Pharmacokinetics, Tissue Distribution, and Druggability Prediction of the Natural Anticancer Active Compound Cytisine N-Isoflavones Combined with Computer Simulation

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Summary

Cytisine N-methylene-(5,7-dihydroxy-4'-methoxy)-isoflavone (CNF2) is a new compound isolated from the Chinese herbal medicine Sophora alopecuroides. Preliminary pharmacodynamic studies demonstrated its activity in inhibiting breast cancer cell metastasis. This study examined the pharmacokinetics, absolute bioavailability, and tissue distribution of CNF2 in rats, and combined computer-aided technology to predict the druggability of CNF2. The binding site of CNF2 and the breast cancer target HER2 were examined with molecular docking technology. Next, ACD/Percepta software was used to predict the druggability of CNF2 based on the quantitative structure-activity relationship (QSAR). Finally, a simple and effective high-performance liquid chromatography (HPLC) method was used to determine plasma pharmacokinetics and tissue distribution of CNF2 in rats. Prediction and experimental results show that compared with the positive control HER2 inhibitor SYR127063, CNF2 has a stronger binding affinity with HER2, suggesting that its efficacy is stronger; and the structure of CNF2 complies with the Lipinski's Rule of Five and has good drug-likeness. The residence time of CNF2 in rats is less than 4 hours, and the metabolic rate is relatively fast; But the absolute bioavailability of CNF2 in rats was 6.6%, mainly distributed in the stomach, intestine, and lung tissues, where the CNF2 contents were 401.20 μg/g, 144.01 μg/g, and 245.82 μg/g, respectively. This study constructed rapid screening and preliminary evaluation of active compounds, which provided important references for the development and further research of such compounds.
**Keywords**  * Sophora alopecuroides; Cytisine-isoflavone derivative; Computer simulation; Pharmacokinetics; Tissue distribution; Druggability
INTRODUCTION

*Sophora alopecuroides* L. is a perennial herb belonging to the family Leguminosae. As a traditional Chinese medicine, *Sophora alopecuroides* was officially included in the 1977 edition of the Chinese Pharmacopoeia. According to past reports, it has medicinal values that include clearing heat, detoxication, dispelling wind, removing dampness, relieving pain and killing insects. 1-3) Recent research findings showed that *Sophora alopecuroides* has anti-arrhythmia, antiviral, anti-inflammatory, and antitumor pharmacological activities. 4-6) The chemical constituents of *Sophora alopecuroides* include organic acids, flavonoids, pigments, and alkaloids, the main active ingredients are alkaloids and flavonoids. 2, 7, 8) Previous studies found that the total alkaloids of *Sophora alopecuroides* (TASa) and flavonoid compounds have significant antitumor activity, can induce cervical cancer cell apoptosis, and can regulate T cells to inhibit tumor growth in mice. 9-12) To determine the exact antitumor activity of *Sophora alopecuroides*, its anticancer active ingredients deserve further study.

Our research group first isolated a new compound of cytisine \(N\)-methylene-(5,7-dihydroxy-4'-methoxy)-isoflavone (CNF2) (Fig. 1) from *Sophora alopecuroides*. It is a novel structural compound formed by the combination of cytisine and isoflavone compounds via methylene. Earlier, we demonstrated through the cell scratch test that CNF2 can inhibit breast cancer cell (MAD-MB-231 and 4T1 cells) metastasis, and it has significant antitumor activity. 13) In related research work on compounds with similar structures, scholars have obtained cytisine-isoflavone derivatives through chemical synthesis, and predict that they will show pharmacological activities of antioxidation and lowering
blood lipids. Therefore, it is urgent that the druggability of the compounds be screened as soon as possible, to improve their development success.

Pharmacokinetic research plays an important role in the research of innovative drug translation and pervades the entire development process. Therefore, it is crucial to predict the pharmacokinetic parameters of potential lead compounds early and accurately. From the “Prodrug Design Concepts” came a proposal to study pharmacokinetics in advance. The idea is to use computer-aided drug design technology to study pharmacokinetics and tissue distribution before conducting in-depth pharmacodynamic experiments. Then, based on the results of theoretical and experimental research, one can quickly determine whether further pharmacological experiments need to be carried out. This not only can improve the targeting and rate of finding new drugs, but also can greatly shorten the development cycle of new drugs and reduce the cost of research and development.

This study uses AutoDockTools-1.5.6 software to predict the efficacy of the compounds CNF2 and human epidermal growth factor receptor-2 (HER2) and use ACD/Percepta software to predict the druggability of CNF2 using the quantitative structure-activity relationship (QSAR). A simple and effective reversed-phase high-performance liquid chromatography (RP-HPLC) method was used to determine the pharmacokinetics and tissue distribution of CNF2 in rats. The plasma concentration-time absorption curves of CNF2 after oral and intravenous administration were obtained according to the noncompartmental model, along with preliminary exploration of the absorption, elimination regularity and absolute bioavailability of the compound, and can quickly screen the activity and druggability of CNF2, which form an important foundation for the development of CNF2 as a lead
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compound.

MATERIALS AND METHODS

**Apparatus** Shimadzu LC-20AT (Shimadzu, Japan) high performance liquid chromatography; TGL-15B high-speed desktop centrifuge (Shanghai Anting Scientific Instrument Factory, China); Bullet Blender rapid tissue cell disruption instrument (Next Advance, America); PHS-3C PH meter (INESA, China); GL224-1SCN analytical electronic balance (one per ten thousand, Sartorius, China); XH-C vortex mixer (Jintan Chengdong Xinrui Instrument Factory, China); KQ-500B ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd., China).

**Chemicals** CNF2 (purity:95%) was prepared by our laboratory. Ofloxacin standard (Internal standard) (Lot: HY19S919, purity≥98.0%) was purchased from Shanghai Hengyuan Biotechnology Co., Ltd. Heparin sodium (batch number: P1356787, USP), sodium chloride, triethylamine, phosphoric acid, diethyl ether and absolute ethanol were analytical reagent, all purchased from Shanghai Titan Technology Co., Ltd. HPLC grade methanol and acetonitrile were purchased from Shanghai Macklin Biochemical Co., Ltd. Ultrapure water is prepared by UPT-II-20T ULUPURE ultrapure water machine (Baoshan District Urban Industrial Park, Shanghai, China).

**Simulation Method**

**Molecular Docking**

The X-ray crystal structure (PDB number: 3PP0, resolution: 2.25 Å) of HER2 was downloaded from the protein database (http://www.rcsb.org), which contains the known HER2 inhibitor SYR127063. For CNF2 and SYR127063, GaussView 5.0 software was
used to draw a schematic diagram of the structure with the ab initio Hartree-Fock method basis set 6-311G to optimize the structure for the optimal configuration of the ligand compound.

The method for docking verification is to use the optimized co-crystal structure for redocking SYR127063 to the active site of the HER2 protein, to select the posture. The parameters for validation involved the root mean square deviation (RMSD) for each ligand co-crystal at the selected binding site. The docking program is preferentially used to predict the results of experimental poses with an RMSD not exceeding 2Å. A lower RMSD indicates that the resulting redocking ligand position was closer to the crystallography results ligand position. 23, 24)

Using the same method as the verification procedure, with the HER2 tyrosine kinase domain 25) as the active site for docking, and using the AutoDockTools-1.5.6 program 26) for semiflexible docking of HER2 and CNF2 to obtain 100 different conformations, the best scoring conformation was obtained by cluster analysis (where RMSD was 2.0 Å). The PyMOL program 27) was used to analyze the interaction of ligand molecules with amino acid residues near the binding region. The main parameters observed during molecular docking 28) include free energy of binding (ΔG), inhibition constant (Ki), amino acid residues, and hydrogen bonds. The free energy of binding and inhibition constant found with the scoring data reflect the affinity of ligands for receptors. In general, a more negative free energy of binding and a lower inhibition constant indicate a higher binding affinity between the ligand and the receptor. 29) The CNF2 conformation results obtained with the most free energy of binding and lowest inhibition constant were compared with the results obtained by docking
verification to determine that CNF2 can be an effective inhibitor of HRE2. 30)

Druggability Prediction

ACD/Percepta (Tri-I Biotech, Shanghai Inc., China) software was used to predict the physicochemical properties and ADME properties of CNF2 based on QSAR.

Experimental Method

HPLC Separation Conditions

The sample solution was separated on a Hypersil ODS C18 (4.6×250 mm, 5 μm) column at 35 °C. The mobile phase consisted of solvent A (0.01% triethylamine solution, phosphoric acid adjusted to pH 7.2) and solvent B (methanol), with the gradient elution at a flow rate of 1 mL/min. The procedure for gradient elution was as follows: 0 min, 20% solvent B; 10 min, 48% solvent B; 25 min, 60% solvent B; 30 min, 90% solvent B. The detection wavelength was 290 nm and the injection volume was 10 μL.

Preparation of Stock Solution, Calibration Standards and Quality Control Sample

CNF2 is dissolved in methanol to obtain a stock solution with a concentration of 1 mg/mL, and the stock solution was gradually diluted with methanol aqueous solution (1:1, v/v) to obtain a working solution of CNF2 having a concentration ranging from 4 to 400 μg/mL. Ofloxacin (IS) was dissolved in methanol to give a stock solution having a concentration of 0.1 mg/mL, and diluted with acetonitrile to obtain an internal standard solution having a final concentration of 4 μg/mL. All solutions were stored in a 4 °C refrigerator.

Plasma and liver tissue calibration standards samples were obtained by diluting the CNF2 working solution with the corresponding blank matrix, respectively. The operation is
as follows: blank plasma or liver homogenate (90 μL) was spiked with the corresponding CNF2 working solution (10 μL) and mixed at a volume ratio of 9:1 to prepare plasma or liver tissue calibration standards samples at a concentration of 0.4-40 μg/mL.

The quality control plasma and liver homogenate samples of low, medium and high levels were prepared by the same method as above. Low quality control (LQC), medium quality control (MQC) and high quality control (HQC) samples were at 1, 6, and 40 μg/mL, respectively. All plasma and liver homogenate samples were stored in a -80 °C freezer until analysis.

Pretreatment of Biological Samples

100 μL of the plasma sample or tissue homogenate sample to be tested was placed in a 1.5 mL Eppendorf tube, 300 μL of acetonitrile (containing 4 μg/mL of IS) was added, vortexed for 1 minute, centrifuged at 9224 g for 3 minutes, the supernatant was taken out and concentrated by evaporation with nitrogen at 40 °C. The obtained residue was reconstituted with 600 μL of an aqueous methanol solution (1:1, v/v), and the mixture was vortexed for 1 minute, centrifuged at 9224 g for 10 minutes. The supernatant was removed and transferred to the injection vial through a 0.22 μm filter membrane for analysis.

Bioanalytical Method Validation

QC samples of rat plasma and liver homogenate were selected according to the FDA analytical method validation guidelines 31, 32) to verify that the method included linearity, stability, precision and accuracy, and extraction recovery.

Pharmacokinetics and Tissue Distribution Studies

Female Sprague-Dawley rats (n = 30, 7-8 weeks old, 200 ± 20 g) used in this experiment
were purchased from Shanghai Slack Laboratory Animals Co., Ltd. (License number: SCXK (Shanghai) 2017-0005). Rats were housed in animal rooms before the experiment and were allowed free access to drinking water and feed. Rats fasted for 14 hours before intragastric administration, to rule out any effect of food on drug absorption. All procedures were carried out after approval by the Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine.

Twelve SD rats were randomly divided into two groups (intravenous [iv] and intravenous [ig]), six in each group. Rats in the first group were injected with CNF2 intravenously (6 mg/kg; dimethyl sulfoxide [DMSO]: normal saline, 6:4, v/v), and rats in the second group were intragastrically administered CNF2 (60 mg/kg; DMSO: PEG400: normal saline, 1:8:1), blood samples of 0.3 mL was collected from the orbital venous at 5, 10, 15, 25, 40, 60, 120, 180, and 240 minutes after administration, respectively. Whole blood samples were placed in Eppendorf tubes containing sodium heparin, mixed immediately, centrifuged at 9224 g for 3 min, and the upper plasma was collected and stored in a -80 °C freezer to be tested.

Eighteen SD rats were randomly divided into three groups of six each, and CNF2 was administered intragastrically at a dose of 60 mg/kg; rats were sacrificed at 5, 15 and 40 minutes after administration, respectively. The heart, liver, spleen, lungs, kidneys, stomach, intestines, brain, pancreas, muscles, fat and uterus were quickly removed. After rinsing the blood from the surface of the tissues with saline, tissues were wiped with a filter paper and collected in an Eppendorf tube, then stored in a -80 °C freezer until testing. The tissue samples were homogenized before they were measured, 0.3 g of each tissue was weighed,
physiological saline was added, so the sample was mixed in an Eppendorf tube at a mass ratio of 1:2, and then it was ground in a tissue cell breaker to prepare a tissue homogenate.

Data Analysis

Pharmacokinetic parameters including area under the plasma concentration-time curve (AUC), peak concentration (C\text{max}), peak time (T\text{max}) and mean residence time (MRT) was calculated from the non-compartment model WinNonlin (version, 5.2). Absolute bioavailability calculation: F = \frac{\text{AUC}_{(0-t)ig} \times \text{Dose}_{ig}}{\text{AUC}_{(0-t)iv} \times \text{Dose}_{iv}} \times 100\%. The data result is expressed as mean±SD.

RESULTS AND DISCUSSION

Molecular Simulation

Molecular Docking

AutoDockTools-1.5.6 was used for redocking to complete the docking verification. The co-crystal ligand for the selected binding site was used to verify the entire binding site, as shown in Fig. 2. The RMSD value obtained by redocking was 1.612Å (<2Å), indicating that the optimized structure is effective for molecular docking with the receptor HER2. Using the ab initio Hartree-Fock method basic set 6-311G to optimize the structure, gives it high credibility.

The AutoDockTools-1.5.6 program was used to predict the binding mode of SYR127063 and CNF2, respectively, to HER2. There were multiple docking conformations at the binding site, and the best conformation was selected for analysis based on cluster analysis (with RMSD 2.0Å). The results are shown in Table 1. It can be seen from Table 1 that compared with SYR127063, CNF2 has more negative free energy of binding and a lower inhibition.
constant, which indicates that CNF2 has a higher binding affinity for the HER2 tyrosine kinase domain than the HER2 inhibitor SYR127063, further indicating that CNF2 has better HER2 kinase inhibitory activity.

The docking results for CNF2 and HER2 are shown in Fig. 3, showing many interactions between CNF2 and amino acid residues near the active site of HER2. The C-4 carbonyl and C-5 hydroxy groups of the isoflavone of CNF2 form strong hydrogen bonds with Gln-799 and Met-801, the amino acid residues in the hinge region of HER2, respectively; meanwhile, the C-7 hydroxyl group forms a hydrogen bond with Thr-862, which is in the back pocket region. In the solvent region, the carbonyl group of the cytisine moiety of CNF2 forms a hydrogen bond with Ser-728. These hydrogen bonds provide orientation for the combination of CNF2 and HER2 and contribute some electrostatic force. In addition, CNF2 forms hydrophobic interactions with residues Leu-726, Gly-727, Gly-729, Val-734, Ala-751, Thr-798, Leu-800, Pro-802, Gly-804, Cys-805, Arg-849, Asn-850, Leu-852, and Asp-863, which contribute a strong van der Waals force to the combination of CNF2 and HER2. Therefore, the interaction between CNF2 and HER2 is mainly hydrophobic, and the binding orientation is locked by hydrogen bonding.

Druggability Prediction

The ACD/Percepta software was used to predict the physicochemical properties and ADME properties of CNF2. The predicted parameter results are shown in Table 2. Lipinski's five rules 33) are empirical rules summarized for oral drugs, and the specific content is as follows: (1) the molecular weight <500 g/moL; (2) the number of hydrogen bond donors <5; (3) the number of hydrogen bond acceptors <10; (4) the lipid-water partition coefficient <5;
the number of rotatable bonds $\leq 10$. When a small molecule drug violates two or more of these criteria, it indicates that the drug has poor oral absorption. It can be seen from Table 2 that the physicochemical properties of CNF2 are conformed to Lipinski's rule, indicating that CNF2 has certain druggability. On the other hand, the prediction results of ADME show that CNF2 does not easily penetrate the blood-brain barrier (BBB) to reach the central nervous system (CNS), indicating that CNF2 is less likely to develop into a CNS drug. Because CNS drugs need to pass through the BBB to produce their effects, and many statistical studies have shown that a successful CNS drug candidate should have a smaller molecular weight (Mr <450). Since CNF2 is highly permeable to Caco-2 cells, it indicates that the compound can be well absorbed by the human gastrointestinal tract. Besides, CNF2 has a strong protein binding capacity for human plasma. If new drugs are developed, the risks of clinical use should be noted.

**Establishment of HPLC Analysis Method in Biological Samples**

**Optimization of Pretreatment Methods for Biological Samples**

We pretreated the biological samples with the classical protein precipitation method and compared the extraction rates of target components in plasma samples with three solvents (methanol, ethanol, and acetonitrile). The results showed that acetonitrile had a higher extraction rate of the target components, better protein removal performance, and the resolution and peak shape of the tested components met the requirements. Therefore, acetonitrile was selected as the protein precipitant.

**Optimization of Chromatographic Separation Conditions**

An HPLC internal standard method was established for the determination of CNF2 in
rats, and ofloxacin was selected as the internal standard. To ensure better separation of CNF2 and IS from endogenous substances in the biological matrix, the mobile phase conditions were optimized. Three mobile phase systems were selected for testing on a Hypersil ODS C18 column, including methanol and water, methanol and 0.1% formic acid water, methanol and 0.01% triethylamine aqueous solution (pH adjusted to 7.2 by phosphoric acid). The results show that in the third system, the peak tailing phenomenon of CNF2 and IS can be improved after adding triethylamine to the aqueous phase. The buffer of PH 6.0-8.0 range buffer can maintain the stable form of the target component, and ensure good chromatographic peak symmetry. Therefore, it was determined that methanol and 0.01% triethylamine aqueous solution (pH adjusted to 7.2 by phosphoric acid) were mobile phase compositions.

Method Validation

Specificity, Linear Range and Lower Limit of Quantification (LLOQ)

To investigate whether the endogenous substances in biological samples interfere with the determination of CNF2, this study selected plasma samples and liver tissue samples to represent the specificity of the analytical method established. HPLC chromatograms of plasma samples and liver tissue samples are shown in Fig. 4. The peak retention times of IS and CNF2 were 14.70 min and 20.42min, respectively, and the peak resolution was good. No endogenous component interference was observed near the analytes in the six test samples of the plasma and liver tissue samples, indicating that the method has good specificity.

Process and analyze calibration curve samples and establish linear regression curves. The linear regression equation was obtained by weighted \((1/x^2)\) least squares method with the
mass concentration of CNF2 as the x-axis and the peak area ratio of CNF2/IS as the y-axis. Calibration curve equation for plasma samples: $y=0.169x-0.000936$, $\gamma^2=0.999$; calibration curve equation for liver tissue samples: $y=0.156x+0.0077$, $\gamma^2=0.999$, which indicates the concentration of CNF2 and the peak area ratio of CNF2/IS have a good linear relationship between 0.4-40 $\mu$g/mL. The LLOQ is 0.4 $\mu$g/mL, which satisfies the requirement that the signal-to-noise ratio (S/N) > 5, and the precision and accuracy are within ±20%.

**Precision and Accuracy**

The precision and accuracy of the analytical method were evaluated using three different concentrations of QC samples (1, 6 and 40 $\mu$g/mL). All samples were prepared for analysis within one day to calculate intra-day precision and accuracy, and preparation analysis was performed for three consecutive days to calculate inter-day precision and accuracy. Precision is expressed as relative standard deviation (RSD), and accuracy (comparison of measured values of QC samples with labeled values) is expressed as relative deviation (RE). The calculation results are shown in Table 3. The intra- and inter-day RSD of the QC plasma samples were less than 10.57% and 7.55%, respectively, and the accuracy was within ±7.5%; the intra- and inter-day RSD of the QC liver tissue samples were less than 8.33% and 8.41%, respectively, and the accuracy was within ±9.5%. The limit requirements comply with US FDA guidelines (precision <15%, accuracy within ±15%), indicating that the method has good precision and accuracy.

**Extraction Recovery**

The QC samples at three concentration levels were selected for the determination of the recovery rate. The analytical response (peak area) of the extracted QC samples was compared
to the response (peak area) of CNF2 spiked to blank plasma. The test results of CNF2 extraction and recovery in rat plasma and liver tissue are shown in Table 4. The extraction recovery of CNF2 in three different concentrations of QC samples (1, 6 and 40 μg/mL) was between 90% and 96%, and the RSD was ≤7.38%. The results show that acetonitrile has high and stable extraction efficiency for CNF2, and can be reproduced within a limited range of variation.

Stability

QC samples were stored under different conditions to test the stability of CNF2. The stability of 4 h at room temperature, storage in a low-temperature refrigerator at -80 °C for one month and subjected to three freeze-thaw cycles was investigated, respectively. Besides, the pretreated sample was placed in an autosampler for 24 hours to investigate post-extraction stability. The stability test results are shown in Table 5. The RSD of all QC samples was <15% and the RE was within ± 15%. The results showed that the concentration of CNF2 remained stable, ensuring the accuracy and reproducibility of the test results.

In summary, the established method validation criteria for HPLC in vivo assays comply with US FDA guidelines, which proves that the method is effective and feasible.

**Preliminary Pharmacokinetics and Bioavailability Studies** The plasma pharmacokinetics of CNF2 in rats were studied by intravenous injection (dose: 6 mg/kg) and intragastric administration (dose: 60 mg/kg). The mean plasma concentration-time curve of CNF2 after intravenous and intragastric administration is shown in Fig. 5. The concentration of CNF2 in plasma was 12.4 ± 2.5 μg/mL after intravenous injection for 5 minutes, and then decreased rapidly; the concentration of CNF2 in plasma reached the highest value after 15
minutes of intragastric administration ($C_{\text{max}}$: 7.7 ± 0.9 μg/mL), and after 4 hours, it was below the LLOQ. The pharmacokinetic parameters obtained from the noncompartment model are summarized in Table 6. The mean residence time of CNF2 in rats after intravenous and intragastric administration was 184.5 ± 3.4 min and 109.1 ± 1.0 min, respectively, which indicated that the retention time of CNF2 in rats was shorter and the clearance was faster. The area under the concentration-time curve (AUC) after intravenous and intragastric administration was 3480.9 ± 173.7 μg/mL·min and 2302.8 ± 67.3 μg/mL·min, respectively. The absolute bioavailability of CNF2 in rats was calculated to be 6.6%. Studies have shown that CNF2 is not well absorbed in rats and has a low absolute bioavailability, probably because all components measured by intravenous administration are in the blood and the absorption mechanism of oral administration is complicated, resulting in low bioavailability.

**Preliminary Tissue Distribution Studies** The distribution of CNF2 in the heart, liver, spleen, lung, kidney, brain, stomach, intestine, pancreas, muscle, fat, and uterus was determined by validated HPLC. The content of CNF2 was determine in rat tissues at different time points (5, 15 and 40 min) after intragastric administration at a dose of 60 mg/kg, and a distribution histogram was plotted to show changes in CNF2 concentration in different tissues. As shown in Fig. 6, CNF2 is rapidly distributed in organ tissues other than brain tissue after a single dose. CNF2 is mainly distributed in the stomach, intestine and lung tissues, and the levels found were 401.20 μg/g, 144.01 μg/g and 245.82 μg/g, respectively, followed by liver, fat, uterus, and kidney, and minimally distributed in the heart and spleen (4.6 μg/g and 5.42 μg/g, respectively). CNF2 is rapidly distributed and cleared in various organ tissues, so that the possibility of accumulation by continuous administration is small, which is consistent
with the change in the plasma concentration-time curve. The content of CNF2 in the stomach and intestine is high, probably because the stomach is the site of administration and the intestine is the main absorption site, which is consistent with the prediction results from human intestinal absorption evaluation. Note that CNF2 was not detected in brain tissue, indicating that the compound does not easily cross the BBB. This further validated the prediction results of ADME, indicating that CNF2 is less likely to have side effects on the central nervous system.

In the present work, based on the prediction results and experimental results, the druggability of CNF2 was comprehensively analyzed. This study used molecular docking technology to examine the interaction between CNF2 and breast cancer target HER2 and compared it with the positive control SYR127063. It was found that the binding of CNF2 to HER2 has a higher negative free energy of binding and a lower inhibition constant. Therefore, it was first suggested that CNF2 may be a HER2 inhibitor. Second, this study that CNF2 has common characteristics of drugs in structure and physicochemical properties through Lipinski's rule of five, which indicated that CNF2 could have druggability. However, from the results of pharmacokinetic parameters and tissue distribution, the pharmacokinetic properties of CNF2 in rats are found to be not ideal, and the absolute bioavailability is only 6.6%, which did not meet the expectations of theoretical prediction; moreover, CNF2 is significantly higher in the three tissues of the liver, gastrointestinal system, and stomach, which indicates that CNF2 does have a good gastrointestinal absorption capacity. Bioavailability is mainly related to lipid solubility, water solubility, and the first-pass effects of the compound. 36) Based on computer simulation results showing that CNF2 has good
physicochemical properties, it is possible to speculate that CNF2 may undergo significant first-pass effects in vivo, resulting in low bioavailability. Traditional pharmacy theory indicates that liver metabolism can cause first-pass effects. In recent years, studies have shown that intestinal epithelial cells also contain a variety of metabolic enzymes and bacterial clusters similar to those found in the liver, so that oral drugs can be metabolized during intestinal absorption. 37-39) Therefore, to determine whether CNF2 undergoes a first-pass effect in rats, future work, can establish intestinal and vascular access port animal models to explore the pharmacokinetic characteristics of different administration routes of CNF2 and quantitatively evaluate the liver and intestinal first-pass effects.

CONCLUSION

In conclusion, the purpose of this study is to explore the druggability and development value of cytisine N-isoflavones. The idea of combining pharmacokinetics with computer simulation technology was used to predict the druggability of CNF2, and some results were obtained. This study provides data support for subsequent work on CNF2 and provides new models and ideas for the development of other active compounds.

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**Conflict of Interest** The authors declare no conflict of interest.
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| Receptor | Ligand   | Free Energy of Binding (kcal/mol) | Ki (µM) | Amino Acids Residue Hydrogen Bonding |
|----------|----------|-----------------------------------|---------|-------------------------------------|
| HER2     | SYR127063 | -9.48                             | 0.1125  | LEU-726, SER-728, GLY-729, VAL-734, ALA-751, LYS-753, MET-774, SER-783, LEU-785, LEU-796, THR-798, GLN-799, LEU-800, MET-801, PRO-802, GLY-804, 1LEU-852, THR-862, ASP-863, PHE-864 |
| HER2     | CNF2     | -10.53                            | 0.01919 | LEU-726, GLY-727, SER-728, GLY-729, VAL-734, ALA-751, THR-798, GLN-799, LEU-800, MET-801, PRO-802, GLY-804, CYS-805, ARG-849, ASN-850, LEU-852, THR-862, ASP-863 |

Ki stands for inhibition constant.
Table 2. Partial Physicochemical Properties and ADME Properties of CNF2 were Obtained on ACD/Labs Percepta Database Software

| Modules                  | Parameter                        | Value     | Evaluation |
|--------------------------|----------------------------------|-----------|------------|
| PhysChem Profiling       | LogP                             | 2.58      | Optimal    |
|                          | Molecular Weight                 | 516.54    | bad        |
|                          | Number of Hydrogen Bond Donors   | 2         | Good       |
|                          | Number of Hydrogen Bond Acceptors| 9         | Good       |
|                          | Number of Rotatable Bonds        | 5         | Good       |
|                          | Rings                            | 6         | Bad        |
|                          | Lipinski                         | 1 violation | Moderate   |
|                          | Solubility                       | 0.96mg/mL | Soluble    |
| ADME Profiling           | Caco-2                           | 68×10⁻⁶cm/s | Highly permeable |
|                          | Binding Rate of Plasma Protein   | 94%       | Extensively bound|
|                          | CNS                              | -3.86     | Non-penetrant |
|                          | HIA                              | 100%      | Highly absorbed |

ADME is an abbreviation for absorption, distribution, metabolism and excretion.
Table 3. Intra- and Inter-day Precision and Accuracy of CNF2 in Rat Plasma and Liver Tissue (n = 5)

| Matrix | Concentration (µg/mL) | Intra-Day (n=5) | Inter-Day (n=5) |
|--------|-----------------------|----------------|---------------|
|        |                      | C<sub>measured</sub> (µg/mL) | Accuracy (RE, %) | Precision (RSD, %) | C<sub>measured</sub> (µg/mL) | Accuracy (RE, %) | Precision (RSD, %) |
| Plasma | 1                     | 1.04±0.11       | 4.00          | 10.57           | 1.06±0.08       | 6.00          | 7.55           |
|        | 6                     | 5.96±0.35       | -0.67         | 5.87            | 5.93±0.21       | -1.17         | 3.54           |
|        | 40                    | 37.15±1.75      | -7.10         | 4.72            | 37.00±0.65      | -7.50         | 2.00           |
| Liver  | 1                     | 1.08±0.09       | 8.00          | 8.33            | 1.07±0.09       | 7.00          | 8.41           |
|        | 6                     | 5.43±0.07       | -9.50         | 1.29            | 5.448±0.61      | -9.2          | 5.14           |
|        | 40                    | 36.84±1.16      | -7.90         | 3.15            | 37.53±6.72      | -6.19         | 4.88           |
Table 4. Extraction Recovery of CNF2 in Rat Plasma and Liver (n = 5)

| Matrix | Concentration (μg/mL) | Extraction recovery (%) | RSD (%) |
|--------|-----------------------|-------------------------|---------|
| Plasma | 1                     | 96                      | 1.88    |
|        | 6                     | 93                      | 1.63    |
|        | 40                    | 94                      | 1.66    |
| Liver  | 1                     | 90                      | 5.85    |
|        | 6                     | 90                      | 6.54    |
|        | 40                    | 91                      | 7.38    |
Table 5. Stability of CNF2 in Rat Plasma and Liver Tissue (n = 5)

| Matrix | Concentration (μg/mL) | Short-term stability at Room temperature | Long-term Stability For One Month | Three Freeze-Thaw Cycles Stability | Autosampler Stability For 24 h |
|--------|-----------------------|----------------------------------------|----------------------------------|----------------------------------|-------------------------------|
|        |                       | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| Plasma | 1                     | 7.64   | 1.33    | 8.72   | 1.86    | 10.78  | 1.17    | 9.52   | 1.53    |
|        | 6                     | -5.21  | 3.77    | -3.25  | 2.93    | -0.85  | 1.56    | -3.73  | 5.35    |
|        | 40                    | -3.49  | 5.22    | -4.92  | 3.69    | -5.24  | 4.16    | -2.51  | 3.86    |
| Liver  | 1                     | -10.63 | 4.27    | -12.70 | 6.41    | -14.00 | 3.26    | -12.66 | 3.43    |
|        | 6                     | -7.41  | 3.88    | -9.08  | 1.47    | -8.58  | 7.29    | -13.33 | 7.12    |
|        | 40                    | -9.60  | 4.67    | -8.08  | 4.03    | -4.80  | 11.82   | -6.85  | 8.67    |
Table 6. Main Pharmacokinetic Parameters of CNF2 After Oral and Intravenous Administration (n = 6)

| Parameters | Unit             | Ig (60mg/kg)       | Iv (6mg/kg)      |
|------------|------------------|--------------------|------------------|
| AUC\(_{0-t}\) | µg/mL·min       | 2302.8±67.3        | 3480.9±173.7     |
| AUC\(_{0-\infty}\) | µg/mL·min       | 2507.9±68.0        | 3588.6±201.4     |
| MRT\(_{0-t}\)  | min              | 109.1±1.0          | 184.5±3.4        |
| MRT\(_{\text{inf}}\) | min             | 611.2±0.9          | 650.4±4.8        |
| CL\(_{z/F}\)   | mL·min\(^{-1}\)·kg\(^{-1}\) | 23.9±2.7          | 1.7±0.4          |
| T\(_{\text{max}}\) | min             | 15.0               | —                |
| C\(_{\text{max}}\) | µg/mL           | 7.7±0.9            | 12.4±2.5         |
Fig. 1. Chemical Structures of the Compound CNF2 (a) and Internal Standard Substance Ofloxacin (b)
Fig. 2. Verification Results of SYR127063 and HER2 (RMSD: 1.612)

(Red: SYR127063 Crystallography Result; Green: SYR127063 Redocking Result)

(Color figure can be accessed in the online version.)
Fig. 3. Docking Results of CNF2 and HER2

(a) CNF2 Position in Tyrosine Kinase Domain of HER2; (b) Interactions Between CNF2 and Amino Acid Residues near the HER2 Active Site.

(Color figure can be accessed in the online version.)
Fig. 4. HPLC Chromatograms of CNF2 in Different Matrices (A) Plasma Samples: (I) Blank Plasma Samples, (II) MQC Plasma Samples and IS, (III) Rat Plasma Samples 40 Minutes after Intragastric Administration; (B) Liver Tissue Samples: (I) Blank Liver Homogenate Samples; (II) MQC Liver Homogenate Samples and IS; (III) Rat Liver Homogenate Samples 40 minutes after Intragastric Administration
Fig. 5. Mean Plasma Concentration-time Curve of CNF2 (6 mg/kg) Injected via Tail Vein and CNF2 (60 mg/kg)

Administered Intragastrically
Fig. 6. CNF2 Content (mean ± SD) in Rat Tissues at 5, 15, and 40 Min after Intragastric Administration (n = 6)

(Color figure can be accessed in the online version.)