A study of the binding between radicicol and four proteins by means of spectroscopy and molecular docking

Ya Gan, Ning Bai, Xitong Li, Shuiting Gao and Ruiyong Wang

Abstract
The interactions between radicicol and four proteins (catalase, trypsin, pepsin, and human serum protein) are investigated by spectroscopic techniques and molecular docking. A static quenching process is confirmed. The binding constant value between radicicol and human serum protein is the largest among the four proteins. Results reveal changes in the micro-environment of the protein by the addition of radicicol. It is found that radicicol shows an inhibitory effect on the activity of proteins (catalase, trypsin, and pepsin). Molecular docking results are consistent with the thermodynamic experimental results. This work provides clues to the elucidation of the mechanisms of the interactions between radicicol and proteins.

Keywords
interactions, molecular docking, protein, radicicol, spectroscopy

Introduction
Radicicol is a natural antifungal macrocyclic compound obtained from the metabolites of cucumber anthracnose using macroporous resin extraction and silica gel column chromatography separation technology; its structure is shown in Figure 1. Early research found that radicicol is a highly effective and specific small molecule heat shock protein 90 (Hsp90) inhibitor. Radicicol can change the conformation of Hsp, thereby inhibiting the specific binding of Hsp90 to the target protein. Radicicol, as a recognized Hsp90 functional inhibitor, has been widely used in biological research and has been used in clinical trials as an antitumor drug. Rohner et al. treated the offspring of Mexican dark-environment cavefish and surface waters with radicicol and detected that the mRNA levels of two Hsp90 inhibitory marker genes, BCL2-associated athanogene-3 (BAG3) and heat shock protein beta-1 (HSPB1), were upregulated, proving that Hsp90 function is inhibited. Radicicol has attracted wide attention from researchers and is an interesting candidate for clinical research.

Interactions between small molecules and proteins can affect their pharmacodynamics and pharmacokinetic properties and may also affect the structure of proteins. Therefore, studies on the interactions between small molecules and proteins at the molecular level are of great significance for understanding the role of these molecules in the human body. Commonly used methods for such studies are UV-Vis absorption spectroscopy, fluorescence spectroscopy, infrared spectroscopy, and circular dichroism (CD). In recent years, mass spectrometry, nuclear magnetic resonance, isothermal titration calorimetry, and other technologies have gradually been utilized for the study of proteins. In our previous work, we studied the interactions between radicicol and the FTO protein and proved that radicicol is an effective inhibitor. Human serum proteins are used as transport and storage proteins in organisms and can be used as transport intermediates for many compounds, providing a basis for the study of some small molecules entering the body. Trypsin plays a very important role in the digestion and decomposition of food, and its level can be used to judge pancreatic function and pathological changes. Pepsin is a type of hydrolase which functions as a
digestion protein and has specificity for certain amino acid sequences. Catalase is an antioxidant and a potential target for some diseases. The study of the interaction between radicicol and proteins has theoretical significance for the design and development of new drugs.

Results and discussion

Fluorescence quenching spectra

The fluorescence spectra of the four proteins in the absence and presence of increasing radicicol concentrations are shown in Figure 2. The concentrations and volumes of radicicol used with the four proteins are identical. It can be seen from the figure that the excitation wavelength is 280 nm and that the maximum emission peaks of the four proteins are all around 340 nm. After adding the same volume of radicicol, the fluorescence of the four proteins exhibited quenching effects, and the fluorescence intensities of the proteins gradually decreased.

The type of fluorescence quenching is usually divided into static quenching and dynamic quenching. To study the quenching types of radicicol on the four proteins, we used the Stern–Volmer equation (1) to calculate the $K_q$ values at three different temperatures to determine the quenching mechanism:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q]$$

where $F_0$ and $F$ are the fluorescence intensities of the four proteins in the presence and absence of radicicol, $K_q$ is the bimolecular quenching constant, $\tau_0$ is the lifetime of the fluorescence in the absence of quencher ($\tau_0 = 10^{-8}$ s), $[Q]$ is the concentration of radicicol, and $K_{sv}$ is the Stern–Volmer quenching constant. Therefore, the above formula can be used to obtain $K_{sv}$ by linear regression of a plot of $F_0/F$ and $[Q]$.

The $F_0/F-[Q]$ curves obtained based on the fluorescence emission spectra of the interactions between radicicol and the four proteins at three temperatures (293, 301, and 309 K) are shown in Figure 3. The quenching constants $K_{sv}$ at different temperatures are evaluated and are given in Table 1. Since the fluorescence lifetime of the biopolymer is $10^{-8}$ s, the quenching rate constant $K_q$ can be calculated based on $K_{sv} = K_q \tau_0$. The reported maximum scattering collision quenching constant is $2 \times 10^{10}$ L mol$^{-1}$ s$^{-1}$. The data

Figure 1. The structure of radicicol.

Figure 2. Effects of radicicol on fluorescence spectra of the four proteins ($T = 301$ K, $pH = 7.4$, $\lambda_{ex} = 280$ nm, from 1 to 9 means the concentration of radicicol is 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, $8.0 \times 10^{-6}$ mol L$^{-1}$, $C_{Hsa} = 3.2 \times 10^{-6}$ mol L$^{-1}$, $C_{Pep} = 3 \times 10^{-7}$ mol L$^{-1}$, $C_{Cat} = 4 \times 10^{-8}$ mol L$^{-1}$, $C_{Tr} = 3.75 \times 10^{-8}$ mol L$^{-1}$).
in Table 1 show that the value of $K_q$ is much larger than the reported maximum scattering collision quenching constant. Therefore, the rate constant of the protein quenching initiated by radicicol is greater than the value of $K_q$ for the quenching mechanism and proves that radicicol is static for the four protein macromolecules. From Table 1, the decrease in $K_q$ with increasing temperature also indicates a static quenching mechanism.

### Binding constants and number of binding sites

The binding constant ($K_A$) and the number of binding sites ($n$) in the static quenching reaction can be obtained according to the double logarithmic equation (2)²⁸,²⁹

$$
\log\left(\frac{F_0 - F}{F}\right) = \log K_A + n \log [Q]
$$

Figure 4 shows the fitting curve for $\log [Q]$ versus $\log [(F_0 - F)/F]$ at different temperatures. The binding constant ($K_A$) and the number of binding sites ($n$) can be obtained by extrapolating the slope and intercept of the curve and are listed in Table 2.

It can be seen from the plots in Figure 4 that a linear fit was obtained between $\log [(F_0 - F)/F]$ and $\log [Q]$. The $K_A$ data in Table 2 show that the order of the binding ability is Hsa > Pep > Cat > Try. The values of $K_A$ are in the range of $10^4$–$10^6$ L mol$^{-1}$, which are in agreement with the common affinities of drugs for proteins.³⁰ The number of binding sites is about 1. The data in Table 2 indicate that the binding constant values decrease with increasing temperature due to a reduction of the stability of the radicicol–protein complex and a change of the surrounding protein caused by the high temperature, which leads to a weakening of the binding ability of radicicol. It was further confirmed that radicicol quenched the fluorescence of the four proteins as static quenching.

### Table 1. Quenching constants ($K_{sv}$), quenching rate constants ($K_q$), and correlation coefficients ($R^2$) of the interactions between radicicol and the four proteins.

| Protein | T (K) | $K_{sv}$ (L mol$^{-1}$) | $K_q$ (L mol$^{-1}$ s$^{-1}$) | $R^2$ |
|---------|-------|-------------------------|-----------------------------|-------|
| Cat     | 293   | $4.85 \times 10^4$      | $4.85 \times 10^{12}$       | 0.9978|
|         | 301   | $3.75 \times 10^4$      | $3.75 \times 10^{12}$       | 0.9834|
|         | 309   | $3.06 \times 10^4$      | $3.06 \times 10^{12}$       | 0.9706|
| Hsa     | 293   | $1.48 \times 10^5$      | $1.48 \times 10^{13}$       | 0.9977|
|         | 301   | $1.44 \times 10^5$      | $1.44 \times 10^{13}$       | 0.9988|
|         | 309   | $1.38 \times 10^5$      | $1.38 \times 10^{13}$       | 0.9919|
| Pep     | 293   | $7.32 \times 10^4$      | $7.32 \times 10^{12}$       | 0.9905|
|         | 301   | $6.60 \times 10^4$      | $6.60 \times 10^{12}$       | 0.9973|
|         | 309   | $5.63 \times 10^4$      | $5.63 \times 10^{12}$       | 0.9933|
| Try     | 293   | $1.87 \times 10^5$      | $1.87 \times 10^{13}$       | 0.9844|
|         | 301   | $1.01 \times 10^5$      | $1.01 \times 10^{13}$       | 0.9971|
|         | 309   | $7.02 \times 10^4$      | $7.02 \times 10^{12}$       | 0.9950|
To determine the type of force between radicicol and the four proteins, the thermodynamic parameters $\Delta H^\theta$ (enthalpy change), $\Delta S^\theta$ (entropy change), and $\Delta G^\theta$ (Gibbs free energy) can be calculated according to the van’t Hoff equation (3) and the thermodynamic equation (4) for comparison:

$$\ln K_a = -\frac{\Delta H^\theta}{R T} + \frac{\Delta S^\theta}{R}$$  \hspace{1cm} (3)

$$\Delta G^\theta = \Delta H^\theta - T\Delta S^\theta$$  \hspace{1cm} (4)

The thermodynamic curves of the interaction between radicicol and the four proteins are shown in Figure 5, and the thermodynamic parameters obtained by calculation are listed in Table 3.

In this study, the formation of a radicicol–protein complex was found to be spontaneous as was evident from the negative $\Delta G$ values. The negative values of $\Delta G$ and the $\Delta S$ values for the interactions of Pep (or Hsa) with radicicol indicated that hydrogen bonding and weak van der Waals interactions are the predominant forces that contribute to the Gibbs free energy change of the interactions. $\Delta G < 0$, $\Delta H < 0$, and $\Delta S > 0$ indicate that the reaction between radicicol and Cat (or Try) is an entropy-driven self-heating reaction, and that the type of forces may be electrostatic and hydrophobic.

**Number of thermodynamic parameters and binding force type**

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$$\ln K_a = -\frac{\Delta H^\theta}{R T} + \frac{\Delta S^\theta}{R}$$  \hspace{1cm} (3)

The effect of radicicol on the conformations of the four proteins

Synchronous fluorescence and three-dimensional (3D) fluorescence are commonly used to study the influence of small molecules on protein conformations. It was reported that the change in the maximum emission position corresponds to the change in polarity around the chromophore molecules. When the wavelength interval ($\Delta \lambda$) between the excitation and emission wavelengths is stable at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of
tyrosine residues or tryptophan residues. The synchronous fluorescence map of the four proteins and radicicol is shown in Figure 6. It can be seen that the fluorescence intensity of tryptophan residues is much greater than that of tyrosine residues. With the continuous addition of radicicol, the fluorescence intensity of the tryptophan residues and tyrosine residues is reduced to varying degrees. When the maximum emission wavelength of Cat and Pep at $\Delta \lambda = 15$ nm remains unchanged and the maximum emission wavelength at $\Delta \lambda = 60$ nm is slightly red-shifted, this is because the addition of radicicol changes the micro-environment of the tryptophan residues around the two proteins. Try and Pep remain unchanged, indicating that the micro-environment of these two amino acid residues is not changed.

The 3D fluorescence spectra are shown in Figure 7. The intensities of Peak 1 and Peak 2 decrease significantly in the presence and absence of radicicol. The uniformity further proves that radicicol can change the micro-environments and conformations of the four proteins.

**CD measurements**

CD can be used to measure the secondary structure of proteins in the far ultraviolet region (190–260 nm). At these wavelengths, the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. The effect of radicicol on the CD spectra of the four proteins is shown in Figure 8. Hsa and Cat have two negative peaks at 208 and 220 nm, which are typical $\alpha$-helices. Both Pep and Try have a strong negative peak, which is a typical $\beta$-sheet. When radicicol binds to proteins, the negative band intensities decreased. This clearly indicated changes in the protein secondary structure, with some apparent loss of helical stability. The shapes of the CD spectra of the proteins in the absence and presence of radicicol were observed to be similar, which indicated that the structures of the proteins were predominantly $\alpha$-helical and $\beta$-sheets even after binding with radicicol. The CD results are expressed in terms of the mean residue ellipticity (MRE) in degree cm$^2$ dmol$^{-1}$ according to equation (5)

$$MRE = \frac{\text{observed(\text{mdeg})}}{C_p n l \times 10}$$

where $C_p$ is the molar concentration of protein, $n$ is the number of amino acid residues of the protein, and $l$ is the path length. The $\alpha$-helix combined protein is calculated from the MRE values at 208 nm using equation (6)

$$\alpha - \text{helix} \% = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000}$$

The conformational changes observed from CD spectroscopic analysis are shown in Table 4. The CD spectra suggest that the secondary structures of the proteins were changed due to the addition of radicicol.

**The effect of radicicol on protein activity**

To reveal whether radicicol can affect the activity of proteins, the effect of radicicol on protein activity in vitro was investigated. The absorbance change was measured at 275 nm (OD275) to study the inhibition rate of pepsin activity. The absorbance change was measured at 253 nm (OD253) to study the trypsin activity inhibition rate. The absorbance change was measured at 240 nm (OD240) to study the inhibition rate of catalase activity. The inhibition rate of protein activity can be calculated with the formula (7)

$$\text{Relative activity rate(\%)} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{blank}}}$$

As showed in Figure 9, the changes in enzyme activity are induced by the addition of radicicol of different concentrations. With the rise of concentration of radicicol, the inhibition rate increases and the activity of the protein is decreased. It can be seen from the figure that there are obvious differences in the inhibitory effects of radicicol on several proteins, and the inhibitory effect on pepsin is the best. These results imply that radicicol can inhibit protein activity when it enters the human body.

**Molecular docking**

The docking results with the lowest energy obtained by molecular docking technology are shown in Figure 10. The amino acid residues that bind to the four proteins are listed.
in Table 5. The analysis has revealed that radicicol is embedded in the four proteins, and that the hydrophobic residues interacting with Hsa are PHE211, TRP214, LEU481, and LEU198. The hydrophobic residues that interact with Cat are PHE160, VAL145, GLY146, and ALA132. The hydrophobic residues that interact with Pep are GLY217, VAL30, and PHE111. The hydrophobic residues that interact with TRY are ILE162, LEU163, PHE181, and MET180. The existence of these hydrophobic residues proves the existence of hydrophobic forces in the interactions between radicicol and the four proteins. In addition, it can be seen from the table that there are hydrogen bonds in the residues of the four proteins that interact with radicicol, but the number of hydrogen bonds is different, which proves that hydrogen bonds also play a certain role in the interactions. This is consistent with the conclusions obtained from thermodynamic data analysis.

Conclusion

This work provides an approach for studying the interactions of four proteins with radicicol using different...
spectroscopic techniques under physiological conditions. The decreasing values of $K_q$ with increase in temperature indicate the presence of a static quenching mechanism. Calculating the binding constant and the number of reactive binding sites at three temperatures shows that Hsa has the strongest binding capacity of the four proteins. According to the relevant laws of thermodynamics, the reaction interactions of radicicol and the four proteins can proceed spontaneously. The results show that the addition of radicicol can change the conformation of the four proteins. The CD spectra revealed that the presence of radicicol decreased the $\alpha$-helix content of Hsa and Cat, meanwhile the presence of radicicol decreased the $\beta$-sheet content of Pep and Try. The spectrophotometric method directly proved that radicicol has an inhibitory effect on the proteins Pep, Cat, and Try. The results of molecular docking support the thermodynamic data results. The different numbers of residues in the docking results of the

**Figure 7.** The three-dimensional fluorescence spectra of the four proteins and the three-dimensional fluorescence spectra after the addition of radicicol at 301 K.
four proteins with radicicol also proved the different binding abilities.

**Experimental**

**Materials and instruments**

The four proteins and radicicol used in this experiment were purchased from Aladdin Reagent Company. Pepsin and catalase were stored in a refrigerator at 4 °C. Try and human serum protein were stored in a refrigerator at −20 °C. Human serum protein and catalase were dissolved in Tris-HCl buffer solution to prepare a stock solution, and gastric protein was dissolved in pH = 2 HCl-CH₃COONa buffer solution. Trypsin was dissolved in PBS buffer to make a stock solution. Radicicol was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. Double distilled water was used, and the reagents used were all analytically pure. The stock solutions were all centrifuged and degassed after preparation. The protein stock solution was stored in a refrigerator at −80 °C, and the radicicol stock solution was stored in a refrigerator at −20 °C and then diluted according to the different experimental requirements.

An Agilent 8453 UV-Vis spectrophotometer (Agilent), a F-4600 fluorescence spectrophotometer (Japan Hitachi), a DZKW-4 constant temperature water bath (Beijing Zhongxing Weiyi), a QL-866 vortex oscillator (Kirin, Haimen City medical instruments), an AUW120D analytical balance (SHIMADZU), and a Centrifuge 5424 small high-speed centrifuge (Ebende, Germany) were used.
**Fluorescence spectra**

The fluorescence spectra of the four proteins, both in the absence and presence of radicicol, were recorded over a wavelength range of 290–450 nm on excitation at 280 nm with 5 nm excitation and emission monochromator slit widths. Fluorescence quenching experiments were carried out at three different temperatures (293, 301, and 309 K) to evaluate the effect of the temperature on the interactions.10

**Synchronous fluorescence spectroscopy**

Synchronization was carried out at room temperature with $\Delta \lambda = 15$ or 60 nm. The scanning range is 240–340 nm; scanning speed is 1200 nm min$^{-1}$.10

**3D fluorescence spectroscopy**

The excitation wavelength and emission wavelength ranges were set to 210–290 nm and 310–380 nm, respectively; the scanning speed is 1200 nm min$^{-1}$.

**CD measurements**

CD measurements were recorded in the far ultraviolet region at room temperature on a CD250 spectrometer. A 1.0-cm quartz cell was used. The molar ratio of protein and radicicol was kept constant, and the spectra of the four proteins were measured in the presence and absence of radicicol. The buffered spectrum is subtracted during scanning, and each sample is measured three times on average. The CD of Cat, Hsa, and Try were measured at 200–260 nm, and the CD of Pep was measured at 190–260 nm.

**Protein activity experiments**

Radicicol was prepared as 2 mM stock solutions with DMSO. Cat was made up as a 0.5 mg mL$^{-1}$ mother liquor with pH = 7 PBS solution, and hydrogen peroxide was used as the substrate.39 Try was prepared as a 10 mg mL$^{-1}$ stock solution, N-benzyol-L-arginine ethyl ester (BAEE) was used as the substrate.38 Pep is made into 20 µM stock solution with pH = 2 HCl-CH$_3$COONa buffer, with hemoglobin as a substrate.37 UV-Vis absorption spectroscopy was used to detect the absorbance changes of the three substrates and to study the changes in protein activity.40

### Table 5. Amino acid residues interacting with the four proteins.

| Protein | Amino acid residue |
|---------|-------------------|
| Cat     | THR360, HIS361, PHE160, VAL145, GLY146, ASN147, GLY146, ALA147, LYS199, LEU481, SER454, LEU198 |
| Hsa     | HOH877, ARG71, TRP214, HOH2024 |
| Pep     | GLN287, ASP215, SER219, THR218, HOH370, LEU163, CYS182, PHE182, HOH181 |
| Try     | GLU13, MET180, ASP165, ILE162, LEU163, PHE182, HOH181 |

**Figure 10.** The docking results of radicicol and four proteins: the figures on the left show the ribbons model of the four proteins and the spherical models of radicicol. The figures on the right show the amino acid residues that interact with radicicol in the four proteins. The dotted lines and the numbers indicate the hydrogen bonds and bond lengths, respectively.
**Molecular docking experiments**

The radicicol structure was generated using ChemDraw, and the molecular docking simulation was performed with the software Autodock4.2, and the Lamarck genetic algorithm was applied to the docking process. Gaussian 09 was used for optimization. The density functional B3LYP was used to perform geometric optimization on the 6-31G (d,p) basis sets of C, H, O, N, and F atoms. The crystal structures of the four proteins are obtained from the Brook Haven Protein Data Bank (http://www.rcsb.org/pdb). The PDBID of human serum protein is 1H9Z, the PDBID of Cat is 4BLC, the PDBID of Pep is 5Pep, and the PDBID of Try is 2ZQ1. After the docking was completed, the optimal combination was found from 250 conformations.

**Declaration of conflicting interests**

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