Cyclic Conversion of the Novel Src Kinase Inhibitor [7-(2,6-Dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine (TG100435) and Its N-Oxide Metabolite by Flavin-Containing Monoxygenases and Cytochrome P450 Reductase

Ahmed Kousba, Richard Soll, Shiyin Yee, and Michael Martin

TargeGen, Inc., San Diego, California

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ABSTRACT:

[7-(2,6-Dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine (TG100435) is a novel multi-targeted Src family kinase inhibitor with demonstrated anticancer activity in preclinical species. Potent kinase inhibition is associated with TG100435 and its major N-oxide metabolite [7-(2,6-dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-[2-(1-oxy-pyrrolidin-1-yl)-ethoxy]-phenyl]-amine (TG100855). The objectives of the current study were to identify the hepatic enzyme(s) responsible for 1) the total metabolic flux of TG100435, 2) the formation of TG100855, and 3) the subsequent metabolism of TG100855. Flavin-containing monoxygenases (FMO) and cytochrome P450 monoxygenases (P450s) contribute to TG100435 total metabolic flux. TG100435 metabolic flux was completely inhibited by methimazole and ketoconazole, suggesting only FMO- and CYP3A4-mediated metabolism. TG100855 formation was markedly inhibited (~90%) by methimazole or heat inactivation (>99%). FMO3 was the primary enzyme responsible for TG100855 formation. In addition, an enzyme mediated retroreduction of TG100855 back to TG100435 was observed. The N-oxidation reaction was approximately 15 times faster than the retroreduction reaction. Interestingly, the retroreduction of TG100855 to TG100435 in recombinant P450 or liver microsomes lacked inhibition by the P450 inhibitors. TG100435 formation in the human liver microsomes or recombinant P450 or liver microsomes lacked inhibition by the P450 inhibitors. TG100435 formation in the human liver microsomes or recombinant P450 increased as a function of cytochrome P450 reductase activity, suggesting potential involvement of cytochrome P450 reductase. The results of this in vitro study demonstrate the potential of TG100435 and TG100855 to be interconverted metabolically. FMO seem to be the major N-oxidizing enzymes, whereas cytochrome P450 reductase seems to be responsible for the retroreduction reaction.

[7-(2,6-Dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine (TG100435) is a novel multi-targeted Src family kinase inhibitor. Src is the prototype member of the Src-family of tyrosine kinases, which comprise 11 highly homologous proteins, including Src, Yes, Fyn, Lyn, Hck, Blk, Brk, Fgr, Frk, Srm, and Yrk (Trevino et al., 2006). Src is dysregulated in several types of cancers, and it is involved in tumor progression and metastases (Summy and Gallick, 2006). Src inhibitors have a potential utility in the treatment of various kinds of cancers. TG100435 is the first Src kinase inhibitor. Src is the prototype member of the Src family kinase inhibitor with demonstrated anticancer activity in preclinical species. Potent kinase inhibition is associated with TG100435 and its major N-oxide metabolite [7-(2,6-dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-[2-(1-oxy-pyrrolidin-1-yl)-ethoxy]-phenyl]-amine (TG100855). The objectives of the current study were to identify the hepatic enzyme(s) responsible for 1) the total metabolic flux of TG100435, 2) the formation of TG100855, and 3) the subsequent metabolism of TG100855. Flavin-containing monoxygenases (FMO) and cytochrome P450 monoxygenases (P450s) contribute to TG100435 total metabolic flux. TG100435 metabolic flux was completely inhibited by methimazole and ketoconazole, suggesting only FMO- and CYP3A4-mediated metabolism. TG100855 formation was markedly inhibited (~90%) by methimazole or heat inactivation (>99%). FMO3 was the primary enzyme responsible for TG100855 formation. In addition, an enzyme mediated retroreduction of TG100855 back to TG100435 was observed. The N-oxidation reaction was approximately 15 times faster than the retroreduction reaction. Interestingly, the retroreduction of TG100855 to TG100435 in recombinant P450 or liver microsomes lacked inhibition by the P450 inhibitors. TG100435 formation in the human liver microsomes or recombinant P450 or liver microsomes lacked inhibition by the P450 inhibitors. TG100435 formation in the human liver microsomes or recombinant P450 increased as a function of cytochrome P450 reductase activity, suggesting potential involvement of cytochrome P450 reductase. The results of this in vitro study demonstrate the potential of TG100435 and TG100855 to be interconverted metabolically. FMO seem to be the major N-oxidizing enzymes, whereas cytochrome P450 reductase seems to be responsible for the retroreduction reaction.

As described by Hu et al. (2007), TG100855 is the major metabolite of TG100435 observed in several preclinical species and in human, dog, rat, and mouse liver microsomes. Although most oxidation reactions lead to inactive metabolites (Carmella et al., 1997; Cashman and Zhang, 2006; Krueger et al., 2006), active metabolites, such as TG100855, have been demonstrated in vitro and in vivo (Krieter et al., 1984; Hu et al., 2007).

The N-oxidation of tertiary amines is a common metabolic pathway, and it has been mainly attributed to both cytochrome P450 monoxygenases (P450s) and flavin-containing monoxygenases (FMO) (Oberly et al., 1997; Tugnait et al., 1997). A few studies attributed these compounds’ N-oxidation to FMO and not P450s. FMO and P450s play an important role in the drug oxidative metabolism. FMO represent the major mammalian non-P450 oxidative enzymes (Rettie et al., 1995). Many more examples of P450-mediated metabolism.
metabolism have been reported compared with examples of FMO metabolism. This could be attributed to FMO thermal liability in the absence of NADPH, the paucity of reported data, the relative similarity in the types of metabolites produced by FMO and P450s, or possibly the use of inadequate bioanalytical methods (Cashman and Zhang, 2006). However, the FMO family has gained increasing attention in the last decade. This may be a consequence of an increased emphasis of drug metabolism in drug development and the recognition that introduction of functional groups such as tertiary amines or sulfides into drug candidates that are metabolized by FMO could help decrease adverse drug-drug interactions (Cashman and Zhang, 2006). Unlike P450s, FMO are not easily induced or readily inhibited by environmental chemicals or drugs (Cashman and Jiang, 2006). Incorporation of tertiary amine into TG100435 provided a liable site for FMO-mediated metabolism.

In the current study, an oxidation-reduction cycling was observed between TG100435 and TG100855. The biological redetermination of several N-oxide metabolites has been reported in the literature. For example, clozapine N-oxide, tamoxifen N-oxide, and phenethyl hydroxylamine have been shown to form clozapin, tamoxifen, and phenethylamine in human liver microsomes, respectively (Pirmohamed et al., 1995; Lin and Cashman, 1997; Parte and Kupfer, 2005). However, very little is known about the associated enzyme(s) responsible for biological retroreduction of various N-oxide metabolites (Parte and Kupfer, 2005).

Therefore, the objectives of the current study were to identify the hepatic enzyme(s) responsible for 1) the total metabolic flux of TG100435, 2) the formation of TG100855, and 3) the subsequent metabolism of TG100855. In vitro determination of drug-metabolizing enzymes helps to predict the potential for in vivo drug-drug interactions, the impact of polymorphic enzyme activity on drug disposition, and the formation of toxic or active metabolites. Data for the in vitro drug metabolism are commonly used in the pharmaceutical industry as one of the main criteria for selecting the compounds that are expected to possess commercially acceptable pharmacokinetic characteristics. The enzymes associated with TG100435 metabolic flux, its N-oxidation to TG100855, and the retrodetermination of TG100855 to TG100435 were identified in human liver microsomes and human recombinant P450s and FMO using selective and nonselective enzyme inhibitors and different incubation conditions.

### Materials and Methods

**Test Materials.** TG100435, TG100855, and a chemical analog used as an internal standard were synthesized at TargeGen, Inc. (San Diego, CA). Ketoconazole, methimazole, 1-aminoxybenzotriazole, furafylline, sulfaphenazole, tranylcypromine, quinidine, midazolam, and methimazole were purchased from Sigma-Aldrich (St. Louis, MO). Pooled mixed gender human liver microsomes and human recombinant P450 isoforms CYP1A2, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 were purchased from In Vitro Technologies (Baltimore, MD). FM01, FM03, and FM05 were purchased from Sigma-Aldrich. Recombinant cytochrome P450 reductase and CYP2C19 were purchased from Invitrogen (Carlsbad, CA).

**Identification of Enzyme(s) Involved in TG100435 Metabolism.** Incubations of TG100435 in recombinant P450 isoforms. The assay conditions were optimized for the incubation time and protein concentrations. TG100435 was incubated at a final concentration of 1 μM in 100 pmol/ml human recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 baculosomes in 0.1 M potassium phosphate buffer, pH 7.4, in the presence or the absence of NADPH regeneration system. The total incubation volume was 0.5 ml. All samples were preincubated in triplicates at 37°C for 5 min. The reactions were started by the addition of TG100435, and samples were then incubated at 37°C for 0 and 60 min. The control samples for assessing the nonenzymatic metabolism of TG100435 as follows: 1) TG100435 in potassium phosphate buffer, 2) TG100435 in potassium phosphate buffer plus NADPH regeneration system, 3) TG100435 in potassium phosphate buffer plus enzyme, and 4) TG100435 in potassium phosphate plus enzyme, where 0.5 ml of 100% ice-cold acetonitrile was added first to terminate the reaction followed by the addition of the NADPH regeneration system. The disappearance of TG100435 following incubation with CYP3A4 was linear for protein concentrations between 50 and 400 pmol/ml and an incubation time of 15 to 120 min.

**Incubations of TG100435 in human liver microsomes.** The metabolic stability of TG100435 was evaluated in pooled mixed gender human liver microsomes. The microsomal activity was confirmed and the experimental conditions were optimized with respect to the incubation time and microsomal protein concentrations. The disappearance of TG100435 was linear for protein concentrations between 0.5 and 2 mg/ml and an incubation time of 10 to 60 min. The human liver microsomes (1 mg/ml) were preincubated with NADPH regeneration system at 37°C for 5 min. The reactions were started by the addition of TG100435 at a final concentration of 1 μM, and samples were then incubated at 37°C for up to 60 min. TG100435 was also incubated in the presence or the absence of the broad-spectrum P450 inhibitor 1-aminoxybenzotriazole (2 mM), the specific CYP3A4 inhibitor ketoconazole (1 μM), or the FM0 competitive inhibitor methimazole (25 μM). The incubation conditions of methimazole were optimized to avoid its potential inhibitory effects on the metabolic activities of TG100435.

**Incubations of TG100435 in cryopreserved hepatocytes.** The metabolic stability of TG100435 was evaluated in human cryopreserved hepatocytes. The hepatocytes were stored in liquid nitrogen until the time of the experiments. After removal from the liquid nitrogen, the vials were uncapped for few
seconds, and then they were recapped and put over ice for 5 min. Thawing was achieved by gently shaking the vials of hepatocytes in a 37°C water bath for up to 90 s. As soon as all contents had been thawed, the vials were placed immediately on ice and suspended in 15 ml of thawing buffer followed by centrifugation at 50 g for 5 min. The supernatant was discarded, and the pellet containing the hepatocytes was resuspended in the incubation buffer to a volume of 1 ml. The hepatocytes viability determined by trypan blue exclusion was 70%. The hepatocytes were further diluted in incubation buffer to yield final hepatocytes concentration/incubation of 5 * 10^5/H11003 10^5 cell/ml. TG100435 (1/H9262 M) was incubated in the diluted hepatocytes in a final mixture volume of 50/H9262 l at 37°C and 95% O2, 5% CO2 for up to 3 h. The control samples included either TG100435 incubation in buffer that did not contain hepatocytes or incubation of buffer that contained hepatocytes where 0.5 ml of 100% ice-cold acetonitrile was added first to terminate the reaction followed by the addition TG100435.

Identification of the Enzyme(s) Responsible for TG100855 Formation. TG100855 formation in human liver microsomes. Initial studies confirmed the activity of FMO in the studied liver microsomes using FMO-selective substrate benzydamine (Stormer et al., 2000; Cashman and Zhang, 2006). The formation of benzydamine-N-oxide following benzydamine incubation in human liver microsomes in the presence or the absence of NADPH regeneration system was evaluated. To determine metabolic enzyme system associated with TG100855 formation, FMO and P450 activities were selectively inhibited following TG100435 incubation in the presence or absence of 1-aminobenzotriazole or methimazole as described above.

The effect of heat sensitivity on TG100855 formation was evaluated using the method described by Ring et al. (1999). Two sets of microsomal incubations were used, where NADPH regeneration system was added to one set and it was omitted from the other set. Both sets of samples were then preincubated for 1 min at 55°C, and then all samples were placed on ice, and TG100435 was added to each sample. The samples without regeneration system were supplemented with the cofactor. All samples were then incubated at 37°C for up to 60 min. TG100855 formation was compared with TG100855 formation in the standard incubations. For the standard incubations, liver microsomes were preincubated at 37°C for 5 min in the presence of NADPH regeneration system, and then the reactions were initiated by the addition of TG100435 and incubated at 37°C for up to 60 min. The activity of P450s and FMO is preserved following microsomal preincubation at high temperature in the presence NADPH regeneration system, whereas only P450 activity is preserved in the absence NADPH regeneration system (Kedderis and Rickert, 1985). The formation of TG100855 following TG100435 incubation in pooled human liver microsomes was linear for protein concentrations between 0.5 and 4 mg/ml and for an incubation time of 10 to 120 min.

TG100855 formation in human recombinant enzymes. To identify the specific FMO isoform(s) involved in TG100855 formation, TG100435 (25/H9262 M final concentration) was incubated in 200/H9262 g/ml human recombinant FMO1, FMO3, or FMO5 baculosomes. The assay conditions were optimized for the incubation time and protein concentrations. The TG100855 formation showed linear increases as a function of FMO1 and FMO3 protein concentrations of 50 to 400 g/ml and an incubation time of 5 to 60 min. The total incubation volume was 0.5 ml. Samples were preincubated at 37°C for 5 min. The reactions were started by the addition of TG100435 and samples were then incubated at 37°C for up to 45 min. The disappearance of TG100435 and the production of TG100855 during incubation were simultaneously evaluated. The nonenzymatic metabolism of TG100435 depletion or TG100855 formation was evaluated using the control sample conditions described above.

The proportional contribution of each individual FMO isoform toward the formation of TG100855 was normalized to the specific isoform content in

**FIG. 2.** Chromatograms of the TIC (A) and spectrum (B) of TG100435 and its metabolite TG100855 in microsomal samples incubated for 0 and 120 min compared with TG100435 and TG100855 synthetic standards. TG100435 (20 μM) was incubated in pooled human liver microsomes (1 mg/ml) as described under Materials and Methods.
human liver microsomes using modification of the approach reported by Rodrigues (1999). Briefly, the slope of the log linear regression analysis of TG100855 formation rate in each individual isoform was multiplied by the isoform content to estimate the corresponding isoform normalized rate (NR). The total normalized rate (TNR) was calculated from the summation of the NR of all isoforms. The TNR was used to estimate the %TNR for each individual isoform by dividing the NR of an isoform by the (TNR × 100). A considerable human liver microsomes interindividual variability in the contents of FMO1 and FMO3 (from 2- to 20-fold) has been reported in the literature (Haining et al., 1997; Overby et al., 1997; Koukouritaki et al., 2002). Koukouritaki et al. (2002) reported FMO1 and FMO3 contents of 0.1 ± 0.3 and 26.9 ± 8.6 pmol/mg microsomal protein, respectively. Overby et al. (1997) reported FMO3 content of 5 to 100 pmol/mg microsomal protein and an FMO5 content that is 3- to 4-fold less than that of FMO3.

Identification of Enzyme(s) Responsible for TG100855 Metabolism into TG100435, Human liver microsomes. Initial studies confirmed the appropriate FMO activity in the used liver microsomes as described above. The formation of TG100435 in pooled human liver microsomes was linear for protein concentrations between 0.5 and 4 mg/ml and an incubation time of 20 to 120 min. To determine metabolic enzyme system associated with TG100435 production from TG100855, the effect of heat sensitivity on TG100435 formation was evaluated as described previously using the method of Ring et al. (1999). TG100435 formation was also evaluated in the presence of the FMO inhibitor methimazole at 0.1 and 1 mM. Additional studies evaluated TG100435 formation in the presence of P450-specific substrate and P450-specific and nonspecific inhibitors. TG100855 (2.5 μM) was incubated in human liver microsomes as described above with a selected probe substrate (at respective K_{m} values) in the presence or the absence of P450-specific inhibitors (Walsky and Obach, 2004). Phenacetin, tolbutamide, methylenoxanthin, dextromethorphan, and midazolam were used as probe substrates for CYP1A2, CYP2C9, CYP2C19, and CYP3A4, respectively. Furafylline, sulfaphenazole, triacylpyrine, quinidine, and ketoconazole were used for the inhibition of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively. 1-Aminobenzotriazole (1 and 10 mM) was also used as nonspecific P450 inhibitor to assess TG100435 formation following the inhibition of all P450s. All inhibitors were used at incubation concentrations that are sufficient to induce 90% or more inhibition of the respective P450 isoform activity. The P450 activities were estimated from the rate of metabolite production as a function of incubation time in the presence or the absence of the P450 inhibitor. The effects of increasing the reducing potency of NADPH (10- fold) in the incubation medium on the rate of TG100855 reduction to TG100435 were evaluated using the procedure described above.

Human recombinant P450 and FMO isoforms. To identify the specific isoform(s) involved in TG100435 formation, TG100855 (2.5 μM final concentration) was incubated in 200 μg/ml human recombinant FMO1, FMO3, or FMO5 and in 100 μM/ml human recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The assay conditions were optimized for the incubation time and protein concentrations. The formation of TG100435 in human recombinant P450 was linear for protein concentrations between 50 and 400 pmol/ml and an incubation time of 20 to 120 min. Samples were treated as described previously. The control samples for assessing the nonenzymatic and NADPH-independent metabolism of TG100855 were prepared as follows: 1) TG100855 in potassium phosphate buffer, 2) TG100855 in potassium phosphate buffer plus NADPH regeneration system, 3) TG100855 in potassium phosphate buffer plus enzyme, and 4) TG100855 in potassium phosphate plus enzyme, where 0.5 ml of 100% ice-cold acetone was added first to terminate the reaction followed by the addition of the NADPH regeneration system. TG100855 (2.5 μM) metabolism to TG100435 was also evaluated in human recombinant P450s in the presence of P450-specific probe substrates and P450-specific and -nonspecific inhibitors as described above.

Human recombinant cytochrome P450 reductase. To evaluate the potential association of human cytochrome P450 reductase with TG100435 formation, TG100855 (2.5 μM final concentration) was incubated in human liver microsomes or in potassium phosphate buffer, pH 7.4, in the presence of increasing concentrations of human cytochrome P450 reductase (0, 100, and 500 pmol/ ml). The assay conditions were optimized for the incubation time and protein concentrations. The total incubation volume was 0.5 ml. The human liver microsomes or the potassium phosphate buffer, pH 7.4, plus human cytochrome P450 reductase and the NADPH were preincubated at 37°C for 5 min. The reactions were started by the addition of TG100855, and samples were then incubated at 37°C for up to 45 min. Samples were treated as described previously, and the production of TG100435 was evaluated.

The reductase activity in human liver microsomes was determined spectrophotometrically by measuring the rate of cytochrome c reduction at 550 nm. A mixture of NADPH regeneration system, 3.3 mM magnesium chloride, and 0.95 mg/ml cytochrome c in 250 mM potassium phosphate, pH 7.4, was preincubated for 5 min at 37°C. The reaction was initiated by the addition of 0.1 mg/ml protein, and the change of absorbance at 550 nm was monitored. An extinction coefficient for reduced cytochrome c at 550 nm of 19.6 μM−1 cm−1 was used to calculate the reductase activity. In human liver microsomes, the reductase activity is 367 ± 10 nmol/min/mg protein. The estimated activity is consistent with the typical value of 150 to 350 nmol/min/mg protein (Rodrigues, 1999; Dudka et al., 2005; Dudka, 2006). The manufacturer-predetermined activity of reductase in CYP1A2, CYP2C9, CYP2C19, CYP2D6, and 3 CYP4A was 2.12, 1.32, 2.1, 0.85, and 1.1 μmol/min/mg, respectively. The recombinant FMO system did not express any measurable reductase activity.

Determination of Kinetic Parameters of TG100855 and TG100435 Formation. The kinetic parameters (Km and V_{max}) of TG100855 and TG100435 formation were determined in human liver microsomes. Preliminary experiments established the linearity of TG100855 and TG100435 metabolism with respect to the incubation time (10–60 min) and the microsomal protein concentrations (0.2–2 mg/ml). The microsomes were preincubated with the NADPH regeneration system for 5 min at 37°C. Increasing concentrations of TG100435 or TG100855 (0.1–40 μM) were added to initiate the reaction, and the incubation was continued for a period of 45 min at 37°C. The total incubation volume was 0.5 ml. The nonenzymatic formation of TG100855 and TG100435 was evaluated, as described above.
All reactions were terminated by the addition of 500 μl of 100% ice-cold acetonitrile to each vial followed by the addition of 20 μl of internal standard at a final concentration of 0.1 μM. The samples were vortexed for 1 min, and then they were centrifuged at 15,000g for 15 min at 23°C. The supernatant was analyzed for measuring TG100435 and/or TG100855 concentration using liquid chromatography/tandem mass spectrometry (LC/MS/MS).

Analytical Methods. LC/MS/MS Waters Quattro LC (Waters, Milford, MA) was used for the analysis of TG100435 and TG100855 as follows: 20 μl of the supernatant was injected by a Leap CTC autosampler onto an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Samples were separated using HPLC column Waters Atlantis T3 (100 × 2.1 mm, 5-μm particle size) and a mobile phase of solvent A (1% formic acid in water) and solvent B (1% formic in 100% acetonitrile) at a flow rate of 0.3 ml/min using LC gradient going from 40 to 65% solvent B for a total run time of 8 min. Samples for the identification of TG100435 or TG100855 metabolites were analyzed at a flow rate of 0.3 ml/min using LC gradient going from 5 to 95% solvent B for a total run time of 35 min using HPLC column Waters SymmetryShield RP18 (150 × 2.1 mm, 3.5-μm particle size). The LC detector uses a starting wavelength of 200 nm and end wavelength of 600 nm. The MicroMass Quattro LC uses electrospray positive (ES+) ionization mode; total ion current (TIC) and multiple reaction monitoring (MRM) modes were used. The limit of detection was 5 and 10 ng/ml for TG100435 and TG100855, respectively, and the inter- and intraday coefficients of variation did not exceed 15%.

Data Analysis. Estimation of TG100435-predicted hepatic clearance. The percentage of TG100435 remaining was used to calculate the half-life (t1/2, minutes) using a log linear regression analysis according the standard pharmacokinetic equation t1/2 = 0.693/slope. The half-life values were scaled to calculate the predicted clearance according to the equations described by Obach (1999). In human liver microsomes, (CLint) = 0.693 × t1/2 × (g liver wt./kg b.wt.) × (ml incubation/number of cells/incubation) × (number of cells/g liver wt.). The human physiological standard value of 21 g/kg for the gram of liver weight per kilogram of body weight and a 45 mg microsomes/g liver value were used (Obach, 1999). In human hepatocytes, (CLint) = 0.693 × t1/2 × (g liver wt./kg b.wt.) × (ml incubation/number of cells/incubation) × (number of cells/g liver wt.). The standard value of the hepatocellularity of 12 × 10⁷ hepatocytes/g was used (Bayliss et al., 1999).

Calculation of kinetic parameters for TG100855 or TG100435 formation. The apparent K_m and V_max of TG100855 or TG100435 formation were estimated using the one- and two-site Michaelis-Menten models. Each model was fitted to the untransformed data of the rate of metabolite formation versus the substrate concentration using the nonlinear regression program Prism version 4.01 (GraphPad Software Inc., San Diego, CA). The transformed data were also analyzed by Lineweaver-Burk plot, 1/substrate concentration (1/S) versus 1/rate of metabolite formation (1/V).

The statistical analysis of the data was limited to the determination of a mean ± S.D. for the samples (n = 3) by using the standard equations available in Excel (Microsoft, Redmond, WA). The values for metabolites formation in the samples were compared with the control values using Student’s unpaired t test. The levels of significance were considered at p ≤ 0.05.

Results

TG100435 Metabolism in Human Recombinant P450, Liver Microsomes, and Hepatocytes. To identify the enzyme responsible for the metabolic flux of TG100435, the study used the major human recombinant P450 isoforms involved in drug metabolism. Incubation of TG100435 for 60 min in 100 pmol/ml human recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, or CYP2E1 displayed metabolic conversion in only the CYP3A4 samples (33 ± 2.5% disappearance of TG100435). There was no observable disappearance of TG100435 following its incubation in CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 in the control samples.

In the human liver microsomes, the use of the P450 inhibitors ketoconazole and 1-amino benzotriazole resulted in approximately 50% inhibition of TG100435 total metabolic flux (Table 1). The in
vitro estimated $t_{1/2}$ values of TG100435 disappearance in human liver microsomes in the presence of ketoconazole or 1-aminobenzotriazole were 290 and 250 min, respectively compared with a $t_{1/2}$ value of 124 min determined in the absence inhibitors (Table 1). The corresponding TG100435 predicted intrinsic clearance value decreased from 2.9 ml/min/kg to 1.3 and 1.4 ml/min/kg in the presence of ketoconazole and 1-aminobenzotriazole, respectively (Table 1). Nonenzymatic depletion of TG100435 was not observed in controls. Incubation of TG100435 in human hepatocytes yielded a predicted intrinsic clearance value of 3 ml/min/kg, which is approximately equal to the predicted value determined in the liver microsomes in the absence of inhibitors (Table 1). Coincubation of TG100435 with a mixture of methimazole and ketoconazole in human liver microsomes resulted in complete inhibition of TG100435 metabolism (Table 1).

**TG100855 Formation in Human Liver Microsomes and Recombinant Enzymes.** Figure 2 presents the TIC and spectrum chromatogram of TG100435 following in vitro metabolism in liver microsomes for 0 and 120 min compared with the chromatogram of the synthetic standards of TG100435 and TG100855. The major metabolite peak occurs at a retention time of 20.05 min and $m/z$ 510.6 (Fig. 2A). This peak displayed an HPLC retention time and a mass spectrum identical to the synthesized TG100855 (Fig. 2). TG100855 was stable under the current experimental conditions and showed no analytical interference with TG100435 (Fig. 2). The CYP3A4 metabolic products were not observed at detectable levels in the current study. This observation is consistent with previously reported results of Hu et al. (2007) who reported that TG100855 was the only metabolite observed in the human liver microsomal incubations.

The metabolic enzyme system associated with TG100855 formation was determined following selective inhibition of FMO and P450 activities. The effects of 1-aminobenzotriazole and methimazole on TG100855 formation are presented in Fig. 3A. Incubation of TG100435 with methimazole or with a mixture of methimazole and 1-aminobenzotriazole resulted in marked inhibition of TG100855 formation (90%) relative to the standard incubations (Fig. 3A). In contrast, incubation of TG100435 with 1-aminobenzotriazole did not result in substantial inhibition of TG100855 formation (18%). TG100855 formation was markedly inhibited (>99%) following microsomal preincubation for 1 min at 55°C in the absence of NADPH regeneration system relative to the standard preincubation system (Fig. 3B). In contrast, there was no inhibition of TG100855 formation (0%) in the samples preincubated for 1 min at 55°C in the presence of NADPH regeneration system compared with the standard preincubation (Fig. 3B). The inhibition of FMO-mediated TG100855 formation was associated with a 50% reduction in the total clearance of TG100435.
The major metabolite peak occurs following preincubation of TG100855 in vitro metabolism in liver microsomes for recombinant Enzymes.

TG100435 in high concentrations (up to 400 pmol/ml) of recombinant TG100855 formation by FMO1, FMO3, and FMO5 was 3.7 times and 154 pmol/min/mg protein, respectively (Fig. 8). The correspondence of the interconversion of TG100855 and TG100435.

The profiles of the simultaneous TG100435 depletion and the formation of TG100855 following TG100435 incubation in recombinant FMO1, FMO3, and FMO5 are shown in Fig. 4, A to C. FMO3 demonstrated 2.3 and 18 times more TG100855 formation than FMO1 and FMO5, respectively (Fig. 4, A–C). Using the range of the reported values for FMO contents in human liver microsomes, the %TNR of TG100855 formation by FMO1, FMO3, and FMO5 was 3.7 ± 4, 94.4 ± 3.6, and 1 ± 0.1%, respectively (Fig. 4D). Incubation of TG100435 in high concentrations (up to 400 pmol/ml) of recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 did not result in any observable TG100855 formation.

**TG100855 Metabolism in Human Liver Microsomes and Recombinant Enzymes.** Figure 5 presents the TIC and spectrum chromatogram of TG100855 in vitro metabolism in liver microsomes for 0 and 120 min compared with chromatogram of the synthetic standards of TG100435 and TG100855. The major metabolite peak occurs at a retention time of 19.28 min and m/z 494.6 (Fig. 5A). This peak showed an HPLC retention time and a mass spectrum identical to the synthesized TG100435 (Fig. 5).

The rate of TG100435 formation following TG100855 preincubation in the human liver microsomes for 5 min at 37°C or 1 min at 55°C in the presence of the absence of NADPH regeneration system is shown in Fig. 6A. TG100435 formation rates in the samples preincubated for 1 min at 55°C were comparable with the formation rates in the samples preincubated for 5 min at 37°C in the presence or the absence of NADPH regeneration system (Fig. 6A). Therefore, heat inactivation of FMO did not inhibit TG100435 formation implying and that FMO is not associated with TG100435 formation. There was no substantial formation of TG100435 in the control samples. As shown in Fig. 6B, TG100435 formation following methimazole addition was higher than its formation in the standard incubations, and it increased as methimazole concentration increased (Fig. 6B).

To identify the enzyme responsible for TG100435 formation, TG100855 was incubated in the major human recombinant P450 and FMO isoforms involved in drug metabolism. TG100435 was produced in all studied P450s, with highest apparent formation by CYP1A2 and CYP2C19. The higher levels of TG100435 formation in CYP1A2 and CYP2C19 may be attributed to the elevated reductase activity in these preparations (~2-fold higher than other isoforms). There was no observable TG100435 formation following TG100855 incubation in FMO1, FMO3, or FMO5.

The effects of the prototypical P450 inhibitors (1-aminobenzotriazole, furafylline, sulfaphenazole, tranylcypromine, quinidine, or ketoconazole) on the formation of TG100435 following TG100855 incubation in human liver microsomes or in human recombinant P450s are presented in Fig. 7, A–C. Although the studied isoform-specific inhibitors demonstrated more than 90% inhibition of P450 activity, there was no observable inhibition of TG100435 formation in human liver microsomes or in recombinant isoforms (Fig. 7, A–C). Interestingly, the formation of TG100435 in the presence of 1-aminobenzotriazole was approximately 3 to 4 times higher than TG100435 formation in the absence of 1-aminobenzotriazole (Fig. 7C). Increasing the 1-aminobenzotriazole concentrations in the microsomal incubation resulted in significant increase in the formation rate of TG100435 (Fig. 7C), accompanied by an increase in TG100855 disappearance. The increased formation rate of TG100435 can be attributed to an accumulation of TG100435 due to inhibition of subsequent P450-mediated metabolism. Increasing NADPH concentrations by 10-fold did not result in any appreciable changes in the formation rate of TG100435 following TG100855 incubation in human liver microsomes (data not shown). The formation TG100435 in human liver microsomes supplemented by cytochrome P450 reductase demonstrated the increase as a function of the reductase concentrations (Fig. 7D). Increasing concentrations of cytochrome P450 reductase in potassium phosphate buffer, pH 7.4, had no effect on the rate of TG100435 formation (data not shown), which may be attributed to the absence of cofactors and reconstituted lipid vesicle system required for reductase activity.

**Determination of Kinetic Parameters of TG100855 or TG100435 Formation in Human Liver Microsomes.** TG100855 formation in human liver microsomes increases as a function of the incubated TG100435 concentrations (Fig. 8). The one-site model provided a better fit for TG100855 formation than the two-site model. The 95% confidence intervals estimated using the one-site model ranged from 85.95 to 223.4 for the \( V_{\max} \), and from 5.685 to 53.87 for the \( K_{m} \). The two-site model yielded 95% confidence intervals ranging from 0 to 4.54 \( \times 10^{7} \) for \( V_{\max1} \) and \( V_{\max2} \), from 0 to 4.19 \( \times 10^{7} \) for \( K_{m1} \), and from 0 to 1.205 \( \times 10^{7} \) for \( K_{m2} \). The calculated \( K_{m} \) and \( V_{\max} \) values of TG100855 formation using the one-site model were 30 \( \mu \)M and 154 pmol/min/mg protein, respectively (Fig. 8). The corresponding intrinsic clearance value (\( V_{\max} / K_{m} \)) was 5.2 \( \mu \)l/min/mg protein. Transformation of these data to Lineweaver-Burk plot shows no evidence of nonlinearity for the N-oxidation reaction (Fig. 8B). The intrinsic clearance for N-oxide formation should theoretically be 50% of the total intrinsic clearance in liver microsomes. The intrinsic clearance value of 5.2 \( \mu \)l/min/mg protein is scaled to 4.7 ml/min/kg hepatic intrinsic clearance, which is equivalent to the intrinsic clearance of TG100435. This deviation from theoretical could be the result of the interconversion of TG100855 and TG100435.
TG100435 formation in human liver microsomes increases as a function of the incubated TG100855 concentrations (Fig. 8). The one-site model provided a better fit for TG100435 formation than the two-site model. The 95% confidence intervals estimated using the one-site model ranged from 4.78 to 7.09 for the $V_{\text{max}}$ and from 10.07 to 24.81 for the $K_m$. The two-site model yielded 95% confidence intervals ranging from 0 to 63.81 for $V_{\text{max1}}$, from 0 to 1434 for $V_{\text{max2}}$, from 0 to 129.7 for $K_{m1}$, and from 0 to 84,126 for $K_{m2}$. The calculated $K_m$ and $V_{\text{max}}$ values of TG100435 formation using the one-site model were $17 \mu$M and 6 pmol/min/mg protein, respectively (Fig. 8). The corresponding intrinsic clearance value ($V_{\text{max}}/K_m$) was 0.34 $\mu$l/min/mg protein. Transformation of these data to Lineweaver-Burk plot shows no evidence of nonlinearity for the retroreduction reaction (Fig. 8C).

Although the observed rate of retroreduction is 15 times slower than the rate of oxidation, the only observed metabolic product for TG100855 was reduction back to TG100435 (Fig. 5). Overall the retroreduction pathway represents a minor component in the metabolism of TG100435.

**Discussion**

The current study identified the enzymes responsible for the total metabolic flux of TG100435, the formation of TG100855, and the retroconversion of TG100855 back to TG100435. In human recombinant enzymes, CYP3A4 seemed to be the only P450 isoform that metabolizes TG100435. The human liver microsomal study supported this observation, where TG100435 P450-mediated metabolism was completely inhibited following ketoconazole or 1-aminobenzotriazole (Table 1). Ketoconazole is usually used to assess CYP3A4 participation in xenobiotics and drug metabolism (Newton et al., 1995; Sai et al., 2000). 1-Aminobenzotriazole is used to assess the general participation of all P450s in the metabolism (Ortiz de Montellano et al., 1984).

Since P450-mediated metabolism accounted only for ~50% of TG100435 total metabolism in human liver microsomes (Table 1), an additional enzyme(s) that is NADPH-dependent and is not susceptible to the P450 inhibitors may be involved in TG100435 metabolism. The similarity of TG100435 predicted clearance values using the human liver microsomes and the human hepatocytes (Table 1) and the absence of TG100435 phase II metabolism imply that TG100435 hepatic clearance may be due to phase I oxidative metabolism. In the liver microsomes, P450s and FMO are the only two NADPH-dependent enzyme systems responsible for xenobiotic oxidation. The complete inhibition of TG100435 metabolism following the mixture of ketoconazole and methimazole suggests that CYP3A4 and FMO contribute to the total metabolic flux of TG100435 (Table 1).

In human liver microsomes, the main metabolite of TG100435 is TG100855 (Fig. 2). Methimazole resulted in ~90% reduction of TG100855 formation relative to the standard incubation (Fig. 3A), suggesting that FMO are involved in the TG100855 formation. Methimazole is a selective competitive substrate with high affinity for FMO, and it is widely used to inhibit FMO activity (Ziegler, 1990; Tugnait et al., 1997). The association of FMO with TG100855 was also demonstrated by microsomal heating in the absence of NADPH.
regeneration system. More than 99% inhibition of TG100855 formation was observed (Fig. 3B). TG100855 formation was preserved (0% inhibition) in the presence of NAPDH regeneration system (Fig. 3B). Human FMO heating liability is a useful method to differentiate FMO from P450s in microsomal incubations (McManus et al., 1987; Grothuesen et al., 1996).

The use of human recombinant FMO indicated that the FMO-dependent TG100855 formation is attributed to FMO1 and FMO3 (major isoform), but not FMO5 (Fig. 4, A–D). Five isoforms of FMO (FMO1−5) have been cloned and sequenced in human (Hines et al., 1994; Cashman, 1995). FMO1 is the predominant isoform in the kidneys, lungs, and fetal liver, whereas FMO3 is the predominant isoform in adult liver. FMO1 and FMO3 have been implicated in the metabolism of many drugs (Cashman, 1995). FMO5 apparently does not catalyze the oxidations of the common FMO substrates, has a narrow substrate specificity, and its role in drug or chemical metabolism has not been yet established (Overby et al., 1995; Cashman and Jhang, 2006). Although the current work focused on the human liver FMO-mediated metabolism of TG100435, human renal FMO1 could contribute to TG100435 extrahepatic clearance due to the high levels of renal FMO1 (47 ± 9 pmol/mg protein) (Yeung et al., 2000).

Interestingly, the main metabolic pathway of TG100855 was its back reduction to the parent compound TG100435 (Fig. 5). Increasing TG100435 formation as a function of methimazole concentration (Fig. 6B) may imply that methimazole prevented the subsequent N-oxidation of the produced TG100855.

As shown in Figs. 3 to 7, TG100435 and TG100855 seem to be metabolically interconverted in cyclic a manner. The levels of either compound will be a sum of the oxidation and reduction reactions. It is difficult to know the exact rate of one reaction without knowing the other. Therefore, the current study investigated the rates and the levels of both compounds simultaneously and in the presence of the absence of various enzyme inhibitors.

Taken together, the linear rates of TG100855 or TG100453 formation demonstrated by Lineweaver-Burk (Fig. 8, B and C) and better fit of the one-site model suggest that a single-enzyme system may be involved in the N-oxidation or the retroreduction reaction. FMO3 seems to be the enzyme associated with the N-oxidation as reflected from its %TNR value of 94.4 ± 3.6% (Fig. 4D). Cytochrome P450 reductase seems to be the enzyme associated with the retroreduction reaction as reflected from increasing TG100435 formation as a function of the supplemented reductase (Fig. 7C) and increasing TG100435 formation in CYP1A2 and CYP2C19, which contain the highest reductase activity among the recombinant isoforms.

In summary, FMO and P450 demonstrated comparable contribution to the total metabolic flux of TG100435. The N-oxidation of TG100435 to TG100855 was only associated with FMO activity. The retroreduction of TG100855 back to TG100435 was primarily mediated by the cytochrome P450 reductase activity. This is the first body of work demonstrating the explicit involvement of cytochrome P450 reductase activity in the xenobiotic retroreductive metabolism.

**References**


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Address correspondence to: Dr. Ahmed Kousba, Department of Pharmaceutical Property Assessment, TargeGen, Inc., 9380 Judicial Dr., San Diego, CA 92121. E-mail: akousba@targegen.com