In vitro metabolism of an estrogen-related receptor γ modulator, GSK5182, by human liver microsomes and recombinant cytochrome P450s

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ABSTRACT: GSK5182 (4-[(Z)-1-[4-(2-dimethylaminoethyloxy)phenyl]-hydroxy-2-phenylpent-1-enyl]phenol) is a specific inverse agonist for estrogen-related receptor γ, a member of the orphan nuclear receptor family that has important functions in development and homeostasis. This study was performed to elucidate the metabolites of GSK5182 and to characterize the enzymes involved in its metabolism. Incubation of human liver microsomes with GSK5182 in the presence of NADPH resulted in the formation of three metabolites, M1, M2, and M3. M1 and M3 were identified as N-desmethyl-GSK5182 and GSK5182 N-oxide, respectively, on the basis of liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis. M2 was suggested to be hydroxy-GSK5182 through interpretation of its MS/MS fragmentation pattern. In addition, the specific cytochrome P450 (P450) and flavin-containing monooxygenase (FMO) isoforms responsible for GSK5182 oxidation to the three metabolites were identified using a combination of correlation analysis, chemical inhibition in human liver microsomes and metabolism by expressed recombinant P450 and FMO isoforms. GSK5182 N-demethylation and hydroxylation is mainly mediated by CYP3A4, whereas FMO1 and FMO3 contribute to the formation of GSK5182 N-oxide from GSK5182. The present data will be useful for understanding the pharmacokinetics and drug interactions of GSK5182 in vivo. Copyright © 2014 John Wiley & Sons, Ltd.

Key words: GSK5182; microsomes; cytochrome P450; flavin-containing monooxygenase; oxidation

Introduction

The orphan estrogen-related receptors (ERRs) are members of the NR3B subfamily of nuclear receptors, which consists of three members: ERRα, ERRβ and ERRγ. The ERRs are closely related to estrogen receptors [1] and are primarily expressed in brain, heart, kidney, liver, pancreas, placenta and skeletal muscle [2,3]. Recent studies have reported that hepatic ERRγ regulates hepatic gluconeogenesis [4,5] and hepatic insulin signaling [6], leading to type 2 diabetes mellitus (T2DM), alcohol-induced oxidative stress and liver injury [7].

Structural studies suggest that the ERRs are constitutively active in the absence of natural ligands, such that small molecule ligands could either further activate or repress ERRγ activity [8,9].
GSK5182, a structural analog of 4-hydroxy-tamoxifen, is a highly selective inverse agonist of ERRγ that does not interact with other nuclear receptors [5,10]. It has been shown to restore impaired insulin signaling by inhibiting diacylglycerol production [6]. In addition, GSK5182 lowers blood glucose levels through the inhibition of hepatic gluconeogenesis and ameliorates hyperglycemia in diabetic mice by inhibiting ERRγ transcriptional activity [4,5].

GSK5182 is currently being evaluated in preclinical studies as a new anti-diabetic agent for T2DM [5]. GSK5182 appears to be relatively non-toxic with an oral LD50 in mice of greater than 1000 mg/kg. Furthermore, no significant toxicities have been observed in lung, liver, spleen, kidney or stomach [5]. The absolute oral bioavailability for GSK5182 is 69.2% at a dose of 10 mg/kg in rats (unpublished data). Taken together, these data suggest that GSK5182 could be a good candidate as a new anti-diabetic drug.

Although much is known about the biological effects of GSK5182, its metabolism in human liver microsomes has not been described. To support our early drug discovery and development efforts, mass spectral structural identification of the major metabolites of GSK5182 was conducted following in vitro incubation of human liver microsomes (HLMs) with the drug. In addition, the drug metabolizing enzymes responsible for GSK5182 metabolism were characterized using a combination of correlation analysis, chemical inhibition of human liver microsomes and metabolism by expressed recombinant P450 and flavin-containing monooxygenase (FMO) isoforms. This information may be of clinical importance to explain individual variations in drug metabolism and pharmacokinetics of GSK5182 for different individuals.

Materials and Methods

Chemicals and reagents

GSK5182, N-desmethyl-GSK5182, 4′-hydroxy-GSK5182 and GSK5182 N-oxide were synthesized by the Daegu-Gyeongbuk Medical Innovation Foundation (Daegu, Korea) and Korea Institute of Science and Technology (Seoul, Korea). Benzydamine, benzydamine N-oxide, clomethiazole, d-glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), ketoconazole, methimazole, α-napthoflavone, β-nicotinamide adenine dinucleotide phosphate (β-NADP+), rosiglitazone, SKF-525A, sulfaphenazole, thio-TEPA, tranylcypromine and quinidine were purchased from Sigma-Aldrich (St Louis, MO). S-Benzylpirvanol was purchased from Ultrafine Chemical Co. (Manchester, UK). Solvents were high performance liquid chromatography-mass spectrometry (LC-MS) grade (Fisher Scientific Co., Pittsburgh, PA) and the other chemicals were of the highest quality available. Pooled HLMs (coded HLM 150), ten different human recombinant P450 isoforms and three different human recombinant FMO isoforms were purchased from BD Biosciences (Woburn, MA). Information regarding protein content, P450 content and enzymatic activity is available at wwwbdbiosciences.com.

Identification of GSK5182 metabolites in human liver microsomes

Incubation mixtures containing 1.0 mg of pooled human liver microsomes (HLM 150, BD Biosciences) and 100 μM GSK5182 in 0.1 M potassium phosphate buffer (pH7.4) were pre-incubated at 37°C for 5 min. The reaction was initiated by adding a NADPH-generating system containing 3.3 mM G6P, 1.3 mM β-NADP+, 3.3 mM MgCl2 and 500 unit/ml G6PDH. The reaction mixtures (final volume 100 μl) were incubated for 60 min at 37°C with agitation. Control incubations were conducted with heat-denatured microsomal preparations (100°C for 30 min). The reaction was terminated by the addition of 50 μl ice-cold acetonitrile followed by centrifugation at 18000 x g for 5 min at 4°C. Aliquots (1 μl) of the supernatant were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the identification of GSK5182 metabolites. In all experiments, GSK5182 was dissolved in methanol. The final concentration of methanol did not exceed 1%.

In vitro metabolism of GSK5182 by human liver microsomes, recombinantly expressed P450 isoforms and recombinantly expressed FMO isoforms

Preliminary experiments showed that the formation of GSK5182 metabolites was linear with
respect to both time over 30 min and to liver microsomal protein concentrations from 0 to 1.0 mg/ml at 37 °C. Therefore, a 15 min incubation time and a 0.1 mg/ml microsomal concentration were selected for these experiments. The incubation mixtures containing either 5 μl of human liver microsomes (2 mg protein/ml), P450 isoforms (500 pmol/ml) or FMO isoforms (5 mg protein/ml) and various concentration of GSK5182 (0–200 μM) in 0.1 M phosphate buffer (pH 7.4) were prewarmed for 5 min at 37 °C. The reaction was initiated by adding the NADPH-generating system described above. After 15 min at 37 °C, the reaction was terminated by the addition of 50 μl of ice-cold acetonitrile containing 60 nM omeprazole as an internal standard (IS) and centrifuged at 20000 × g for 5 min at 4 °C. Aliquots of the supernatant were analysed by LC-MS/MS.

**LC-MS/MS analysis of GSK5182 and its metabolites**

A Thermo Vantage triple quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled with a Thermo Accela HPLC system (ThermoFisher Scientific) was used to identify GSK5182 and its metabolites. Separation was performed on a Luan C18 column (2 mm i.d. × 50 mm, 5 μm, 100 Å, Phenomenex, Torrance, CA) with an isocratic mobile phase consisting of acetonitrile and water (20/80, v/v) containing 0.1% formic acid. The flow rate was 0.2 ml/min. The total run time was 15 min. For identification of the metabolites, mass spectra were recorded by electrospray ionization with a positive mode. The optimum operating conditions were determined to be as follows: capillary temperature, 350 °C; vaporizer temperature, 300 °C; sheath gas pressure, 35 Arb; auxiliary gas, 10 Arb; nitrogen gas flow rate, 81/min; spray voltage, 4000 V. Analytical data were processed by Xcalibur (version 2.1, ThermoFisher Scientific).

For quantification of the metabolites, the mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) with the following gradient conditions: 20% A at initial state, A was linearly increased from 20% to 40% over 5 min, held at 40% A for an additional 0.1 min, A was linearly decreased from 40% to 20% over 3 min, and then maintained for re-equilibration for 5 min at a flow rate of 0.2 ml/min. The total run time was 8 min. Quantification was performed by selected reaction monitoring (SRM) of the [M + H]+ ion and the related product ion for each metabolite, using an IS to establish peak area ratios. Quadrupoles Q1 and Q3 were set at unit resolution. Detection of the ions was performed by monitoring the transitions of m/z 418 → 72 for GSK5182 (collision energy 20 eV), 434 → 72 for hydroxy-GSK5182 and GSK5182 N-oxide (collision energy 22 eV), 404 → 58 for N-desmethyl-GSK5182 (collision energy 20 eV), 326 → 102 for benzodamone N-oxide (collision energy 15 eV) and 342 → 203 for omeprazole (IS, collision energy 17 eV). Analytical data were processed by Xcalibur software (version 2.1, ThermoFisher Scientific). The lower limits of quantification for metabolites were 0.2 nM. The coefficient of variation of inter-assay precision for the analyte was less than 15%.

**Chemical inhibition studies with human liver microsomes**

The inhibitory effects of known P450 and FMO inhibitors on the formation of N-desmethyl-GSK5182, hydroxyl-GSK5185 and GSK5182 N-oxide were evaluated to determine the P450 and FMO isoforms involved in the metabolic pathway. The P450 isoform-selective inhibitors used were α-naphthoflavone (5 μM) for CYP1A2, tramcyromine (5 μM) for CYP2A6, thio-TEPA (5 μM) for CYP2B6, rosiglitazone (10 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, S-benzylpirvanol (5 μM) for CYP2C19, quinidine (5 μM) for CYP2D6, clomethiazole (20 μM) for CYP2E1 and ketoconazole (1 μM) for CYP3A. SKF-525A (100 μM) and methimazole (100 μM) were also used as P450 and FMO inhibitors, respectively. Incubations were performed with inhibitor, pooled human liver microsomes (HLM 150, 0.1 mg/ml) and GSK5182 (10 μM). Activities in the presence of the inhibitors were compared with that of the inhibitor-free controls. Except for the addition of P450 and FMO isoform-selective inhibitors, all other incubation conditions and LC-MS/MS analytical conditions were similar to those described above.

**Correlation experiments**

GSK5182 (10 μM) was incubated with human liver microsomes from 15 different livers to test the correlation of GSK5182 metabolism with the activity...
of individual P450s. After adding the internal standard and centrifugation as described above, aliquots of the supernatant were analysed on an LC-MS/MS system. The activity of each P450 isoform was determined using LC-MS/MS as described previously [11]. The isoform-specific reaction markers used to determine the activity of each P450 were: phenacetin O-deethylation with 50 μM phenacetin (CYP1A2), coumarin 7-hydroxylation with 5 μM coumarin (CYP2A6), bupropion hydroxylation with 50 μM bupropion (CYP2B6), paclitaxel 6′-hydroxylation with 10 μM paclitaxel (CYP2C8), tolbutamide 4-methylhydroxylation with 100 μM tolbutamide (CYP2C9), S-mephenytoin 4-hydroxylation with 100 μM S-mephenytoin (CYP2C19), dextromethorphan O-demethylation with 5 μM dextromethorphan (CYP2D6), chlorzoxazone 6-hydroxylation with 50 μM chlorzoxazone (CYP2E1) and midazolam 1′-hydroxylation with 5 μM midazolam (CYP3A). The activity of the FMO enzyme was determined using LC-MS/MS as described previously with some modification [12]. In brief, the incubation mixtures containing human liver microsomes (0.1 mg protein/ml) and benzydamine (20 μM) in phosphate buffer were pre-warmed for 5 min at 37 °C. The reaction was initiated by adding the NADPH-generating system described above. After 20 min at 37 °C, the reaction was terminated by the addition of 50 μl of ice-cold acetonitrile containing 60 nM omeprazole as an internal standard (IS), and centrifuged at 20000 × g for 5 min at 4 °C. Aliquots of the supernatant were analysed by LC-MS/MS. The correlation coefficients between the formation rates of N-desmethyl-GSK5182, hydroxyl-GSK5182 and GSK5182 N-oxide from GSK5182, and the activity of each P450 isoform were calculated by parametric regression analysis (SAS version 8.01; SAS Institute, Cary, NC). A value of $p < 0.05$ was considered statistically significant.

**Results and Discussion**

GSK5182, an ERRγ inverse agonist, is a new therapeutic candidate for the treatment of type 2 diabetes [4]. This study was performed in support of early drug discovery and development efforts in order to identify the metabolic pathway of GSK5182 in HLMs.

![Figure 1. Extracted ion chromatograms of GSK5182 (A) and its three metabolites; N-desmethyl-GSK5182 (B), 3-hydroxy-GSK5182 (C) and GSK5182 N-oxide (C) obtained by LC-MS/MS analysis of the human liver microsomal incubates with GSK5182 in the presence of a NADPH generating system. The retention times for N-desmethyl-GSK5182, 3-hydroxy-GSK5182 and GSK5182 N-oxide were approximately 7.0, 5.0 and 12.4 min, respectively.](image-url)
Following the incubation of GSK5182 with HLMs in the presence of a NADPH generating system, three metabolites (M1, M2 and M3) were detected by LC-MS/MS analysis (Figures 1 and 2) as well as HPLC-UV analysis at 280 nm (Supplementary Figure S1). The retention times for GSK5182, M1, M2 and M3 in LC-MS/MS analysis were approximately 8.1, 7.0, 5.0 and 12.4 min, respectively. When pooled HLMs (1.0 mg protein/ml) were incubated with 10 μM GSK5182 at 37°C, 61.2% of the GSK5182 remained after 1 h. The percentages of M1, M2 and M3 were about 26.2%, 0.3% and 12.3%, respectively based on the quantitation data obtained from calibration curve (Figure 1). The metabolites M1 and M3, respectively, having a protonated molecular ion [M+H]+ at m/z 404 and 434, gave a characteristic fragment ion at m/z 58 and 88 indicating loss of one methyl group and gain of an oxygen atom from a N,N-dimethylethaneamine group (m/z 72) (Figures 3B

![Figure 2. MS/MS spectra of GSK5182 (A) and its three metabolites; N-desmethyl-GSK5182 (B), 3-hydroxy-GSK5182 (C) and GSK5182 N-oxide (D) obtained by LC-MS/MS analysis of the human liver microsomal incubates with GSK5182 in the presence of a NADPH generating system](image-url)
and 3D), whereas that of GSK5182 showed a fragment ion at \( m/z \) 72 (Figure 3A). Similarly, the MS/MS spectrum of M1 and M3 collision-induced fragmented protonated ions gave \( m/z \) 152 and 182, whereas that of GSK5182 showed a fragment ion at \( m/z \) 166, which was also observed in the MS/MS spectrum of toremifene [13], a structural analog of GSK5182. Based on these results, M1 and M3 were identified as N-desmethyl-GSK5182 and GSK5182 N-oxide, respectively. To confirm the structure of GSK5182 N-oxide, titanium (III) chloride was used to reduce GSK5182 N-oxide [14]. After titanium chloride treatment, the M3 peak in the LC-MS/MS chromatogram almost disappeared, suggesting that M3 is an N-oxide metabolite of GSK5182 (Supplementary Figure S2). Metabolite M2 gave a protonated molecular ion at \( m/z \) 434, one oxygen atom more than the parent compound. The MS/MS spectrum of M2 gave fragment ions at \( m/z \) 166 and 72 which are also found in GSK5182, indicating that the hydroxylation site is not at the \( N,N \)-dimethyl-2-phenoxyethaneamine group (Figure 3C). To elucidate the exact structure of M2, MS/MS/MS (MS3) analysis of the fragments at \( m/z \) 362 and 225 was performed using an ion trap mass spectrometer (ThermoFisher LTQ system). The MS3 spectrum of \( m/z \) 362 ion gave a characteristic ion at \( m/z \) 215, whereas that of GSK5182 showed a fragment ion at \( m/z \) 199, indicating that the oxygen atom was inserted on the phenolic moiety of GSK5182 (Supplementary Figure S3).

The MS3 spectrum of the \( m/z \) 225 ion gave a characteristic ion at \( m/z \) 147, whereas that of GSK5182 showed a fragment ion at \( m/z \) 131. The exact hydroxylation site in the phenolic ring of M2 could not be determined. However, it can be predicted that the hydroxylation site in the phenolic ring of M2 might be in the ortho- rather than in the meta-position based on previous findings showing that 4-hydroxy-tamoxifen, one of the metabolites of tamoxifen, is preferentially metabolized to 3,4-dihydroxy-tamoxifen [15]. 3,4-Dihydroxy-toremifene was also found in human urine after oral administration of toremifene to a healthy male volunteer, whereas 2,4-dihydroxy-toremifene was not detected [16]. Based on these results, M2 is most likely 3-hydroxy-GSK5182, rather than 2-hydroxy-GSK5182. Similar metabolites have been found in studies of the metabolism of tamoxifen and toremifene [17,18]. The structures of N-desmethyl-GSK5182 (M1), 3-hydroxy-GSK5182 (M2) and GSK5182 N-oxide (M3) were confirmed by product ion scan mass spectra of those compounds and/or co-chromatography with authentic standard.

Next, the metabolizing enzymes responsible for the biotransformation of GSK5182 were characterized using HLMs and recombinant P450 and FMO isoforms. GSK5182 was metabolized by HLMs in the presence of a NADPH generating system but was not metabolized in the absence of NADPH, indicating that GSK5182 metabolism is P450 and/or FMO dependent. The data indicate that
N-desmethyl- and 3-hydroxy-GSK5182 formation from GSK5182 is mainly catalysed by the CYP3A4 isoform, whereas GSK5182 N-oxide formation is mainly by the FMO1 isoform.

First, the formation rates of N-desmethyl- and 3-hydroxy-GSK5182 were inhibited by SKF-525A (~85%), a general P450 inhibitor [19] and by ketoconazole (95–97%), a selective CYP3A inhibitor [20], whereas that of GSK5182 N-oxide was inhibited (69%) by methimazole, a general FMO inhibitor (Figure 4) [21].

Second, GSK5182 N-demethylation was predominately mediated by CYP3A4, with little contribution of CYP3A5 (Figure 5A). Recombinant CYP3A4 metabolized GSK5182 to 3-hydroxy-GSK5182, whereas other P450 and FMO isoforms did not (Figure 5B). The identification of CYP3A4 as the catalyst of GSK5182 hydroxylation may allow us to use GSK5182 to probe CYP3A4 enzyme activity. The substrates of CYP3A that are used for inhibition studies are midazolam, testosterone and nifedipine (FDA guidance, http://www.fda.gov/cder/guidance/index.htm). However, midazolam 1′-hydroxylation, testosterone 6β-hydroxylation and nifedipine dehydrogenation are catalysed by CYP3A5 as well as CYP3A4 isoforms [22].

GSK5182 N-oxide was mainly formed by FMO1, with a minor contribution from FMO3 and CYP3A4 (Figure 5C). Therefore, the inhibition of GSK5182 N-oxidation by ketoconazole (Figure 4) could be explained by a minor contribution of CYP3A to GSK5182 N-oxide formation (Figure 5C). Previous studies have reported that tamoxifen, a structural analog of GSK5182, is also metabolized to N-desmethyl-tamoxifen mainly by CYP3A and to tamoxifen N-oxide by FMO1 [23,24]. Dehal and Kuper [15] also reported that 4-hydroxytamoxifen, a metabolite of tamoxifen, was further metabolized to 3,4-dihydroxytamoxifen mainly by CYP3A4, similar to 3-hydroxy-GSK5182 formation from

Figure 4. Effects of cytochrome P450 isoform-selective inhibitors and an FMO inhibitor on the metabolism of GSK5182 by human liver microsomes. GSK5182 (10 μM) was incubated with pooled human liver microsomes (HLM 150, 0.1 mg/ml) in the presence of various inhibitors at 37 °C for 15 min. The chemical inhibitors used were as follows: a-naphthoflavone (5 μM) for CYP1A2, tranylcypromine (5 μM) for CYP2A6, thio-TEPA (5 μM) for CYP2B6, rosiglitazone (10 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, S-benzylmirkanol (5 μM) for CYP2C19, quinidine (5 μM) for CYP2D6, clomethiazole (20 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A, SKF 525-A (100 μM) for cytochrome P450 and methimazole (100 μM) for FMO. Data are averages of remaining activity relative to the control metabolite formation rate determined from triplicate samples.

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GSK5182. These findings suggest that the metabolic pathway of GSK5182 (Figure 6) was similar to that of tamoxifen (Supplementary Figure S4). The N-demethylation of toremifene having a similar
structure to GSK5182 is also mediated by CYP3A4 [26]. The levels of involvement of other recombinant P450 and FMO isoforms in the formation of GSK5182 metabolites were negligible.

Third, midazolam 1′-hydroxylation activity, a marker of CYP3A activity [27], in 15 individual HLMs exhibited a high correlation with the formation rates of N-desmethyl- and 3-hydroxy-GSK5182 (r = 0.60) (Table 1). The significant correlation observed between the activity of CYP2C9 and N-desmethyl- and 3-hydroxy-GSK5182 formation in the panel of HLMs tested may not be caused by the actual involvement of CYP2C9 in GSK5182 metabolism. Because our recombinant experiments and chemical inhibition study do not support a significant role of CYP2C9 in GSK5182 metabolism, the observed correlation is probably derived from the significant correlation between the activity of CYP2C9 and CYP3A (Pearson r = 0.71, p < 0.01) in the bank of human livers tested (Supplementary Figure S5). This intra-P450 activity correlation in the panel of HLMs used was previously reported by Li et al. (2003). They also observed a significant correlation (p < 0.05) between the activities of CYP3A-mediated midazolam 1′-hydroxylation and CYP2C9-mediated diclofenac 4′-hydroxylation in the HLMs bank [26]. Benzydamine N-oxidation activity, a marker of FMO activity [28] in 15 individual HLMs exhibited a high correlation with the formation rates of GSK5182 N-oxide formation (r = 0.82) (Table 1). In addition, there was no correlation between GSK5182 N-oxidation and nine other P450 markers, supporting the conclusions that GSK5182 N-oxide formation is mainly mediated by FMO. Based on these findings, the metabolic pathway for GSK5182 metabolism by human liver microsomes is proposed in Figure 6.

In conclusion, this study demonstrates that N-desmethyl- and 3-hydroxy-GSK5182, and GSK5182 N-oxide are the major metabolites of GSK5182 in human liver microsomes. The chemical inhibition study, correlation study and incubation study with P450 and FMO enzymes showed that CYP3A4 and FMO1 were responsible for the formation of these three metabolites from GSK5182.

Acknowledgements

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Conflict of Interest

There were no conflicts of interest.

References


Table 1. Correlation of formation rates of N-desmethyl-GSK5182, 3-hydroxy-GSK5182 and GSK5182 N-oxide from GSK5182 (10 μM) with the P450 and FMO marker activities in HLMs (n = 15)

<table>
<thead>
<tr>
<th>Activity</th>
<th>P450 isoforms</th>
<th>N-Desmethyl- GSK5182</th>
<th>3-Hydroxy- GSK5182</th>
<th>GSK5182 N-oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin O-deethylation</td>
<td>CYP1A2</td>
<td>0.12 (p = 0.66)</td>
<td>0.13 (p = 0.64)</td>
<td>0.20 (p = 0.47)</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2A6</td>
<td>0.01 (p = 0.97)</td>
<td>0.04 (p = 0.89)</td>
<td>0.08 (p = 0.78)</td>
</tr>
<tr>
<td>Bupropion hydroxylation</td>
<td>CYP2B6</td>
<td>0.24 (p = 0.38)</td>
<td>0.25 (p = 0.38)</td>
<td>0.30 (p = 0.47)</td>
</tr>
<tr>
<td>Paclitaxel 6ε-hydroxylation</td>
<td>CYP2C8</td>
<td>0.24 (p = 0.41)</td>
<td>0.21 (p = 0.46)</td>
<td>0.14 (p = 0.28)</td>
</tr>
<tr>
<td>Tolbutamide 4-methylhydroxylation</td>
<td>CYP2C9</td>
<td>0.77 (p &lt; 0.01)</td>
<td>0.75 (p &lt; 0.01)</td>
<td>0.77 (p = 0.62)</td>
</tr>
<tr>
<td>S-Mephenytoin 4-hydroxylation</td>
<td>CYP2C19</td>
<td>0.01 (p = 0.97)</td>
<td>0.02 (p = 0.95)</td>
<td>0.24 (p = 0.39)</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>CYP2D6</td>
<td>0.37 (p = 0.17)</td>
<td>0.34 (p = 0.22)</td>
<td>0.02 (p = 0.95)</td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>CYP2E1</td>
<td>0.09 (p = 0.75)</td>
<td>0.07 (p = 0.81)</td>
<td>0.38 (p = 0.16)</td>
</tr>
<tr>
<td>Midazolam 1′-hydroxylation</td>
<td>CYP3A</td>
<td>0.60 (p = 0.02)</td>
<td>0.60 (p = 0.02)</td>
<td>0.15 (p = 0.59)</td>
</tr>
<tr>
<td>Benzydamine N-oxidation</td>
<td>FMO</td>
<td>0.04 (p = 0.89)</td>
<td>0.10 (p = 0.73)</td>
<td>0.82 (p &lt; 0.01)</td>
</tr>
</tbody>
</table>

Data were analysed using Pearson’s parametric correlation test. The activity of each P450 isoform was determined using the respective specific substrate probe reaction, as described previously [11]. The activity of FMO was determined using benzydamine, a FMO selective substrate, as described previously [25].


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**Supporting information**

Additional supporting information may be found in the online version of this article at publisher’s web site:

**Figure S1.** HPLC chromatogram of human liver microsomal incubates with GSK5182. GSK5182 and its metabolites (N-desmethyl-GSK5182 (M1), 3-hydroxy-GSK5182 (M2) and GSK5182 N-oxide (M3)) were monitored at 280 nm.

**Figure S2.** SRM chromatogram of GSK5182 N-oxide (M3) obtained by LC-MS/MS analysis of the human liver microsomal incubates with GSK5182 in the presence of a NADPH generating system (before (A) and after (B) titanium (III) chloride treatment).

**Figure S3.** MS/MS/MS (MS3) spectra and fragmentation schemes of 4′-hydroxy-GSK5182 (A) and 3-hydroxy-GSK5182 (B) obtained using ion trap mass spectrometry.

**Figure S4.** Metabolic pathway of tamoxifen in human liver microsomes.

**Figure S5.** Correlation analysis between the CYP3A-mediated midazolam 1′-hydroxylation activity and GSK5182 N-demethylation (A), 3-hydroxylation (B) and tolbutamide 4-hydroxylation (C) and between the FMO-mediated benzydamine N-oxidation and GSK5182 N-oxide formation (D) in 15 HLMs.