Pharmacokineti
c, Distribution, Metabolism, and Excretion of (Z)-2-Amino-1,5-Dihydro-1-Methyl-5-[4-(Mesyl)Benzylidene]-4H-Imidazo
l-4-One Mesilate (ZLJ-601) in Sprague-Dawley Rats

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Key Words
Pharmacokinetic • ZLJ-601 • Tissue distribution • Metabolism • Excretion • COX/5-LOX inhibitor

Abstract
(Z)-2-amino-1,5-dihydro-1-methyl-5-[4-(mesyl)benzylidene]-4H-imidazo
l-4-one mesilate (ZLJ-601) is an imidazolone COX/5-LOX inhibitor, which has excellent anti-inflammatory activity with an improved gastrointestinal safety profile. The purpose of this study was to evaluate the in vivo absorption, distribution, metabolism, and excretion of ZLJ-601 in Sprague-Dawley rats. After intravenous or intragastric administration to rats, the concentration of ZLJ-601 in plasma, bile, urine, feces and various types of tissues was detected by LC-MS. We also conducted the identification of metabolites using tandem mass spectrometry. After the intravenous administration, the t1/2 ranged from 38.71 to 42.62 min and the AUC increased in a dose-proportional manner. After oral dosing, the plasma level of ZLJ-601 peaked at 28.33 min, having a Cmax value of 0.26 mg/l, and the bioavailability was only 4.92%. The highest tissue concentration of ZLJ-601 was observed in lung and kidney, but it was not found in brain.

The majority of unchanged ZLJ-601 was excreted in urine (~35.87%) within 36 h. Two main metabolites are the hydroxylation product and the glucuronide conjugate of the hydroxylation product.

Introduction
Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of arthritis and rheumatism to improve pain, stiffness and physical function [1]. However, the use of conventional NSAIDs is limited by their serious side effects such as gastrointestinal (GI) hemorrhage and ulceration, renal failure and asthma [2–4]. The mechanism of action of conventional NSAIDs is attributed to the inhibition of cyclo-oxygenase (COX), but the inhibition of COX pathways may increase the transformation of arachidonic acid by the 5-lipoxygenase (5-LOX) pathway [5]. Leukotrienes produced by 5-LOX can enhance the vascular permeability of inflammatory sites, exert chemotactic activity and increase the adhesion and migration of leukocytes and neutrophils in injured tissue.
[6]. Furthermore, LTs promote the development of GI damage, which is the most troublesome side effect of NSAIDs [7]. Therefore, compounds which inhibit both COX and 5-LOX (dual inhibitors) may prove to be more potent anti-inflammatory agents with a reduced risk of gastrointestinal pathology [8–10].

Several COX/5-LOX dual inhibitors, such as licofelone, tenidap and CI-1004, have reached clinical development. Among these dual inhibitors, 3,5-di-tert-butyl-4-hydroxy benzylidene moiety and five- or six-membered lactones or heterocycles, such as KME-4, E-5110 and CI-1004, showed excellent anti-inflammatory activity [11–13]. Furthermore, the phenol moiety they possess results not only in inhibiting both COX and 5-LOX, but disturbs other redox enzyme systems in the liver [14]. To develop alternative anti-inflammatory agents, we conducted exploratory research focusing on replacement of the phenol moiety in these molecules with a methylsulfonylphenyl moiety [15], the common pharmacophore of COX-2 inhibitors [16]. Our work led us to the discovery of the novel anti-inflammatory drug ZLJ-601 from a series of imidazoline COX/5-LOX inhibitors we had synthesized.

(Z)-2-amino-1,5-dihydro-1-methyl-5-[4-(mesyl) benzylidene]-4H-imidazol-4-one mesilate (ZLJ-601) (fig. 1) is a potent inhibitor of COX and 5-LOX. It inhibits cyclo-oxygenase in human whole blood, with an IC$_{50}$ = 0.73 for COX-1 and 0.31 μM for COX-2, and it also suppressed the activity of 5-LOX in the rat basophilic leukemia (RBL-1) cell lysate (IC$_{50}$ = 0.32 μM) and in intact cells (IC$_{50}$ = 1.06 μM). Furthermore, no gastrointestinal ulcers were found with the anti-inflammatory dose in normal rats. Compared with the conventional NSAIDs, this compound not only showed equivalent efficacy but also an advantageous gastrointestinal safety profile [17].

Despite significant pharmacological activity, the pharmacokinetics of ZLJ-601 have not been reported. In this study, we investigated the absorption, distribution and excretion of ZLJ-601 in rats for the first time through monitoring ZLJ-601 by liquid chromatography mass spectrometry (LC-MS), and two predominant metabolites of ZLJ-601 were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Fig. 1. Chemical structure of compound ZLJ-601.

### Materials and Methods

**Chemicals and Reagents**

ZLJ-601 (98.6% purity) was provided by the Center of Drug Discovery of China Pharmaceutical University. Ketotifen, used as the internal standard, was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile of liquid chromatographic grade were purchased from Tedia Company (Fairfield, Ohio, USA). β-Glucuronidase (041K70321, type B-1, from bovine liver) was purchased from Sigma-Aldrich (St. Louis, Mo., USA). All other chemicals and reagents were of analytical grade and commercially available.

**Animal**

Sprague-Dawley rats (weighing 200 ± 20 g) were purchased from the Experimental Animal Center of Zhejiang province. Rats were housed under controlled conditions (22 ± 2°C, 50% relative humidity) with a control light-dark cycle. They were allowed to acclimate to standard housing and environmental conditions for 1 week prior to study. Feed and water were provided ad libitum. All studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

**Pharmacokinetics Study**

Twenty-four Sprague-Dawley rats (12 male and 12 female) were randomly assigned to four treatment groups and received a single intravenous administration of ZLJ-601 (in a solution of saline) at 2.5, 5 and 10 mg/kg, respectively, or intragastric administration of 50 mg/kg. Blood samples were collected predose and at 2, 5, 10, 20, 30, 60, 90, 120 and 180 min postdose into heparinized tubes for the i.v. group and 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min for the i.g. group. Plasma was isolated from the blood samples by centrifugation and then stored at –70°C until analysis.

Rat plasma samples (100 μl) were spiked with 10 μl internal standard (ketotifen, 2 μg/ml) and extracted with 600 μl of ethyl acetate by vortexing for 10 min. The organic and aqueous phases were separated by centrifugation at 12,000 g for 3 min. The upper organic phase was transferred to another tube and evaporated to dryness at 50°C. The residue was dissolved in 100 μl acetonitrile and vortexed. A 10-μl aliquot of the solution was injected onto the LC-MS system for analysis.

The pharmacokinetic parameters were calculated with Drug and Statistics version 2.0 program (Anhui Provincial Center for Drug Clinical Evaluation, China) with a noncompartmental model. Absolute bioavailability (F) of ZLJ-601 in rats was calculated using the equation: F = (AUCig × Div)/(AUCiv × Dig) × 100%, where AUCig and AUCiv are the AUC values after intragastric and intravenous administration of ZLJ-601, respectively, and Dig and Div are the doses used for intragastric and intravenous administration, respectively.
injected only with saline served as blank controls. Tissue samples of saline and then homogenized. Tissues from rats after dosing (6 rats at one time) and washed with saline, and blood samples were collected into heparinized polythene tubes before the time of dosing. Blood samples were allowed to clot for 1 h at room temperature and then centrifuged at 12,000 × g. The supernatant (10 mL) was injected and centrifuged at 12,000 g for 10 min. The supernatant (10 μL) was injected into LC-MS/MS for analysis. The feces samples were added to triple volume acetonitrile to generate fecal homogenates. Tissues from rats injected with saline served as blank controls. Tissue samples after homogenization were stored at −70°C until further analysis and the preparation process was just like that described above for plasma.

**Tissue Distribution Study**

Eighteen male rats were i.v. administered a single dose of ZLJ-601 at 5 mg/kg in a solution of saline through the tail vein. Tissues (heart, liver, spleen, lung, kidney, stomach, intestine, testis, muscle, and brain) were promptly removed at 10, 60, and 120 min after dosing (6 rats at one time) and washed with saline, and blood samples were collected into heparinized polythene tubes before tissue removal. Each tissue sample was diluted with triple volumes of saline and then homogenized. Tissues from rats injected with saline served as blank controls. Tissue samples after homogenization were stored at −70°C until further analysis and the preparation process was just like that described above for plasma.

**Excretion Study**

Six male rats were housed in separate metabolic cages and received a single intravenous administration of 5 mg/kg ZLJ-601 dissolved in saline after staying in the metabolic cages for 24 h. Food and water were provided ad libitum throughout the whole experiment period. Cumulative bile samples were collected before and during the time intervals from 0 to 0.5, 0.5 to 1, 1 to 2, 2 to 4, 4 to 6, 6 to 8 and 8 to 12 h after dosing.

Feces were weighed and homogenized (feces:water ratio of 1:4, w/v) and the volume of urine and bile samples was measured before storage at −70°C. The samples were then subjected to the same procedure as described for the plasma samples.

**LC-MS Analysis**

Plasma, tissue and excretion samples were analyzed by LC-MS. A Shimadzu (Kyoto, Japan) LC-MS-2010A liquid chromatograph-mass spectrometer equipped with a SIL-HTC autosampler, two LC-10AVP pumps and an electrospay-ionization (ESI) interface was used for LC-MS analyses. Data acquisition and processing were accomplished using Shimadzu LC-MS Solution (version 3.20 with Windows XP operating system). Separation was carried out on a Shimadzu VP-ODS (150 × 2.0 mm, i.d. 5 μm, Shimadzu, Japan) coupled with a SecurityGuard C18 guard column (4 × 3.0 mm, Phenomenex, Torrance, Calif., USA) maintained at 40°C. The mobile phase consisted of 85% water (containing 0.1% formic acid) and 15% acetonitrile at a flow rate of 0.2 ml/min. The MS conditions were: curved desolvation line (CDL), temperature 250°C, heater block 200°C, nebulizing gas 1.5 l/min, detector voltage 1.6 kV. The injection volume was 10 μL. LC-ESI-MS was performed in the positive selected-ion monitoring (SIM) mode. [M+H]+ ions were detected at m/z 280.3 for ZLJ-601 and m/z 310.4 for the internal standard (ketotifen).

**Identification of Metabolites**

In order to identify the in vivo metabolites of ZLJ-601, the bile, urine and feces samples from different subjects and time intervals were pooled. For the bile and urine samples, they were processed by adding triple volumes of acetonitrile and the mixture was vortexed for 1 min and centrifuged at 12,000 g for 10 min. The supernatant (10 μL) was injected into LC-MS/MS for analysis. The feces samples were added to triple volume acetonitrile to generate fecal homogenates by tissue grinder, and then centrifuged at 12,000 g for 10 min. The supernatants were vaporized to dryness, and the residues were reconstituted in acetonitrile for LC-MS/MS analysis.

An aliquot (150 μL) of bile sample was spiked with 50 μL β-glucuronidase (2,000 U), prepared in the sodium acetate buffer (pH 5.0), and incubated in a shaking water bath at 37°C for 12 h. After incubation, the hydrolysate was terminated with triple volumes of acetonitrile, and samples were processed for analysis as described above.

**Qualitative Analysis with LC-MS/MS**

The LC system consisted of an LC210AD binary pump system, a SIL10AD autosampler and a CTO 10A oven (Shimadzu, Kyoto, Japan). The analytical column was Shimadzu Shim-pack VP-ODS (column size 5 μm, 150 × 2.0 mm), coupled with a SecurityGuard C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, Calif., USA). The mobile phase consisted of water (containing 0.1% formic acid, mobile phase A) and acetonitrile (mobile phase B) with rate set at 0.2 ml/min. The autosampler temperature was maintained at 4°C and injection volume was 10 μL. The composition of gradient elution increased from 5 to 50% of mobile phase B over 3 min, and maintained at 50% B for 1.5 min, followed by change to the initial condition and re-equilibrated.
The temperature of the column was maintained at 40 °C. The Thermo Scientific TSQ Quantum MS/MS system was equipped with an electro-spray ionization interface. The spray voltage was set at 4,000 V for the positive mode. The temperature of capillary was maintained at 350 °C. The fluid was nebulised by high-purity nitrogen, and sheath gas and auxiliary gas were set at 30 and 10 arbitrary units, respectively. The product ions were generated by collision-induced dissociation of the selected precursor ions using ultra purity argon (Ar) as collision gas (pressure set at 1.5 mTorr).

**Results**

**Pharmacokinetics**

There was no gender difference in the pharmacokinetics of ZLJ-601 (data not show). The mean plasma concentration-time curves are shown in figure 2 and the pharmacokinetic parameters of ZLJ-601 in rats after intravenous and oral administration are presented in tables 1 and 2. After intravenous administration, the $t_{1/2}$ were 42.62, 38.71 and 38.98 min, respectively, and the AUC increased in a dose-proportional manner from 78.59 mg·l$^{-1}$·min for 2.5 mg/kg to 365.85 mg·l$^{-1}$·min for 10 mg/kg. After oral dosing at 50 mg/kg, plasma levels of ZLJ-601 peaked at 28.33 min, having a $C_{max}$ value of 0.26 mg/l after administration, and the bioavailability was only 4.92%.

![Fig. 3. The ratio of tissue concentrations compared with the plasma concentration at 10, 60 and 120 min after intravenous administration of 5 mg/kg ZLJ-601. Means ± SD for n = 6 rats are shown.](image)

**Distribution of ZLJ-601**

The tissues and plasma concentrations at 3 time points after the intravenous administration of 5 mg/kg ZLJ-601 are summarized in table 3. The ratios of tissue concentration compared with the plasma concentration (T/P) are shown in figure 3. After i.v. administration, ZLJ-601 was found in all tissues examined except brain and the high-

**Table 1.** Pharmacokinetic parameters for ZLJ-601 in rats after intravenous administration

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>AUC$_{(0→t)}$, mg·l$^{-1}$·min</th>
<th>MRT$_{(0→t)}$, min</th>
<th>$t_{1/2}$, min</th>
<th>CL, l·min$^{-1}$·kg$^{-1}$</th>
<th>Vz, l/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>78.59 ± 8.98</td>
<td>38.40 ± 5.72</td>
<td>42.62 ± 11.32</td>
<td>0.03 ± 0.00</td>
<td>1.96 ± 0.43</td>
</tr>
<tr>
<td>5</td>
<td>134.09 ± 30.39</td>
<td>35.85 ± 6.51</td>
<td>38.71 ± 5.53</td>
<td>0.04 ± 0.01</td>
<td>2.17 ± 0.59</td>
</tr>
<tr>
<td>10</td>
<td>365.85 ± 98.21</td>
<td>38.96 ± 7.48</td>
<td>38.98 ± 11.22</td>
<td>0.03 ± 0.01</td>
<td>1.66 ± 0.61</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n = 6.

**Table 2.** Pharmacokinetic parameters for ZLJ-601 in rats after oral administration

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>AUC$_{(0→t)}$, mg·l$^{-1}$·min</th>
<th>$T_{max}$, min</th>
<th>$C_{max}$, mg/l</th>
<th>F, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>65.99 ± 13.56</td>
<td>28.33 ± 4.08</td>
<td>0.26 ± 0.03</td>
<td>4.92 ± 1.37</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n = 6.
est tissue levels of ZLJ-601 were detected in the lung and kidney. The concentration was below the limit of detection of the assay in heart at 120 min and in testis at 60 and 120 min.

**ZLJ-601 in Bile, Urine and Feces**

The concentration in bile, urine and feces samples were detected after the i.v. administration of 5 mg/kg of ZLJ-601. The mean recovery of ZLJ-601 in bile, urine and feces was 16.00, 35.87 and 1.45%, respectively, with the urine being the major route of excretion (fig. 4, 5). ZLJ-601 was almost completely excreted within 12 h in bile and 36 h in urine and feces. The excretion speed was fast at first and then decreased with increasing time in bile and urine, and in the first 4 h we did not detect ZLJ-601 in feces.

**Identification of Metabolites**

Two metabolites (M1 and M2) were detected both in bile and urine (fig. 6), while in feces no metabolite was observed. M1, M2 and parent compound were characterized by LC–MS/MS analysis in the positive mode. The parent compound ZLJ-601 eluted at around 1.66 min with a protonated molecular ion at m/z 280 (fig. 7). M1 eluted at 1.14 min and had a protonated molecular ion at m/z 296 (fig. 8), which was 16 Da higher than that of ZLJ-601. Therefore, M1 was proposed as the hydroxylated metabolites of ZLJ-601. However, the exact site of hydroxylation was not identified. M2 eluted at 2.20 min with a protonated molecular ion at m/z 472 (fig. 9), which was 176 Da higher than that

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**Table 3.** Concentration of ZLJ-601 in plasma and tissues of rats injected i.v. with a 5-mg/kg dose of ZLJ-601 (each value is the mean of six samples)

<table>
<thead>
<tr>
<th>Time</th>
<th>Plasma μg/ml</th>
<th>Brain μg/g</th>
<th>Heart μg/g</th>
<th>Liver μg/g</th>
<th>Spleen μg/g</th>
<th>Lung μg/g</th>
<th>Kidney μg/g</th>
<th>Stomach μg/g</th>
<th>Intestine μg/g</th>
<th>Testis μg/g</th>
<th>Muscle μg/g</th>
<th>Fat μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>1.82</td>
<td>ND</td>
<td>1.59</td>
<td>2.44</td>
<td>1.02</td>
<td>36.13</td>
<td>18.73</td>
<td>1.16</td>
<td>3.04</td>
<td>0.29</td>
<td>0.51</td>
<td>0.60</td>
</tr>
<tr>
<td>60 min</td>
<td>0.26</td>
<td>ND</td>
<td>0.28</td>
<td>0.54</td>
<td>0.42</td>
<td>30.16</td>
<td>3.18</td>
<td>0.25</td>
<td>2.39</td>
<td>ND</td>
<td>0.11</td>
<td>0.20</td>
</tr>
<tr>
<td>120 min</td>
<td>0.09</td>
<td>ND</td>
<td>ND</td>
<td>0.63</td>
<td>0.50</td>
<td>7.51</td>
<td>7.39</td>
<td>0.38</td>
<td>0.76</td>
<td>ND</td>
<td>0.08</td>
<td>0.25</td>
</tr>
</tbody>
</table>

ND = Value is below the limit of detection of the assay.
of M1. After spiking bile with \(\beta\)-glucuronidase at 37°C for 12 h, the peak area of M2 markedly decreased. Therefore, M2 was identified as the glucuronide conjugate of M1. An- other peak in figure 8 eluted at 2.13 min was likely to be the losing glucuronide product ion of M2 (m/z is also 296) due to the similar retention time with M2 under the same chromatographic condition.

**Discussion**

Many companies have conducted drug-discovery pro- grams to develop COX-2 selective inhibitors (coxibs) to improve gastrointestinal safety because COX-2 is directly linked to the inflammatory process. However, all the coxibs are burdened with a very low but unequivocal risk
to induce life-threatening or fatal cardiovascular events [18]. The COX/5-LOX inhibitor combines the advantages of traditional NSAIDs and coxibs such as good anti-inflammatory activity and gastrointestinal and cardiovascular safety. ZLJ-601 is a promising candidate drug screened from many imidazolone COX/5-LOX dual inhibitors we have synthesized due to excellent anti-inflammatory effects in various models [17, 19].

To our knowledge, this is the first report on the pharmacokinetics, distribution, metabolism, and excretion of...
ZLJ-601. Before determining the concentration of ZLJ-601, we validated the analysis method and the selectivity, precision, accuracy and stability were acceptable. In the pharmacokinetic study, the t1/2 range was from 38.71 to 42.62 min after intravenous administration, indicating that ZLJ-601 was rapidly eliminated from plasma in vivo. The AUC increased in a dose-proportional manner after intravenous administration and good linearity of the kinetics of ZLJ-601 was observed. The bioavailability is only 4.92% which is consistent with the polar properties and poor retention in chromatographic column, suggesting that it is not suitable for taking orally. However, the reason for its poor bioavailability is not clear and further studies such as the membrane permeability in the Caco-2 cell line and first-pass effect are necessary to investigate the exact reason.

The ZLJ-601 was well distributed to tissues but not found in brain, suggesting that it did not cross the blood–brain barrier efficiently. The highest tissue level was found in lung and it is consistent with the study result of licofelone, another COX/5-LOX inhibitor [20]. The concentration in kidney was also very high and it may be related to the major excretion that occurred in this tissue.

In the excretion study, because the labeled ZLJ-601 was not available, we did not carry out an excretion mass balance study by examining the total radioactivity and regretfully only focused on the parent compound. Thus, the objective of the excretion study was to identify the main route of elimination and the recovery of unchanged drug in bile, urine and feces. The majority of the i.v. administered dose (approximately 35.87% in 36 h) was recovered in the urine and it was the primary route of excretion. In addition, the total recovery of the intact parent compound is relatively low, indicating that a metabolite does exist.

An LC-MS/MS method was developed to identify the major metabolites through comparison of the molecular ion and enzyme hydrolysis even when authentic metabolite standards are not available as described by Li et al. [21]. The metabolic pathway for ZLJ-601 involved mono-hydroxylation and additional glucuronide conjugation of the hydroxylated metabolite, but we were not sure on which position the hydroxylation occurred. No glucuronide conjugate was present in feces, suggesting that hydrolysis of the glucuronide metabolite occurred in the GI tract.

**Conclusions**

In summary, ZLJ-601 can eliminate quickly from blood and its bioavailability is low and that it is readily distributed to most tissues but cannot cross the blood–brain barrier efficiently. The major excretion route of the parent drug is in the urine and the main metabolites are the hydroxylation product and the glucuronide conjugate of the hydroxylation product.

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**References**


