Metabolism and Pharmacokinetics of a Novel Src Kinase Inhibitor TG100435 ([7-(2,6-Dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine) and Its Active N-Oxide Metabolite TG100855 ([7-(2,6-Dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-{4-[2-(1-oxy-pyrrolidin-1-yl)-ethoxy]-phenyl}-amine)


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ABSTRACT:
TG100435 ([7-(2,6-dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine) is a novel multitargeted, orally active protein tyrosine kinase inhibitor. The inhibition constants ($K_i$) of TG100435 against Src, Lyn, Abl, Yes, Lck, and EphB4 range from 13 to 64 nM. TG100435 has systemic clearance values of 20.1, 12.7, and 14.5 ml/min/kg and oral bioavailability of 74%, 23%, and 11% in mouse, rat, and dog, respectively. Four oxidation metabolites of TG100435 have been found in human, dog, and rat in vitro and in vivo. The ethylpyrrolidine N-oxide of TG100435 is the predominant metabolite (TG100855; [7-(2,6-dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-{4-[2-(1-oxy-pyrrolidin-1-yl)-ethoxy]-phenyl}-amine) in human, dog, and rat. TG100855 is 2 to 9 times more potent than the parent compound. Flavin-containing monooxygenases are the primary enzymes mediating the biotransformation. Significant conversion of TG100435 to TG100855 has been observed in rat and dog after oral administration. Systemic exposure of TG100855 is 1.1- and 2.1-fold greater than that of TG100435 in rat and dog after oral dosing of TG100435. Since TG100435 is predominantly converted to the more potent N-oxide metabolite across species in vivo and in vitro, the overall tyrosine kinase inhibition in animal models may be substantially increased after oral administration of TG100435.

The Src kinase family consists of a group of nonreceptor protein tyrosine kinases (PTKs) including Src, Yes, Fyn, Lyn, Hck, Blk, Brk, Fgr, Frk, Srm, Lck, and York (Trevino et al., 2006). Src PTKs play critical roles in a variety of cellular signal transduction pathways regulating diverse processes including cell survival, proliferation, motility, adhesion, and transformation. Elevated or constitutive activation of Src kinase is commonly observed in tumors, most notably in colon and breast cancer, but also occurs in other tumor types, including pancreatic cancer (Lutz et al., 1998). Overexpression of Src PTKs has been associated with tumorigenesis, metastasis, and invasion; consequently, Src family kinases have become very important biological targets in oncological drug development. Small molecule kinase inhibitors have shown great promise as a new class of therapeutics and exhibit much less toxicity than currently used chemotherapeutic agents (Levitzki and Mishani, 2006).

Nitrogen-containing small molecules are the most common of all of the organic compounds of pharmacological interest. The functionalities of nitrogen provide flexibility in the drug design toward proper potencies and physical properties. However, the multiple oxidation states of nitrogen increase the metabolic instability of drug candidates (Cho and Lindeke, 1988). N-Oxidation is a common biotransformation of aliphatic tertiary amine-containing compounds. The pharmaceutical and toxicological importance of this metabolic pathway has been widely studied. The nitrogen-centered oxidation of tertiary amine drugs is commonly considered as a detoxification pathway resulting in nontoxic and biologically inactive metabolites (Carmella et al., 1997; Cashman and Zhang, 2006; Krueger et al., 2006). The benign nature of tertiary amine N-oxides under normal physiological conditions has been used as a prodrug approach to selectively elicit cytotoxic events associated with hypoxic conditions in solid tumor...
cells (Skálová et al., 2000; Cerecetto and González, 2001; Patterson, 2002). Although N-oxidation of the aliphatic tertiary amine-containing drug compounds has been widely identified and studied, the biochemical activity of such N-oxide metabolites is not commonly reported.

We report here a novel multitargeted, orally active PTK inhibitor, TG100435 ([7-(2,6-dichloro-phenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-(2-pyrrolidin-1-yl-ethoxy)-phenyl]-amine)) and its biochemically more potent N-oxide metabolite, TG100855 ([7-(2,6-dichlorophenyl)-5-methyl-benzo[1,2,4]triazin-3-yl]-[4-[2-(1-oxy-pyrrolidin-1-yl)-1-ethoxy]-phenyl]-amine). The structural design and activity of TG100435 against human tumor cells have been described elsewhere (Noronha et al., 2006, 2007). The N-oxide metabolite of TG100435 has been identified in vitro and in vivo rat and dog in addition to in vitro human samples. The biochemical potencies of TG100435 and TG100855 are evaluated for six protein tyrosine kinases. Enzymatic conversion of TG100435 to TG100855 is investigated using mouse liver microsome. The pharmacokinetic properties of TG100435 and TG100855 are also characterized in the mouse, rat, and dog.

**Materials and Methods**

**Compounds.** TG100435 and TG100855 were synthesized at TargeGen, Inc. (San Diego, CA) (Fig. 1). Structures were confirmed by 1H NMR and mass spectra (Tables 1 and 2; Fig. 2). For the pharmacokinetic studies, both TG100435 and TG100855 were formulated in a 5:5:10:80 mixture of Solutol HS15/polyethylene glycol 400/ethanol/water for i.v. and i.p. administration and formulated in Physal 50PG (American Lecithin, Oxford, CT) for p.o. dosing.

**Determination of Inhibition Constant $K_i$ against a Series of Kinases.** $K_i$ values for TG100435 and TG100855 against Src, Lyn, Abl, Yes, Lck, and EphB4 were determined using a luminescence-based kinase assay. These recombinant kinases were obtained from Invitrogen (Carlsbad, CA). The assays were performed in 96-well plates at room temperature. Each well contained 40 µl of 75 mM Tris buffer (pH 7.2), containing 95 mM MgCl$_2$, 1.5 mM EGTA, 0.35 mM Triton X-100, and 10 µM β-mercaptoethanol, and an appropriate amount of the PTK was added such that the assay was linear over 60 min. Varying amounts of peptide substrate in water were then added in the presence of a series of different concentrations of either TG100435 or TG100855. The reactions were initiated by addition of ATP to a final concentration of 3 µM. After 60 min, the reactions were terminated by adding 50 µl of Kinase-Glo reagent (Promega, Madison, WI). Luminosity was measured using an Ultra S384 instrument (Tecan, Durham, NC). A control without peptide substrate was used for a zero point. Enzymatic reaction rates were derived by calculating the difference between kinase-catalyzed and noncatalyzed reactions at a specific compound concentration. $K_i$ values were derived from rate data by noncompetitive enzyme kinetics curve fitting using Prism software (Version 4; GraphPad Software, San Diego, CA).

**In Vitro Mouse Liver Microsomal Evaluation on N-Oxidation of TG100435.** The contribution of FMO and cytochrome P450 to N-oxidation of TG100435 in mouse liver microsome was evaluated via heat deactivation of FMO (Grothuesen et al., 1996; Cashman, 2005). One set of mouse liver microsomal samples was preincubated with a NADPH-regenerating system (0.4 mM NADP, 4.2 mM glucose 6-phosphate and 1.2 unit/ml glucose-6-phosphate dehydrogenase) at 37°C for 1 min, and then 10 µM TG100435 was added for continuous incubation. The other set of mouse liver microsomal samples was preheated without the NADPH-regenerating system at 55°C for 1 min and then incubated with 10 µM TG100435 and the NADPH-regenerating system at 37°C. The formation of TG100855 was monitored after 0, 10, 20, 30, 45, and 60 min of incubation using LC/MS/MS after protein precipitation by cold acetonitrile.

**Pharmacokinetic Studies of TG100435 and TG100855.** Male Sprague-Dawley rats (300 g), male BALB/c mice (25 g), and male and female Beagle dogs (8 kg) were used in the studies. Animals were fasted overnight for single p.o. administration and not fasted for single i.v. or i.p. dosing. Rats were dosed with TG100435 at 25 or 40 mg/kg or TG100855 at 25 mg/kg in the p.o. studies and administered TG100435 or TG100855 at 5 mg/kg in the i.v. studies. In the i.p. dosing, rats were dosed with TG100855 at 5 mg/kg. Each dosing group consisted of five rats. Serial blood sampling at 5, 15, and 30 min, and 1, 3, 6, 24, and 48 h postdose for the i.v. dose groups, at 0.5, 1, 3, 5, 8, 24, 30, and 48 h postdose for the p.o. and i.p. dose groups was used to establish the rat pharmacokinetics. Sodium heparin was used as anticoagulant in blood samples. The plasma samples were obtained by centrifugation for 10 min.

Mice were dosed with TG100435 at 25 or 30 mg/kg, or TG100855 at 25 mg/kg in the p.o. studies and administered TG100435 or TG100855 at 5 mg/kg in the i.v. studies. In the i.p. dosing, mice were dosed with TG100855 at 5 mg/kg. Composite blood sampling ($n = 3$ animals per time point) at 5, 15, and 30 min, and 1, 3, 6, 24, and 48 h postdose for the i.v. dose groups, and at 0.5, 1, 3, 5, 8, 24, 30, and 48 h postdose for the p.o. and i.p. dose groups was used to establish the mouse pharmacokinetics.

Dogs were administered TG100435 at 5 mg/kg in the i.v. study or 25 mg/kg in the p.o. study. Each dosing group consisted of two male and two female dogs. Serial blood sampling at 5, 15, and 30 min and 1, 3, 5, 7, 12, 24, 36, and 48 h postdose for the i.v. dose group and at 0.5, 1, 3, 5, 8, 12, 24, 36, and 48 h postdose for the p.o. dose group was used to establish the dog pharmacokinetics. Dog urine samples were collected from the i.v. study with a 12-h interval at 12 h, 24 h, and 48 h.

**Sample Preparation for Metabolite Identification.** In vitro samples. TG100435 (80 µM) was incubated with rat, dog, or human liver microsome (In Vitro Technologies, Inc., Baltimore, MD; 8 mg protein/ml) in 10 mM phosphate buffer (pH 7.4) in the presence of the NADPH-regenerating system for 4 h at 37°C. The reactions were terminated by adding cold acetonitrile and the supernatants were concentrated for analysis. Two controls were prepared: one without the test compound and the other without the NADPH-regenerating system.

In vivo samples. Plasma was collected from the pharmacokinetic studies of TG100435 in dogs (i.v. and p.o.) and rats (p.o.) as described previously. Aliquots of the plasma samples of dogs and rats collected from different time points were pooled and processed using protein precipitation by adding acetonitrile. The supernatants were concentrated for analysis. Aliquots of the dog urine from different periods in the dog i.v. study were combined and processed as plasma samples by adding acetonitrile. The supernatant was concentrated for analysis.

**Metabolite Identification.** Metabolites were identified using a triple quadrupole LC/MS/MS system. The HPLC system consisted of two Shimadzu...
LC-10AD pumps, a Shimadzu DGU-3A degasser, a Shimadzu CTO-10A column heater, a Shimadzu SCL-10A controller, an Agilent 1100 series DAD detector (Agilent Technologies, Palo Alto, CA), and a Leap Technologies (Naperville, IL) CTC HTS autosampler. Samples were separated on a Zorbax SB C-18 (3.5-μm particle size, 50 mm × 2.1 mm) column using a 20-min, 27% to 37% linear gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.25 ml/min and column temperature of 40°C. An API3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) was interfaced via an electrospray ionization probe with HPLC. Positive modes were used in all of the analyses. Precursor ion scans of 98, 395, and 397 were used as survey scans to determine potential biotransformation at ethylpyrrolidine moiety or a methylbenzotriazine core of TG100435 (Fig. 2). Mass spectra of metabolites were obtained using product ion scans. Multiple reaction monitoring (MRM) was used to confirm the presence of the metabolites in different samples.

Quantitative Analysis of Pharmacokinetic Samples. Plasma samples from mouse, rat, and dog were prepared by protein precipitation using acetonitrile containing an internal standard. The supernatants were analyzed using the same LC/MS/MS system described above. A Phenomenex (Torrance, CA) Synergi Max-RP column (2-μm particle size, 20 mm × 2.1 mm) was used for separation with a 1-min, 10% to 100% linear gradient of 0.05% trifluoroacetic acid in water and 0.05% trifluoroacetic acid in acetonitrile. TG100435 and TG100855 were quantitatively monitored using positive-mode MRM of 494.2/98.1 and 510.2/98.1, respectively. Pharmacokinetic parameters were calculated using the WinNonlin program (version 4.01; Pharsight Corporation, Mountain View, CA) with noncompartmental model analysis, and the area under the curve (AUC) was estimated by linear trapezoidal integration.

**Results**

**Identification of N-Oxide Metabolites of TG100435.** In positive ion mode, the product ion mass spectrum of TG100435 at m/z 494 (M + H)⁺ displayed six structurally characteristic fragment ions (Fig. 2a). The ethylpyrrolidine moiety of TG100435 demonstrated the characteristic fragments of m/z 98, 395, 84, and 71 and the corresponding fragments of m/z 395, 408, and 423 associated with the methylbenzotriazine core. Based on the characteristic mass spectrum, metabolites were identified by determining changes in fragmentation patterns at part B, the ethylpyrrolidine moiety, or part A, the methylbenzotriazine core. The precursor ion scans of the fragments 98, 395, or 397 were used as survey scans to detect possible metabolites. Metabolites M1 to M4 were detected. Using product ion scan, mass fragment patterns of those metabolites were obtained (Table 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Ion</th>
<th>Fragment Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG100435</td>
<td>494</td>
<td>423, 408, 395, 367, 98, 84, 71</td>
</tr>
<tr>
<td>M1 (TG100855)</td>
<td>510</td>
<td>423, 395, 367, 116, 114, 98, 84, 71</td>
</tr>
<tr>
<td>M2</td>
<td>528</td>
<td>492, 421, 406, 393, 98, 84, 71</td>
</tr>
<tr>
<td>M3</td>
<td>544</td>
<td>421, 393, 116, 114, 98, 84</td>
</tr>
<tr>
<td>M4</td>
<td>526</td>
<td>423, 408, 399, 397, 368, 130, 112, 87</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Ion</th>
<th>Fragment Ions</th>
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<tbody>
<tr>
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<td>423, 408, 395, 367, 98, 84, 71</td>
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<td>M3</td>
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</tr>
<tr>
<td>M4</td>
<td>526</td>
<td>423, 408, 399, 397, 368, 130, 112, 87</td>
</tr>
</tbody>
</table>

(1H NMR of TG100435 and TG100855 in DMSO-d₆ (500 MHz))

<table>
<thead>
<tr>
<th>Compound</th>
<th>δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG100435</td>
<td>1.68 2.52 2.64 2.78 4.07 7.00 7.50 7.6–7.7 7.94 8.06 10.86</td>
</tr>
<tr>
<td>TG100855</td>
<td>2.19 3.47 3.55 2.62 3.80 4.51 7.06 7.51 7.6–7.7 7.96 8.06 10.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of H*</th>
<th>Multiplet (J, Hz)</th>
<th>δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG100435</td>
<td>4H (a)</td>
<td>br s</td>
<td>4.07</td>
</tr>
<tr>
<td>TG100855</td>
<td>2H (f)</td>
<td>br s</td>
<td>4.07</td>
</tr>
</tbody>
</table>

δ, chemical shift; H, proton; br, broad; s, singlet; d, doublet; t, triplet; J, coupling constant.

*(a)–(j), position of protons labeled in Fig. 1.*

**Fig. 2.** Product ion mass spectra and fragment patterns of TG100435 (molecular ion m/z 494) (a) and its active metabolite TG100855 (molecular ion m/z 510) (b).
N-oxidation at the ethylpyrrolidine (TG100855). Both HPLC retention time and mass spectrum confirmed that M1 was TG100855.

The metabolite M2 with a mass increase of 34 from parent compound was found in the dog p.o. sample using the precursor scan of 98. The product ion mass spectrum indicated that the addition of mass 34 occurred at part A of the parent compound because the fragment pattern of \( m/z \) 98, 84, and 71 remained and the \( m/z \) 395 and 423 shifted 2 units to \( m/z \) 393 and 421 (Table 2). The metabolite M3 with a mass increase of 50 from parent compound was detected in the rat liver microsomal sample. Its product ion mass spectrum gave a fragment pattern similar to that of M2 but had fragments 112 and 114 indicating \( N \)-oxidation at the ethylpyrrolidine moiety. The metabolite M4 with a mass increase of 32 from parent compound was found in the in vivo dog p.o. sample. Its product ion mass spectra indicated the addition of two oxygen atoms at the ethylpyrrolidine moiety. The fragments 112 and 130 in the mass spectrum indicated oxidative ring-opening of the pyrrolidine ring.

The presence of these metabolites in each sample was confirmed using the MRM method to analyze all of the in vitro and in vivo samples. The metabolites identified in the in vitro and in vivo samples are listed in Table 3. Oxidation was the major metabolic biotransformation for TG100435 in human, dog, and rat. This oxidation occurred at both part B, the ethylpyrrolidine moiety, and part A, the methylbenzotriazine core of the parent compound.

M1 was the only metabolite detected in trace amounts in the human liver microsomal sample using mass spectrometry. M1 was the predominant metabolite in the rat liver microsomal, rat, and dog in vivo samples detected at 190 to 400 nm of the photodiode array (Fig. 3, a and b). M4 was also present in the in vivo dog samples but at a reduced level compared with M1 (Fig. 3b).

Enzymatic Potency of TG100435 and TG100855. TG100435 displayed biochemical potency against Src, Lyn, Abl, Yes, Lck, and EphB4 with \( K_i \) values ranging from 13 to 64 nM (Table 4). Since TG100855 was the predominant metabolite of TG100435, a synthetic standard was generated and its kinase inhibition constants were determined (Table 4). Compared with TG100435, TG100855 was 2 to 9 times more potent against the same set of PTKs.

N-Oxidation of TG100435 in the in Vitro Study. The contribution of FMO and P450 on the \( N \)-oxidation of TG100435 was distinguished by deactivating FMO through preheating mouse liver microsome without NADPH. Compared with the nonheated mouse liver microsomal system, the initial formation rate of TG100855 was reduced significantly (Fig. 4).

Pharmacokinetics of TG100435 and Its Active Metabolite TG100855 in Mouse, Rat, and Dog. Pharmacokinetic parameters of TG100435 and TG100855 after single doses in mouse, rat, and dog are listed in Tables 5, 6, and 7. After a single bolus injection of 5 mg/kg TG100435, the compound showed low to moderate systemic absorption with peak plasma concentrations ranging from 19.3 ± 3.2 to 62.5 ± 1.5 ng/mL in mouse, rat, and dog, respectively. The elimination half-lives were in the range of 1.5 to 2.5 h, indicating rapid clearance from the bloodstream.

![Fig. 3. Chromatograms of rat p.o. (a) and dog p.o. (b) samples at UV 190 to 400 nm. Metabolite peaks were determined by comparing with controls; the retention time of TG100435 is 14.1 min.](image-url)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>M Retention Time</th>
<th>Dog Liver Microsome</th>
<th>Human Liver Microsome</th>
<th>Rat Liver Microsome</th>
<th>Rat p.o.</th>
<th>Dog p.o.</th>
<th>Dog i.v.</th>
<th>Dog Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>M+16 15.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>M+34 6.3</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>M3</td>
<td>M+50 7.1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>M4</td>
<td>M+32 12.3</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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</table>

+, found; −, not found.

![TABLE 3](image-url)

<table>
<thead>
<tr>
<th>Metabolites of TG100435 identified in rat, dog, and human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite M Retention Time</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td>M2</td>
</tr>
<tr>
<td>M3</td>
</tr>
<tr>
<td>M4</td>
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![TABLE 4](image-url)

<table>
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<tr>
<th>Inhibition constants of TG100435 and TG100855 to different protein tyrosine kinases</th>
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</thead>
<tbody>
<tr>
<td>PTK</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Src</td>
</tr>
<tr>
<td>Lyn</td>
</tr>
<tr>
<td>Abl</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>Lck</td>
</tr>
<tr>
<td>EphB4</td>
</tr>
</tbody>
</table>
clearance, a large volume of distribution, and a long half-life in all tested animals. Oral bioavailability of TG100435 was high in mouse but low in rat and dog. Absorption of TG100435 was slow in those animals, as peak plasma levels were reached at 5 to 8 h.

Formation of TG100855 was monitored in the oral studies of TG100435 in mouse, rat, and dog. A significant amount of TG100855 was formed after a single oral administration of 25 or 30 mg/kg TG100435 in either dog or rat (Fig. 5, b and c), but much less was detected in mouse after a single oral dosing of 40 mg/kg TG100435 (Fig. 5a). The AUC ratios of TG100855 to TG100435 were 2.1, 1.1, and 0.38 in dog, rat, and mouse, respectively.

After a single i.v. dose of TG100855 in mouse and rat, TG100855 showed clearance similar that of its parent compound in rat but lower clearance in mouse. Its volume of distribution and half-life were much less than that of its parent compound in both mouse and rat. TG100855 was partially converted back to TG100435 after dosing in different routes. There was no significant difference in mean AUC values of TG100855 and the converted TG100435 after i.v. and i.p. doses in both mouse and rat. Oral bioavailability of TG100855 was low in both animals, but its oral absorption was more rapid than that of TG100435, since peak plasma levels were reached at 1 and 3.5 h for mouse and rat, respectively. After oral administration of TG100855, the mean AUC of the converted TG100435 was larger than those of TG100855. The AUC ratios of the converted TG100435 to TG100855 were 1.93 and 1.87 in rat and mouse, respectively. Mean plasma concentration-time profiles obtained from both intravenous and oral dosing of TG100435 or TG100855 in mouse and rat are demonstrated in Figs. 6 and 7. The

### TABLE 5

Mean (S.D.) pharmacokinetic parameters of TG100435 and TG100855 following 5 mg/kg i.v. and i.p. dosing in mice and rats

<table>
<thead>
<tr>
<th>Species</th>
<th>Route</th>
<th>Compound</th>
<th>C&lt;sub&gt;0&lt;/sub&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;0-1009&lt;/sub&gt; (h ng)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>V&lt;sub&gt;d&lt;/sub&gt; (l/kg)</th>
<th>CL (ml/min/kg)</th>
<th>AUC&lt;sub&gt;0-1009&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (ng h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>i.v.</td>
<td>TG100435</td>
<td>873</td>
<td>4140</td>
<td>5.4</td>
<td>1.2</td>
<td>15.6</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>TG100855</td>
<td>9030</td>
<td>5350</td>
<td>0.9</td>
<td>2.8</td>
<td>11.6</td>
<td>1390</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>TG100855</td>
<td>4100</td>
<td>6520</td>
<td>2.8</td>
<td>2.6</td>
<td>11.6</td>
<td>1400</td>
</tr>
<tr>
<td>Rat</td>
<td>i.v.</td>
<td>TG100435</td>
<td>1650 (840)</td>
<td>7030 (2170)</td>
<td>8.9 (1.9)</td>
<td>9.4 (1.7)</td>
<td>12.7 (3.5)</td>
<td>539 (171)</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>TG100855</td>
<td>12,200 (3980)</td>
<td>6050 (386)</td>
<td>1.6 (0.2)</td>
<td>1.9 (0.4)</td>
<td>13.8 (0.9)</td>
<td>539 (171)</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>TG100855</td>
<td>1080 (806)</td>
<td>4990 (577)</td>
<td>2.3 (1.4)</td>
<td>3.3 (2.3)</td>
<td>17.0 (2.0)</td>
<td>498 (62)</td>
</tr>
</tbody>
</table>

<sup>a</sup> AUC of TG100435 converted from TG100855 after dosing with TG100855.

### TABLE 6

Mean (S.D.) pharmacokinetic parameters of TG100435 and TG100855 following 25 mg/kg p.o. administration in mice and rats

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-1009&lt;/sub&gt; (h ng)</th>
<th>AUC&lt;sub&gt;0-last&lt;/sub&gt; (h ng)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>TG100435</td>
<td>618</td>
<td>8</td>
<td>15,300</td>
<td>15,000</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>TG100855</td>
<td>171</td>
<td>1</td>
<td>939</td>
<td>912</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>TG100435&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113</td>
<td>7</td>
<td>1760</td>
<td>1740</td>
<td>6.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat</td>
<td>TG100435</td>
<td>348 (82)</td>
<td>8</td>
<td>8010 (3850)</td>
<td>7980 (3810)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>TG100855</td>
<td>160 (85)</td>
<td>3.5 (1.9)</td>
<td>470 (145)</td>
<td>467 (150)</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>TG100435&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0 (31.8)</td>
<td>4.0 (2.0)</td>
<td>906 (437)</td>
<td>881 (431)</td>
<td>3.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> TG100435 converted from TG100855 after dosed with TG100855.
<sup>b</sup> Oral bioavailability from the converted TG100435.

### TABLE 7

Mean (S.D.) pharmacokinetic parameters of TG100435 and TG100855 following 5 mg/kg i.v. and 25 mg/kg p.o. administration in dogs

<table>
<thead>
<tr>
<th>Route</th>
<th>Compound</th>
<th>C&lt;sub&gt;0&lt;/sub&gt; (ng/ml)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-1009&lt;/sub&gt; (h ng)</th>
<th>V&lt;sub&gt;d&lt;/sub&gt; (l/kg)</th>
<th>CL (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>TG100435</td>
<td>1660 (335)</td>
<td>15.1 (1.6)</td>
<td>5800 (158)</td>
<td>18.7 (2.2)</td>
<td>14.5 (0.4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Route</th>
<th>Compound</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-1009&lt;/sub&gt; (h ng)</th>
<th>AUC&lt;sub&gt;0-last&lt;/sub&gt; (h ng)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.o.</td>
<td>TG100435</td>
<td>102 (11)</td>
<td>4.0 (1.0)</td>
<td>3109 (812)</td>
<td>2536 (502)</td>
<td>10.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> AUC of TG100855 converted from TG100435 (same p.o. study of Fig. 5c).
intravenously administered compounds declined biexponentially in both animals.

**Discussion**

Oxidation is the primarily observed metabolic process of TG100435 in vitro and in vivo. N-Oxidation at the tertiary amine of the ethylpyrrolidine moiety (M1, TG100855) has been found in all samples of the study. This metabolite is the predominant metabolite in rat and dog, and the only one observed in the human liver microsomal sample. N-Oxidation of either aliphatic or aromatic amines by microsomal enzymes is well documented. For example, the N-oxides of the aliphatic amines in tamoxifen (Foster et al., 1980), imipramine (Bickel, 1972), and the imipramine-related compounds amitriptyline (Beckett, 1971), chlorpromazine (Bickel, 1972), and N,N-dimethyl-5H-dibenzo-[a,d]cycloheptene-Δ2,γ-propylamine (Belvedere et al., 1974) are well established. N-Oxidation is mediated by both cytochrome P450 and FMO (Miwao and Walsh, 1988). It has been found that N-oxidation of a potent 5-hydroxytryptamine2D receptor agonist, L-755,606, was mediated primarily by FMO3, whereas P450 had minimal involvement in the N-oxidative pathway in humans (Prueksaritanont et al., 2000). FMO3 is also the most active isoform in N-oxidation of trimethylamine in humans (Lang et al., 1998). It has
bepridil (Wu et al., 1992), and prolintane (Rucker et al., 1992). It is
proposed that the ring-opening metabolites are formed through alicy-
clic hydroxylation at the \( N \)-c of the pyrrolidine ring (Wu et al., 1988).

The mass spectra of M2 and M3 do not provide sufficient infor-

The role of pharmacologically active metabolites is a concern in
drug discovery and development. Active metabolites can contribute
significantly to the overall therapeutic and adverse effects of drugs. To
fully understand the mechanism of action of drugs, it is important to
recognize the biotransformation and pharmacokinetics of active me-
tabolites. TG100855 has much higher biochemical potency than its
parent compound, but its oral bioavailability is poor. This makes

TG100435 is a potent multi-tyrosine kinase inhibitor. Its \( N \)-oxide
metabolite TG100855 is even more potent biochemically than its
parent compound. Only a few \( N \)-oxide metabolites of drug compounds
have been reported to have high potency. For example, the \( N \)-oxide
metabolite of roflumilast shows equal potency to its parent compound
in attenuating allergen-induced bronchoconstriction in guinea pigs
(Bundschoh et al., 2001). The \( N \)-oxide of a drug is usually less active
than its parent compound. Many \( N \)-oxide metabolites are found to be
not active. For example, the \( N \)-oxide of an antitumor agent, azonafide,
is much less potent and cytotoxic than its parent compound (Uematsu
et al., 1989). The \( N \)-oxides are rapidly excreted out in the urine since
FMO generally converts nucleophilic heteroatom-containing chemi-
cals and drugs into harmless, polar, readily excreted metabolites
(Cashman and Zhang, 2006). Therefore, \( N \)-oxidation is often consid-
ered as a detoxication mechanism for tertiary nitrogen-containing
drugs. The high potency of TG100855 against multi-tyrosine kinases
presents a quite different potential of \( N \)-oxide metabolites. Because
the \( N \)-oxidation of TG100435 results in a much more potent metab-
olite, this metabolism will not decrease but potentially maintain or
even increase inhibition to tyrosine kinases.

The pharmacokinetic profile of TG100435 has been determined in
mouse, rat, and dog. TG100435 has low or moderate clearance in
those species compared with hepatic blood flow. However, the oral
bioavailability of TG100435 is much lower in rat and dog than in
mouse. The low oral bioavailability is a result of significant
\( N \)-oxidation of TG100435 in rat and dog. It has been reported that male
rat liver microsome contains twice the amount of FMO3 as male
mouse liver microsome (Ripp et al., 1999). This is consistent with our
observation that there is more conversion of TG100435 to TG100855
in male rat liver microsome than in male mouse liver microsome (data
not shown). On account of the fact that much more TG100435 is
\( N \)-oxidized in rat and dog, the observed oral bioavailability of
TG100435 is much lower in rat and dog than in mouse. If the
converted TG100855 were taken into account, the overall oral avail-
ability of active compounds (TG100435 plus TG100855) would be
increased to approximately 30% or 50% in dog or rat. Moreover,
because TG100855 is 2 to 9 times more potent than its parent
compound, the overall effect of inhibition to Src kinases in rat and
dog will be equivalent to or even greater than that in mouse after oral
dosing.

TG100855 has similar or lower clearance compared with
TG100435 in rat and mouse. The oral bioavailability of this metab-
olite is very low in both mouse and rat, although TG100855 is more
potent. After both i.v. and p.o. dosing of TG100855, TG100855 is
partially converted back to TG100435 in both mouse and rat. The
back-conversion is more evident in oral administration. Since i.p.
administration of TG100855 exhibits complete bioavailability in
mouse and \( >80\% \) bioavailability in rat (Tables 5 and 6), the intestinal
first-pass metabolism may be the primary contributor for the back-
conversion. Back-conversion of metabolites to parent compounds is
not uncommon and has been found between several drug compounds
and their metabolites in rat (Ebling and Jusko, 1986; Kuo et al., 1993;
Wong et al., 1996). The reduction of tertiary amine \( N \)-oxides through
different enzymes has been extensively documented. It is believed that
cytochrome P450 is partly responsible for \( N \)-oxide reduction, and this
reduction appears to be relatively nonspecific with respect to substrate
structures (Cho, 1988; Skálová et al., 2000).

The pharmacological activity of TG100435 and its active metab-
olite is a concern in drug discovery and development. Active metab-
lites can contribute significantly to the overall therapeutic and adverse
effects of drugs. To fully understand the mechanism of action of drugs, it is important to
recognize the biotransformation and pharmacokinetics of active me-
tabolites. TG100855 has much higher biochemical potency than its
parent compound, but its oral bioavailability is poor. This makes

**FIG. 7.** a, plasma concentration versus time profile of TG100435 (○),
TG100855 (■), and the converted TG100435 (△) after a 5 mg/kg intravenous dose of
TG100435 or TG100855 in rat. b, plasma concentration versus time profile of
TG100435 (○), TG100855 (■), and the converted TG100435 (△) after a 25 mg/kg
oral dose of TG100435 or TG100855 in rat.
TG100855 inappropriate as an oral drug. However, its parent compound, TG100435, has reasonably high overall oral bioavailability (TG100435 plus TG100855) and converts predominantly to TG100855 in vitro and in vivo. As a result, after in vivo oral administration of TG100435, the overall exposure of TG100435 and TG100855 is high. Because TG100435 converts to the more potent metabolite, this may potentially increase overall inhibition to PTKs, thus affecting efficacy of TG100435 in in vivo models. A further investigation of this finding is needed.

In summary, TG100435 is a novel multitargeted protein tyrosine kinase inhibitor. This small molecule inhibitor has low to moderate systemic clearance in mouse, rat, and dog. The oral bioavailability of TG100435 is high in mouse but low in rat and dog. The low bioavailability in rat and dog is due to significant biotransformation of TG100435 to its metabolite TG100855. TG100855 is 2 to 9 times more potent than its parent compound. Since TG100435 is predominately converted to the more potent N-oxide metabolite across species in vivo and in vitro, the overall tyrosine kinase inhibition in animal models may be substantially increased after oral administration of TG100435.

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References