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Screening of epidermal growth factor receptor inhibitors in natural products by capillary electrophoresis combined with high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method for screening of inhibitors to epidermal growth factor receptor (EGFR) in natural product extracts with capillary electrophoresis (CE) in conjunction with high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is reported. The method was established by employing 5-carboxyfluorescein labeled substrate peptide, two commercially available EGFR inhibitors OSI-744 and ZD1839, and a small chemical library consisted of 39 natural product extracts derived from the Traditional Chinese Medicines. Biochemical assay of crude natural product extracts was carried out by using CE equipped with a laser induced fluorescence detector. The CE separation allowed an accurately quantitative measurement of the phosphorylated product, hence the measurement of the enzymatic activity as well as the inhibition kinetics. The hits are identified if the peak area of the phosphorylated product is reduced in comparison with the negative control. The active constituents in the natural product extract were then identified by an assay-guided isolation with HPLC–MS/MS system. With the method, the flavonoids component of the *Lycopus lucidus* extract, namely quercetin and rutin were identified to be the active ingredients. Their IC_{50} values were determined as 0.88 μM and 10.1 μM, respectively. This result demonstrated a significant merit of our method in the identification of the bioactive compounds in natural products.

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1. Introduction

The epidermal growth factor receptor (EGFR) is a member of ErbB family of receptors tyrosine kinases [1,2]. Upon binding with epidermal growth factor [1], EGFR is induced to form homodimer, and subsequently its intrinsic tyrosine kinase domain is activated to initiate the intracellular signaling transduction [3]. It has been discovered that dysregulations of EGFR (overexpression) associates with the pathogenesis and the progression of different carcinoma types [4,5]. EGFR inhibitors, such as gefitinib and erotinib have been clinically used for cancer treatment [6–8]. However, the clinical efficacy of EGFR inhibitors in EGFR-mutant non-small-cell lung cancer (NSCLC) is limited by the development of drug-resistance mutations [9–11]. Therefore, to find novel mutant-selective EGFR inhibitors for developing new drugs for treatment of cancer is urgently needed [12,13].

The rapidly growing interest in protein kinase drug discovery has prompted the development of numerous kinase assay technologies, which can be currently classified into three categories: radiometric assays, phospho-antibody-dependent fluorescence/luminescence assays, phospho-antibody independent fluorescence/luminescence assays [14–17]. Although most of these technologies are amenable to the high throughput screening, a few of them are able to utilize for kinetic and mechanistic studies. Further, these methods are labor-intensive, time-consuming, high background and low signal to noise ratio. Due to various limitations of each technique, none of them can be truly universal [14]. Therefore, the universal assay technologies free of radioisotopes and custom reagents such as phospho-specific antibodies are badly needed.

Fortunately, capillary electrophoresis (CE) may represent relatively ideal technology which has been utilized for enzyme inhibitor screening [18–24]. Synthetic peptides with specific

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sequences are commonly used to as the substrates for protein kinase inhibitors screening. And CE has proven to be a versatile tool for peptide separation [25,26]. The CE-based enzyme assay method has a unique advantage to produce the high quality assay data. This is because the biochemical assay is integrated into the separation process of CE to avoid the detective interference from the substrate as well as from the complex sample matrix. Moreover, the peak areas of the enzyme reaction in product can be accurately measured, hence the highly accurate measurement of enzymatic inhibition and the inhibition kinetics [27–29]. CE-based screening method has several other advantages, such as minute requirement of reagents and test compounds, automation, and short analysis time.

Natural products and their derivatives have long been used as the most productive resources of new drug discovery because of their great diversity of the chemical structures and better drug-like properties compared to the synthetic compounds. About 60% and 70% of anti-cancer and anti-infection drugs originated from the natural resources [31]. However, in the past decade, research into the natural products has declined in the pharmaceutical industry [32,33]. This is because all the high-throughput screening technologies require the pure compounds [34]. To purify the natural compounds is a time-consuming and laborious process.

Most recently, we proposed a strategy for simplifying the process of screening of bioactive compounds in nature products [23,30,35]. The crude extract of natural product can be directly assayed by CE, meantime, HPLC is utilized as a high throughput purification platform to provide the purified compounds for tracking the active components. Meanwhile, the structure elucidation of the components can be feasibly performed by HPLC–MS/MS analysis.

Here, we further extend our drug discovery strategy for screening of EGFR inhibitors in natural products. An effective and robust CE-based enzyme assay with laser-induced fluorescence detection was developed to screen 39 natural extracts derived from the Traditional Chinese Medicines. The extract from *Lycopus lucidus* was identified to be active. In combination with HPLC–MS/MS, the component of the extract flavonoids, namely quercetin and rutin were verified to be the active ingredients.

2. Experimental

2.1. Reagents and chemicals

Recombinant EGFR (695-end) was purchased from SignalChem (Richmond, Canada). Adenosine 5′-triphosphate disodium salt (ATP), sodium fluorescein, dithiothreitol (DTT), β-glycerol-phosphate disodium, 3-morpholinopropanesulfoinc acid (MOPS), bovine serum albumin (BSA), boric acid, dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Steinheim, Germany). $MnCl_2.4H_2O$, $MgCl_2$, ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), NaCl and NaOH were from Aladdin Reagent (Shanghai, China). The gefitinib (ZD1839) and erlotinib (OSI-744) were purchased from Selleckchem Chemicals (Houston, TX, USA). 5-Carboxyfluorescein-labeled peptide (Ala-Asp-Glu-Tyr-Leu-Ile-Pro-Gln-Gln) (F-EGFR) was obtained from Anaspec (San Jose, CA, USA). The peptide was designed according to the C-terminal sequence from amino acid residue 889–997 which forms the EGFR autophosphorylation site. Therefore, the peptide is denoted F-EGFR, and its phosphorylated product is denoted pF-EGFR.

The CE running buffer was composed of 200 mM boric acid buffer (adjusted to pH 9.0 with NaOH solution). The EGFR solution (10 μg/mL) was prepared in 25 mM MOPS (pH 7.2) containing 12.5 mM β-glycerol-phosphate, 20 mM $MgCl_2$, 12.5 mM $MnCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT and 50 μg/mL BSA. The substrate solution was prepared by dissolving a certain amount of F-EGFR, ATP and sodium fluorescein in 25 mM MOPS to give the final concentrations of each component: 10 μM F-EGFR, 100 μM ATP and 1×10^{-7} M sodium fluorescein. Sodium fluorescein was employed as an internal standard for correcting the variation of the injection volume. All solutions were freshly prepared in each day.

All natural product extracts listed in Table 1 were prepared from herbs. Briefly, the herbs were ground into a fine powder, and then ultrasonically extracted with 70% (v/v) ethanol for three times. After filtration and removal of the solvent by rotary evaporation, the natural compound extracts were obtained. Quercetin and rutin were purchased from Yuanye Biological (Shanghai, China).

Table 1
A library consisted of natural compound extract for inhibitor screening.

Sample	Inhibition (%)	Sample	Inhibition (%)
Radix ophiopogonis	0	*Rhizoma curcumae*	0
Flos carthami	0	*Fructus aurantii immaturus*	0
Semen persicae	0	*Rhizoma chuanxiong*	0
Fructus forsythia	0	*Radix bupleuri*	0
Radix notoginseng	0	*Radix achyranthis bidentatae*	0
Fructus gardeniae	0	*Reed rhizome*	0
Rhizoma corydalis	0	*Schisandra*	0
Ramulus cinnamomi	0	*Cortex Phellodendri*	0
Rhizoma coptidis	0	*Lignum dalbergiae odoriferae*	0
Radix ginseng rubra	0	*Rheum palmatum*	0
Radix salviae miltiorrhizae	0	*Rhizoma Anemarrhenae*	0
Radix scutellariae	0	*Coix Seed*	0
Radix angelicae sinensis	0	*Honeylocust*	0
Thunder Gold Vine	0	*Radix paeoniae rubra*	0
Rhizoma fagopyri dibotryis	0	*Rhizoma Acori Tatarinowii*	0
Herba hedyotis diffusae	0	*Radix platycodi*	0
Herba scutellariae babratae	0	*Radix Rehmanniae*	0
Semen ziziphi spinosae	0	*Cortex cinnamomi*	0
Radix puerariae	0	*Lycopus lucidus*	76
Spica prunellae	0	OSI-744	91

The concentration of OSI-744 was 100 nM, the concentration of natural product extracts were 0.5 mg/mL.

2.2. Instrumentation

2.2.1. Capillary electrophoresis

All CE experiments were performed on a P/ACE MDQ CE system (Beckman Coulter, CA, USA) equipped with laser-induced fluorescence detector. A 488 nm semiconductor laser was used as an excitation source, and the emission of fluorescence was monitored at 520 nm. The CE separations were performed on a fused silica capillary with a dimension of 50 μm I.D. (370 μm O.D.) and a total length of 31 cm (effective length of 20.5 cm) (Polymicro Technologies, Phoenix, AZ, USA).

A new capillary was pretreated by flushing 0.1 M NaOH for 30 min, followed by flushing with the deionized water and separation buffer under a pressure of 0.21 MPa for 5 min each. Between two runs, the capillary was rinsed sequentially with 1 M NaCl, 0.1 M NaOH, deionized water, and the running buffer at a pressure of 0.21 MPa for 1 min each. The samples were injected by 1379 Pa for 3 s from the inlet of the capillary. A voltage of 15 kV was applied to separate pF-EGFR from F-EGFR and the sodium fluorescein (internal standard). The temperature of the capillary cartridge was set as 25 °C.

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