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

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ABSTRACT

Apicidin, a potential oral chemotherapeutic agent, possesses potent anti-histone-deacetylase activity. After oral administration, the total bioavailability of apicidin is known to be low (14.2%–19.3%). In the present study, we evaluated the factors contributing to the low bioavailability of apicidin by means of quantitative determination of absorption fraction and first-pass metabolism after oral administration. Apicidin was given to rats by five different routes: into the femoral vein, duodenum, superior mesenteric artery, portal vein, and carotid artery. Especially, the fraction absorbed ($F_a$) and the fraction that is not metabolized in the gut wall ($F_X$) were separated by injection of apicidin via superior mesenteric artery, which enables bypassing the permeability barrier. The $F_X$ was 45.9% ± 9.7%, the $F_a$ 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. Although 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 the 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., Whitehouse Station, NJ) and Romidepsin (Gloucester Pharmaceutical Inc., Cambridge, MA) have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma. More than 15 HDAC inhibitors including Panobinostat (LBH589), Entinostat (MS-275), Belinostat (PXD101), Givinostat (ITF2357), Mocetinostat (MGCD0103), and Abexinostat (PCI-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 a monotherapy or in combination with other anticancer 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 that has potential to be developed as an oral chemotherapeutic agent with potent antiproliferative and cytodifferentiation activities (Darkin-Rattray et al., 1996). It has been shown to exhibit antitumor 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 tumor necrosis factor–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 nonfasting 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.

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ABBREVIATIONS: AUC, area under the time-concentration curve; CL, clearance; $F_X$ fraction absorbed; $F_a$ fraction not metabolized in the gut wall; $F_L$, hepatic bioavailability; $F_P$, pulmonary bioavailability; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; ITF2357, Givinostat; LBH589, Panobinostat; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MGCD0103, Mocetinostat; MRT, mean residence time; MS-275, Entinostat; PE, polyethylene; P-gp, P-glycoprotein; PCI-24781, Abexinostat; PXD101, Belinostat.
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 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 extrahepatic 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, distinguishing 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 analogs with improved bioavailability. Nevertheless, experimental separation of the fraction absorbed—that is, the net transport of unchanged drug into the gastrointestinal tract (FX)—and the fraction that is not metabolized through the gut wall (FC) in vivo is not easily achieved, and most of the literature reports them together as gastrointestinal bioavailability (FX FC) (Raaf 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).

Our study quantitatively evaluated 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 in vivo approach for the quantitative determination of FX and FC by comparing the area under the time-concentration curve (AUC) obtained via different routes of administration. In vitro and in situ studies were also conducted to confirm the results from the in vivo experiment and to further assess the factors contributing to the in vivo absorption such as, passive diffusion, efflux by P-glycoprotein (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). The other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

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, South Korea) and water. The animals were maintained at a temperature of 22–24°C with a 12-hour 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 Immobilized Artificial Membrane Chromatographic Assay. The absorption potential of apicidin by passive diffusion was evaluated by the immobilized artificial membrane (IAM) chromatographic assay described previously elsewhere (Yoon et al., 2004). Briefly, high-performance liquid chromatography (HPLC) analysis was performed using a Waters 2695 separations module and Waters 2996 photo diode array detector (Waters Corporation, Milford, MA). The retention time of apicidin (tR) and the holdup time of the column (tH) were determined using the IAM.PC/DD 2 Drug-Discovery column (Regis Technologies, Morton Grove, IL). The isocratic mobile phase consisted of acetonitrile and Dulbecco’s phosphate-buffered saline (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 (KIAM) were determined for 28 commercial drugs, and the obtained KIAM was modified by the power of the compound molecular weight (KIAM/MW) to improve the correlation between the KIAM and the dose absorbed in humans (Fp). The human Fp was obtained from the literature (Worland et al., 1984; Sugano et al., 2001; Zhu et al., 2002). The relationship between modified KIAM (KIAM/MW) and Fp was best described by the following sigmoidal equation:

\[
F_p(\%) = 98.01 \times \left(1 - e^{-4.305 \times \text{KIAM}/\text{MW}}\right) ^{3.00}
\]  

(1)

where KIAM is the IAM capacity factor and MW is the molecular weight of the compound. The Fp for apicidin by passive diffusion was calculated using eq. 1.

 Determination of Intestinal Permeability by Single-Pass Perfusion. The effect of 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 anesthetized 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, South 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-minute intervals from 50 minutes to 120 minutes 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 minutes, 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 (Peff) was calculated by the single-pass perfusion approach:

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

(2)

where C_in and C_out are the inlet and outlet concentrations at steady state, Q_in is the perfusion rate, R and L are the intestinal radius (1.375 mm) and length (10 cm), respectively, and 2πRL 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 liter. 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 liter. Portions of the simulated gastric and intestinal juices (900 μl each) were preincubated in a water bath for 5 minutes at 37°C, and then 100 μl of apicidin dissolved in dimethylsulfoxide (100 g/ml) was added to each simulated gastric juice and intestinal juices, separately. Samples were taken at 15, 30, 60, and 120 minutes and kept at −20°C until analysis.

In Vivo Pharmacokinetic Study. The rats were anesthetized 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 dimethylsulfoxide: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 intraduodenal administration, rats were anesthetized and cannulated after 12 hours of 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) was inserted into the superior mesenteric artery. Epoxy glue (Krazy Glue, Columbus, OH) 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) was inserted into the portal vein. Epoxy glue (Krazy Glue) 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 the 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 before and at 5, 10, 15, and 30 minutes, and 1, 1.5, 2, 3, 4, and 6 hours after injection of apicidin. All experiments were performed under anesthetized conditions. To maintain anesthesia, ketamine and xylazine (22:5.25 mg/kg) were administered via the intraperitoneal route every 1 hour after initial anesthesia, and the animal was placed on a heating pad to maintain 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 1500g for 10 minutes and were stored at −20°C until analysis.

Quantification of Apicidin and Its Metabolites by Liquid Chromatography Coupled with Tandem Mass Spectrometry. Apicidin and its metabolites demethylated apicidin, hydroxylated apicidin, dihydroxylated apicidin, glucurononidated apicidin, glucurononidated monohydroxyapicidin, and glucurononidated dihydroxyapicidin were assayed by a modification of a liquid chromatography coupled with tandem mass spectrometry method (LC-MS/MS) method previously reported elsewhere (Shin et al., 2005). Briefly, 100 μl of the sample was mixed with 100 μl of the internal standard solution (trazodone 100 ng/ml in methanol) and precipitated by addition of 800 μl 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 minutes and centrifuged at 2840g for 10 minutes. An aliquot of the supernatant (10 μl) was then injected into the LC-MS/MS.

The 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 C18 column (150 × 2.0 mm i.d., 5 μm) with a SecurityGuard column (4 × 2.0 mm i.d.) (Phenomenex, Torrance, CA). The isotropic 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 minutes.

The HPLC system was coupled to an API 2000 triple-quadrupole mass spectrometer equipped with a turbo electrospray ionization (ESI) source (AB MDS Sciex, Toronto, Ontario, Canada). The electrospray ionization source was operated in a positive mode. The selected multiple reaction monitoring transitions of the precursors to the product ion were 624.5 → 84.2 for apicidin, 610.5 → 84.2 for demethylated apicidin, 640.5 → 84.2 for hydroxylated apicidin, 656.5 → 84.2 for dihydroxylated apicidin, 800.5 → 84.2 for glucurononidated apicidin, 816.5 → 84.2 for glucurononidated monohydroxyapicidin, 832.5 → 84.2 for glucurononidated dihydroxyapicidin, and 372.1 → 176.1 for trazodone.

Pharmacokinetic Analysis. The serum concentration of apicidin versus time data was analyzed by the noncompartmental method using WinNonLin (Pharsight, Cary, NC). The absolute oral bioavailability (F) was calculated by the following equation:

\[
F = \frac{D_{o}}{D_{p}} \times \frac{AUC_{po}}{AUC_{vi}}
\]

The fraction absorbed (FX) was calculated as follows:

\[
FX = \frac{D_{veneary artery}}{D_{po}} \times \frac{AUC_{po}}{AUC_{veneary artery}}
\]

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

\[
F_G = \frac{D_{portal vein}}{D_{veneary artery}} \times \frac{AUC_{veneary artery}}{AUC_{portal vein}}
\]

\[
F_L = \frac{D_{portal vein}}{D_{veneary artery}} \times \frac{AUC_{veneary artery}}{AUC_{portal vein}}
\]

\[
F_F = \frac{D_{portal vein}}{D_{veneary artery}} \times \frac{AUC_{veneary artery}}{AUC_{portal vein}}
\]

where AUC is the area under the serum apicidin concentration versus 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 analysis of variance (ANOVA) followed by Scheffe’s post hoc test among more than two means for unpaired data. P < 0.05 was considered statistically significant.

Results

Quantification of Apicidin and Its Metabolites in Biologic 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 biologic fluids. The biologic 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 minutes, respectively. None of the potential phase II metabolites, such as glucurononidated apicidin, glucurononidated monohydroxyapicidin, or glucurononidated dihydroxyapicidin, were detected in the serum samples.

Determination of Passive Absorption Potential of Apicidin Using IAM Chromatography. The relationship between the fraction of dose absorbed in humans (FX%) and the IAM capacity factors (KIAM) of 28 commercial drugs is shown in Fig. 1. Based on this relationship, the equation derived to predict the passive absorption potential had 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 (t_v = 190.7 minutes), 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/MW_2.05 = 32.22), and the fraction of dose absorbed in humans by passive diffusion (FX%) 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 in the intestinal permeability of apicidin was evaluated by in situ single-pass intestinal perfusion. The effective permeability (P_{eff}) of apicidin through the duodenum was compared in the presence or absence of the potent P-gp inhibitor elacridar. The P_{eff} of apicidin in the duodenum in the presence of 1 μM of elacridar was statistically significantly higher than that in the absence of elacridar (17.83 ± 4.68 × 10−5 versus 5.08 ± 4.11 × 10−5 cm/s, P < 0.05). This finding suggests that apicidin is a substrate of P-gp and that P-gp may play an important role in the intestinal permeability of apicidin.
Absorption and First-Pass Metabolism 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 hours of incubation in simulated gastric and intestinal juices. Apicidin was found to be stable (99.5% ± 3.4%) in the simulated intestinal juice over the 2-hour incubation period. Apicidin was also stable in the simulated gastric juice (pH = 1.2) for 1 hour, but its concentration decreased to 85.0% at the end of the incubation.

Pharmacokinetics of Apicidin and Its Metabolites in Anesthetized Rats after Intravenous Bolus Injection. The average concentration versus time profiles of apicidin, demethylated apicidin, and hydroxylated apicidin obtained after i.v. injection of apicidin at 2 mg/kg in anesthetized rats are shown in Fig. 2. The serum apicidin concentration versus time profile shows multiexponential decline. Both demethylated apicidin and hydroxylated apicidin were detected from the first blood sampling time (5 minutes) after i.v. administration of apicidin. Although the demethylated apicidin concentrations decreased in parallel to the apicidin concentrations, the hydroxylated apicidin concentrations increased initially, reached a maximum concentration at approximately 0.6 hours, and decreased at a slower rate (Fig. 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 (t1/2) of demethylated apicidin (1.8 ± 0.3 hours) was comparable to that of apicidin (1.9 ± 0.2 hours). The serum concentrations of demethylated apicidin extrapolated to time zero (C0) and the AUC were 8.4% and 8.6% of the corresponding values found for apicidin. The t1/2 of hydroxylated apicidin (2.6 ± 0.4 hours) 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 concentrations versus time profiles obtained after intraduodenal administration of apicidin at doses of 5 and 10 mg/kg in anesthetized rats are shown in Fig. 3. Apicidin was detected in serum from the first blood sampling time (5 minutes) and reached its maximum concentration within 1 hour in both groups of rats. The concentration versus 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 (Cmax) and the AUC of apicidin increased as the dose was increased. The observed Cmax values were 341.6 ± 85.2 and 924.4 ± 121.9 ng/ml and the AUCs were 725.7 ± 152.5 and 2110.5 ± 397.2 ng·h/ml after intraduodenal administration of 5 and 10 mg/kg, respectively. The t1/2 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 (CLs/F) at doses of 5 or 10 mg/kg were 119.0 ± 26.6 and 81.1 ± 14.4 ml/min/kg, respectively. The systemic clearance corrected by oral bioavailability (CLs) was not significantly different between the 5 and 10 mg/kg doses (27.4 versus 27.1 ml/min/kg), and these values were significantly lower than those obtained after i.v. injection (Table 1).

**TABLE 1.**

<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>t1/2 (h)</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>2.6 ± 0.4*</td>
</tr>
<tr>
<td>C0 (ng/ml)</td>
<td>1585.9 ± 362.5</td>
<td>1338.8 ± 26.2</td>
<td>46.6 ± 26.1</td>
</tr>
<tr>
<td>AUC (ng·h/ml)</td>
<td>126.5 ± 73.8</td>
<td>108.5 ± 17.8</td>
<td>181.1 ± 37.2</td>
</tr>
<tr>
<td>CLs (ml/min/kg)</td>
<td>26.1 ± 1.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>3.2 ± 0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vm (l/kg)</td>
<td>4.3 ± 0.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MRTs (h)</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 versus hydroxylated apicidin.
At both doses, the 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 ± 0.7 and 3.1 ± 0.9 hours, respectively) was longer than those of apicidin (1.3 ± 0.4 and 1.8 ± 0.7 hours) and demethylated apicidin (1.4 ± 0.3 and 2.0 ± 0.6 hours). The $T_{\text{max}}$ of hydroxylated apicidin (1.1 ± 0.3 and 1.1 ± 0.2 hours for 5 and 10 mg/kg dose, respectively) occurred later than those of apicidin and demethylated apicidin. The AUC of demethylated and hydroxylated apicidin obtained after intraduodenal administration of 5 mg/kg dose was 96.3 ± 13.6 ng/ml and 199.2 ± 33.2 ng·h/ml, respectively, and they were 13.3% and 27.5% of apicidin, respectively. The AUC of demethylated and hydroxylated apicidin after intraduodenal administration of 10 mg/kg dose was 294.7 ± 68.2 ng/ml and 576.2 ± 182.0 ng·h/ml, respectively, and they were 14.0% and 27.3% of apicidin, respectively, which was significantly greater than 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 versus 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 Fig. 4. The serum concentrations of apicidin after 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 hours, respectively) were all comparable with that found after i.v. injection (1.9 ± 0.2 hours). However, there were significant differences between the AUC values after intraduodenal versus superior mesenteric arterial injection, superior mesenteric arterial versus portal venous injection, and portal venous versus i.v. injection. The AUC of apicidin obtained after superior mesenteric arterial injection (891.1 ± 155.3 ng·h/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 ($F_{\text{Total}}$), absorption ($F_X$), gut wall ($F_G$), hepatic ($F_H$), and pulmonary ($F_L$) bioavailability and the first-pass elimination fraction of apicidin. The calculated fraction absorbed ($F_X$) was 45.9% ± 9.7%. The calculated fraction not metabolized in the single passage through the gut wall was 70.9% ± 8.1%; that is, 29.1%

**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 ± S.D.).

<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>$t_{1/2}$ (h)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>341.6 ± 85.2</td>
<td>48.9 ± 9.0</td>
</tr>
<tr>
<td>AUC$_{\text{g-i}}$ (ng·h/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>$V_{\text{d}/F}$ (l/kg)</td>
<td>13.3 ± 2.9</td>
<td>—</td>
</tr>
<tr>
<td>$MRT_{\text{g-i}}$ (h)</td>
<td>2.1 ± 0.6</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>
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% ± 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 × 0.706 × 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—that is, into the femoral vein, duodenum, superior mesenteric artery, portal vein, and carotid artery—allowed the estimation of the fraction absorbed and first-pass metabolism in the gut, liver, and lung in vivo (Fig. 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 after intraduodenal 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% ± 8.1%; 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% ± 9.7%.

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 uses 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% ± 9.7%). Although IAM chromatography is a simple and potentially useful method for screening a large number of

![Fig. 4](image-url) Average serum concentration of apicidin versus time curves obtained after superior mesenteric arterial, portal venous, and intra-arterial injections (2 mg/kg each) and oral administration (5 mg/kg) in anesthetized rats. *Serum concentrations obtained after oral administration was normalized to a 2 mg/kg dose. Data are presented as the average ± S.D.

### 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 ± S.D.).

<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} (h)</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^a</td>
<td>799.7 ± 180.6^b</td>
<td>1616.7 ± 418.3</td>
</tr>
<tr>
<td>AUC (ng•h/ml)</td>
<td>891.1 ± 155.3^b</td>
<td>632.0 ± 72.5^b</td>
<td>1233.3 ± 84.4</td>
</tr>
<tr>
<td>CL_{m} (ml/min/kg)</td>
<td>38.6 ± 8.3^a</td>
<td>53.4 ± 6.8^a</td>
<td>27.1 ± 2.0</td>
</tr>
<tr>
<td>V_{ss} (l/kg)</td>
<td>5.5 ± 1.4^a</td>
<td>4.9 ± 1.1</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.4 ± 0.6</td>
<td>1.6 ± 0.4^b</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

^aP < 0.05 versus i.v. injection.

^bP < 0.05 versus portal venous injection.
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-sized 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 the 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 ($P_{eff}$) of apicidin co-perfused with elacridar was more than 3 times higher than that without elacridar, indicating that 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 that 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 hours in simulated intestinal fluid and 1 hour in simulated gastric fluid. After oral administration of apicidin, the average $T_{max}$ ranged from 0.8–0.9 hours; therefore, it is likely that the absorption of apicidin was complete within 2 hours after oral administration. Moreover, given the gastric emptying half-life of 6.29 ± 1.01 minutes 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 ($F_X = 45.9\% \pm 9.7\%$) 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 extrahepatic metabolism. One of the sites responsible for potential extrahepatic 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,

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Calculation} & \text{Bioavailability ($\%$)} & \text{First-pass elimination fraction ($\%$)} \\
\hline
F_{Tota} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 & 77.0 \\
F_{X} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 & 77.0 \\
F_{G} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 & 77.0 \\
F_{H} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 & 77.0 \\
F_{L} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 & 77.0 \\
\hline
\end{array}
\]

$TABLE 4.$ Total ($F_{Tota}$), 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.

\[
\begin{array}{|c|c|}
\hline
\text{Calculation} & \text{Bioavailability ($\%$)} \\
\hline
F_{Tota} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 \\
F_{X} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 \\
F_{G} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 \\
F_{H} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 \\
F_{L} = \frac{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}}{D_{\text{portal}} \times \text{AUC} \text{prenasogamy}} & 23.0 \pm 4.8 \\
\hline
\end{array}
\]
The role of biliary and urinary excretion of apicidin seems to be minor as well (Shin et al., 2006). Therefore, an extrahepatic elimination such as pulmonary metabolism may be suspected. In this study, the pulmonary bioavailability was calculated by dividing the AUC after i.v. injection by the AUC after intra-arterial injection. No statistically significant difference was found between the two dosing routes, suggesting that the contribution of the lung metabolism may be insignificant to the overall systemic clearance of apicidin (FL = 102.8% ± 7.4%).

To determine the extent of oral absorption, we introduced apicidin into the duodenum. Most drug absorption takes place predominantly within the upper duodenum and jejunum (Thummel et al., 1996). Intraduodenal 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. Interanimal variability is also thought to be less. After intraduodenal injection of apicidin at doses of 5 and 10 mg/kg, a trend of a more than dose-proportional increase in AUC values was observed, and the clearance (CL/F) obtained after oral administration of 5 mg/kg was statistically significantly higher than that of 10 mg/kg (119.9 ± 26.6 versus 81.4 ± 14.4 ml/min/kg). In our previous study, however, no saturable disposition was observed over the i.v. dose range of 0.5–4.0 mg/kg (Shin et al., 2006). The serum concentrations obtained after oral administration of 5 or 10 mg/kg in our present study were within the ranges of the observed serum concentrations in our 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-g-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 intraduodenal administration at 5 mg/kg was used for calculating the oral bioavailability, because 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 with that found in the previous report after i.v. administration of apicidin to conscious rats (Shin et al., 2006). In the literature, anesthesia with ketamine/xylazine reduced the heart rate and blood pressure of ICR (Institute of Cancer Research) mice (Furukawa et al., 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 such that systemic clearance and elimination half-life of apicidin might be changed. Comparison of the pharmacokinetic profiles and pharmacokinetic parameters of apicidin in anesthetized versus conscious rats are presented in the supplemental data (Supplemental Fig. 1; Supplemental Table 1).


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