Title:
Quantitative Determination of Absorption and First-Pass Metabolism of Apicidin, a Potent Histone Deacetylase Inhibitor

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Abbreviations:
$F_X$, fraction absorbed; $F_G$, fraction that is not metabolized in the gut wall; $F_H$, hepatic bioavailability; $F_L$, pulmonary bioavailability; P-gp, P-glycoprotein; LC, liquid chromatography; MS/MS, tandem mass spectrometry; HDACs, histone deacetylases; AUC, area under the time-concentration curve; CL, clearance.
Apicidin is a potential oral chemotherapeutic agent, which possesses potent anti-histone deacetylases activity. Following oral administration, the total bioavailability of apicidin was known to be low (14.2 - 19.3%). In the present study, the factors contributing to the low bioavailability of apicidin were evaluated by quantitative determination of absorption fraction and first pass metabolism after oral administration. Apicidin was given to rats by five different routes, i.e., intra-femoral vein, duodenum, superior mesenteric artery, portal vein, and carotid artery. Especially, the fraction absorbed ($F_X$) and the fraction that is not metabolized in the gut wall ($F_G$) were separated by injection of apicidin via superior mesenteric artery, which enables to bypass the permeability barrier. The $F_X$ was 45.9 ± 9.7%, the $F_G$ was 70.9 ± 8.1% and the hepatic bioavailability ($F_H$) was 70.6 ± 12.3%, while the pulmonary first-pass metabolism was minimal ($F_L = 102.8 ± 7.4$%), indicating that intestinal absorption was the rate determining step for oral absorption of apicidin. The low $F_X$ was further examined in terms of passive diffusion and transporter mediated efflux by in vitro immobilized artificial membrane (IAM) chromatographic assay and in situ single pass perfusion method, respectively. While the passive diffusion potential of apicidin was high (98.01%) by the IAM assay, the in situ permeability was significantly enhanced by the presence of a P-glycoprotein (P-gp) inhibitor, elacridar. These data suggest that the low bioavailability of apicidin was mainly attributed to the P-gp efflux consistent with the limited $F_X$ measured in vivo experiment.
INTRODUCTION

Inhibitors of histone deacetylases (HDACs) have been identified as a novel class of chemotherapeutics for the treatment of various cancers. To date, two of the HDAC inhibitors, Vorinostat (Merck&Co., Inc) and Romidepsin (Gloucester Pharmaceutical Inc.) have been approved by the FDA for the treatment of cutaneous T-cell lymphoma. More than fifteen HDAC inhibitors including Panobinostat (LBH589), Entinostat (MS-275), Belinostat (PXD101), Givinostat (ITF2357), Mocetinostat (MGCD0103), and PCK-24781 have been tested in preclinical and clinical studies (Tan et al., 2010; Kim and Bae, 2011). Currently, 104 clinical studies involving HDAC inhibitors have been completed and over 100 trials are ongoing to develop these inhibitors as monotherapy or in combination with other anti-cancer agents. This mechanism of cancer treatment has been recognized as a promising new direction in developing novel anticancer agents. Moreover, some of these HDAC inhibitors are orally available, which is of particular merit in the new drug development.

Apicidin is one of the HDAC inhibitors which has potential to be developed as an oral chemotherapeutic agent with potent anti-proliferative and cyto-differentiation activities (Darkin-Rattray et al., 1996). It has been shown to exhibit anti-tumor effects in several human cancer cell lines, including leukemia (Kwon et al., 2002), ovarian (Ahn et al., 2012), endometrial (Ahn et al., 2010), oral squamous (Ahn et al., 2011), cervical (Han et al., 2000; Luczak and Jagodzinski, 2008), and breast (Han et al., 2000) cancer cell lines in vitro and in vivo (Lai et al., 2009). The therapeutic potential of apicidin also appears promising in combination therapy with other agents such as docetaxel (Buoncervello et al., 2012), doxorubicin (Lai et al., 2009), and TNF-related apoptosis-inducing ligand (Park et al., 2009). However, only a few reports are available about the pharmacokinetic characteristics of apicidin. In our previous study, the oral bioavailability of apicidin was found to be 19.3 and 14.2% in fasting and non-fasting rats, respectively (Shin et al., 2006). A fundamental understanding of the absorption processes as
well as the factors responsible for the relatively low oral bioavailability is critical for apicidin to be developed as an orally available chemotherapeutic agent.

The absorption process can be affected by a number of factors such as solubility, permeability, transporters, and metabolism. After oral administration, a drug must pass through the gastrointestinal lumen, transit through the gut wall, and withstand the initial degradation by intestinal mucosal enzymes. It may be metabolized while penetrating through the gut wall and the drug that reaches the portal vein may be affected by hepatic metabolism or biliary extraction. The portion escaping the hepatic elimination may also be subject to extra-hepatic metabolism. Moreover, efflux transporters and drug metabolizing enzymes often work together for xenobiotics, which may confound the interpretation of the in vivo experiments. The relative contributory roles of these factors leading to the poor bioavailability of apicidin are still unclear. Especially, to distinguish between the contributions of gut wall absorption and first pass metabolism, which compose the gastrointestinal bioavailability, will provide useful information for the development of novel apicidin analogues with improved bioavailability. Nevertheless, experimental separation of the fraction absorbed, i.e., net transport of unchanged drug into the gastrointestinal tract \((F_X)\), and fraction that is not metabolized through the gut wall \((F_D)\) in vivo is not easily achieved, and most literature reported them together as gastrointestinal bioavailability \((F_X \cdot F_D)\) (Raoof et al., 1996; Hashimoto et al., 1998; Mihara et al., 2001; Choi et al., 2006; Hanada et al., 2008; Bae et al., 2009; Gertz et al., 2011).

The present study was aimed to quantitatively evaluate the contributory roles of the absorption process and first-pass metabolism of apicidin to its oral bioavailability after oral administration. We administered apicidin by various routes, including the femoral vein, duodenum, superior mesentery artery, portal vein, and carotid artery to distinguish the fractions absorbed through the gut wall and metabolized at different sites after oral administration. Particularly, superior mesenteric arterial injection of apicidin allowed us to determine the in vivo
gastrointestinal absorption and gut wall first-pass metabolism separately. To best of our knowledge, this is the first report of the \textit{in vivo} approach for the quantitative determination of $F_X$ and $F_G$ by comparing AUC obtained by different routes of administration. \textit{In vitro} and \textit{in situ} studies were also conducted to confirm the results from the \textit{in vivo} experiment and further assess the factors contributing to the \textit{in vivo} absorption such as passive diffusion, efflux by P-gp and gastrointestinal stability.
MATERIALS AND METHODS

Chemicals and reagents

Apicidin was prepared from *Fusarium sp.* strain KCTC 16677 according to a method described previously (Park et al., 1999). HPLC grade acetonitrile, ethanol and t-butyl methyl ether were purchased from J.T. Baker (Phillipsburg, NJ, USA). The other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Sprague-Dawley rats (8 – 10 weeks old, body weight 220 – 272 g) were kept in plastic cages with free access to standard rat diet (Samyang, Seoul, Korea) and water. The animals were maintained at a temperature of 22 – 24°C with a 12 hr light-dark cycle and relative humidity of 50 ± 10% before the experiment. All animal studies were approved by the ethics committee for the treatment of laboratory animals at Sungkyunkwan University and Catholic University of Daegu.

Determination of passive absorption potential by immobilized artificial membrane (IAM) chromatographic assay

The absorption potential of apicidin by passive diffusion was evaluated by the immobilized artificial membrane (IAM) chromatographic assay described previously (Yoon et al., 2004). Briefly, HPLC analysis was performed using a Waters 2695 separations module and Waters 2996 photo diode array detector (Waters, MA, USA). The retention time of apicidin (t_r) and the hold-up time of the column (t_0) were determined using the IAM.PC.DD 2 Drug-Discovery column (Regis, IL, USA). The isocratic mobile phase consisted of acetonitrile and Dulbecco’s phosphate buffered saline (DPBS) (80:20 v/v). The flow rate of the mobile phase was 1.0 mL/min and the column oven temperature was 37°C. Apicidin was detected at 210 nm. The IAM
capacity factors ($K_{IAM}$) were determined for 28 commercial drugs and the obtained $K_{IAM}$ was modified by the power of the compound molecular weight ($K_{IAM} \cdot MW^n$) to improve the correlation between $K_{IAM}$ and dose absorbed in humans ($F_a$). The human $F_a$ was obtained from literature (van de Waterbeemd et al., 2003; Sugano et al., 2001; Zhu et al., 2002; Worland et al., 1984). The relationship between modified $K_{IAM}$ ($K_{IAM} \cdot MW^n$) and $F_a$ was best described by the following sigmoid equation:

$$F_a(\%) = 98.01 \times (1 - e^{-4.306 \times \frac{K_{IAM}}{MW^{0.30}}} )^{3.00} \quad \text{(Eq. 1)}$$

where $K_{IAM}$ is the IAM capacity factor and $MW$ is the molecular weight of the compound. The $F_a$ for apicidin by passive diffusion was calculated using the above equation.

**Determination of intestinal permeability by single pass perfusion**

The effect of P-glycoprotein (P-gp) on the intestinal absorption of apicidin was evaluated by means of a single pass perfusion method in the absence or presence of the P-gp inhibitor elacridar. After overnight fasting, the rats were anaesthetized by intraperitoneal injection of Zoletil 50® (20 mg/kg, Virbac Laboratories, Carros, France). The abdomen was opened and the intestinal segment of the duodenum (10 cm length from the end of the stomach) was isolated and cannulated at both ends with a silicone tube (2 mm i.d. Daihan Scientific Co., Wonjoo, Korea). The duodenal segment was rinsed with 37°C saline to clear the segment before perfusion. Perfusate was prepared by dissolving apicidin at a concentration of 10 μM in normal saline in the absence or presence of elacridar (1 μM). The perfusion rate was 0.2 mL/min, and the outlet perfusates were collected at 15 min intervals from 50 min to 120 min after initiation of infusion. Collected samples were stored at -20°C until analysis. To maintain anesthesia, Zoletil 50® was administered via the intraperitoneal route every 30 min and the animal was placed on a heating pad to maintain the body temperature. The abdominal incision area was stapled to
prevent loss of fluid and hypothermia. The effective permeability \( P_{\text{eff}} \) was calculated by the single pass perfusion approach as below:

\[
P_{\text{eff}} = -Q_{\text{in}} \times \ln\left(\frac{C_{\text{out}}}{C_{\text{in}}}\right)2\pi RL
\]

(Eq. 2)

where \( C_{\text{in}} \) and \( C_{\text{out}} \) are the inlet and outlet concentrations at steady state, \( Q_{\text{in}} \) is the perfusion rate, \( R \) and \( L \) are the intestinal radius (1.375 mm) and length (10 cm), respectively, and \( 2\pi RL \) represents the available area for the membrane permeability (Kararli, 1995; Liao et al., 2005).

**Stability of apicidin in artificial gastric and intestinal juices**

Simulated gastric juice was prepared by dissolving 2 mg of sodium chloride and 35% hydrochloric acid in distilled water to a total volume of 1 L. Simulated intestinal juice was prepared by mixing 250 mL of 0.2 mol/L potassium phosphate and 118 mL of 0.2 mol/L sodium hydroxide in distilled water to a total volume of 1 L. Portions of the simulated gastric and intestinal juices (900 \( \mu \)L each) were pre-incubated in a water bath for 5 min at 37°C, and then 100 \( \mu \)L of apicidin dissolved in DMSO (100 g/mL) was added to each simulated gastric juice and intestinal juices, separately. Samples were taken at 15, 30, 60, and 120 min and kept at -20°C until analysis.

**In vivo pharmacokinetic study**

The rats were anaesthetized by intraperitoneal injection of ketamine and xylazine (90:10 mg/kg) and cannulated with a polyethylene (PE) tubing (0.58 mm i.d., 0.96 mm o.d., Natsume, Tokyo, Japan) in the right jugular vein and the intended administration routes of femoral vein, duodenum, superior mesenteric artery, portal vein, or carotid artery. Apicidin was dissolved in DMSO:PEG400:isotonic saline mixture (15:65:20 v/v) and injected into the different administration sites.
The femoral vein was cannulated in rats receiving i.v. bolus injection. Apicidin was injected at a dose of 2 mg/kg (1 mL/kg) into the femoral vein (n = 4 rats). An abdominal incision was also made in these rats to preserve the same experimental conditions as in the other groups.

For intra-duodenal administration, rats were anaesthetized and cannulated after 12 hr fasting. The abdomen was opened, and a PE tubing (0.58 mm i.d., 0.96 mm o.d., Natsume, Tokyo, Japan) was inserted through the stomach with the end of the PE tubing located at the duodenum. To prevent reflux of the administered apicidin, the pylorus was ligated. Apicidin was then injected at doses of 5 and 10 mg/kg (1, 2 mL/kg) via the duodenal cannula (n = 4 and 5, respectively).

For superior mesenteric arterial injection, an abdominal incision was made to expose the superior mesenteric artery. A 29-gauge needle bent at 60° angle attached to a PE tubing (0.28 mm i.d., 0.61 mm o.d., Natsume, Tokyo, Japan) was inserted into the superior mesenteric artery. Epoxy glue (Krazy Glue, Krazy Glue, IL, USA) was applied to prevent bleeding. Apicidin was injected at a dose of 2 mg/kg (1 mL/kg) into the superior mesenteric artery (n = 5).

For portal venous injection, an abdominal incision was made to expose the portal vein. A PE tube (0.4 mm i.d., 0.8 mm o.d., Natsume, Tokyo, Japan) was inserted into the portal vein. Epoxy glue (Krazy Glue, Krazy Glue, IL, USA) was applied to prevent bleeding. Apicidin was injected at a dose of 2 mg/kg (1 mL/kg) into the portal vein (n = 5).

For intra-arterial injection, the left carotid artery was cannulated and apicidin was injected at a dose of 2 mg/kg (1 mL/kg, n = 6). An abdominal incision was made to preserve the same experimental conditions as in the other experiments.

Venous blood samples were collected prior to and at 5, 10, 15, 30 min, 1, 1.5, 2, 3, 4, and 6 hr after injection of apicidin. All experiments were performed under anaesthetized conditions. To maintain anesthesia, ketamine and xylazine (22.5:2.5 mg/kg) were administered via the intra-peritoneal route every 1 hr after initial anesthesia and the animal was placed on a
heating pad to maintain a body temperature. The abdominal incision area was stapled to prevent loss of fluid and hypothermia. Serum samples were obtained by centrifugation of the blood samples at 1,500 \( \times g \) for 10 min and were stored at -20°C until analysis.

**Quantification of apicidin and its metabolites by LC-MS/MS**

Apicidin and its metabolites, demethylated apicidin, hydroxylated apicidin, di-hydroxylated apicidin, glucuronidated apicidin, glucuronidated monohydroxyapicidin, and glucuronidated dihydroxyapicidin, were assayed by a modification of the previously reported LC-MS/MS method (Shin et al., 2005). Briefly, 100 \( \mu L \) of the sample was mixed with 100 \( \mu L \) of the internal standard solution (trazodone 100 ng/mL in methanol) and precipitated by addition of 800 \( \mu L \) of the mobile phase (acetonitrile:water containing 10 mM ammonium acetate = 85:15 v/v). The samples were mixed on a vortex mixer for 10 min and centrifuged at 2840 \( \times g \) for 10 min. An aliquot of the supernatant (10 \( \mu L \)) was then injected into the LC-MS/MS.

HPLC analysis was performed using a Shimadzu 10Avp HPLC system (Kyoto, Japan) consisting of SCL-10Avp system controller, LC-10ADvp pump, SIL-10ADvp auto sampler, CTO-10Avp column oven, and DGU-14A degasser. Analytes were separated on a Gemini C\(_{18}\) column (150 x 2.0 mm i.d., 5 \( \mu m \)) with a SecurityGuard column (4 x 2.0 mm i.d.) (Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisted of acetonitrile and 10 mM ammonium acetate in water (85:15 v/v). The flow rate of the mobile phase was 0.2 mL/min and the column oven temperature was 35°C. The total run time was 4 min.

The HPLC system was coupled to an API 2000 triple-quadrupole mass spectrometer equipped with turbo ion spray ionization (ESI) source (AB MDS Sciex, Toronto, Canada). The ESI source was operated in a positive mode. The selected MRM transitions of the precursors to the product ion were 624.5 \( \rightarrow 84.2 \) for apicidin, 610.5 \( \rightarrow 84.2 \) for demethylated apicidin, 640.5


Pharmacokinetic analysis

The serum concentration of apicidin vs. time data were analyzed by the noncompartmental method using WinNonlin (Pharsight, Cary, NC, USA). The absolute oral bioavailability ($F$) was calculated by the following equation:

$$
F = \frac{D_{po}}{AUC_{po}} \cdot \frac{AUC_{po}}{AUC_{iv}}
$$

(Eq. 3)

The fraction absorbed ($F_X$) was calculated as follow:

$$
F_X = \frac{D_{mesentary artery}}{D_{po}} \cdot \frac{AUC_{po}}{AUC_{mesentary artery}}
$$

(Eq. 4)

Fractions of the administered dose that escaped the first-pass metabolism by gut wall ($F_G$), liver ($F_H$), and lung ($F_L$) were calculated as follows:

$$
F_G = \frac{D_{portal vein}}{D_{mesentary artery}} \cdot \frac{AUC_{mesentary artery}}{AUC_{portal vein}}
$$

(Eq. 5)

$$
F_H = \frac{D_{iv}}{D_{portal vein}} \cdot \frac{AUC_{portal vein}}{AUC_{iv}}
$$

(Eq. 6)

$$
F_L = \frac{D_{ia}}{D_{iv}} \cdot \frac{AUC_{iv}}{AUC_{ia}}
$$

(Eq. 7)

where $AUC$ is the area under the serum apicidin concentration vs. time curve from time zero to infinity and $D$ is the administered dose. The subscript refers to the route of administration.

Statistical analysis

The obtained parameters were compared by unpaired $t$-test between the two means for
unpaired data or one-way ANOVA followed by Scheffe’s post hoc test among more than two means for unpaired data. P values <0.05 were considered as statistically significant.
RESULTS

Quantification of apicidin and its metabolites in biological fluids by LC-MS/MS

The LC-MS/MS method used to determine apicidin concentrations was optimized and applied to analyze apicidin and its metabolites in various biological fluids. The biological samples were pretreated with one-step protein precipitation, and the assay was validated over a linear concentration range from 2 – 1000 ng/mL.

After the rats were given apicidin, apicidin and its metabolites including demethylated apicidin and hydroxylated apicidin were detected in serum samples. The retention times of apicidin, demethylated apicidin, hydroxylated apicidin, and dihydroxylated apicidin were 2.81, 2.63, 2.34, and 2.07 min, respectively. None of the potential phase II metabolites, e.g., glucuronidated apicidin, glucuronidated monohydroxylapicidin, and glucuronidated dihydroxyapicidin were detected in serum samples.

Determination of passive absorption potential of apicidin using IAM chromatography

The relationship between the fraction of dose absorbed in humans (Fa%) and the IAM capacity factors (KIAM) of 28 commercial drugs is shown in Figure 1. Based on this relationship, the equation which can be used to predict the passive absorption potential was derived with excellent correlation (Eq. 1, r=0.958). The passive absorption potential of apicidin was then calculated from this equation. Apicidin was highly retained on the amphiphilic phospholipids covalently bound to aminopropyl silica particles on IAM (tᵣ=190.7 min), suggesting that it would strongly interact with the cell membrane and demonstrate a high intestinal permeability. Based on the retention time, the IAM capacity factor of apicidin corrected by the power of the compound molecular weight was calculated (KIAM/MW².05=32.22) and the fraction of dose absorbed in humans by passive diffusion (Fa%) was predicted as 98.01%, indicating that the passive diffusion mediated permeability of apicidin would be very high.
Determination of intestinal permeability of apicidin using single pass perfusion

The role of P-gp on the intestinal permeability of apicidin was evaluated by in situ single pass intestinal perfusion. The effective permeability ($P_{\text{eff}}$) of apicidin through the duodenum was compared in the presence or absence of a potent P-gp inhibitor, elacridar. The $P_{\text{eff}}$ of apicidin in the duodenum in the presence of 1 μM of elacridar was significantly higher than that in the absence of elacridar ($17.83 \pm 4.68 \times 10^{-5}$ vs. $5.08 \pm 4.11 \times 10^{-5}$ cm/s, $p<0.05$). This finding suggests that apicidin is a substrate of P-gp and that P-gp may play a significant role in the intestinal permeability of apicidin.

Stability of apicidin in simulated gastric and intestinal juices

The stability of apicidin in gastric and intestinal fluids was examined by monitoring changes in apicidin concentration during 2 hr-incubation in the simulated gastric and intestinal juices. Apicidin was found to be stable (99.5 ± 3.4%) in the simulated intestinal juice over the 2-hr incubation period. Apicidin was also stable in the simulated gastric juice (pH = 1.2) for 1 hr but its concentration was decreased to 85.0% at the end of the incubation.

Pharmacokinetics of apicidin and its metabolites in anesthetized rats after i.v. bolus injection

The average concentration of apicidin, demethylated apicidin, and hydroxylated apicidin vs. time profiles after a single i.v. bolus injection of apicidin at 2 mg/kg in anesthetized rats are shown in Figure 2. The serum apicidin concentration vs. time profile shows multi-exponential decline. Both demethylated apicidin and hydroxylated apicidin were detected from the first blood sampling time (5 min) after i.v. administration of apicidin. While demethylated apicidin concentrations decreased in parallel to apicidin concentrations, hydroxylated apicidin...
concentrations increased initially, reached a maximum concentration at approximately 0.6 hr and decreased at a slower rate (Figure 2).

The noncompartmental pharmacokinetic parameters of apicidin, demethylated apicidin, and hydroxylated apicidin obtained after i.v. injection of apicidin are shown in Table 1. The average terminal elimination half-life \( (t_{1/2}) \) of demethylated apicidin \( (1.8 \pm 0.3 \text{ hr}) \) was comparable to that of apicidin \( (1.9 \pm 0.2 \text{ hr}) \). The serum concentrations of demethylated apicidin extrapolated to time zero \( (C_0) \) and the \( AUC \) were 8.4% and 8.6% of the corresponding values found for apicidin. The \( t_{1/2} \) of hydroxylated apicidin \( (2.6 \pm 0.4 \text{ hr}) \) was significantly longer than that of apicidin, indicating that the elimination rate of hydroxylated apicidin was slower than the metabolite formation rate.

Pharmacokinetics of apicidin and metabolites in anesthetized rats after intraduodenal administration

The average apicidin and metabolite concentration vs. time profiles obtained after intraduodenal administration of apicidin at doses of 5 and 10 mg/kg in anesthetized rats are shown in Figure 3. Apicidin was detected in serum from the first blood sampling time (5 min) and reached its maximum concentration within 1 hr in both groups of rats. The concentration vs. time profiles of demethylated apicidin were in parallel with those of apicidin. However, the decrease in serum hydroxylated apicidin concentrations appeared to be slower than that of apicidin; this observation was similar to what was found after i.v. injection.

The noncompartmental pharmacokinetic parameters of apicidin, demethylated apicidin, and hydroxylated apicidin obtained after intraduodenal administration of apicidin at doses of 5 and 10 mg/kg are summarized in Table 2. The maximum serum concentration \( (C_{\text{max}}) \) and the \( AUC \) of apicidin increased as the dose was increased. The observed \( C_{\text{max}} \) values were 341.6 \pm 85.2 and 924.4 \pm 121.9 \text{ ng/mL} \) and the \( AUCs \) were 725.7 \pm 152.5 and 2,110.5 \pm 397.2 \text{ ng hr/mL} \).
after intraduodenal administration of 5 and 10 mg/kg, respectively. The $t_{1/2}$ of apicidin after intraduodenal administration was not significantly different from that obtained after i.v. injection (Tables 1-2). The systemic clearances after intraduodenal administration of apicidin ($CL_d/F$) at doses of 5 or 10 mg/kg were $119.0 \pm 26.6$ and $81.1 \pm 14.4$ mL/min/kg, respectively. The systemic clearance corrected by oral bioavailability ($CL_s$) was not significantly different between the 5 and 10 mg/kg doses ($27.4$ vs. $27.1$ mL/min/kg), and these values were comparable to that obtained after i.v. injection ($26.5 \pm 1.6$ mL/min/kg). The average absolute oral bioavailability of apicidin were $23.0 \pm 4.8\%$ and $33.4 \pm 6.3\%$ at 5 and 10 mg/kg doses, respectively.

At both doses, serum concentrations of demethylated apicidin decreased in parallel with those of apicidin and the $t_{1/2}$ was comparable between apicidin and demethylated apicidin. In contrast, the $t_{1/2}$ of hydroxylated apicidin at 5 and 10 mg/kg doses ($2.3 \pm 0.7$ and $3.1 \pm 0.9$ hr, respectively) were longer than those of apicidin ($1.3 \pm 0.4$ and $1.8 \pm 0.7$ hr) and demethylated apicidin ($1.4 \pm 0.3$ and $2.0 \pm 0.6$ hr). The $T_{max}$ of hydroxylated apicidin ($1.1 \pm 0.3$ and $1.1 \pm 0.2$ hr for 5 and 10 mg/kg dose, respectively) occurred later than those of apicidin and demethylated apicidin. The $AUC$s of demethylated and hydroxylated apicidin obtained after intraduodenal administration of 5 mg/kg dose were $96.3 \pm 13.6$ ng/mL and $199.2 \pm 33.2$ ng·hr/mL, respectively, and they were 13.3% and 27.5% of apicidin, respectively. The $AUC$s of demethylated and hydroxylated apicidin after intraduodenal admininstration of 10 mg/kg dose were $294.7 \pm 68.2$ ng/mL and $576.2 \pm 182.0$ ng·hr/mL, respectively, and they were 14.0% and 27.3% of apicidin, respectively, which are significantly greater than those obtained after i.v. injection.

**Pharmacokinetics of apicidin in anesthetized rats after superior mesenteric arterial, portal venous, and carotid arterial injection**

The mean apicidin concentration vs. time profiles obtained after superior mesenteric arterial, portal venous, and intra-arterial (left carotid arterial) injection of apicidin at a dose of 2
mg/kg in anesthetized rats in comparison with that after i.v. injection are shown in Figure 4. The serum concentrations of apicidin following different routes of administration declined in parallel with that after i.v. injection. The noncompartmental pharmacokinetic parameters of apicidin obtained after portal venous, superior mesenteric arterial, and intra-arterial injection of apicidin at a dose of 2 mg/kg are shown in Table 3.

The $t_{1/2}$ obtained after superior mesenteric arterial, portal venous, and arterial injection (1.4 ± 0.5, 1.9 ± 0.3, 1.3 ± 0.4 hr, respectively) were all comparable with that found after i.v. injection (1.9 ± 0.2 hr). However, there were significant differences between AUC values after intra-duodenal vs. superior mesenteric arterial injection, superior mesenteric arterial vs. portal venous injection, and portal venous vs. i.v. injection. The $AUC$ of apicidin obtained after superior mesenteric arterial injection (891.1 ± 155.3 ng/hr/mL) was significantly lower than that found after portal venous injection. The $C_0$ and $AUC$ of apicidin obtained portal venous injection was significantly lower than those obtained after i.v. injection. The pharmacokinetic parameters obtained after intra-arterial injection of apicidin were not significantly different from those after i.v. injection.

**Determination of the gastrointestinal, hepatic, and pulmonary first-pass metabolism and overall bioavailability**

Table 4 summarizes the estimation of total ($F_{total}$), absorption ($F_A$), gut wall ($F_G$), hepatic ($F_H$), and pulmonary ($F_L$) bioavailability and the first-pass elimination fraction of apicidin. The calculated fraction absorbed ($F_A$) was 45.9 ± 9.7%. The calculated fraction not metabolized in the single passage through the gut wall was 70.9 ± 8.1%, i.e. 29.1% of the permeated apicidin through gut wall was metabolized by the gut wall first-pass extraction. The calculated hepatic bioavailability was 70.6 ± 12.3%. Conversely, 29.4% of apicidin given into the portal vein was eliminated by the liver first-pass metabolism. There was no significant difference in $AUC$
between two different dosing routes, indicating that the pulmonary first-pass metabolism was negligible \( F_L = 102.8 \pm 7.4\% \).

Taken together, 45.9\% of the administered apicidin was absorbed into enterocytes, and 29.1\% of the absorbed apicidin was metabolized by the gut wall first-pass extraction upon duodenal administration. Only 32.5\% of the administered dose reached the portal vein, and 29.4\% of that was extracted by the liver first-pass metabolism while the rest (70.6\%) escaped the hepatic metabolism. As a consequence, the overall bioavailability was calculated to be 23.0\% \( (0.459 \times 0.709 \times 0.706 \times 100\%) \).
DISCUSSION

This study examined the pharmacokinetics of apicidin and quantitatively determined the factors contributing to its oral bioavailability, including the absorption and first-pass metabolism at different stages of the oral absorption. Administering apicidin into different sites, i.e., intra-femoral vein, -duodenum, -superior mesenteric artery, -portal vein, and -carotid artery allowed estimation of the fraction absorbed and first-pass metabolism in the gut, liver, and lung in vivo (Figure 5).

The gastrointestinal bioavailability \( (F_X F_G) \) consists of the fraction absorbed \( (F_X) \) and the fraction that is not metabolized during passage through the gut wall \( (F_G) \). However, few in vivo experimental strategies are available to distinguish the absorption and first-pass metabolism in the gut. Most studies determined the total gastrointestinal bioavailability \( (F_X F_G) \) (Raoof et al., 1996; Hashimoto et al., 1998; Mihara et al., 2001; Choi et al., 2006; Hanada et al., 2008; Bae et al., 2009; Gertz et al., 2011), or minimized the interference of the other by using a model drug of which metabolism is negligible (Kagan et al., 2010) or specific inhibitors (Shirasaka et al., 2011). Although predictions can be made by in vitro methods, a simple extrapolation from in vitro to in vivo has considerable limitations because various factors may affect the absorption and metabolism in the gastrointestinal tract. Separate quantification of absorption and metabolism in the gut wall was enabled in the present study by comparing the drug exposure following intra-duodenal and superior mesenteric arterial administration of apicidin. The mesenteric artery is responsible for blood supply to the gastrointestinal tract. Especially, the superior mesenteric artery supplies the arterial blood to major drug absorption sites, including the duodenum and jejunum. Thus, apicidin injected into the mesenteric artery bypasses the permeation process and the apicidin injected via this route is considered to be fully absorbed into the gut. This experimental design allows the separate determination of the fraction absorbed into the gut and
the fraction metabolized during passage through the gut wall (Kwan, 1997). The fraction of drug passing through the gut wall without being metabolized ($F_G$), separate from the effect of gastrointestinal absorption, could be obtained by dividing the $AUC$ obtained after superior mesenteric arterial injection by the $AUC$ after portal venous injection. The $F_G$ was calculated to be $70.9 \pm 8.1\%$, i.e., $29.1\%$ of absorbed apicidin was thought to be metabolized by the gut wall first-pass extraction. The calculated fraction absorbed ($F_X$) was $45.9 \pm 9.7\%$.

In order to assess the factors contributing to the fraction absorbed ($F_X$) of apicidin, a series of $in$ $vitro$ and $in$ $situ$ studies were conducted. Membrane permeability through the gut wall is determined by various mechanisms including passive diffusion and carrier mediated uptake or efflux. The most important factor for passive diffusion is the affinity between drug and membrane. The IAM chromatographic assay utilizes the interaction between drug and amphiphilic phospholipids covalently bound to aminopropyl silica particles (Pidgeon et al., 1995; Yang and Lundahl, 1995; Ong et al., 1996; Caldwell et al., 1998; Taillardat-Bertschinger et al., 2003) which represent the cell membrane. The membrane permeability can be predicted from the chromatographic affinity as represented by the IAM capacity factor ($K_{IAM}$). The membrane permeability of apicidin predicted by IAM chromatographic assay was $98.01\%$, which was higher than the value obtained by the $in$ $vivo$ study ($F_X=45.9 \pm 9.7\%$). Although the IAM chromatography is a simple and potentially useful method for screening a large number of compounds, the limitation of this method is that the predicted membrane permeability is mostly governed by the lipophilicity of the drug. Thus, the permeability prediction for small size hydrophilic drugs or substrates for membrane transporters may be limited (Balimane et al., 2000). In case of apicidin, the significantly higher permeability prediction based on the IAM chromatographic assay than the $in$ $vivo$ estimation might be due to the involvement of efflux transporters such as P-gp.

To assess potential involvement of P-gp in the absorption of apicidin, a single pass perfusion was performed in the presence or absence of the P-gp inhibitor, elacridar. Elacridar,
an acridone-carboxamide derivative, is the third generation P-gp inhibitor developed by Glaxo laboratories. It binds to the allosteric site of P-gp at the nanomolar range. The effective permeability ($P_{	ext{eff}}$) of apicidin co-perfused with elacridar was more than 3 times higher than that without elacridar, indicating P-gp mediated efflux may be responsible for the limited in vivo permeability. This result is also consistent with the literature report of the involvement of P-gp in the resistance mechanism of apicidin in osteosarcoma cells in vitro (Okada et al., 2006).

Another factor which may contribute to the lower fraction absorbed ($F_x$) is the potential degradation of apicidin in the gut. When the stability was determined in simulated gastric juice (pH = 1.2) and simulated intestinal juice (pH = 6.8), apicidin was found stable for up to 2 hr in simulated intestinal fluid and 1 hr in simulated gastric fluid. After oral administration of apicidin, the average $T_{\text{max}}$ ranged from 0.8 – 0.9 hr and, therefore, it is likely that the absorption of apicidin was complete within 2 hr after oral administration. Moreover, given the gastric emptying half-life of 6.29 ± 1.01 min in rats (Franklin, 1977), most apicidin in solution was quickly removed from stomach. Therefore, instability may not be a major contributing factor to the absorption of apicidin. Taken together, the fraction absorbed of apicidin ($F_x$=45.9 ± 9.7%) may be mainly limited by P-gp, although the permeability by passive diffusion is extensive.

The drug that escapes the hepatic metabolism may be subject to extra-hepatic metabolism. One of the sites responsible for potential extra-hepatic metabolism is the lung (Heinemann and Fishman, 1969). The total systemic clearance of apicidin in conscious rat was reported to be 15.7 mL/min (Shin et al., 2006), which was significantly higher than the liver blood flow (11.9 mL/min) (Bernareggi and Rowland, 1991). The role of biliary and urinary excretion of apicidin seems to be minor as well (Shin et al., 2006). Therefore, an extra-hepatic elimination such as pulmonary metabolism may be suspected. In this study, the pulmonary bioavailability was calculated by dividing $AUC$ after i.v. injection by $AUC$ after intra-arterial injection. No significant difference was found between the two dosing routes, suggesting the
contribution of the lung metabolism may be insignificant to the overall systemic clearance of apicidin ($F_L = 102.8 \pm 7.4\%$).

To determine the extent of oral absorption, apicidin was introduced into the duodenum in the present study. Most drug absorption takes place predominantly within the upper duodenum and jejunum (Thummel et al., 1996). Intra-duodenal administration allows a direct contact of apicidin with the main absorption site, and the absorption is less affected by other factors such as drug transit and the presence of food in the gastrointestinal tract. Inter-animal variability is also thought to be less. Following intra-duodenal injection of apicidin at doses of 5 and 10 mg/kg, a trend of more than dose-proportional increase in $AUC$ values was observed and the clearance ($CL_d/F$) obtained after oral administration of 5 mg/kg was significantly higher than that of 10 mg/kg ($119.9 \pm 26.6$ vs. $81.4 \pm 14.4$ mL/min/kg). In our previous study, however, no saturable disposition was observed over the i.v. dose range of 0.5 – 4 mg/kg (Shin et al., 2006). The serum concentrations obtained after oral administration of 5 or 10 mg/kg in the present study were within the ranges of the observed serum concentrations in the previous study. It is possible that the absorption processes including gastrointestinal absorption, intestinal, or hepatic first-pass metabolism may be saturated at an oral dose of 10 mg/kg, whereas systemic metabolism may not. At higher oral doses, P-gp mediated efflux could be saturated, which leads to an increase in the absorption fraction followed by higher drug exposure with dose increase. Thus, the $AUC$ obtained after intra-duodenal administration at 5 mg/kg was used for calculating the oral bioavailability, since no or minimal absorption saturation may occur at this dose.

In this study, the superior mesenteric arterial and portal venous injection studies were conducted in anesthetized rats. Literature information on the pharmacokinetics of apicidin following i.v. and oral administration were available only from conscious rats (Shin et al., 2006). To compare the pharmacokinetics of apicidin under the same conditions, i.v. and oral administration studies were conducted in anesthetized rats in the present study. Thus, it was possible to examine the effect of anesthesia on the pharmacokinetics of apicidin. Upon i.v.
injection of apicidin in anesthetized rats, a significantly longer terminal elimination half-life of apicidin with no changes in the volume of distribution (i.e., a smaller systemic clearance) was observed in this study compared to that found in the previous report following i.v. administration of apicidin to conscious rats (Shin et al., 2006). In the literature, anesthesia with ketamine/xylazine reduced heart rate and blood pressure of ICR mice (Furukawa et al., Laboratory Animal Science, 1998). Organ clearance is a product of the extraction ratio and the organ blood flow. Thus, alterations in the organ blood flow may affect the organ clearance so that systemic clearance and elimination half-life of apicidin might be changed. Comparison of the pharmacokinetic profiles and pharmacokinetic parameters of apicidin in anesthetized vs. conscious rats are presented in the Supplemental Data (Supplemental Figure 1, Supplemental Table 1).
CONCLUSIONS

The gastrointestinal absorption of apicidin and the sequential first-pass metabolism in the gut, liver, and lung were quantitatively determined after various routes of administration in rats. Our results demonstrate that the absorption of apicidin is limited by P-gp mediated efflux as well as by first-pass gut wall and hepatic metabolism despite the high potential of passive diffusion. The present study would provide useful experimental strategy to understand absorption processes of a drug and guide to develop new analogues with optimized oral bioavailability.
DMD #56713

Authorship Contributions

Participated in research design: B.S.S., S.D.Y., T.H.K., S.H.J, S.S.


Wrote or contributed to the writing of the manuscript: B.S.S., S.D.Y., T.H.K., J.B.B., C.B.L., J.C.S., J.H.C., K.Y.W., S.H.J., S.S.
REFERENCES


DMD #56713


FOOTNOTES

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Beom Soo Shin and Sun Dong Yoo contributed equally to this work.
Legends for Figures

Figure 1. Relationship between the fraction of dose absorbed in humans ($F_a\%$) and $K_{IAM}/MW$ for 28 commercial drugs (○) and apicidin(●). Solid line represents the relationship between $F_a\%$ and $K_{IAM}$ expressed by a sigmoid equation, $F_a(\%) = 98.01 \times (1 - e^{-4.308 \cdot \frac{K_{IAM}}{MW^{3.00}}})^{3.00}$.

Figure 2. Average serum concentration of apicidin, demethylated apicidin, and hydroxylated apicidin vs. time curves after i.v. injection of apicidin at 2 mg/kg in anaesthetized rats. Data are presented as the average ± SD (n = 4).

Figure 3. Average serum concentration of apicidin, demethylated apicidin, and hydroxylated apicidin vs. time curves after intra-duodenal administration of apicidin at 5 mg/kg (n = 4, panel A) and 10 mg/kg (n = 5, panel B) in anaesthetized rats. Data are presented as the average ± SD.

Figure 4. Average serum concentration of apicidin vs. time curves obtained after superior mesenteric arterial, portal venous, and intra-arterial injections (2 mg/kg each) and oral administration (5 mg/kg) in anaesthetized rats. *Serum concentrations obtained after oral administration was normalized to a 2 mg/kg dose. Data are presented as the average ± SD.

Figure 5. Schematic representation of the absorption processes following oral administration.
Table 1. Noncompartmental pharmacokinetic parameters of apicidin, demethylated apicidin, and hydroxylated apicidin obtained after i.v. injection of apicidin (2 mg/kg) in anesthetized rats (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Apicidin (n = 4)</th>
<th>Demethylated apicidin (n = 4)</th>
<th>Hydroxylated apicidin (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>2.6 ± 0.4 *</td>
</tr>
<tr>
<td>$C_0$ (ng/mL)</td>
<td>1585.9 ± 362.5</td>
<td>133.8 ± 26.2</td>
<td>46.6 ± 26.1</td>
</tr>
<tr>
<td>$AUC_\infty$ (ng·hr/mL)</td>
<td>1262.5 ± 73.8</td>
<td>108.5 ± 17.8</td>
<td>181.1 ± 37.2</td>
</tr>
<tr>
<td>$CL_s$ (mL/min/kg)</td>
<td>26.5 ± 1.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$V_{ss}$ (L/kg)</td>
<td>3.2 ± 0.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$V_z$ (L/kg)</td>
<td>4.3 ± 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$MRT_\infty$ (hr)</td>
<td>2.0 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>4.1 ± 0.6</td>
</tr>
</tbody>
</table>

$p<0.05$ for apicidin vs. hydroxylated apicidin.
Table 2. Noncompartmental pharmacokinetic parameters of apicidin, demethylated apicidin, and hydroxylated apicidin obtained after intraduodenal administration of apicidin at 5 and 10 mg/kg doses in anesthetized rats (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dose = 5 mg/kg</th>
<th>Dose = 10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apicidin (n = 4)</td>
<td>Demethylated apicidin (n = 4)</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>$T_{max}$ (hr)</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>341.6 ± 85.2</td>
<td>48.9 ± 9.0</td>
</tr>
<tr>
<td>$AUC_{\infty}$ (ng hr/mL)</td>
<td>725.7 ± 152.5</td>
<td>96.3 ± 13.6</td>
</tr>
<tr>
<td>$CL/F$ (mL/min/kg)</td>
<td>119.0 ± 26.6</td>
<td>-</td>
</tr>
<tr>
<td>$V_z/F$ (L/kg)</td>
<td>13.3 ± 2.9</td>
<td>-</td>
</tr>
<tr>
<td>$MRT_{\infty}$ (hr)</td>
<td>2.1 ± 0.6</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>
Table 3. Noncompartmental pharmacokinetic parameters of apicidin after portal venous, superior mesenteric arterial, and intra-arterial injection of apicidin (2 mg/kg) in anesthetized rats (mean ± SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Portal venous injection (n = 5)</th>
<th>Mesenteric arterial injection (n = 5)</th>
<th>Intra-arterial Injection (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>1.9 ± 0.3</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>$C_0$ (ng/mL)</td>
<td>957.8 ± 384.0*</td>
<td>799.7 ± 180.6</td>
<td>1616.7 ± 418.3</td>
</tr>
<tr>
<td>$AUC_{\infty}$ (ng·hr/mL)</td>
<td>891.1 ± 155.3*</td>
<td>632.0 ± 72.5#</td>
<td>1233.3 ± 84.4</td>
</tr>
<tr>
<td>$CL_s$ (mL/min/kg)</td>
<td>38.6 ± 8.3*</td>
<td>53.4 ± 6.8#</td>
<td>27.1 ± 2.0</td>
</tr>
<tr>
<td>$V_{ss}$ (L/kg)</td>
<td>5.5 ± 1.4*</td>
<td>4.9 ± 1.1</td>
<td>2.3± 0.5</td>
</tr>
<tr>
<td>$MRT_{\infty}$ (hr)</td>
<td>2.4 ± 0.6</td>
<td>1.6 ± 0.4#</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

* $p<0.05$ vs. i.v. injection.
# $p<0.05$ vs. portal venous injection.
Table 4. Total ($F_{Total}$), absorption ($F_X$), gut wall ($F_G$), hepatic ($F_H$), and pulmonary ($F_L$) bioavailability and the first-pass elimination fraction of apicidin administered duodenally to anesthetized rats.

<table>
<thead>
<tr>
<th></th>
<th>$F_{Total}$</th>
<th>$F_X$</th>
<th>$F_G$</th>
<th>$F_H$</th>
<th>$F_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculation</td>
<td>$F = \frac{D_{iv}}{D_{po}} \cdot \frac{AUC_{po}}{AUC_{iv}}$</td>
<td>$F_X = \frac{D_{mesentery}}{D_{po}} \cdot \frac{AUC_{po}}{AUC_{mesentery}}$</td>
<td>$F_G = \frac{D_{portal \text{ vein}}}{D_{mesentery}} \cdot \frac{AUC_{mesentery}}{AUC_{portal \text{ vein}}}$</td>
<td>$F_H = \frac{D_{portal \text{ vein}}}{D_{portal \text{ vein}} - D_{iv}} \cdot \frac{AUC_{portal \text{ vein}}}{AUC_{po}}$</td>
<td>$F_L = \frac{D_{portal \text{ vein}}}{D_{w}} \cdot \frac{AUC_{w}}{AUC_{po}}$</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>23.0 ± 4.8</td>
<td>45.9 ± 9.7</td>
<td>70.9 ± 8.1</td>
<td>70.6 ± 12.3</td>
<td>102.8 ± 7.4</td>
</tr>
<tr>
<td>First-pass elimination fraction (%)</td>
<td>77.0</td>
<td>54.1</td>
<td>29.1</td>
<td>29.4</td>
<td>≈ 0</td>
</tr>
</tbody>
</table>
Figure 1

Fraction of dose absorbed (%) vs. \((K_{IAM}/MW^{2.05})^5\)

Apicidin
Figure 2

A graph showing the concentration (ng/ml) over time (hr) for Apicidin, Demethylated Apicidin, and Hydroxylated Apicidin.
Figure 3

A

B
Figure 4

![Graph showing apicidin concentration over time for different administration methods.]

- **Intraarterial injection** (n=5)
- **Intravenous injection** (n=4)
- **Portal venous injection** (n=5)
- **Mesenteric arterial injection** (n=5)
- **Oral administration** (n=4)
Figure 5

Metabolized in lung \(1 - F_L = E_L\)

Intra-arterial injection

Intravenous injection

Other tissues

Metabolized in liver \(1 - F_H = E_H\)

Liver

Portal venous injection

Metabolized in gut wall \(1 - F_G = E_G\)

Gut wall

To feces \(1 - F_X = E_X\)

Gut Lumen

Mesenteric arterial injection

Oral administration
Title: Quantitative Determination of Absorption and First-Pass Metabolism of Apicidin, a Potent Histone Deacetylase Inhibitor

Authors: Beom Soo Shin, Sun Dong Yoo, Tae Hwan Kim, Jurgen B Bulitta, Cornelia B Landersdorfer, Jeong Cheol Shin, Jin Ho Choi, Kwon-Yeon Weon, Sang Hoon Joo, Soyoung Shin

Journal title: Drug Metabolism and Disposition

Supplemental Figure 1. Average serum apicidin concentration vs. time curves obtained after i.v. injection of apicidin (2 mg/kg) in anesthetized (n=4) and conscious rats (n=5).
Supplemental Table 1. Noncompartmental pharmacokinetic parameters of apicidin obtained after i.v. injection of apicidin at 2 mg/kg dose in conscious and anesthetized rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conscious rats (n=5)</th>
<th>Anesthetized rats (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t_{1/2}) (hr)</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>(C_0) (ng/mL)</td>
<td>1235.6 ± 203.8</td>
<td>1585.9 ± 362.5</td>
</tr>
<tr>
<td>(AUC_{\infty}) (ng·hr/mL)</td>
<td>498.1 ± 74.4</td>
<td>1262.5 ± 73.8*</td>
</tr>
<tr>
<td>(CL_s) (mL/min/kg)</td>
<td>68.0 ± 9.3</td>
<td>26.5 ± 1.6*</td>
</tr>
<tr>
<td>(V_{ss}) (L/kg)</td>
<td>2.7 ± 0.5</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>(MRT_{\infty}) (hr)</td>
<td>0.7 ± 0.1</td>
<td>2.0 ± 0.2*</td>
</tr>
</tbody>
</table>

*\(p<0.05\) vs. conscious rat.

†Data were obtained from literature (Shin et al., 2006).