

Drug Interactions of Imperatorin and Curcumin on Macitentan *in vitro* and *in vivo*

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Purpose: The purpose of this study was to establish an *in vitro* incubation system and an *in vivo* model to investigate the potential kinetic interactions of macitentan with imperatorin and curcumin, and to validate the potential inhibitory mechanisms using molecular docking.

Methods: *In vitro*, the enzyme kinetic profile of macitentan was explored in rat liver microsomes (RLM) and human liver microsomes (HLM). Furthermore, molecular docking technique was used to study the sites of action of macitentan, imperatorin, and curcumin with CYP 3A4. *In vivo*, the pharmacokinetic parameters of macitentan were investigated in Sprague-Dawley (SD) rats administered the drug orally, both as a single agent and in combination with imperatorin and curcumin.

Results: *In vitro*, the results indicated that imperatorin and curcumin could inhibit the metabolism of macitentan, with IC_{50} values of 6.58 μ M and 10.86 μ M in RLM and 6.97 μ M and 5.71 μ M in HLM, respectively. And in the study of inhibition type, in RLM, the inhibition types of imperatorin and curcumin on macitentan were mixed and non-competitive, respectively; in HLM, the inhibition types of imperatorin and curcumin on macitentan were both mixed. Furthermore, additional molecular docking studies demonstrated that both imperatorin and curcumin occupied the CYP3A4 site. *In vivo*, the result showed significant increases in $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, T_{max} , $t_{1/2}$, and C_{max} for macitentan while a decrease in $CL_{z/F}$ when combined with imperatorin. The metabolite ACT-132577 exhibited substantial increases in $t_{1/2}$, T_{max} , and C_{max} . Combined with curcumin, the $AUC_{(0-\infty)}$ and T_{max} of macitentan were significantly increased, while $CL_{z/F}$ was significantly decreased. Conversely, the metabolite ACT-132577 exhibited a substantial decrease in $CL_{z/F}$, accompanied by notable increases in $AUC_{(0-\infty)}$ and T_{max} .

Conclusion: *In vitro* and *in vivo* studies revealed that imperatorin and curcumin exhibited inhibitory effects on the metabolism of macitentan. Furthermore, molecular docking revealed that the metabolic inhibition of macitentan by imperatorin and curcumin occurred through binding to the site on CYP3A4. However, further investigation is necessary to ascertain whether this phenomenon will occur in humans.

Keywords: pharmacokinetics, molecular docking, UPLC-MS/MS, inhibition mechanism

Introduction

Pulmonary arterial hypertension (PAH) is a rare cardiovascular disease characterized by a pathology of high pulmonary arteries and increased pulmonary vascular resistance, which ultimately leads to increased right ventricular afterload, right heart failure and functional decline.^{1,2} This condition eventually progresses to cardiac arterial hypertension.³ The endothelin (ET) pathway plays a pivotal role in the pathogenesis of PAH.⁴ In the context of PAH pathophysiology, the activation of endothelin A (ETA) receptors has been shown to promote vasoconstriction, smooth muscle proliferation, and fibrosis.^{5,6} In contrast, endothelin B (ETB) receptors have been observed to mediate endothelium-dependent vasodilatation, involving the release of nitric oxide, and pro-fibrotic responses.^{7,8} This intricate receptor complexity underscores the necessity for targeted therapeutic modulation. For the treatment, ET receptor antagonists (ERAs) have become important therapeutic agents for PAH.⁹

Macitentan is a novel dual (ETA and ETB) ERA that has been approved for the treatment of PAH.¹⁰ By blocking the endothelin system, it improves hemodynamics, right ventricular hypertrophy and survival.¹¹ First, macitentan has been shown to inhibit vasoconstriction and remodeling by inhibiting ET-1-mediated vascular smooth muscle contraction through the inhibition of the ETA receptor and the reduction of smooth muscle cell proliferation and migration.¹² Furthermore, the simultaneous inhibition of ETA and ETB receptors has been shown to reduce the expression of inflammatory and pro-fibrotic factors, thereby delaying the progression of PAH.¹³ Macitentan is based on the structure of bosentan, with the sulphonamide portion replaced by an aminosulfonyl portion that is more efficacious, better tolerated and safer, and reduces the risk of liver injury.¹⁴

Studies have demonstrated that macitentan is primarily metabolized by CYP3A4 to the pharmacologically active metabolite ACT-132577 (also known as N-despropyl macitentan) and the inactive metabolite ACT-373898.¹⁵ The active metabolite, ACT-132577, is of particular importance. Some factors affecting enzyme activity, such as drug combinations, may inhibit drug metabolism and increase plasma exposure, which may result in increased adverse drug reactions.

Imperatorin is a naturally occurring furanocoumarin that has been demonstrated to reduce cardiac hypertrophy, attenuate myocardial fibrosis and thereby improve pulmonary oedema.^{16–18} It is used clinically in a wide range of cardiovascular patients, especially those suffering from hypertension, hyperlipidaemia and diabetes mellitus.^{19,20} Curcumin is a natural polyphenolic compound extracted from turmeric,²¹ which has the potential to reverse the development of PAH and pulmonary vascular remodeling by preserving mitochondrial function in VSMCs and delaying pulmonary fibrosis.²² A frequent adverse effect of macitentan is hepatic injury.²³ Prior research has indicated that imperatorin exerts a protective effect against acetaminophen-induced hepatic injury, and curcumin has been demonstrated to have a protective effect against liver ischemia/reperfusion (I/R) injury.^{24,25} Consequently, it may be hypothesized that the combination of macitentan with imperatorin or curcumin could potentially reduce the risk of liver injury adverse effects and hepatotoxicity. Therefore, the combination of imperatorin and curcumin with PAH drugs has potential possibilities. However, imperatorin and curcumin share the same metabolic enzyme, CYP3A4, with macitentan. This suggests the possibility of CYP3A4-mediated drug-drug interactions between macitentan and imperatorin or curcumin.

The aim of this study was to investigate how imperatorin and curcumin interact with macitentan and the potential mechanisms of inhibition. The present study established two *in vitro* incubation systems, including rat liver microsomes (RLM) and human liver microsomes (HLM) incubation systems to assess the inhibitory effects and potential mechanisms of imperatorin and curcumin on macitentan metabolism. The results of molecular docking also predict the underlying mechanisms. Furthermore, an *in vivo* pharmacokinetic study in rats was performed to evaluate the possible pharmacokinetic interactions of imperatorin and curcumin with macitentan.

Materials and Methods

Chemicals and Reagents

Macitentan (Figure 1A),²⁶ its main metabolite ACT-132577 (Figure 1B),²⁶ imperatorin (Figure 1C),²⁷ curcumin (Figure 1D),²⁸ and bosentan (used as internal standard, IS) were bought from Shanghai Canspec Scientific Instruments Co., Ltd. (Shanghai, China). The HLM used in the experiment was purchased from iPhase Pharmaceutical Services Co., Ltd. (Jiangsu, China). For reagents, we bought nicotinamide adenine dinucleotide phosphate (NADPH) from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China), and methanol and acetonitrile in LC grade from Merck (Darmstadt, Germany). Formic acid was purchased from Anaqua Chemicals Supply (ACS, USA). Ultrapure water was prepared using a Milli-Q water purification system from Millipore (Bedford, MA, USA). All other chemicals and reagents used were also of LC grade.

Instruments and Analytical Conditions

Chromatographic separations were conducted on an UPLC-MS/MS system comprising a Waters Xevo TQ-S triple quadrupole tandem mass spectrometer (Milford, MA, USA) with an electrospray ionization source and a Waters Acquity UPLC I-Class system. An Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm) was utilized to facilitate the separation of the compounds of interest and potential interferences. The mobile phase was consisted of a 0.1% formic acid solution

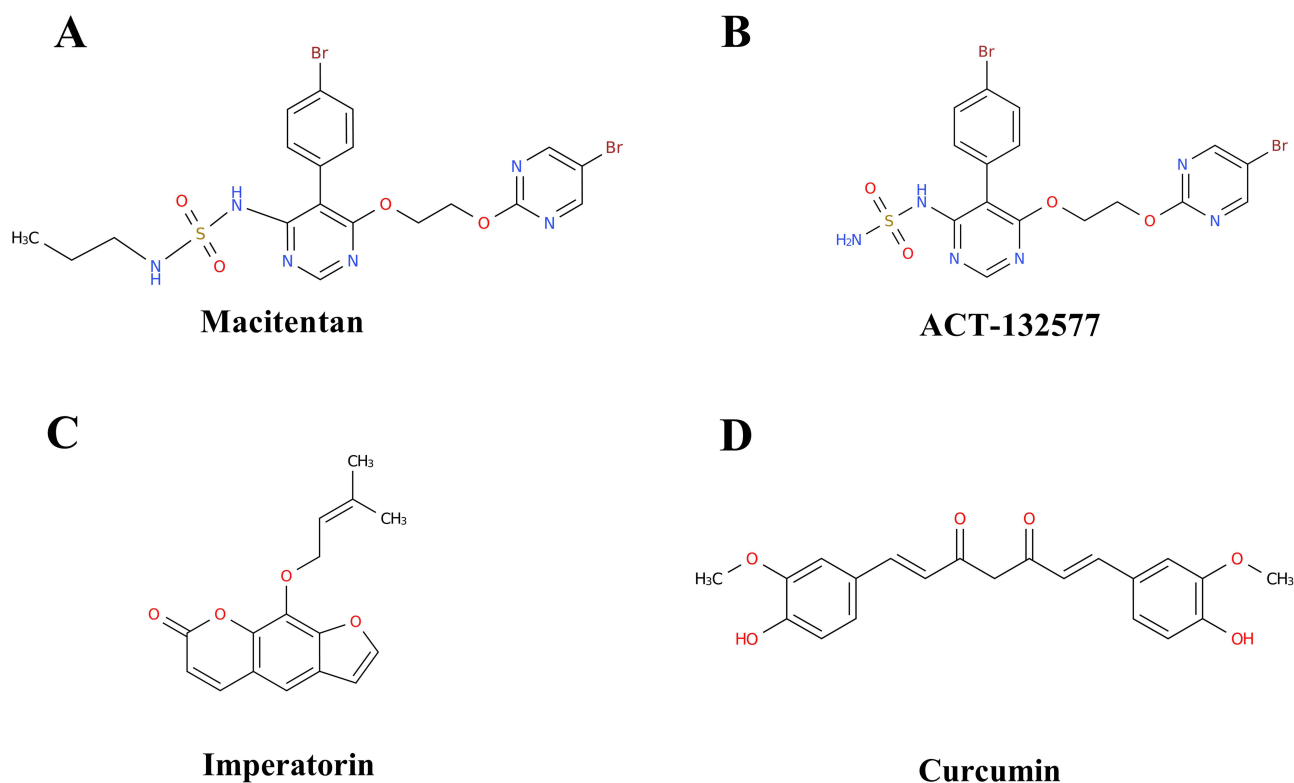


Figure 1 Schematic chemical structures of macitentan (**A**), ACT-132577 (**B**), imperatorin (**C**), and curcumin (**D**).

(solution A) and acetonitrile (solution B). The gradient elution procedure was as follows: 0–0.5 min (90% solution A), 0.5–1.0 min (90%–10% solution A), 1.0–1.4 min (10% solution A), 1.4–1.5 min (10%–90% solution A), 1.5–2.0 min (90% solution A). A single sample was injected into the UPLC-MS/MS at a volume of 2.0 μ L and eluted at a flow rate of 0.40 mL/min for 2.0 min. The temperature of the autosampler was maintained at 10°C, while the temperature of the column was maintained at 40°C.

The samples were quantified by a Waters Xevo TQ-S triple quadrupole tandem mass spectrometer. The levels of macitentan, ACT-132577 and IS were detected in positive mode using multiple reaction monitoring (MRM). The data were collected and analyzed using MassLynx 4.1 software (Waters Corp, Milford, MA, USA). Typical UPLC-MS/MS chromatograms in rat plasma samples were presented in Figure 2.

Rat Liver Microsomes (RLM)

Six rat livers were weighed and rinsed three times with phosphate-buffered saline (PBS). They were then cut into small pieces. The samples were homogenized in a homogenizer using PBS-0.25 mM sucrose buffer and subsequently subjected to ultracentrifugation at 11,000 rpm for 15 min at 4°C, this process was repeated twice. Subsequently, the precipitate was discarded, and the supernatant was collected. The supernatant was then removed by centrifugation at $75,600 \times g$ for 2 h at 4°C. The supernatant was then discarded, and the precipitate was collected and diluted with a volume of cold PBS that was between two and three times the original volume. This solution was then pipetted repeatedly with a pipette gun. The diluted solution was then poured back into the same centrifuge tube to homogenize it. Finally, the solution was dispensed and stored in a container at -80°C . The Bradford Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used to measure the protein concentration.²⁹

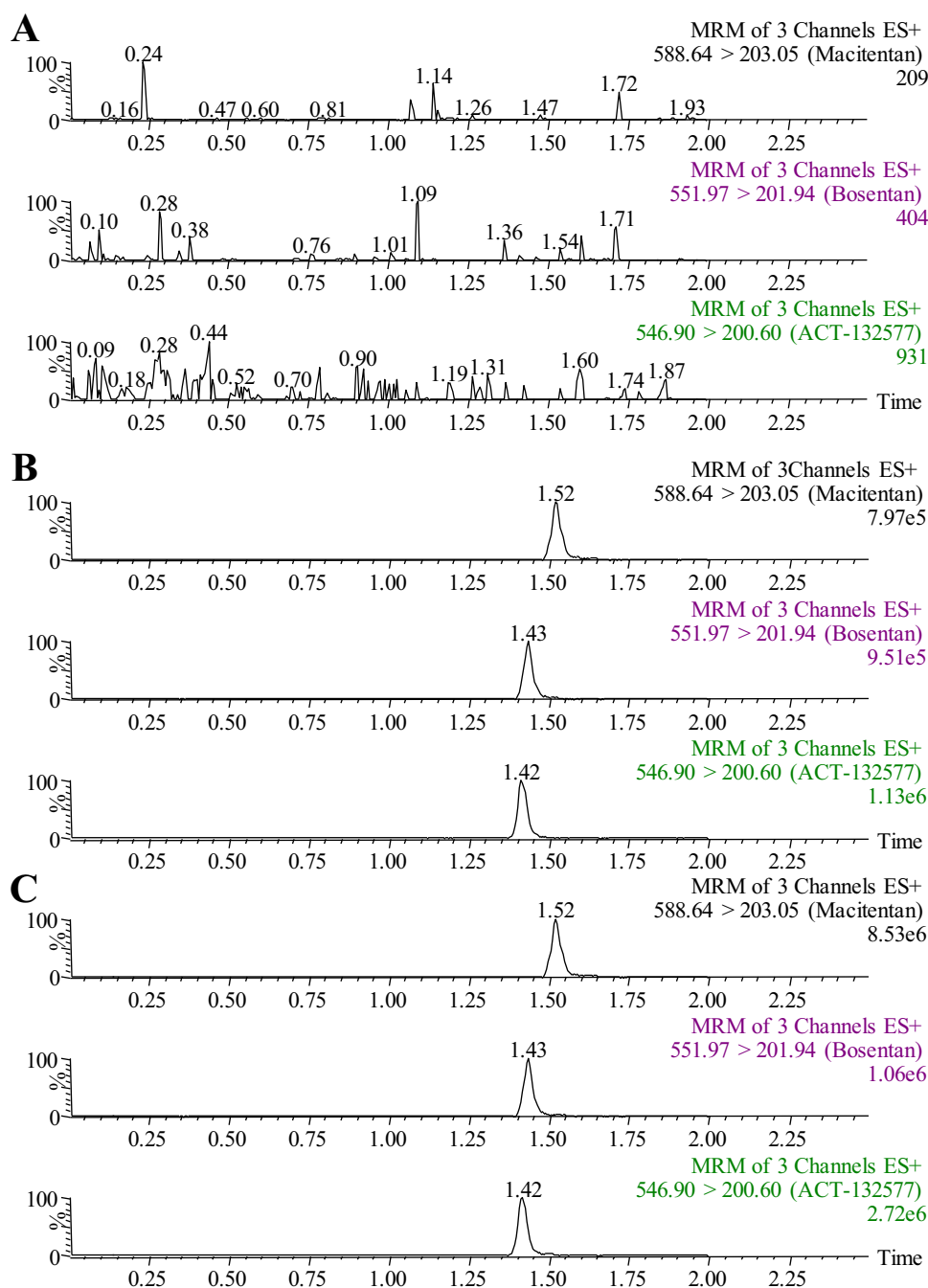


Figure 2 Representative MRM chromatograms: **(A)** blank plasma sample without analyte and IS; **(B)** blank plasma sample containing analyte and IS; and **(C)** rat plasma sample after oral administration of 10 mg/kg of macitentan alone.

Abbreviation: MRM, multiple reaction monitoring.

In vitro Experiment

The experimental design was informed by extant published literature.²⁹ The 190 μ L primary incubation system was consisted of PBS (pH 7.4, 100 mM), different concentrations of macitentan (1, 5, 10, 20, 50, 100, 150, 200 μ M), and RLM or HLM (0.3 mg/mL). The enzyme reaction was initiated by the addition of 10 μ L of NADPH (1 mM), following a 5 min preincubation at 37°C. The samples were incubated for 30 min and stored in a refrigerator at -80°C. Following the termination of the reaction, 400 μ L acetonitrile and 20 μ L of the IS working solution were added to the system. The

samples were then vortexed for 2 min and subsequently centrifuged in a pre-cooled centrifuge for 10 min. Thereafter, 100 μ L of the supernatant was aspirated for the quantification of the metabolite of macitentan by UPLC-MS/MS. The objective was to determine the Michaelis constant (K_m) of macitentan.

The 190 μ L primary incubation system was consisted of PBS (pH 7.4, 100 mM), macitentan (concentration in RLM: 8.81 μ M, concentration in HLM: 68.30 μ M), imperatorin/curcumin (0.01, 0.1, 1, 10, 25, 50, 100 μ M), and RLM or HLM (0.3 mg/mL). The samples were prepared in triplicate. Subsequent samples were also subjected to the aforementioned method in order to determine the half-maximum inhibitory concentration (IC_{50}) of imperatorin/curcumin on macitentan.

In addition, IC_{50} shift experiments were performed to verify whether the inhibition of macitentan by imperatorin and curcumin was time-dependent. The pre-incubation system was contained of PBS, macitentan (8.81 μ M), imperatorin/curcumin (0.01, 0.1, 1, 10, 25, 50, 100 μ M), and RLM (0.3 mg/mL). The samples were prepared in triplicate. The samples were divided into two groups: one group was pre-incubated without NADPH, and the other group was pre-incubated with NADPH. Following a 30-min pre-incubation period, macitentan (8.81 μ M) was added to the samples, and the samples were then incubated for an additional 30 min. Thereafter, the samples were transferred to -80°C . Subsequent samples were treated in an identical manner to the aforementioned samples.

In order to illustrate the inhibitory effects of imperatorin and curcumin on macitentan *in vitro*, a series of concentration gradients were established based on the IC_{50} and K_m values. In RLM, the concentration of macitentan ranged from 2.20 to 17.61 μ M, the concentration of imperatorin ranged from 0 to 6.53 μ M, and the concentration of curcumin ranged from 0 to 21.72 μ M. In HLM, the concentration of macitentan ranged from 17.08 to 136.60 μ M, the concentration of imperatorin ranged from 0 to 6.97 μ M, and the concentration of curcumin ranged from 0 to 5.71 μ M. The samples were prepared in triplicate. The incubation mixtures were prepared as previously described, and the samples were handled in accordance with the aforementioned methodology.

In vivo Pharmacokinetic Experiments

All male Sprague-Dawley (SD) rats (200–220 g) were sourced from the Animal Experimental Center of the First Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). The entirety of the experiment was overseen by Institutional Animal Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University, and was granted ethical approval with the ethical review number: WYYY-IACUC-AEC-2024-042. This experiment was conducted according to the Regulations and Rules of “Guidelines for Ethical Review of the Welfare in Laboratory Animals” (2018). The rats were maintained in the SPF environment, provided with standard feed and water *ad libitum*, and fasted for 12 h prior to the pharmacokinetic experiments. The SD rats were housed under controlled temperature conditions, maintained within a range of $18\text{--}29^{\circ}\text{C}$, with a maximum daily temperature fluctuation of 3°C . The relative humidity was kept at a range of 40%–70%, and the light exposure was alternated between 12 h of light and 12 h of darkness. A total of fifteen SD rats were randomly divided into three groups ($n = 5$). In the report by Patricia N. Sidharta et al, plasma concentrations were found to be intermediate when 100 mg of macitentan was administered to humans at doses ranging from 0.2 to 600 mg.³⁰ Therefore, the conversion of human to animal doses was performed by means of body surface area normalization. In this regard, rats were administered 10 mg/kg of macitentan orally. The doses of imperatorin and curcumin referenced by previous authors have been documented in the extant literature.^{31,32} Group A was served as the control group and was received 10 mg/kg of macitentan. Groups B and C were the experimental groups and were received 30 mg/kg of imperatorin and 20 mg/kg of curcumin, respectively. Drug administration was conducted via gavage. At the commencement of the experiment, 30 mg/kg of imperatorin and 20 mg/kg of curcumin were administered to Group B and C, respectively. 30 min later, 10 mg/kg of macitentan was administered to all three groups. Macitentan, imperatorin, and curcumin were all dissolved in corn oil to create homogeneous solutions and their concentrations were 2, 6 and 4 mg/mL, respectively. The precise volume of the administered drug for rats was 5.0 mL/kg. Blood samples were collected from the tail vein at 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24, and 36 h, and were centrifuged (13,000 rpm, 5 min, 4°C). Finally, 100 μ L of the supernatant was aspirated and stored temporarily in a -80°C refrigerator. Prior to analysis, the samples were thawed and 10 μ L of IS working solution and 300 μ L of acetonitrile were added. The samples were vortexed for 2 min and centrifuged (13,000 rpm, 10 min, 4°C). The 100 μ L aliquot of the supernatants were

aspirated for quantitative analysis by UPLC-MS/MS. After the experiment, all rats were euthanized by intravenous injection of 150 mg/kg phenobarbital.

Molecular Docking

The protein structures were obtained from the Protein Data Bank (PDB ID: 5TE8; <https://www.rcsb.org/>), and the three-dimensional sdf-format files for macitentan, imperatorin, curcumin, midazolam (probe substrate of CYP3A4) and ketoconazole (strong CYP3A4 inhibitor) were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The three-dimensional protein conformation was optimized using PyMOL, with the removal of chain B, chain C, the original ligand and water, and the addition of hydrogen atoms using AutoDock Tools 1.5.6. Semi-flexible docking was performed using AutoDock 4.2.6, with a maximum output of 50 gestures. The best scoring conformations were visualized using PyMOL.

Data Analysis

In vitro enzyme kinetic parameters, including K_m , IC_{50} , and inhibition constant (K_i), were calculated using GraphPad Prism 8.3 software. All relevant curves, including plasma concentration-time graphs, were plotted. Individual pharmacokinetic parameters were calculated in vivo in rats using Drug and Statistics (DAS) 2.0 software. Statistical analyses between groups were performed using independent samples *t*-test in SPSS 25.0 software, with a significance level of $P < 0.05$.

Results

Optimization of Analytical Conditions

As illustrated in Figure 2, the chromatographic and mass spectrometric conditions were effectively optimised for the quantitative analysis of macitentan and ACT-132577 utilising a dependable and efficient UPLC-MS/MS analytical methodology. The peaks of macitentan, IS and ACT-132577 were observed at 1.52, 1.43 and 1.42 min, respectively, and the three analytes were successfully separated without any interference from other peaks. The chromatographic conditions were optimised in the course of the experiment. The separation was conducted on an Acquity BEH C18 column (2.1 mm \times 50 mm, 1.7 μ m). The mobile phase was comprised of a 0.1% solution of formic acid (solution A) and acetonitrile (solution B) at a flow rate of 0.40 mL/min, with a gradient elution time of 2.0 min. It was established that a solution of 0.1% formic acid and acetonitrile yielded the optimal response and peak shape. The brief elution time and high efficiency met the requirements for a multi-sample assay.

The mass spectrometry conditions were also optimised, and the mass spectral information was obtained from the ESI source in both positive and negative ion modes. The highest peak intensities of the analytes were observed in the positive ion mode. The mass spectrometry analysis of macitentan, IS and ACT-132577 revealed that the most prevalent protolated molecules were observed at m/z 588.64, 551.97, and 546.90, respectively. Additionally, the most abundant fragment ion spectra appeared at m/z 203.05, 201.94, and 200.60, respectively.

Effects of Imperatorin and Curcumin on the Metabolism of Macitentan in vitro

The effects of imperatorin and curcumin on the metabolism of macitentan in vitro were evaluated in two models (RLM and HLM). The results were presented in Figure 3–6, which showed Michaelis-Menten plots, IC_{50} curves (including IC_{50} curves for RLM), Lineweaver-Burk plots for RLM and HLM. The K_m values of macitentan in RLM and HLM were 8.81 μ M (Figure 3A) and 68.30 μ M (Figure 3B), respectively. The IC_{50} values of imperatorin and curcumin in RLM were 6.58 ± 1.14 μ M (Figure 4A) and 10.86 ± 0.13 μ M (Figure 4B), respectively. In HLM, the IC_{50} values of imperatorin and curcumin were 6.97 ± 0.07 μ M (Figure 4C) and 5.71 ± 0.15 μ M (Figure 4D), respectively. The above data illustrated that imperatorin strongly inhibited the metabolism of macitentan in both RLM and HLM, whereas curcumin inhibited the metabolism of macitentan more strongly in HLM than in RLM. IC_{50} shift experiments demonstrated that the IC_{50} values in the presence of imperatorin were 2.80 ± 0.04 μ M and 1.25 ± 0.12 μ M for pre-incubation without and with NADPH, respectively, with an IC_{50} shift fold of 2.24 (Figure 4E). In the presence of curcumin, the IC_{50} values in the pre-incubation solution with and without

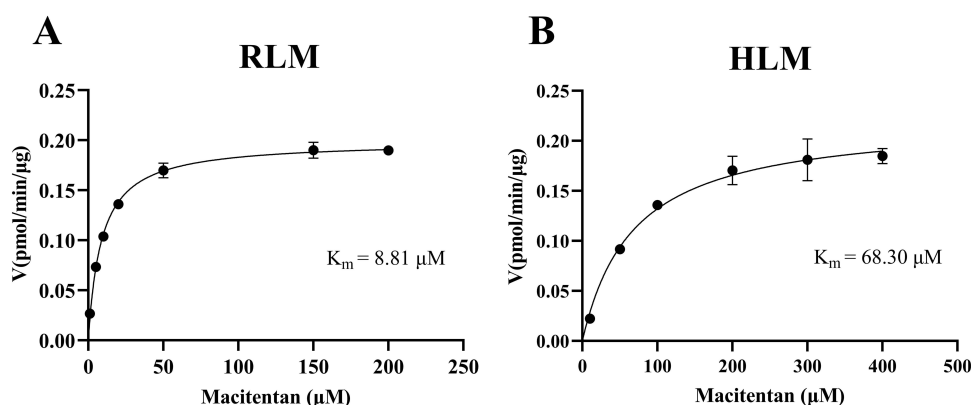


Figure 3 Michaelis-Menten plots for macitentan in RLM (A) and HLM (B). V, velocity of enzyme catalysis; K_m , Michaelis-Menten constant.

NADPH were $5.69 \pm 0.25 \mu\text{M}$ and $9.00 \pm 0.24 \mu\text{M}$, respectively, with an IC_{50} shift fold of 0.63 (Figure 4F). Their IC_{50} shift folds were less than 10, which could be considered to be a non-time-dependent inhibition.³³

Therefore, the mechanism of the inhibition of macitentan in different microsomes incubation systems was further explored (Figure 5 and 6). In RLM, the Lineweaver-Burk plots of imperatorin and curcumin demonstrated that the type of metabolic inhibition of macitentan by imperatorin and curcumin were mixed and non-competitive, respectively. In HLM, the Lineweaver-Burk plots of imperatorin and curcumin demonstrated that the type of metabolic inhibition of macitentan by imperatorin and curcumin were mixed. In summary, both imperatorin and curcumin could inhibit macitentan metabolism in vitro.

Effects of Imperatorin and Curcumin on the Metabolism of Macitentan in vivo

Figure 7 illustrated the mean plasma concentration versus time curves of macitentan and ACT-132577 in different SD rats, and Table 1 and Table 2 presented the main pharmacokinetic parameters. The $\text{AUC}_{(0-t)}$, $\text{AUC}_{(0-\infty)}$, $t_{1/2}$, T_{\max} , $\text{CL}_{z/F}$, and C_{\max} of macitentan were significantly altered following imperatorin administration in comparison to the control group. In particular, the $\text{AUC}_{(0-t)}$, $\text{AUC}_{(0-\infty)}$, and T_{\max} were increased by approximately one-fold. The C_{\max} of macitentan was increased by 0.56-fold, while the $t_{1/2}$ was also correspondingly prolonged (from $2.64 \pm 0.40 \text{ h}$ to $3.40 \pm 0.53 \text{ h}$). In addition, the $\text{CL}_{z/F}$ was significantly reduced by approximately 0.46-fold. For the metabolite ACT-132577, both $t_{1/2}$, T_{\max} and C_{\max} exhibited significant alterations. Its $t_{1/2}$ was shortened from $6.73 \pm 0.63 \text{ h}$ to $5.51 \pm 0.71 \text{ h}$. Following curcumin administration, macitentan exhibited significant increases in $\text{AUC}_{(0-\infty)}$ and T_{\max} by 0.34-fold and 1.8-fold, respectively, and a significant decrease in $\text{CL}_{z/F}$ (from $0.41 \pm 0.06 \text{ L/h/kg}$ to $0.31 \pm 0.07 \text{ L/h/kg}$). Although the $t_{1/2}$ values were not statistically different, there was a tendency for the $t_{1/2}$ to be prolonged (from $2.64 \pm 0.40 \text{ h}$ to $4.02 \pm 1.92 \text{ h}$). For the metabolite ACT-132577, there were significant increases in $\text{AUC}_{(0-\infty)}$ and T_{\max} , and a significant decrease in $\text{CL}_{z/F}$. The $t_{1/2}$ was found to be prolonged from $6.73 \pm 0.63 \text{ h}$ to $8.98 \pm 3.13 \text{ h}$. These results indicated that both imperatorin and curcumin significantly inhibited the metabolism of macitentan in SD rats, and the inhibitory effect of imperatorin on the plasma exposure of macitentan was more significant in comparison to curcumin.

Molecular Docking

To further validate the inhibitory effects of imperatorin and curcumin on macitentan, molecular docking analyses were performed. As illustrated in Figure 8A and B, imperatorin (cyan), curcumin (slate), and macitentan (salmon) all spontaneously bind to the active site of CYP3A4. Molecular docking analyses demonstrated that macitentan exhibited hydrogen bonding interaction forces with GLU-374 (3.6 Å) and LEU-216 (1.8 Å), while imperatorin formed a hydrogen bond with PHE-304 (3.6 Å). Curcumin was also able to bind to HEM-601 (2.3 Å), ARG-372 (3.0 Å), LEU-216 (2.8 Å) and ALA-305 (2.0 Å) to form one hydrogen bond and ASP-214 (1.8 Å and 3.0 Å) to form two hydrogen bonds, respectively. The binding energies of the macitentan, imperatorin and curcumin to CYP3A4 were -9.24 kcal/mol , -6.60 kcal/mol and -9.13 kcal/mol , respectively. As illustrated in Figure 8C, midazolam could bind to CYP3A4 with a binding energy of -8.44 kcal/mol , whereas ketoconazole could bind to GLU-374 (2.7 Å), ALA-370 (3.4 Å), and PHE-213 (2.5

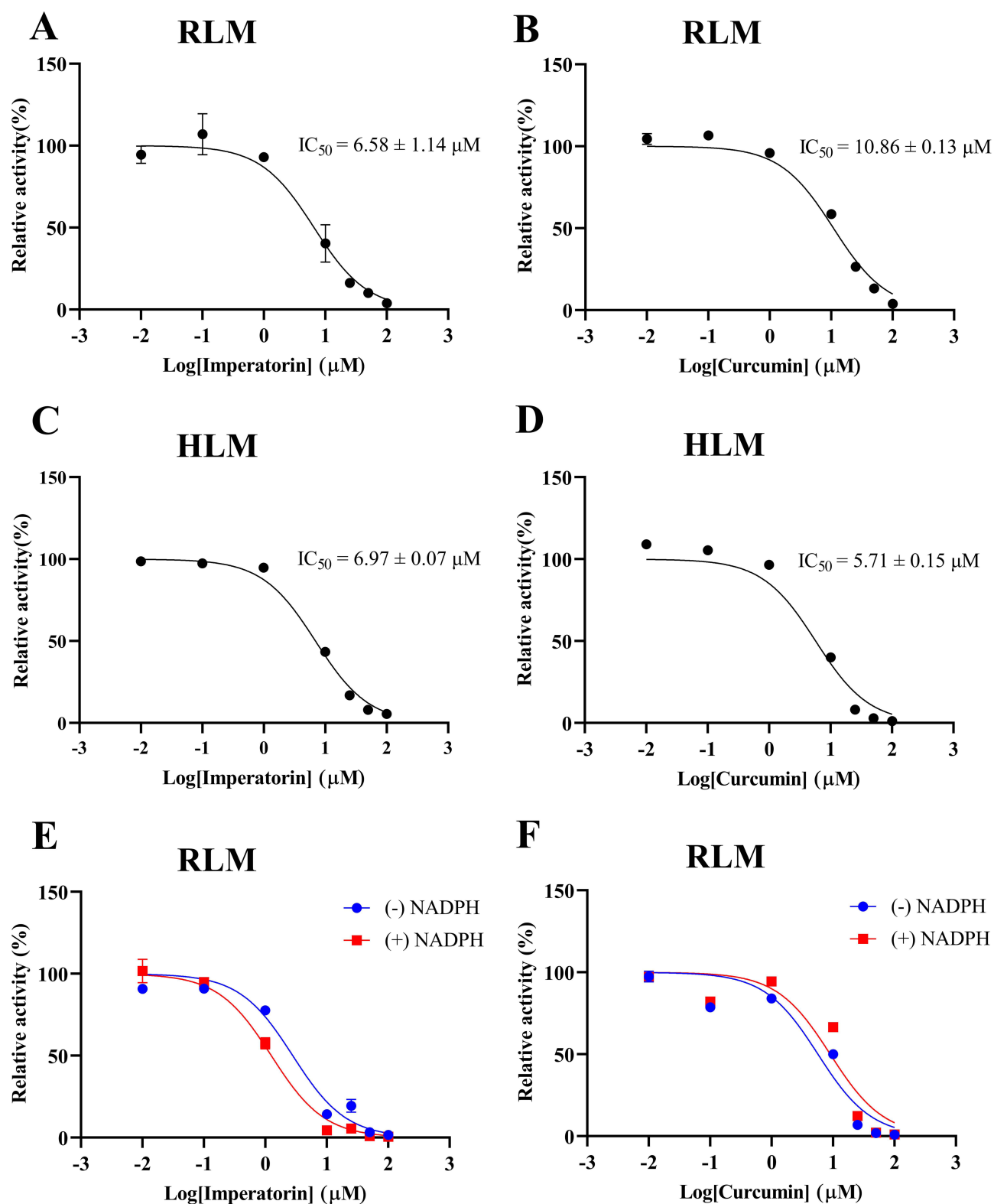


Figure 4 IC_{50} curves of imperatorin and curcumin on macitentan metabolism in RLM (**A** and **B**) and HLM (**C** and **D**). IC_{50} shift curves of imperatorin and curcumin on macitentan metabolism in RLM (**E** and **F**).

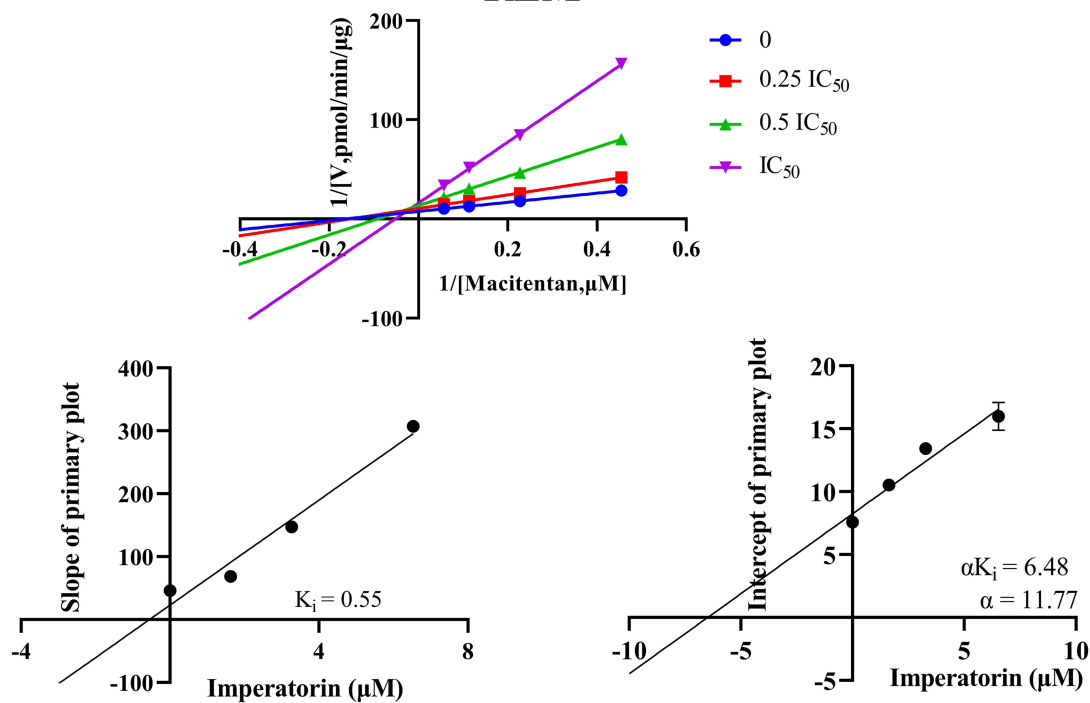
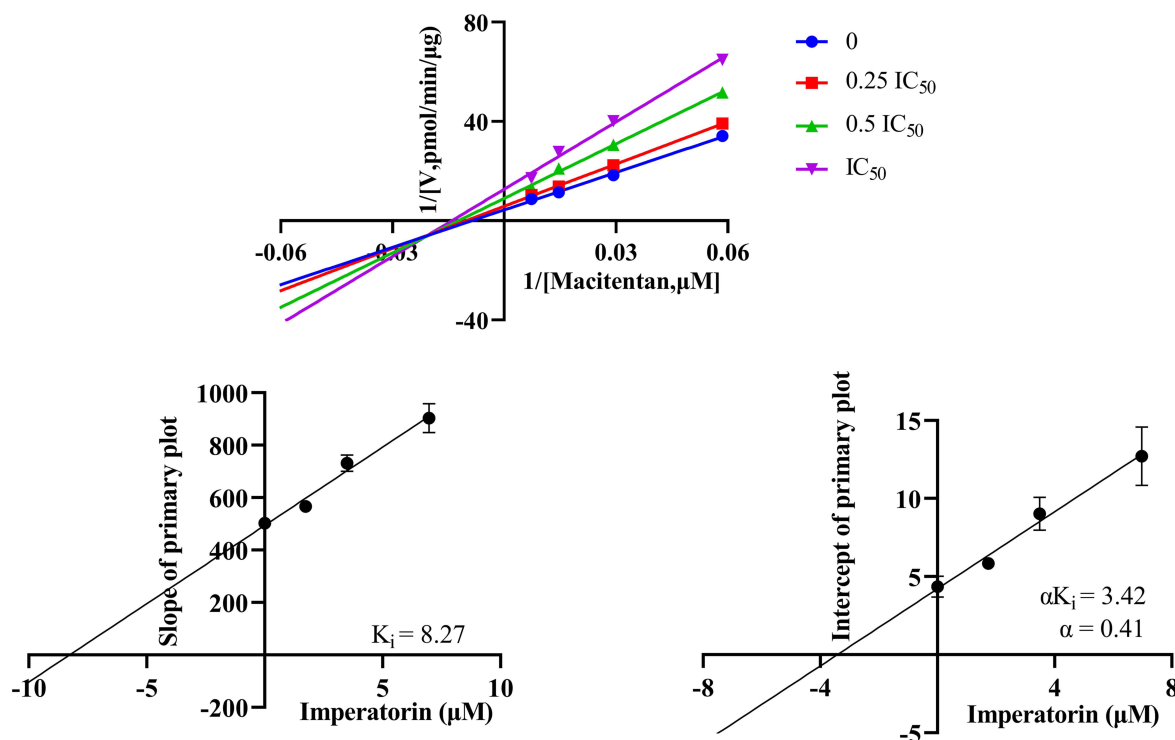
A**RLM****B****HLM**

Figure 5 In RLM (A) and HLM (B), Lineweaver-Burk plots, K_i secondary plots and αK_i secondary plots of inhibition of macitentan metabolism by different concentrations of imperatorin.

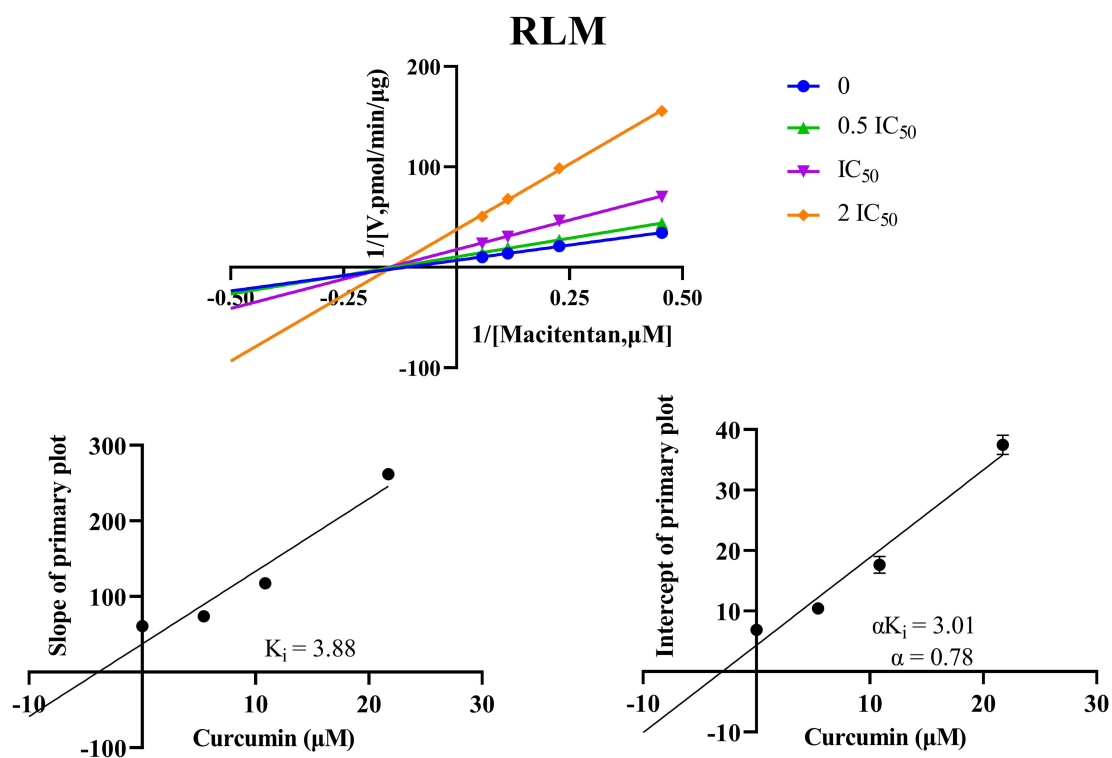
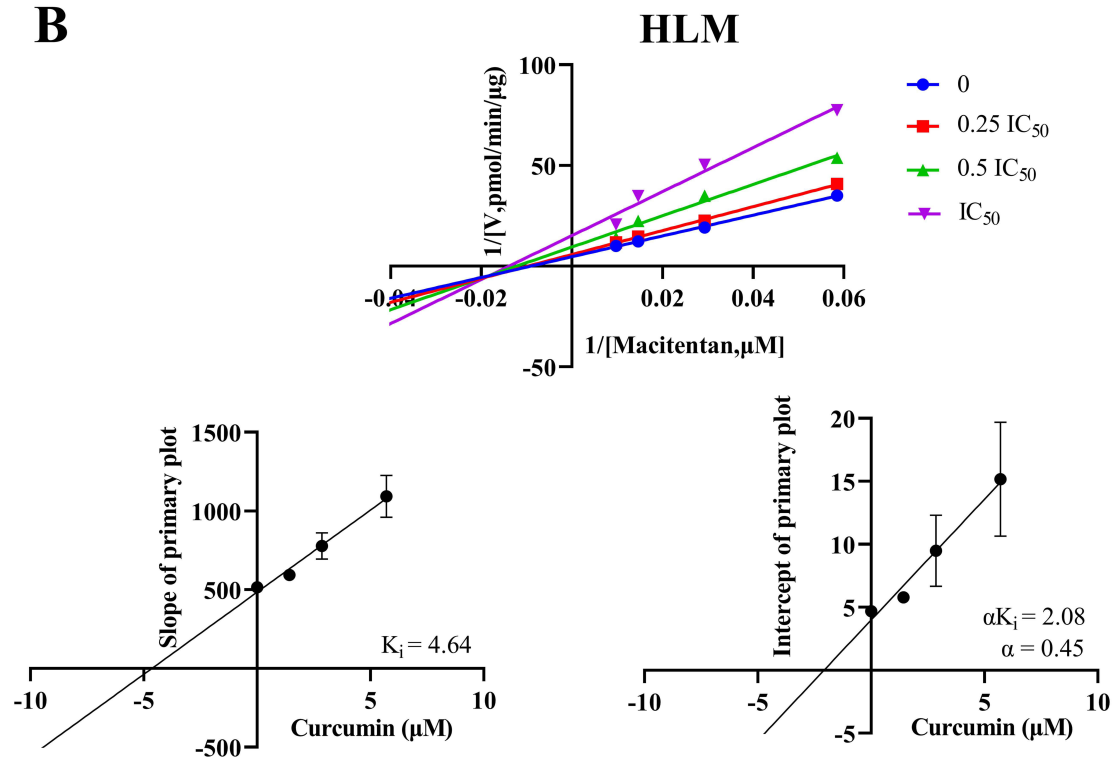
A**B**

Figure 6 In RLM (A) and HLM (B), Lineweaver-Burk plots, K_i secondary plots and αK_i secondary plots of inhibition of macitentan metabolism by different concentrations of curcumin.

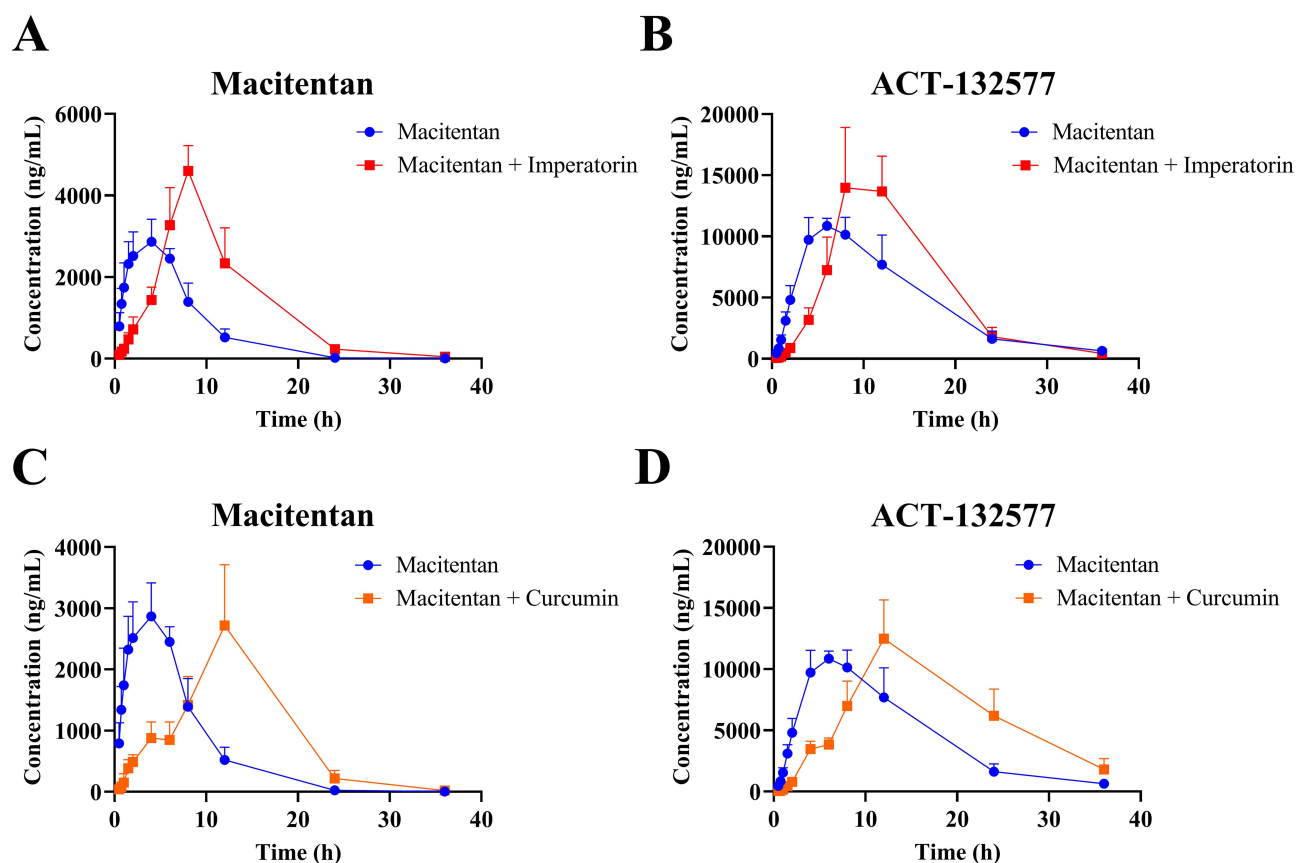


Figure 7 Mean plasma concentration-time curves of macitentan (**A** and **C**) and its metabolite ACT-132577 (**B** and **D**) in different groups of SD rats (n = 5).

Å), forming a total of three hydrogen bonds with a binding energy of -9.52 kcal/mol. Furthermore, macitentan, imperatorin, and curcumin were docked with CYP3A4. The docking results demonstrated that the distances from the action groups of macitentan, imperatorin, and curcumin to the ferrous ions in hemoglobin were 12.7, 7.2, and 3.6 Å (Figure 9A–C). It was observed that imperatorin and curcumin exhibited a closer proximity to ferrous ions in comparison to macitentan, indicating that imperatorin and curcumin possess a potentially stronger affinity for CYP3A4 compared to macitentan. This observation may provide a crucial rationale for the observed inhibition of macitentan metabolism by imperatorin and curcumin in HLM.

Table 1 Main Pharmacokinetic Parameters of Macitentan in Different Groups of SD Rats (n = 5, Mean \pm SD)

Parameters	Macitentan	Macitentan + Imperatorin	Macitentan + Curcumin
AUC _(0-t) (ng/mL*h)	24921.35 \pm 3248.66	46,323.84 \pm 7234.96**	33,121.39 \pm 7153.26
AUC _(0-∞) (ng/mL*h)	24926.96 \pm 3248.80	46,420.58 \pm 7303.56**	33,292.87 \pm 7011.28*
t _{1/2} (h)	2.64 \pm 0.40	3.40 \pm 0.53*	4.02 \pm 1.92
T _{max} (h)	4.00 \pm 1.41	7.60 \pm 0.89**	11.20 \pm 1.79***
CL _{z/F} (L/h/kg)	0.41 \pm 0.06	0.22 \pm 0.04***	0.31 \pm 0.07*
C _{max} (ng/mL)	3073.59 \pm 346.46	4787.35 \pm 388.31***	2775.68 \pm 902.31

Notes: * P < 0.05, ** P < 0.01, *** P < 0.001, compared with macitentan group.

Abbreviations: AUC, area under the plasma concentration-time curve; t_{1/2}, elimination half-life; T_{max}, peak time; CL_{z/F}, plasma clearance; C_{max}, maximum plasma concentration.

Table 2 Main Pharmacokinetic Parameters of ACT-132577 in Different Groups of SD Rats (n = 5, Mean \pm SD)

Parameters	Macitentan	Macitentan + Imperatorin	Macitentan + Curcumin
AUC _(0-t) (ng/mL*h)	164940.45 \pm 29,868.59	198,014.86 \pm 42,067.42	222,049.13 \pm 50,075.25
AUC _(0-∞) (ng/mL*h)	171175.79 \pm 30,840.23	201,388.16 \pm 43,129.35	250,911.48 \pm 59,657.73*
t _{1/2} (h)	6.73 \pm 0.63	5.51 \pm 0.71*	8.98 \pm 3.13
T _{max} (h)	5.60 \pm 1.67	10.40 \pm 2.19**	12.00 \pm 0.00**
CL _{z/F} (L/h/kg)	0.06 \pm 0.01	0.05 \pm 0.01	0.04 \pm 0.01*
C _{max} (ng/mL)	11296.37 \pm 552.61	15,788.04 \pm 3663.57*	12,492.86 \pm 3165.15

Notes: * P < 0.05, ** P < 0.01, compared with macitentan group.

Abbreviations: AUC, area under the plasma concentration-time curve; t_{1/2}, elimination half-life; T_{max}, peak time; CL_{z/F}, plasma clearance; C_{max}, maximum plasma concentration.

Discussion

Macitentan, the most recent treatment option for PAH, is metabolized *in vivo* by CYP3A4, with the main metabolite being the pharmacologically active N-despropyl macitentan (ie, ACT-132577).^{34,35} This metabolite has recently been shown to be efficacious in the treatment of recalcitrant hypertension.³⁶ A previous study demonstrated that the combination of macitentan with ketoconazole, a potent CYP3A4 inhibitor, resulted in a significant increase in macitentan plasma exposure, with a two-fold increase in both AUC_(0-t) and AUC_(0-∞).³⁷ This enhanced the oral bioavailability of macitentan. In the presence of rifampicin, a CYP3A4 inducer with a strong effect, the plasma exposure of macitentan was reduced by approximately four times, which may affect the overall pharmacological activity of macitentan *in vivo*.³⁸ Given that macitentan shares the same binding enzyme with imperatorin and curcumin and has the potential to be used in combination in clinical practice, it is of great interest to explore whether there is a drug-drug interaction between macitentan and imperatorin/curcumin.

Firstly, *in vitro* studies were conducted to assess the enzyme kinetic parameters of macitentan using different microsome models. In this study, two hepatic models, RLM and HLM, were employed to investigate the changes on the metabolism of macitentan in the presence of imperatorin or curcumin. The results of the RLM and HLM experiments demonstrated that imperatorin and curcumin inhibited the metabolism of macitentan *in vitro*. However, the degree of inhibition differed between the two models. Imperatorin demonstrated a robust inhibitory effect in both RLM and HLM, with comparable inhibitory potency. In contrast, curcumin exhibited a more pronounced inhibitory effect on macitentan in HLM, with an IC₅₀ value half of that observed in RLM. Additionally, the inhibitory mechanism was investigated, revealing distinct inhibition patterns of macitentan in RLM and HLM. In RLM, imperatorin exhibited mixed-type inhibition, while curcumin exhibited non-competitive inhibition. In HLM, both imperatorin and curcumin exhibited mixed-type inhibition. In one report, imperatorin demonstrated robust inhibition of CYP3A4 (IC₅₀ = 0.5 μ M) and a mixed-type inhibition pattern,³⁹ which aligned with our findings. The IC₅₀ value for human CYP3A4 inhibition by curcumin was 16.30 μ M, indicating a markedly weaker inhibitory effect than that of imperatorin.⁴⁰ This differed from the results of our study in HLM, where the IC₅₀ values of curcumin and imperatorin were found to be similar. This discrepancy may be attributed to individual differences in CYP enzymes involved in metabolism. Furthermore, IC₅₀ shift experiments revealed that the inhibition of macitentan by imperatorin and curcumin would not be time-dependent.

Subsequently, *in vivo* pharmacokinetic studies were conducted in rats to further validate the inhibitory effects of imperatorin and curcumin on macitentan. A previous study reported that following oral administration of 3 mg/kg containing 60 μ Ci/kg ¹⁴C-macitentan to rats, the peak concentration of macitentan was found to be lower than that of the metabolite M6 (ie, ACT-132577), with C_{max} of 0.34 nmol/mL and 0.69 nmol/mL, respectively.⁴¹ It was observed that following oral administration of 10 mg/kg of macitentan to rats, the C_{max} of macitentan was lower than the prototype concentration of ACT-132577, which was consistent with the trend reported in the aforementioned literature. In the presence of imperatorin, the AUC_(0-t) and AUC_(0-∞) of macitentan were significantly increased, with a corresponding prolongation of the t_{1/2} to 3.40 \pm 0.53 h. The T_{max} was observed to extend to 7.60 \pm 0.89 h. The results demonstrated that the C_{max} was significantly increased, while the CL_{z/F} was significantly decreased. The increase in macitentan exposure may be due to the fact that imperatorin inhibited macitentan metabolism. Additionally, the t_{1/2}, T_{max} and C_{max} of ACT-132577 exhibited significant alterations. In the

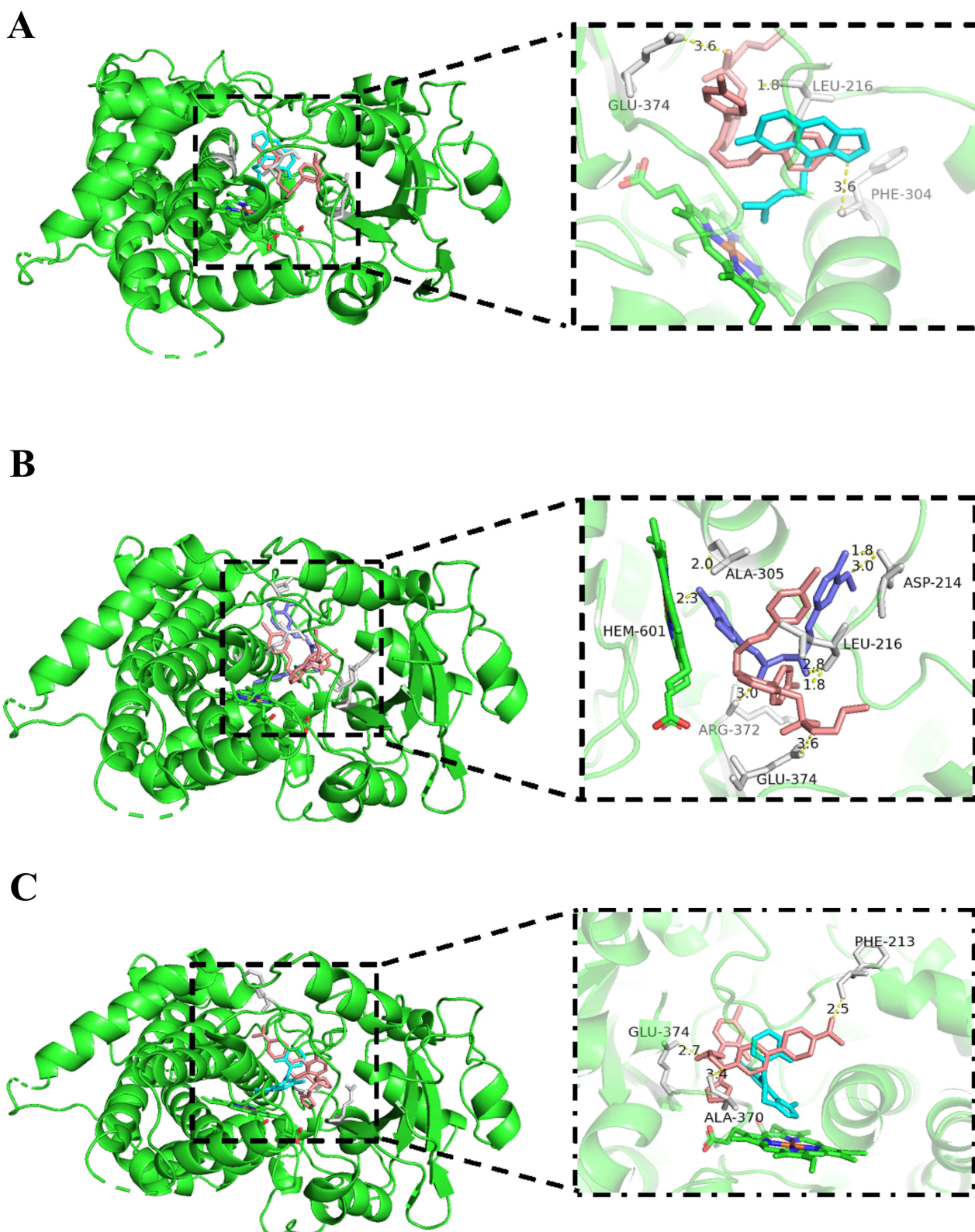


Figure 8 Molecular docking: **(A)** Binding sites of macitentan and imperatorin to CYP3A4; **(B)** binding sites of macitentan and curcumin to CYP3A4. (macitentan: salmon, imperatorin: cyan, curcumin: slate, and the residues are shown in gray.); **(C)** binding sites of midazolam and ketoconazole to CYP3A4. (midazolam: cyan, imperatorin: salmon, and the residues are shown in gray.).

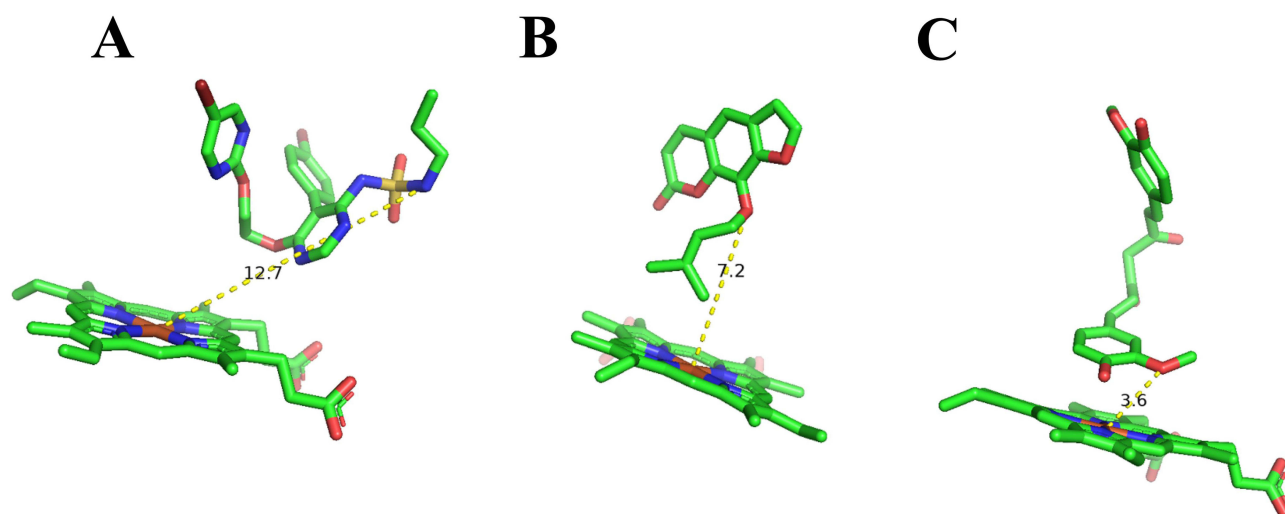


Figure 9 Schematic representation of the distances of macitentan (A), imperatorin (B), and curcumin (C) to ferrous ions in the heme moiety in CYP3A4.

presence of curcumin, the $AUC_{(0-\infty)}$ of macitentan was significantly increased, the T_{max} was significantly prolonged, while the $CL_{z/F}$ was significantly decreased. The $AUC_{(0-\infty)}$ of ACT-132577 was significantly increased, and the T_{max} was also prolonged to 12.00 ± 0.00 h, which was almost the same as that of macitentan. Prolongation of the $t_{1/2}$ of macitentan was observed when administered concomitantly with either imperatorin or curcumin. This phenomenon can be attributed to the inhibition of the activity of metabolic enzymes, leading to a reduced rate of macitentan metabolism. Consequently, an increased residence time was attained *in vivo*, resulting in an augmented plasma exposure and, thereby, a lengthened $t_{1/2}$. Although macitentan is primarily metabolized to ACT-132577 by CYP3A4, it can also be metabolized to ACT-132577 via CYP2C8, CYP2C9, and CYP2C19 (though with a lesser contribution compared to CYP3A4).⁴² In the presence of imperatorin or curcumin, CYP3A4 activity mainly be inhibited, allowing macitentan to be metabolized via the CYP2C8, CYP2C9, and CYP2C19 pathways. This phenomenon may have contributed to the increased concentration of ACT-132577. The metabolism of macitentan was significantly inhibited in the presence of imperatorin/curcumin, resulting in increased bioavailability, indicating that a larger proportion of macitentan may be converted to ACT-132577. The reduced clearance of macitentan may be attributed to two primary factors. First, in terms of metabolism, imperatorin/curcumin had been shown to inhibit the CYP3A4 enzyme. Second, in regard to transport, the hepatic uptake of macitentan was dependent on OATP1B1/1B3,⁴³ and curcumin exhibited an inhibitory effect on the OATP1B1/OATP1B3 activity,⁴⁴ while imperatorin demonstrated no significant inhibitory effect on OATP1B1/OATP1B3 activity, according to the available reports.⁴⁵ Therefore, it can be hypothesized that curcumin may cause a decrease in the clearance of macitentan by partly inhibiting OATP1B1/OATP1B3. The significant multi-fold increase in the T_{max} of ACT-132577 may be attributed to several potential mechanisms. First, it is hypothesized that imperatorin and curcumin themselves may potentially inhibit the CYP3A4 enzyme,^{39,46} and that this inhibition may result in a delay in the conversion of macitentan to ACT-132577. Second, it is hypothesized that changes in gastrointestinal motility, pH, or efflux transporter protein interactions may slow the absorption of macitentan, thereby delaying ACT-132577 formation and resulting in a delayed time to peak.⁴⁷ Furthermore, ACT-132577 may undergo enterohepatic recycling, whereby ACT-132577 is reabsorbed from the intestine after biliary excretion,^{41,48} a process that may also result in a prolonged T_{max} . Further studies are warranted to elucidate the precise mechanisms underlying this observation.

Since our results indicated that imperatorin and curcumin inhibited the metabolism of macitentan in rats, therefore, elevated plasma concentrations of macitentan may result in an augmented probability of adverse reactions, such as headache, nasopharyngitis, and anemia. Nonetheless, the present study is not without limitations, and the results observed in rats must be extrapolated with caution to humans, as interspecies differences in drug metabolism (particularly those related to CYP3A4 activity) and the absence of clinical validation at present constitute significant study limitations.⁴⁹ Consequently, the necessity for further clinical pharmacokinetic studies is evident.

A study found that when imperatorin was co-administered with diazepam, which is mainly metabolized by CYP2C19 and CYP3A4, the $AUC_{(0-12h)}$ and C_{max} were significantly increased, while the apparent clearance $CL_{z/F}$ was significantly decreased.⁵⁰ A further in vivo pharmacokinetic study in rats revealed that the presence of curcumin (at doses of 2 and 8 mg/kg) resulted in a 35.1% and 50.8% increase in the AUC of etoposide (a main liver metabolic enzyme that is CYP3A4), respectively, accompanied by a notable rise in its plasma exposure.⁵¹ These findings suggested that imperatorin and curcumin significantly inhibited the metabolism of drugs by CYP3A4, which was in line with our findings.

Furthermore, imperatorin docking was achieved at a higher binding energy (−6.60 kcal/mol), while curcumin docking was achieved at a lower binding energy (−9.13 kcal/mol), which was associated with its relative in vitro inhibition ability (in HLM, imperatorin: $IC_{50} = 6.97 \mu M$, $K_i = 8.27 \mu M$, curcumin: $IC_{50} = 5.71 \mu M$, $K_i = 4.64 \mu M$). Moreover, both imperatorin and curcumin formed hydrogen bonds with different amino acid residues on CYP3A4. In conclusion, imperatorin and curcumin binded to distinct sites on CYP3A4, thereby inhibiting the metabolic action of this enzyme on macitentan. In addition, our findings revealed a closer proximity and stronger affinity of imperatorin and curcumin to ferrous ions in hemoglobin, which may serve as a significant factor contributing to the inhibition of macitentan metabolism by imperatorin and curcumin in HLM.

Conclusion

In conclusion, imperatorin and curcumin were found to inhibit the metabolism of macitentan in vitro, although the degree of metabolism varied in different systems. In SD rats, a significant increase in plasma exposure of macitentan in vivo further confirmed the inhibitory potency of imperatorin and curcumin. Furthermore, molecular docking demonstrated that the metabolic inhibition of macitentan by imperatorin and curcumin was achieved by binding to the site on CYP3A4. Nevertheless, the results of experiments conducted on rats cannot be accurately extrapolated to humans due to inherent differences between species. Consequently, further validation of this inhibitory effect through the study of human pharmacokinetics may be necessary.

Disclosure

The authors report no conflicts of interest in this work.

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