A high expression EGFR/cell membrane chromatography and online high performance liquid chromatography/mass spectrometry method for screening EGFR antagonists from Rhizoma Polygoni Cuspidati

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Abstract The epidermal growth factor receptors (EGFRs) in some tumor cells are significant targets for drug discovery. In this work, we have developed an EGFR cell membrane chromatography and online high performance liquid chromatography/mass spectrometry system for screening active component from Rhizoma Polygoni Cuspidati. As a result, resveratrol from Rhizoma Polygoni Cuspidati was found to be the active component acting on EGFR like gefitinib. There was a good relationship between their inhibiting effects on EGFR secretion and HEK293 EGFR cell growth in vitro. The EGFR/CMC-online-HPLC/MS system demonstrated fast and effective characteristics for screening leading compounds from traditional Chinese medicine.

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1. Introduction

The epidermal growth factor receptor (EGFR)-mediated signaling can promote the cells into a continuous and uncontrolled dividing state, which leads to produce more malignant cells and augmenting tumor. Therefore, EGFR has become an important target for screening anti-tumor inhibitors. Some effective anti-EGFR drugs have been applied in clinic. As drug-targeting examples, there had an efficacy and potential prognostic value of gefitinib for non-small-cell lung cancer and recurrent endometrial cancer, trastuzumab for breast cancer, cetuximab for colorectal cancer in clinic treatment. Natural medicinal plants including traditional Chinese medicine are an important resource for the discovery of leading compounds. Modern pharmacological studies had shown that traditional Chinese medicine such as *Rhizoma Polygoni Cuspidati* (Huzhang in Chinese) had inhibiting effects on a variety of malignant tumors. However, little information has been mentioned on screening active components and investigating action domains by means of a new analytical technique and method. Cell membrane chromatography (CMC) is a kind of bionic affinity chromatography in which the membrane receptors were prepared as cell membrane stationary phase (CMSP). It has widely been used for studying drug–receptor interactions and screening target components from complex samples such as traditional Chinese medicines. In this paper, a high expression EGFR/cell membrane chromatography and online high performance liquid chromatography/mass spectrometry method was established and used for screening active component from Huzhang. Furthermore, the acting sites, docking extents and biological effects of active components were also investigated.

2. Materials and methods

2.1. Reagents and materials

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium acetate was obtained from Fuchen Chemical Reagents Factory (Tianjin, China). Ammonia, ethanol and chloroform were obtained from Xi’an Chemical Reagents Factory (Xi’an, China). Water was purified by Chengfu Ultrapure Technology Co., Ltd. (Chengdu, China). Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Gefitinib (purity > 99.2%) was purchased from Nanjing Ange Pharmaceutical Co., Ltd. (Nanjing, China). Resveratrol, tamsulosin and nitrendipine were prepared from the National Institute for the Pharmaceutical and Biological Products of China (Beijing, China). RPC was purchased from the TCM Store (Xi’an, China).

2.2. Preparation of EGFR/CMC column

The cultured EGFR cells were washed thrice with PBS by centrifuging at 110 × g for 10 min at 4 °C. Tris–HCl (50 mmol/L, pH = 7.4) was added to produce EGFR cell suspension, which was ruptured by supersonic procedure for 30 min. The resulting homogenate was centrifuged at 1000 × g for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × g for 20 min at 4 °C. The precipitation was suspended in 10 mL tris–HCl (50 mmol/L, pH = 7.4), and the suspension was centrifuged at 12,000 × g again. EGFR cell membrane suspension in 5 mL distilled water was obtained. According to the literatures, EGFR cell membrane stationary phase (CMSP) was prepared by the adsorption of cell membrane suspension (5 mL) on the activated silica (0.05 g) under the vacuum and agitation conditions at 4 °C. The EGFR CMSP was packed into the column (10 mm × 2.0 mm I.D.) using a wet packing procedure. Other chromatographic conditions included ammonium acetate (10 mmol/L, pH = 7.4) as a mobile phase with 0.2 mL/min flow rate, UV detector and column temperature at 37 ± 0.5 °C.

2.3. Apparatus

A high performance liquid chromatography mass spectrometer (HPLC/MS, Shimadzu Corporation, Kyoto, Japan) including three LC-20AD pumps, DGU-20A3 degasser, SIL-20A autosampler, CTO-20A column oven, SPD-20A UV/vis detector, SPD-M20A diode array detector, LCMS2010EV mass spectrometry and LC/MS solution workstation. A VICIAG 10G-0911V 10-port 2-pos valve (Valco Instrument Co. Inc., Houston, USA) was used as the column switcher (CS) and two Shim-pack VP-ODS pre-columns (10 mm × 2.0 mm I.D., 5 μm, Shimadzu Corporation, Kyoto, Japan) were used as the enrichment columns. A high expression EGFR/CMC column (10 mm × 2.0 mm I.D.) was used as the 1st D column and a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm I.D., 5 μm, Kyoto, Japan) as the 2nd D column.

2.4. Preparation of standard solutions

Standard stock solutions of gefitinib, tamsulosin and nitrendipine (1 mg/mL each) were separately prepared in methanol. A mixed standards solution (0.01 mg/mL) of gefitinib, tamsulosin and nitrendipine was prepared in methanol. Standard stock solution of resveratrol (1 mg/mL each) was prepared in methanol. The working solutions (0.01 mg/mL) were diluted with the mobile phase freshly.

2.5. Preparation of analytical samples

The PRC extract was obtained by following the following procedures. 100 g dried PRC powder was extracted with 500 and 300 mL 70% ethanol twice for 2 h each time. The solvents were combined and recovered to obtain 2.7 g PRC extract. Sample solution of PRC extract (1 mg/mL each) was prepared in methanol, and stored at 4 °C in dark. The working solution (0.1 mg/mL) was diluted with a mobile phase freshly.

2.6. Operating conditions

HPLC conditions include a VP-ODS column (150 mm × 2.0 mm I.D., 5 μm), a mobile phase of methanol–water–0.1% ammonia (55:45, v/v) with 0.2 mL/min flow rate and column temperature at 37 °C. MS conditions are given as follows: nebulizer gas (N2, purity > 99.99%) flow rate, 1.5 L/min; drying gas (N2, purity > 99.99%) pressure, 0.1 MPa; interface temperature, 250 °C; heat block temperature, 200 °C; detector voltage, 1.25 kV.
The EGFR/CMC model was combined with HPLC/MS by means of a 10-port column switcher in an online way. Each sample was injected into the EGFR/CMC model, and then the elution curve \((C_{1D})\) of each sample was recorded and related fractions were switched onto two ODS pre-columns (PC1 and PC2) at the same time. Each enriched fraction was successively eluted into the HPLC/MS system for analysis, and HPLC/MS chromatograms \((C_{2D})\) of each fraction were obtained.

2.7. Analysis of samples

This EGFR/CMC-online-HPLC/MS system was applied for screening EGFR antagonists in gefitinib as a control drug. The standard solution and PRC extract prepared above were analyzed.

2.8. Cell growth assay

The effect of resveratrol on EK293/EGFR viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were harvested and plated in 96-well plates at a concentration of \(1 \times 10^4\) cells/well. After 24 h incubation at 37°C, cells were treated with resveratrol at various concentrations for 48 h. Then, 20 μL of MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h. After the supernatant was discarded, 150 μL of DMSO was added to each well, and the optical density of cells was determined with a microplate reader (Bio-RAD Instruments, USA) at 490 nm and expressed as absorbance values.

2.9. Western blot analysis

The HEK293/EGFR cells treated with resveratrol for 48 h were prepared by extracting proteins with RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Switzerland) on ice. The epidermal growth factor (EGF) was added in the cell culture medium for 15 min before extracting proteins. Protein concentration was determined by BCA protein quantitation kit according to the manufacturer’s instructions. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) loading 30 μL of

![Figure 1 Chromatograms of standard solution and RPC extract using an EGFR/CMC-online-HPLC/MS method. (a) HPLC/MS chromatogram of the mixed standards solution (Cs); EGFR/CMC chromatogram of the mixed standards solution (C1D); R1 fraction enriched by CS and analyzed by HPLC/MS as gefitinib (g), and R0 fraction identified as tamsulosin (t) and nitrendipine (n) (C2D). (b) HPLC/MS chromatogram of RPC extract (Cs). EGFR/CMC chromatogram of RPC extract (C1D); R0 and R1 are the fractions of RPC extract on EGFR/CMC and enriched by CS and were analyzed by HPLC/MS, the chromatograms were recorded as C2D. The peak R1,1 of R1 fraction was identified as resveratrol (r). (c) HPLC/MS chromatogram of resveratrol standard solution (Cs). EGFR/CMC chromatogram of resveratrol standard solution (C1D); HPLC/MS chromatogram of R1 fraction (C2D), and the retention peak (R1) was identified as resveratrol (r).](image-url)
cell lysates per lane. After electrophoresis, separated proteins were transferred to nitrocellulose membrane and blocked by 5% non-fat milk in TBST buffer for 2 h. After then, the membranes were incubated with primary antibodies (anti-Erk, anti-phospho-Erk and anti-β-actin were used as primary antibodies) at 1:1000 dilutions in 5% non-fat milk overnight at 4°C with continuous agitation, and then secondary antibodies conjugated with horseradish peroxidase at 1:5000 dilution for 2 h at room temperature according to the manufacturer’s recommended protocol31. Finally, the blots were detected by enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotechnology, USA) and analyzed using Quantity one®. 1D analysis software (Version 4.4, Bio-Rad, USA).

3. Results and discussion

3.1. Reliability of the EGFR/CMC-online-HPLC/MS system

The EGFR/CMC-online-LC/MS system developed in this study was suitable for qualitative analysis of active components from the complex samples. The brief scheme of the system has been demonstrated in our previous work25,26. In this system, the components were recognized by the EGFR/CMC model and then identified by the HPLC/MS from a complex sample in an online way. A mixed standard solution containing gefitinib, tamsulosin and nitrendipine was used to verify the selectivity of the EGFR/CMC-online-HPLC/MS system (Fig. 1a). Of the three standards, only gefitinib is selectively acting on EGFR. The chromatogram of the mixed standard solution on EGFR/CMC is presented in Fig. 1a C1D. Two fractions (indicated by dotted lines) were sequentially extracted onto pre-columns and then switched onto column C18DS for chromatographic separation (Fig. 1a C2D) and MS identification. This experiment demonstrated that gefitinib was specifically retained by the EGFR/CMC system from the solution of mixed standards and could be simultaneously analyzed by HPLC/MS.

3.2. Applications

This system was used for screening active components from traditional Chinese medicines such as RPC. Chromatograms of the RPC extract obtained using the EGFR/CMC-online-LC/MS method are shown in Fig. 1b. The retention fraction (R1) in the EGFR/CMC model (Fig. 1b C1D), and the fraction was assayed using the LC/MS system online for further separation and identification. As shown in Fig. 1b C2b, the main component of the R1 fraction (peak R1-1) was identified as resveratrol. Compared with Fig. 2b C8, resveratrol can be identified from the RPC extract.
Development of a system for screening EGFR antagonists

To further verify the screening results above, the resveratrol solution was analyzed using the EGFR/CMC-online-LC/MS method. The standard solution of resveratrol (0.01 mg/mL) was injected into the EGFR/CMC model. As shown in Fig. 1c, the main retention fraction in the EGFR/CMC model was identified as resveratrol.

3.3. Biological trials

In this method, the EGFR/CMC model that used high EGFR expression EGFR cell membrane as stationary phase can selectively recognize EGFR antagonists such as gefitinib. Gefitinib was also used as a control drug for the biological activity of the cellular level verification. MTT results showed that resveratrol measured by high expression of EGFR-dependent manner inhibited cell proliferation (Fig. 2a) and phosphorylation (Fig. 2b), as well as effectively reduced the downstream signaling molecules (Fig. 2c) expression and blocked signal transduction.

4. Conclusions

EGFR/CMC-online-HPLC/MS system, the use of high expression of EGFR/CMC model, increasing the target component ‘identification’ of the selectivity and sensitivity, online in conjunction with the HPLC/MS identification enhancing the system’s qualitative features, is a complex system from the lead compounds in drug discovery for the fast and effective screening system.

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References


