Identification of a Novel CYP2C19-Mediated Metabolic Pathway of S-Citalopram in Vitro

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ABSTRACT:

Systemic exposure of the antidepressant S-citalopram (escitalopram, SCIT) differs several-fold according to variable cytochrome P450 CYP2C19 activity, demonstrating the importance of this enzyme for the metabolic clearance of SCIT in vivo. However, previous studies have indicated that the involvement of CYP2C19 in formation of the metabolite N-desmethyl S-citalopram (SDCIT) is limited. Therefore, the purpose of the present in vitro study was to investigate to what extent the CYP2C19-mediated clearance of SCIT was due to a metabolic pathway different from N-desmethylation and to identify the product(s) of this possible alternative metabolic reaction. CYP2C19-mediated metabolism of SCIT was investigated using recombinant Supersomes expressing human CYP2C19. Initial experiments showed that approximately half of the CYP2C19-mediated clearance of SCIT was accounted for by the N-desmethylation pathway. Subsequent experiments identified that, in addition to SDCIT, the propionic acid metabolite of SCIT (SCIT PROP) was formed by CYP2C19 in vitro. Formation of SCIT PROP accounted for 35% of total CYP2C19-mediated clearance of SCIT (calculated as the ratio between metabolite formation rate and substrate concentration at low substrate concentration). Moreover, analysis of samples from six CYP2C19-genotyped patients treated with SCIT indicated that differences in serum concentrations of SCIT between CYP2C19 genotypes may be due to a combined effect on SCIT PROP and SDCIT formation. Identification of SCIT PROP as a metabolic pathway catalyzed by CYP2C19 might explain why impaired CYP2C19 activity has a substantially larger effect on SCIT exposure than estimated from in vitro data based solely on formation of SDCIT.

The selective serotonin reuptake inhibitor citalopram (CIT, 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofurane-5-carbonitrile) is a racemic compound for which pharmacological activity resides mainly in the S-enantiomer (escitalopram, SCIT) (Hyttel et al., 1992). Pure SCIT was introduced as an individual drug in 2001 and is currently widely used in the treatment of depression.

Several studies have shown that CYP2C19 is important for the metabolic clearance of SCIT in vivo (Bondolfi et al., 1996, 2000; Herrlin et al., 2003; Rudberg et al., 2006, 2008). Pharmacogenetic studies have shown that carriers of variant alleles encoding defective CYP2C19 metabolism obtain up to 6-fold higher systemic exposure of SCIT compared with individuals homozygous for the wild-type allele (Herrlin et al., 2003; Rudberg et al., 2008). Moreover, drug interaction studies have demonstrated that plasma concentrations of SCIT increased significantly subsequent to the addition of a CYP2C19 inhibitor (fluvoxamine or fluoxetine) to the treatment regimen (Bondolfi et al., 1996, 2000).

In vitro studies have shown that CYP2C19 mediates the formation of N-desmethyl S-citalopram (SDCIT) (Kobayashi et al., 1997; Rochat et al., 1997; Olesen and Linnet, 1999; von Moltke et al., 2001). However, CYP3A4 and CYP2D6 also catalyze this reaction in vitro, and it has been shown that less than 40% of the N-desmethylation clearance of SCIT in vivo is accounted for by CYP2C19 (Kobayashi et al., 1997; von Moltke et al., 2001). Because the systemic exposure of SCIT is up to 6-fold higher in individuals with deficient CYP2C19 activity (Rudberg et al., 2008), the fraction of SCIT clearance that is mediated by CYP2C19 needs to be substantially larger than 40%. Thus, CYP2C19 is likely to be involved in the formation of metabolites other than SDCIT.

The involvement of CYP2C19 in pathways besides N-desmethylation is supported by a previous study investigating the pharmacokinetics of racemic CIT in relation to the CYP2C19 phenotype (Sindrup et al., 1993). This study indicated that a larger fraction of the CYP2C19-mediated clearance of this compound was represented by metabolic reactions other than N-desmethylation. However, no previous studies have, to our knowledge, been able to identify CYP2C19-mediated metabolites of SCIT besides SDCIT.

ABBR EVIATIONS: CIT, citalopram; SCIT, S-citalopram; P450, cytochrome P450; SDCIT, N-desmethyl S-citalopram; LC, liquid chromatography; MS/MS, tandem mass spectrometry; ALP, alprenolol; Clint, intrinsic clearance; UPLC, ultraperformance liquid chromatography; MRM, multiple reaction monitoring; SDDCIT, N-didesmethyl S-citalopram; SCIT NO, S-citalopram N-oxide; SCIT PROP, S-citalopram propionic acid; TDM, therapeutic drug monitoring.
The purpose of the present study was to investigate to what extent the CYP2C19-catalyzed clearance of SCIT was due to a metabolic pathway different from N-desmethylation. Furthermore, we aimed to identify the product(s) of this possible alternative metabolic reaction.

Materials and Methods

Materials and Reagents. Reference substances of CIT and the available metabolites N-desmethyl citalopram, N-didemethyl citalopram, citalopram N-oxide, and citalopram propionic acid for LC-MS/MS analysis and pure enantiomer of S-citalopram (SCIT) for incubations were gifts from Lundbeck AS (Copenhagen, Denmark). The pure enantiomer of SCIT for incubations was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Baculovirus-infected insect microsomes expressing human recombinant CYP2C19, P450 reductase, and cytochrome b5 (Supersomes) and micromoles without P450 activity (control Supersomes) were purchased from BD Gentest (Woburn, MA). NADPH was purchased from Sigma-Aldrich (St. Louis, MO).

In Vitro Metabolism Experiments. Methanol standard solutions of SCIT and SDCIT were evaporated to dryness and redissolved in incubation matrix, which consisted of 118 mM Tris-H2SO4 (pH 7.5), 3.3 mM MgCl2, and 1.6 mM NADPH. Before preheating, reactions were initiated by addition of ice-cold Supersomes. Incubations were performed in a shaking bath at 57°C. Reactions were terminated by mixing the incubation 1:1 (v/v) with precipitation solution [acetonitrile-methanol (90:10) with alloprenol (ALP, internal standard) final concentration 330 nM]. The samples were kept on ice for at least 30 min before centrifugation at 1600×g for 10 min at 4°C (Universal 32R centrifuge; Hettig Zentrifugen, Tuttlingen, Germany) and withdrawal of supernatants. All experiments were performed with the same lot of Supersomes.

Initially, substrate depletion experiments were performed to quantify the fraction of total intrinsic clearance (total CLint) of SCIT by recombinant CYP2C19 Supersomes that was attributable to formation of SDCIT. To investigate the potential subsequent metabolism of SDCIT by CYP2C19, substrate depletion experiments with SDCIT were also performed. The substrate (SCIT or SDCIT, 1 μM) was incubated with 50 pmol/ml recombinant CYP2C19 in a final incubation volume of 700 μl. Sample aliquots of 50 μl were collected at fixed time intervals (2.5–40 min). Before the experiments, linear depletion of SCIT was observed. Incubation with control Supersomes without P450 activity was performed similarly, using protein concentrations equal to those for active Supersomes. Three separate experiments were performed.

Calculations of intrinsic clearance (CLint) of SCIT and SDCIT by recombinant CYP2C19 Supersomes (described below) indicated that a substantial fraction of total CLint of SCIT could not be accounted for by the N-desmethylation pathway. Samples for the qualitative search for other metabolites of SCIT with recombinant CYP2C19 Supersomes were investigated with regard to available metabolites of SCIT other than SDCIT, i.e., SDDCIT, SCIT NO, and SCIT PROP. SDDCIT and SCIT NO were analyzed by the UPLC-MS/MS method described for SCIT and SDCIT quantification. Detection was performed by MRM in the positive mode at m/z 297 → 109 (SDDCIT) and 341 → 109 (SCIT NO). The retention times were 2.38 min for SDDCIT and 2.54 min for SCIT NO. SCIT PROP was analyzed on a TSQ Quantum Access mass analyzer after separation on a Dionex Ultimate 3000 high-performance liquid chromatograph. Data acquisition was performed with XCalibur 2.0.7 (ThermoFinn, Oslo, Norway). Chromatographic separation was performed on a BioBasic C8 column (300 Å, 5 μm, 1 mm × 50 mm; Holger, Oslo, Norway) at room temperature using a gradient from 25 to 33% of mobile phase B within 24 min at 1.00 ml/min (mobile phase A: 5% methanol in 10 mM ammonium acetate, pH 4.8; mobile phase B: 95% methanol in 10 mM ammonium acetate, pH 4.8). The retention time for SCIT PROP was 15 to 16 min, and total run time was 40 min. Spray voltage was 5 kV, capillary temperature was 270°C, and collision gas pressure (argon) was 1.6 mtorr. Collision energy was 17 V. SCIT PROP was detected by MRM in the negative mode at the following m/z transitions: 311 → 267, 311 → 239, and 311 → 237 (all SCIT PROP). It was assumed that the fragmentation involved elimination of the carboxylic and propionic acid groups.

When we searched for possible unknown metabolites of SCIT formed by CYP2C19, precursor ion scans of MS/MS fragments of the available metabolites were performed. Before the precursor ion scans, samples were separated on a BioBasic C8 column (300 Å, 5 μm, 1 mm × 50 mm; Waters, Milford, MA). Chromatographic separation was performed with an Acquity UPLC BEH Shield RP18 column (1.7 μm, 1.0 × 100 mm; Waters) by gradient elution (18–45% acetonitrile in 10 mM ammonium acetate buffer, pH 4.8, within 2.8 min at 0.2 ml/min) at 40°C. The retention times were 2.21 min for ALP, the internal standard, 2.47 min for SCIT, and 2.53 min for SCIT. Total run time was 5 min. Capillary voltage was 0.9 kV, source temperature and desolvation temperatures were 100 and 450°C, respectively, and collision gas pressure (argon) was 4.8 × 10−2 mbar. Cone voltages were 32, 28, and 35 V, and collision energy was 20, 16, and 17 eV for SCIT, SDCIT, and ALP, respectively. Detection was performed by multiple reaction monitoring (MRM) in the positive electrospray ionization mode at the following m/z transitions: 325 → 262 (SCIT), 311 → 262 (SDCIT), and 250 → 116 (ALP). Formation of the m/z 262 fragment of SCIT and SDCIT was supposed to involve loss of the fluorine and amino groups. Processing was performed with QuanLynx software (version 4.1; Waters). Calibration curves ranged from 100 to 1250 nM for SCIT and from 20 to 1250 nM for SDCIT. Data points of the calibration curves were weighted by 1/γ, and R² values were >0.99 (α = 5). Intra- and interassay accuracy and precision were less than 11% (α = 5) for both substances. All measured concentrations were within the range of the calibration curves.

Search for CYP2C19-mediated metabolites other than SDCIT. Incubations of SCIT with recombinant CYP2C19 Supersomes were investigated with regard to available metabolites of SCIT other than SDCIT, i.e., SDDCIT, SCIT NO, and SCIT PROP. SDDCIT and SCIT NO were analyzed by the UPLC-MS/MS method described for SCIT and SDCIT quantification. Detection was performed by MRM in the positive mode at m/z 297 → 109 (SDDCIT) and 341 → 109 (SCIT NO). The retention times were 2.38 min for SDDCIT and 2.54 min for SCIT NO. SCIT PROP was analyzed on a TSQ Quantum Access mass analyzer after separation on a Dionex Ultimate 3000 high-performance liquid chromatograph. Data acquisition was performed with XCalibur 2.0.7 (ThermoFinn, Oslo, Norway). Chromatographic separation was performed on a BioBasic C8 column (300 Å, 5 μm, 1 mm × 50 mm; Holger, Oslo, Norway) at room temperature using a gradient from 25 to 33% of mobile phase B within 24 min at 1.00 ml/min (mobile phase A: 5% methanol in 10 mM ammonium acetate, pH 4.8; mobile phase B: 95% methanol in 10 mM ammonium acetate, pH 4.8). The retention time for SCIT PROP was 15 to 16 min, and total run time was 40 min. Spray voltage was 5 kV, capillary temperature was 270°C, and collision gas pressure (argon) was 1.6 mtorr. Collision energy was 17 V. SCIT PROP was detected by MRM in the negative mode at the following m/z transitions: 311 → 267, 311 → 239, and 311 → 237 (all SCIT PROP). It was assumed that the fragmentation involved elimination of the carboxylic and propionic acid groups.

When we searched for possible unknown metabolites of SCIT formed by CYP2C19, precursor ion scans of MS/MS fragments of the available metabolites were performed. Before the precursor ion scans, samples were separated as described for the MRM detection of SCIT PROP. By injection of standard solutions of CIT, N-desmethyl citalopram, N-didemethyl citalopram, citalopram N-oxide, and citalopram propionic acid, their presence was confirmed with the chosen precursor ion scan set at 6.0, 7.2, 7.2, 9.1, and 15 min, respectively.

Calculations of CLint. Substrate depletion. Substrate depletion data from each separate experiment were fitted to the monoeponential decay model (eq. 1):

\[ S_t = S_0 e^{-k t} \]  

where \( S_t \) is the substrate concentration at time \( t \), \( S_0 \) is the initial substrate concentration, and \( k \) is the depletion rate constant (min⁻¹). The data points were weighted by 1/γ. Visual inspection of the residual plots was used to determine the time range for which the substrate depletion followed monoeponential decay (plots not shown). For each separate experiment, \( k \) was estimated from data points in the monoeponential time range, and total CLint of substrate by recombinant CYP2C19 Supersomes was calculated (eq. 2):

\[ \text{Total CL}_{\text{int}} = \left( k \times 1000 \right) / \text{pmol CYP2C19/ml} \]  

Providing that the substrate concentration is markedly less than the \( K_m \) value (<10%). \( \text{CL}_{\text{int}} \) due to the formation of a specific metabolite may be determined as the ratio of metabolite formation rate (v) and substrate concentration (S) (eq. 3) (Houston and Kenworthy, 2000; Nath and Atkins, 2006):
Therefore, in addition to depletion of the substrate, the formation of metabolites was measured in the substrate depletion experiments with SCIT. For the separate time intervals of an experiment, \( v \) was divided by the SCIT concentration (\( S \)) at the beginning of the time interval. Mean \( v/S \) for the time intervals was used as an estimate of the CL_{int} attributable to formation of a specific metabolite. Only time intervals in the monoeponential time range of the SCIT depletion curves were used.

**Substrate saturation.** \( v \) as a function of \( S \) was fitted to equations for classic Michaelis-Menten enzyme kinetics, substrate activation, and substrate inhibition (eqs. 4–6) (Houston and Kenworthy, 2000; Venkatakrishnan et al., 2001) by nonlinear regression. Data points were weighted by \( 1/v \), and visual inspection of the graph of the fits and Akaike’s information criterion was used to choose the appropriate model (Venkatakrishnan et al., 2001; Motulsky and Christopoulos, 2003). Enzyme kinetic parameters for metabolite formation were estimated (maximum metabolite formation rate \( V_{\text{max}} \), Michaelis-Menten constant \( K_m \), substrate concentration at half the maximal rate \( S_{\text{50}} \), Hill coefficient \( A \), and substrate inhibition constant \( K_{\text{si}} \)):

\[
\text{Michaelis-Menten: } v = \frac{V_{\text{max}} \times S}{(K_m + S)} \tag{4}
\]

\[
\text{Substrate activation: } v = \frac{V_{\text{max}} \times S^A}{(S_{\text{50}}^A + S^A)} \tag{5}
\]

\[
\text{Substrate inhibition: } v = \frac{V_{\text{max}} \times S}{(K_m + S + (S^2/K_{\text{si}}))} \tag{6}
\]

Curve-fitting was performed using GraphPad Prism (version 4.03; GraphPad Software Inc., San Diego, CA). CL_{int} based on substrate saturation experiments was calculated as follows (eq. 7):

\[
\text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_m} \tag{7}
\]

**Samples from Patients with Various CYP2C19 Genotypes.** The Department of Psychopharmacology, Diakonhjemmet Hospital (Oslo, Norway) performs P450 genotyping and therapeutic drug monitoring (TDM) of psycho- tropic drugs as part of the clinical follow-up of psychiatric patients. Information about the CYP2C19 genotype of patients treated with SCIT was drawn from TDM files, and serum samples from six patients genotyped as CYP2C19*1/*17 (ultrarapid metabolizers, \( n = 2 \)), CYP2C19*1/*1 (extensive metabolizers, \( n = 2 \)), or CYP2C19*2/*2 (poor metabolizers, \( n = 2 \)) were randomly selected. Serum concentrations of SCIT and SDCIT, which were determined previously, were retrieved from the TDM files, and the samples were reanalyzed with regard to CYP2C19-mediated metabolites besides SDCIT. Information about drug dose, patient age and gender, coprescribed drugs, time between last drug intake and serum sampling, and recent dose adjustment was obtained from the requisition forms. Before reanalysis, serum samples were purified by protein precipitation (~150 \( \mu \)l of serum and 300 \( \mu \)l of acetonitrile-methanol (90:10) before centrifugation at 1800 \( g \) for 10 min at 4°C. Supernatants (225 \( \mu \)l) were evaporated to dryness and redissolved in mobile phase A (50 \( \mu \)l). To adjust for differences in drug dosages, serum concentrations were divided by daily drug dose, i.e., dose-adjusted serum concentrations (nmol/l per mg). Mean dose-adjusted serum concentrations and...
mean metabolite/parent ratios (SDCIT/SCIT and SCIT PROP/SCIT) were compared among CYP2C19 genotypes.

Results

Contribution from the \(N\)-Desmethylation Pathway to CYP2C19-Mediated Clearance of SCIT. Substrate depletion of SCIT (1 \(\mu\)M) in human recombinant CYP2C19 Supersomes showed monoeponential decay between 2.5 and 20 min (eq. 1; Fig. 1A), and total \(\text{CL}_{\text{int}}\) of SCIT was estimated to be \(0.559 \pm 0.041 \mu\text{l/min/pmol} \) CYP2C19 (eq. 2, mean ± S.E.M.). \(\text{CL}_{\text{int}}\) due to formation of SDCIT was calculated to be \(0.240 \pm 0.009 \mu\text{l/min/pmol} \) CYP2C19 (eq. 3, mean ± S.E.M.), and accounted for 43% of total \(\text{CL}_{\text{int}}\) of SCIT. Figure 2A shows the recovery of SCIT and SDCIT at the fixed time points (2.5–20 min) of the SCIT depletion experiments, illustrating an unexplained loss of substrate at all time points, i.e., depleted SCIT not recovered as SDCIT.

Incubation of SDCIT (1 \(\mu\)M) with recombinant CYP2C19 Supersomes produced only a minor substrate loss. The data points (2.5–20 min) were fitted to the monoeponential decay model (eq. 1; Fig. 1B). Total \(\text{CL}_{\text{int}}\) of SDCIT was estimated to be \(0.060 \pm 0.002 \mu\text{l/min/pmol} \) CYP2C19 (eq. 2, mean ± S.E.M.), indicating that the potential subsequent metabolism of SDCIT by CYP2C19 was minor. Hence, a substantial fraction (~50%) of the total \(\text{CL}_{\text{int}}\) of SCIT by recombinant CYP2C19 Supersomes was not accounted for by the \(N\)-desmethylation pathway. There was no substrate loss (SCIT nor SDCIT) in incubations with control Supersomes without P450 activity.

Search for CYP2C19-Mediated Metabolites Other than SDCIT. Incubation of SCIT (1 \(\mu\)M) with recombinant CYP2C19 Supersomes was investigated with regard to available metabolites of SCIT (MRM analysis) and possibly unknown metabolites (precursor ion scan analysis). MRM analysis showed that the incubation contained a substantial amount of SCIT PROP. A precursor ion scan in negative mode of \(m/z\) 267, a MS/MS fragment of SCIT PROP, produced one peak (15.3 min), which corresponded to SCIT PROP (Fig. 3A). A similar chromatogram was obtained for \(m/z\) 237, another MS/MS fragment of SCIT PROP. MRM of SDDCIT and SCIT NO showed that only trace amounts of these metabolites were formed (data not shown). An MS/MS fragment common for SCIT, SDCIT, SCIT NO, and SDDCIT (\(m/z\) 109) was identified in the positive mode. A precursor ion scan of \(m/z\) 109 produced one peak (7.3 min) (Fig. 3B). Inspection of the mass spectrum averaged between 6.8 and 7.8 min confirmed that the peak consisted mainly of SDCIT, with some unmetabolized SCIT in front of the peak (data not shown). Besides this, only trace amounts of SDDCIT and SCIT NO were seen in the MS spectrum. To summarize, these experiments indicated that, in addition to SDCIT, SCIT PROP was formed in incubations of SCIT with recombinant CYP2C19 Supersomes. There was no indication of metabolites of SCIT other than SDCIT and SCIT PROP being present in the incubations in considerable amounts.

Contribution from SCIT PROP Formation to CYP2C19-Mediated Clearance of SCIT. To estimate the contribution of the SCIT PROP formation to the substrate loss of SCIT, an additional substrate depletion experiment (1 \(\mu\)M) was performed. The HPLC-MS/MS method for detection of SCIT PROP described previously (see Materials and Methods) was evaluated with regard to quantitative determination of SCIT PROP. Before the analysis, 150 \(\mu\)l of the supernatants was evaporated to dryness and redissolved in 50 \(\mu\)l of mobile phase A. The method’s performance was satisfactory as shown from its evaluation: quantification of SCIT PROP was performed from linear plots of peak area versus concentration of calibration standards (15–500 nM) in incubation matrix (\(R^2 > 0.99, n = 5\)). Intra- and interassay accuracy and precision were less than 12.5% (\(n = 4–5\)). This method was also used for quantification of SCIT PROP in serum samples from six patients. Of all samples analyzed, SCIT PROP concentrations were below the calibration curve in only four samples. However, they were above the limit of detection (4 nM, signal/noise ratio = 3:1) and were quantified by extrapolation of the calibration curves.
SCIT PROP was detected at all time points of the SCIT depletion experiment (Figs. 2B and 4A). Total CLint of SCIT in this experiment was 0.415 µL/min/pmol CYP2C19 (eq. 2), of which 35% (0.144 ± 0.012 µL/min/pmol CYP2C19) was attributable to the formation of SCIT PROP (eq. 3; Table 1). CLint due to SDCIT formation was 0.210 ± 0.014 µL/min/pmol CYP2C19 (eq. 3; Table 1A; Fig. 5). CLint due to SDCIT formation accounted for 51% of the total CLint of SCIT in this experiment. Hence, based on the substrate saturation experiments, CLint due to SDCIT formation was 69% compared with CLint due to SCIT PROP formation. In a substrate depletion experiment with SDCIT (1 µM), only trace amounts of SCIT PROP were detected (Fig. 4B). No SCIT PROP was present in incubations of SCIT (1 µM) without Supersomes (Fig. 4C).

After the discovery of SCIT PROP as a metabolite formed by recombinant CYP2C19 Supersomes, formation rates of SDCIT and SCIT PROP were evaluated over a wide concentration range of SCIT (1–1000 µM, substrate saturation experiments). Plots of metabolite formation rate (V) versus substrate concentration (S) showed that formation of SDCIT followed classic Michaelis-Menten kinetics (eq. 4; Fig. 6A), whereas formation of SCIT PROP was best described by the substrate inhibition model (eq. 6; Fig. 6B). Estimates of enzyme kinetics parameters for SDCIT and SCIT PROP formation are listed in Table 1. The Km value for SCIT PROP formation was lower than that for SDCIT formation [53 ± 12 versus 130 ± 7 µM, respectively (mean ± S.E.M.)], but the Vmax for SCIT PROP formation was estimated to be less than one-tenth of the Vmax for SDCIT formation. Hence, based on the substrate saturation experiments, CLint (eq. 7) was 5.2 times higher for SDCIT formation than for SCIT PROP formation. However, the curve fits for SCIT PROP formation were not satisfactory. The R2 values were low (0.64–0.85), and inspection of the residual plots indicated non-normality in the distribution of the data points (plots not shown). The model for SCIT PROP formation based on substrate saturation experiments might therefore give poor estimates of enzyme kinetic parameters for SCIT PROP formation (Table 1).

**Serum Concentrations of SCIT PROP in Relation to CYP2C19 Genotype.** SCIT PROP was determined in six CYP2C19-genotyped patients for whom serum samples had been received for TDM of SCIT (Table 2). None of the patients had recently changed their SCIT dose or used drugs known to interact with CYP2C19 enzyme activity. Dose-adjusted serum concentrations of SCIT PROP were low in the patients carrying the defective CYP2C19*2/*2 genotype compared with patients carrying functional CYP2C19 alleles (CYP2C19*1/*1 and CYP2C19*17/*17). Serum concentrations of SCIT PROP exceeded those of SCIT in all but one patient. The highest serum concentration of SCIT PROP (14.3 nmol/l per mg) was measured in a patient carrying the ultrarapid CYP2C19*17/*17 genotype. Figure 7 shows mean dose-adjusted serum concentrations of SCIT, SDCIT, and SCIT PROP (Fig. 7A) and mean metabolite/parent ratios (Fig. 7B) in relation to CYP2C19 genotype. Mean dose-adjusted serum

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**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate depletion</th>
<th>SDCIT</th>
<th>SCIT PROP</th>
</tr>
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<tbody>
<tr>
<td>CLint (µL/min/pmol of CYP2C19) (eq. 3)</td>
<td>0.210 ± 0.014</td>
<td>0.144 ± 0.012</td>
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<tr>
<td>Substrate saturation</td>
<td></td>
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<tr>
<td>Model</td>
<td>Michaelis-Menten (eq. 4)</td>
<td>Substrate inhibition (eq. 6)</td>
</tr>
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<td>R² value</td>
<td>0.95 ± 0.98</td>
<td>0.75 ± 0.90</td>
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<tr>
<td>Vmax (µmol/min/pmol of CYP2C19)</td>
<td>24 ± 1.0</td>
<td>1.9 ± 0.43a</td>
</tr>
<tr>
<td>Km (µM)</td>
<td>130 ± 7</td>
<td>53 ± 12a</td>
</tr>
<tr>
<td>Km (µM)</td>
<td>N.A.</td>
<td>1067 ± 218a</td>
</tr>
<tr>
<td>CLint (µL/min/pmol of CYP2C19) (eq. 7)</td>
<td>0.183 ± 0.002</td>
<td>0.035 ± 0.004</td>
</tr>
</tbody>
</table>

N.A., not applicable.

* Indicates poor estimates due to unsatisfactory curve fits.
explain the substantially larger effect of impaired CYP2C19 metabolism on SCIT exposure in vivo, compared with the effect that has been estimated from in vitro studies, which were based solely on the formation of SDCIT (Bondolfi et al., 1996, 2000; Kobayashi et al., 1997; von Moltke et al., 2001; Herrlin et al., 2003; Rudberg et al., 2008).

Carboxylic acid metabolites of amines are supposed to be formed in a multistep sequence involving an aldehyde intermediate (Sladek et al., 1989). Formation of SCIT PROP by CYP2C19 is consistent with previous reports indicating that both the formation of aldehyde intermediates and the subsequent transformation to the corresponding carboxylic acids might be catalyzed by P450 enzymes (Watanabe et al., 1990; Zhang et al., 2000). However, two previous in vitro studies have investigated the formation of CIT PROP by human liver preparations, without providing indications for the involvement of P450 enzymes in this reaction (Rochat et al., 1998; Olesen and Linnet, 1999). This may be explained by the substrate saturation experiments in the present study, which indicated that CYP2C19-catalyzed formation of SCIT PROP exhibited substrate inhibition kinetics, whereas formation of SDCIT followed Michaelis-Menten kinetics. Atypical kinetic profiles are suggested to occur when two substrate molecules can bind within the active site of the enzyme simultaneously (Tracy and Hummel, 2004). The kinetic profiles for formation of SDCIT and SCIT PROP in the present study indicate that at high concentrations of SCIT, CYP2C19 favors formation of SDCIT in preference to SCIT PROP. Because the two previous studies investigated CIT PROP formation at quite high substrate concentrations (100–500 μM), a substrate inhibition phenomenon may explain the discrepancy with regard to the involvement of P450 enzymes in CIT PROP formation. However, because of the high concentrations required, substrate inhibition is unlikely to be of importance in vivo (Houston and Kenworthy, 2000). At low substrate concentrations, which are comparable to those reached in liver tissue during CIT treatment (Olesen and Linnet, 1999), the present study showed that a considerable amount of SCIT PROP was formed by CYP2C19.

CLint due to formation of separate metabolites was calculated from both substrate depletion experiments (CLint = v/S) and substrate saturation experiments (CLint = V_{max}/K_{m}). Based on the substrate depletion experiment, CLint due to SCIT PROP formation was approximately 70% of the CLint due to SDCIT formation. In contrast, when calculated from substrate saturation experiments, CLint was only 19% for SCIT PROP formation compared with SDCIT formation. However, the curve fits for SCIT PROP formation in the substrate saturation experiments were not satisfactory (Fig. 6B). The latter model might therefore give poor estimates of enzyme kinetic parameters for the formation of this metabolite (Table 1), which could explain the divergence of the estimates of CLint due to SCIT PROP formation based on substrate saturation and substrate depletion experiments. On the basis of the above-mentioned reasons, calculation of CLint based on the substrate depletion experiment was considered as the most reliable estimate of CLint due to SCIT PROP formation.

In addition to the in vitro metabolism experiments, serum concentrations of SCIT, SDCIT, and SCIT PROP were determined in samples from six patients with various CYP2C19 genotypes undergoing treatment with SCIT. In line with a former pharmacogenetic study (Rudberg et al., 2008), a several-fold difference in mean serum concentrations of SCIT was observed between patients with the defective CYP2C19*2/*2 genotype compared with patients carrying functional CYP2C19 alleles (CYP2C19*1/*1 and *17/*17). Furthermore, the metabolite/parent ratios for both SDCIT and SCIT PROP were lower in the CYP2C19*2/*2 subgroup and higher in the CYP2C19*17/*17 subgroup compared with the CYP2C19*1/*1 subgroup.
group. Because clearance of SDCIT and SCIT PROP seems to be independent of CYP2C19 activity (Fig. 1B) (Oyehaug et al., 1984; Dalgaard and Larsen, 1999; Olesen and Linnet, 1999; von Moltke et al., 2001), the shift in metabolite/parent ratios may reflect altered formation rates of these metabolites according to CYP2C19 genotype. Formation rates of SDCIT and SCIT PROP being higher in patients carrying the ultrarapid CYP2C19 genotype and lower in patients with the defective CYP2C19 genotype is in accordance with the present in vitro study, showing that CYP2C19 catalyzes the formation of both these metabolites. This finding indicates that altered CYP2C19 activity in vivo affects SCIT elimination through a combined effect on SCIT PROP and SDCIT formation. Identification of SCIT PROP as a metabolic pathway catalyzed by CYP2C19 might explain why impaired CYP2C19 activity has shown a substantially larger effect on SCIT exposure than what has been estimated from in vitro data based solely on the formation of SDCIT (Kobayashi et al., 1997; von Moltke et al., 2001; Rudberg et al., 2008).

Based on the substrate depletion experiments, CLint due to SCIT PROP formation was approximately 70% of the CLint accounted for by SCIT formation. However, in line with previous studies (Bondolfi et al., 1996, 2000; Herrlin et al., 2003; Rudberg et al., 2006, 2008), the finding can be explained by previous studies reporting that CYP3A4 and CYP2D6 also catalyze the formation of SDCIT (Kobayashi et al., 1997; Rochat et al., 1997; Olesen and Linnet, 1999; von Moltke et al., 2001; Yu et al., 2003). Contrary to SCIT, the mean serum concentration of SCIT PROP was lower in the patients carrying the CYP2C19*2/*2 genotype than in the patients carrying functional CYP2C19 alleles. Although not directly comparable because of stereoselective metabolism of CIT (Rochat et al., 1995a, 1997; Sidhu et al., 1997; Olesen and Linnet, 1999; von Moltke et al., 2001; Yu et al., 2003), reduced exposure of the propionic acid metabolite in patients with impaired CYP2C19 activity is supported by a previous study reporting lower serum concentrations of racemic CIT PROP in six patients with a CYP2C19 poor metabolizer phenotype compared with CYP2C19 extensive metabolizers (Bauermann et al., 1996). Thus, in comparison to the N-desmethylation pathway, it seems that formation of SCIT PROP is more specific for CYP2C19.

The fact that SCIT PROP was also present in serum samples from patients carrying the defective CYP2C19 genotype shows that enzymes besides CYP2C19 are also able to catalyze the formation of this metabolite. Previous studies indicate that the initial step of aldehyde formation might be mediated by monoamine oxidase (Rochat et al., 1995a, 1997; Sidhu et al., 1997; Olesen and Linnet, 1999; von Moltke et al., 2001; Herrlin et al., 2003), reduced exposure of the propionic acid metabolite in patients with impaired CYP2C19 activity is supported by a previous study reporting lower serum concentrations of racemic CIT PROP in six patients with a CYP2C19 poor metabolizer phenotype compared with CYP2C19 extensive metabolizers (Bauermann et al., 1996). Thus, in comparison to the N-desmethylation pathway, it seems that formation of SCIT PROP is more specific for CYP2C19.

### Table 2
Demographics and serum concentrations of SCIT, SDCIT, and SCIT PROP in six CYP2C19-genotyped patients treated with SCIT

<table>
<thead>
<tr>
<th>CYP2C19</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19*1/*1</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>33</td>
<td>25</td>
<td>33</td>
<td>41</td>
<td>30</td>
<td>48</td>
</tr>
<tr>
<td>Daily dose (mg)</td>
<td>30</td>
<td>30</td>
<td>25</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sampling time (h)</td>
<td>20</td>
<td>24</td>
<td>24</td>
<td>23</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>Dose-adjusted serum concentrations (nmol/l per mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCIT</td>
<td>3.2</td>
<td>1.3</td>
<td>4.4</td>
<td>5.5</td>
<td>5.0</td>
<td>13.0</td>
</tr>
<tr>
<td>SDCIT</td>
<td>4.6</td>
<td>2.5</td>
<td>2.9</td>
<td>1.9</td>
<td>1.3</td>
<td>3.0</td>
</tr>
<tr>
<td>SCIT PROP</td>
<td>14.3</td>
<td>3.6</td>
<td>9.2</td>
<td>3.4</td>
<td>3.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Relative dose-adjusted serum concentrations</td>
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<tr>
<td>SCIT</td>
<td>0.45</td>
<td>1</td>
<td>0.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDCIT</td>
<td>1.51</td>
<td>1</td>
<td>0.91</td>
<td></td>
<td></td>
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<tr>
<td>SCIT PROP</td>
<td>1.42</td>
<td>1</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative metabolite/parent ratios</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDCIT/SCIT</td>
<td>3.45</td>
<td>1</td>
<td>0.50</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SCIT PROP/SCIT</td>
<td>2.69</td>
<td>1</td>
<td>0.32</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

F, female; M, male.
To summarize, the present study showed that CYP2C19 was able to catalyze the formation of SCIT PROP from SCIT in vitro. Our data suggested that this metabolic pathway accounted for approximately one-third of the in vitro clearance of SCIT by CYP2C19. Moreover, analysis of six serum samples indicated that the differences in serum concentrations of SCIT between patients genotyped as CYP2C19*1/*1, CYP2C19*1/*1, and CYP2C19*2/*2 (poor metabolizers, n = 2), CYP2C19*1/*1 (extensive metabolizers, n = 2), or CYP2C19*17/*17 (ultrarapid metabolizers, n = 2).

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References


We thank Niclas Lunder at the Department of Psychopharmacology and Siri Johannessen at the School of Pharmacy for skilled assistance during the laboratory work.

FIG. 7. A, mean dose-adjusted serum concentrations (nmol/l per mg) of SCIT, SDCIT, and SCIT PROP and mean metabolite/parent ratios (SDCIT/SCIT and SCIT PROP/SCIT) (B) in six patients genotyped as CYP2C19*17/*17, CYP2C19*17/*17, and CYP2C19*1/*1 (poor metabolizers, n = 2), CYP2C19*1/*1 (extensive metabolizers, n = 2), or CYP2C19*17/*17 (ultrarapid metabolizers, n = 2).


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