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EGFR/cell membrane chromatography-online-high performance liquid chromatography/mass spectrometry method for screening EGFR antagonists from *Radix Angelicae Pubescentis*

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The intracellular kinase domains of the epidermal growth factor receptor (EGFR) in some tumor cells are significant targets for drug discovery. We have developed a new EGFR cell membrane chromatography (EGFR/CMC)-online-high performance liquid chromatography/mass spectrometry (HPLC/MS) method for screening anti-EGFR antagonists from medicinal herbs such as *Radix Angelicae Pubescentis*. In this study, the HEK293 EGFR cells with high expression of EGFR were used to prepare cell membrane stationary phase (CMSP) in the EGFR/CMC model. The retention fractions on the EGFR/CMC model were directly analyzed by combining a 10 port columns switcher with a HPLC/MS system online. As a result, osthole from *Radix Angelicae Pubescentis* was found to be the active component acting on EGFR like dasatinib as the control drug. There was a good relationship between their inhibiting effects on EGFR secretion and HEK293 EGFR cell growth *in vitro*. This new EGFR/CMC-online-HPLC/MS method can be applied for screening anti-EGFR antagonists from TCMs, for instance, *Radix Angelicae Pubescentis*. It will be a useful method for drug discovery with natural medicinal herbs as a leading compound resource.

EGFR cell membrane chromatography, high performance liquid chromatography/mass spectrometry, anti-EGFR antagonists, *Radix Angelicae Pubescentis*

1 Introduction

Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein, is widely distributed in mammalian epithelial cells [1]. Clinical studies have shown that EGFR is overexpressed in a variety of malignant tumors such as breast cancer [2], lung cancer [3], and colon cancer [4]. The activation of EGFR would promote tumor cell proliferation [5], angiogenesis [6] and metastasis and inhibit apoptosis [7]. In recent years, EGFRs as screening targets have been investigated for the development of anti-cancer drugs [8]. Monoclonal antibody [9] and panitumumab [10], which targets the extracellular domain of EGFR, are the cases. EGFR antagonists, gefitinib, dasatinib and erlotinib [11], which act on the intracellular ATP binding sites, are also widely used clinically. High EGFR expression levels have been identified in A431 cells [12]. Therefore, A431 will be effective EGFR-rich target cells for screening leading compounds of EGFR antagonists [13]. Since the 21st century, a variety of screening methods or techniques have been used, for example, a direct drug screen based on target receptors or enzymes[14, 15]and a virtual screen using computer-aided drug design [16, 17] have been used for investigation of leading compounds and drugs. However, the aforementioned methods and techniques would be effective only if a large number of pure compounds were supplied by the compound libraries which guarantee the varieties of com-

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pound structures. This is a significant limitation of these screening models for directly indentifying active compounds or components from a complex sample.

Since the establishment of cell membrane chromatography (CMC), much research has been done on the basic methodology [18–21], interactions between drugs and membrane receptors [22–26] as well as screening of active components from traditional Chinese medicines (TCMs) [27–33]. On this basis, we developed a high expressed EGFR/CMC model, combined it with a HPLC/MS system by means of an online column switching technique to develop a new method of two-dimensional (2D) liquid chromatography which performs recognition-separation-identification in one successive procedure. Its application in screening anti-EGFR components from *Radix Angelicae Pubescentis* was also studied.

2 Experimental

2.1 Chemicals and materials

Dasatinib (purity > 99.2%) was purchased from Nanjing Ange Pharmaceutical Co., Ltd. (Nanjing, China). Osthole (purity > 99.2%) was obtained from the National Institute for the Pharmaceutical and Biological Products of China (Beijing, China). Fetal calf serum was ordered from Lanzhou Minghai Co., Ltd. (Lanzhou, China). Penicillin (800000IU/ bottle) was from Harbin Pharmaceutical Group Co., Ltd. General Pharm. Factory (Harbin, China). Streptomycin (1000000IU/bottle) was supplied by Merro Pharmaceutical Co., Ltd. (Dalian, China). GIBCO MEM medium and trypsin were obtained from Invitrogen Corporation (Grand Island, N.Y., USA). HPLC grade methanol was purchased from Burdick & Jackson (Morris, NJ, USA). Ultrapure water was prepared in the laboratory. Other reagents used are of analytical grade.

2.2 Instrumentation

The HPLC mass spectrometer system (HPLC/MS, Shimadzu Corporation, Kyoto, Japan) included three LC-20AD pumps, a DGU-20A3 degasser, a SIL-20A autosampler, a CTO-20A column oven, a SPD-20A UV/VIS detector, a SPD-M20A diode array detector (DAD), LCMS2010EV mass spectrometry and a LCMS solution workstation.

A VICIAG 10G-0911V 10 port 2-pos valve (Valco Instrument Co., Inc., Houston, USA) was used as the column switcher and two Shim-pack VP-ODS pre-columns (10 × 2.0 mm I.D., 5 μm, Shimadzu Corporation, Kyoto, Japan) were used as the enrichment columns. An EGFR-CMC column (10 × 2.0 mm I.D.) was used as the first dimension column and a Shimadzu Shim-pack VP-ODS column (150 × 2.0 mm I.D., 5 μm, Kyoto, Japan) as the second dimension column.

2.3 Cell culture of highly expressed EGFR and preparation of stationary phase

The HEK293 EGFR cell line with high expression of EGF [34] was cultured in the minimal essential medium (MEM; contained 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. Cells from exponentially growing cultures (approximately 80% coverage) were harvested using 0.25% trypsin. The cells were resuspended to a single cell suspension with a small portion of MEM. Cell counting was performed to ensure the amount of cells was no less than 1×10^7. The cell suspension was centrifuged at 110 × g for 10 min, and the supernatant was discarded. Tris-HCl (50 mM, pH 7.4) hypotonic solution was added to produce a cell suspension. The cells were then ruptured by ultrasonication for 30 min. The resulting homogenate was centrifuged at 1000 × g for 10 min. The pellet was discarded and the supernatant was centrifuged at 12000 × g for 20 min at 4 °C. The precipitation was suspended in 10 mL Tris-HCl (50 mM, pH 7.4), and the suspension was centrifuged at 12000 × g again. Cell membrane suspension was then obtained by adding 5 mL physiological saline solution to the precipitate and was reserved.

According to the previously reported methods [19], 0.05 g silica was activated at 105 °C for 30 min and used as a carrier. It was then homogenized with the cell membrane suspension, by adding the mixture slowly to it under vacuum and with agitation at 4 °C. The mixture obtained was packed into the column by a wet method to yield an EGFR/CMC column (10 mm × 2.0 mm I.D.).

2.4 Chromatography and MS conditions

EGFR/CMC model (the first dimension): mobile phase (M_1) of water with a flow rate of 0.2 mL/min at the column temperature of 37 °C; UV detection, 320 nm.

The HPLC system (the second dimension): mobile phase (M_2) of methanol: 0.1% aqueous formic acid (60:40, *V*/*V*) with a flow rate of 0.2 mL/min at the column temperature of 37 °C; DAD detection, 320 nm; electrospray ionization (ESI). MS conditions were as follows: nebulizer gas, N_2 (purity 99.999%); flow rate, 1.5 L/min; detector voltage, 1.5 kV; *m*/*z* scan range, 50–1000 *m*/*z*; scan mode: positive ionization mode; detector voltage, 1.5 kV; heat block temperature, 200 °C; interface temperature, 250 °C.

VP-ODS enrichment columns: The first enrichment column (EC_1) and the second enrichment column (EC_2) were combined by means of an online 10-port column switcher. EC_1 and EC_2 can be alternately in either the first or the second dimension system. Alternation of enrichment and elution can be achieved by this parallel connection of two enrichment columns, which enables the online enrichment of the eluent from the first dimension.

1 μL of injection was performed after 2 h of chroma-

tographic system balance. Any retention fraction, which was "recognized" on the first dimension CMC, was enriched onto an enrichment column. The enriched fraction was then alternately eluted into the second dimension VP-ODS system for "separation and identification".

2.5 Sample preparation

The sample from *Radix Angelicae Pubescentis* was comminuted and screened through a 16-mesh sieve. 50 g of *Radix Angelicae Pubescentis* powder was extracted with ethanol for 2 h twice. The solvents were combined and filtered. The solvent was evaporated in a 60 °C water bath, and recovered to obtain 10.1 g *Radix Angelicae Pubescentis* extract.

Storage solutions of *Radix Angelicae Pubescentis* extract and osthole (1 mg/mL each) were separately prepared in ethanol. 0.1 mg/mL sample solution of *Radix Angelicae Pubescentis* extract was prepared by diluting the storage solution with methanol. 0.01 mg/mL sample solution of osthole was obtained by adding methanol to the storage solution.

2.6 Cell growth assay

The effects of oxymatrine and matrine on A431 viability were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were harvested during the exponential growth period by 0.25% trypsin, and prepared to single cell suspension with different media (containing 10% FBS). Cells were plated in 96-well plates at a concentration of 2×10^4 cells/well, with the volume of 200 μL per well. The medium was discarded after 24 h incubation at 37 °C with 5% CO_2. The cells were treated with media of various osthole or xanthotoxin concentrations for 48 h, with 20 μL in each well, and the blank medium was used for the control group. 200 μL serum-free medium was used to replace the old one. 20 μL of MTT (5 mg/mL) was added to each well and the plates were incubated for 4 h at 37 °C. After the supernatant was discarded, 150 μL DMSO was added to each well and shaken for 10–15 min. The absorbance values were determined using a microplate reader (Bio-RAD instruments, USA) at 490 nm.

$$\text{survival rate\%} = \frac{\text{absorbance of experiment group}}{\text{absorbance of control group}} \times 100\%$$

$$\text{Inhibitive rate\%} = (1 - \text{survival rate}) \times 100\%$$

2.7 Analysis of protein expression of EGFR

Enzyme-linked immunosorbent assay (ELISA) was applied. Hek293/EGFR cells were collected during the exponential growth period by 0.25% trypsin, and prepared to single cell suspension with the medium (containing 10% FBS). Cells were placed in 6-well plates at a concentration of 5×10^4 cells/well. Drugs were treated by group after 24 h incubation at 37 °C with 5% CO_2. Osthole and xanthotoxin interacted with the cells for 48 h, the proteins were then decomposed, and lysates were used to determine the amount of EGFR. Procedures of the assay were conducted according to instructions of ELISA reagent. The reaction began after the samples were added to each well, and working solutions of antibodies and enzyme-labeled antibodies were then added for continued reaction. The working solution of substrates was added in the end, and the absorbance values were determined at 450 nm to determine the amount of EGFR.

3 Results and discussion

3.1 System validation of the EGFR/CMC-online-HPLC/MS method

The EGFR/CMC-online-HPLC/MS method developed in this study was suitable for qualitative analysis of active components from the complex samples. As shown in Figure 1, at position A, the first retention fraction recognized in the

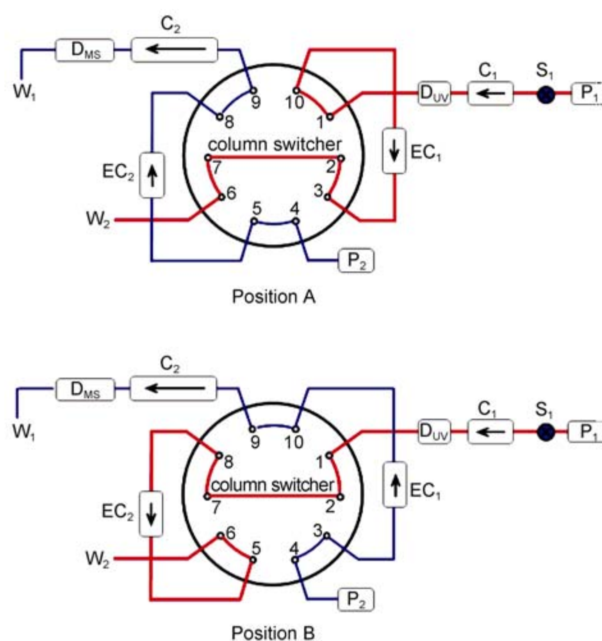

Figure 1 Brief scheme of the EGFR/CMC-online-HPLC/MS method. Position A: Affinity recognition procedure using the EGFR/CMC model with the first retention fraction enriched onto an ODS pre-column (EC_1) after the EGFR/CMC column (C_1) from a complex sample (S_1); Establishing the equilibrium procedure of the HPLC/MS system from another ODS pre-column (EC_2) to an analytical column (C_2). B: Analytical identification procedure using the HPLC/MS system with the first extracted fraction analyzed using C_{ODS}-HPLC/MS to identify their chemical structures; The second retention fraction (if there was) was enriched onto another extraction column (EC_2) after the EGFR/CMC column (C_1) from the same sample (S_1). Note: D_{UV}: ultra-violet detector, D_{MS}: mass spectrographic detector, P_1 and P_2: pumps.

EGFR/CMC model was extracted onto an ODS pre-column (EC_1), and then at position B, the extracted components were pumped into an ODS analytical column (C_{ODS}) for qualitative analysis. At the same time, the second retention fraction was pumped onto another ODS pre-column (EC_2) and into the C_{ODS} for analysis, alternately.

C_{18} VP-ODS pre-column has better retentive ability than EGFR/CMC column. Therefore, eluted fraction from EGFR/CMC system could be well reserved when it was used as the enrichment column in the first dimension. The retention fraction was easy to be washed out and pumped into the analytical column for analysis in the second dimension. In this alternative mode, on-line enrichment of retentive components could be achieved, and the sensibility was improved correspondingly.

Dasatinib standard solution was used to verify the ability of the EGFR/CMC-online-HPLC/MS system to "recognize" and identify target components. As shown in Figure 2, the retention time of dasatinib was 11.5 min, the selected fraction R_1 (between the two dotted lines) was extracted onto EC_1, and then switched into C_2 for chromatographic separation and MS identification (Figure 2(B)). Data from the second dimensional chromatography and mass spectrometry confirmed that R_1 was dasatinib. The repeatability and detection limit were studied as well. Intraday and interday accuracy were studied by repeated injection of 5 μL 0.1 μg/mL dasatinib standard solution using the retention time as indication. The result was 0.58% and 2.44% ($n = 5$), respectively. The detection limit was 2 ng (S/N = 3).

Dasatinib, which is a micromolecular EGFR-specified antagonist, exhibited great retention in this system. Because dasatinib could bind to EGFR receptors which are abundant in CMC stationary phase, it can be recognized and retained by EGFR/CMC. Accordingly, this system is suitable for recognition, analysis and identification of active components which interact with EGFR.

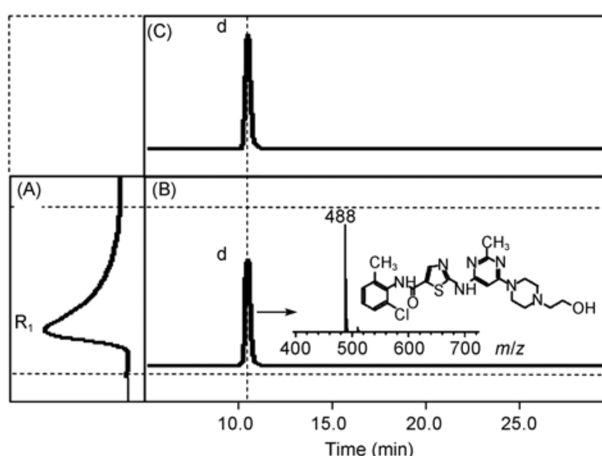

Figure 2 Chromatograms of dasatinib using an EGFR/CMC-online-HPLC/MS method. (A) EGFR/CMC chromatogram of dasatinib; (B) HPLC/MS chromatogram of the corresponding fraction (between two dotted lines in A_{431}/CMC chromatogram) extracted onto the PC_1, and identified as dasatinib; (C) HPLC/MS chromatogram of dasatinib standard solution.

3.2 Screening target components from *Radix Angelicae Pubescentis*

EGFR/CMC-HPLC/MS was applied for screening anti-EGFR antagonists from TCM *Radix Angelicae Pubescentis*. Chromatograms of the *Radix Angelicae Pubescentis* extract obtained using the EGFR/CMC-online-HPLC/MS method are shown in Figure 3, in which R_0 was non-retentive fraction and R_1 was retentive fraction. Each fraction was assessed using the HPLC/MS system online for further separation and identification. As shown in Figure 3(B), the peak R_{1-1}, a main component of the R_2 fraction, was identified as osthole. Compared with Figure 3(C), osthole can be identified as from the *Radix Angelicae Pubescentis* extract under the same chromatographic condition. Peak R_{1-1} was assigned to osthole in the chromatography of *Radix Angelicae Pubescentis* extract.

To further verify the screening results above, the standard solution of osthole was analyzed using the EGFR/CMC-online-HPLC/MS method. Figure 4 shows the chromatography of osthole standard solution in EGFR/CMC system with the retention time of 4.8 min. The retention fraction R_1 was enriched and pumped into HPLC/MS system for further analysis (Figure 4(B)). Data from the second dimension and MS indicated R_1 was osthole.

The results demonstrated great retention of osthole in this system, which indicated the ability to interact with EGFR.

3.3 Correlation with pharmacological effect

Inhibitory effect on cell growth: The stationary phase of EGFR/CMC is the membrane with a high level of EGFR, thus EGFR antagonist dasatinib could be selectively recognized. As shown in Figure 2(A), dasatinib demonstrated significant retention activity in the EGFR/CMC model, and the retention fraction was assessed using the HPLC/MS system online for further separation and MS identification (Figure 2(B)). Osthole, which can interact with EGFR, was discovered from *Radix Angelicae Pubescentis* by using this EGFR/CMC-online-HPLC/MS method. To further verify the activity of osthole, cell growth of HEK293/EGFR cell and expression of EGFR were studied using MTT method.

Inhibitory effect on the expression of EGFR: As shown in Figure 5, osthole and dasatinib have inhibited HEK293/EGFR cell growth in a dose-dependent manner. The expression of EGFR protein in HEK293/EGFR cell was inhibited by osthole, which was not as effective as dasatinib, too. The retention time of dasatinib on EGFR/CMC was 11.5 min, compared with 4.8 min of osthole, which indicated a stronger interaction of dasatinib with EGFR. The retention behaviors of the two were correlated to their pharmacological activities.

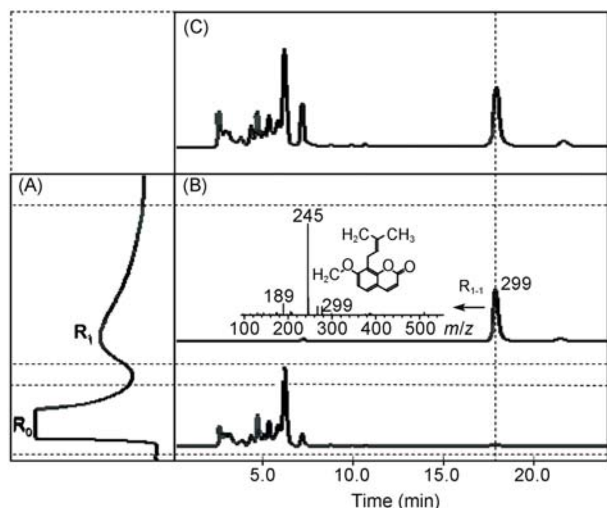

Figure 3 Chromatograms of *Radix Angelicae Pubescentis* extract using an EGFR/CMC-online-HPLC/MS method. (A) EGFR/CMC chromatogram of *Radix Angelicae Pubescentis* extract including R_0 and R_1 fractions (between two dotted lines); (B) HPLC/MS chromatogram of R_0 fraction with a main retention peak (R_1) identified as osthole; (C) HPLC/MS chromatogram of *Radix Angelicae Pubescentis* extract.

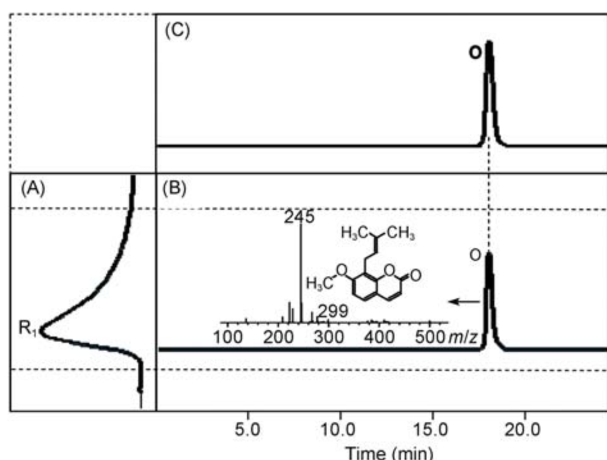

Figure 4 Chromatograms of the osthole standard solution using an EGFR/ CMC-online-HPLC/MS method. (A) EGFR/CMC chromatogram of the osthole standard solution including R_1 fraction (between two dotted lines); (B) HPLC/MS chromatogram of R_1 fraction with solely one retention peak (O) identified as osthole; (C) HPLC/MS chromatogram of the osthole standard solution.

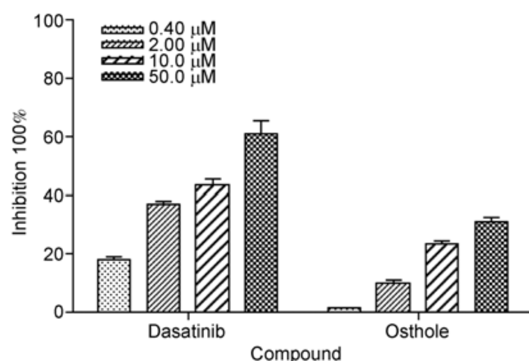

Figure 5 The inhibitory effects of osthole on HEK293 EGFR cell growth.

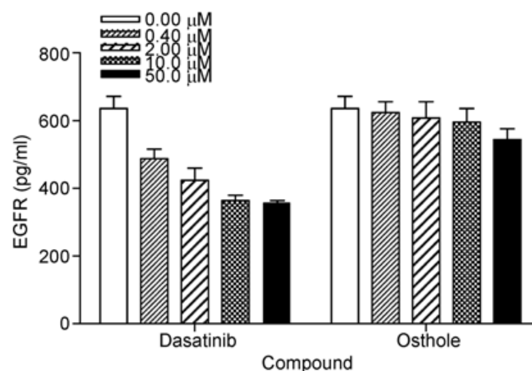

Figure 6 The inhibitory effects of osthole on EGFR secretion.

4 Conclusions

In summary, we have for the first time established a new EGFR/CMC-online-HPLC/MS method for screening EGFR antagonists from a complex system. The selectivity, sensibility and specificity were increased. This method can efficiently drive the screening process by combining specific recognition via the EGFR/CMC model with accurate identification via the HPLC/MS online system, which improved the automatic screening process. It will be a useful method in drug discovery with natural medicinal herbs as leading compounds.

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