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Revealing multi-binding sites for taspine to VEGFR-2 by cell membrane chromatography zonal elution

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ABSTRACT

A new high-expression vascular endothelial growth factor receptor-2 (VEGFR-2) cell membrane chromatography (CMC) method was developed to investigate the affinity of ligands for VEGFR-2. An HEK293 VEGFR-2/CMC system was applied to specifically recognize ligands acting on VEGFR-2. Sorafenib was used as a mobile phase additive to evaluate the effect of the marker's concentration on the retention of sorafenib and taspine, respectively. The relationship among the retention, the types of binding sites and the affinity of taspine binding to VEGFR-2 has also been concerned. The retention behavior indicated that sorafenib had two major binding regions on VEGFR-2, and that taspine might act as a multi-target VEGFR-2 inhibitor with similar biological activity to sorafenib. The equilibrium dissociation constants (K_D) obtained from the model are $(5.25 \pm 0.31) \times 10^{-7}$ and $(9.88 \pm 0.54) \times 10^{-5}$ mol L^{-1} for sorafenib at the high- and low-affinity sites, respectively, and the corresponding values for taspine are $(3.88 \pm 0.31) \times 10^{-6}$ and $(7.04 \pm 0.49) \times 10^{-5}$ mol L^{-1}. The two types of binding sites contributed about a 1:2 ratio on the retention of taspine.

1. Introduction

Vascular endothelial growth factor receptors (VEGFRs), a family of tyrosine kinase receptors, are composed of an extracellular ligand-binding domain, a hydrophobic transmembrane domain and an intracellular kinase domain [1]. VEGFR-2, a subtype of VEGFR, plays an important role in tumor growth and metastasis [2], and is of increasing interest as a potential new target for the study of tumorigenesis and signal transduction [3]. Abnormal activation of VEGFR-2 leads to several disorders including cancer [4,5]. Sorafenib, sunitinib and vandetanib are three small molecular VEGFR inhibitors, available for the oral treatment of several neoplasms, including colon, lung, breast, kidney, medullary thyroid and pancreatic cancer [6–8].

Taspine, a bioactive ingredient present as the free alkaloid in the sap of the tree *Croton lechleri* [9,10], has been isolated from the radix of *Caulophyllum robustum* using cell membrane chromatography (CMC) [11]. As an aporphine alkaloid, taspine has many pharmacological actions including anti-inflammatory [9], wound healing [12,13] and cytotoxic activity [14]. In a previous study, we demonstrated that taspine can inhibit tumor angiogenesis [11] and one of its mechanisms of action may involve inhibition of VEGFR-2 and the proliferation and migration of vascular endothelial cells [15,16]. Accordingly, considerable efforts to design, synthesize and evaluate taspine derivatives for cancer research have been made in recent years [17,18].

There have been several studies of the binding characteristics of flavonoids and proteins using fluorescence quenching techniques [19,20]. These methods possess high sensitivity but cannot reveal the dynamic process of drug–protein interaction and lack the ability of drug screening *in vitro*. Based on receptor affinity chromatography [21,22], an online method coupling CMC with liquid chromatography mass spectrometry (LC/MS) was used to screen three alkaloids from *Aconitum carmichaeli* Debx. acting on VEGFR-2 [23,24]. However, the strength of this binding and the number of sites involved in the interaction are still areas of controversy. It has been demonstrated that CMC is an effective method for characterizing the affinity between ligands and membrane receptors [25,26]. The measurement of equilibrium dissociation constants (K_D) has been carried out on the premise that the ligand interacts reversibly with a single type of binding site on the receptor. In fact, interactions between ligands and protein have been reported to occur at two types of binding regions in many cases [27–30]. However, these studies have almost entirely been restricted to the protein rather than receptor, and few reports have focused on the retention–affinity relationship of ligands binding to receptors.

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In this study, a new CMC approach was used to study sorafenib–taspine competition involving small injections of sorafenib and taspine, respectively, while a known concentration of sorafenib was used as the mobile phase additive. This rapid and accurate method has been validated for calculating K_D values between taspine and VEGFR-2. Based on a modified competitive equation, the type of competition occurring at the binding sites was determined by examining how the mobile phase concentration of sorafenib affects the retention of the injected sorafenib and taspine, respectively. The proportion of binding sites contributed on the retention of taspine has also been evaluated.

2. Experimental

2.1. Chemicals and reagents

Sorafenib tosylate, sunitinib malate and vandetanib as standard drugs were provided by the Nanjing Ange Pharmaceutical Co., Ltd (Nanjing, China). Taspine was from the Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University (Xi'an, China). Dulbecco's Minimal Essential Medium (DMEM) and G418 were purchased from Invitrogen Corporation (Grand Island, NE, USA). The human VEGFR-2 ELISA kit was provided by Cusabio Biotech (Wuhan, China). Methanol was of LC grade (Honeywell, NJ, USA). Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical (Qingdao, China). C_{18} Solid-Phase Extraction (SPE) columns were purchased from Supelco (500 mg/3 mL, Sigma–Aldrich, Bellefonte, PA, USA). All other reagents and solvents were of analytical reagent grade and were used without further purification unless noted otherwise.

2.2. Apparatus and conditions

CMC analysis was performed on a Shimadzu LC-20A instrument that consisted of two LC-20AD pumps, a DGU-20A3 degasser, an SIL-20A autosampler, a CTO-20A column oven, and an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The data were acquired using LCsolution software version 2.2 (Shimadzu, Kyoto, Japan).

The CMC mobile phase consisted of phosphate-buffered saline (PBS, pH 7.4) delivered at a flow rate of 0.2 mL min^{-1}. The HPLC conditions were a Dikma C_{18} column (150 × 4.6 mm, 5 μm), and a mobile phase of methanol–water–triethylamine (70:29.5:0.5, v/v/v, pH adjusted to 6.0 with acetic acid) at a flow rate of 1.0 mL min^{-1}. All measurements were performed with photodiode array detection at 37 °C.

2.3. Preparation of standard solutions

Stock solutions (2.0 mg mL^{-1}) of sorafenib, sunitinib, vandetanib and taspine were prepared by separately dissolving the standard drugs in methanol. Standard solutions at various concentrations were prepared by diluting each of the stock solutions with methanol.

2.4. Cell culture and preparation of the cell membrane stationary phase (CMSP)

The recombinant eukaryotic plasmid pcDNA3.1(+)-VEGFR-2 was transfected into HEK293 cells using a cationic liposome. G418-resistant clones were obtained and confirmed as positive by RT-PCR, and the VEGFR-2 protein expression located on the cell membrane was confirmed by immunofluorescence staining. The HEK293 VEGFR-2 cell line with high expression of VEGFR-2 was constructed and cultured in DMEM (contained 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin and 0.30 mg mL^{-1} G418). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2, and cells from exponentially growing cultures (10^7) were harvested using trypsin and incubated for 10 min at 4 °C. The high-expression VEGFR-2 CMSP was prepared as described previously [26]. The HEK293 (non-transfected) CMSP was also obtained by the same procedure. The VEGFR-2 protein in HEK293 and HEK293 VEGFR-2 cell membrane suspensions before and after adsorption on activated silica was determined by using a commercially available VEGFR-2 ELISA kit.

2.5. VEGFR-2/CMC-offline-HPLC applications

A mixed standard solution containing sunitinib, vandetanib and sorafenib (0.1 mg mL^{-1}) was used to validate the specificity of the HEK293 VEGFR-2/CMC-offline-HPLC system. Fractions retained by the VEGFR-2/CMC column were concentrated separately and then injected into the HPLC system. The HEK293 CMSP was also applied to differentiate the non-specific from specific binding.

2.6. Zonal elution

Solutions of sorafenib ranging in concentration from 1.46 × 10^{-7} to 1.46 × 10^{-5} mol L^{-1} in PBS (5 mM, pH 7.4) were pumped through a VEGFR-2/CMC column, while standard solutions of sorafenib and taspine (each 0.1 mg mL^{-1}) were injected separately onto the column. The breakthrough curves of sorafenib at several different concentrations were recorded, and the retention factors (k') of the ligands were obtained. In the case of the ligands competing for two types (primary and secondary) of binding sites, the k' values of the ligands were obtained by Eq. (1) proposed by Hage [27,30], where k'_1 and k'_2 denote binding to the primary (high-affinity) and secondary (low-affinity) binding sites, respectively.

$$k' - X = k'_1 + k'_2 = \frac{K_{1M}[R]_{1s}}{K_{1D}V_m([L]_m + K_{1M})} + \frac{K_{2M}[R]_{2s}}{K_{2D}V_m([L]_m + K_{2M})} \quad (1)$$

$[L]_m$ and $[R]_s$ are the concentrations of the marker in the mobile phase and binding sites on the immobilized receptors, respectively; V_m is the dead volume of the column; and K_D and K_M are the equilibrium dissociation constants of the analyte and the marker, respectively. The term X was introduced to eliminate errors obtained by iterative testing [28]. Subscripts 1 and 2 denote the retention resulting from binding to high- and low-affinity sites, respectively. We assumed that at lower marker concentrations, the ligand acted competitively on both types of binding sites; as the concentration increased, the high-affinity sites were saturated, and the competition occurred exclusively at the low-affinity sites (Fig. 1). According to this assumption, Eq. (2) was obtained from Eq. (1) as:

$$k'_2 \approx k' - X = \frac{K_{2M}[R]_{2s}}{K_{2D}V_m([L]_m + K_{2M})} \quad (2)$$

Eq. (2) can be converted into a linear equation:

$$\frac{1}{k'_2} = \frac{K_{2D}V_M}{K_{2M}[R]_{2s}}[L]_m + \frac{K_{2D}V_M}{[R]_{2s}} \quad (3)$$

After the theoretical calculation of k'_2 at the initial phase, k'_1 values were calculated by subtracting k'_2 from k' as follows:

$$k'_1 = k' - X - k'_2 = \frac{K_{1M}[R]_{1s}}{K_{1D}V_m([L]_m + K_{1M})} \quad (4)$$

Eq. (4) can also be converted into a linear equation:

$$\frac{1}{k'_1} = \frac{K_{1D}V_M}{K_{1M}[R]_{1s}}[L]_m + \frac{K_{1D}V_M}{[R]_{1s}} \quad (5)$$