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Prediction of drug-drug plasma protein binding interactions of resveratrol in combination with celecoxib and leflunomide by molecular docking combined with an ultrafiltration technique

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The present study is aimed at computational prediction of the molecular interactions between resveratrol, celecoxib, leflunomide and human serum albumin (HSA) and then investigates the plasma protein binding of resveratrol combined with celecoxib or leflunomide by an ultrafiltration technique. Molecular operating environment (MOE, 2008.10) software package was used to explore molecular interactions between the drugs and HSA. Molecular docking was adopted to predict the interactions between resveratrol and other drugs and then the ultrafiltration technique was used to verify the docking results. In *in vitro* experiments, a mixture of resveratrol and celecoxib or leflunomide was added to rat plasma for determination of the plasma protein binding rate. Molecular docking results have shown that resveratrol interacts with HSA mainly through hydrogen bond and π-π stacking, while celecoxib and leflunomide bind only with the hydrogen bond. Celecoxib or leflunomide, even at high tested doses, did not affect the plasma protein binding of resveratrol, thus suggesting pharmacological suitability of the investigated combinations.

Keywords: resveratrol, celecoxib, leflunomide, molecular docking, plasma protein binding, ultrafiltration

Resveratrol (3,5,4′-trihydroxystilbene) is a polyphenolic compound (1). Owing to its anti-inflammatory and immunomodulatory properties, resveratrol has the potential to treat inflammatory diseases (2). Rheumatoid arthritis (RA) is a common autoimmune disease, where resveratrol was shown to inhibit the overproduction of matrix metalloproteinase (MMPs) and receptor activator of nuclear factor-κB ligand (RANKL) (3). Besides, resveratrol inhibited the activity of cyclooxygenase (COX) 1 and COX-2 (4). Resveratrol also

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attenuated TNF-α-induced production of IL-1β and MMP-3 *via* inhibition of the PI3K-Akt signaling pathway in RA fibroblast-like synoviocytes (FLS) (5). In summary, resveratrol may play an important role in the prevention and treatment of RA.

Previous studies have shown that the very low oral bioavailability of resveratrol might be attributed to its structure and poor water solubility (6). Very low concentrations of free drug in plasma may also be due to its high binding to plasma or tissue proteins. The mean plasma protein binding (PPB) of resveratrol is 98.3 % in humans (7). Pharmacokinetic properties, such as the hepatic metabolism rate, renal excretion rate, membrane transport rate and distribution volume, are functions of the ratio of the unbound drug fraction (8). Therefore, investigation of pharmacokinetic parameters, such as plasma protein binding of resveratrol in combination with other drugs, is of great importance.

It is well known that non-steroidal anti-inflammatory drugs (NSAIDs) can alleviate pain and stiffness, and control the symptoms of RA. The highly liposoluble drug celecoxib, a selective COX-2 inhibitor, has analgesic and anti-inflammatory effects in patients with rheumatoid arthritis (9). A study has recently shown the synergistic effect of combining resveratrol with celecoxib on reduction of all tumor parameters in *N*-methyl-*N*-nitrosourea (NMU)-induced breast cancer in rats (10). Leflunomide is a disease-modifying anti-rheumatic drug (DMARD) for the treatment of RA. In clinics, leflunomide was found to be therapeutically effective and safe in active RA patients throughout progression of the disease (11).

Molecular docking provides a detailed view of the drug-target interaction and predicts drug-drug plasma protein binding interactions. Herein, we introduce a potentially widely applicable and accurate drug target identification strategy to predict drug-drug interactions. The predicted molecular docking results will thereafter be verified by *in vitro* plasma protein binding experiments.

EXPERIMENTAL

Animals

Male Sprague-Dawley (SD) rats, weighing 220–250 g, were purchased from the Animal Department of Anhui Medical University (Hefei, Anhui Province, China) and acclimatized to our animal house for at least a week before the experiments. All manipulations with animals were carried out in the morning to minimize the effects on circadian rhythm. Abdominal aorta blood was harvested from ten SD rats. A blood sample of 8 mL was withdrawn from each rat into an anticoagulant tube to obtain drug-free blank rat plasma. All rats were anesthetized with sodium pentobarbital to minimize suffering and were sacrificed, followed by cervical dislocation after blood collection.

The study was approved by the Experimental Animal Ethics Committee of the Anhui University of Chinese Medicine, Hefei, PRC.

Drugs and reagents

Resveratrol was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Celecoxib and leflunomide were purchased from National Institutes for Food and Drug Con-

trol (Beijing, China). Purity of reference compounds was > 98 % according to HPLC (provided by Aladdin Reagent Co., Ltd., and National Institutes for Food and Drug Control). Chromatographic grade methanol was obtained from Fisher Scientific (USA). Deionized water was purified using a Milli-Q system (Millipore, USA). Centrifugal filter units (Centrifree® YM-10 regenerated cellulose membrane, MWCO 10K) were obtained from Millipore (USA). All other chemicals and solvents used in the study were of reagent grade or better.

HPLC analysis and validation

HPLC analyses were performed on an Agilent 1200 series HPLC system (Agilent Technologies, USA). An HC-C18 column (4.6 × 250 mm, 5 μm) was used with column temperature set to 30 °C. The mobile phase consisting of distilled water and methanol (38:62, *V*/*V*) was used at a flow rate of 0.8 mL min^{-1}. Twenty-five μL of the sample were injected into the HPLC system for analysis. Analytes were detected at 300 nm.

Specificity. – Specificity was evaluated by comparing the chromatograms of blank plasma, resveratrol, resveratrol and celecoxib or leflunomide, blank plasma spiked with resveratrol and celecoxib or leflunomide (Figs. S1 and S2).

Calibration, recovery and precision. – Stock solutions of resveratrol (1 mg mL^{-1}) and celecoxib (10 mg mL^{-1}) or leflunomide (10 mg mL^{-1}) were prepared in methanol. Stock solutions were protected from light and kept at 4 °C until use. Stock solutions were diluted with methanol to prepare working solutions just prior to use.

Linearity was assessed by assaying calibration curves at eight concentration levels in the range of 0.1–80 μg mL^{-1} in blank rat plasma. Resveratrol concentration in the sample was calculated from the peak area using the linear regression equation obtained from the calibration curve.

Recovery of resveratrol (%) was calculated from the concentration of blank plasma samples with added resveratrol and resveratrol alone.

For precision tests, intra-day variation was measured by assaying each sample three times on the same day, while inter-day variance was also measured but over three consecutive days.

Stability. – For the stability test, blank rat plasma with resveratrol was incubated in a water bath at 37 °C for 1, 2, 3 and 4 h.

Molecular docking between resveratrol, celecoxib, leflunomide and human serum albumin (HSA)

Interactions of resveratrol, celecoxib, leflunomide and human serum albumin (HSA) were docked by the MOE (2008.10) software package. Structures of resveratrol, celecoxib and leflunomide were drawn by Chemdraw software (Fig. 1) and the three-dimensional crystal structure of HSA (PDB ID: 1HA2) was obtained from the Protein Data Bank (PDB) (www.rcsb.org/pdb) (12).

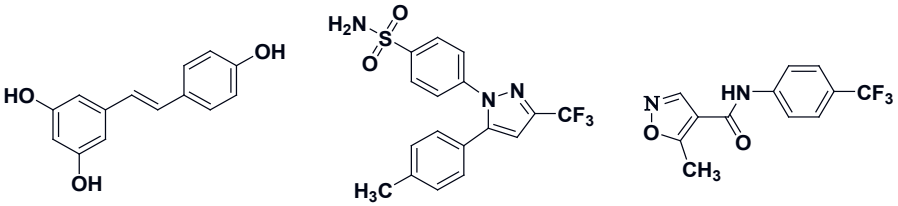

Fig. 1. Chemical structures of resveratrol, celecoxib and leflunomide.

Molecular docking experiments were performed as previously described, with some modifications (13). Briefly, 3D structures of resveratrol, celecoxib and leflunomide were optimized by adding hydrogens and were energy minimized. When correct conformations and minimum energy structures were obtained, ligands were allowed to be flexible. Water molecules were removed from the target and the 3D protonation of HSA was carried out using the MOE (2008.10) software package. The self-ligand was removed from HSA and the partial charge was minimized using the MMFF94 force field. Predicted drugs were docked with the hydrophobic pocket of HSA, parameter London dG was retained 10, and the interaction energy scores and binding mode with amino acid residues were automatically given.

Plasma protein binding of resveratrol alone and combined with celecoxib or leflunomide

Optimization of the ultrafiltration conditions can affect plasma protein binding. Firstly, the incubation temperature and time can influence the speed and processes of drug-protein binding equilibrium (14). Plasma protein binding of resveratrol was performed at incubation times of 15, 30, 45 and 60 min at 4 or 37 °C. Conditions of 30 min and 37 °C were chosen as optimal incubation conditions. Secondly, the rate and time of centrifugation were also important for influencing the ultrafiltration (24). The centrifugal rate and time were measured at 6000, 8000 and 10000 *g* for 10 min, and at centrifugal rates of 8000 *g* for 5, 10 and 20 min. The results showed that 8000 *g* and 20 min were the optimal centrifugal conditions.

Plasma protein binding of resveratrol *in vitro* was studied by an ultrafiltration method (15). In brief, 30 μL of resveratrol solution (250, 500, 1000 μg mL^{-1}) was spiked to 570 μL of blank rat plasma in amber vials to get the final 12.5, 25.0 and 50.0 μg mL^{-1}, resp. The samples were incubated at 37 °C for 30 min to achieve equilibrium. Five hundred μL of samples was transferred to the centrifugal filter unit and then centrifuged at 8000 *g* for 20 min.

Thirty μL of resveratrol solution (250, 500, 1000 μg mL^{-1}) and 30 μL of celecoxib or leflunomide solution (500, 5000 μg mL^{-1}) were spiked to 540 μL of blank rat plasma in amber vials to yield the final resveratrol (12.5, 25.0, 50.0 μg mL^{-1}) and celecoxib or leflunomide (25, 250 μg mL^{--1}). The ultrafiltration steps are described as mentioned above.

Finally, 100 μL of plasma sample without ultrafiltration and 100 μL of filtered sample were spiked with 100 μL of methanol. Twenty-five μL of the testing sample was injected into the HPLC system for analysis. Percentage of plasma protein binding (PPB) of the drug was calculated.

RESULTS AND DISCUSSION

HPLC method: specificity, standard curve, recovery, precision, stability

Retention times of resveratrol, leflunomide and celecoxib were approx. 4.8, 16.0 and 23.8 min, resp. No significant endogenous peaks interfering with resveratrol were obtained in blank plasma (Figs. S1 and S2 in Supplementary material).

Calibration curves of resveratrol of rat plasma after ultrafiltration were linear over the range from 0.05 to 100 μg mL^{-1} (0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μg mL^{-1}). The regression line equation for resveratrol was $y = 37.68x - 5.62$; $R = 0.9999$, $n = 3$.

Recovery of resveratrol after ultrafiltration at 0.5, 5.0 and 50.0 μg mL^{-1} was 97.2 ± 2.8, 96.3 ± 1.2 and 96.7 ± 3.9 %, resp. (Table SI in Supplementary material).

Intra-day and inter-day RSDs were all lower than 6 % (Table SII in Supplementary material).

Resveratrol was found stable in plasma at 37 and 4 °C (Table SIII in Supplementary material). Consequently, these results indicate that the analytical method was suitable for the present study.

Molecular interactions between resveratrol, celecoxib, leflunomide and HSA

This is the first time that molecular docking is adopted to predict drug-drug plasma protein binding interactions of resveratrol. Molecular interactions between resveratrol and HSA include H-bond formation and π-π stacking. Resveratrol binds HSA *via* hydrogen bond formation at Arg 117 and *via* π-π stacking at Tyr 161. Celecoxib binds HSA *via* hydrogen bond formation at Arg 485, and leflunomide forms an intermolecular hydrogen bond with HSA at Arg 10 (Table I, Fig. 2). Although resveratrol, celecoxib and leflunomide act on the same target, the site of interaction is different. This indicates that the three tested drugs act at different HSA sites. Therefore, resveratrol, celecoxib and leflunomide may have no competitive inhibitory interaction in clinical settings.

Molecular docking of drugs with HSA is mainly based on clinical application, while determination of the plasma protein binding of resveratrol combined with celecoxib or leflunomide is a basic study. Therefore, the molecular operating environment (MOE, 2008.10) software package was used to explore molecular interactions between the drugs and HSA, predicting the interaction between resveratrol and celecoxib or leflunomide, and then the ultrafiltration technique was used to verify the docking results. In *in vitro* experiments, a mixture of resveratrol and celecoxib or leflunomide was added to rat plasma for determination of the plasma protein binding rate. Molecular docking of HSA and the drugs was used for prediction; the crystal structure of RSA is not clear yet.

Table I. Interactions between resveratrol, celecoxib, leflunomide and HSA

Chemical	Interaction energy (kcal mol^{-1})	Number of binding sites to residues	Residues involved in H-bond formation	Arene-Arene
Resveratrol	–10.3795	2	Arg 117	Tyr 161
Celecoxib	–11.5363	1	Arg 485	–
Leflunomide	–9.3051	1	Arg 10	–

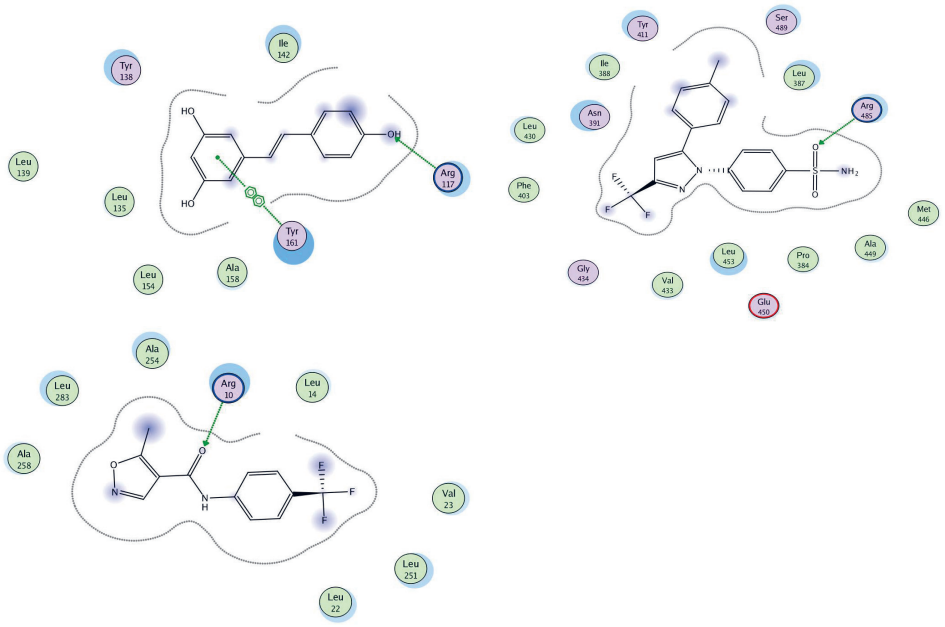

Fig. 2. 2D pictures of the docked conformations of HSA and: a) resveratrol, b) celecoxib, c) leflunomide.

Rat plasma protein binding of resveratrol

As shown in Table II, the plasma protein binding of resveratrol at 37 °C was consistently > 98 % over three concentrations of 12.5, 25.0 and 50.0 μg mL^{-1}.

Effects of low and high concentrations of celecoxib

Protein binding of resveratrol (12.5, 25.0 and 50.0 μg mL^{-1}) in the presence of celecoxib (25 μg mL^{-1}) were 98.1 ± 1.2, 98.6 ± 1.0 and 98.9 ± 0.8 %, resp. Protein binding of resveratrol (12.5, 25.0 and 50.0 μg mL^{-1}) in the presence of celecoxib (250 μg mL^{-1}) was 97.8 ± 1.3, 98.4 ± 0.9 and 98.8 ± 1.1 %, resp. The results demonstrate that either low or higher concentration of celecoxib is unlikely to affect the plasma protein binding of resveratrol (Table III).

Table II. Rat plasma protein binding of resveratrol

Drug	Added conc. (μg mL^{-1})	Measured conc. (μg mL^{-1})	Ultrafiltrate conc. (μg mL^{-1})	Plasma protein binding rate (%)
Resveratrol	12.50	12.21 ± 0.68	0.21 ± 0.02	98.3 ± 1.0
	25.00	24.72 ± 0.97	0.34 ± 0.02	98.6 ± 1.2
	50.00	50.97 ± 1.98	0.47 ± 0.03	99.0 ± 0.8

Mean ± SD, n = 4.

Table III. Effect of celecoxib on plasma protein binding of resveratrol

	Resveratrol			
Celecoxib (μg mL^{-1})	Added conc. (μg mL^{-1})	Measured conc. (μg mL^{-1})	Ultrafiltrate conc. (μg mL^{-1})	Plasma protein binding rate (%)
	12.50	12.36 ± 0.75	0.23 ± 0.02	98.1 ± 1.2
25	25.00	25.15 ± 1.15	0.36 ± 0.03	98.6 ± 1.0
	50.00	51.54 ± 1.83	0.53 ± 0.03	98.9 ± 0.8
250	12.50	12.43 ± 0.87	0.29 ± 0.02	97.8 ± 1.3
	25.00	25.05 ± 1.02	0.38 ± 0.03	98.5 ± 0.9
	50.00	50.79 ± 1.56	0.61 ± 0.02	98.8 ± 1.1

Mean ± SD, $n = 4$.

Table IV. Effect of leflunomide on plasma protein binding of resveratrol

	Resveratrol			
Leflunomide (μg mL^{-1})	Added conc. (μg mL^{-1})	Measured conc. (μg mL^{-1})	Ultrafiltrate conc. (μg mL^{-1})	Plasma protein binding rate (%)
	12.50	12.40 ± 0.79	0.26 ± 0.02	97.9 ± 1.1
25	25.00	25.05 ± 1.35	0.39 ± 0.04	98.4 ± 1.3
	50.00	50.74 ± 1.84	0.58 ± 0.08	98.9 ± 1.0
250	12.50	12.37 ± 0.89	0.32 ± 0.02	97.4 ± 0.8
	25.00	24.64 ± 1.13	0.41 ± 0.03	98.3 ± 0.7
	50.00	49.59 ± 1.49	0.67 ± 0.09	98.6 ± 1.2

Mean ± SD, $n = 4$.

Effects of low and high concentrations of leflunomide

Results presented in Table IV show that neither the low nor high concentration of leflunomide is likely to affect the plasma protein binding of resveratrol.

In this study, we found that plasma protein binding of resveratrol was not affected if combined with celecoxib or leflunomide, presumably because of the different binding sites with plasma proteins.

CONCLUSIONS

In the present study, molecular docking was adopted to predict the drug-drug plasma protein binding interactions of resveratrol, and then the ultrafiltration technique was used to verify the docking results. Results demonstrate that resveratrol alone has a high binding

rate with rat plasma proteins, even if combined with celecoxib or leflunomide. These results indicate that the proposed combination therapy might be pharmacologically feasible. However, the drawback of the study is the lack of more extensive investigation of different relevant pharmacokinetic parameters of resverastrol and its combination with celecoxib or leflunomide. Further studies will focus on drug-drug interactions of resveratrol with human plasma proteins. Thus, more in-depth studies are needed to improve the experimental technology and enrich research indicators.

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The results for selectivity, recovery and precision of the method and stability of resveratrol are given in Supplementary material. It is available upon request.

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