Supporting Information

Unveiling the interaction profile of rosmarinic acid and its bioactive substructures with serum albumin

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Table of Contents

A. Characterization of salvianic acid .......................................................... 32
   A1. HPLC chromatograms from the purification of salvianic acid .................. 32
   A2. 1HNMR spectrum of salvianic acid .................................................. 54
   A3. HSQC-HMBC spectrum of salvianic acid .......................................... 54

B. Stern-Volmer analysis of the fluorescence spectroscopic data ...................... 76

C. Characterization of the BSA interaction with rosmarinic, caffeic and salvianic acid through STD and NOESY NMR ........................................................... 76
   C1. Epitope mapping of the BSA interaction with caffeic and salvianic acid .... 76
      C1a. Epitope mapping of the BSA-caffeic acid interaction ....................... 76
      C1b. Epitope mapping of the BSA-salvianic acid interaction .................... 76
C2. NOESY spectra of rosmarinic, caffeic and salvianic acid in the presence or absence of BSA

C2a. NOESY spectra of rosmarinic acid

C2b. NOESY spectra of caffeic acid

C2c. NOESY spectra of salvianic acid

C3. STD NMR data of competitive experiments

C3a. STD NMR spectra and STD amplification factors of warfarin

C3b. STD NMR spectra and STD amplification factors of ibuprofen

B3c. STD amplification factors of rosmarinic acid in the presence of warfarin/ibuprofen

C3d. STD amplification factors of caffeic acid in the presence of warfarin/ibuprofen

C3e. STD amplification factors of salvianic acid in the presence of warfarin/ibuprofen

D. Isothermal titration calorimetry

E. Molecular Docking to the main active sites of BSA

E1. Interaction of rosmarinic acid with Sudlow site I

E2. Interaction of salvianic acid with Sudlow site I

E3. Interaction of warfarin with Sudlow site I

E4. Interaction of ibuprofen with Sudlow site II

E5. Interaction of ibuprofen with Sudlow site II

A. Characterization of salvianic acid

A1. HPLC chromatograms from the purification of salvianic acid
Figure S1. HPLC chromatograms from the purification of salvianic acid. (1): NaOH 0.1 M, 1 h; (2): NaOH 1.0 M, 4 h; (3): NaOH 1.0 M, 8 h; (4): NaOH 1.0 M, 12 h; (5): NaOH 1.0 M, 4 h; (6): NaOH 1.0 M, 8 h; (7): NaOH 0.1 M, 4 h; The desired product’s elution time is at 12.6 min, caffeic acid is eluted at 21 min and non-hydrolyzed rosmarinic acid is eluted at 27 min with gradient elution system MeOH–H₂O containing 0.1 percent TFA (40-100% MeOH).
Figure S2. $^1$HNMR spectrum of salvianic acid in DMSO-$d_6$, 298K, recorded at 500 MHz.

A3. HSQC-HMBC spectrum of salvianic acid
Figure S3. HSQC-HMBC overlay of salvianic acid in DMSO-d$_6$, 298K, recorded at 500 MHz.

B. Stern-Volmer analysis of the fluorescence spectroscopic data

Figure S3.4. F$_0$/F as a function of A) Rosmarinic B) Caffeic and C) Salvianic acid concentration for the calculation of the Stern–Volmer quenching constant, $K_{SV}$ at room temperature.

Table S1. The quenching constants of BSA in the presence of rosmarinic, caffeic and salvianic acid.

| Compound      | $K_{SV} (10^{4} \text{ L mol}^{-1})$ | $K_{q} (10^{13} \text{ L mol}^{-1} \text{ s}^{-1})$ | $R^2$ |
|---------------|------------------------------------|-------------------------------------------------|-------|
| Rosmarinic acid | 42.4                               | 4.2                                             | 0.99  |
| Caffeic acid   | 17.1                                | 1.7                                             | 0.99  |
| Salvianic acid | 14.3                                | 1.4                                             | 0.95  |
**BC.** Characterization of the BSA interaction with rosmarinic, caffeic and salvianic acid through STD and NOESY NMR.

**B1C1.** Epitope mapping of the BSA interaction with caffeic and salvianic acid

**B1aC1a.** Epitope mapping of the BSA-caffeic acid interaction

**Figure S4S5.** (a) $^1$H NMR reference spectrum of the complex caffeic acid (2 mM)-BSA (20 μM) in Tris-$d_1$ buffer 10 mM, pH=7.4 with 600 μl $D_2$O. (b) STD difference NMR spectrum of the complex caffeic acid-BSA. The percentage values show the STD$_{AMP}$ for all the protons of caffeic acid.

**B1bCJb.** Epitope mapping of the BSA-salvianic acid interaction
Figure S56. (a) $^1$H NMR reference spectrum of the complex salvianic acid (2 mM)-BSA (20 μM) in Tris-d$_{11}$ buffer 10 mM, pH=7.4 with 600 μL D$_2$O. (b) STD difference NMR spectrum of the complex salvianic acid-BSA. The percentage values show the STD$_{AMP}$ for all the protons of salvianic acid.

B2C2. NOESY spectra of rosmarinic, caffeic and salvianic acid in the presence or absence of BSA
**Figure S6S7.** Expanded region of NOESY spectrum of the rosmarinic acid, recorded with mixing time of 800 ms at 500 MHz, showing the presence of negative-positive NOEs of free ligand (different sign than the diagonal).
Figure S7S8. Expanded region of tr- NOESY spectrum of the complex rosmarinic acid-BSA, recorded with mixing time of 800 ms at 500 MHz, showing the presence of positive negative NOEs of bound ligand (same sign as the diagonal).

Figure S8S9. Expanded region of NOESY spectrum of the caffeic acid, recorded with mixing time of 800 ms at 500 MHz, showing the presence of negative positive NOEs of free ligand (different sign than the diagonal).
Figure S9. Expanded region of tr-NOESY spectrum of the complex caffeic acid-BSA, recorded with mixing time of 800 ms at 500 MHz, showing the presence of positive negative NOEs of bound ligand (same sign as the diagonal).

82c2c. NOESY spectra of salvianic acid
Figure S40-S11. Expanded region of NOESY spectrum of the salvianic acid, recorded with mixing time of 800 ms at 500 MHz, showing the presence of negative and positive NOEs of the free ligand (different sign than the diagonal).
Figure S11S12. Expanded region of tr- NOESY spectrum of the complex salvianic acid-BSA, recorded with mixing time of 800 ms at 500 MHz, showing the presence of positive negative NOEs of bound ligand (same sign as the diagonal).

**B3C3.** STD NMR data of competitive experiments

**B3aC3a.** STD NMR spectra and STD amplification factors of warfarin
Figure S12S13. (a) 1H NMR reference spectrum of the complex warfarin (2 mM)-BSA (50 μM) in PBS buffer 10 mM, pH=7.4 with 600 μl D₂O. (b) STD difference NMR spectrum of the complex warfarin-BSA.

B3b. STD NMR spectra and STD amplification factors of ibuprofen

Figure S13S14. (a) 1H NMR reference spectrum of the complex ibuprofen (2 mM)-BSA (20 μM) in Tris-d₁₁ buffer 10 mM, pH=7.4 with 600 μL D₂O. (b) STD difference NMR spectrum of the complex ibuprofen-BSA.

B3c. STD amplification factors of rosmarinic acid in the presence of warfarin/ibuprofen

Table S1S2. STDAMP factors occurring from the titrations of rosmarinic acid with warfarin.

| Proton/Concentration | STDAMP factors for Rosmarinic Acid |
|----------------------|-----------------------------------|
|                      | 0 mM warfarin | 2 mM warfarin | 4 mM warfarin | 6 mM warfarin | 8 mM warfarin |
| H7                   | 27.32%        | 26.54%        | 24.87%        | 22.32%        | 20.85%        |
| H5                   | 22.25%        | 21.32%        | 19.98%        | 17.65%        | 16.39%        |
| H2                   | 27.85%        | 26.39%        | 22.88%        | 23.33%        | 18.91%        |
| H6                   | 22.65%        | 21.31%        | 20.83%        | 19.47%        | 18.09%        |
| H2                   | 34.87%        | 34.60%        | 28.91%        | 22.87%        | 22.01%        |
Table S2S3. STDAMP factors occurring from the titrations of rosmarinic acid with ibuprofen.

| Protons/Concentration | STDAMP Factors of Rosmarinic Acid |
|-----------------------|----------------------------------|
|                       | 0 mM ibuprofen | 2 mM ibuprofen | 4 mM ibuprofen |
| H5                    | 19.09%          | 18.09%          | 14.51%          | 13.80%          | 13.31%          |
| H6                    | 24.20%          | 23.53%          | 19.66%          | 15.57%          | 7.79%           |
| H7a                   | 7.32%           | 6.08%           | 3.11%           | 4.91%           | 3.55%           |
| H7b                   | 20.32%          | 19.85%          | 12.99%          | 11.98%          | 9.86%           |

Table S3S4. STDAMP factors occurring from the titrations of caffeic acid with warfarin.

| Protons/Concentration | STDAMP Factors for Rosmarinic Acid |
|-----------------------|----------------------------------|
|                       | 0 mM warfarin | 2 mM warfarin | 4 mM warfarin | 6 mM warfarin | 8 mM warfarin |
| H3                    | 26.43%          | 25.7%          | 22.68%          | 18.32%          | 4.95%           |
| H5                    | 26.43%          | 25.19%          | 10.68%          | 9.80%           | 3.99%           |
| H8                    | 12.65%          | 11.44%          | 9.41%           | 8.21%           | 8.18%           |
| H9                    | 14.87%          | 13.15%          | 11.19%          | 11.14%          | 9.02%           |
| H2                    | 15.32%          | 14.44%          | 4.95%           | 4.88%           | 4.57%           |

Table S4S5. STDAMP factors occurring from the titrations of caffeic acid with ibuprofen.

| Protons/Concentration | STDAMP Factors for Caffeic Acid |
|-----------------------|--------------------------------|
|                       | 0 mM ibuprofen | 2 mM ibuprofen | 4 mM ibuprofen |
| H3                    | 11.48%          | 12.43%          | 23.10%          |
| H5                    | 19.65%          | 20.17%          | 21.62%          |
| H8                    | 20.51%          | 22.83%          | 38.22%          |
| H9                    | 25.76%          | 28.83%          | 35.23%          |
| H2                    | 22.87%          | 23.40%          | 38.41%          |

Table S3S3. STD amplification factors of caffeic acid in the presence of warfarin/ibuprofen

Table S4S4. STDAMP factors occurring from the titrations of caffeic acid with warfarin.

Table S5S5. STDAMP factors occurring from the titrations of caffeic acid with ibuprofen.

Table S3S3. STD amplification factors of salvianic acid in the presence of warfarin/ibuprofen
**Table S56.** STDAMP factors occurring from the titrations of salvianic acid with warfarin.

| Proton/Concentration | STDAMP factors for Salvianic acid | 0 mM warfarin | 2 mM warfarin | 4 mM warfarin |
|----------------------|----------------------------------|---------------|---------------|---------------|
| H8                   | 25.11%                           | 0             | 0             |               |
| H5                   | 27.94%                           | 0             | 0             |               |
| H9                   | 33.78%                           | 0             | 0             |               |
| H3a                  | 13.49%                           | 0             | 0             |               |

**Table S667.** STDAMP factors occurring from the titrations of salvianic acid with ibuprofen.

| Protons/Concentration | STDAMP factors for Salvianic acid | 0 mM ibuprofen | 2 mM ibuprofen | 4 mM ibuprofen |
|-----------------------|----------------------------------|---------------|---------------|---------------|
| H3a                   | 0.19%                            | 0.26%         | 0.46%         |

**Figure S45S15.** (a) $^1$H NMR reference spectrum of the complex ibuprofen (2 mM)-BSA (20 μM), including salvianic acid 0.2 mM, in Tris-d$_{11}$ buffer 10 mM, pH=7.4 with 600 μl D$_2$O. STD difference NMR spectrum of the complex ibuprofen-BSA, including:
(b) 0.2 mM salvianic acid (c) 0.4 mM salvianic acid (d) 0.8 mM salvianic acid (e) 1 mM salvianic acid (f) 1.5 mM salvianic acid (g) 2 mM salvianic acid (h) 3 mM salvianic acid.

**Table S7S8.** STD<sub>AMP</sub> factors occurring from the titrations of the complex ibuprofen-BSA with salvianic acid.

| Proton/Concentration | 8.2 mM | 0.4 mM | 0.8 mM | 1 mM | 1.50 mM | 2 mM | 3 mM |
|----------------------|--------|--------|--------|------|--------|------|------|
| H8                   | 0.05%  | 0.06%  | 0.10%  | 0.14%| 0.17%  | 0.19%| 1.31% |
| H5                   | 0.05%  | 0.07%  | 0.10%  | 0.16%| 0.21%  | 0.22%| 1.63% |
| H4                   | 0.06%  | 0.09%  | 0.14%  | 0.19%| 0.20%  | 1.18%|      |
| H2 minor             | 0%     | 0.08%  | 0.13%  | 0.17%| 0.23%  | 0.23%| 2.64% |
| H2 major             | 0%     | 0.01%  | 0.09%  | 0.10%| 0.13%  | 0.15%| 1.17% |
| H3b                  | 0.01%  | 0.02%  | 0.04%  | 0.04%| 0.10%  | 0.36%| 0.96% |
| H3a                  | 0.01%  | 0.02%  | 0.02%  | 0.07%| 0.11%  | 0.15%| 0.83% |

**Graph S1.** STD<sub>AMP</sub> factor values of representative protons of Ibuprofen, from the complex Ibuprofen (2 mM)- BSA (20 μM), which is titrated with salvianic acid.

**CD.** Isothermal titration calorimetry
**Figure S15-S16.** Isothermal titration calorimetry measurement for the interaction of BSA with Salvianic acid. The isotherm plot (up) and the integrated curve (down) are presented.

**DE. Molecular Docking to the main active sites of BSA**

**Table S8-S9.** Binding energies of the studied molecules in Sudlow site I in BSA.

| Bovine Serum Albumin (PDB : 4F5S) | Sudlow Site I – Site IIA Docking Score (kcal/mol) |
|----------------------------------|--------------------------------------------------|
| Rosmarinic acid                  | -13.545                                          |
| Caffeic acid                     | -11.583                                          |
| Salvianic acid                   | -9.849                                           |
| Warfarin                         | -9.922                                           |
| Ibuprofen                        | -6.048                                           |
Interaction of rosmarinic acid with Sudlow site I

Figure S16S17. Binding interactions of rosmarinic acid in Sudlow site I of BSA.

Interaction of salvianic acid with Sudlow site I
**Figure S17S18.** Binding interactions of salvianic acid in Sudlow site I of BSA.

**D4E4. Interaction of warfarin with Sudlow site I**

Warfarin shows a very favored binding value (Docking Score = -9.922 kcal / mol) in Sudlow site I (Table S8S9). Warfarin in its best pose conformation (Figure S18S19, S19S20) forms H – bonds with four residues of the protein (Arg194, Arg198, His241, Arg256). In addition, the formation of a salt bridge between Arg217 and the enol group of the ligand contributes to the stabilization of the molecule in the binding site. Importantly, there are strong coulombic interactions between Sudlow site I and warfarin and this makes the molecule to adopt preferable binding to this site.
**Figure S18**. Best pose of warfarin in Sudlow site I. The four favorable hydrogen bonds, the salt bridge between Arg217 and the enol group of the ligand can explain its highly favorable binding.

**Figure S19**. Binding interactions of warfarin in Sudlow site I of BSA.

**D5ES**. Interaction of ibuprofen with Sudlow site II

Docking calculations, using empirical scoring functions, expressed a value of free energy binding in Sudlow site II equal to -9.769 kcal/mol (Table S8S9). Ibuprofen
in its best pose conformation (Figure S20S21, S21S22) forms H- bonds with three residues of the protein (Ser488, Asn390, Arg409). In addition, the formation of the salt bridge between the carboxylic group of the ligand and Lys413 plays a key role in the stabilization of the complex. In the low value of free energy, the orientation of the aromatic ring towards the hydrophobic residues (i.e. Leu452, Leu456, Leu406, Leu429, Val432, Cys391, Cys436, Cys437, Phe394, Phe402) plays an important role too.

Figure S20S21. Best pose of ibuprofen in Sudlow site II. The three favorable hydrogen bonds and the salt bridge formed between Lys413 and the carboxylic group of the ligand can explain its highly favorable binding.
Figure S21. Binding interactions of ibuprofen in Sudlow site II of BSA.