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Screening bioactive compounds with multi-targets from *Rhodiola crenulata* by a single column containing co-immobilized beta$_2$-adrenergic receptor and voltage dependent anion channel isoform 1

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ABSTRACT

The pursuit of drugs having improved therapeutic efficacy necessitates increasing research on new assays for screening bioactive compounds with multi-targets. This work synthesized a chromatographic stationary phase containing co-immobilized beta$_2$-adrenergic receptor (β_2-AR) and voltage dependent anion channel isoform 1 (VDAC-1) to achieve such purpose. Specific ligands of the two receptors (e.g. salbutamol, methoxyphenamine, ATP and NADH) were utilized to characterize the specificity and bioactivity of the column. Validated application of the stationary phase was performed by screening multi-target compounds of *Rhodiola crenulata* using high performance affinity chromatography coupled with ESI-Q-TOF-MS. By zonal elution, we identified salidroside as a bioactive compound simultaneously binding to β_2-AR and VDAC-1. The compound exhibited the binding sites of 1.0×10^{-7} and 4.0×10^{-7} M on the β_2-AR and VDAC-1. On these sites, the association constants were calculated to be 3.3×10^4 and 1.0×10^4 M^{-1}. Molecular docking indicated that the binding of salidroside to the two receptors occurred on Ser^{169} and $Phe^{255}$of β_2-AR, and the channel wall of VDAC-1. Taking together, we concluded that the column containing co-immobilized receptors has potential for screening bioactive compounds with multi-targets from complex matrices including traditional Chinese medicines.

1. Introduction

As the most productive sort of natural products, traditional Chinese medicines (TCMs) have become a crucial alternative for lead discovery [1,2]. However, the screening of bioactive compounds remains open due to the complexity of TCMs and the lack of high throughput methodologies [3]. Among the reported compounds, the leads selectively binding to multiple targets have proved to be promising owning to their improved therapeutic efficiency, safety and resistance profiles [4,5]. This necessitates the increasing research on new assays for the pursuit of bioactive compounds with multi-targets from TCMs.

Over the past few decades, numerous technologies have been developed to screen bioactive compounds from complex system [6]. These assays include high performance liquid chromatography-tandem mass spectrometry [7], gene and protein chip [8], computer aided virtual screening [9] and pharmacological function-guided approaches [10]. Despite a contribution to bioactive compounds screening, these methods are limited ascribed to the low efficiency, complicated operation, heavy workload and technical difficulties [11]. As an alternative, high performance affinity chromatography (HPAC) has been developed by the utilization of immobilized biologically-related targets as stationary phases [12,13]. Advances in HPAC have accelerated the screening of bioactive compounds owning to the fusion of high-resolution and high-selectivity. Wang et al. have combined an A431/cell membrane chromatography with online-HPLC/MS for screening epidermal growth factor receptor antagonists from *Radix sophorae flavescentis* [14]. More specific stationary phases have also been achieved by immobilizing purified receptors onto solid supports [15], including β_2-adrenergic receptor (β_2-AR), voltage dependent anion channel isoform 1 (VDAC-1) and cannabinoid receptor (CB1/CB2). Their application has been achieved by screening the bioactive compounds from *Semen sinapis*, *Rheum officinale Baill* and *Zanthoxylum clava-herculis* [16,17]. Wainer and co-workers have immobilized solubilized rat forebrain tissue on an artificial membrane liquid chromatographic stationary phase. Although the presence of co-immobilized *N*-methyl D-aspartate (NMDA), γ-aminobutyrate ($GABA_A$) and nicotinic (NCT)

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receptors [18], the application remains open in terms of screening bioactive compounds from complex matrices. Our previous work has introduced column switching technique into the couple of two columns containing diverse types of receptor for pursuing the compound with multi-targets [19]. Broad application of such designation is hindered because of the relatively expensive instruments and tedious operation.

This work intends to develop a simple method for multi-target compound exploration by preparing a column containing co-immobilized β_2-AR and VDAC-1. Successful application was achieved by screening the bioactive compounds binding to two receptors from *Rhodiola crenulata*. The interaction between the result compounds and the two receptors was validated by zonal elution, injection amount dependent method and site-directed molecular docking. The results demonstrated that the co-immobilized β_2-AR and VDAC-1 is useful for screening multi-target compounds from complex matrices.

2. Experimental

2.1. Materials and instruments

Polystyrene microspheres with amino group (NH_2-20140925, 8.0 μm, 300 Å) were purchased from Knowledge & Benefit Sphere Tech. Co. Ltd. (Suzhou, China). The microspheres were dried overnight at 60 °C. Ampicillin and kanamycin were acquired from Amresco (Solon, USA). Ni^{2+}-chelated Sepharose 6 Fast Flow column and quaternary Sepharose Fast Flow prepacked column were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Reference standards of prazosin, promethazine, salbutamol, clorprenaline, methoxyphenamine, curcumin, fluoxetine and salidroside were obtained from the National Institute for Food and Drug Control of China (Beijing, China). The medicinal materials of *Rhodiola crenulata* was obtained from Tong Ren Tang herbal store (Xi'an, China) and identified by Professor Fang Minfeng (School of Life Sciences, Northwest University, Xi'an, China). HPLC grade methanol and acetonitrile were obtained from Thermo Fisher Scientific (Pittsburgh, USA).

An AKTA10 system equipped with UNICORN 5.20 software (GE Healthcare, Uppsala, Sweden) was utilized to purify the target receptors. The ZZXT-A packing machine was purchased from Dalian Yilite Analytic Instruments Company (Dalian, China). The extract of *Rhodiola crenulata* was screened on an Agilent 1260 Infinity HPLC system (Santa Clara, CA, USA). An Agilent 1200 HPLC apparatus coupled with an Agilent 6520 Quadrupole Time-of-Flight (QTOF) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) was applied to identify the bioactive compounds collected from the receptor column.

2.2. Preparation of herbal extract and standard solutions

Dry *Rhodiola crenulata* powder (1.2 g) was extracted by ultrasonic method using water (30 mL) as the solvent. The optimized sonicating conditions were: a processing time of 1.0 h; an output power of 180 W and a fixed frequency of 40 kHz. The obtained aqueous extract was filtered for HPAC analysis. Standard stock solutions (2.0 mmol/L) of prazosin, promethazine, salbutamol, clorprenaline and methoxyphenamine were prepared by ultrapure water. Curcumin and fluoxetine were dissolved in methanol to prepare the solutions (2.0 mmol/L).

2.3. Purification of the receptors

β_2-AR was purified using a reported method [20]. Briefly, the *Sus scrofa* cDNA sequence of β_2-AR (Refseq: MN_ 001128436) was cloned into the *Eco*RI/*Hin*dIII with restriction sites of pET32a. This produced a plasmid of pET32a-β_2-AR with a Histidine tag. The recombinant protein was expressed in *Escherichia coli* BL21(DE3). The purification of the receptor was achieved by Ni^{2+}-chelated Sepharose 6 Fast Flow column and quaternary Sepharose Fast Flow prepacked column under non-denaturalization condition. The recombinant plasmid of pET28b-VDAC-1 was constructed by inserting the cDNA fragment of *Homo sapiens* VDAC-1 (NM_003374) into the expression vector pET28b through *Xho*I/*Nhe*I sites [21]. This plasmid was transfected into *Escherichia coli* BL21 (DE3) to express His-tagged VDAC-1. The receptor in the inclusion bodies was purified using on-column method. The two proteins were desalted by dialysis and analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Immobilization of β_2-AR and VDAC-1

The purified β_2-AR and VDAC-1 were immobilized on the surface of amino microspheres by a previous method [22]. In this case, 1.33 g dry amino microspheres were activated by 0.27 g N, *N′*-carbonyldiimidazole using acetonitrile as the solvent. The activated amino microspheres were suspended in 10 mL phosphate buffer (20 mmol/L, pH 7.4) containing equal molar β_2-AR and VDAC-1. The suspensions were stirred for 3.0 h in an ice-cold water bath. Subsequent removal of the un-reacted imidazole residues was accomplished by adding 0.1 g glycine ethylester into the suspension to trigger a 30 min stirring. The amount of two receptors attached on the microspheres was determined by detecting the concentration of receptors in the solution pro- and post-immobilization through bicinchoninic acid assay (BCA). The result microspheres were packed into a stainless steel column (50 × 4.6 mm) under a pressure of 4×10^7 Pa. Two control columns containing single immobilized receptor (β_2-AR or VDAC-1) were prepared under the same condition.

2.5. Bioactivity of the immobilized receptors

The bioactivity of immobilized receptors was evaluated by the retention behaviors of specific ligands including salbutamol, methoxyphenamine, ATP and NADH. The detection wavelengths for the six drugs were set at 251, 300, 276, 213, 260 and 260 nm, respectively. The flow rate was 0.2 mL/min. Sodium nitrite was utilized to determine the void time of chromatographic system because of ignorable binding to the two receptors.

2.6. Screening of bioactive compounds with multiple targets

An aliquot of 5.0 μL *Rhodiola crenulata* extract was loaded onto the column containing the co-immobilized β_2-AR and VDAC-1 to recognize the bioactive compounds with multi-targets. The mobile phase was phosphate buffer (20 mmol/L, pH 7.4) and the flow rate was 0.2 mL/min. The peaks with retention time longer than 3.2 min were separately collected and dried under vacuum environment. The residuals were dissolved by mobile phase (methanol-water) and identified by HPLC-ESI-Q-TOF-MS. Mass spectrometric identification was performed in negative ion mode. The optimized mass spectrometric conditions included: a mass-to-charge (m/z) scanning range from 100 to 1000, a nebulizing gas pressure of 35 psig, a capillary voltage of 4500 V, a fragmentation voltage of 100 V, a dry gas temperature of 350 °C and the flow rate of nebulization gas was 9.0 L/min. The data were processed by Mass Hunter Acquisition Software (version: B.04.00).

2.7. Binding interaction between the bioactive compounds and two receptors

The binding interaction between the compounds of interest and the two receptors were pursued by zonal elution [23]. Clorprenaline, methoxyphenamine, curcumin and fluoxetine were applied as competitors due to their canonical binding to the corresponding receptor. The mobile phase concentrations of the competitors were 10, 20, 40, 50 and 70 μmol/L for clorprenaline, 20, 30, 40, 50 and 60 μmol/L for methoxyphenamine, 1, 10, 20, 30 and 40 μmol/L for curcumin, and 10, 20, 30, 40 and 50 μmol/L for fluoxetine. When a steady baseline was achieved, we loaded the compounds of interest onto the column to determine their retention times in triplicate.