Effect of 8-alkylberberine homologues on erythrocyte membrane

Yang Yong*a, Ye Xiao-lib, Zhang Bao-shunc & Li Xue-gangc

*aDepartment of Pharmaceutical Science, Huaihua Medical College, Huaihua 418 000, China
bSchool of Life Science, Southwest University, Chongqing, 400 715, China
cChemistry Institute of Pharmaceutical Resources, School of Pharmaceutical Science, Southwest University, Chongqing, 400 715, China

Received 1 November 2010; revised 17 February 2011

8-alkylberberine homologues (Ber-C8-n, where n indicates carbon atom number of gaseous normal alkyl at 8 position, n =0, 2, 4, 6, 8, 10, 12, or 16) were synthesized and their effects on the hemolysis of rabbit erythrocyte, the fluidity of membrane and the fluorescence of membrane protein were investigated by fluorescence analysis technique. Ber-C8-n with medium length alkyl (4< n<10) exhibited obvious hemolysis effect on rabbit erythrocyte when their concentration exceed 1.25×10⁻⁴ mol/L, and Ber-C8-8 displayed the highest hemolysis effect among all tested homologues. All of Ber-C8-n influenced the fluidity of erythrocyte membrane to different extents, which exhibited an obvious dose-effect relationship. The effect of Ber-C8-n on fluidity increased as the length of alkyl chain was elongated and decreased gradually when the alkyl carbon atoms exceeded 8. The fluorescence of erythrocyte membrane protein was quenched by Ber-C8-n, which showed a similar changing tendency on membrane fluidity. Experiments in vitro suggested that disturbing effects of Ber-C8-n on the conformation and function of membrane protein leaded to the changes of membrane fluidity and stability, and then the membrane was broken down.

Keywords: 8-alkylberberine homologues (Ber-C8-n), Cell membrane, Fluorescence of membrane protein, Membrane fluidity

Berberine is a natural active component extracted from Chinese herbal medicine Coptidis rhizoma and exerts antidiarrhoeal1 and anti-inflammatory activities2. 8-alkylberberine homologues (Ber-C8-n, where n indicates carbon atom number of alkyl at 8 position, n =0, 2, 4, 6, 8, 10, 12 or 16) are derivatives synthesized by substituted with gaseous normal alkyl chain at C 8 position of berberine3. The antimicrobial activity of Ber-C8-n was tested in vitro and structure-activity relationships were evaluated4. The antimicrobial activity of Ber-C8-n increased as the length of aliphatic chain was elongated and then decreased gradually when the alkyl chain exceeded 8 carbon atoms. 8-octylberberine displayed the highest antimicrobial activity among all derivatives investigated4. Disturbing the stability of cell membrane and/or destructing the conformation of membrane protein are common mechanism of antibacterial drugs exerting the pharmaceutical effects5. Strong interaction would be occurred between berberine and protein, which may arise mostly hydrophobic binding6,7. Higher the antimicrobial activity of Ber-C8-n is, the stronger its effect on the conformation of bovine serum albumins (BSA)8. Modifying the conformation of membrane protein and the arrangement of membrane lipid would be one of important factors of antimicrobial drugs exerting the pharmaceutical effects9.

In order to explain the cause of antibacterial activity of Ber-C8-n with varying length of alkyl chain, the interaction between Ber-C8-n and the erythrocyte membrane lipid and protein has been investigated by fluorescence analysis technique, to understand the interaction between Ber-C8-n and membrane protein or lipid.

Materials and Methods
Synthesis and identification of 8-alkylberberine homologues—Seven 8-alkylberberine homologues (Ber-C8-n) were synthesized from berberine (Ber-C8-0)5. They include 8-ethylberbrine (Ber-C8-2), 8-butylberbrine (Ber-C8-4), 8-hexylberbrine (Ber-C8-6), 8-octylberbrine (Ber-C8-8), 8-decylberbrine (Ber-C8-10), 8-dodecylberbrine (Ber-C8-12) and 8-cetylberbrine (Ber-C8-16). Structures of all compounds were identified by means of elemental analysis, UV, NMR, melting point, etc. The purity was analyzed by HPLC and was found to be more than 98%. 
Preparation of erythrocyte and erythrocyte membrane\textsuperscript{9,10}—With fresh rabbit blood as material, erythrocyte and erythrocyte membrane were obtained\textsuperscript{9}. The separated erythrocyte were diluted with isotonic phosphate buffered saline (PBS, 5 mmol/L, pH 7.4) and purified erythrocyte membrane with hypotonic phosphate buffer (PB, 5 mmol/L, pH 7.4), and stored at \(-40^\circ\text{C}\). The protein content of purified erythrocyte membrane was determined by kjeldahl nitrogen determination device.

Determination of erythrocyte hemolysis—Red-blood cell diluted with PBS (5 mmol/L, pH 7.4) by 1\% was added into the same volume of Ber-C8-n solution with different concentration including \(5\times10^{-4}\), \(2.5\times10^{-4}\), \(1.25\times10^{-4}\), \(6.25\times10^{-4}\), \(0.625\times10^{-5}\), \(0.312\times10^{-5}\), \(1.56\times10^{-5}\) and 0 mol/L. The mixed solution was incubated for 30 min at room temperature, and centrifuged at 2000 \(g\) for 10 min. The supernatant was separated and the heme content was detected at 579 nm by UV-1800 spectrophotometer. The extent of hemolysis of red-blood cell was indicated with the content of heme released into supernatant.

Determination of the fluidity of erythrocyte membrane—Cell membrane was diluted into the suspension with 0.2 mg/ml protein by PB (5 mmol/L, pH 7.4). Cell membrane suspension was mixed with three times of Ber-C8-n solution diluted by PB (5 mmol/L, pH 7.4) with different concentration including \(3.12\times10^{-5}\), \(1.56\times10^{-5}\), \(7.8\times10^{-6}\), \(3.9\times10^{-6}\), \(1.95\times10^{-6}\) and 0 mol/L. Above solution was incubated for 1 h at 30°C. The mixed solution was added into 2 mL 1,6-diphenyl-1,3,5-hexatriene (DPH) at a concentration of \(10^{-6}\) mol/L and further incubated at 37°C for 30 min. Suspensions without DPH were used as reference blanks at the same concentration. \(I\) value (fluorescence intensity) was determined by a Hatchi F-4500 fluorescence spectrophotometer which was equipped with a variable temperature controller. Excitation and emission wavelengths were fixed at 362 and 432 nm respectively. Measurements were corrected by subtraction of the corresponding blank value and by the \(G\) factor. The P value (degree of fluorescence polarization) was calculated according to formula as follows\textsuperscript{10}:

\[
P = \frac{I_{0,0} - GI_{0,90}}{I_{0,0} + GI_{0,90}} = \frac{I_{90,0}}{I_{90,90}} \quad \ldots (1)
\]

where \(G\) is the corrected factor, \(I_{0,0}\) is fluorescence intensity when both of the polarizer and analyzer were vertical, and \(I_{0,90}\) is fluorescence intensity when polarizer was vertical and analyzer was horizontal.

Determinations of fluorescence spectra of membrane protein—Same cell membrane suspension (0.2 mg/ml of protein content) was mixed with same volume of Ber-C8-n solution with different concentration. The mixed solution was incubated for 1 h at 30°C. The emission spectra of mixed solution after incubating were scanned within 300 to 500 nm at 295 nm excitation\textsuperscript{9}.

Results

Effect of Ber-C8-n on the erythrocyte hemolysis—The absorbance of solution at 579 nm was indicated the extent of erythrocyte hemolysis and detected data of supernatant is shown in Fig. 1. Results showed that the hemolysis effect were not obvious below a concentration of \(1.25\times10^{-4}\) mol/L.

![Fig. 1—Effect of Ber-C8-n on the erythrocyte hemolysis](image-url)
Hemolysis effects of some components, particularly that of Ber-C8-8 and Ber-C8-6 strengthened when the concentration was more than $1.25 \times 10^{-4}$ mol/L. Compared among homologues, the hemolysis effect was strengthened with the elongating of alkyl chain at C8 position and then weakened gradually when alkyl chain was more than 8 carbon atoms, which was similar to the rule of their antimicrobial activities. 8-octylberberine (Ber-C8-8) which showed highest antimicrobial activity exhibited maximal hemolysis effect among tested 8-alkylberberine homologues.

Effect of Ber-C8-n on the fluidity of erythrocyte membrane—DPH (1, 6-Diphenyl-1, 3, 5-hexatriene) is a molecule with flat long shape. It may exhibit strong fluorescence in hydrophobic alkyl chain in membrane lipid bilayer and not do so in hydrophilic membrane surface. DPH is usually used as fluorescence probe to determine the membrane fluidity. Membrane fluidity can be evaluated by detecting the identity of polarized light (I value) and calculating fluorescence polarization ($P$) of membrane. The change of $P$ value indicates that the arrangement of alkyl chain was disordered.

The $P$ values of membrane declined with increasing concentrations of Ber-C8-n (Fig. 2). Results indicated that Ber-C8-n can change the membrane fluidity. With the elongation of the carbon chain, the effect of Ber-C8-n on $P$ value of membrane increased and then decreased gradually when the alkyl chain exceeded 8 carbon atoms. Ber-C8-8 exhibited highest effect on $P$ value in tested components. This may suggest that Ber-C8-n could affect the fluidity of membrane lipid, and even make lipid arrangement disordered. The stronger the antimicrobial activity of Ber-C8-n was, the more seriously the arrangement of membrane lipid was disturbed.

Effect of Ber-C8-n on the fluorescence spectra of membrane protein—Membrane proteins are important structural elements and account for about 50% of erythrocyte membrane. The stability or activity of membrane is related to the conformation of membrane protein. Three amino acids including tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) can emit fluorescence in protein. The fluorescence spectra of above amino acid residues may change on varying the chemical environment such as protein conformation. Therefore membrane protein conformation can be determined by fluorescence analysis method. Tyr residue was used for detecting fluorescence of the membrane protein, which is located outside and inside of membrane.

The effects of Ber-C8-0 (berberine), Ber-C8-8 and Ber-C8-16 on fluorescence of Tyr residues are presented in Fig. 3. Results indicated that the fluorescence intensity of Tyr residues dramatically quenched with the increasing of Ber-C8-n concentration. All homologues tested could quench the fluorescence of Tyr residue but to lesser extents. It is
suggested that the environment of Tyr residue in membrane protein or conformation of membrane protein would be significantly changed by Ber-C8-n.

The fluorescence quenching effects of all tested homologues are shown in Fig. 4. The quenching effect on fluorescence intensity of membrane protein increased with the elongation of alkyl chain and weakened gradually when the alkyl chain exceeded 6 or 8 carbon atoms. Similarly to the antimicrobial activity of Ber-C8-n, Ber-C8-8 exhibited strongest fluorescence quenching effect on Tyr residue of membrane protein.

Discussion

The antimicrobial activity of Ber-C8-n increased as the length of aliphatic chain was elongated and then decreased gradually when the alkyl chain exceeded 8 carbon atoms, 8-octylberberine displayed the highest antimicrobial activity of all compounds. Present results showed that the Ber-C8-n with higher antimicrobial activity would have stronger effect on hemolysis, membrane fluidity and protein conformation. Results suggested that the effect on membrane is directly related to the antimicrobial mechanism of Ber-C8-n. What is the key effect of Ber-C8-n exerting antimicrobial activity, on membrane protein conformation or on membrane fluidity? In order to analyze and contrast two kind of effect of Ber-C8-n on membrane, the experiment data was processed according to Stern-Volmer equation

\[ \frac{S_0}{S} = 1 + (kq \tau)C \]  

\[ P = k_p C + P_0 \]  

where \( S_0 \) and \( S \) are the fluorescence intensity without or with Ber-C8-n, respectively, \( \tau \) is the endogenous fluorescence longevity of biomacromolecule (about \( 10^{-8} \) s), \( C \) is the concentration of Ber-C8-n, and \( k_q \) is the dynamic quenching velocity constant of biomolecule. Higher the \( k_q \) value the stronger is the fluorescence quenching ability or effect on protein conformation. The \( k_q \) of Ber-C8-n on Tyr residue of membrane protein was calculated and is presented in Table 1. According to Fig. 4 and Eq. (2).

\[ P = k_p C + P_0 \]  

where \( P \) and \( P_0 \) are P value without or with Ber-C8-n, \( C \) is the concentration Ber-C8-n and \( k_p \) is the polarization velocity constant of P value. Higher the \( k_p \) value is, stronger is the effect on membrane fluidity.

In Table 1, IC\(_{50}\) is half effective concentration of Ber-C8-n, IC\(_{50(q)}\) indicated the IC\(_{50}\) of fluorescence quenching of membrane protein, IC\(_{50(p)}\) indicated the IC\(_{50}\) of decreasing P value of membrane fluidity. From Table 1, two effect of Ber-C8-n on membrane fluidity and membrane protein conformation, respectively, exhibited almost same rule as their antimicrobial activity. Results indicated that the antimicrobial activity of Ber-C8-n was related with their effects on membrane protein and membrane fluidity. It may be suggested that the stronger the effect of homologue on membrane, the higher was its antimicrobial activity.

A previous study has reported that an interaction existed between berberine and purified protein and it was suggested to be interaction. An interaction between Ber-C8-n and bovine serum albumins (BSA) was observed. Previous result indicated that the Ber-C8-n with higher antimicrobial activity would have stronger effect on protein conformation. Antimicrobial abilities of Ber-C8-n were related to the

<table>
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<tr>
<th>Compound</th>
<th>( k_q \times 10^{12} ) L/mol/S</th>
<th>IC(_{50(q)}) ( \times 10^{-2} ) mmol/L</th>
<th>( k_p \times 10^{3} ) L/mol</th>
<th>IC(_{50(p)}) ( \times 10^{-2} ) mmol/L</th>
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<tr>
<td>Ber-C8-0</td>
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<td>0.71</td>
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<td>0.62</td>
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<td>3.27</td>
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Table 1—The index of effect of Ber-C8-n on membrane protein and membrane fluidity

Fig. 4—Effect of Ber-C8-n on the fluorescence intensity of Tyr residue of membrane protein
action intensity between tested homologue and BSA which is also a purified protein. The cause why Ber-C8-8 exhibited the highest antimicrobial activity in tested homologues, is that it have optimal lipophilia molecule structure and may exerted hydrophobic binding action with protein. It is well known that membrane protein is an important membrane structure composition and accounts for about 50% in erythrocyte membrane. Membrane fluidity can be affected by many factors such as protein content, rigidity and orderliness of membrane protein in membrane. When the conformation of membrane protein is changed, the rigidity and orderliness of membrane protein will be affected, and then membrane fluidity may be changed. It has been proved that that the changes of membrane conformation could induce the modification of membrane fluidity. Therefore, the effect of Ber-C8-n on membrane conformation, which induced the change of membrane fluidity, would be a important factor for Ber-C8-n exerting antimicrobial effect.

Acknowledgement

Thanks are due to Hunan Ministry of Education (10C0273) and Huaihua Medical College (ky0903) for financial assistance.

References