pioglitazone on the CYP2C mediated metabolism of arachidonic acid in the formation of epoxyeicosatrienic acids would be a mechanism explaining some adverse effect of glitazones. These hypotheses need to be tested in further studies.
Introduction

Pioglitazone is a thiazolidinedione insulin sensitizer acting as an agonist on peroxisome proliferator-activated receptor gamma (PPARγ). It is usually used in combination with other oral antidiabetics. The drug is metabolized extensively in the human liver via hydroxylation and oxidation by the cytochrome P450 enzyme system. Four primary (M-I, M-II, M-IV and M-V) and two secondary metabolites (M-III and M-VI) have been described, with M-III being a product of M-IV. The main pioglitazone metabolites found in human serum are M-III and M-IV (see Figure 1) which both are pharmacologically active [1].

Information on the role of individual human CYP enzymes in pioglitazone metabolism is incomplete and partially contradictory. Jaakkola et al. [2] found that in vitro pioglitazone at a single concentration of 1 µM is metabolised primarily by CYP2C8 and to a lesser extent by CYP3A4, but as a limitation to this work no enzyme kinetic constants were reported, and data on disappearance of pioglitazone and on formation of M-IV in the incubations were not always consistent. In vivo interaction studies showed that gemfibrozil, a selective CYP2C8 inhibitor, considerably decreased pioglitazone clearance, while the potent CYP3A4 inhibitor itraconazole had no effect at all [3]. An interaction of pioglitazone with human CYP2C8 and also with CYP2C9 is also supported by studies examining the inhibitory effects of thiazolidinediones, albeit with conflicting results. While in one study in vitro, all three thiazolidinediones commercially available at this time (i.e. troglitazone, pioglitazone and rosiglitazone) were potent inhibitors of CYP2C8, with K(i) values between 1.7 and 5.6 µM [4], in another study, pioglitazone and rosiglitazone (50 µM) only slightly inhibited activities of CYP2C enzymes [5].
Assuming a major role of CYP2C8 in the metabolism of pioglitazone, individual activity of this enzyme may affect pioglitazone metabolism. CYP2C8 is genetically polymorphic and several CYP2C8 alleles causing functional effects on enzyme activity have been described [6]. The CYP2C8*3 allele encodes a protein with two linked amino acid substitutions Arg139Lys (416G>A) and Lys399Arg (1196A>G), and this protein showed markedly decreased oxidation capacity in vitro for paclitaxel (K_M and V_max could not be determined) and arachidonic acid (turnover rate diminished to 1/3) [6]. CYP2C8*3 is partially, but not completely, linked to the CYP2C9*2 variant [7]. In vivo data on the role of the CYP2C8*3 allele however are conflicting, reporting lower metabolism of R-ibuprofen [8], but no difference between carriers of the CYP2C8*1/*1 genotype and CYP2C8*3/*3 in 97 patients treated with paclitaxel [9]. In a clinical study with repaglinide, another hypoglycemic drug known to be a CYP2C8 substrate, 6 heterozygous carriers of CYP2C8*3 had an even higher oral clearance and lower plasma concentrations compared with wild-type carriers [10]. These findings were replicated in a further study from the same group in 10 heterozygous carriers showing a 50 % increase in oral total repaglinide clearance compared to that in wild-type [11], whereas in another study in 36 volunteers (11 heterozygous and one homozygous for the CYP2C8*3 allele) no effect of the genotype was seen [12]. Pioglitazone however [13], as well as the closely related rosiglitazone [14], also a CYP2C8 substrate, showed a higher clearance in carriers of the CYP2C8*3 allele in clinical studies. Altogether, there seems to be a discrepancy between in vitro data describing a lower activity of the CYP2C8*3 variant compared with the wild-type protein and several in vivo clinical data showing higher oral clearance in *3 allele carriers.

Thus, the objective of the present study was to further assess the role of different human CYP enzymes in the formation of M-IV, the main primary metabolite of pioglitazone in humans, and to address the respective effect of CYP2C8 (and CYP2C9) variants.
**Methods:**

Human cytochrome P450 enzymes from various sources were incubated with pioglitazone (Takeda Pharmaceuticals Co.Ltd., Osaka, Japan). The following sets of incubations were carried out:

a) Human liver microsomes (in-house preparations of individual donors, for details see [15]) at 16.5, 88 and 659 µM pioglitazone concentrations for 5, 10, 15 and 20 min to see time dependency of metabolism; protein concentration approx. 2 mg/ml

b) Human liver microsomes (in-house preparation of individual donors, n=3, and commercial pooled preparations representing a pool from 46 individuals (n=2; Becton Dickinson GmbH, Heidelberg, Germany) at a pioglitazone concentration range between 2 and 220 µM to characterize enzyme kinetics including the estimation of kinetic parameters; protein concentration approx. 2 mg/ml

c) Expressed human CYP enzymes at a pioglitazone concentration range between 2 and 400 µM to identify the main enzymes mediating the formation of M-IV; protein concentration approx. 0.5 mg/ml. The following enzymes were tested: CYP1A2, CYP2A6, CYP2B6, CYP2C9*1(Arg144), CYP2C9*2(Cys144), CYP2C9*3(Leu359), CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 (SUPERSOMES™, Becton Dickinson, Heidelberg, Germany). All enzymes were the high-activity wild type variants, with the exception of CYP2C9 where the three variants CYP2C9*1, *2 and *3 were tested; (CYP2C8 variants are not commercially available). Enzymes were co-expressed with NADPH-cytochrome P450 reductase, and in the case of CYP2E1 additionally with cytochrome b₅ co-expression

d) Expressed human CYP2C8*1 at paclitaxel concentrations of 1 to 50 µM and pioglitazone concentrations between 2 and 160 µM to assess an inhibitory effect of pioglitazone on a standard CYP2C8 mediated reaction.
e) Human liver microsomes (10 Stuttgart liver bank preparations of individual donors with CYP2C8*3/*3: n=5; and with CYP2C8*1/*1: n=5) at pioglitazone concentrations of 2, 8, 20, 50 and 88 µM; protein concentration 0.5 mg/ml, to test for differences between CYP2C8 genotypes. The CYP2C8*3/*3 samples were identified by genotyping for CYP2C8*3 in DNA from 288 human liver microsome donors using PCR-RFLP as described earlier (Dai et al., 2001); the five CYP2C8*1/*1 samples tested were selected randomly from the large group with this genotype. PCR for analysis of CYP2C8*3 resulted in a 347 bp fragment which was digested by BseRI to 310 and 37-bp fragments in the presence of the wild type allele and was not restricted in the CYP2C8*3 allele. To control for CYP2C9 genotype, CYP2C9*2 and *3 variants were also identified using a published method [16].

**Incubation conditions.** All incubations were carried out in duplicate in 50 mM TRIS buffer (pH 7.4) containing a NADPH regenerating system, consisting of 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM MgCl₂ (Becton Dickinson GmbH, Heidelberg, Germany). The NADPH regenerating system, buffer, paclitaxel (where applicable) and human liver microsomes (HLM) or recombinant enzymes were premixed at 4 °C, and incubations were started by the addition of pioglitazone and transfer of the sample to a 37 °C shaking water bath. Stock solutions of pioglitazone were prepared in acetonitrile/methanol (1:1, v/v). Incubation volume was 200µl; the content of the acetonitrile/methanol mixture in all incubations was 2 %. The enzymatic reaction was stopped by removing the sample from the water bath, immediate addition of 200 µl ice cold acetonitrile, vortexing and cooling on ice.

**Quantification of pioglitazone and metabolites.** After addition of 30 µl internal standard (1 mM rosiglitazone in acetonitrile-methanol (1:1, v/v)) to the sample, the solution was
vortexed and centrifuged for 10 min at 4°C by 3500 rpm. A 300 µl aliquot of the supernatant was evaporated to dryness, reconstituted in 100 µl mobile phase and transferred into autosampler vials. Concentrations of pioglitazone and its active metabolites M-III and M-IV were quantified by use of a reversed phase HPLC method. Chromatography was performed on a Zorbax RX-C8 (250x4.6mm, 5 µm) column (Agilent, Santa Clara, CA, USA) operated at a temperature of 25 °C. The mobile phase consisted of 42.5% acetonitrile and 57.5% water (v/v), to which 3 ml acetic acid per L was added, with subsequent pH adjustment to 5.2 with 25% NH₃ solution. The flow of mobile phase was 1.2 ml/min. Analytes were quantified using UV absorption at 269 nm. The method was validated using standard procedures in terms of specificity, linearity, sample stability, lower limit of quantification (LLOQ), precision and accuracy. LLOQ for M-III and M-IV were 0.1 and 0.3 µM (corresponding to 25 and 75 pmol/min/nmol CYP for recombinant CYPs or 10 and 30 pmol/min/mg protein for HLM), respectively. LLOQ for pioglitazone was 1.5 µM. No stability problems were observed. Intra- and inter-subject precision and accuracy were better than 10 % from LLOQ up the highest concentrations observed.

Quantification of 6α-OH-paclitaxel. The paclitaxel 6α-hydroxylase activity was determined by minor modification of a published method [17]. Separation was achieved using a Macherey-Nagel Nucleodur C18 Gravity 4.6 x 250 mm, 5 µm particle size HPLC column (Düren, Germany), maintained at 30 °C with a linear gradient of 45 % mobile phase A (10 % methanol), 55 % mobile phase B (100 % methanol) decreasing to 35 % phase A over 20 min, then held for a further 5 min with 35 % phase A, 65 % phase B and at a flow rate of 1.0 mL/min. The product was detected by its UV absorbance at 230 nm. Retention times of 6α-hydroxypaclitaxel and paclitaxel were 18 min and 21 min, respectively. Intra- and inter-subject precision and accuracy were better than 12 % from LLOQ up the highest concentrations observed.
Estimation enzyme kinetic parameters for formation of M-IV and statistics. Enzyme kinetics were assessed by nonlinear regression analysis using SigmaPlot statistical program with Enzyme Kinetics Module (version 9.01, Systat Software, San Jose, CA, USA). Standard equations were used for the relationship between substrate concentration and metabolite formation [15]. Models tested include a single binding site for pioglitazone, two binding sites and substrate inhibition. The Akaike information criterion was used to select between models that could be fitted to the data. To compare between human liver microsomes preparations with different genotypes, the t-test was used.
Results:

Formation of M-IV in human liver microsomes was linear with time at all pioglitazone concentrations for a duration of incubation up to 20 min. Interestingly, the mean ± SD formation rate of 11.6 ± 1.1 pmol M-IV/mg protein/min for the highest pioglitazone concentration tested (659 µM) was much lower than the formation rate of 55.6 ± 4.3 pmol M-IV/mg protein/min for 88 µM pioglitazone and even lower than M-IV formation at the low concentration of 16.5 µM pioglitazone (23.2 ± 4.9 pmol M-IV/mg protein/min).

This behaviour was seen both in human liver microsomes and in isolated expressed CYPs. Increasing pioglitazone concentrations were related to increasing M-IV formation rates up to approximately 100 µM, whereas at higher pioglitazone concentrations, a decrease of metabolite formation rate was observed (Fig. 2). Nonlinear regression analysis showed that this behaviour was fully compatible with substrate inhibition with a substrate inhibitory constant in the low µM range. In order to reliably estimate the kinetic parameters of a substrate inhibition model, even higher pioglitazone concentrations would have been needed, which could not be used because of solubility limitations. Therefore, those parts of the curve with increasing metabolite formation rates were used to estimate apparent enzyme kinetic parameters. A single binding site provided excellent fits in all cases and gave relatively uniform results for several human liver microsome preparations of different sources. Mean ± SD Km was 67 ± 25 µM, and V_max reached 190 ± 121 pmol/min/mg protein (Table 1).

In incubations with individual CYPs and CYP2C9 variants, several wild type CYPs were able to form M-IV amounts above the limit of quantification, including CYP2C8, CYP1A2, CYP2D6, and CYP2C19 (Figure 3, Table 2). Formation rate was highest in incubations with CYP2D6. Wild type CYP2A6, CYP2B6, CYP2C9(*1), CYP2E1, CYP3A4 and CYP3A5 did not form quantifiable amounts of M-IV. In contrast to CYP2C9*1, CYP2C9*2 was able to
form M-IV, albeit at a relatively low rate, whereas for CYP2C9*3 no activity was seen. None of the isolated enzymes formed quantifiable concentrations of the secondary M-III metabolite when incubated with pioglitazone.

The activity of expressed human CYP2C8 with regard to paclitaxel 6α-hydroxylation fully corresponded to the specifications of the manufacturer and to published data [17], with Km values of approximately 10 µM paclitaxel. This activity however was completely unaffected by addition of pioglitazone at concentrations up to 160 µM (results not shown).

Seven of the 288 liver microsome samples of the Stuttgart liver bank examined carried the CYP2C8*3/*3 genotype, of which 2 did not carry the CYP2C9*2/*2 genotype. The 5 samples with the combined CYP2C8*3/*3 and CYP2C9*2/*2 genotype were chosen for incubations with pioglitazone to compare to microsomes which carried homozygous wild type alleles for both enzymes. Mean ± SD V max values were 464 ± 188 vs. 370 ± 225 pmol M-IV/min/mg protein, mean Km ± SD were 21 ± 12 vs. 10 ± 5 µM pioglitazone for microsomes with wild type alleles (n=5) compared to those with the combined CYP2C8*3/*3 / CYP2C9*2/*2 genotype (n=5), respectively. Corresponding intrinsic clearance values were 25 ± 4 and 35 ± 9 pmol M-IV/min/mg protein/µM pioglitazone. The difference between intrinsic clearance values was statistically significant (p<0.05).
**Discussion**

The present in vitro study based on enzyme kinetics showed that several enzymes including CYP2C8 are relevant for the formation of the main primary metabolite M-IV of pioglitazone and confirmed the more rapid glitazone metabolism observed in carriers of the *CYP2C8*\(^*3\) variant in vivo. It also generated a hypothesis to explain the differences between *CYP2C8* genotypes and to explain some adverse effects of pioglitazone.

Among the wild type CYP enzymes tested, CYP2C8, CYP1A2 and CYP2D6 were the most important ones, based on intrinsic clearance values (Table 2). The major role of CYP2C8 corresponds to another report obtained by different methods [2] and to the result of the in vivo interaction study with gemfibrozil [3]. In contrast to other reports however [2], no role for CYP3A4 in the metabolism of pioglitazone was seen in the present study. While the enzyme may still be involved in the formation of minor metabolites, the lack of an effect of itraconazole on pioglitazone metabolism in vivo [3] also supports that CYP3A4 is not important for pioglitazone metabolism. The roles of CYP1A2 and CYP2D6, the wild type enzymes with intrinsic clearance values for M-IV formation similar to CYP2C8, remain to be assessed further. A relevant contribution of CYP1A2 to pioglitazone metabolism in vivo suggests that pioglitazone clearance may be increased in smokers [18]; also, differences in pioglitazone pharmacokinetics between CYP2D6 genotypes may be present.

While we could not compare the in vitro activity of isolated CYP2C8 variants because CYP2C8*3 was not available, we found a lower intrinsic clearance for M-IV formation in microsomes from donors homozygous for *CYP2C8*\(^*1\) compared to those homozygous for *CYP2C8*\(^*3\). This is an apparent contradiction to low in vitro activity of CYP2C8*3 reported for other substrates (see Introduction section), but is in accordance with the more rapid
metabolism observed in carriers of the \textit{CYP2C8*3} variant in vivo of pioglitazone itself [13], but also of rosiglitazone [14], and some other CYP2C8 substrates [19]. There are two possible explanations taking both findings into account, including (i) a higher expression of the \textit{CYP2C8*3} variant compared to the wild type allele, and/or (ii) an additional contribution of other enzyme and their respective variants which belong to the haplotype represented by the \textit{CYP2C8*3} allele. In both cases, the lower activity of the CYP2C8*3 variant protein could be (more than) compensated. Our results provide evidence for the latter explanation. All of the microsomes homozygous for \textit{CYP2C8*3} in our study were also homozygous carriers of the \textit{CYP2C9*2} variant, to which it is partially linked [7]. Indeed, we found that CYP2C9*2 has a relatively high activity for M-IV formation, whereas wild type CYP2C9*1 and CYP2C9*3 were essentially inactive. This was a surprising result but held true in repeated experiments with different batches of the enzymes. The effect of the CYP2C9*2 variant on substrate turnover is ambiguous and may depend on the substrate [19]. However, in most cases, CYP2C9*2 had a slightly lower activity than the wild type protein, and a higher activity compared to CYP2C9*1 has not been reported for any substrate so far. Whether CYP2C9*2 is able to metabolise other CYP2C8 substrates remains to be investigated.

Despite maintenance of identical incubation conditions for all pioglitazone concentrations, including the same concentrations of organic solvents, we observed a decrease of M-IV formation for pioglitazone concentrations above 100 µM (Fig. 2), whereas no time dependent loss of activity was seen. This phenomenon was present in all but one enzyme preparations with quantifiable pioglitazone turnover. The observation, which may be explained by potent substrate inhibition of CYP enzymes by pioglitazone, suggests that the drug may bind reversibly to different subdomains of the binding site, and that the affinity to a subdomain at which pioglitazone is not metabolized is higher than that to the subdomain which mediates formation of M-IV. Inhibitory effects of pioglitazone on CYPs were also reported by others
[4,5,20]; however, we did not find any inhibitory effect on paclitaxel metabolism in expressed human CYP2C8. The clinical role of CYP inhibition by pioglitazone, however, is not clear. In vivo, the metabolism of repaglinide, another CYP2C8 substrate, was not impaired by pioglitazone coadministration [21]. Taking the putative mechanism into account, it appears that pioglitazone inhibition of CYP enzymes is substrate selective. Thus, it remains to be tested to which extent pioglitazone interacts with other CYP2C8 substrates in vivo, including the metabolism of endogenous substrates such as arachidonic acid to form epoxyeicosatrienic acids (EETs) in vivo and in vitro. This is a possible link to adverse effects of glitazones such as fluid/sodium retention and edema [22, 23] because EETs inhibit sodium re-absorption in the proximal tubulus [24, 25].

In conclusion, it appears that pioglitazone interacts with CYP2C enzymes in several different ways, and a detailed understanding of the underlying mechanisms is required to predict clinical consequences for carriers of CYP2C8 and/or CYP2C9 variants and for drug-drug interactions. Further studies are required to test the hypotheses generated by the present study.
Figures

Figure 1: Main metabolic pathways of pioglitazone [1].

minor phase I metabolites

\[
\text{pioglitazone} \xrightarrow{\text{OH}} \text{metabolite M-IV} \xrightarrow{\text{OH}} \text{metabolite M-III}
\]
**Figure 2:** Formation of the pioglitazone metabolite M-IV in a human liver microsome sample. Triangles represent results of individual incubations. The lower formation rates seen at higher substrate concentrations were compatible with substrate inhibition. Therefore, apparent enzyme constants were calculated for pioglitazone concentrations below 100 µM only.
Figure 3: Formation of the pioglitazone metabolite M-IV by different human recombinant CYP enzymes. Symbols represent means of duplicate incubations. As in human liver microsomes, lower formation rates were seen at substrate concentrations above 100 µM (not shown).
Tables

Table 1: Enzyme kinetic constants for formation of M-IV from pioglitazone in human liver microsomes. Parameters were estimated assuming a single pioglitazone binding site, disregarding substrate concentrations above 100 µM.

<table>
<thead>
<tr>
<th>type of human liver microsomes</th>
<th>apparent Km [standard error of estimate] (µM)</th>
<th>apparent V_max [standard error of estimate] (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-12 single</td>
<td>79 [32]</td>
<td>393 [96]</td>
</tr>
<tr>
<td>HAS002 single</td>
<td>104 [35]</td>
<td>177 [35]</td>
</tr>
<tr>
<td>452161 pooled</td>
<td>52 [21]</td>
<td>183 [36]</td>
</tr>
<tr>
<td>mean</td>
<td>67</td>
<td>190</td>
</tr>
<tr>
<td>SD</td>
<td>25</td>
<td>121</td>
</tr>
</tbody>
</table>

Km, Michaelis-Menten constant; V_max, maximal metabolite formation rate
Table 2: Enzyme kinetic constants for formation of M-IV from pioglitazone in commercial preparations of expressed human CYP enzymes. Parameters were estimated assuming a single pioglitazone binding site, disregarding substrate concentrations above 100 µM. No metabolite formation exceeding the lower limit of quantification (0.3 µM M-IV, corresponding to 75 pmol/min/nmol CYP P450) was observed for wild type CYP2A6, CYP2B6, CYP2C9(*1), CYP2E1, CYP3A4 and CYP3A5, and for the CYP2C9 variant *3.

<table>
<thead>
<tr>
<th>CYP enzyme</th>
<th>apparent Km [standard error of estimate] (µM)</th>
<th>apparent V_{max} [standard error of estimate] (pmol/min/nmol CYP)</th>
<th>Clint (pmol M-IV/min/nmol CYP/µM pioglitazone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8*1</td>
<td>29.5 [4.4]</td>
<td>1702 [109]</td>
<td>58</td>
</tr>
<tr>
<td>CYP1A2*1</td>
<td>29.4 [6.0]</td>
<td>1713 [151]</td>
<td>58</td>
</tr>
<tr>
<td>CYP2D6*1</td>
<td>132.8 [40]</td>
<td>7098 [1453]</td>
<td>53</td>
</tr>
<tr>
<td>CYP2C19*1</td>
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<td>782 [52.8]</td>
<td>40</td>
</tr>
<tr>
<td>CYP2C9*2</td>
<td>4.3 [1.1]</td>
<td>144.5 [8.9]</td>
<td>34</td>
</tr>
</tbody>
</table>

K_{m}, Michaelis-Menten constant; V_{max}, maximal metabolite formation rate; Clint, intrinsic clearance
References:


