Investigating the interactions of benzoylaconine and benzoylhypacoitine with human serum albumin: experimental studies and computer calculations

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Abstract

Benzoylaconine (BAC) and benzoylhypacoitine (BHA), which are derived from Aconitine (AC) and hypacoitine (HA), are commonly used in Traditional Chinese Medicine (TCM). The pharmacokinetics of drugs can be affected by their binding to proteins. Therefore, it is necessary to investigate drug-protein interactions. In this study, multi-spectroscopic, molecular docking and dynamics simulation were applied to investigate the interaction mechanisms of human serum albumin (HSA) with BAC/BHA. The binding constants ($K_a$) of the HSA-BAC/BHA systems at 298 K were $1.701 \times 10^5$ and $1.876 \times 10^5$ M$^{-1}$, respectively. Stable BAC/BHA-HSA complexes were formed under the static quenching mode. Hydrogen bonds and van der Waals forces are the main driving forces during the spontaneous process, and BAC/BHA were suggested embedding into the site I of HSA. Circular dichroism (CD) spectroscopy revealed a reduction of 1.9% and 1.4% in the $\alpha$-helical structure content of HSA induced by BAC/BHA. The molecular docking found that the electronic structure domains of the nitrogen and benzene ring skeletons were critical in the complex formation. Furthermore, computer simulations provided insights into the stability changes and illustrated that the residues such as TRP-214, LEU-219, and LEU-238 acted as key residues in the binding process between aconitine analogs and HSA.
Key words: Alkaloids, Human serum albumin, Molecular docking, Molecular dynamics simulation.

1. Introduction

The benzoylaconine (BAC) and benzoylhypacoitine (BHA) are monoester-diterpenoid alkaloids (MDAs) (see Fig. 1) derived from Aconitine (AC) and hypacoitine (HA) [1, 2]. They are commonly utilized in Traditional Chinese Medicine (TCM) herbal remedies and have been employed to treat various ailments [3, 4]. BAC/ BHA possesses high pharmacological activities in anti-inflammatory, analgesic, anti-tumor, cardiotonic, and immune-boosting treatment [5, 6]. However, recent studies have revealed that BAC/ BHA has toxic effects on the cardiovascular and nervous systems, resulting in muscle weakness and respiratory failure [7-9]. Therefore, more attention should be paid to the formulation guidelines when using these compounds for medicinal purposes.

Plasma proteins play a vital role in enhancing the pharmacokinetic properties of medications [10]. These carrier proteins, such as HSA and BSA, can facilitate the transportation and distribution of pharmaceuticals throughout the body via the bloodstream [11, 12]. Hence, serum medicinal chemistry is a vital method for investigating TCM in vitro [13, 14]. Notably, research on the binding of natural alkaloids to proteins has fueled significant attention [15]. For instants, Khan et al [16] found that coralyne can bind to site I of HSA and BSA and cause partial unfolding of the protein structure. Kesavan et al. [17] also investigated the binding interaction of Luotonin A and its affixed acceptor molecules with BSA. They discovered that the affixed acceptor molecule 1 bound optimally to BSA. However, there are few studies on the interactions between MDAs and serum albumin. Previous researchers have revealed that BHA and BAC have similar high pharmacological activities, while BHA exhibits a higher level of toxicity than BAC [18]. The combination of drugs and HSA may affect the drug's pharmacokinetics, including its metabolism and efficacy in the human body [19]. Comprehensive studies on the binding interactions between BHA/BAC and HSA are urgently necessary for potential pharmacological assessments and drug safety evaluations.

In this study, we utilized multi-spectral analysis and computational approaches to characterize
the interaction mechanism between BAC/BHA and HSA and to predict the optimal binding mode. Our focus was on the binding site, conformation of HSA, binding force, and quenching mechanism. This work could aim in understanding the transport and pharmacological properties of BAC and BHA, and serves as a reference for further studies on the interactions between alkaloids and proteins.

Fig.1 The chemical structures of BAC (A) and BHA (B).

2 Experimental

2.1 Chemicals

HSA (S12018) was purchased from Shanghai Yuanye Bio-Technology (Shanghai, China) and prepared as solution by ultrapure water with a concentration of $1.0 \times 10^{-6}$ M. BAC and BHA (purity ≥ 98%) were obtained from Yousi scientific Co., Ltd. (Shanghai, China). BAC and BHA were dissolved in methanol (1mM), subsequently diluted by ultrapure water to a final concentration of $1 \times 10^{-5}$ M and then stored in refrigerators. Tris-HCl buffer (0.05 M) was used to adjust the pH to 7.4. For this investigation, Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) provided all of the chemicals with AR grade.

2.2 Experimental methodologies

2.2.1 Fluorescence measurements

Fluorescence measurements were performed using an F-4600 fluorescence spectrophotometer (Hitachi, Japan) equipped with a thermostatically controlled quartz cell (SD-300). The experiments were conducted at temperatures of 298, 303, and 308 K. Emission signals were recorded between 290 and 450 nm, with an excitation wavelength of 280 nm. Quenching
experiments were carried out by titrating varying concentrations of BAC/BHA (0.098-0.82 μM) into HSA, which was maintained at a constant concentration (1.0 μM). Equation (1) is applied to correct for the internal filtering effect of fluorescence intensity [20, 21]:

\[ F_{\text{cor}} = F_{\text{obs}} \exp \frac{A_{\text{ex}} + A_{\text{em}}}{2} \]  

(1)

Here, \( F_{\text{obs}} \) and \( F_{\text{cor}} \) express the fluorescence intensity before and after correction. \( A_{\text{ex}} \) and \( A_{\text{em}} \) stand for the absorbance values of the maximum excitation and emission wavelength, respectively.

In 3D fluorescence experiments, the HSA to BAC/BHA concentration ratio was maintained at 1:1. Excitation wavelengths ranged from 200 to 300 nm, and emission wavelengths ranged from 300 to 450 nm.

Synchronous fluorescence measurements were conducted using scan intervals (Δλ) of 15 nm (in the range of 265 to 350 nm) and 60 nm (in the range of 220 to 350 nm), providing insights into changes in the microenvironment of tyrosine (TYR), tryptophan (TRP).

CD spectra were measured using a Chirascan spectropolarimeter (Applied Photophysics, England) equipped with a 1 mm cell. The spectra were obtained within a wavelength range of 200 to 260 nm, with a scan rate of 100 nm/min. The SELCON3 program was utilized to determine the relative contents of α-helix, β-sheet, β-turn, and random coil in the secondary conformation of HSA.

### 2.2.2 Markers displacement measurements

To assist determine the specific binding site of BSA/BHA on HSA, site probes for sites I, II, and III were chosen to be phenylbutazone, ibuprofen, and digitoxin, respectively [22]. Ibuprofen, digoxin, and HSA all had molar ratios of 1:1 whereas phenylbutazone had a 1:0.5 molar ratio to HSA. Meanwhile, the HSA concentration was stabilized at 1.0 μM. Various concentrations of BAC/BHA (0.098-0.82 μM) were added to the systems, and their steady-state fluorescence was measured at 25°C. The binding constants were calculated using equation (1), and the binding sites of BAC/BHA were determined based on the changes in their binding constants [23].
2.2.3 UV–vis absorption spectra

The absorption spectra were scanned in the range of 190-340 nm using Hitachi U-3900H. The concentration of HSA was kept at 1.0 μM and the concentration range of BAC/BHA was 0-1.23 μM. Meanwhile, to obtain accurate absorbance data, the background values of the Tris-HCl buffer were deducted.

2.2.4 Molecular docking study

The binding of BAC/BHA to HSA was simulated using a vacuum the LeDock program. The detailed crystal structures of HSA were provided by the Protein Data Bank (PDB) (http://www.rcsb.org/pdb) (PDB code: 1AO6). The 3D structure of BAC and BHA were obtained from the ChemSpider (CS) database (CSID: 10226897 and 64863496, http://www.chemspider.com) and then the energy was minimized with MMFF94 force field in Chem3D. The grid center at coordinates x = 42.8, y = 47.9, z = 47.0, box size was 30 Å × 30 Å × 30 Å, and grid spacing was 1 Å. Docking was performed with default parameters of 20 runs. Finally, an analysis of docking results was obtained from PyMOL2.3 and Discovery Studio 2018 client software [24].

2.2.5 Molecular dynamics simulation

The molecular dynamics simulations were conducted using the Gromacs 2016.3 program with the CHARMM36 all-atom force field [25]. The simulation system was a dodecahedral box, and free HSA, HSA-BAC, and HSA-BHA complexes dissolved with TIP3P water molecules (edge length > 1 nm). 12 sodium ions are used instead of water molecules to keep the system charge neutral. NVT and NPT ensembles were applied in turn to equilibrate the free HSA, HSA-BAC, and HSA-BHA systems. Subsequently, the systems were then subjected to a simulation run with a time interval of 2 fs and a duration of 100 ns at constant pressure and temperature (300 K, 1 atm). The energy values were determined together with other parameters, such as the radius of gyration (Rg), root means square deviations (RMSD), and root means square fluctuations (RMSF) [26, 27].
3 Results and discussion

3.1 Fluorescence tests

The binding mechanism between HSA and BAC/BHA was determined by steady-state fluorescence spectroscopy. As displayed in Fig. 2, the maximum emission wavelength ($\lambda_{em}$) of HSA was around 344nm when excited at a wavelength ($\lambda_{ex}$) of 280nm. The fluorescence intensity of HSA reduced with the increase of BAC/BHA, indicating that the fluorescence signals of chromophores in HSA were quenched by BAC/BHA [28].

![Effect of BAC(A) and BHA (B) on fluorescence spectroscopy of HSA at 280 nm. ([HSA]:1.0 μM; [BAC] and [BHA] a-f: 0, 0.10, 0.29, 0.47, 6.5 and 0.82 μM, $\lambda_{ex}$ = 280 nm, pH =7.4 at 298 K.)](image)

3.1.1 Quenching mechanism of fluorescence

Dynamic and static quenching can be distinguished as follows: 1) Dynamic quenching is caused by the excited fluorophores colliding with the quencher; 2) Static quenching occurs when the fluorophore and the quencher form a complex with no fluorescence emission [29]. Since the stability of the complex was reduced at high temperatures, the $K_{SV}$ values were decreased with the rise of temperature in static quenching [30]. The $K_{SV}$ and quenching rate constant ($K_q$) are derived based on the Stern Volmer equations (2) and (3):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (2)$$

$$K_q = \frac{K_{SV}}{\tau_0} \quad (3)$$

Where, the fluorescence signals in the free HSA and HSA-BAC/BHA systems are $F_0$ and $F$, respectively.
[Q] represents the quencher concentration, and $\tau_0$ is the integral lifetime of tryptophan (~6 ns) [27, 31].

As indicated in Table 1, the $K_{SV}$ values decreased from 298 K to 308 K, and the $K_q$ values of the HSA-BAC/BHA systems (~$10^{13}$ M$^{-1}$s$^{-1}$) were remarkably higher than the maximum constant of collisional quenching ($2 \times 10^{10}$ M$^{-1}$s$^{-1}$) [32, 33]. These results provide evidence that the binding of BAC/BHA to HSA followed a static quenching, instead of dynamic quenching.

Table 1 quenching parameter of the HSA-BAC and HSA-BHA systems.

<table>
<thead>
<tr>
<th>system</th>
<th>T</th>
<th>$K_{SV}$ (10$^5$ M$^{-1}$)</th>
<th>$K_q$ (10$^{13}$ M$^{-1}$ s$^{-1}$)</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-BAC</td>
<td>298K</td>
<td>1.320 ± 0.027</td>
<td>2.200 ± 0.044</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>303K</td>
<td>1.230 ± 0.017</td>
<td>2.011 ± 0.092</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>308K</td>
<td>1.160 ± 0.026</td>
<td>1.933 ± 0.044</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>298K</td>
<td>1.357 ± 0.025</td>
<td>2.261 ± 0.042</td>
<td>0.996</td>
</tr>
<tr>
<td>HSA-BHA</td>
<td>303K</td>
<td>1.287 ± 0.012</td>
<td>2.144 ± 0.019</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>308K</td>
<td>1.143 ± 0.031</td>
<td>1.906 ± 0.051</td>
<td>0.998</td>
</tr>
</tbody>
</table>

3.1.2 Binding parameters analysis

To quantitatively assess the binding interaction between protein and ligands, the binding constants ($K_a$) and number of binding sites ($n$) were simultaneously calculated by the double-logarithm regression curve, following equation (4) [34]:

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

In this formula, $F$ and $F_0$ are the fluorescence intensity of protein in the presence or absence of quencher, respectively; [Q] represents the concentration of quencher.

As shown in Table 2, the $K_a$ values of BAC/BHA to HSA ranged from $1.039 \times 10^5$ to $1.876 \times 10^5$ M$^{-1}$, which were much higher than the weak to moderate binding constant ($10^2$ to $10^4$ M$^{-1}$) [33, 35]. These findings reveal that there is a strong affinity between BAC/BHA and HSA.
Fig. 3 Double-Logarithmic curves of BAC (A) and BHA (B) interaction with HSA quenching under the different temperatures.

Besides, the $n$ between the HSA-BAC/BHA complex was approximately one, suggesting HSA contained a single high-affinity site for both BAC and BHA [36]. Notably, the HSA-BHA complex exhibited superior stability than the HSA-BAC complex, as indicated by higher $K_a$ values. Thus, BHA has a longer duration in the plasma and maintenance of drug action [37].

3.1.3 Evaluation of thermodynamic parameters

It is well-accepted that hydrogen bonds, van der Waals forces, hydrophobic interactions, and electrostatic interactions were the most important intermolecular forces involved in the ligand-protein binding process. The thermodynamic enthalpy change ($\Delta H^\circ$) and entropy change ($\Delta S^\circ$) are reliable indicators of the primary forces involved in the interactions between small molecules and proteins [38]. The binding force between HSA and BAC/BHA was determined by thermodynamic parameters, and calculated as the following equation [39]:

$$\ln K_a = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

Here, R stands for the gas constant (8.314 J mol$^{-1}$ K$^{-1}$) and T stands for Kelvin temperature.

The negative $\Delta G^\circ$ values in Table 2 illustrate that the combined interactions of the HSA and BAC/BHA were spontaneous process at all temperatures [40]. Remarkably, the $\Delta G^\circ$ values for the HSA-BHA system were consistently smaller than those of the HSA-BAC system, indicating a more stable binding mode between BHA and HSA. The formation of the HSA-BAC/BHA
complex was mainly driven by enthalpy as $|T\Delta S^o| < |\Delta H^o|$ [41]. Meanwhile, the negative $\Delta H^o$ values, coupled with negative $\Delta S^o$, show that van der Waals interactions and hydrogen bonds were the primary forces involved in the binding of BAC/BHA to HSA.

Table 2 The number of binding site ($n$), binding constant ($K_a$) and thermodynamic parameters for HSA-BAC and HSA-BHA systems.

<table>
<thead>
<tr>
<th>system</th>
<th>$T$ (K)</th>
<th>$K_a$ (10$^5$ M$^{-1}$)</th>
<th>$R^2$</th>
<th>$n$</th>
<th>$\Delta H^o$ (kJ.mol$^{-1}$)</th>
<th>$\Delta S^o$ (J. K$^{-1}$.mol$^{-1}$)</th>
<th>$\Delta G^o$ (kJ.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-BAC</td>
<td>298K</td>
<td>1.701 ± 0.070</td>
<td>0.997</td>
<td>1.016</td>
<td>-37.68 ± 1.71</td>
<td>-26.36 ± 5.64</td>
<td>-29.84 ± 0.101</td>
</tr>
<tr>
<td>HSA-BAC</td>
<td>303K</td>
<td>1.309 ± 0.035</td>
<td>0.991</td>
<td>1.003</td>
<td>-29.68 ± 0.066</td>
<td></td>
<td>-29.68 ± 0.066</td>
</tr>
<tr>
<td>HSA-BAC</td>
<td>308K</td>
<td>1.039 ± 0.058</td>
<td>0.996</td>
<td>0.996</td>
<td>-29.58 ± 0.142</td>
<td></td>
<td>-29.58 ± 0.142</td>
</tr>
<tr>
<td>HSA-BHA</td>
<td>298K</td>
<td>1.876 ± 0.044</td>
<td>0.999</td>
<td>1.021</td>
<td>-39.34 ± 1.41</td>
<td>-31.13 ± 4.66</td>
<td>-30.08 ± 0.058</td>
</tr>
<tr>
<td>HSA-BHA</td>
<td>303K</td>
<td>1.430 ± 0.005</td>
<td>0.998</td>
<td>1.008</td>
<td>-29.90 ± 0.009</td>
<td></td>
<td>-29.90 ± 0.009</td>
</tr>
<tr>
<td>HSA-BHA</td>
<td>308K</td>
<td>1.120 ± 0.037</td>
<td>0.999</td>
<td>0.999</td>
<td>-29.77 ± 0.083</td>
<td></td>
<td>-29.77 ± 0.083</td>
</tr>
</tbody>
</table>

3.2 Identification of binding sites
Markers displacement measurements were conducted to certify the binding pocket of BAC/BHA on HSA. As is known to all, site I located on subdomain IIA can be probed by phenylbutazone, site II and site III are indicated by digitoxin and ibuprofen, respectively [42]. Experimental results are presented in Fig. 4 and Table S1.
The $K_a$ values of the HSA-BAC and HSA-BHA complexes decreased significantly by 36.71% and 34.22% with the addition of phenylbutazone, respectively. And the $K_a$ values were minor changed in the presence of digitoxin and ibuprofen. The observations suggest that site I on subdomain IIA was the primary binding pocket for BAC/BHA.

3.3 Alterations in the microenvironment and conformation of HSA

3.3.1 UV-visible absorption: structural investigations

UV-visible absorption measurements are commonly utilized to investigate structural changes during the interaction of proteins with ligands. The secondary structure of HSA is defined by the spatial arrangement of its peptide backbone atoms. Thus, any alterations in this peptide backbone can affect the secondary structure of HSA. The absorption at 201 nm and 278 nm were the characteristic absorption of the backbone structure and aromatic amino acids of HSA [43, 44].

![Graph showing Flod change (%) of binding constants with diverse probes agents of BAC and BHA.](image-url)
Fig. 5 UV-vis spectra of HSA and their BAC/BHA complexes, the concentration of HSA was kept at 1.0 μM and the concentration range of BAC/BHA was 0 ~ 1.23 μM.

From Fig. 5, the absorption at 201 nm was reduced, suggesting a disturbance in the secondary structure of HSA [37]. While, the decrease of the peak at 278 nm indicates that BAC/BHA had formed a complex with HSA, leading to the alteration of the microenvironment surrounding the aromatic residues. These significant alterations observed in the spectrum suggest a static quenching mechanism [45, 46]. This finding is in accordance with the results of the fluorescence experiments.

3.3.2 Synchronous fluorescence: the changes of hydrophobicity

The characteristic fluorescence signals of TYR and TRP can be distinguished by using synchronous spectra with 15 and 60 nm steps [47]. Fig. 6 shows the synchronous fluorescence...
spectroscopy results for the HSA-BAC/BHA systems. By adding BAC/BHA, the maximum emission wavelength of the TRP residue exhibited a slight red shift, while the maximum peak position of the TYR residue was unchanged. These results suggest that the microenvironment of TRP residues increased, while the microenvironment near TYR remained unchanged [26].

![Fig. 6 Synchronous fluorescence spectra of HSA with BAC (A and B) and BHA (C and D): Δλ = 15 and Δλ = 60 nm.](image)

HSA (1.0×10^{-6} M); BAC and BHA ranging from (0-8.18×10^{-7} M) in Tris-HCl buffer, pH = 7.4 at 298 K.

3.3.3 Circular dichroism spectra: alteration of secondary structure

The CD spectroscopy of HSA was conducted to obtain more precise data on the protein's secondary structure [48]. The α-helix conformation in HSA exhibits unique absorption characteristics, as demonstrated by two distinct negative peaks at 208 and 222 nm,
corresponding to $\pi$-$\pi^*$ and $n$-$\pi^*$ transitions [49]. Fig. 7 shows that both peaks at 208 and 222 nm were present in the HSA- BAC/BHA system, and the shape and position of the negative peaks remained unchanged. Hence, HSA maintained its predominantly helical structure in the presence of BAC/BHA. Meanwhile, the addition of drugs resulted in a reduction in the intensity of two negative signals, suggesting the conformation of HSA was changed [50, 51].

![CD fluorescence spectra of the free HSA, HSA-BAC and HSA-BHA complexes.](image)

**Fig. 7 CD fluorescence spectra of the free HSA, HSA-BAC and HSA-BHA complexes.**

[HSA] = 1.0 μM, [BAC/BHA] = 1.0 μM.

The secondary structure contents of HSA, which were calculated by the SELCON3 program, are summarized in Table 3. The addition of BAC and BHA resulted in a 1.9% and 1.4% decrease in the $\alpha$-helical contents of HSA, respectively. Conversely, there was an increase in the $\beta$-sheet, $\beta$-turn, and random coil content. It is reported that this transition from an ordered structure to a random coil signifies the partial unfolding of HSA [52].

**Table 3** The secondary structure content for HSA were calculated by the SELCON3 program with or without BAC and BHA.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-helix (%)</th>
<th>$\beta$-sheet (%)</th>
<th>$\beta$-turn (%)</th>
<th>random coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>63.50</td>
<td>3.900</td>
<td>11.10</td>
<td>20.50</td>
</tr>
</tbody>
</table>

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4476825
<table>
<thead>
<tr>
<th></th>
<th>HSA-BHA (1:1)</th>
<th>HSA-BAC (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strength</td>
<td>62.10</td>
<td>61.60</td>
</tr>
<tr>
<td>Force</td>
<td>5.20</td>
<td>5.70</td>
</tr>
<tr>
<td>Concentration</td>
<td>12.40</td>
<td>12.90</td>
</tr>
<tr>
<td>Duration</td>
<td>22.00</td>
<td>22.50</td>
</tr>
</tbody>
</table>

The more disruption to the conformation of HSA, and its transport capacity becomes weaker.

Since BHA exerted less influence on the secondary structure of HSA, it caused a smaller impact on the transport capacity of HSA. Also, BHA has a longer duration of drug action than BAC. Therefore, BHA is more potentially available as a slow-release drug than BAC.

3.4 Computer technology

3.4.1 Molecular docking studies: binding forces

Investigating the intermolecular interactions between proteins and ligands using molecular docking is essential [53]. The binding pattern of the HSA-BAC/BHA complex was further investigated by applying the LeDock program. As displayed in Fig. 8 (A), the BAC molecule was attached to TRP214 through an alkyl bond, and TRP-214 interacted with BHA through van der Waals forces. TRP-214 was determined to play a primary role in generating the fluorescence signal of HSA [54]. These interactions shed light on the reason behind the fluorescence quenching of HSA induced by BAC/BHA. Moreover, three conventional hydrogen bonds and four carbon-hydrogen bonds were identified within the hydrophobic cavity of the HSA-BAC complex. In contrast, there are only two conventional hydrogen bonds existed in the hydrophobic pocket of the HSA-BHA complex. These hydrogen bonds are crucial for maintaining the structural stability of the HSA-BAC/BHA complex. Residues such as PHE-211, LUE-219, GLN-196, and ASP-451 interacted with the BAC/BHA through van der Waals forces. Meanwhile, residues such as GLN-196, PHE-211, and ASP-415 were attached to BAC/BHA by forming hydrophobic interactions. These results illustrate that the formation of the HSA-BAC/BHA complex is primarily driven by hydrogen bonds, hydrophobic interactions, and van der Waals forces. These findings align with the results of the thermodynamic assay.
Fig. 8 The binding model for HSA-BAC (A) and HSA-BHA systems (B).

Furthermore, the hydrophobic residues LEU-238, ARG-257, LEU-260, ALA-290, and ILE-291 can attach to the benzene ring through pi-cation and pi-alkyl bonds. The hydrophilic residues LYS-199 and LYS-195 form alkyl bonds with the alkyl groups surrounding nitrogen. This outcome implies that the electronic structure of the nitrogen and benzene ring in the aconitum skeleton were key parts of the formation of the HSA-BAC/BHA complex [55]. Thus, the attachment of amino acid residues to benzene rings or alkyl groups depends on the hydrophobicity or hydrophilicity of the residues.

3.4.2 Molecular dynamics simulations analysis: conformational changes of HSA

The dynamics property, such as stability, rigidity, and microenvironment, of free HSA, HSA-BAC, and HSA-BHA complex were evaluated by molecular dynamics (MD) simulations [56, 57]. Fig. 9 (A) and (B) show the root mean square deviation (RMSD) and the radius of gyration...
(Rg) values for the skeletons of free HSA and HSA-BAC/BHA complex. The RMSD fluctuations for the HSA-BAC/BHA systems stabilized after 10 ns, indicating that the HSA-BAC/BHA complex converged to a steady state during the simulation [58]. Meanwhile, the mean RMSD values for the HSA-BAC/BHA complex (0.512 ± 0.076 nm, 0.502 ± 0.067 nm) were slightly larger than those for free HSA (0.460 ± 0.051 nm). Moreover, the Rg values of free HSA and its complex with BAC/BHA reached stability after 30 ns. From 30 to 100 ns, the mean Rg values increased in the order of free HSA (2.680 ± 0.013 nm), HSA-BAC complex (2.709 ± 0.020 nm), and HSA-BHA complex (2.747 ± 0.023 nm). These findings indicate that binding to BAC/BHA induced conformational changes in HSA, resulting in increased relaxation of its peptide chain and larger internal space for HSA [58]. These structural alterations may have implications for the binding affinity of HSA to other molecules and its physiological functions.

The root means square fluctuation (RMSF), which is widely applied to assess biomolecule flexibility and protein mobility [59], was obtained in Fig. 9 (C). Binding to BAC/BHA led to reduced RMSF values for most HSA residues, indicating decreased flexibility upon complex formation. This decrease in flexibility was attributed to the weakening of the α-helix structure, potentially affecting the overall structural stability of the protein [60]. Furthermore, the mean RMSF value of the HSA-BAC system (0.230 ± 0.084 nm) was smaller than that of the HSA-BHA system (0.238 ± 0.087 nm). This outcome suggests that the impact of BAC on the flexibility of HSA was more notable, primarily because of its stronger impact on the structural conformation of HSA. These findings are consistent with the observations from circular dichroism (CD) spectra.
Fig. 9 The RMSD (A), RMSF (B), Rg (C) of free HSA, HSA-BAC and HSA-BHA systems.

Moreover, the energy contribution of each residue in HSA was decomposed and performed in Fig. S2. It is apparent that the residues that significantly contribute to interactions (< -3.5 kJ/mol or > 3.5 kJ/mol) were predominantly located in Suldow's site I of HSA. This finding indicates a critical role for site I in complex formation. Then, TRP-214 (-9.0270 kJ/mol) and LUE-238 (-7.3961 kJ/mol) were identified as the amino acid residues that make the highest energy contribution to the formation of the HSA-BAC and HSA-BHA complexes, respectively. Table 4 provides further insight into the specific residues involved in the binding of BAC/BHA to HSA. Residues TRP-214, LEU-219, LEU-238, and ALA-291 positively affect the binding of BAC/BHA to HSA, while GLU-153 and GLU-292 have a negative impact on their binding. This phenomenon suggests that these residues were key residues for the binding of aconitine analogs to HSA. Residues GLN-196, ARG-222, and HIS-22 play a facilitative or
inhibitory role in the binding of BAC to HSA. LYS-195, ARG-218 and LEU-260 residues may have a positive or negative impact on the binding of BHA to HSA. This difference can be attributed to the subtle structural differences between BAC and BHA. Interestingly, LYS-199 exhibited opposing roles in the combination process of BAC and BHA with HSA, which can be attributed to its alkyl interactions with BAC and BHA at distinct positions. Therefore, slight structural distinctions can give rise to distinct optimal binding modes, leading to divergent roles for a few residues.

Table 4 Energy decomposition of major residues for HSA-BAC and HSA-BHA complexes.

<table>
<thead>
<tr>
<th>Residue</th>
<th>HSA-BAC Binding energy (kJ/mol)</th>
<th>Residue</th>
<th>HSA-BHA Binding energy (kJ/mol)</th>
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<tr>
<td>GLU-153</td>
<td>15.5046</td>
<td>GLU-153</td>
<td>5.1798</td>
</tr>
<tr>
<td>LYS-199</td>
<td>7.7739</td>
<td>LYS-199</td>
<td>-6.4663</td>
</tr>
<tr>
<td>TRP-214</td>
<td>-9.0270</td>
<td>TRP-214</td>
<td>-6.4208</td>
</tr>
<tr>
<td>LEU-219</td>
<td>-6.1481</td>
<td>LEU-219</td>
<td>-5.8241</td>
</tr>
<tr>
<td>LEU-238</td>
<td>-7.8344</td>
<td>LEU-238</td>
<td>-7.3961</td>
</tr>
<tr>
<td>ALA-291</td>
<td>-5.4305</td>
<td>ALA-291</td>
<td>-6.5446</td>
</tr>
<tr>
<td>GLU-292</td>
<td>4.7048</td>
<td>GLU-292</td>
<td>9.1211</td>
</tr>
<tr>
<td>ASP-451</td>
<td>10.0425</td>
<td>ASP-451</td>
<td>8.9426</td>
</tr>
<tr>
<td>GLN-196</td>
<td>-3.9701</td>
<td>LYS-195</td>
<td>-5.5332</td>
</tr>
<tr>
<td>ARG-222</td>
<td>4.0773</td>
<td>ARG-218</td>
<td>-5.4346</td>
</tr>
<tr>
<td>HIS-242</td>
<td>-3.6255</td>
<td>LEU-260</td>
<td>-4.8721</td>
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</table>

4.Conclusion

In this study, we used spectral methods and computer techniques to evaluate the binding interactions between BAC/BHA and HSA. The formation of the HSA-BAC/BHA complex led to static fluorescence quenching, and the HSA-BHA complex displayed higher stability compared to the HSA-BAC complex. Moreover, hydrogen bonds and van der Waals forces facilitated the spontaneous binding of BAC/BHA to site I in HSA. Meanwhile, the microenvironment around TRP residues has altered and the conversion in the secondary structure of HSA from an ordered to a disordered state. Molecular docking results indicate that
in addition to hydrogen bonding and van der Waals forces, hydrophobic interactions were also
essential in the formation of the HSA-BAC/BHA complex. And the electronic structure domains
of the benzene ring and the alkyl groups surrounding the nitrogen are crucial regions for the
formation of complexes. The outcomes from molecular dynamics simulation further
demonstrated that BAC/BHA caused the HSA to partially unfold and the site I was the primary
home to the residues contributing the most to the binding energy. Meanwhile, residues such as
TRP-214, LEU-219, LEU-238 and ALA-291 played a key role in the binding of BAC/BHA to
HSA. This study serves to elucidate the distribution and metabolism of BAC/BHA and provides
novel insights into their therapeutic effects. This finding also provides a basis for rational drug
selection.

Acknowledgments

This study was financially supported by Fundamental Research Funds for the Central
Universities: South-Central MinZu University (Nos. CPT22027, PTZD 22006), and
Undergraduates’ Training Program for Innovation and Entrepreneurship for South-Central
MinZu University (No. GCX2238).
References


[47] L. Khalili, G. Dehghan, A comparative spectroscopic, surface plasmon resonance, atomic force microscopy and molecular docking studies on the interaction of plant derived conferone with serum


Supplementary Materials

Investigating the interactions of benzoylaconine and benzoylhypacoitine with human serum albumin: experimental studies and computer calculations

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Fig. S1 Stern-Volmer curves of BAC(A) and BHA(B) interaction with HSA quenching under the different temperature.

Fig. S2 Contribution of each residue to the binding energy for BAC (A) and BHA (B).
<table>
<thead>
<tr>
<th>system</th>
<th>probe</th>
<th>$K_a$ (10^5 M⁻¹)</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-BAC</td>
<td>none</td>
<td>1.701 ± 0.070</td>
<td>1.016</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>Phenylbutazone 1:05</td>
<td>1.077 ± 0.027</td>
<td>0.998</td>
<td>0.995</td>
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<tr>
<td></td>
<td>Ibuprofen 1:1</td>
<td>1.666 ± 0.046</td>
<td>1.024</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>Digitoxin 1:1</td>
<td>1.680 ± 0.014</td>
<td>1.021</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>1.876 ± 0.044</td>
<td>1.021</td>
<td>0.999</td>
</tr>
<tr>
<td>HSA-BHA</td>
<td>Phenylbutazone 1:0.5</td>
<td>1.234 ± 0.016</td>
<td>0.993</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Ibuprofen 1:1</td>
<td>1.849 ± 0.032</td>
<td>1.023</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>Digitoxin 1:1</td>
<td>1.837 ± 0.047</td>
<td>1.021</td>
<td>0.999</td>
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</table>