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Dual-mixed/CMC model for screening target components from traditional Chinese medicines simultaneously acting on EGFR & FGFR4 receptors

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ABSTRACT

Radix Salviae Miltiorrhiae (also known as DanShen (DS) in China), a popular herbal drug in traditional Chinese medicine (TCM) for promoting blood circulation and treating blood stasis, has been reported to possess potential anti-tumor effects. The aim of the study was to develop an effective and practical method for screening and identifying bioactive compounds from *Radix Salviae Miltiorrhiae*. In this work, the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptors 4 (FGFR4) dual-mixed/cell membrane chromatography (CMC) coupled with high performance liquid chromatography-electrospray ionization-ion trap-time of flight-multistage mass spectrum (HPLC-ESI-IT-TOF-MSn) was established and successfully used to identify the active components from *Radix Salviae Miltiorrhiae*. Salvianolic acid C (SAC), tanshinone I (Tan-I), tanshinone IIA (Tan-IIA), and cryptotanshinone (C-Tan) were identified as bioactive components with EGFR and FGFR4 activities. MTT and kinase assay were performed to investigate inhibitory effects of these compounds against EGFR and FGFR4 cells growth *in vitro*. Both cell viability and kinase activity showed that cryptotanshinone acting on EGFR receptor and tanshinone IIA acting on FGFR4 receptor. In conclusion, the EGFR & FGFR4 dual-mixed/CMC can simultaneously screen the bioactive components from TCMs that act on both EGFR and FGFR4 receptors, which significantly improve the efficiency of specific bioactive components identification from a complex system.

1. Introduction

Traditional Chinese medicine (TCM) has been used for prevention and treatment of human diseases for thousands of years, which has the characteristics of numerous components. Bioactive components are the basis of traditional Chinese medicine pharmacodynamics and the important source of new drug development [1–4]. More than 100 new products are being developed clinically to treat a wide range of diseases, including angiogenic diseases and cancer [5,6]. *Radix Salviae Miltiorrhiae* (the dried root and rhizome of *Salvia miltiorrhiza Bge*) is known for promoting blood circulation, treating blood stasis, and other cardiovascular diseases in TCM. The roots of *Salvia miltiorrhiza* primarily contain fat-soluble diterpenoids, water-soluble phenolic acids, flavonoids, triterpenoids and sterols. *Salvia miltiorrhiza* can prevent reperfusion injury, myocardial ischemia and myocardial infarction by improving microcirculation and reducing myocardial oxygen consumption [7–9]. The lipid-soluble ingredients of *Salvia miltiorrhiza* are reported to possess anti-tumor activity [10,11]. The discovery of the new anti-tumor agents from *Salvia miltiorrhiza* have increased the clinical applications of this phytomedicine.

Malignancy is a dreadful disease that imparts huge socio-economic burden to the patients [12–14]. Recently, the targeted antitumor therapies have gained popularity because of their lower adverse effects and excellent efficacies compared to conventional chemotherapeutic agents. Epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor 4 (FGFR4), the two important drug targets for developing newer anti-cancer therapies, are distributed in vascular endothelial cells, hematopoietic stem cells and macrophages. These factors mediate endothelial cell proliferation, chemotaxis of endothelial cells and increase vascular permeability [15,16]. The upregulation of vascular endothelial EGFR and FGFR4 can accelerate the formation of new blood vessels around the tumors to meet the nutritional needs for tumor growth [17–20].

Cell membrane chromatography (CMC) is a novel technique for

Abbreviations: TCM, traditional Chinese medicine; DS, *Radix Salviae Miltiorrhiaeis*; EGFR, epidermal growth factor receptor; FGFR4, fibroblast growth factor receptors 4; CMC, cell membrane chromatography; HPLC-ESI-IT-TOF-MSn, high performance liquid chromatography-electrospray ionization-ion trap-time of flight-multistage mass spectrum; SAC, salvianolic acid C; Tan-I, tanshinone I; Tan-IIA, tanshinone IIA; C-Tan, cryptotanshinone; CMSP, cell membrane stationary phase; DAD, diode array detector; CDL, curve desolvation line; OD, optical density; RSD, relative standard deviation

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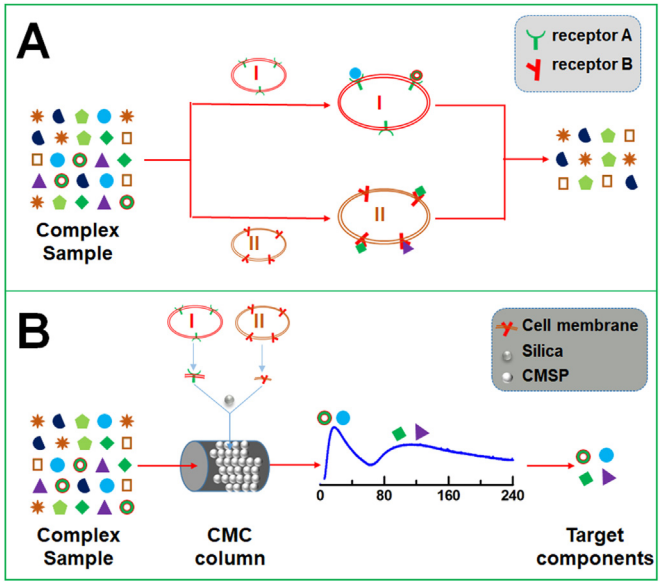

Fig. 1. A strategic overview for the screening of targeted components by dual-mixed/CMC.

screening bioactive components binding on specific receptor in complex systems. In CMC, active tissue or cell membrane is immobilized on a specific carrier-surface to prepare a cell membrane stationary phase (CMSP) [21,22]. The CMC column is wet-packed, and a buffer solution is used as the mobile phase. The drug is used as a solute or added to the mobile phase and chromatographed under dynamic conditions to study the interactions between drug and receptor on CMSP. In conventional cell membrane chromatography, a single-cell membrane chromatography only interacts with the same type of ligand [23,24]. A newer CMC model can combine two different receptors, which utilizes two or more different types of cells and simultaneously identify multiple receptor-specific bioactive components.

In this study, we established an EGFR & FGFR4 dual-mixed/CMC model which can screen bioactive components both via EGFR and FGFR4 receptors. The model was coupled with HPLC-ESI-IT-TOF-MSn and applied to selectively screen the bioactive components of *Radix Salviae Miltiorrhizae* which can target and interact EGFR and FGFR4 receptors simultaneously. The graphical abstract of this study is shown in Fig. 1.

2. Methods

2.1. Chemicals and reagents

Sorafenib (≥98%) and gefitinib (≥98%) were procured from Nanjing Ange Pharmaceutical Co., Ltd. (Nanjing, China). Salvianolic acid C (≥98%, Lot#: 150929) and tanshinone I (≥98%, Lot#: 150915) were purchased from Chengdu Must Bio-Technology CO., Ltd. (Chengdu, China). Tanshinone IIA (≥98%, Lot#: 20150816-2) and cryptotanshinone (≥98%, Lot#: 20151021) were procured from ChenGuang Bio-tech. Co., Ltd. (Baoji, China). Terbutaline and tamsulosin hydrochloride were from Sigma Chemical (St. Louis, MO, USA). *Radix Salviae Miltiorrhiae* was purchased from a TCM store (Xi'an, China), and authenticated by the Department of Pharmacognosy, Xi'an Jiaotong University (Xi'an, China). Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical (Qingdao, China). HPLC-grade methanol was supplied by Thermo Fisher Scientific (Pittsburgh, USA). The ultra-pure water was prepared using a MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Instruments and conditions

The dual-mixed/CMC system was combined with the HPLC-ESI-IT-TOF-MSn establishing a two-dimensional system using a VICIAG 10G-0911V 10 port 2-position valve (Valco Instrument Co. Inc., Houston, USA) and two Shimpack VP-ODS pre-columns (10 mm × 2.0 mm i.d., 5 μm, Shimadzu Corporation, Kyoto, Japan). The first dimension dual-mixed/CMC system contained an LC-20AD pump, a DGU-20A3 degasser, an SIL-20A auto-sampler, a CTO-20AC column oven, a SPD-M20A diode array detector (DAD), while the second dimension (HPLC-ESI-IT-TOF-MSn) contained two LC-20AD pumps, an SPD-M20A diode array detector, an ESI-IT-TOF-MS mass spectrometer, a CBM-20A communication bus module, and LCMS Solutions work station containing LCMS solution Formula Predictor (Shimadzu Corporation, Kyoto, Japan). The EGFR & FGFR4 dual-mixed/CMC column (10.0 mm × 3.0 mm I.D., 5 μm, described later in the section of "Preparation of EGFR & FGFR4 dual-mixed/CMC model") was used as the first dimensional column and was packed using the RPL-10ZD column loading machine (Dalian Replete Science and Technology Co., Ltd., Dalian, China). A Shimadzu Shim-pack VP-ODS column (150 mm × 4.6 mm I.D., 5 μm, Kyoto, Japan) was employed as the second dimensional column.

For the first dimensional system, ultrapure water was used as the mobile phase with a flow rate of 0.2 mL/min, column temperature was kept at 37 °C, DAD detection. For the second dimensional system, a gradient elution with 0.1% (v/v) acetic acid (A) and methanol (B) at the flow rate of 1.0 mL/min was maintained. The gradient elution started at 10% B and increased linearly to 30% B over 0–5 min; then to 55% B over 5–8 min; to 70% B over 8–30 min; to 75% B over 30–45 min; and to 90% B over 45–50 min. The column temperature was maintained at 37 °C, with DAD detector. The MS settings were as follows: nebulizer gas, N_2, purity > 99.999%, flow rate 1.5 L/min; drying gas, N_2, purity > 99.999%, pressure at 109 kPa; interface, ESI source; curve desolvation line (CDL) temperature, 200 °C; heat block temperature, 200 °C; interface voltage, 4.5 kV; detector voltage, 1.57 kV; CID gas, He, purity > 99.999%; CID energy 50%; ion accumulation, 30.0 ms; scan mode, positive ionization; automatic precursor ion selection; scanning range, *m/z* 100 to *m/z* 1000.

2.3. Standard solution and sample preparation

Gefitinib, sorafenib, tanshinone IIA, salvianolic acid C, tanshinone I, cryptotanshinone, terbutaline and tamsulosin hydrochloride standard stock solutions (1 mg/mL) were prepared in methanol and stored at −20 °C in the dark until use. Standard stock solutions were diluted using mobile phase to suitable concentrations prior use.

The *Radix Salviae Miltiorrhiae* total extracts were obtained as follows: about 0.3 g *Radix Salviae Miltiorrhiae* powder was refluxed with 50 mL methanol for 1 h at 65 °C. The filtrate was transferred into a clean round-bottomed flask, and the residue was refluxed in 50 mL methanol for 0.5 h at 65 °C. The filtrates were mixed and concentrated in a rotary evaporator to obtain the *Radix Salviae Miltiorrhiae* total extract. The *Radix Salviae Miltiorrhiae* total extract was finally dissolved in 2 mL methanol, filtered using a Millipore filter (0.45 μm) before use. Sample solution was stored at 4 °C in the dark.

2.4. Preparation of EGFR & FGFR4 dual-mixed/CMC model

EGFR HEK293 & FGFR4 HEK293 cells were constructed in our laboratory [25,26]. EGFR & FGFR4 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin under sterile conditions at 37 °C in a humidity atmosphere with 5% CO_2. The EGFR & FGFR4 dual-mixed/CMC column was prepared according the methods described in previous reports [22–24]. After 80% cell-growth was achieved, EGFR HEK293 cells (5×10^7) and FGFR4 HEK293 cells