Clopidogrel Markedly Increases the Plasma Concentrations of the CYP2C8 Substrate Pioglitazone

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Running Title: Effect of Clopidogrel on the Pharmacokinetics of Pioglitazone

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Abbreviations. AUC, area under the plasma concentration-time curve; CI, confidence interval; C_{max}, peak plasma concentration; CYP, cytochrome P450; fm_{CYP2C8}, fraction metabolized by CYP2C8; M-III, ketopioglitazone; M-IV, hydroxypioglitazone; OATP, organic anion transporting polypeptide; PPAR-γ, peroxisome proliferator-activated receptor γ; t_{max}, time to C_{max}; $t_{½}$ half-life

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Abstract

The glucose-lowering drug pioglitazone undergoes hepatic cytochrome P450 (CYP) 2C8-mediated biotransformation to its main metabolites. The antiplatelet drug clopidogrel is metabolized to clopidogrel acyl-β-D-glucuronide, which was recently found to be a strong time-dependent inhibitor of CYP2C8 in humans. Therefore, we studied the effect of clopidogrel on the pharmacokinetics of pioglitazone. In a randomized crossover study, ten healthy volunteers ingested either 300 mg of clopidogrel on day 1, and 75 mg on days 2 and 3, or placebo. Pioglitazone 15 mg was administered 1 h after placebo and clopidogrel on day 1. Plasma concentrations of pioglitazone, clopidogrel, and their main metabolites were measured up to 72 h. Clopidogrel increased the area under the plasma concentration-time curve ($AUC_{0-\infty}$) of pioglitazone 2.1-fold ($P<0.001$, 90% CI 1.8-2.6) and prolonged its half-life ($t_{1/2}$) from 6.7 to 11 h ($P=0.002$). The peak concentration of pioglitazone was unaffected but the concentration at 24 h was increased 4.5-fold (range 1.6 to 9.8; $P<0.001$, 90% CI 3.17-6.45) by clopidogrel. The M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio was 49% ($P<0.001$, 90% CI 0.40-0.59) of that during the control phase, indicating that clopidogrel inhibited the CYP2C8-mediated biotransformation of pioglitazone. Clopidogrel increases the exposure to pioglitazone by inhibiting its CYP2C8-mediated biotransformation. In consequence, use of clopidogrel may increase the risk of fluid retention and other concentration-related adverse effects of pioglitazone.

Introduction

The thiazolidinedione pioglitazone is an oral glucose-lowering drug that acts by activating the peroxisome proliferator-activated receptor γ (PPAR-γ) (Diamant and Heine, 2003; Waugh et al., 2006). Pioglitazone is well absorbed, has an oral bioavailability of about 80%, and is extensively metabolized to active and inactive metabolites in the liver (Eckland and Danhof, 2000). The main metabolites found in human plasma, hydroxypioglitazone (M-IV), and its secondary metabolite, ketopioglitazone (M-III), contribute to the pharmacological activity (Eckland and Danhof, 2000). Pioglitazone is metabolized mainly by cytochrome P450 (CYP) 2C8 and to a minor extent by CYP3A4 (Jaakkola et al., 2006a). Certain drugs and genetic factors can have a clinically significant effect on hepatic CYP2C8 activity (Backman et al., 2016), and therefore such factors can alter the exposure to pioglitazone (Deng et al., 2005; Jaakkola et al., 2005; Jaakkola et al., 2006b; Tornio et al., 2008; Aquilante et al., 2013b; Kadam et al., 2013), potentially changing its efficacy and risk for concentration-related adverse effects. For example, the fibric acid derivative gemfibrozil, which is a strong CYP2C8 inhibitor, raises the exposure to pioglitazone more than 3-fold (Deng et al., 2005; Jaakkola et al., 2005; Aquilante et al., 2013b) and the product information of Actos warns about risks of their combination (European Medicines Agency, 2014).

Antithrombotic agents, such as the ADP-receptor inhibitor clopidogrel, are commonly used in patients with type 2 diabetes mellitus. In a retrospective epidemiological study, concomitant use of clopidogrel was strongly associated with myotoxic adverse effects of cerivastatin, which is a substrate for CYP2C8 (Mück, 2000; Backman et al., 2002; Floyd et al., 2012). More recently, therapeutic doses of clopidogrel were shown to markedly increase the plasma concentrations and blood glucose-lowering effect of the meglitinide analog repaglinide (Tornio et al., 2014). Inhibition of CYP2C8 by clopidogrel metabolite, clopidogrel acyl-β-D-glucuronide, was suggested to be the main mechanism of the clopidogrel-repaglinide interaction. Therefore, and as the metabolism of pioglitazone is mainly CYP2C8-mediated (Gillies and Dunn, 2000; Scheen, 2007; Tornio et al., 2012), we found it important to investigate the effect of clopidogrel on the pharmacokinetics of pioglitazone.

Materials and methods

Subjects and study design. Ten healthy nonsmoking volunteers (4 women, 6 men; age range, 20-35 years; body mass index range, 19.2-28.9 kg/m^2) participated in the study after giving written informed consent. Their health was confirmed by medical history, clinical examination, and routine laboratory tests before entering the study. All participants had normal blood platelet counts and hemoglobin values. None of the subjects used oral contraceptives or other continuous medication. The study protocol was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (record number 259/13/03/00/2014), and the Finnish Medicines Agency Fimea (EudraCT number 2013-003891-11). In a randomized, placebo-controlled, crossover study, the subjects ingested as pretreatment either placebo (Placebo tablets, University Pharmacy, Helsinki, Finland) or clopidogrel (Plavix, Sanofi-Aventis, Paris, France) once daily at 8 AM for three days with 150 ml water. Clopidogrel dose was 300 mg (four 75 mg tablets) on day 1 followed by 75 mg on days 2 and 3. The wash-out period between the placebo and clopidogrel phases was three weeks. On day 1 of pretreatment, 15 mg pioglitazone (Actos 15 mg tablet, Takeda Europe, London, UK) was administered at 9 AM. On the days of pioglitazone intake, the volunteers had fasted overnight, and a standard warm meal was served 3 h, and snacks 7 and 10 h after the administration of pioglitazone. The use of grapefruit products was not allowed for one week before and during the study, and the use of other drugs from one week before to one week after the study. Use of alcohol was prohibited the day before and on the days of pioglitazone administration.

Sampling. Timed blood samples were drawn from a cannulated forearm vein, or by venipuncture before the administration of pretreatment, and 5 min before and 1, 2, 3, 4, 5, 7, 9, 12, 24, 48 and 72 h after the administration of pioglitazone. The blood samples were collected into ethylenediaminetetraacetic acid (EDTA) containing tubes, which were placed on ice immediately after sampling. Plasma was separated within 30 min and stored at -70 °C until analysis. The samples for the determination of clopidogrel and its metabolites were treated with 2-bromo-3'-methoxyacetophenone

within 30 seconds of blood sample collection in whole blood EDTA samples, in order to derivatize the active metabolite of clopidogrel, as previously described (Delavenne et al., 2010).

Determination of drug concentrations. The concentrations of pioglitazone, M-III and M-IV were measured with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an AB Sciex API 2000 tandem mass spectrometer (Framingham, MA), as previously described, with slight modifications (Jaakkola et al., 2005; Kawaguchi-Suzuki et al., 2014). Briefly, the plasma (100 μl) proteins were precipitated with a volume of 300 μl of acetonitrile containing 1% formic acid and the internal standards, and the supernatant phospholipids were removed using HybridSPE Phospholipid cartridges (30 mg, SUPELCO, Bellefonte, PA). The resulting filtrate was evaporated and the residue was reconstituted in 80 μl of mobile phase, and injected (5 μl) to liquid chromatography instrument. Chromatography was performed on XTerra RP C18 column (3.9x100 mm; Waters Corporation, Milford, MA) using gradient elution. The mobile phase consisted of 10 mM ammonium acetate (pH 9.0, adjusted with 25% ammonia solution) and acetonitrile. Deuterated forms of pioglitazone, M-III and M-IV served as internal standards. The mass spectrometer was operated in positive electrospray mode, and the mass-to-charge (m/z) ion transitions $[M+H]^+$ monitored were 357 to 134 for pioglitazone, 371 to 148 for M-III, and 373 to 150 for M-IV. The limit of quantification was 0.2 ng/ml for pioglitazone and M-III, and 0.5 ng/ml for M-IV. The day-to-day coefficient of variation (CV) was below 15% for all analytes at relevant concentrations. Clopidogrel, the clopidogrel active *cis*-5-thiol metabolite, clopidogrel carboxylic acid, and clopidogrel acyl-β-D-glucuronide were analyzed with a Nexera UPLC instrument (Shimadzu, Kyoto, Japan) connected to an AB Sciex Qtrap 5500 mass spectrometer (Framingham, MA), as previously described (Holmberg et al., 2014; Tornio et al., 2014; Itkonen et al., 2015).

Pharmacokinetics. The peak plasma concentration (C_{max}), time to C_{max} (t_{max}), AUC_{0-72h} and $AUC_{0-\infty}$, and half-life ($t_{1/2}$) were calculated for pioglitazone, clopidogrel, and their metabolites (AUC_{0-13h} for

clopidogrel and its metabolites) by standard noncompartmental methods using Phoenix WinNonlin, version 6.4 (Certara, Princeton, NJ).

Static in vitro–in vivo predictions of the clopidogrel-pioglitazone interaction. Previously published K_I, and k_{inact} values of clopidogrel acyl-β-D-glucuronide for CYP2C8 were used to predict the in vivo clopidogrel-pioglitazone interaction with the following equation (Mayhew et al., 2000; Jones and Hall, 2002; Tornio et al., 2014):

$$\frac{AUC_I}{AUC} = \frac{1}{\dfrac{fm_{CYP2C8}}{1+\dfrac{k_{inact}\times[I]}{K_I \times k_{deg}}}+(1-fm_{CYP2C8})}$$, where AUC_I and AUC are the areas under the

concentration–time curve of pioglitazone with clopidogrel (inhibitor) and placebo (control), respectively; fm_{CYP2C8} expresses the fraction of pioglitazone dose metabolized by CYP2C8; and k_{deg} is the rate constant of hepatic CYP2C8 degradation in the absence of the inhibitor. In our predictions, a half-life of 22 h was used for CYP2C8, corresponding to a k_{deg} of 0.00053 1/min (Backman et al., 2009). The predictions were calculated for clopidogrel acyl-β-D-glucuronide concentrations ranging from 0 to 10 μM and for fm_{CYP2C8} values of 0.60, 0.65, 0.75, and 0.80. The predictions were then related to the median unbound plasma peak ($C_{max,u}$) and average ($C_{avg,u}$) concentrations of clopidogrel observed in the clinical study, assuming an unbound fraction of 10% (Tornio et al., 2014), where the average concentration was calculated by dividing AUC_{0-13h} by 13 h. Clopidogrel and its other metabolites were not included in the predictions because of their negligible significance (Tornio et al., 2014).

Genotyping. Buffy coats were prepared from 9 ml whole blood EDTA samples after plasma separation. Genomic DNA was extracted from the buffy coats using the Maxwell 16 LEV Blood DNA Kit on a Maxwell 16 Research automated nucleic acid extraction system (Promega, Madison, WI). The participants were genotyped for the *CYP2C8*2* (c.805A>T), *3* (c.416G>A and c.1196A>G), and

4 (c.792C>G) alleles with a custom TaqMan OpenArray genotyping panel on a QuantStudio 12K

Flex real-time PCR system (Life Technologies, Carlsbad, CA). Haplotypes containing the reference

allele at each position were designated as *CYP2C8*1*.

Statistical analysis. Based on the pharmacokinetic results of previous drug interaction studies with

pioglitazone, the number of subjects was estimated to be adequate to detect a 30% change in the AUC

of pioglitazone and its active metabolites between the control and clopidogrel phases, with a power of

at least 80% (α level 5%). The results are expressed as geometric means and geometric mean ratios

with geometric CV or 90% confidence intervals (CIs) unless stated otherwise. Logarithmic

transformation was used for pharmacokinetic variables, except t_{max}, before statistical analysis. The

pharmacokinetic variables were compared by repeated-measures analysis of variance with treatment

phase as a within-subjects and treatment sequence as a between-subjects factor, with pairwise

comparisons with the Fisher's least significant difference method. The t_{max} data were compared using

the Wilcoxon signed rank test. Correlations between the AUC_{0-13h} of clopidogrel, or its metabolites,

and the fold-increase in pioglitazone $AUC_{0-\infty}$ were quantified as Kendall's correlation coefficients and

tested using Kendall's test. The M-IV-to-pioglitazone metabolic ratio and pioglitazone $AUC_{0-\infty}$ of

*CYP2C8*3* carriers were compared with those of *CYP2C8*3* noncarriers by independent-samples *t*-

tests. The *P*-values below 0.05 were considered statistically significant. Statistical analyses were

performed using SPSS Statistics for Windows version 22.0 (IBM Corporation, Armonk, NY).

Results

Pioglitazone. Clopidogrel increased the $AUC_{0-\infty}$ of pioglitazone 2.1-fold ($P<0.001$, 90% CI of the geometric mean ratio 1.8-2.6) and prolonged the mean elimination $t_{1/2}$ of pioglitazone from 6.7 to 11 h ($P=0.002$, 1.7-fold, 90% CI 1.4-2.0), compared with placebo (Fig. 1a; Table 1). The $AUC_{0-\infty}$, and $t_{1/2}$ of pioglitazone were increased in all 10 subjects by clopidogrel, the largest individual $AUC_{0-\infty}$ increase being 3.3-fold (Fig. 2a). Clopidogrel had no effect on pioglitazone C_{max}, but the pioglitazone concentration at 24 h (C_{24}) was 4.5-fold higher (range 1.6 to 9.8; $P<0.001$) during clopidogrel than during the placebo phase. The t_{max} occurred later in the clopidogrel phase ($P=0.025$).

Pioglitazone metabolites. The C_{max} of the metabolites M-IV and M-III were 61% ($P=0.007$, 90% CI 0.48-0.79) and 58% ($P=0.003$, 90% CI 0.46-0.74) of that during the placebo phase, respectively (Table 1). However, as the $t_{1/2}$ of both metabolites were prolonged by clopidogrel, their $AUC_{0-\infty}$ values were not significantly different between the clopidogrel and placebo phase (Table 1). In the clopidogrel phase, the M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio was 49% ($P<0.001$, 90% CI 0.40-0.59) of that during the placebo phase.

***CYP2C8* genotype.** One of the subjects had the *CYP2C8*1/*2* genotype, three had the *CYP2C8*1/*3* genotype, two had the *CYP2C8*1/*4* genotype, and the rest (N=4) had the *CYP2C8*1/*1* genotype. The subjects with the *CYP2C8*1/*3* genotype had the highest M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio in the placebo phase ($P<0.001$) (Fig. 2b). They also had a greater decrease in the M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio, caused by clopidogrel, than did the noncarriers of *CYP2C8*3* ($P=0.015$), and there was no statistically significant difference between genotypes in this ratio in the clopidogrel phase ($P=0.676$) (Fig. 2b).

Pharmacokinetics of clopidogrel and in vitro-in vivo correlations. The AUC_{0-13h} of parent clopidogrel, clopidogrel's active *cis*-5-thiol metabolite, clopidogrel carboxylic acid and clopidogrel acyl-*β*-D-glucuronide varied greatly between individuals (Fig. 3, Table 2). There were no significant

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correlations between the AUC_{0-13h} of clopidogrel, or its metabolites, and the fold-increase in pioglitazone $AUC_{0-\infty}$ (data not shown). The prediction model indicated that for substrates with an fm_{CYP2C8} of 60% to 80%, lower than 1 μM static concentrations of clopidogrel acyl-β-D-glucuronide could produce a similar 2.1-fold increase in AUC as in the present clopidogrel-pioglitazone study (Fig. 4). Assuming 10% unbound fraction, the median $C_{max,u}$ and median $C_{avg,u}$ (0-13 h) of clopidogrel acyl-β-D-glucuronide after the 300 mg dose of clopidogrel were 0.85 and 0.26 μM, as indicated by the upper and lower borders of the grey area in Figure 4.

Discussion

In the present study, clinically used doses of clopidogrel increased the exposure to pioglitazone more than 2-fold, and the C_{24}, corresponding to the trough pioglitazone concentration during a typical once daily clinical use, was increased 4.5-fold by clopidogrel. In the clopidogrel phase, the metabolite M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio was 49% of that during the placebo phase, consistent with strong inhibition of CYP2C8 by clopidogrel acyl-β-D-glucuronide. The sum of the $AUC_{0-\infty}$ values of pioglitazone, M-IV and M-III was larger during the clopidogrel than during the placebo phase. Despite some inaccuracies in the extrapolated AUC fractions, this suggests that clopidogrel markedly increases the net exposure to pioglitazone and its active metabolites. This drug-drug interaction is therefore likely to increase the risk of concentration-dependent adverse effects of pioglitazone.

In in vitro experiments, clopidogrel and its metabolites have been shown to inhibit drug metabolizing enzymes, e.g., CYP2B6, CYP2C8 and CYP3A4, and drug transporters, e.g., organic anion transporting polypeptide (OATP) 1B1, suggesting that clopidogrel can cause changes in the pharmacokinetics of other drugs (Richter et al., 2004; Turpeinen et al., 2005; Floyd et al., 2012; Tamraz et al., 2013; Tornio et al., 2014). Recently, it was reported that clopidogrel use is associated with markedly elevated risk for cerivastatin-induced rhabdomyolysis (Floyd et al., 2012). Also, clopidogrel and its 2-oxo, carboxylic acid and acyl-β-D-glucuronide metabolites were found to inhibit CYP2C8 in vitro, but the inhibitory effects were not strong enough to explain a clinically relevant interaction by clopidogrel (Floyd et al., 2012). In a subsequent study, clopidogrel was found to greatly increase the exposure to the glucose-lowering drug repaglinide (Tornio et al., 2014). In addition, clopidogrel acyl-β-D-glucuronide was identified as a potent time-dependent inhibitor of CYP2C8, whereas clopidogrel and its 2-oxo and carboxylic acid metabolites showed only relatively weak direct inhibition in vitro (Tornio et al., 2014). The active cis-5-thiol metabolite of clopidogrel is very unstable and not commercially available. Therefore, the contribution of this metabolite to CYP2C8 inhibition could not be estimated accurately (Tornio et al., 2014). Both cerivastatin and repaglinide are substrates for CYP2C8, as well as for CYP3A4, and OATP1B1 (Mück, 1998; Kantola et al., 1999;

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Backman et al., 2002; Wang et al., 2002; Niemi et al., 2003; Kajosaari et al., 2005; Niemi et al., 2005).

According to a recent study, clopidogrel does not cause a significant change in the disposition of

simvastatin or its active acid form in humans (Itkonen et al., 2015). As simvastatin is among the most

sensitive drugs to changes in CYP3A4 and OATP1B1 activity (Neuvonen et al., 1998; Backman et al.,

2000; Ichimaru et al., 2001; Prueksaritanont et al., 2003; Pasanen et al., 2006; Niemi et al., 2011),

clopidogrel is unlikely to have a clinically relevant effect on CYP3A4 or OATP1B1 activity (Itkonen

et al., 2015). These findings indicate that inhibition of CYP2C8 by clopidogrel acyl-β-D-glucuronide

is the main mechanism of the clopidogrel-cerivastatin and clopidogrel-repaglinide interactions (Floyd

et al., 2012; Tornio et al., 2014; Itkonen et al., 2015). This conclusion is also supported by a recent

case report, which suggests that clopidogrel significantly reduces the metabolic clearance of the

CYP2C8 substrate anticancer agent paclitaxel (Bergmann et al., 2015).

According to in vitro studies, pioglitazone can be metabolized by several CYP

enzymes, e.g., CYP2C8 and CYP3A4 (Jaakkola et al., 2006a; Scheen, 2007; Tornio et al., 2012).

However, the strong CYP3A4 inhibitor itraconazole has had no effect on the pharmacokinetics of

pioglitazone, whereas the strong CYP2C8 inhibitor gemfibrozil has increased the AUC$_{0-\infty}$ of

pioglitazone 3- to 4-fold (Deng et al., 2005; Jaakkola et al., 2005; Aquilante et al., 2013b). Also

trimethoprim, which is a weak inhibitor of CYP2C8, has raised the exposure to pioglitazone 1.4-fold

(Tornio et al., 2008). Thus, in vivo in humans, CYP2C8 is a crucial enzyme in the biotransformation

of pioglitazone, accounting for about 70-75% of the total elimination of pioglitazone, and the

contribution of CYP3A4 to its elimination seems to be negligible. Furthermore, the pharmacokinetics

of pioglitazone is not affected by the *SLCO1B1* c.521T>C polymorphism, indicating that pioglitazone

is not a substrate of OATP1B1 (Kalliokoski et al., 2008). Therefore, pioglitazone can be used as a

selective probe substrate to study the function of CYP2C8 in vivo (Tornio et al., 2012; Backman et al.,

2016). The average 2.1-fold increase in pioglitazone AUC by clopidogrel in the current study is in

agreement with the results of earlier drug interaction studies between pioglitazone and CYP2C8

inhibitors (Deng et al., 2005; Jaakkola et al., 2005; Tornio et al., 2008). The result also conforms well

with the recent prediction that daily dosing of clopidogrel 75 mg in steady state causes 60%-85%

inhibition of CYP2C8 activity (Tornio et al., 2014).

The previous static predictions of the effects of clopidogrel and its metabolites on CYP2C8 activity indicated that during standard dosing of clopidogrel, the reversible CYP2C8 inhibitory effects of clopidogrel and clopidogrel carboxylic acid are negligible compared to the mechanism-based CYP2C8-inhibitory effect of clopidogrel acyl-β-D-glucuronide (Tornio et al., 2014). Accordingly, we used the reported mechanism-based CYP2C8 inhibitory constants of the glucuronide metabolite to predict the effect of clopidogrel on the AUC of a CYP2C8 substrate with an fm_{CYP2C8} of 60% to 80%. On the basis of studies with gemfibrozil (Deng et al., 2005; Jaakkola et al., 2005; Aquilante et al., 2013b), this fm_{CYP2C8} range is likely to include the fm_{CYP2C8} of pioglitazone in most individuals. Our predictions showed that clopidogrel acyl-β-D-glucuronide concentrations corresponding to its peak or average (0-13 h) unbound plasma concentrations after a 300 mg clopidogrel dose can cause a similar AUC increase as we observed in the present study. It should be noted, however, that this was not a static steady-state study, and that due to the 75 mg doses of clopiodogrel given on days 2 and 3, the concentrations of clopidogrel acyl-β-D-glucuronide during most of the 72 h study period were much lower than the average 0-13 h concentration. Therefore, it is likely that the unbound concentrations of clopidogrel acyl-β-D-glucuronide are much higher at the CYP2C8 enzyme site than in plasma.

Although clopidogrel increased the $AUC_{0-\infty}$ of pioglitazone, it did not increase pioglitazone C_{max}. This could be explained by the high oral bioavailability and limited first-pass metabolism of pioglitazone (Eckland and Danhof, 2000). Similarly, a three-day pretreatment with the strong time-dependent CYP2C8 inhibitor gemfibrozil had no effect on pioglitazone C_{max}, even though it increased the $AUC_{0-\infty}$ by 3.2-fold (Jaakkola et al., 2005). It should be noted that CYP2C8 inhibition by clopidogrel probably takes place quite rapidly, despite the time-dependent mechanism (Tornio et al., 2014). Also the inactivation of CYP2C8 by gemfibrozil (via formation of an acyl glucuronide metabolite) is a rapid process, as indicated by a 90% decrease in the AUC of a CYP2C8-dependent metabolite, and a nearly maximum increase in the AUC of the CYP2C8 substrate repaglinide when ingested 1 h after 600 mg of gemfibrozil (Honkalammi et al., 2011). Theoretically, a prolonged absorption or an increased distribution volume of pioglitazone, together with its impaired metabolism, could also explain the unchanged C_{max} despite the increased $AUC_{0-\infty}$.

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The C_{max} values of pioglitazone metabolites IV and III occurred considerably later than the C_{max} of pioglitazone. Consistent with impaired and prolonged metabolite formation, clopidogrel prolonged further the t_{max} values of these metabolites. Yet, due to prolonged half-lives of these metabolites, their $AUC_{0-\infty}$ values were not changed by clopidogrel. Interestingly, in the study of Jaakkola et al. (2005), gemfibrozil caused changes very similar to those caused by clopidogrel in the time-courses of pioglitazone and its M-IV and M-III metabolite concentrations. Thus, it is reasonable to assume, that also the further metabolism of M-IV and M-III is partially dependent on the activity of CYP2C8.

The increase in the $AUC_{0-\infty}$ of pioglitazone by clopidogrel varied between 1.1- and 3.3-fold in the homogenous group of 10 healthy volunteers. However, the extent of the interaction did not correlate significantly with the plasma concentrations (AUC_{0-13h}) of clopidogrel, or its *cis*-5-thiol, carboxylic acid and acyl-*β*-D-glucuronide metabolites. Previous studies have shown that the AUC of pioglitazone is about 30% lower in *CYP2C8*3* carriers than in noncarriers, and that gemfibrozil increases pioglitazone exposure somewhat more in *CYP2C8*3* carriers than in noncarriers (Tornio et al., 2008; Aquilante et al., 2013a; Aquilante et al., 2013b; Kadam et al., 2013). In the present study, the AUC of pioglitazone was about 40% lower in the three individuals with the *CYP2C8*1/*3* genotype than in *CYP2C8*3* noncarriers during the placebo phase. Furthermore, the M-IV-to-pioglitazone $AUC0-\infty$ ratio, an index of the CYP2C8-mediated metabolic clearance of pioglitazone, was highest in these three individuals. The inhibitory effect of clopidogrel on this metabolic ratio was greatest in carriers of the *CYP2C8*3* allele, and consequently the difference between genotypes in the metabolic ratio disappeared in the clopidogrel phase. This finding is biologically plausible, since the contribution of CYP2C8 to the metabolism of pioglitazone should be greatest in individuals carrying the increased activity *CYP2C8*3* allele. Overall, these findings suggest that carriers of the *CYP2C8*3* allele are more susceptible than noncarriers to the interaction between clopidogrel and pioglitazone. However, it should be noted that the sample size of the present study was not sufficient to draw any firm conclusions concerning these genotype-effects, and a clinical study with an adequate statistical power is needed to confirm this hypothesis.

The results of the current study have clear clinical implications, although the study was performed in healthy volunteers. The 300 mg loading dose of clopidogrel followed by 75 mg daily is the recommended dosing regimen. Only a single low 15 mg therapeutic dose of pioglitazone was administered for safety reasons. However, the pharmacokinetics of pioglitazone is linear and similar in patients with type 2 diabetes mellitus to that in healthy volunteers (Eckland and Danhof, 2000). Moreover, it is unlikely that there would be a major difference in clopidogrel pharmacokinetics between healthy subjects and patients with diabetes. Thus, the result of our pharmacokinetic interaction study, i.e., markedly increased pioglitazone exposure by clopidogrel, should be transferable to diabetic patients irrespective of the pioglitazone dose.

Pioglitazone has a generally favourable side-effect profile, but like rosiglitazone, it can cause dose-related fluid retention, and this is more likely to occur when pioglitazone is used in combination with insulin or sulphonylureas (Nesto et al., 2003). Fluid retention, in turn, can worsen the symptoms of congestive heart failure and even cause pulmonary edema (Waugh et al., 2006). Pioglitazone, either alone or in combination with other glucose-lowering drugs, is often used in conjunction with ADP-receptor inhibitors, e.g., clopidogrel, in the treatment of patients with type 2 diabetes mellitus with cardiovascular co-morbidity. It is reasonable to assume that addition of clopidogrel to a pioglitazone-containing drug regimen can markedly raise pioglitazone exposure, in some patients even more than 3-fold. Furthermore, as clopidogrel prolongs the $t_{1/2}$ of pioglitazone, its trough concentrations are likely to increase even more, up to 10-fold. At least theoretically, the uninterrupted high pioglitazone exposure could increase the risk of fluid retention even more than the daily fluctuating exposure, which includes low concentrations before the next dosing.

Glucuronide metabolites have only recently gained attention as perpetrators of drug-drug interactions (Backman et al., 2016). In addition to clopidogrel acyl-β-D-glucuronide, gemfibrozil 1-O-β-glucuronide and deleobuvir acyl glucuronide (M829/2) have been identified as time-dependent inhibitors of CYP2C8 (Shitara et al., 2004; Ogilvie et al., 2006; Tornio et al., 2014; Sane et al., 2016). The active site of CYP2C8 is relatively large (Schoch et al., 2004) and can accommodate these glucuronide metabolites, allowing their metabolism to a reactive species that inactivates CYP2C8. The present findings corroborate the previous report of the CYP2C8-mediated clopidogrel-repaglinide

interaction, in the sense that clopidogrel, via its glucuronide metabolite, is able to cause strong inhibition of CYP2C8 activity. Thus, glucuronide metabolites of some other drugs may also cause unexpected drug interactions with pioglitazone and other CYP2C8 substrates (Backman et al., 2016).

In conclusion, our findings indicate that clopidogrel is a clinically relevant CYP2C8 inhibitor, and concomitant use of clopidogrel and pioglitazone may therefore increase the efficacy and risk of concentration-related adverse effects of pioglitazone. Thus, the combination of pioglitazone and clopidogrel should be used with caution, and dose reduction of pioglitazone should be considered if these two drugs are used concomitantly. Clopidogrel is likely to increase the exposure to other drugs mainly metabolized by CYP2C8, e.g., rosiglitazone.

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Authorship contributions

Participated in research design: Itkonen, Tornio, M. Neuvonen, P. J. Neuvonen, Niemi, Backman.

Conducted experiments: Itkonen, Tornio, M. Neuvonen, P. J. Neuvonen, Niemi, Backman.

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Wrote or contributed to the writing of the manuscript: Itkonen, Tornio, M. Neuvonen, P. J. Neuvonen, Niemi, Backman.

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Footnotes

Figure legends

Figure 1. The effect of clopidogrel (300 mg on day 1, followed by 75 mg on days 2 and 3) on the mean plasma concentrations of pioglitazone (**A**), its primary metabolite, M-IV (**B**), and its secondary metabolite, M-III (**C**). On day 1 of clopidogrel (solid circles) and placebo (open circles) phases, pioglitazone (15 mg) was administered to 10 healthy individuals 1 h after first dose of pretreatment. Data are presented as geometric means with 90% confidence intervals. For clarity, some error bars have been omitted. Inset depicts the same pioglitazone data on semilogarithmic scale.

Figure 2. The effect of clopidogrel on individual area under the plasma concentration time curve from zero to infinity ($AUC_{0-\infty}$) of pioglitazone (**A**) and individual M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio (**B**) in 10 healthy subjects after ingestion of 15 mg of pioglitazone in a crossover study during the two study phases. Pioglitazone was ingested 1 h after placebo on day 1 and 1 h after clopidogrel 300 mg. Open circles, *CYP2C8*3* noncarriers (*N*=7); solid circles, individuals with the *CYP2C8*1/*3* genotype (*N*=3). The M-IV-to-pioglitazone metabolic ratio and pioglitazone $AUC_{0-\infty}$ of *CYP2C8*3* carriers were compared with those of *CYP2C8*3* noncarriers by independent-samples *t*-tests. The subject with the nearly unchanged pioglitazone $AUC_{0-\infty}$ had an exceptionally slow pioglitazone absorption in the clopidogrel phase.

Figure 3. The mean plasma concentrations of clopidogrel (**A**)**,** and its *cis*-5-thiol (**B**), carboxylic acid (**C**), and acyl-*β*-D-glucuronide (**D**) metabolites after clopidogrel 300 mg in 10 healthy volunteers. Data are presented as geometric means with 90% confidence intervals.

Figure 4. Static prediction model for multiples of increase in the AUC of pioglitazone (AUC_I/AUC) in relation to the plasma concentrations of unbound clopidogrel acyl-*β*-D-glucuronide, as described in Methods. The median maximum concentration and the median average concentration (0-13 h) of clopidogrel acyl-*β*-D-glucuronide (assuming 10% unbound fraction) after clopidogrel 300 mg were 0.85 and 0.26 μM, respectively, as indicated by the upper and lower borders of the grey area. The

fm_{CYP2C8} expresses the fraction of pioglitazone dose metabolized by CYP2C8. The horizontal dashed

grey line represents the observed median fold increase in pioglitazone AUC in the present study.

Tables

Table 1. Pharmacokinetic variables of pioglitazone, and its metabolites M-IV and M-III in 10 healthy volunteers, who ingested placebo on days 1, 2 and 3, or 300 mg clopidogrel on day 1, and 75 mg clopidogrel on days 2 and 3, and a single 15 mg dose of pioglitazone 1 h after the administration of placebo or clopidogrel on day 1.

Data are given as geometric mean with geometric coefficient of variation, t_{max} as median with range. The geometric mean ratios between the two phases are given with 90% CI.

Variable	Placebo phase (control)	Clopidogrel phase	Ratio to control (90% CI)
Pioglitazone			
C_{max} (ng/ml)	642 (38)	656 (46)	1.02 (0.77-1.35)
C_{24} (ng/ml)	39.6 (82)	179 (42)***	4.52 (3.17-6.45)
t_{max} (h)	2.0 (2.0-4.0)	3.0 (2.0-4.0)*	
$t_{\frac{1}{2}}$ (h)	6.7 (14)	11 (37)**	1.66 (1.35-2.04)
AUC_{0-72h} (ng·h/ml)	5580 (39)	11700 (36)***	2.09 (1.69-2.59)
$AUC_{0-\infty}$ (ng·h/ml)	5580 (39)	12000 (36)***	2.14 (1.76-2.61)
M-IV			
C_{max} (ng/ml)	263 (25)	162 (30)*	0.61 (0.48-0.79)
t_{max} (h)	12 (9.0-24)	24 (24-48)*	
$t_{\frac{1}{2}}$ (h)	21 (15)	38 (55)*	1.74 (1.30-2.32)
AUC_{0-72h} (ng·h/ml)	10200 (19)	8050 (26)	0.79 (0.65-0.97)
$AUC_{0-\infty}$ (ng·h/ml)	11600 (19)	12200 (32)	1.05 (0.90-1.22)
M-IV-to-pioglitazone $AUC_{0-\infty}$ ratio	2.08 (25)	1.02 (26)***	0.49 (0.40-0.59)
M-III			
C_{max} (ng/ml)	83.7 (37)	48.8 (31)**	0.58 (0.46-0.74)
t_{max} (h)	12 (9.0-24)	24 (12-24)**	
$t_{\frac{1}{2}}$ (h)	22 (19)	40 (60)*	1.84 (1.36-2.48)
AUC_{0-72h} (ng·h/ml)	3150 (40)	2400 (32)*	0.76 (0.62-0.93)
$AUC_{0-\infty}$ (ng·h/ml)	3630 (40)	3820 (50)	1.05 (0.89-1.24)

* $P<0.05$ vs. placebo, ** $P<0.005$ vs. placebo, *** $P<0.001$ vs. placebo

Table 2. Pharmacokinetic variables of clopidogrel, clopidogrel active *cis*-5-thiol metabolite, clopidogrel carboxylic acid and clopidogrel acyl-*β*-D-glucuronide in 10 healthy volunteers after 300 mg (day 1) of clopidogrel, which was ingested 1 h before pioglitazone.

Data are given as median with range.

| Variable	Clopidogrel phase
Clopidogrel	
C_{max} (ng/ml)	1.34 (0.67-32.1)
t_{max} (h)	1.5 (0.92-3.0)
$t_{1/2}$ (h)	2.8 (1.5-5.4)
AUC_{0-13h} (ng·h/ml)	3.97 (1.61-53.9)
Clopidogrel active *cis*-5-thiol metabolite	
C_{max} (ng/ml)	34.7 (15.7-72.6)
t_{max} (h)	0.92 (0.92-1.5)
$t_{1/2}$ (h)	2.9 (1.1-5.9)
AUC_{0-13h} (ng·h/ml)	39.4 (21.4-85.5)
Clopidogrel carboxylic acid	
C_{max} (ng/ml)	11,400 (5,640-15,900)
t_{max} (h)	0.92 (0.92-1.5)
$t_{1/2}$ (h)	3.3 (2.5-6.1)
AUC_{0-13h} (ng·h/ml)	30,900 (16,700-55,600)
Clopidogrel acyl-*β*-D-glucuronide	
C_{max} (ng/ml)	4,120 (1,390-6,640)
t_{max} (h)	1.5 (0.92-2.0)
$t_{1/2}$ (h)	3.0 (2.3-4.4)
AUC_{0-13h} (ng·h/ml)	16,500 (5,330-31,200)
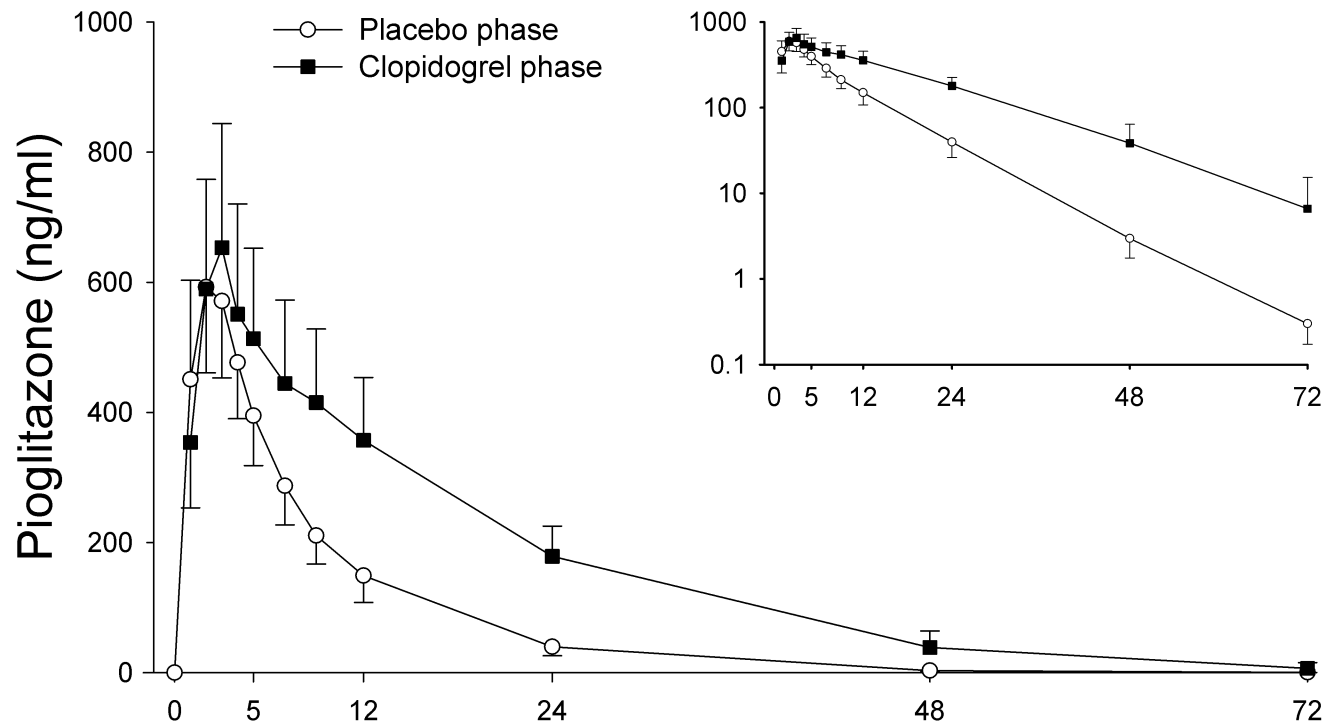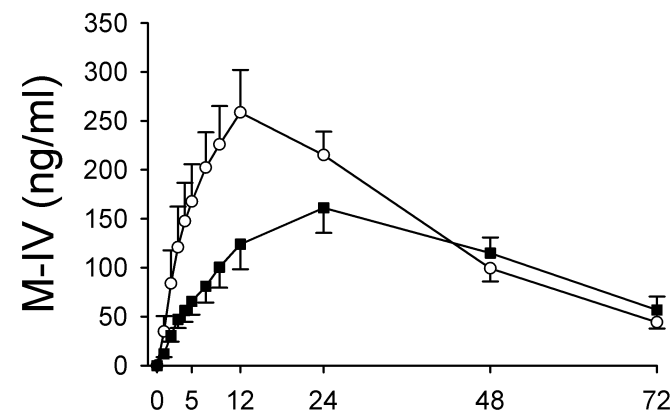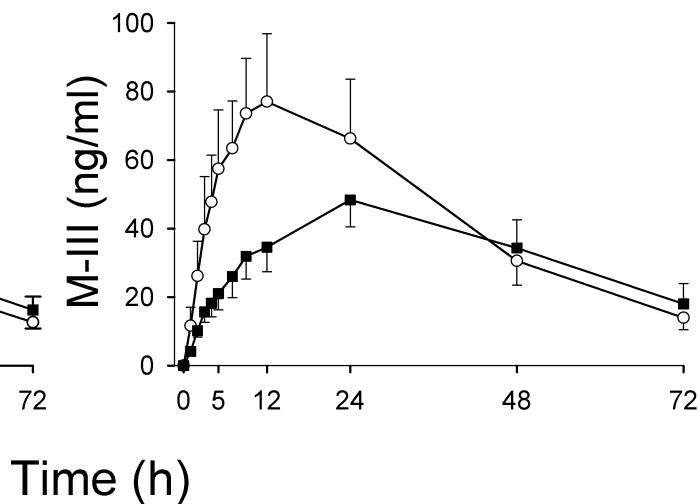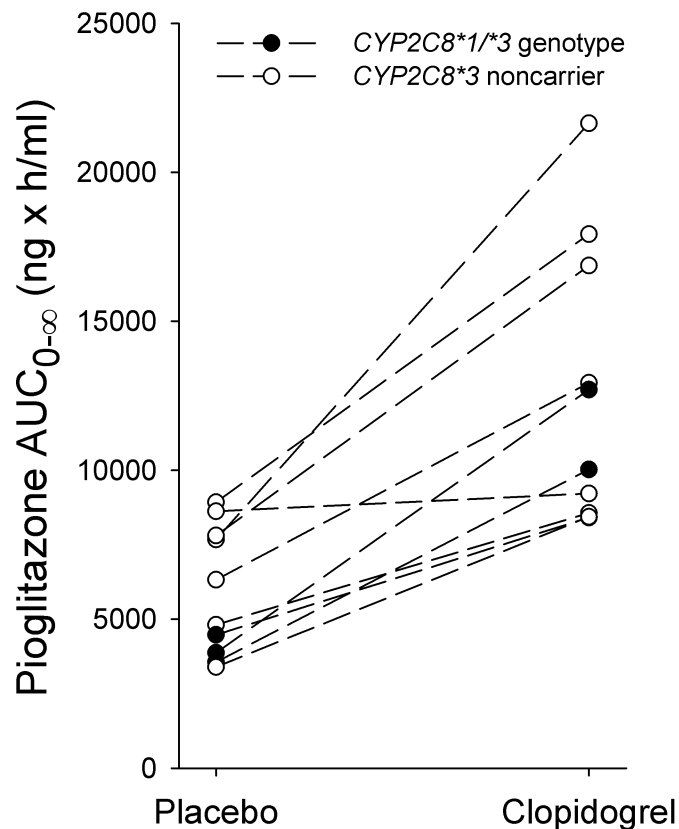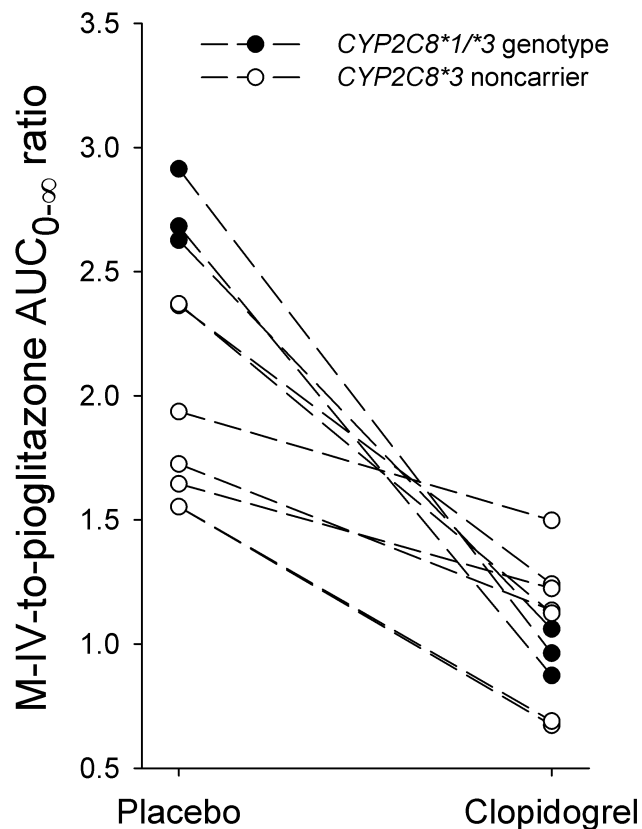
Figure 1

Figure 2

Figure 3

Figure 4