Thyroid hormones (THs) are known to influence all major metabolic pathways including growth, development, and lipids metabolism. The involvement of THs in lipids metabolism was first reported in 1930,11 and since then, accumulating evidence has shown that THs lower cholesterol levels in patients with hypercholesterolemia.2−4) TH synthesis and secretion are strictly regulated by the hypothalamic/pituitary/thyroid (HPT) axis, and their physiological action is modulated via two homologous TH receptors, TRα and TRβ, which belong to the nuclear receptor superfamily of ligand-dependent transcription factors.9) Because TH receptors are ubiquitously expressed in humans, plasma levels of circulating TH affect various physiological pathways and are associated with a number of pathological states such as hyperthyroidism, which is characterized by decreased serum cholesterol and triacylglycerides (TG) levels, body weight loss, tachycardia, palpitation, muscle wasting, and osteoporosis in postmenopausal women.6)

Both academia and industry are making numerous efforts to separate TH beneficial effects such as cholesterol lowering and body weight loss from their other hormonal functions.5,7−10) Studies in TR-mutant mice suggest that TRβ is responsible for the cholesterol-lowering effect of TH.11−13) In addition, TRβ is predominantly expressed in the liver, where it accounts for approximately 80% of T3-binding TRs.14) Therefore a number of liver-selective TRβ agonists have been developed. Among them, GC-1 (Fig. 1) and KB2115 showed promising clinical trial results, but never reached the market due to undesirable side effects.10) Considering their drawbacks, we were interested in their influences on thyroid-stimulating hormone (TSH). Thus GC-1 dose-dependently reduces plasma TSH levels within the therapeutic dose range in rats.5,10) KB2115 also reduces plasma TSH levels at its minimum therapeutic dose of 100μg daily in humans.15) As described above, TSH is secreted from the anterior pituitary gland and promotes TH synthesis by stimulating a number of thyroid genes via binding to the TSH receptor in the thyroid gland. TSH secretion is also regulated by thyrotropin-releasing hormone (TRH) via binding to TRH receptors. Both TRH and TSH secretion are negatively regulated by THs (thyroxine: T4; triiodothyronine: T3).18−20) Overall, TH synthesis and secretion are controlled under a negative feedback system, which maintains physiological concentrations of TH. It is therefore suggested that suppression of TSH production results in hypothyroidism.21) At least, there would be a significant concern over long-term use of GC-1 or KB2115.

Very recently, we reported that the novel TRβ-selective agonists SKL-12846 and SKL-13784 reduce cholesterol levels without affecting thyroid-stimulating hormone (TSH) in cholesterol-fed rats.11,12) Our aim in this study was to elucidate what sets apart these SKL-compounds as TRβ agonists with no effect on TSH. To this end, we determined SKL-compounds pharmacokinetics and tissue distribution in normal rats and compared them to those of GC-1, a liver-selective TRβ agonist with concomitant effect on TSH. The present study explains why SKL-12846 and SKL-13784 have beneficial effects on lowering lipids without affecting heart rate and TSH production at the therapeutic dose in cholesterol-fed rats. In addition, we found that SKL-13784 shows no sign of escape phenomenon in fructose-fed rats. These results demonstrate the advantages of extremely high liver specificity to TRβ agonists. However, SKL-13784 has been found significantly to reduce endogenous T3 levels at doses lower than its lipid-lowering dose, which may raise concerns over this compound’s ability to alter thyroid hormone metabolism in the liver. While the mechanism by which SKL-13784 reduces endogenous T3 levels is still unclear, our results would help design better liver-selective TRβ modulators.

**Key words** thyroid hormone receptor β; thyroid stimulating hormone; lipid-lowering effect; GC-1

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**In Vivo Evaluation of 1-Benzyl-4-aminoindole-Based Thyroid Hormone Receptor β Agonists: Importance of Liver Selectivity in Drug Discovery**

Naoki Takahashi,a Yukiyasu Asano,a Koji Maeda,a and Nobuhide Watanabe*a,d


Received November 24, 2013; accepted March 9, 2014

We recently reported that the novel thyroid hormone receptor β (TRβ) selective agonists SKL-12846 and SKL-13784 reduce blood cholesterol levels without affecting thyroid-stimulating hormone (TSH) in cholesterol-fed rats.11,12) Our aim in this study was to elucidate what sets apart these SKL-compounds as TRβ agonists with no effect on TSH. To this end, we determined SKL-compounds pharmacokinetics and tissue distribution in normal rats and compared them to those of GC-1, a liver-selective TRβ agonist with concomitant effect on TSH. The present study explains why SKL-12846 and SKL-13784 have beneficial effects on lowering lipids without affecting heart rate and TSH production at the therapeutic dose in cholesterol-fed rats. In addition, we found that SKL-13784 shows no sign of escape phenomenon in fructose-fed rats. These results demonstrate the advantages of extremely high liver specificity to TRβ agonists. However, SKL-13784 has been found significantly to reduce endogenous T3 levels at doses lower than its lipid-lowering dose, which may raise concerns over this compound’s ability to alter thyroid hormone metabolism in the liver. While the mechanism by which SKL-13784 reduces endogenous T3 levels is still unclear, our results would help design better liver-selective TRβ modulators.

**Key words** thyroid hormone receptor β; thyroid stimulating hormone; lipid-lowering effect; GC-1

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**Fig. 1. Chemical Structures of SKL-12846, SKL-13784, and GC-1**

The authors declare no conflict of interest.  
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levels as well as plasma levels of endogenous T₃ and T₄ in fructose-fed rats.

MATERIALS AND METHODS

Chemicals SKL-12846,²²¹ SKL-13784,²²¹ and GC-1²³ were prepared in our laboratories as previously described.

Animals Male Sprague-Dawley (SD) rats (Charles River Japan, Kanagawa, Japan) were housed under standard conditions with a 12-h light/dark cycle and allowed free access to water and a commercial diet (NMF, Oriental Yeast, Japan) for at least 5 d. All animal procedures were approved by the Sanwa Kagaku Kenkyusho Institutional Animal Care and Use Committee, and were conducted in accordance with institutional guidelines that complied with “Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour, and Welfare of Japan” (2006).

PK in Normal Rats Rats (n=2–3) were fasted overnight 1 d before the experimental day. SKL-12846 and SKL-13784 were prepared in saline at a concentration of 1 mg/1 mL/kg for intravenous (i.v.) bolus injection into the tail vein, and in 5% gum arabic solution (GA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a concentration of 30 mg/5 mL/kg for oral administration (per os (p.o.)). Blood samples (approximately 200 µL) were collected from the jugular vein under diethyl ether anesthesia using a heparinized syringe at 5 (i.v. only), 10 (i.v. only), 15, and 30 min and 1, 2, 3, 4, 6, 8, and 24 h post-dosing. Plasma was obtained by centrifugation of the samples at 4°C and stored at −70°C until analysis.

Tissue Concentrations of Drugs in Normal Rats Eleven male SD rats were assigned to tissue sampling at 0.5 (GC-1 only), 2 (SKL-compounds only), 4, 8 (SKL-compounds only), and 24 h after drug administration. SKL-12846 (30 mg/kg), SKL-13784 (30 mg/kg), or GC-1 (5.0 mg/kg) was orally given at the indicated doses, using 5% GA suspension vehicle. Blood samples were collected from the jugular vein of each animal with a heparinized syringe under diethyl ether anesthesia. The rats were then killed by cervical dislocation and the brain, liver, and heart immediately dissected out and gently washed with ice-cold saline.

Fructose-Induced Hypertriglyceremic Rats SD rats were divided into 8 groups (n=10): a baseline group fed normal diet (NMF, Oriental Yeast, Japan), a control group fed fructose-enriched diet (F2HFrD, Oriental Yeast, Japan), and 6 groups fed fructose-enriched diet and treated with SKL-13784 at 0.06, 0.2, 0.6, 2, and 6 mg/kg/d or bezafibrate at 60 mg/kg/d as a positive control. SKL-13784 or bezafibrate was administered daily by oral gavage for 4 weeks, using 0.5% CMC-Na suspension vehicle. Fructose feeding was continued throughout the experimental period. After 6 h of the drug treatment on days 14 and 28, blood samples for chemical analyses were collected from the retro-orbital sinus of each animal. After 18 h of blood sampling on day 28, blood samples were collected under diethyl ether anesthesia, then the rats were killed by cervical dislocation and the liver was immediately dissected out and gently washed with ice-cold saline. Total cholesterol (TC) and TG levels were analyzed by enzyme assays, and T₃ and T₄ levels were determined by chemiluminescent immunoassay (CLIA).

Sample Analysis The dissected tissues were homogenized with ice-cold distilled water using a Multi-beads shacker (Yasui Kikai, Osaka, Japan) and were stored at −80°C until analysis. An aliquot (20 µL) of each sample was mixed with 20 µL MeOH (containing the internal standard) and 30 µL acetonitrile, which was vortexed, then centrifuged at 14000 rpm for 5 min. The supernatant was diluted with 10 µL of 50 mM ammonium acetate, and injected into a LC/MS/MS system. Chromatography was performed on reversed-phase high-performance liquid chromatography (HPLC) column (YMC Pack Pro C18, 2.0×50 mm; YMC, Kyoto, Japan) using a NANOSPACE system (Shiseido, Tokyo, Japan). The mobile phases consisted of 5 mM ammonium acetate and acetonitrile with gradients. The mass spectrometer (TSQ7000; Thermo Fisher Scientific, Waltham, MA, U.S.A.) was operated by electrospray ionization in positive ion mode monitoring, and detection of SKL-compounds was achieved in the multiple reaction monitoring mode. The selected reaction monitoring transitions were 381→233 for SKL-12846 and 381→233 for SKL-13784. Peak areas were calculated by Xcalibur® software (Thermo Fisher Scientific).

PK Analysis Plasma concentration versus (vs.) time data of SKL-12846 and SKL-13784 were analyzed by non-compartmental method using Phoenix WinNonlin software (Pharsight, Mountain View, CA, U.S.A.). The maximum concentration (C_max) and time of C_max (T_max) were determined by visual inspection of the observed plasma concentration vs. time profiles. The area under the concentration–time curve (AUC) after i.v. or p.o. administration was calculated by the trapezoidal rule. Total plasma clearance (CL_p) was calculated by dividing the i.v. dose by AUC iv, and the volume of distribution (V_d) was calculated by multiplying the CL_p by the mean residence time. Half-life (t½) of the terminal log-linear phase was calculated as 0.693/elimination rate constant. Oral bioavailability (BA) expressed as a percentage was calculated by taking the ratio of the dose-normalized AUC values after p.o. doses to those after i.v. doses. Tissue-to-plasma concentration ratio was calculated by dividing tissue concentration by the corresponding plasma concentration.

Statistical Analysis Differences among the control group, SKL-13784-treated groups, and bezafibrate-treated groups were analyzed by Student t-test for two groups or by Dunnett’s test for multiple comparisons with a control group. p<0.05 was assumed significant. Data are given as the mean± standard error of the mean (S.E.M.).

RESULTS

PK Parameters and BA PK parameters of SKL-12846 and SKL-13784 in rats are summarized in Table 1. As shown in Fig. 2, both compounds were declined with triphasic response characterized by first two rapid elimination phases, followed by a slow terminal phase after i.v. administration. The CL_p of both compounds was moderate (1.00/L/h/kg for SKL-12846, 1.39/L/h/kg for SKL-13784) compared with hepatic blood flow in rats.²⁴ The T½ of SKL-13784 was approximately 3-fold greater than that of SKL-12846. After an oral dose of 30 mg/kg, SKL-12846 and SKL-13784 were rapidly absorbed with a T_max of 0.33 and 0.50 h to reach a C_max of 4.61 and 5.32 µM, respectively. SKL-13784 achieved a higher C_max of 5.32 µM, while SKL-12846 showed a longer T½ of 6.92 h. Exposure (AUC_0→∞) was slightly greater for SKL-12846...
with a value of 15.8 h·µM. The BA values of SKL-12846 and SKL-13784 were similar (20.8, 22.1%, respectively).

Plasma and Tissue Concentrations Tissue concentrations after single oral dosing with SKL-12846 (30 mg/kg), SKL-13784 (30 mg/kg), or GC-1 (5.0 mg/kg) are summarized in Table 2. For all test compounds, the highest concentrations were observed in the liver (L) followed by plasma (P), heart (H), and brain (B). A high L/H ratio was observed with SKL-compounds with values of more than 100 at all time points, while the maximum L/H ratio for GC-1 was 40.5. Concentrations of SKL-compounds in the brain were considerably low, with SKL-13784 levels below the lower limit of quantitation (BLQ) 4 h after dosing. On the other hand, GC-1 concentration remained relatively constant in the brain at concentrations ranging from 0.24 to 0.34 µM. Consistent with T_{max} data, the concentrations of SKL-compounds gradually decreased in all tissues from 2 to 24 h after administration.

Effects of SKL-13784 on Plasma Lipids and TH in Fructose-Fed Rats Fructose feeding produced a significant increase in plasma TC, TG, and Glu levels compared with normal controls (Fig. 3). Notably, TG levels were approximately 3-fold higher in fructose-fed rats than in normal rats.

### Table 1. Pharmacokinetic Parameters of SKL-12846 and SKL-13784 after Intravenous (1 mg/kg) and Oral (30 mg/kg) Administration in SD Rats

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose, i.v./p.o. (mg/kg)</th>
<th>t_{1/2} (h)</th>
<th>CL_{p} (L/h/kg)</th>
<th>V_{ss} (L/kg)</th>
<th>AUC_{i.v.} (h·µM)</th>
<th>p.o. t_{1/2} (h)</th>
<th>C_{max} (µM)</th>
<th>AUC_{p.o.} (h·µM)</th>
<th>T_{max} (h)</th>
<th>BA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKL-12846</td>
<td>1.0/30</td>
<td>6.76</td>
<td>1.00</td>
<td>2.38</td>
<td>2.48</td>
<td>6.92</td>
<td>4.61</td>
<td>15.8</td>
<td>0.33</td>
<td>20.8</td>
</tr>
<tr>
<td>SKL-13784</td>
<td>1.0/30</td>
<td>9.40</td>
<td>1.39</td>
<td>6.55</td>
<td>1.81</td>
<td>5.16</td>
<td>5.32</td>
<td>12.0</td>
<td>0.50</td>
<td>22.1</td>
</tr>
</tbody>
</table>

a) Male SD rats were acclimated to experimental conditions 7–14d before use, and had free access to food and water throughout the acclimatization period. The animals were fasted overnight, and SKL-13784 or SKL-12846 was administered intravenously via the tail vein or orally (by gavage) at the indicated doses (n=2–3) as a solution in saline and 5%GA. Blood samples were taken periodically and the plasma was analyzed by LC-MS-MS with quantitation against a standard curve. AUC, area under the plasma concentration curve; BA, bioavailability; CL_{p}, plasma clearance; C_{max}, maximum plasma concentration; t_{1/2}, half-life; T_{max}, time to reach C_{max}; V_{ss}, steady-state volume of distribution. Values represent the mean (n=2–3).

### Table 2. Plasma and Tissue Concentrations of SKL-Compounds and GC-1 after Oral Administration in SD Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SKL-13784 (30 mg/kg)</th>
<th>SKL-12846 (30 mg/kg)</th>
<th>GC-1 (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (h)</td>
<td>Time (h)</td>
<td>Time (h)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Plasma (P) (µM)</td>
<td>0.455</td>
<td>0.335</td>
<td>0.186</td>
</tr>
<tr>
<td>Liver (L) (µM)</td>
<td>19.7</td>
<td>16.0</td>
<td>4.60</td>
</tr>
<tr>
<td>Heart (H) (µM)</td>
<td>0.110</td>
<td>0.0604</td>
<td>0.0454</td>
</tr>
<tr>
<td>Brain (B) (µM)</td>
<td>0.0143</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
</tbody>
</table>

a) Male SD rats were acclimated to experimental conditions 7–14d before use, and had free access to food and water throughout the acclimatization period. The animals were fasted overnight, and SKL-13784 or SKL-12846 was administered orally (by gavage) at the indicated doses (n=1) as a solution in 5%GA. Blood samples were taken periodically and the plasma was analyzed by LC-MS-MS with quantitation against a standard curve. b) Not determined. c) Below the low limit of quantitation (LLOQ was 0.0124 µM).
controls, reaching $240\pm37.9\text{mg/dL}$ after 2 weeks' feeding. Bezafibrate at a dose of $60\text{mg/kg}$ daily produced significant reduction in TC and TG levels, but had no effect on Glu levels, compared with treated controls. More strikingly, treatment with SKL-13784 dose-dependently decreased plasma and hepatic TG levels, achieving normal plasma and hepatic TG levels at the dose of 2.0 and $6.0\text{mg/kg}$ daily, respectively, after 4 weeks' treatment. SKL-13784 at the doses of 2.0 and $6.0\text{mg/kg}$ significantly reduced Glu levels, but had no effect on TC levels even at the highest dose. In general, these effects of SKL-13784 were more pronounced after 4 weeks' treatment than after 2 weeks. Treatment with bezafibrate significantly decreased plasma $T_3$ levels, whereas SKL-13784 produced a dose-dependent decrease in plasma $T_4$ levels with significant effect at doses below its TG-lowering dose range. Measurement of plasma and liver SKL-13784 levels 24h after the final treatment revealed an average liver-to-plasma concentration ratio ($K_p$) of 55, which was greater than expected from the tissue distribution study (Table 3).

Table 3. Plasma Concentrations of SKL-13784 in Fructose-Fed Rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Plasma (nM)</th>
<th>Liver (nM)</th>
<th>$K_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>$1.60\pm0.38$</td>
<td>$112\pm33$</td>
<td>70</td>
</tr>
<tr>
<td>2.0</td>
<td>$2.50\pm0.90$</td>
<td>$141\pm29$</td>
<td>56</td>
</tr>
<tr>
<td>6.0</td>
<td>$8.13\pm3.20$</td>
<td>$314\pm100$</td>
<td>39</td>
</tr>
</tbody>
</table>

Values are the mean±S.E.M. ($n=5$). After 24h of the final drug treatment, blood was collected from the inferior vena cava and analyzed.
DISCUSSION

Oral administration of SKL-compounds resulted in very rapid absorption and considerably smaller systemic exposure (C_{\text{max}} and AUC) than generally expected from a dose of 30 mg/kg (Table 1). Moderate CL_p and V_s values suggest that the short exposure was at least partly due to first liver uptake. Especially, considering the moderate TR\/β agonist potency of SKL-13784 (50% effective concentration (EC_{50}) for TR\/β transcriptional activity in HepG2 cells: 0.22 \mu m for SKL-12846; 1.3 \mu m for SKL-13784), it is hard to explain from the present data that oral administration at the dose of 30 mg/kg once daily can produce excellent cholesterol-lowering effects in cholesterol-fed rats. Additionally, the observed triphasic decline in plasma SKL-compounds following i.v. administration suggests the presence of a tissue compartment responsible for selective partitioning of SKL-compounds. These findings indicate a marked tissue distribution in the liver for SKL-compounds.

Our investigation of SKL-compounds and GC-1 tissue concentrations revealed an L/H ratio following treatment with GC-1 of 40.5 and 24.8 at 0.5 and 4 h after dosing, respectively (Table 2). These values seem to be greater than previously reported (L/H ratio of about 17 at 1 h after i.v. administration in SD rats).{^{15}} The discrepancy between these results is probably due to differences in both the route of administration and sampling time. To rule out influence of administration route, we used oral administration in our experiment. SKL-compounds showed greater L/H ratios than GC-1. This large L/H ratio would explain that SKL-compounds did not increase heart rate and heart weight, a sensitive cardiac toxicity index, even at a 5-fold higher cholesterol-lowering dose in rats,{^{22}} because the diminished effect of GC-1 on heart rate is thought partly to derive from its L/H ratio.{^{15}} As for L/B ratio, we found a more pronounced difference between SKL-compounds and GC-1. This may derive from difference in capability of first liver uptake, because all compounds showed poor brain penetration. In other words, it is speculated that SKL-compounds underwent aggressive liver uptake, whereby their circulating levels remained low. Subsequently, concentrations of SKL-compounds in brain were negligible thanks to poor brain penetration. Collectively, SKL-compounds’ large L/B ratio may explain their ability to lower cholesterol levels without suppressing TSH. It should be noted that elimination of GC-1 was found to be slow in the brain, which may lead to a narrow margin for TSH suppression in rats.

Fructose-feeding is known to increase secretion of very-low-density lipoproteins (VLDL) TG, resulting in hypertriglyceridemia in rats.{^{25}} TH directly and indirectly reduces low-density lipoproteins (VLDL) TG, resulting in hypertriglyceridemia in rats. {^{25}} TH directly and indirectly reduces low-density lipoproteins (VLDL) TG, resulting in hypertriglyceridemia in rats. Therefore, it is suggested that this glucose lowering may involve other mechanisms, although no comparison between body weight and Glu levels was made to support this hypothesis.

Given SKL-13784 tissue distribution in normal rats, brain concentration of this compound in fructose-fed rats was expected to be negligible (Table 3). Consistent with this, no escape phenomenon was observed with all doses of SKL-13784, suggesting that SKL-13784 did not produce TSH suppression. Nonetheless, SKL-13784 dose-dependently reduced total T_4 levels with significant effect at doses below its TG-lowering dose, but had no effect on total T_3 levels (Figs. 3D,E). One plausible explanation is provided in previously reported results that the liver-targeted TR agonist MB07811 is metabolized to an active form MB07344 predominantly in the liver by CYP3A. As with SKL-13784, MB07811 reduces plasma lipid and T_4 levels in DIO mice without affecting T_3 levels or TSH. The concomitant reduction in T_4 levels is thought due to enhanced metabolism of endogenous T_4, because MB07811 increases expression of hepatic type 1 deiodinase (DII), which metabolizes T_4 to T_3, reverse T_3, and 3,3’-diiodothyronine (T_3D3, E). One plausible explanation is provided in previously reported results that the liver-targeted TR agonist MB07811 is metabolized to an active form MB07344 predominantly in the liver by CYP3A. As with SKL-13784, MB07811 reduces plasma lipid and T_4 levels in DIO mice without affecting T_3 levels or TSH. The concomitant reduction in T_4 levels is thought due to enhanced metabolism of endogenous T_4, because MB07811 increases expression of hepatic type 1 deiodinase (DII), which metabolizes T_4 to T_3, reverse T_3, and 3,3’-diiodothyronine (T_3D3, E). These results indicate that TR\/β plays a critical role in the regulation of TH metabolism in the liver. It is however unknown to what extent altered TH metabolism has an impact on the potential usefulness of liver-selective TR\/β agonists. Further long-term studies would provide useful information.

In conclusion, the present study explains why SKL-12846 and SKL-13784 have beneficial effects on lipid without affecting heart rate and TSH production at therapeutic doses in cholesterol-fed rats. In addition, we found that SKL-13784 shows no sign of escape phenomenon in fructose-fed rats. These results demonstrate the advantages of extremely high liver specificity to TR\/β agonists. However, as with MB07811, SKL-13784 significantly reduces endogenous T_4 levels at doses lower than its lipid-lowering dose, which may raise concerns over this compound’s ability to alter TH metabolism in the liver. While the mechanism by which SKL-13784 reduces endogenous T_4 levels is still unclear, our results in this study would help design better liver-selective TR\/β modulators.

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