**Regular Article**

**Comparative Analysis of In Vitro and In Vivo Pharmacokinetic Parameters Related to Individual Variability of GTS-21 in Canine**

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**Summary:** To clarify the cause of the canine individual variability in plasma concentration after oral administration of GTS-21, we evaluated in vitro the metabolism to 4-OH-GTS-21 in liver microsomes of the same individuals from in vivo pharmacokinetic study. First, we applied to the Michaelis-Menten kinetic parameters to a dispersion model, and compared hepatic availability ($F_H$) and hepatic clearance ($CL_H$) values from in vitro with bioavailability ($F$), hepatic plasma flow ($Q_{PH}$), and plasma clearance ($CL_P$) values from in vivo. The ratios of $CL_H$ to $Q_{PH}$ were ranged 0.74 to 0.94, suggesting that GTS-21 is a hepatic plasma flow-limiting drug. A significant correlation of $F_H$ and $F$ in the four dogs ($r = 0.995, p = 0.005$) indicates that the variability is predominantly caused by GTS-21 4-demethylase activity. Second, we specified the cytochrome P450 (CYP) enzymes that are involved with the metabolism by chemical inhibition. α-Naphthoflavone, furafylline, quinidine, quinine, and troleandomycin significantly inhibited GTS-21 4-demethylase activity. Thus CYP1A, CYP2D15, and CYP3A12 were involved with 4-demethylation. The variability in control activity decreased on addition of α-naphthoflavone and furafylline. Third, we quantified the contents of CYP1A and CYP3A12 by enzyme-linked immunosorbent assay. The content of CYP1A was consistent with GTS-21 4-demethylase activity. We concluded that canine liver CYP1A causes the individual variability in GTS-21 plasma concentration after oral administration.

**Key words:** dog; GTS-21; individual variability; intrinsic clearance; hepatic availability; CYP1A

**Introduction**

Canine models have the advantages of providing consecutive data on the plasma concentration of individuals, allowing the use of formulations,1,2) ease of handling, and an abundance of background data. Dogs thus are widely used for pharmacokinetic and toxicokinetic studies. However, they occasionally show individual variability in the plasma concentration after oral administration.3) The variability often complicates the analysis of the results. In some cases, it may be necessary to repeat or add a study, delaying research and development. To make matters worse, one may have to abandon the development of valuable drug candidates in the pre-clinical stage due to the variability. Thus, we consider it important to clarify the cause of the variability. Clarification would provide precious information for the selection of appropriate animals to evaluate results more accurately.

The model compound, GTS-21, (Fig. 1, also referred to as DMXB or DMXBA), (E)-3-(2,4-dimethoxybenzylidene)-3,4,5,6-tetra-hydro-2,3'-bipyridine dihydrochloride, has been developed to treat Alzheimer’s disease4) and schizophrenia.5)

In a previous work,6) we evaluated the absorption ratio ($F_A$), intestinal availability ($F_G$), and hepatic availability ($F_H$) in three dogs. GTS-21 was completely absorbed but lost by first-pass effects of passage through the gut wall and liver. The first-pass effect of liver (Mean ± S.D., 0.100 ± 0.063) is larger than that of gut wall (0.455 ± 0.006), and dominates the individual variability in plasma concentration. GTS-21 is mainly metabolized to 4-OH-GTS-21 (Fig. 1) and conjugated glucuronide.7) We therefore focused on in vitro metabolism of...
GTS-21 to 4-OH-GTS-21 in the liver microsomes of individuals, for which we had obtained in vivo pharmacokinetic data.

The purpose of our study is the elucidation of GTS-21 O4-demethylase activity as the main cause of variable pharmacokinetics of GTS-21 in dogs. To estimate the contribution of the metabolism to total clearance, we subjected Michaelis-Menten kinetic parameters of four dogs to a scaling procedure.8) A comparison of the in vivo pharmacokinetic parameters with the in vitro kinetic parameters revealed that the individual variability in plasma concentration was predominantly caused by GTS-21 O4-demethylase activity. Next, we specified the cytochrome P450 (CYP) enzymes that are involved with the metabolism by chemical inhibition, and quantified the contents of key enzymes by enzyme-linked immunosorbent assays (ELISA).

Methods

1. Chemicals

GTS-21 and 4-OH-GTS-21 were synthesized at Chemical Lab, Taiho Pharmaceutical Co., Ltd. (Hanno, Japan). α-Naphthoflavone, sulfaphenazole, troleandomycin, quinidine, and quinine were purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). Coumarin and p-nitrophenol were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Furafylline, anti-rabbit IgG HRP-linked antibody, anti-dog CYP1A serum, and anti-dog CYP3A12 serum for ELISA were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). The products of anti-dog serum contain microsome standard and antiserum. All other reagents were of the highest purity commercially available. Water was purified with Milli-Q (Millipore, Tokyo, Japan).

2. In Vivo Pharmacokinetics of GTS-21

The Taiho Animal Investigation Committee approved the study protocol. Four male beagle dogs (30 months old; Dog-1, 15.0 kg; Dog-2, 11.4 kg; Dog-3, 11.6 kg; Dog-4, 14.4 kg) were purchased from Covance Research Product Inc. (Kalamazoo, MI). They were fasted with free access to water for 16 h before and fasted 4 h after administration. GTS-21 was dissolved in distilled water (2 mg/ml) into an amber glass container due to the photosensitivity of GTS-21. The dogs were administered GTS-21 (1 mg/kg) intravenously. Blood (2 ml) was collected with a heparinized syringe from the jugular vein at 5, 10, 15, 30, 45, 60, 120, and 240 min after administration. Also in the fasted condition, dogs were administered GTS-21 (1 mg/kg) orally, then tap water (30 ml) was flushed into the stomach. Blood (2 ml) was collected with a heparinized syringe from the jugular vein at 15, 30, 45, 60, 90, 120, and 150 min after administration. The washout period was more than one week. Plasma was separated by centrifugation at 1,200 g, 4°C, for 15 min and stored at −80°C until analysis. Plasma sample preparations (0.25 ml) and the HPLC conditions were previously described.9) Maximum plasma concentration (Cmax, ng/ml), time to reach Cmax (tmax, min), area under the plasma concentration versus time curve zero to infinity (AUC0–∞, ng·h/ml), elimination half-life (t1/2, min), plasma clearance (CL), ml/min/kg, and bioavailability (F) were calculated with a non-compartment model using Win-Nonlin Ver. 1.1 (Pharsight, Apex, NC). The elimination phase was analyzed by weighing 1/y automatically.

3. Preparation of Dog Liver Microsomes

Three months after the pharmacokinetic study, the four dogs were weighed (Dog-1, 15.1 kg; Dog-2, 11.6 kg; Dog-3, 11.6 kg; Dog-4, 14.3 kg) and euthanized under thiopental anesthesia (0.5 ml/kg, i.v.). The livers were removed. Subsequent procedures were conducted in an ice-packed container. Livers were homogenized in the mixture buffer of 0.1 M potassium phosphate buffer (pH 7.4), 0.125 M KCl, and 1 mM EDTA.10) The homogenates were first centrifuged at 9,000 g for 20 min, and then the supernatants were centrifuged at 105,000 g for 60 min. The microsomal pellets were re-suspended in the mixture buffer, and then centrifuged at 105,000 g for 60 min. The pellets were re-suspended in the mixture buffer. The microsomal suspensions of the four dogs were individually stored at −80°C. Protein concentration was determined at 595 nm using a Bio-Rad Protein Assay Kit (Tokyo, Japan) with bovine serum albumin as a standard. Microsome contents were calculated using Eq. (1).

Microsome contents = Microsome concentration × Volume Liver weight Liver weight for microsome preparation × Body weight

4. GTS-21 O4-Demethylase Activity in Dog Liver Microsomes

The procedures were conducted under yellow light. GTS-21 was dissolved in water. The microsomal mixture (200 μl, n = 3) contained 40 μg protein, 0.1 M potassium phosphate buffer (pH 7.4), 25 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 1 mM NADPH, and...
GTS-21 (1, 2, 5, 10, 20, 50, 100, and 200 μM). The mixtures without NADPH solution were pre-incubated for 1 min at 37°C, then added NADPH solution. The mixtures were incubated for 1 min. The reaction was stopped by addition of ice-cold 10% HClO₄ (200 μl), and the samples vortexed for 5 s. The samples were centrifuged at 1,200 g for 5 min at 4°C. The supernatants (40 μl) were injected into an HPLC system. HPLC conditions were as follows: analytical column, Hydrosphere C18 (3 μm, 100 × 4.6 mm i.d., YMC, Kyoto, Japan); column temperature, 40°C; mobile phase, 0.06% trifluoroacetic acid/acetonitrile (19/81); detection, UV-405 nm; flow rate, 1.0 ml/min.

5. Protein Binding of GTS-21 in Microsomal Incubation and Plasma

The microsomal mixtures without NADPH (1–200 μM GTS-21, n = 4) were centrifuged at 105,000 g, and 37°C for 65 min. The mixtures were de-proteinized, and the supernatants were injected into the HPLC system. Plasma spiked with 6.6 μM GTS-21 (200 μl, n = 4) was incubated at 37°C for 15 min, then centrifuged at 194,000 g, and 37°C for 22 h. Supernatants were de-proteinized, and the supernatants were injected into the HPLC system. The mean unbound fractions of microsomal incubation were calculated by regression analysis.

6. Application of Michaelis-Menten Kinetic Parameters Using a Scaling Procedure

Hepatic plasma flow ($Q_{PH}$, ml/min/kg) was (1-Hematocrit) multiplied by hepatic blood flow. Alometric Eq. (2) is shown below. The hematocrit value was determined by the cumulative pulse height detection method using an automatic hematology analyzer NE-600 (SYSMEX, Tokyo, Japan).

$$Q_{PH} = (1 - \text{Hematocrit}) \times \frac{0.150 \times \text{Body weight}^{0.840}}{\text{Body weight}} \times 1000.$$  

(2)

Michaelis-Menten kinetic parameters of a two- enzymatic model, Eq. (3), maximum velocity ($V_{max1}$ and $V_{max2}$, nmol/min/mg protein) and Michaelis constant ($K_{m1}$ and $K_{m2}$, μM), were calculated by the damping Gauss-Newton method in MULTI. Eadie-Hofstee plots were drawn using the unbound concentration of GTS-21, [S]₀, and the metabolic velocity of 4-OH-GTS-21, v.

$$v = \frac{V_{max1} \cdot [S]_o}{K_{m1} + [S]_o} + \frac{V_{max2} \cdot [S]_o}{K_{m2} + [S]_o}.$$  

(3)

Hepatic intrinsic clearance ($CL_{H, int}$, ml/min/kg) was calculated from Eq. (4).

$$CL_{H, int} = \left(\frac{V_{max1} + V_{max2}}{K_{m1} + K_{m2}}\right) \times \text{Microsome Contents.}$$  

(4)

The predicted values, $F_H$ and hepatic clearance ($CL_H$, ml/min/kg), were calculated by applying $Q_{PH}$, plasma unbound fraction ($f_u$), $CL_{H, int}$, and a dispersion number ($D_N$) of 0.17 to the dispersion model, Eqs. (5) and (6).

$$F_H = \frac{4a}{(1 + a)^2 \cdot \exp [(a - 1)/2D_N] - (1 - a)^2 \cdot \exp [-(a + 1)/2D_N]}.$$  

(5)

$$CL_H = \frac{Q_{PH} \left[1 - (1 + a)^2 \cdot \exp [(a - 1)/2D_N] - (1 - a)^2 \cdot \exp [-(a + 1)/2D_N]\right]}{(1 + 4a) \cdot CL_{H, int}}.$$  

(6)

In these Eqs., where $a = (1 + 4R_N \cdot D_N)^{1/2}$, where $R_N$ (efficiency number) = $f_u \cdot CL_{H, int} / Q_{PH}$.

7. Chemical Inhibition Study of GTS-21 O4-Demethylase Activity

α-Naphthoflavone, furafylline, coumarin, sulfaphenazole, quinidine, quinine, p-nitrophenol, and troleandomycin were dissolved in 2% dimethylsulfoxide. Microsomal mixtures (200 μl containing 0.5% dimethylsulfoxide, n = 2) contained 50 μg protein, 0.1 M potassium phosphate buffer (pH 7.4), 25 mM KCl, 50 μM EDTA, 5 mM MgCl₂, 2 mM NADPH, 131 μM GTS-21, and 10 μM chemical inhibitor. When α-naphthoflavone or a mechanism-based inhibitor (furafylline or troleandomycin) was included, the mixtures without GTS-21 were pre-incubated for 15 min at 37°C, then GTS-21 was added and incubated for 1 min. When the other inhibitor was included or not (control), the mixtures without NADPH were pre-incubated for 1 min at 37°C, then NADPH was added and incubated for 1 min. Subsequent procedures were the same as the determination of GTS-21 O4-demethylase activity.

8. Enzyme Linked Immunosorbent Assay for Canine CYP1A and 3A12

The contents of cytochrome P450 were determined by the usual method. The contents of CYP1A and CYP3A12 were quantified according to Eguchi et al. The microsomal suspension was diluted with phosphate buffered saline to 6.6–10 mg/ml for CYP1A quantification and 2.5–4.0 mg/ml for CYP3A12 quantification, respectively. To prepare TMB solution, 15 mg 3,3′,5,5′-tetramethylbenzidine (TMB) was dissolved in 15 ml dimethylsulfoxide, and then added to 85 ml buffer (0.1 M citric acid-0.2 M Na₂HPO₄, pH 4.5). Just before use, H₂O₂ was added to the TMB solution at 0.012%.

9. Statistics

Statistics were calculated with EXSAS Ver. 5.00 (Arm
Corporation, Osaka, Japan). Correlation between actual $F$ and predicted $F_H$ was analyzed by Pearson’s correlation. Significant differences are expressed by *, $p < 0.05$. Differences among means with and without chemical inhibitor were analyzed by Dunnet’s multiple comparison. One group was α-naphthoflavone, coumarin, sulphanphenoazone, quinidine, $p$-nitrophenol, and troleandomycin. The other group was furafylline and quinine. Significant differences are represented by $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

**Results**

1. **Pharmacokinetics of GTS-21 in Four Dogs**

GTS-21 (1 mg/kg) was orally and intravenously administered to four fasted dogs. Plasma concentrations in all dogs peaked within 30 min and GTS-21 was eliminated rapidly with a $t_{1/2}$ of 29 to 47 min. The pharmacokinetic parameters, $t_{max}$, $C_{max}$, $AUC_{0-\infty}$, and $t_{1/2}$, of GTS-21 are shown in Table 1. The elimination rate from plasma in individuals varied little, but a 7-fold difference of GTS-21 are shown in Table 1. The elimination rate within the range 1 to 200 was larger in Dogs-1, 3, and 4 than Dog-2 and 4. The ratios of Dogs-1 to 4 were 0.143, 0.125, 0.033, and 0.020, respectively. In contrast, less than a 2-fold difference was observed after intravenous administration. The $t_{1/2}$ values in all dogs were consistent with those after oral administration. The $F$ values of Dogs-1 to 4 were 0.143, 0.125, 0.033, and 0.020, respectively.

2. **GTS-21 O4-Demethylase Activity in Dog Liver Microsomes**

The metabolic velocities of GTS-21 O4-demethylation were larger in Dogs-1, 3, and 4 than Dog-2 and within the range 1 to 200 μM. As shown in Fig. 2, Eadie-Hofstee plots of all dogs showed biphasic kinetics. The Michaelis-Menten kinetic parameters, $V_{max}$ and $K_m$, were analyzed with a two-enzymatic model. These parameters are listed in Table 2. The $K_m$ value of Dog-1 was larger than those of Dogs-2, 3 and 4. The ratios of $V_{max}$ to $K_m$ of Dogs-3 and 4 were 2-fold more than the ratios of Dogs-1 and 2.

3. **Hepatic Clearance and Hepatic Availability Using a Scaling Procedure**

The $CL_{H,int}$ values were calculated by applying Michaelis-Menten kinetic parameters to Eq. (4). The difference of $CL_{H,int}$ was 3-fold between maximum and minimum values. The hematocrit values of Dogs-1 to 4 were 0.453, 0.538, 0.482, and 0.428, respectively. The $Q_{PH}$ values were calculated by applying the hematocrit values and body weight of the in vivo pharmacokinetic study to Eq. (2). The unbound fraction in microsomal incubation increased with the concentration of GTS-21. The regression equation was as follows: $y = x^{0.0474} \times \exp (-0.344)$, where $x$ is substrate concentration ($r = 0.948$). The mean unbound fraction in plasma, $f_u$, was 0.21. The predicted values, $F_H$ and $CL_{H}$, were calculated using a dispersion model of Eqs. (5) and (6). These parameters and $F$ and $CL_P$ from the in vivo study are listed in Table 3. The ratios of predicted $CL_{H}$ to $Q_{PH}$ of Dogs-1 to 4 were 0.74, 0.77, 0.94, and 0.93, respectively. In addition, the ratios of $CL_{H}$ to $CL_P$ of Dogs-1 to 4 were 0.81, 0.50, 0.66, and 1.1, respectively. The individual variability of predicted $F_H$ corresponded well with that of $F$, a significant correlation ($r = 0.995$, $p = 0.005$) being obtained.

![Fig. 2. Eadie-Hofstee profiles of GTS-21 O4-demethylase activity in dog liver microsomes.](image)

Table 1. Pharmacokinetic parameters of GTS-21 after its administration at 1 mg/kg to four dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dog-1</th>
<th>Dog-2</th>
<th>Dog-3</th>
<th>Dog-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{max}$ (min)</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>92.0</td>
<td>68.7</td>
<td>25.9</td>
<td>20.4</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (ng·h/ml)</td>
<td>148.3</td>
<td>88.3</td>
<td>22.2</td>
<td>21.6</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>47</td>
<td>32</td>
<td>29</td>
<td>33</td>
</tr>
</tbody>
</table>

B) Intravenous administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dog-1</th>
<th>Dog-2</th>
<th>Dog-3</th>
<th>Dog-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-\infty}$ (ng·h/ml)</td>
<td>1034</td>
<td>706</td>
<td>673</td>
<td>1080</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>48</td>
<td>31</td>
<td>32</td>
<td>43</td>
</tr>
</tbody>
</table>

Dosages for Dog-1 to 4 were 15.0, 11.4, 11.7, and 14.4 mg, respectively.

Table 2. Michaelis-Menten kinetic parameters of GTS-21 O4-demethylation in dog liver microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dog-1</th>
<th>Dog-2</th>
<th>Dog-3</th>
<th>Dog-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (nmol/min/mg protein)</td>
<td>1.41</td>
<td>0.49</td>
<td>1.72</td>
<td>1.57</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>3.62</td>
<td>1.32</td>
<td>1.85</td>
<td>1.94</td>
</tr>
<tr>
<td>$V_{max}$ (nmol/min/mg protein)</td>
<td>3.82</td>
<td>3.94</td>
<td>3.27</td>
<td>6.45</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>328</td>
<td>210</td>
<td>103</td>
<td>331</td>
</tr>
</tbody>
</table>

The $K_m$ and $V_{max}$ values were derived from fitting the triplicate data to a two-enzymatic model for the Michaelis-Menten equation.
Fig. 3. Effect of 10 μM chemical inhibitors on GTS-21 O4-demethylase activity in dog liver microsomes. The concentration of GTS-21 was 131 μM. Samples were incubated at 37 °C for 1 min. Values represent the mean of duplicate measurements as a percentage of control. One assay group was α-naphthoflavone, coumarin, sulfaphenazole, quinidine, p-nitrophenol, and troleandomycin. The other assay group was furafylline and quinine. Significant differences were analyzed by Dunnet's multiple comparison: * , p < 0.05; ** , p < 0.01; *** , p < 0.001.

Table 3. Comparison of in vitro and in vivo kinetic parameters

<table>
<thead>
<tr>
<th>Data</th>
<th>Parameter</th>
<th>Dog-1</th>
<th>Dog-2</th>
<th>Dog-3</th>
<th>Dog-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLH,int (ml/min/kg)</td>
<td>135</td>
<td>130</td>
<td>322</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>0.258</td>
<td>0.229</td>
<td>0.062</td>
<td>0.066</td>
</tr>
<tr>
<td>in vitro</td>
<td>CLH (ml/min/kg)</td>
<td>13.0</td>
<td>11.8</td>
<td>16.3</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>QpH (ml/min/kg)</td>
<td>17.5</td>
<td>15.3</td>
<td>17.4</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.143</td>
<td>0.125</td>
<td>0.033</td>
<td>0.020</td>
</tr>
</tbody>
</table>

The mean F was 0.21. Both the FH and the CLH were calculated using a dispersion model (Dn, 0.17).

4. Effect of CYP Chemical Inhibitors on GTS-21 O4-Demethylation

To specify the canine CYP enzymes catalyzing GTS-21 O4-demethylation, the effects of chemical inhibitors were tested. The results from the addition of 10 μM chemical inhibitors are shown in Fig. 3. The inhibitors are α-naphthoflavone (inhibitor of CYP1A), furafylline (CYP1A), coumarin (CYP2A), sulfaphenazole (CYP2C), quinidine (CYP2D15), quinine (CYP2D15), p-nitrophenol (CYP2E1) and troleandomycin (CYP3A12). α-Naphthoflavone, furafylline, quinidine, quinine, and troleandomycin significantly inhibited GTS-21 O4-demethylase activity in all dog liver microsomes. Coumarin, sulfaphenazole, and p-nitrophenol did not inhibit the activity. Sulfaphenazole in Dog-3 microsomes significantly inhibited the activity because the standard errors of all activities were small.

The individual variability, 46.1% (%R.S.D.), in control GTS-21 O4-demethylase activity for 0.87, 0.86, 1.68, and 1.74 nmol/min/mg protein of Dogs-1 to 4
Table 4. Contents of CYP enzymes in dog liver microsomes quantified by ELISA

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>CYP1A (pmol/mg protein)</th>
<th>CYP3A12 (pmol/mg protein)</th>
<th>Cytochrome P450 (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog-1</td>
<td>18.7</td>
<td>66.0</td>
<td>604</td>
</tr>
<tr>
<td>Dog-2</td>
<td>20.2</td>
<td>62.8</td>
<td>618</td>
</tr>
<tr>
<td>Dog-3</td>
<td>42.4</td>
<td>56.4</td>
<td>516</td>
</tr>
<tr>
<td>Dog-4</td>
<td>40.0</td>
<td>64.5</td>
<td>529</td>
</tr>
</tbody>
</table>

The contents of cytochrome P450 were quantified by CO reduced difference spectral assay.

decreased 16.3% on addition of furafylline (0.72, 0.64, 0.93, and 0.83 nmol/min/mg protein). But the variability did not decrease on addition of quinine (58.4% vs. control, 37.9%), quinine (72.4% vs. control, 46.1%), or troleandomycin (42.5% vs. control, 37.9%). Similarly, the individual variability decreased on addition of α-naphthoflavone (8.1% vs. control, 37.9%).

5. Contents of Canine CYP1A and CYP3A12

CYP1A, CYP3A12, and cytochrome P450 contents and mean, S.D., and %R.S.D. are summarized in Table 4. The cytochrome P450 contents of Dogs-1 to 4 indicate little individual variability. The CYP1A contents of Dogs-1 and 2 were half those of Dogs-3 and 4. The %R.S.D. for CYP1A was notably greater (49.0%) than that of CYP3A12 (6.8%) and cytochrome P450 (9.1%).

Discussion

The focus of our study was to resolve the cause of canine individual variability in the plasma concentration of GTS-21 after oral administration. To this end, we applied the in vitro Michaelis-Menten kinetic parameters in the metabolism to O4-demethylation, and compared the predicted $F_H$ and $CL_H$ values from in vitro with the actual $F$, $CL_F$, and $Q_{P_H}$ values from in vivo. In addition, we specified by chemical inhibitors and quantified the enzymes that are involved in the metabolism.

Four dogs were administered GTS-21 orally, the $AUC_{0-\infty}$ values of Dogs-1 and 2 being 4 to 7-fold larger than those of Dogs-3 and 4. Similarly, the $C_{max}$ values of Dogs-1 and 2 were larger than those of Dogs-3 and 4, but the $t_{1/2}$ values did not differ. In a previous work, the bioavailability of GTS-21 in three dogs was 0.072, 0.021, and 0.037, respectively. The absorption ratio was close to 1, and the intestinal availability ranged from 0.449 to 0.461. The hepatic availability was estimated at 0.170, 0.047, and 0.083. We considered that the first-pass effect through the liver is the key step. We therefore shifted our attention to the metabolism, namely, GTS-21 O4-demethylase activity in the liver microsomes.

To estimate the initial metabolic velocity accurately, we investigated the linearity of the reaction time for GTS-21 O4-demethylase activity. The formation of 4-OH-GTS-21 was fast enough to necessitate a 1-min incubation. Also, a 1-min preincubation at 37°C was adequate for thermal equilibration. Relatively low $CL_H$ values, 135 and 130 ml/min/kg, were obtained from liver microsomes of Dogs-1 and 2 that showed high $C_{max}$ and $AUC_{0-\infty}$ values in vivo. High $CL_H$ values, 322 and 337 ml/min/kg, were obtained from those of Dogs-3 and 4 that showed low $C_{max}$ and $AUC_{0-\infty}$ values.

Prior to scaling, we paid attention to the following three points; 1) choice of an appropriate scaling model, 2) protein binding on microsomal incubation, and 3) application of hepatic plasma flow rate. We chose a dispersion model because it is most suitable for high clearance drugs. Iwatsubo et al. reported that the $F_H$ of high clearance drugs would be underestimated by a well-stirred model and overestimated by a parallel-tube model.

Next, we took protein binding into account at every substrate concentration in the microsomal incubation, and recalculated free GTS-21. The free GTS-21 increased from 0.71 to 0.91 with the concentration of 1 to 200 μM. Finally, to compare actual $CL_F$ with predicted $CL_H$ we applied $Q_{P_H}$ instead of hepatic blood flow, and obtained predicted $CL_H$ and $F_H$.

The ratio of $CL_H$ to $Q_{P_H}$ ranged 0.74 to 0.94, suggesting that GTS-21 is a hepatic plasma flow-limiting drug. With GTS-21 it is easy to obtain a first-pass effect, and the elimination of GTS-21 is apt to be affected by the hepatic plasma flow rate. The $t_{1/2}$ values after oral administration in individuals were not different in spite of the large individual variability of $C_{max}$ or $AUC_{0-\infty}$. This finding supports that the metabolism of GTS-21 depends on hepatic plasma flow. The ratio of $CL_H$ to $CL_F$ varied from 0.50 to 1.1, suggesting that GTS-21 eliminated primarily via metabolism to O4-demethylation.

Although the $AUC_{0-\infty}$ after intravenous administration in Dog-4 was highest in four dogs, the variability was small. On the contrary, $AUC_{0-\infty}$ after oral administration in Dog-4 was lowest. The $AUC_{0-\infty}$ after oral administration is decreased by the first-pass effect through the liver. The $F_H$ of a high clearance drug is more influenced by $f_a \cdot CL_H$, than $Q_{P_H}$. There was no individual variability in $f_a$ in all dogs. Therefore the variability of the $CL_{H,int}$ reflects that of $F_H$.

A discrepancy between $CL_H$ and $CL_F$ may be attributed to the excretory clearance of GTS-21 via bile and the non-actual measurement of $Q_{P_H}$ values.

A significant correlation of $F_H$ and $F$ in the four dogs indicates that the individual variability in the plasma
concentration of GTS-21 is predominantly caused by GTS-21 O4-demethylase activity in the liver microsomes. We did not investigate the intestinal metabolism in small intestine because \( F_o \) values were not variable in previous work.\(^9\) The products of \( F_i \) multiplied by the mean \( F_o \) value, 0.455\(^10\) are 0.117, 0.104, 0.028, and 0.030 of Dog-1 to 4, respectively. The values well correspond to \( F \) values, 0.143, 0.125, 0.033, and 0.020.

Consequently, we considered that the variability in the plasma concentration of GTS-21 depends on the magnitude of \( CL_{H,\text{int}} \), which is first extracted from the liver. Also, we considered that scaling model without blood-plasma partition is suitable for the relationship between in vivo and in vitro pharmacokinetic parameters.

The authors acknowledge that the specificity of chemical inhibitors to canine cytochrome P450 is limited. Canine CYP1A catalyzes phenacetin O-deethylation.\(^{16,17}\) The activity is inhibited by furafylline.\(^{16}\) Also, canine CYP1A catalyzes ethoxyresorufin O-deethylation,\(^{18}\) and the activity is inhibited by both \( \alpha \)-naphthoflavone and furafylline.\(^{18}\) Coumarin 7-hydroxylase activity in dog is lower than that in human, whereas coumarin can act as an inhibitor of canine CYP2A.\(^{16-20}\) Diclofenac 4'-hydroxylation can be evaluated as a canine CYP2C substrate. This activity is inhibited by sulfaphenazole.\(^{18}\) Quinidine and quinine are specific inhibitors of canine CYP2D15.\(^{21,22}\) \( \beta \)-Nitrophenol hydroxylation is catalyzed by canine CYP2E1,\(^{23}\) and can be used as an inhibitor. Troleandomycin is a potent inhibitor of canine CYP3A12.\(^{24,25}\)

Biphasic Eadie-Hofstee plots suggest at least two CYP enzymes that are involved with GTS-21 O4-demethylation. To detect high and low affinity CYP enzymes, we chose a high concentration of GTS-21 (131 \( \mu \)M). \( \alpha \)-Naphthoflavone, furafylline, quinidine, quinine, and troleandomycin significantly inhibited GTS-21 O4-demethylase activity in all dogs. We specified three enzymes, CYP1A, CYP2D15, and CYP3A15 were involved with O4-demethylation. Among the three enzymes, only CYP1A inhibitors markedly decreased the individual variability in the residual activities. This indicates that CYP1A is a predominant enzyme that controls the individual variability of GTS-21 O4-demethylation.

Dog expresses CYP3A12 and CYP3A26.\(^{26}\) The antidog CYP3A12 serum only crosses with CYP3A12.\(^{14}\) On the other hand, anti-dog CYP1A serum crosses with both CYP1A1 and CYP1A2. As the results of ELISA, the contents of CYP1A for Dogs-1 and 2 were found to be half of those for Dogs-3 and 4 (see Table 4). The finding was consistent with the difference in GTS-21 O4-demethylase activity. This suggests that GTS-21 O4-demethylase activity is dependent on the content of CYP1A. In other words, the individual difference of the activity is based on quantitative changes, not qualitative ones. The %R.S.D. for CYP3A12 (6.8%) was markedly smaller than that of CYP1A (49.0%). We considered that the content of CYP3A12 did not influence GTS-21 O4-demethylase activity at all. Taking into consideration of the residual activity in addition of chemical inhibitors, we did not quantify the content of CYP2D15. Because drugs were not administered to the four dogs during in vivo and in vitro studies, enzyme induction or inhibition in the dogs would not be altered.

As results of above findings, we considered that the contents of canine liver CYP1A caused the individual variability in GTS-21 plasma concentration after oral administration. Although in vivo probe drugs of canine CYP1A are not established so far, we should be cautious the variability in plasma concentration when a high clearance drug that is predominantly metabolized by CYP1A is administered to dogs orally.

In conclusion, comparison in vivo with in vitro pharmacokinetic parameters from a scaling procedure taking into account the protein binding to matrix is useful for characterizing GTS-21 as a hepatic plasma flow-limiting drug. We clarified that the canine individual variability in the plasma concentration of GTS-21 after oral administration was caused by a first-pass effect that depended on GTS-21 O4-demethylase activity in liver microsomes. As the results of chemical inhibition study and ELISA, canine CYP1A plays an important role in determining the individual variability of GTS-21 O4-demethylation.

References


