



Immobilised Histidine Tagged β_2 -Adrenoceptor Oriented by a Diazonium Salt Reaction and Its Application in Exploring Drug-Protein Interaction Using Ephedrine and Pseudoephedrine as Probes

Qian Li¹, Liujiào Bian¹, Xinfeng Zhao¹, Xiaokang Gao¹, Jianbin Zheng², Zijian Li³, Youyi Zhang^{3*}, Ru Jiang⁴, Xiaohui Zheng^{1*}

1 College of Life Sciences, Northwest University, Xi'an, Shaanxi, China, **2** Institute of Analytical Science, Northwest University, Xi'an, Shaanxi, China, **3** Institute of Vascular Medicine, Peking University; Third Hospital and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, China, **4** School of Pharmacy, The Fourth Military Medical University, Xi'an, Shaanxi, China

Abstract

A new oriented method using a diazonium salt reaction was developed for linking β_2 -adrenoceptor (β_2 -AR) on the surface of macroporous silica gel. Stationary phase containing the immobilised receptor was used to investigate the interaction between β_2 -AR and ephedrine plus pseudoephedrine by zonal elution. The isotherms of the two drugs best fit the Langmuir model. Only one type of binding site was found for ephedrine and pseudoephedrine targeting β_2 -AR. At 37 °C, the association constants during the binding were $(5.94 \pm 0.05) \times 10^3/M$ for ephedrine and $(3.80 \pm 0.02) \times 10^3/M$ for pseudoephedrine, with the binding sites of $(8.92 \pm 0.06) \times 10^{-4} M$. Thermodynamic studies showed that the binding of the two compounds to β_2 -AR was a spontaneous reaction with exothermal processes. The ΔG° , ΔH° and ΔS° for the interaction between ephedrine and β_2 -AR were $-(22.33 \pm 0.04)$ kJ/mol, $-(6.51 \pm 0.69)$ kJ/mol and 50.94 ± 0.31 J/mol·K, respectively. For the binding of pseudoephedrine to the receptor, these values were $-(21.17 \pm 0.02)$ kJ/mol, $-(7.48 \pm 0.56)$ kJ/mol and 44.13 ± 0.01 J/mol·K. Electrostatic interaction proved to be the driving force during the binding of the two drugs to β_2 -AR. The proposed immobilised method will have great potential for attaching protein to solid substrates and realizing the interactions between proteins and drugs.

Citation: Li Q, Bian L, Zhao X, Gao X, Zheng J, et al. (2014) Immobilised Histidine Tagged β_2 -Adrenoceptor Oriented by a Diazonium Salt Reaction and Its Application in Exploring Drug-Protein Interaction Using Ephedrine and Pseudoephedrine as Probes. PLoS ONE 9(4): e94955. doi:10.1371/journal.pone.0094955

Editor: Eugene A. Permyakov, Russian Academy of Sciences, Institute for Biological Instrumentation, Russian Federation

Received: December 15, 2013; **Accepted:** March 21, 2014; **Published:** April 18, 2014

Copyright: © 2014 Li et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by National Nature Science Foundation of China (Nos. 21075097, 21005060), Innovative Research Team of Shaanxi Province (No. 2013KCT-24) and the Ministry of Science and Technology of the People's Republic of China (No. 2013YQ170525; subproject: 2013YQ17052509). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zhangyy@bjmu.edu.cn (YZ); zhengxh@nwu.edu.cn (XZ)

Introduction

The interactions between a protein and a drug are of growing interest due to the role in elucidating protein functions, explaining drug action mechanisms and discovering novel drug candidates [1,2]. G-protein coupled receptors (GPCRs) are the largest and most diverse superfamily of integral membrane receptors due to their crucial role in curing varied diseases [3–5] and being the most important class of drug targets [6].

Currently, a wide range of methods have been developed for investigating the drug-protein interaction, including ultrafiltration [7], mass spectrometry [8], X-ray crystallography [9], nuclear magnetic resonance [10], surface plasmon resonance [11], affinity capillary electrophoresis [12] and computer-aided methods [13]. Although contributing greatly to the exploration of drug-protein interactions, these approaches need to be improved to overcome the limitations of long analysis time, requirements for samples with high concentrations or purities, potential interfering substance from the sample and relatively specialized equipment [14]. High performance affinity chromatography (HPAC) is another widely

used method for the study of drug-protein interaction [15]. The method often involves immobilization of a protein on a solid matrix to construct affinity stationary phase.

Synthesising an assay for attaching a protein to a solid matrix is a key factor for accomplishing an HPAC method. Physical absorption and random immobilised methods are commonly reported ways to synthesis an affinity stationary phase [16,17]. The types of stationary phases constructed by these methods have been confirmed to generate the inevitable issue of losing bioactive binding sites of the protein during the immobilisation procedure. Oriented immobilisation has proved feasible to address this issue in previous publications [18,19] and should be further used to synthesise HPAC stationary phases attributed to the particularly important role in life sciences [20,21].

Beta₂-adrenoceptor (β_2 -AR), one of the members of the GPCRs, is a crucial target of many drugs for fighting obesity and ailments of the heart and respiratory system [22]. Ephedrine and pseudoephedrine (Figure 1) are isomers widely used for treating diseases of the respiratory system. Both ephedrine and pseudoephedrine exert their therapeutic action through the same

signal pathway involving β_2 -AR [23]. The two drugs have varied potency *in vivo* although they have similar structures and the same acting mechanism. One reason for this may be the different binding affinities of the two ligands to β_2 -AR. This work is firstly designed to establish an oriented immobilised method for fixing his-tagged fusion β_2 -AR on a solid matrix to synthesise an HPAC stationary phase. Further work is performed to investigate the binding behaviours of ephedrine and pseudoephedrine to β_2 -AR by HPAC methods.

Theory

Zonal elution

The binding of ephedrine and pseudoephedrine to the oriented immobilised β_2 -AR was determined by zonal elution. The theory assumes that a known concentration of a competing agent (I) continuously passes through a column containing an immobilised ligand (L) while the injecting volume of an analyte (A) is small enough to be neglected compared with the concentration of I in the mobile phase. Assuming I and A have direct competition at a single binding site on L and the interactions have association/dissociation kinetics, Eq. (1) can be used to calculate the binding of A and I to L [24,25].

$$\frac{1}{k' - X} = \frac{V_m K_I [I]}{K_A m_L} + \frac{V_m}{K_A m_L} \quad (1)$$

where X is the capacity factor for A due to the existence of a second type of binding site on the column, and k' is the overall capacity factors of A ascribed to all the types of sites. In this case, the equation assumes the capacity factors of A produced by the individual sites are additive, and an independent estimate of the capacity factors due to the second type of site can be obtained, allowing for the capacity factor attributed to the first type of site 1 (Site 1) to be measured by $k_1' = k' - X$.

When the column is equilibrated with an eluent at concentration [A] and [I] equal to zero, and the retention of compound A is injected as a perturbation, Eq. (2) is used to determine the binding of A to L:

$$\frac{1}{\sqrt{k'}} = \frac{K_A}{\sqrt{K_A m_L}} [A] + \frac{1}{\sqrt{K_A m_L}} \quad (2)$$

Assuming a single type of binding site, a linear expression can be obtained by plotting $1/\sqrt{k'}$ as a function of [A] to determine m_L and K_A . For two types of binding sites, a second term is added to the k' expressions.

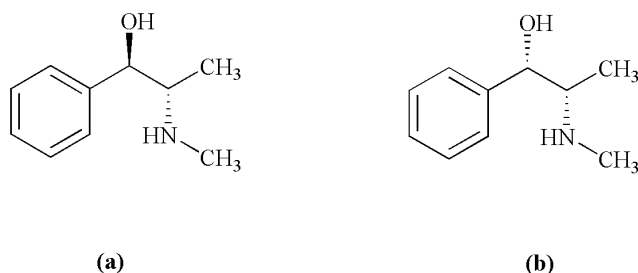


Figure 1. Chemical structures of ephedrine and pseudoephedrine. (a), 1R,2S-ephedrine; (b), 1S,2S-pseudoephedrine. doi:10.1371/journal.pone.0094955.g001

Thermodynamic study

The thermodynamic interaction between β_2 -AR and ephedrine plus pseudoephedrine was described by the van't Hoff equation, listed as Eq. (3) and (4).

$$\ln K_A = \frac{-\Delta H^\theta}{RT} + \frac{\Delta S^\theta}{R} \quad (3)$$

$$\Delta G^\theta = \Delta H^\theta - T\Delta S^\theta \quad (4)$$

In these equations, R is the ideal gas constant (8.31 J/mol/K), and T is the temperature in Kelvin. According to the semi-empirical rules summarised by Ross et al. [26], when $\Delta G^\theta < 0$, $\Delta H^\theta > 0$ and $\Delta S^\theta > 0$, hydrophobic interaction is the main interaction force during the binding process of a drug to protein. If $\Delta G^\theta < 0$, $\Delta H^\theta < 0$ and $\Delta S^\theta > 0$, electrostatic interactions mainly contribute to the interaction. If $\Delta G^\theta < 0$, $\Delta H^\theta < 0$ and $\Delta S^\theta < 0$, Van der Waals forces or hydrogen bonds are the major forces of the binding.

Materials and Methods

Chemicals and instruments

Ephedrine (1R,2S-ephedrine, 171241) and pseudoephedrine (1S,2S-pseudoephedrine, 171237) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Fatty acid and globulin free bovine serum albumin (BSA, A0281) was obtained as lyophilized powder from Sigma-Aldrich (St. Louis, MO, USA). The Quaternary Sepharose Fast Flow pre-packed column (16×25 mm), Ni²⁺ Sepharose 6 Fast Flow (10×58 mm) and Sephadex G25 pre-packed column (10×100 mm) were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Macroporous silica gel (SPS 300-7, pore size 300 Å, particle size 7.0 mm) was from Fuji Silysia Chemical Company Limited (Tokyo, Japan).

The HPLC system employed in this work consisted of a binary pump, a column oven from Agilent Technologies (Santa Clara, USA) and a diode array detector (Waldbronn, Germany). The data were collected and processed by a Chemstation 5.2 software installation.

Expression and purification of β_2 -AR

The full gene of *Sus scrofa* β_2 -AR (Refseq:MN_001128436) was cloned in our previous work [27] and the protein was expressed in *E. coli* BL21 (DE3) with an N-terminal His-tag. The transformed *E. coli* strain was grown overnight at 37 °C in 50 mL Luria-Bertani (LB) medium containing 100 µg/L penicillin. When the value of OD₆₀₀ reached 0.4–0.6, isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 2.0 mM, and the culture was incubated at 30 °C for 6.0 h. The cells containing His-tagged β_2 -AR were separated from the culture media by centrifugation at 6000×g for 30 min at 4.0 °C, and the supernatant was discarded. Falcon tubes containing the cell pellets were placed into a dry ice/ethanol bath for 3–5 min and were transferred to cold water for 60 s to melt the ice along the walls of the tubes. The pellet was broken free by a sterile plastic spatula and suspended in 200 mL neutral lysis buffer (50 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl and 0.5 mM EDTA) in a 500-mL flask. Lysozyme (0.5 mg/mL) was added to the buffer and incubated for 2.0 h on ice with agitation to digest the cell wall. The *E. coli* cells were lysed using 20 mL of a 10× Mg/Mn salt

solution (100 mM MgCl_2 and 10 mM MnCl_2). DNA was removed by DNase (10 $\mu\text{g}/\text{mL}$) with incubation for 30 min at RT. The resulting *E. coli* lysate (approximately 260 mL) was placed into a dialysis bag (10 kD molecular weight cut-off) in 4 L buffer (10 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA). The dialysis buffer was changed twice over a 16–24 h period. At the end of the dialysis, the cell debris was removed as a pellet by centrifugation at $10000 \times g$ for 30 min at 10 °C.

Dialysed and centrifuged recombinant β_2 -AR supernatant was applied to a Ni^{2+} Sepharose 6 Fast Flow column that had been equilibrated with dialysis buffer (10 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA). The column was eluted with an increasing salt gradient of up to 500 mM imidazole in buffer A (20 mM potassium phosphate, pH 7.4, containing 500 mM NaCl) at a speed of 1.0 mL/min. The fraction of interest was collected by eluting the column using 50% buffer B (buffer A in the presence of 500 mM imidazole, pH 7.4). Further purification of the collected fraction was performed on the Quaternary Sepharose Fast Flow column equilibrated with 18% buffer C (buffer A containing 800 mM NaCl, pH 7.4). The β_2 -AR solution was collected by gradient elution from the column using buffer C ranging from 18%–40% with a flow rate of 5.0 mL/min. The solution was finally desalted by the Sephadex G25 column.

Oriented immobilisation of β_2 -AR

The β_2 -AR was oriented immobilised on macroporous silica gel using a diazo coupling method (Figure 2). In detail, aminopropyl-bonded silica was synthesised according to the method in our previous report [28]. Two grams of aminopropyl-bonded silica gel was suspended in a *p*-nitrobenzaldehyde methanol solution (18.2 mg/L, 100 mL) through stirring for 48 h at 60 °C. The resulting gel was filtrated by a sintered filter funnel and washed three times with methanol. The remnant methanol was removed by drying the gel in a vacuum oven at 60 °C for 12 h. The double bond in the produced gel was reduced to a single bond through reaction between the gel and 100 mL 8.6 $\mu\text{g}/\text{mL}$ NaBH_4 at room temperature for 12 h [29], using water as a solvent. After being filtrated and washed three times by water, the product was dried until the weight remained constant at 110 °C under vacuum. The nitro group in the gel was reduced to an amino group by mixing the gel with a SnCl_2 ethanol solution (8.1 mg/L, 100 mL) and reacting for 6 h at 90 °C. The suspension was cooled to 0 °C and alkalinised with 50 mL 5% NaHCO_3 , followed by a sequential cleaning with saturated NaCl and distilled water [30]. The produced phenylamine group in the gel was then transformed to its diazonium form through reaction with excess HCl (20 mM, 30 mL), in which a freshly prepared NaNO_2 solution (20 mM) was added dropwise until reaching the turning point of potassium iodide starch paper changing to blue [31]. The whole procedure for preparing the diazonium salt was performed at 4 °C, and a post stirring for 10 min was needed to accomplish the reaction. The purified His-tagged β_2 -AR was immobilised on the resulting gel by immediately adding the receptor (61.67 $\mu\text{g}/\text{mL}$, 10 mL) solution into the suspension and reacting for 2 h at 4 °C. Finally, the gel was washed three times with phosphate buffer (20 mM, pH 7.4).

Chromatography

The column containing the oriented immobilised β_2 -AR was prepared by packing the stationary phase into a stainless steel column (2.1 mm \times 5.0 cm) using the slurry method at a pressure of 4.0×10^7 Pa. Phosphate buffer (20 mM, pH 7.4) was used as the slurry solution. The control column containing exposed silica gel

and attached histidine was prepared using the same synthesis method as that of the β_2 -AR column.

The mobile phases were prepared by adding 0 to 40 μM of ephedrine hydrochloride as the competing agent to 20 mM phosphate buffer (pH 7.4). All of the mobile phases were filtrated with 0.45 μm Millipore HV filters and degassed by ultrasonication before HPAC analysis. Working solutions of ephedrine and pseudoephedrine were prepared by dissolving the drugs in phosphate buffer (20 mM, pH 7.4) to a concentration of 1.0 mM. The detection wavelength was 220 nm with an injection of 5.0 μL for each drug. The flow rate was 0.2 mL/min for all of the analyses. Under the proposed chromatographic conditions, the void time was 1.6 min using sodium nitrite as the injection solute. The capacity factor (k') was calculated by detecting the retention time in triplicate for each injected sample using the equation $k' = (t_R - t_0)/t_0$. When the injection solute was pseudoephedrine, Eq. (1) was used to plot the curve of $1/(k' - X)$ and $[A]$, whereas Eq. (2) was used to achieve the plot between $1/\sqrt{k'}$ and $[A]$ when ephedrine was used as the analyte.

Thermodynamic study

The thermodynamic investigation of ephedrine and pseudoephedrine on oriented immobilised β_2 -AR was performed at 10, 20, 30, 37 and 45 °C. Eq. (3) and (4) were used to calculate the free energy, enthalpy and entropy during the binding of the two drugs to the receptor. The semi-empirical rules by Ross et al. [26] were utilised to reveal the binding mechanism process of the two drugs to β_2 -AR.

Molecular docking

The sequence of *Sus scrofa* β_2 -AR (Accession Number: AF000134) was obtained from Genbank. The query sequence of the target receptor was searched find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program [32] against Protein Data Bank database. The three dimensional homology model was calculated using crystal structural coordinate of template on the basis alignment of target and template sequence of β_2 -AR according to the procedures illustrated by Discovery Studio 2.5 (DS 2.5, Accelrys Software Inc., San Diego, CA). Structural evaluation of the receptor was performed using two programs named PRO-CHECK and Verify 3D. Once the three dimensional model was generated, energy minimisation was carried out for the receptor before dockings. Structure of ephedrine and pseudoephedrine were constructed by DS 2.5. CDOCKER program, implemented in the software platform of DS, was used for the docking study. The pose cluster radius was set to 0.5 with a value of top hits as 10.

Results and Discussion

Purification of his-tagged β_2 -AR

The total protein in the desalted β_2 -AR solution was determined by bicinchoninic acid (BCA). Using BSA as a standard, the calibration curve was plotted to assay the total protein in the solution. The linear regression equation was $y = 0.0002x + 0.2702$, with a correlation coefficient of 0.9957. The concentration of the total protein in the β_2 -AR solution was (61.67 ± 4.48) $\mu\text{g}/\text{mL}$. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the molecular weight of the protein in the desalted β_2 -AR solution (Figure 3). The lanes of the desalted β_2 -AR solution presented only one clear band corresponding to the band of the marker with a molecular weight of 66.5 kDa, based on which the molecular weight of the protein in the desalted β_2 -AR solution is believed to be 66.5 kDa [30]. The purity of β_2 -

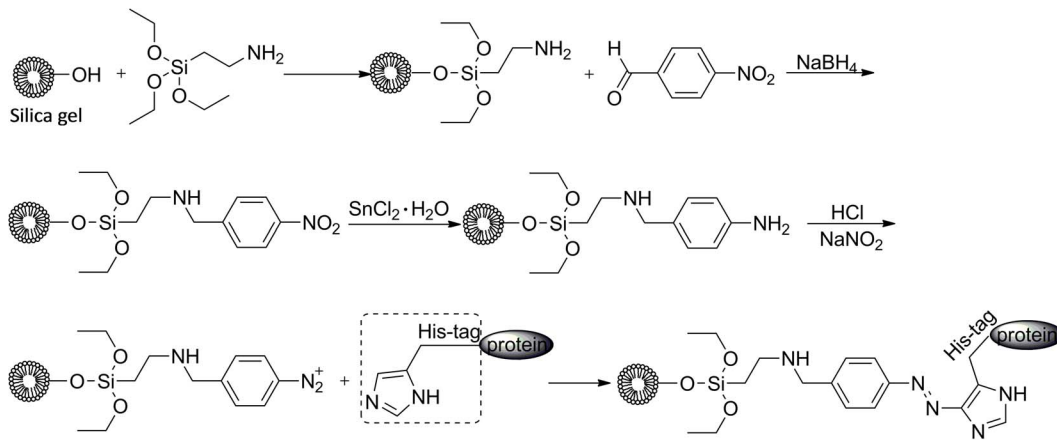


Figure 2. Diagram for oriented attaching His-tagged β_2 -AR on the surface of diazonium salt coated silica gel.
doi:10.1371/journal.pone.0094955.g002

AR in the solution was calculated to be 95% of the total protein by densitometry quantification of the lane using the BandScan software. Furthermore, the desalted β_2 -AR solution was also analysed by exclusion-size chromatography, which did not present any interference of the peak with a molecular weight of 66.5 kDa and gave a purity of 96% for the peak corresponding to β_2 -AR. Taken together, the concentration of β_2 -AR was approximately 59 $\mu\text{g}/\text{mL}$.

Immobilisation of His-tagged β_2 -AR

Random and oriented immobilised methods are the main assays for attaching a protein to solid substrates. Comparing with random methods, the proposed approach is oriented, and thus, it had the properties of better maintaining the bioactive characteristics and increasing the binding sites of the protein [33]. The concentration of the immobilised His-tagged β_2 -AR on silica gel was calculated by detecting the free concentration of β_2 -AR in the

reaction solution before and after the diazonium reaction through the bicinchoninic acid method. The amount of β_2 -AR attached on the gel was 200.17 ± 7.47 pmol/g. This value was much greater than for the random immobilisation reported earlier [24,33]. The commonly reported oriented method for protein immobilisation included the use of metal chelate resin [34] and horseradish peroxidase [35]. This work developed a new oriented method through the reaction between the histidine tag in the protein and the diazonium salt activated silica gel. Compared with the previous two types of oriented methods, the proposed assay had more potency in varied protein immobilisation, attributed to the lack of structure selectivity of the protein.

Specificity and stability of β_2 -AR column

The specificity of the oriented β_2 -AR column was tested by determining the retention times of specific drugs on the receptor. The retention times of the specific ligands, including salbutamol, clorprenaline, tobuterol and bambuterol were 5.0, 9.9, 12.5 and 49.1 min, respectively, whereas the specific ligands of the β_1 -adrenoceptor and α_1 -adrenoceptor, esmolol and terazosin, both gave retention times of 1.6 min on the column (Figure 4A). The varied retention times illustrated that His-tagged β_2 -AR still had the specificity for recognising its ligands after the immobilisation and packing procedures. Meanwhile, the rank order of the retention times of salbutamol, tulobuterol and clenbuterol is in line with the order of their association constants calculated by the radio-ligand binding assay [36]. These data confirms that it is reasonable to characterise the β_2 -AR column using the retention times of the ligand targets of the receptor.

The stability of β_2 -AR was investigated by detecting the retention times of salbutamol, clorprenaline and tobuterol for 30 consecutive days. The relative standard deviations (RSD) of the retention times on those days were 2.43%, 1.48% and 3.13%, indicating that the retention times of the drugs remained stable over the 30 days. The peak profiles were also stable over the 30 days. In conclusion, the β_2 -AR column had good stability for at least 30 days. The specific drugs of β_2 -AR give retention times approximate to the void time (Table 1) on the control column, which indicates that the retention of the specific ligands on the β_2 -AR column is a consequence of the β_2 -AR presentation but not a nonspecific interaction with the silica gel.

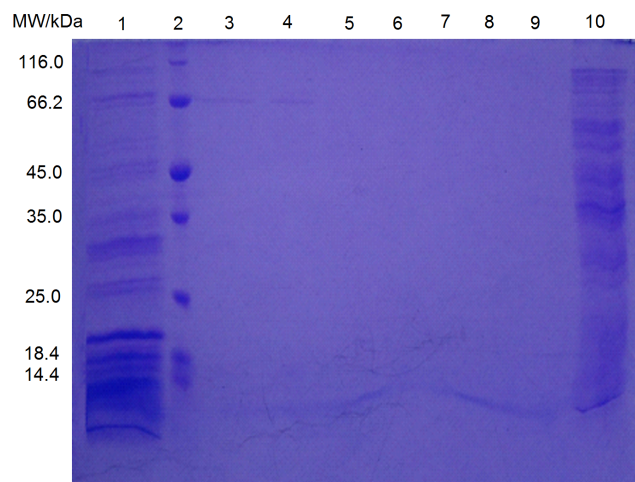


Figure 3. SDS-PAGE analysis of β_2 -AR solution. Lane 1, collection from Ni^{2+} Sepharose 6 Fast Flow column eluted by 50% buffer B; Lane 2, protein molecular weight marker (the molecular weights from top to bottom are 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa, respectively); Lane 3–4, collection from Quaternary Sepharose Fast Flow column gradient eluted by 18%–40% buffer C; Line 5–9, collection from Quaternary Sepharose Fast Flow column eluted by 50%, 60%, 70%, 80% and 90% buffer C; Lane 10, β_2 -AR supernatant.
doi:10.1371/journal.pone.0094955.g003

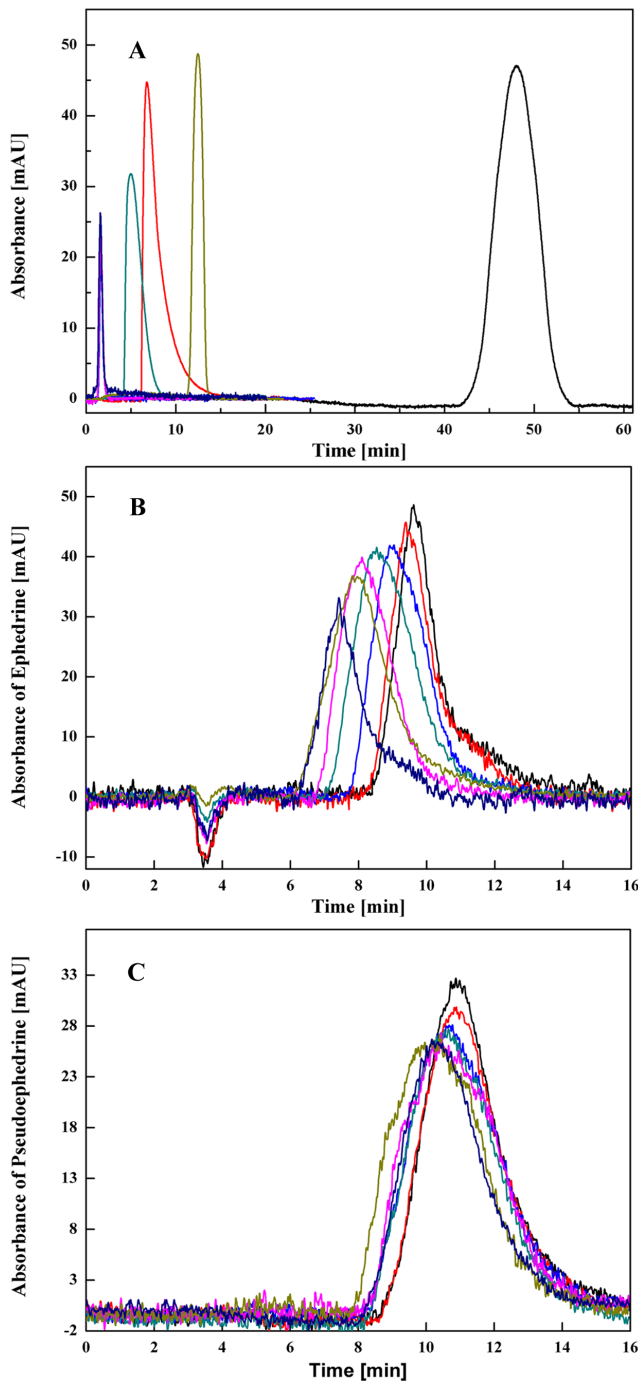


Figure 4. Representative chromatograms of control drugs, ephedrine and pseudoephedrine. A, Control drugs were eluted from β_2 -AR column using 20 mM phosphate buffer (pH 7.4) as mobile phase at 0.2 mL/min. Non-retained peaks: sodium nitrite, terazosin, esmolol. Retained peaks: salbutamol, clorprenaline, tobuterol, bambuterol (left to right); B, Ephedrine eluted from β_2 -AR column when the mobile phase containing ephedrine at different concentration (from right to left: 4, 8, 12, 20, 24, 30 and 34 μ M); C, Pseudoephedrine eluted from β_2 -AR column when the mobile phase containing ephedrine at different concentration (from right to left: 2, 4, 8, 12, 16, 24 and 30 μ M). doi:10.1371/journal.pone.0094955.g004

Self competitive studies

Self-competitive studies were performed where ephedrine served as both analyte and competing agent at 37 °C (Figure 4B). In this case, the injection volume of the analyte was 5.0 μ L of 40 μ M ephedrine, and the competitive agent in the mobile phase was 4, 8, 12, 20, 24, 30 or 34 μ M ephedrine. Triplicate injections were performed for each concentrations of the competitive agent in the mobile phase. The capacity factors of ephedrine in the β_2 -AR column are summarised in Table 2. The capacity factors of ephedrine gave a negative response to the concentrations of ephedrine in the mobile phase. Meanwhile, the capacity factors did not reach saturated adsorption during the tested concentrations, providing a situation of the response in the linear scope of the isotherm curve. According to eq. (2), plotting the curve of $1/\sqrt{k'}$ against the concentrations of ephedrine in the mobile phase at 37 °C (Figure 5A), a good linear relationship is obtained with a regression equation of $y = (2.58 \pm 0.02) \times 10^3 x + (0.43 \pm 0.01)$ and a correlation coefficient of 0.9962 over the seven data points, providing a proof of one type of binding site for ephedrine binding to β_2 -AR. Based on which ephedrine is believed to have only one type of binding site on the β_2 -AR column. By dividing the intercept by the slope of Figure 5A, the association constant of ephedrine on the column was calculated to be $(5.94 \pm 0.05) \times 10^3 / \text{M}$ (Table 3). Through the slope of Figure 5A and the values of the association constant, the concentration of the binding site for ephedrine targeting β_2 -AR was estimated to be $(8.92 \pm 0.06) \times 10^{-4}$ M.

Direct competitive study

A direct competitive study was conducted using ephedrine as a mobile phase additive and pseudoephedrine as an injection solute at 37 °C (Figure 4C). As shown in Table 2, a decreasing capacity factor of pseudoephedrine was found when the concentration of ephedrine in the mobile phase was increased. The capacity factors of pseudoephedrine were not saturated during the tested concentrations, which indicated that the pseudoephedrine adsorption properties were in line with the Langmuir model. Eq. (1) was used to plot the curve between the pseudoephedrine capacity factors and the concentrations of ephedrine in the mobile phase. Using Eq. (1), a linear relationship was obtained between the $1/(k'-X)$ of pseudoephedrine and the concentrations of ephedrine, as shown in Figure 5B. The value of X was calculated to be 2.49 ± 0.26 by non-linear regression. By applying the slope and intercept of the linear curve in Figure 5B, the association constant of ephedrine was determined to be $(5.98 \pm 0.24) \times 10^3 / \text{M}$, as shown in Table 3. This value was in good agreement with the association constant obtained by the self-competitive studies. According to the hypothesis of zonal elution assay, the concentration of the binding sites for pseudoephedrine was $(8.92 \pm 0.06) \times 10^{-4}$ M based on the value of ephedrine calculated by self-competitive studies. Using the binding sites and the intercept of Figure 5B, the association constant for pseudoephedrine binding to β_2 -AR was determined to be $(3.80 \pm 0.02) \times 10^3 / \text{M}$, as shown in Table 3.

The association constant of ephedrine is greater than that of pseudoephedrine even though the structures are similar. The difference between the structures of the two drugs is the configuration of the amino group, which produces the different spatial selectivity of the two drugs to the binding sites on β_2 -AR during the interactions with the receptor. Although the association constants of the two drugs have the same order of magnitude, the exact values are varied due the high repeatability and precision of the HPLC assay, on which the spatial position of the amino groups are believed to contribute greatly to the binding of the two drugs

Table 1. Retention times of β_2 -AR specific ligands on different columns.

| Ligand | Retention times (min) | | |
|---------------|-----------------------|----------------------|---------------------------|
| | Exposed silica gel | Histidine coated gel | Immobilized β_2 -AR |
| Salbutamol | 1.65 | 1.62 | 5.0 |
| Tobutanol | 1.58 | 1.67 | 9.9 |
| Clorprenaline | 1.71 | 1.64 | 12.5 |
| Bambuterol | 1.56 | 1.59 | 49.1 |

Notes: All the capacity factors are determined triplicate at 37 °C. The void time is measured as 1.6 min using sodium nitrite as an injection.
doi:10.1371/journal.pone.0094955.t001

and β_2 -AR. This explanation can be confirmed by the research of Vansal et al. [37] who found that *1S,2R*-ephedrine has a maximal response relative to isoproterenol (EC₅₀) of 72 μ M, whereas *1S,2S*-pseudoephedrine gives an EC₅₀ value of 309 μ M on β_2 -AR. Taken together, it is concluded that the varied affinity of the two drugs to β_2 -AR may be one of the key factors for their different pharmacological potency through the signal pathway involving β_2 -AR.

Thermodynamic studies

Thermodynamic studies between the two drugs and β_2 -AR were performed at 10, 20, 30, 37 and 45 °C. Zonal elution was used to calculate the association constants of ephedrine and pseudoephedrine at those temperatures. According to eq. (3), linear relationships were found by plotting the $\ln K_A$ of ephedrine and pseudoephedrine against $1000/T$, with correlation coefficients of 0.9716 and 0.9771 (Figure 6), respectively. The ΔH^θ and ΔS^θ for ephedrine binding to β_2 -AR were calculated to be $-(6.51 \pm 0.69)$ kJ/mol and 50.94 ± 0.31 J/mol·K. By eq. (4), the free energy during the binding was $-(22.33 \pm 0.04)$ kJ/mol (Table 4), indicating a spontaneous reaction during the binding procedure. For the interaction between ephedrine and β_2 -AR, $\Delta H^\theta < 0$, $\Delta S^\theta > 0$ and $\Delta G^\theta < 0$, which confirmed an exothermal process synergistically controlled by increasing entropy and decreasing enthalpy. During the process, ΔH^θ contributed 29.15% to ΔG^θ , whereas ΔS^θ accounted for 70.85% of the contribution to ΔG^θ at 37 °C. Electrostatic interaction was believed to be the main force of the binding of ephedrine to β_2 -AR.

The values of ΔH^θ , ΔS^θ and ΔG^θ for the binding of pseudoephedrine to β_2 -AR were $-(7.48 \pm 0.56)$ kJ/mol, 44.13 ± 0.01 J/mol·K and $-(21.17 \pm 0.02)$ kJ/mol (Table 4). Similar to the interaction between ephedrine and β_2 -AR, the binding of pseudoephedrine to the receptor was found to be a spontaneous reaction due to the negative value of ΔG^θ . The binding interaction proved to be mainly driven by electrostatic forces because $\Delta H^\theta < 0$, $\Delta S^\theta > 0$ and $\Delta G^\theta < 0$ during the binding. The free energy for the binding of ephedrine and β_2 -AR is more negative than that of pseudoephedrine, indicating that ephedrine more easily targets β_2 -AR. This may be another reason for the fact that the two drugs have varied pharmacological potency.

The association constants of ephedrine and pseudoephedrine binding to β_2 -AR decreased with increasing temperature, whereas the binding sites presented a positive response to an increasing temperature. When the temperature changed from 10 °C to 45 °C, the association constant of the two drugs decreased from $7.17 \times 10^3/M$ to $5.18 \times 10^3/M$ and from $4.93 \times 10^3/M$ to $3.36 \times 10^3/M$, respectively. Meanwhile, the binding sites increased from 8.07×10^{-4} M to 9.48×10^{-4} M during the interaction, and

the capacity factors of the two drugs on the β_2 -AR column diminished from 5.40 to 4.63 and from 6.36 to 5.60 when the concentrations were 4.0 μ M. The association constants decreased 1.4 and 1.5 fold, whereas the binding sites increased 1.2 fold. At the same time, the capacity factor diminished 1.2 fold. The retention behaviour of a solute on an affinity column ascribed to the synergistic contribution of the association constant and the binding sites. For ephedrine and pseudoephedrine binding to β_2 -AR, the decreasing retention is mainly attributed to the diminishing association constants. The reason for this phenomenon may be related to the conformation change induced by the temperature.

In the literature, it is reported that β_2 -AR has three types of binding sites. One is the salt bridge between the amine group of the ligand and the carboxylate side chain of Asp¹¹³ in the third hydrophobic domain. The other two types of binding interactions proceed through hydrogen bonds between the hydroxyl side chain of Ser²⁰⁴ and the *meta*-hydroxyl group of the ligand or between the hydroxyl side chain of Ser²⁰⁷ and the *para*-hydroxyl group of the ligand [38]. Ammonium salts mainly interact with the first type of binding site [39]. Being members of the group of ammonium salts, electrostatic interaction is believed to be the driving force during the binding of the two drugs to β_2 -AR. In this presentation, both ephedrine and pseudoephedrine gave a thermodynamic profile of $\Delta G^\theta < 0$, $\Delta H^\theta < 0$ and $\Delta S^\theta > 0$. This result confirmed that electrostatic interaction is the main force during the bindings. Thermodynamic studies in this work illustrated the same point to aforementioned report, which paves the way to the application of the thermodynamic method in revealing the binding mechanism of the interaction between ligands and the receptor.

As noted in the literature [37], stereoselective and rank differences exist among the direct effects of ephedrine isomers on β_2 -AR; *1R,2S*-ephedrine is the most potent. In this case, the affinity of *1R,2S*-ephedrine binding to β_2 -AR should be stronger than the other isomers. In this work, the association constants of *1R,2S*-ephedrine and *1S,2S*-pseudoephedrine were calculated as $(5.94 \pm 0.05) \times 10^3 M^{-1}$ and $(3.80 \pm 0.02) \times 10^3 M^{-1}$, respectively. This result is notable ascribed to the high agreement with aforementioned report. Waldeck et al [39] has discovered that ephedrine has a marked positive inotropic effect on the papillary muscle with a pD₂ of about 5.95, which corresponds an dissociation constant of 11200 nM for the drug binding to β_2 -AR. Subsequent studies by Samama et al [40] and Seifert et al [41] have reported an dissociation constant of 8000 nM for *1R,2S*-ephedrine binding to wild-type β_2 -AR and a value of 5100 ± 1300 nM to β_2 -ARGs α_s . Based on the association constant in this work, the dissociation constant for *1R,2S*-ephedrine to the receptor was 168000 nM. Since the receptors used in these work are cloned with various gene sequence, it is expected that the exact values of dissociation constant for the drug differ from each other.

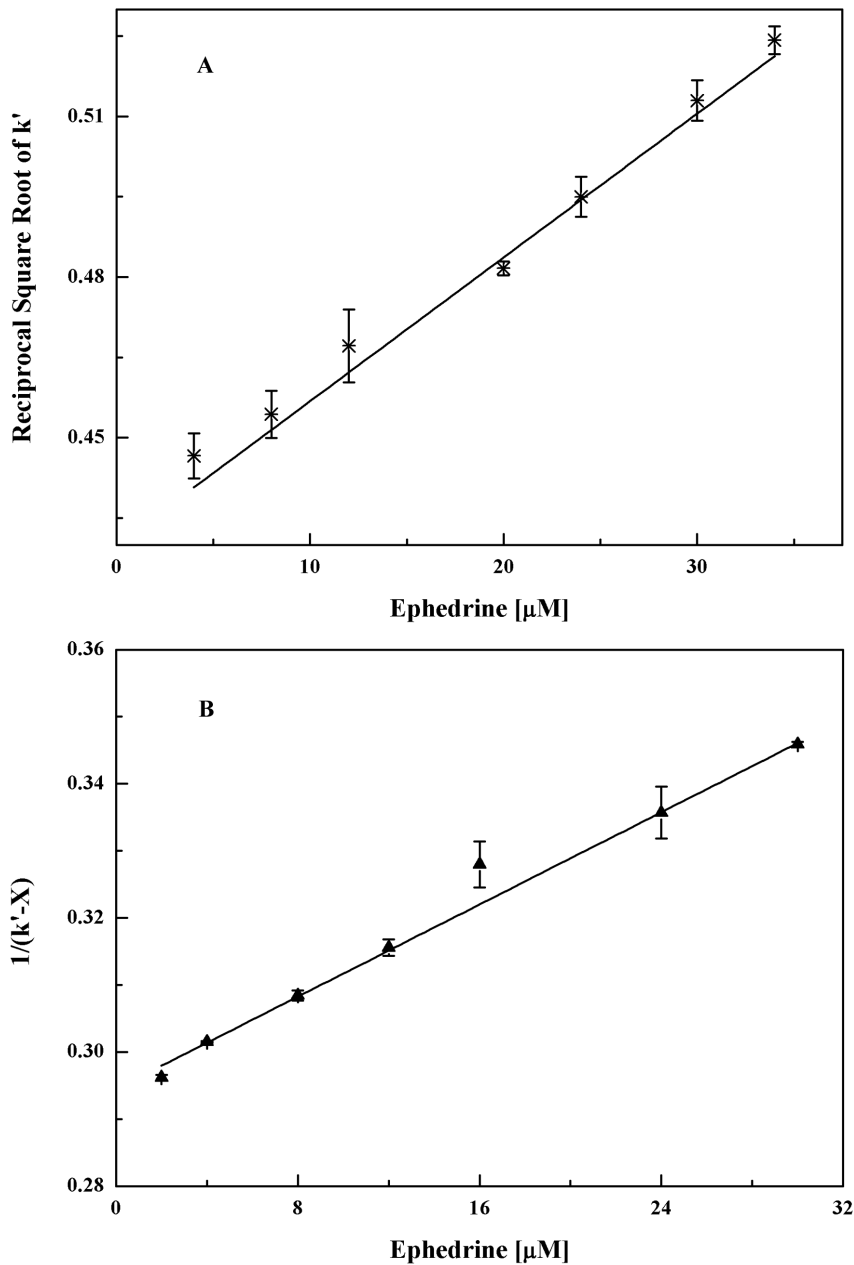


Figure 5. Change in $1/\sqrt{k'}$ of ephedrine and $1/(k'-X)$ of pseudoephedrine with mobile-phase concentrations of ephedrine. Data were from an oriented immobilised β_2 -AR column at 37 °C. The equations of the best fit lines were $y = (2.58 \pm 0.02) \times 10^{-3}x + (0.43 \pm 0.01)$ for ephedrine with a coefficient of 0.9962 and $y = (1.76 \pm 0.07) \times 10^{-3}x + (0.29 \pm 0.01)$ for pseudoephedrine with a coefficient of 0.9915. The value of X used for the plot of pseudoephedrine was set at (2.49 ± 0.26) . A, Ephedrine at different concentrations was added in the mobile phase. The injection solute was 5.0 nmol of ephedrine; B, Ephedrine at different concentrations was added in the mobile phase while 5.0 nmol of pseudoephedrine was injected as analytes. doi:10.1371/journal.pone.0094955.g005

Molecular docking

One template had been identified (PDB ID: 3KJ6, methylated β_2 -AR-Fab complex) through homology searches in PDB by comparing and aligning the target sequence with that in PDB. The sequence identity was 50%. Followed by structural-based alignment between query and target receptor, C-alpha atoms were selected for the model building ascribed to the highest identity (Figure 7A). Under the desired modelling condition, ten annotated model structure of the receptor were predicted. The best model structure of β_2 -AR superimposed to the template was believed to be pigAR.BL00010001 (Figure 7B). It showed the lowest value in

PDF (Probability Density Functions) Total Energy (-2866.93), PDF Physical Energy (-5004.22) and DOPE (Discrete Optimized Protein Energy) score (-31734.13). These results indicated that the best model of β_2 -AR compared to the other models. The structural evaluation of β_2 -AR model revealed that stereochemical and geometrical parameters incorporated in PROCHECK were satisfied in this model. The Ramachandran Plot of the receptor model calculated by PROCHECK was showed in Figure 7C. It was notable that most of the amino acid residues of β_2 -AR distributed in the favoured region. Further verification of the model by Verify 3D confirmed that a good consistent of β_2 -AR

Table 2. The capacity factors of ephedrine and pseudoephedrine at 37 °C.

| Ephedrine (μM) | $k'e$ | Ephedrine (μM) | $k'p$ |
|-----------------------------|-----------------|-----------------------------|-----------------|
| 4 | 5.01 ± 0.02 | 2 | 5.87 ± 0.01 |
| 8 | 4.84 ± 0.02 | 4 | 5.81 ± 0.01 |
| 12 | 4.58 ± 0.03 | 8 | 5.73 ± 0.01 |
| 20 | 4.31 ± 0.01 | 12 | 5.66 ± 0.01 |
| 24 | 4.08 ± 0.01 | 16 | 5.54 ± 0.03 |
| 30 | 3.80 ± 0.01 | 24 | 5.47 ± 0.03 |
| 34 | 3.64 ± 0.01 | 30 | 5.38 ± 0.01 |

Notes: $k'e$ and $k'p$ represent the capacity factors of ephedrine and pseudoephedrine respectively. $k'e$ is determined when ephedrine serves both injecting solute and competitive agents with the concentrations of 4, 8, 12, 20, 24, 30 and 34 μM . While $k'p$ is obtained by direct competitive study for which pseudoephedrine is used as an injection and ephedrine acts as competitive agents with the concentrations of 2, 4, 8, 12, 16, 24 and 30 μM .

doi:10.1371/journal.pone.0094955.t002

Table 3. The association constants of ephedrine and pseudoephedrine binding to β_2 -AR at 37°C.

| Injection analyte | |
|----------------------------------------------------------------------|----------------------------------------------------------------------------|
| Ephedrine | Pseudoephedrine |
| $K_A \text{ ephedrine} = (5.94 \pm 0.05) \times 10^3 \text{ M}^{-1}$ | $K_A \text{ ephedrine} = (5.98 \pm 0.24) \times 10^3 \text{ M}^{-1}$ |
| | $K_A \text{ pseudoephedrine} = (3.80 \pm 0.02) \times 10^3 \text{ M}^{-1}$ |

doi:10.1371/journal.pone.0094955.t003

with the respective amino acid due to 65.30% of the residues having an averaged compatibility of an atomic model (3D) with its own amino acid sequence (1D).

Site directed docking results showed that only one type of binding domain is engaged in the interaction between the two

drugs and β_2 -AR (Figure 7D). Asp⁷⁹ (corresponding to the reported Asp¹¹³ of the reported β_2 -AR) proved to be the exact site for the two isomers binding to the receptor. Meanwhile, no hydrogen bond was found during this interaction. It is widely known that Asp is an acidic amino acid with an isoelectric point of

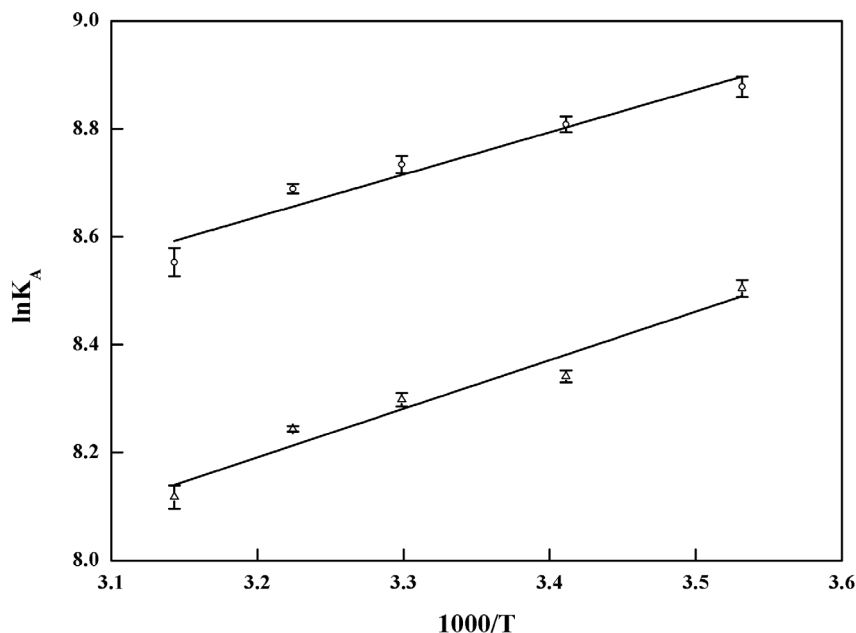


Figure 6. Van't Hoff plots for the interaction of ephedrine and pseudoephedrine binding to β_2 -AR. Circle, best-fit line with an equation of $y = (0.78 \pm 0.08)x + (6.12 \pm 0.28)$ for ephedrine and a coefficient of 0.9716. Triangle, Best-fit curve for pseudoephedrine with an equation of $y = (0.90 \pm 0.07)x + (5.31 \pm 0.23)$ and a coefficient of 0.9771. All the experiments were performed at 10, 20, 30, 37 and 45 °C.

doi:10.1371/journal.pone.0094955.g006

Table 4. The free energy and association constants of ephedrine and pseudoephedrine in different temperature.

| Temperature (K) | Ephedrine | | Pseudoephedrine | |
|-----------------|-------------------------------------------------------|-----------------------|-------------------------------------------------------|-----------------------|
| | Association constant ($\times 10^3 \text{ M}^{-1}$) | ΔG^0 (kJ/mol) | Association constant ($\times 10^3 \text{ M}^{-1}$) | ΔG^0 (kJ/mol) |
| 283.15 | 7.17 ± 0.14 | | 4.94 ± 0.08 | |
| 293.15 | 6.69 ± 0.10 | $-(22.33 \pm 0.04)$ | 4.19 ± 0.05 | $-(21.17 \pm 0.02)$ |
| 303.15 | 6.21 ± 0.10 | | 4.02 ± 0.05 | |
| 310.15 | 5.94 ± 0.05 | | 3.80 ± 0.02 | |
| 318.15 | 5.18 ± 0.13 | | 3.35 ± 0.07 | |

doi:10.1371/journal.pone.0094955.t004

2.98. In this case, Asp possesses negative charge in a solution with pH of 7.4. While in the same environment, both ephedrine and pseudoephedrine exist as positive ions. Based on these points, we concluded that the binding of the two drugs to β_2 -AR is driven by

electrostatic interaction. This result is in good agreement with the results from thermodynamic investigation in this work and the report by Strader et al [42–43].

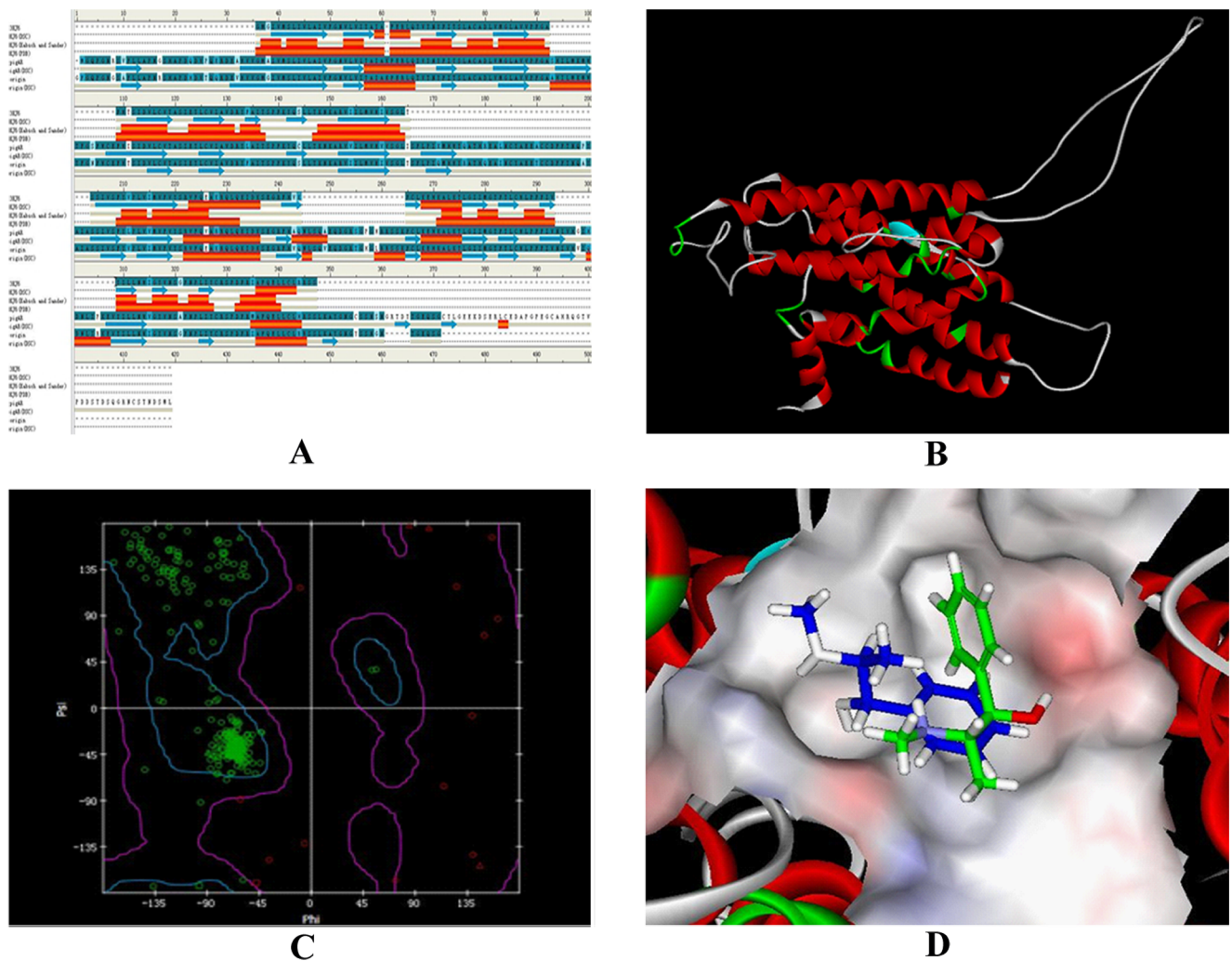


Figure 7. Three dimensional model of *Sus scrofa* β_2 -AR by homology modelling and its binding to ephedrine and pseudoephedrine. A, Structural-based alignment of β_2 -AR (PDB ID: 3KJ6). The sequence shaded in cyan represents sequence similarity. Red colour means the transmembrane domain; B, The best predicted model of β_2 -AR (pigAR.BL00010001); C, Ramachandran plot of the β_2 -AR model calculated by PROCHECK; D, Docking complexes overview of ephedrine/ β_2 -AR and pseudoephedrine/ β_2 -AR. Green illustrates Ephedrine. Blue illustrates Pseudoephedrine.

doi:10.1371/journal.pone.0094955.g007

Conclusion

A diazo coupling method was developed for oriented immobilising β_2 -AR onto the surface of macroporous silica gel. Compared with random immobilised assays, the current method proves to have the advantages of better maintaining the bioactive characteristics of β_2 -AR due to the greater amount of the immobilised protein on the gel and the increased binding sites on the surface of the receptor for recognising its ligands. The stationary phase prepared by the oriented method was also applied in exploring the interaction between β_2 -AR and two drugs, ephedrine and pseudoephedrine. The varied association constants of the two drugs are believed to be the key reason for their different

pharmacological potency. The binding of the two drugs to β_2 -AR was mainly driven by the electrostatic interaction between the amino group in the structure of the drugs and the carboxylate side chain of Asp¹¹³ in the third hydrophobic domain of the receptor. The oriented method is potential in preparing stationary phases containing other receptors for HPAC to study drug-receptor interaction.

Author Contributions

Conceived and designed the experiments: LB X. Zheng YZ. Performed the experiments: QL XG. Analyzed the data: JZ ZL RJ. Wrote the paper: X. Zhao QL.

References

- Bertucci C (2010) Characterization and modulation of molecular recognition processes: Protein/Protein interaction in drug discovery. *J Biotechnol* 150: s30. doi: 10.1016/j.jbiotec.2010.08.088
- Chautard E, Thierry-Mieg N, Ricard-Blum S (2009) Interaction networks: From protein functions to drug discovery. *A review Pathol. Biol.* 57: 324–333. doi: 10.1016/j.patbio.2008.10.004
- Wang X, Bosonca AM, Fernandez-Patron C (2012) Metalloproteinases: key and common mediators of multiple GPCRs and candidate therapeutic targets in models of hypertensive cardiac disease. *Drug Discovery Today* 9: e103–e108. doi: 10.1016/j.ddmod.2012.04.003
- Zencir S, Ovec M, Dobson MJ, Banerjee M, Topcu Z, et al. (2011) Identification of brain-specific angiogenesis inhibitor 2 as an interaction partner of glutaminase interacting protein. *Biochem Biophys Res Commun* 411: 792–797. doi: 10.1016/j.bbrc.2011.07.029
- Verburg-van Kemenade BML, Van der Aa LM, Chadzinska M (2013) Neuroendocrine-immune interaction: Regulation of inflammation via G-protein coupled receptors. *Gen Comp Endocr* 188: 94–101. doi: 10.1016/j.ygcen.2012.11.010
- Wang CIA, Lewis RJ (2013) Emerging opportunities for allosteric modulation of G-protein coupled receptors. *Biochem Pharmacol* 85: 153–162. doi: 10.1016/j.bcp.2012.09.001
- Wang CG, Williams NS (2013) A mass balance approach for calculation of recovery and binding enables the use of ultrafiltration as a rapid method for measurement of plasma protein binding for even highly lipophilic compounds. *J Pharm Biomed Anal* 75: 112–117. doi: 10.1016/j.jpba.2012.11.018
- Zinn N, Hopf C, Drewes G, Bantscheff M (2012) Mass spectrometry approaches to monitor protein-drug interactions. *Methods* 57: 430–440. doi: 10.1016/j.jymeth.2012.05.008
- Zhu LL, Yang F, Chen LQ, Meehan EJ, Huang MD (2008) A new drug binding subsite on human serum albumin and drug-drug interaction studied by X-ray crystallography. *J Struct Biol* 162: 40–49. doi: 10.1016/j.jsb.2007.12.004
- Sousa FFO, Luzardo-Álvarez A, Blanco-Méndez J, Martín-Pastor M (2012) NMR techniques in drug delivery: Application to zein protein complexes. *Int J Pharm* 429: 41–48. doi: 10.1016/j.ijpharm.2012.09.046
- Hubbard RE (2011) Structure-based drug discovery and protein targets in the CNS. *Neuropharmacology*, 60: 7–23. doi: 10.1016/j.neuropharm.2010.07.016
- Busch MHA, Carels LB, Boelens HFM., Kraak JC, Poppe H (1997) Comparison of five methods for the study of drug-protein binding in affinity capillary electrophoresis. *J Chromatogr A* 777: 311–328. doi: 10.1016/S0021-9673(97)00369-5
- Werner T, Morris MB, Dastmalchi S, Church WB (2012) Structural modelling and dynamics of proteins for insights into drug interactions. *Adv. Drug. Deliver. Rev.* 64: 323–343. doi: 10.1016/j.addr.2011.11.011
- Mallik R, Yoo MJ, Briscoe CJ, Hage DS (2010) Analysis of drug–protein binding by ultrafast affinity chromatography using immobilized human serum albumin. *J Chromatogr A* 1217: 2796–2803. doi: 10.1016/j.chroma.2010.02.026
- Tong ZH, Schiel JE, Papastavros E, Ohnmacht CM, Smith QR, et al. (2011) Kinetic studies of drug–protein interactions by using peak profiling and high-performance affinity chromatography: Examination of multi-site interactions of drugs with human serum albumin columns. *J Chromatogr A* 1218: 2065–2071. doi: 10.1016/j.chroma.2010.10.070
- D'Souza SF, Godbole SS (2002) Immobilization of invertase on rice husk using polyethylenimine. *J Biochem Biophys Methods* 52: 59–62. doi: 10.1016/S0165-022X(02)00032-5
- Rizzi SC, Hubbell JA (2005) Recombinant protein-co-PEG networks as cell-adhesive and proteolytically degradable hydrogel matrices. Part I: development and physicochemical characteristics. *Biomacromolecules* 6: 1226–1238. doi: 10.1021/bm049614c
- Wang ZH, Jin G (2003) Feasibility of protein A for the oriented immobilization of immunoglobulin on silicon surface for a biosensor with imaging ellipsometry. *J Biochem. Biophys Methods* 57: 203–211. doi: 10.1016/S0165-022X(03)00109-X
- Sassolas A, Blum LJ, Leca-Bouvier BD (2012) Immobilization strategies to develop enzymatic biosensors. *Biotechnol Adv* 30: 489–511. doi: 10.1016/j.biotechadv.2011.09.003
- Kang JH, Choi HJ, Hwang SY, Han SH, Jeon JY, et al. (2007) Improving immunobinding using oriented immobilization of an oxidized antibody. *J Chromatogr A* 1161: 9–14. doi: 10.1016/j.chroma.2007.05.023
- Turková J (1999) Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. *J Chromatogr B* 722: 11–31. doi: 10.1016/S0378-4347(98)00434-4
- Johnson M (2005) Molecular mechanisms of β_2 -adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol* 117: 292–296. doi: 10.1513/pats.200504-027SR
- Vansal SS, Feller DR (1999) Direct Effects of Ephedrine Isomers on Human β -Adrenergic Receptor Subtypes. *Biochem Pharmacol* 58: 807–810. doi: 10.1016/S0006-2952(99)00152-5
- Zhao XF, Zheng XH, Wei YM, Bian LJ, Wang SX, et al. (2009) Thermodynamic study of the interaction between terbutaline and salbutamol with an immobilized β_2 -adrenoceptor by high-performance liquid chromatography. *J Chromatogr B* 877: 911–918. doi: 10.1016/j.jchromb.2009.02.031
- Hage DS, Noctor TAG, Wainer IW (1995) Characterization of the protein binding of chiral drugs by high-performance affinity chromatography interactions of R- and S-ibuprofen with human serum albumin. *J Chromatogr A* 693: 23–32. doi: 10.1016/0021-9673(94)01009-4
- Ross PD, Subramanian S (1981) Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry* 20: 3096–3102. doi: 10.1021/bi00514a017
- Li MY, Li SF, Wang SX, Zheng XH, Bian LJ (2009) Cloning and sequence analysis of beta2-adrenergic receptor gene (written in Chinese). *Biotechnology* 19: 4–6.
- Zheng XH, Zhao XF, Yang R, Wang SX, Wei YM, et al. (2008) β_2 -adrenoceptor affinity chromatography and its application in the screening of the active compounds from semen armeniaca amarum. *Chin Sci Bull* 53: 842–847. doi: 10.1007/s11434-007-0510-8
- Christopher FB, Ross AW (2005) Platinum-catalyzed intramolecular hydroamination of unactivated olefins with secondary alkylamines. *J Am Chem Soc* 127: 1070–1071. doi: 10.1021/ja043278q
- XU LH, Wang SX, Zheng XH, Bian LJ (2010) Two-step chromatographic method for the separation and purification of recombinant porcine β_2 -adrenoceptors. *Chin J Chromatogr* 28: 374–378.
- Dolan PL, Wu Y, Ista LK, Metzberg RL, Nelson MA, et al. (2001) Robust and efficient synthetic method for forming DNA microarrays. *Nucleic Acids Res.* 29: E107–7. doi: 10.1093/nar/29.21.e107
- Basic Local Alignment Search Tool (BLAST) website. Available: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Accessed 2010 Jun 3
- Zhao XF, Li Q, Bian LJ, Zheng XH, Zheng JB, et al. (2012) Using immobilized G-protein coupled receptors to screen bioactive Traditional Chinese Medicine compounds with multiple targets. *J Pharm Biomed Analysis*, 70: 549–552 doi: 10.1016/j.jpba.2012.05.004
- Pack SH, Bachas LG, Schramm W (1993) Defined analyte-enzyme conjugates as signal generators in immunoassays. *Anal Biochem* 210: 145–154.
- Hale JE (1995) Irreversible, oriented immobilization of antibodies to cobalt-aminodiacetate resin for use as immunoaffinity media. *Anal Biochem* 231: 46–49. doi: 10.1006/abio.1995.1501
- Baker JG (2010) The selectivity of β -adrenoceptor agonists at human β_1 -, β_2 -, β_3 -adrenoceptors. *Br J Pharmacol* 160: 1048–1061. doi: 10.1111/j.1476-5381.2010.00754.x
- Vansal SS, Feller DS (1999) Direct effects of ephedrine isomers on human beta-adrenergic receptor subtypes. *Biochem Pharmacol* 58: 807–810. doi: 10.1016/S0006-2952(99)00152-5
- Zhang WS, Li AL (1999) *Medicinal Chemistry*. Beijing: Higher Education Press. 279 p.
- Waldeck B, Widmark E (1985) The interaction of ephedrine with β -adrenoceptors in tracheal, cardiac and skeletal muscles. *Clin Exp Pharmacol P* 12: 439–442. doi: 10.1111/j.1440-1681.1985.tb00891.x

40. Samama P, Cotecchia S, Costa T, Lefkowitz RJ (1993) A mutation-induced activated state of the β_2 -adrenergic receptor. *J Biol Chem* 268: 4625–4636
41. Seifert R, Wenzel-Seifert K, Lee TW, Gether U, Sanders-Bush, et al. (1998) Different effects of Gs α splice variants on β_2 -adrenoreceptor-mediated signalling. *J Biol Chem* 273: 5109a–5116. doi: 10.1074/jbc.273.9.5109
42. Strader CD, Candelore MR, Hill WS, Sigal IS, Dixon RAF (1989) Identification of two serine residues involved in agonist activation of the β -adrenergic receptor. *J Biol Chem* 264: 13572–13578.
43. Strader CD, Sigal IS, Dixon RAF (1989) Structural basis of β -adrenergic receptor function. *FASEB J* 3: 1825–1832