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# A novel lead of P-selectin inhibitor: Discovery, synthesis, bioassays and action mechanism

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ABSTRACT

By docking 126 derivatives of β-carboline-3-carboxylic acid, tetrahydro-β-carboline-3-carboxylic acid and indoloquinolizine into the active pocket of P-selectin (2-(3-(hydroxymethyl)-9*H*-pyrido[3,4-*b*]indol-1-yl)ethyl)-L-phenylalanine (HMCEF) was assigned a novel inhibitor. ELISA and flow cytometry experiments showed that HMCEF effectively down-regulated P-selectin expression and supported the rationality of the computer assistant screening, while UV spectrum experiments demonstrated that HMCEF directly bound to P-selectin. In vivo HMCEF dose dependently inhibited the rats and mice to form thrombus and had a minimal effective dose of 20 nmol/kg, dose dependently inhibited inflammatory response of mice and had a minimal effective dose of 20 nmol/kg. The decrease of serum TNFα and IL-8 of the treated mice was proposed to be the action mechanism of HMCEF inhibiting thrombosis and inflammation. All data imply that HMCEF is a novel lead of P-selectin inhibitor.



Vascular thrombosis is involved in the onset of diabetes mellitus, metabolic syndrome, atherosclerosis and hypertension,[1] while inflammation promotes the onset of cardiovascular disease and tipping the haemostatic balance towards vascular thrombosis, thereby inflammation and blood coagulation are linked.[2] P-selectin mediates the rolling of blood cells on the surface of the endothelium, and triggers the adhesion of leukocytes to the platelets, endothelial cells and other leukocytes at injury sites and inflammation tissues.[3] The P-selectin level in the serum of the patients with coronary heart disease is abnormally high, thus the decrease of serum level of P-selectin provides a potential target for treating vascular thrombosis and related diseases,[4] and the discovery of P-selectin inhibitors is of clinical importance.

β-Carbolines, such as β-carboline-3-carboxylic acids and tetrahydro-β-carboline-3-carboxylic acids, are anti-thrombotic active in vitro and in vivo.[5–10] A derivative of β-carboline-3-carboxylic acid is recently presented as an anti-inflammatory agent, and the anti-thrombotic activity of another derivative is correlated with the down-regulation of P-selectin.[11] These findings imply that β-carboline should be a scaffold for designing novel lead of P-selectin inhibitors that possess both anti-thrombotic and anti-inflammatory activities. As a going on interest in P-selectin inhibitors, this Letter docked β-carboline-3-carboxylic acids, tetrahydro-β-carboline-3-carboxylic acids and indoloquinolizines,[9,10,12–17] totally 126 derivatives of our compound bank, into the active pocket of P-selectin. In the integration of computational information the binding energies were compared, the contributions of the pharmacophores of 126 derivatives to binding energies were estimated, the pharmacophores having significant contributions to binding energies were selected, and (2-(3-(hydroxymethyl)-9*H*-pyrido[3,4-*b*]indol-1-yl)ethyl)-L-phenylalanine (HMCEF) was theoretically constructed. HMCEF was then also docked into the active pocket of P-selectin, the binding energy, −7.76 kcal/mol, of HMCEF was approved to be lower than those of all 126 derivatives. In this context, the design, synthesis and evaluation of HMCEF as a novel lead of P-selectin inhibitor having anti-thrombotic and anti-inflammatory double actions are presented here.

In molecular docking the structure of P-selectin was treated as rigid and prepared by AutoDock 4.0. The grid box dimensions were set to 22.5 Å × 30 Å × 30 Å using a grid spacing of 0.375 Å for two average structures. Energy-minimized 3D conformations of 126 derivatives were treated as flexible, prepared with AutoDock 4.0 and docked into the active sites of the average structure of P-selectin (PDB: 1G1R). This allows automated docking of flexible ligands to a rigid receptor with certain flexible residues. Lamarckian genetic algorithm was used to find the appropriate binding positions, orientations, and conformations of 126 derivatives in the binding site of the average structure. The global optimization

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was started with parameters of a population of 100 randomly positioned individuals. The maximum number of energy evaluations was increased to $2.5 \times 10^7$ and the maximum number of generations in the Lamarckian genetic algorithm was increased to $2.7 \times 10^5$. The Solis and Wets local search was performed with a maximum number of 3000. In each simulation, 200 runs were carried out for each derivative. The resulted 200 conformations of each derivative were scored by the lowest binding energy and clustered using a root mean square tolerance of 2.0 Å. It is worthy to mention that for computational docking the carboxyl of 126 derivatives and HMCEF, as well as the side chain of Lys84 in the active site of P-selectin were deprotonized, i.e., the docking was processed in neutral condition.

As seen in Figure 1, the carboline ring of HMCEF is settled by $Ca^{2+}$ (red ball) in the manner of π–π interactions,[18] thereby 6 hydrogen bonds occur, of which the first one is from the interaction of pyrrole N of HMCEF and the H of side chain $NH_2$ of Asn82, the second one is from the interaction of hydroxyl O of HMCEF and the H of side chain $NH_2$ of Asn83, the third one is from the interaction of hydroxyl H of HMCEF and the O of acyl group of Asn83, the fourth one is from the interaction of the H of Phe moiety of HMCEF and the O of acyl group of the side chain of Asn83, the fifth one is from the interaction of the carboxyl H of Phe moiety of HMCEF and the acyl O of the side chain of Asn83, and the sixth one is from the interaction of the carboxyl O of Phe moiety of HMCEF and the H of side chain $NH_2$ of Lys84.

In the preparation of HMCEF a seven-step reaction procedure and corresponding reaction conditions were used (Scheme 1). Compound **1** as a Pictet–Spengler condensation derivative was synthesized in 89% yield.[19] The yields of compounds **2**, **3**, **4**, **5** and **6** were 80%, 61% 61%, 50% and 69%, respectively. The total yield of HMCEF was 9%. The data imply that this seven-step reaction procedure is able to provide HMCEF in easy available condition and good yield.

To examine the in vitro effect of HMCEF on P-selectin expression, arachidonic acid (AA) activated platelets were treated with normal saline (NS) and HMCEF (20, 200 and 2000 nM) to perform the quantitative ELISA experiments.[20] Figure 2A indicates that the P-selectin expressed on the platelets treated with NS is equal to that expressed on the platelets treated with 20 nM HMCEF and is significantly higher than that expressed on the platelets treated with 200 nM HMCEF, suggesting that the minimal effective concentration of HMCEF inhibiting P-selectin expression is 200 nM. Besides, the P-selectin expressed on the platelets treated with 200 nM and 2000 nM of HMCEF are equal to each other, suggesting HMCEF down-regulating P-selectin expression is capable of saturation.

To further examine the in vitro effect of HMCEF on P-selectin expression the AA-activated platelets were labeled with PE-anti-CD62P and treated with NS or HMCEF (20, 200 and 2000 nM). In the performance of the flow cytometry experiments,[21] the level of P-selectin expression was represented with MFI (mean fluorescence intensities) and the values are shown in Figure 2B–F. Figure 2B indicates that the MFI of the platelets treated with NS is significantly higher than that of the platelets treated with 20 nM of HMCEF, suggesting that the minimal effective concentration of HMCEF inhibiting P-selectin expression is 20 nM. The MFI of the platelets treated by HMCEF is decreased with the concentration been increased from 20 nM to 200 nM and to 2000 nM. Therefore HMCEF concentration dependently inhibits the platelets to express P-selectin. Figure 2C–F indicate that the platelets treated with NS, and HMCEF at 20 nM, 200 nM and 2000 nM are 1621, 871, 754 and 665, respectively, suggesting a concentration dependent action of HMCEF. Thus the concentration dependent decrease of the fluores-

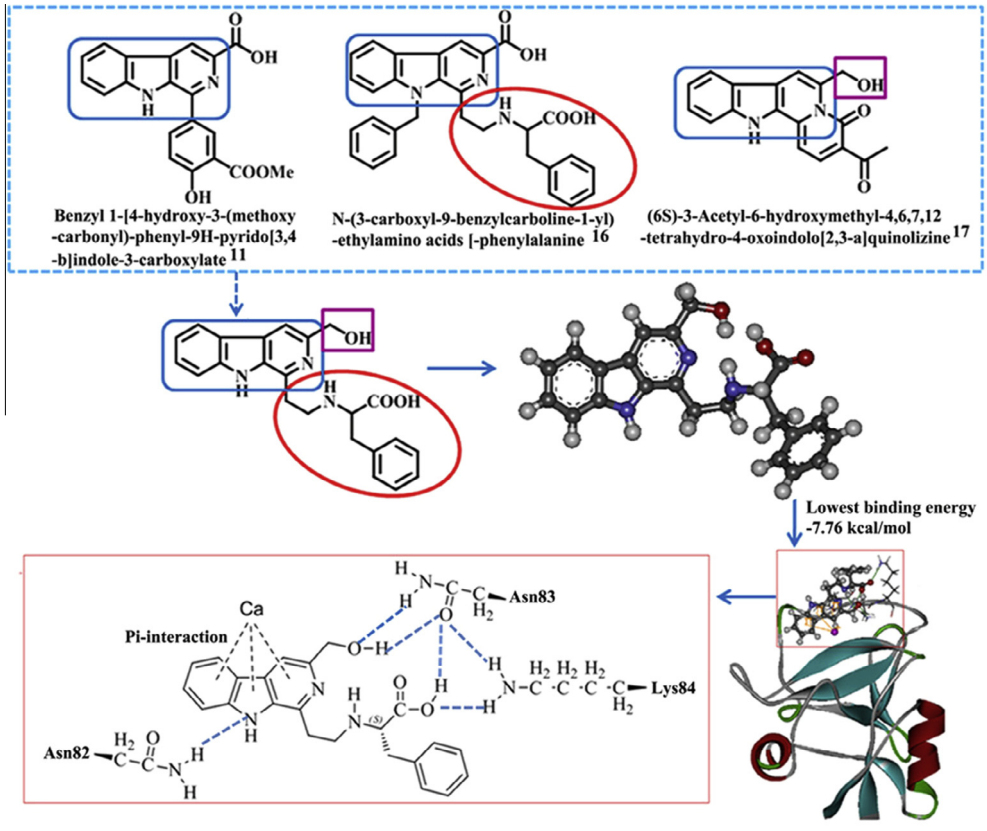
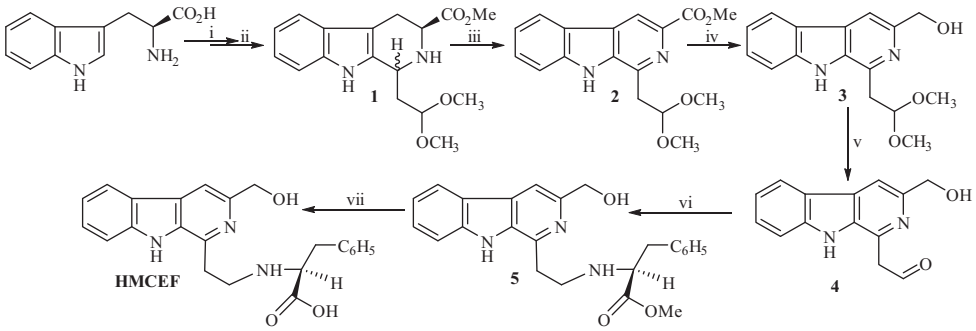



**Figure 1.** HMCEF design and detailed interactions between HMCEF and the active site of P-selectin.




**Scheme 1.** Synthesis of HMCEF. Reagents and conditions: (i) Thionyl chloride and $CH_3OH$; (ii) hydrochloric acid, 1,1,3,3-tetramethoxypropane and $CH_3OH$; (iii) potassium permanganate in DMF; (iv) lithium aluminum hydride and anhydrous THF; (v) hydrochloric acid, acetic acid and $H_2O$; (vi) NaOH and L-PheOMe; then, $NaBH_3CN$; (vii) NaOH and $CH_3OH$.

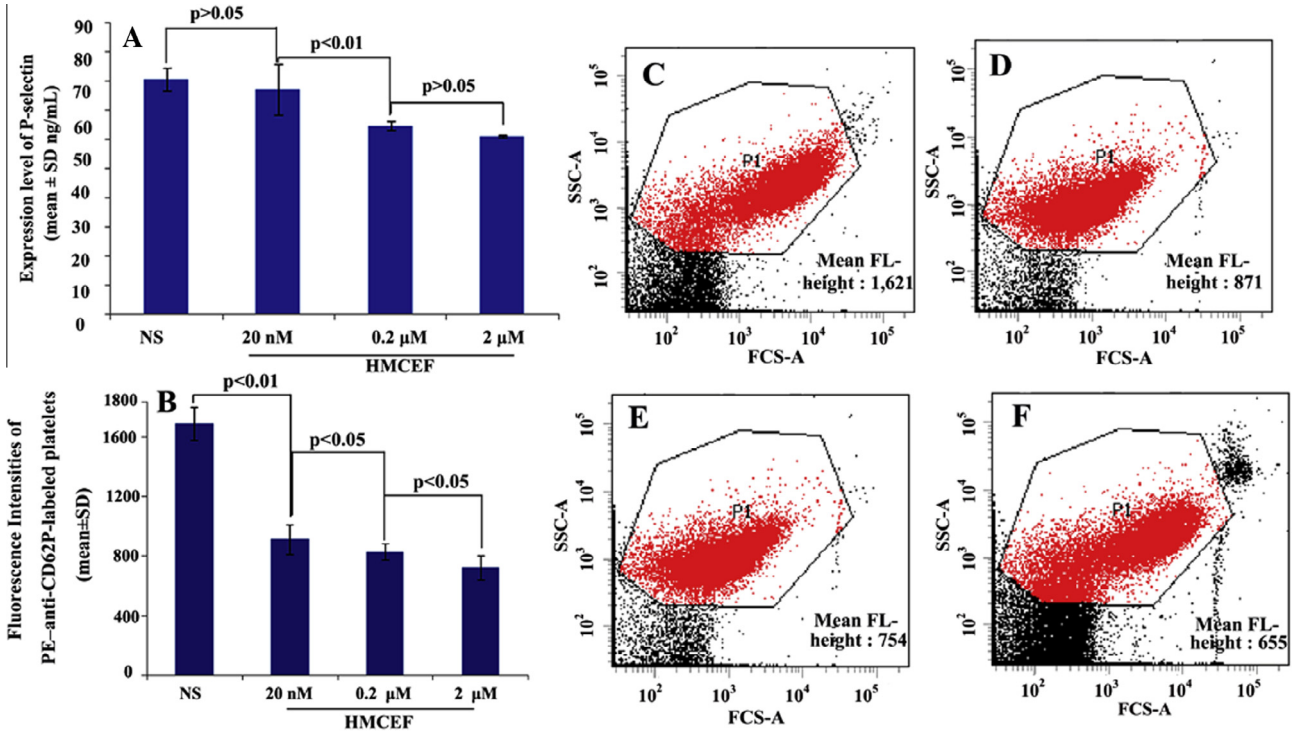



**Figure 2.** Effects of HMCEF on P-selectin expression. (A) P-selectin expression levels of AA-activated platelets treated with HMCEF at three concentrations, $n = 5$; (B) The quantitative description of MFI, $n = 3$; (C) MFI of platelets treated with NS; (D) MFI of platelets treated with 20 nM of HMCEF; (E) MFI of platelets treated with 200 nM of HMCEF; (F) MFI of platelets treated with 2000 nM of HMCEF. MFI of unlabeled AA-activated platelets (background) was 14.

cence intensity matches the concentration dependent down-regulation of P-selectin expression.

The binding of HMCEF towards P-selectin was mirrored with the comparisons of the UV spectra of sample diluent alone, HMCEF in sample diluent (0.18 mM), P-selectin in sample diluent (300 ng/mL), and P-selectin in sample diluent (300 ng/mL) plus 10 μL of HMCEF in sample diluent (final concentration 0.18, 0.26, 0.34 and 0.42 mM).[11,22] Figure 3A indicates that sample diluent alone, HMCEF in sample diluent (0.18 mM), and P-selectin in sample diluent (300 ng/mL) give distinct UV spectrum. The UV spectrum of sample diluent shows a strong wide band, the wavelength of maximal absorbance is 273.8 nm and the absorbance is 2.355. The spectrum of HMCEF in sample diluent consists of four bands, for band I the wavelength of maximal absorbance is 277.8 nm and the absorbance is 0.438, for band II the wavelength of maximal absorbance is 295.0 nm and the absorbance is 0.392, for band III the wavelength of maximal absorbance is 341.4 nm and the absorbance is 0.385, for band IV the wavelength of maximal absorbance is 351.6 nm and the absorbance is 0.370. The spectrum of P-selectin in sample diluent shows a weak wide band, the wavelength of maximal absorbance is 274.8 nm and the absorbance is 0.137. Figure 3B gives a set of UV profiles and visualizes the UV spectra of 2 mL solution of P-selectin in sample diluent (300 ng/mL) plus 10 μL solution of HMCEF in sample diluent (final concentration 0.18, 0.26, 0.34 and 0.42 mM). As seen, 2 mL of P-selectin (300 ng/mL) plus 10 μL of HMCEF (final concentration 0.18 mM) results in a new spectrum of three bands, of which the wavelength of maximal absorbance occur at 295.4 nm (absorbance, 0.415), 340.8 nm (absorbance, 0.355) and 352.0 nm (absorbance, 0.339), respectively, while the band of P-selectin and band I of HMCEF disappear. The increase of the final concentration of HMCEF (from 0.18 mM to 0.42 mM) induces the absorbance of three bands gradual increase. It could be seen, the increase of HMCEF's concentration significantly changes the UV spectrum, such as the band at

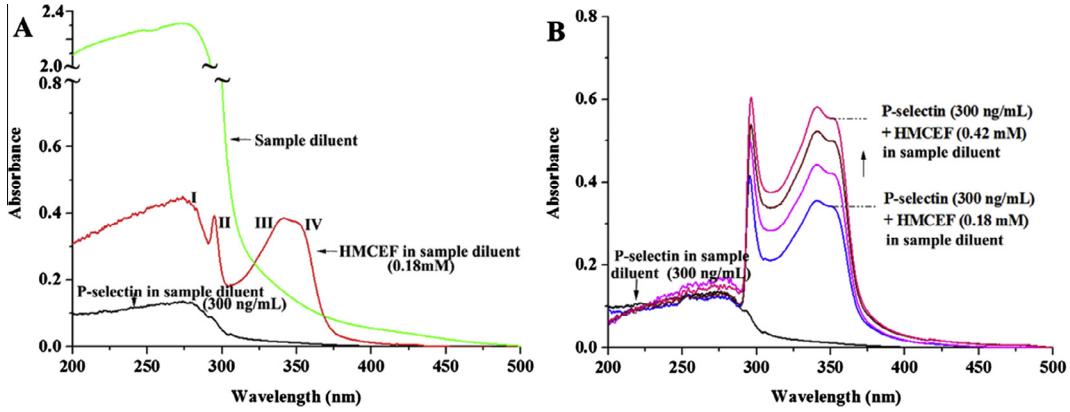



**Figure 3.** The UV spectra of the P-selectin/HMCEF system. (A) The UV spectra of sample diluent, P-selectin in sample diluent (final concentration 300 ng/mL) and HMCEF in sample diluent (final concentration 0.18 mM), respectively; (B) the UV spectra of P-selectin alone and P-selectin/HMCEF system.

295.4 nm (absorbance, 0.415) shows a marked red shift (~1.4 nm), the band at 340.8 nm (absorbance, 0.355) shows a hyperchromic effect of ~51.2% and a bathochromic shift of 0.6 nm, the band at 352.0 nm (absorbance, 0.339) shows a marked red shift of ~1.4 nm.

The changes of the UV spectra of P-selectin and HMCEF induced by adding of HMCEF obviously reflect a direct interaction of HMCEF with P-selectin, and mirror the docking of HMCEF towards the active pocket of P-selectin. Taking the in vitro effect of HMCEF on AA-activated platelets to express P-selectin into account this direct interaction should be responsible for HMCEF down regulating P-selectin expression.

In consistent with the literatures,[23,13,24,25] the effect of HMCEF on P-selectin expression could be understood as a three-step process. (1) In the presence of AA the platelets are activated and P-selectin inside the α-granule moves to the surface membrane of the platelets within a few minutes. (2) On the surface membrane, HMCEF blocks the interaction between P-selectin and natural ligands, such as P-selectin glycoprotein ligand 1 (PSGL-1). (3) This discontinues P-selectin to be shed from the surface membrane, and no more soluble P-selectin appears in blood circulation.

The relationships between the blood level of P-selectin and various cardiovascular diseases lead to this in vivo anti-thrombotic evaluation,[26,27] both rat model and mouse model were used, the thrombus weight represents the antithrombotic activity and the data are shown in Figure 4. Figure 4A indicates that on rat thrombosis model the thrombus weights of the rats orally treated by HMCEF are progressively decreased with the doses are from 2 nmol/kg increased to 20 nmol/kg and finally to 200 nmol/kg, suggesting a dose dependent action of HMCEF. Besides, the thrombus weight of the rats treated with 20 nmol/kg of HMCEF is significantly lower than that of NS orally treated rats, suggesting the minimal effective dose of HMCEF is 20 nmol/kg, while the thrombus weight of the rats treated with 200 nmol/kg of HMCEF is equal to that of the rats orally treated with 167 μmol/kg of aspirin, suggesting the activity of HMCEF is 835 folds higher than that of aspirin.

Figure 4B indicates that on mouse thrombosis model the thrombus weight of the mice treated with 200 nmol/kg HMCEF is significantly lower than that of the mice treated with NS and is equal to that of the mice treated with 240 μmol/kg of aspirin, suggesting that on mouse model the anti-thrombotic activity of HMCEF is 1200 folds higher than that of aspirin. Thus the data of Figure 4 support the success of HMCEF therapy on two thrombosis models and ensure HMCEF would be a validated candidate of novel lead of anti-thrombotic drug.

The reciprocal relationship of thrombosis with inflammation leads to this evaluation,[28,29] the in vivo anti-inflammation activities of HMCEF were evaluated on xylene-induced ear edema mouse model, and the ear edema represented the activity.[30] Figure 5A indicates that the xylene-induced ear edema of the mice orally treated with NS is significantly higher than that of the mice orally treated with 20 nmol/kg of HMCEF. This means that the minimal effective dose of HMCEF inhibiting the inflammation response of the mice is 20 nmol/kg. Besides, with the increase of HMCEF's dose the ear edema of the mice gradually decreases. Furthermore, the ear edema of the mice orally treated with 0.11 mmol/kg of aspirin is equal to that of the mice orally treated with 200 nmol/kg of

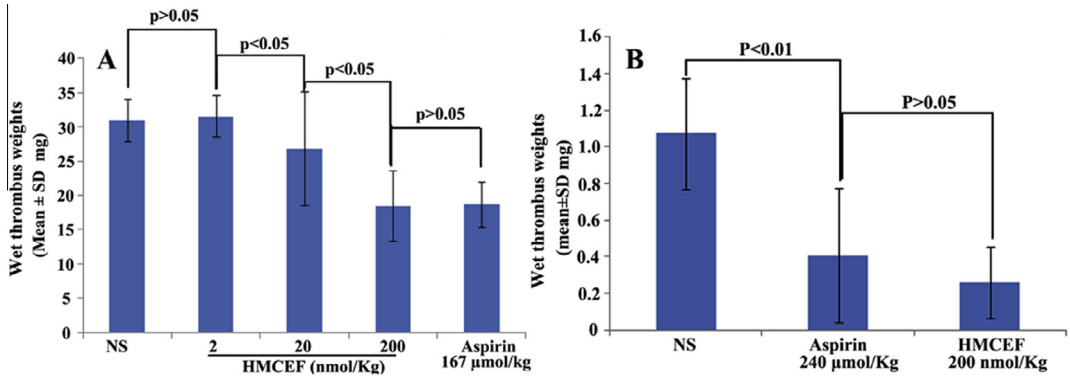



**Figure 4.** Anti-thrombotic activities of HMCEF in vivo. (A) On rat model HMCEF dose-dependently inhibits the rats to form thrombus, $n = 12$; (B) On mouse model 200 nmol/kg of HMCEF effectively inhibits the mice to form thrombus, $n = 12$.

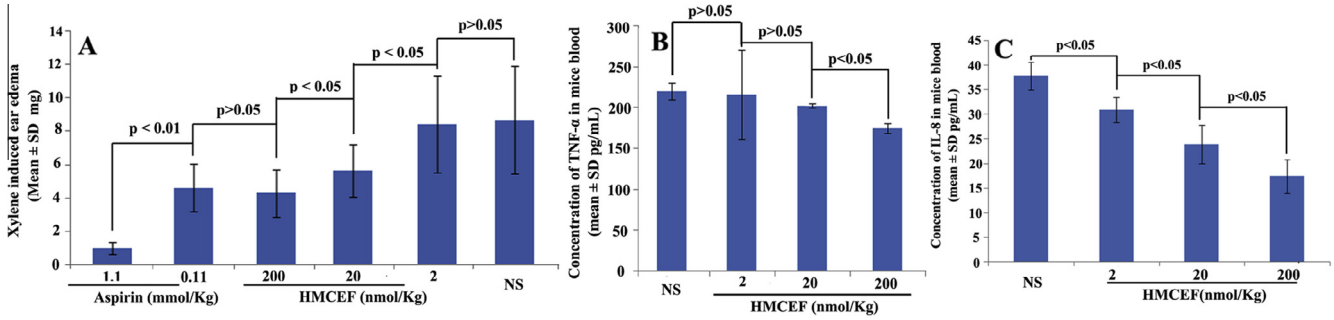



**Figure 5.** Dose-dependent effect of HMCEF on mouse inflammation, serum TNFα and IL-8, $n = 12$. (A) Dose-dependent effect of HMCEF on ear edema mice; (B) effect of 200 nmol/kg of HMCEF on serum TNFα of ear edema mice; (C) dose-dependent effect of HMCEF on serum IL-8 of ear edema mice.

HMCEF. This suggests that on xylene-induced ear edema mouse model the anti-inflammation activity of HMCEF is 550 folds higher than that of aspirin.

TNFα and IL-8 are the biomarker of both inflammation and thrombosis, and can be released from P-selectin induced monocytes.[31–33] These lead to the measurements of serum TNFα and IL-8 of HMCEF treated inflammatory mice and the data are also shown in Figure 5. Figure 5B indicates that the serum concentration of TNFα of ear edema mice orally treated with NS is equal to that of ear edema mice orally treated with 2 and 20 nmol/kg of HMCEF. This suggests that at 2 nmol/kg and 20 nmol/kg of doses HMCEF is unable to decrease the serum concentration of TNFα of ear edema mice. At 200 nmol/kg of dose, however, the serum concentration of TNFα of ear edema mice treated with HMCEF is significantly lower than that of the mice treated with NS. This suggests that the minimal effective dose of HMCEF to decrease the serum concentration of TNFα of ear edema mice is 200 nmol/kg. Figure 5C indicates that the serum concentration of IL-8 of ear edema mice orally treated with NS is significantly higher than that of ear edema mice orally treated with 2 nmol/kg of HMCEF. This means that the minimal effective dose of HMCEF decreases the serum concentration of IL-8 of ear edema mice is 2 nmol/kg. Besides, with the increase of the dose of HMCEF the serum concentration of IL-8 of treated ear edema mice gradually decreases. This means that HMCEF dose dependently decreases the serum concentrations of IL-8 of ear edema mice, which matches the dose dependent anti-inflammation and anti-thrombotic actions. Thus it could be hypothesized that via inhibiting serum IL-8 HMCEF dose dependently exhibits anti-thrombotic and anti-inflammation actions.

In conclusions, these results of ELISA experiment, flow cytometry measurement and UV determination confirm that P-selectin active pocket docking based computer screening of 126 derivatives of β-carboline-3-carboxylic acid and tetrahydro-β-carboline-3-carboxylic acid rationally lead to the discovery of HMCEF as a novel lead of P-selectin inhibitor, the dose dependent in vivo anti-thrombotic and anti-inflammatory actions reflect the utility of HMCEF as a novel lead of P-selectin inhibitor, dose dependent anti-thrombotic and anti-inflammation actions match the dose dependent decrease of serum IL-8, and suggests that by decreasing serum IL-8 HMCEF inhibits inflammatory response and thrombosis.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.061.

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