Journal of Chromatography A, 1388 (2015) 267–273

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Screening of mammalian target of rapamycin inhibitors in natural product extracts by capillary electrophoresis in combination with high performance liquid chromatography–tandem mass spectrometry

Yanmei Zhang[a], Feng Li[a], Mingxia Li[b], Jingwu Kang[a,b,*]

[a] Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, State Key Laboratory of Bio-organic and Natural Products Chemistry, Lingling Road 345, Shanghai 200032, China
[b] ShanghaiTech University, Yueyang Road 319, Shanghai 200031, China

ARTICLE INFO

Article history:
Received 29 December 2014
Received in revised form 9 February 2015
Accepted 9 February 2015
Available online 16 February 2015

Dedicated to Prof. Weile Yu on the occasion of her 90th birthday.

Keywords:
mTOR inhibitor
Natural products
CE
HPLC–MS/MS

ABSTRACT

In this study, capillary electrophoresis (CE) combined with HPLC–MS/MS were used as a powerful platform for screening of inhibitors of mammalian target of rapamycin (mTOR) in natural product extracts. The screening system has been established by using 5-carboxyfluorescein labeled substrate peptide F-4EBP1, a known mTOR inhibitor AZD8055, and a small chemical library consisted of 18 natural product extracts. Biochemical screening of natural product extracts was performed by CE with laser induced fluorescence detection. The CE separation allowed a quantitative measurement of the phosphorylated product, hence the quantitation of enzymatic inhibition as well as inhibition kinetics. The hits are readily identified as long as the peak area of the phosphorylated product is reduced in comparison with the negative control. Subsequent assay-guided isolation of the active natural product extract was performed with HPLC–MS/MS to track the particular active components. The structures of the identified active components were elucidated by the molecular ions and fragmentaion information provided by MS/MS analysis. The CE-based assay method only requires minute pure compounds, which can be readily purified by HPLC. Therefore, the combination of CE and HPLC–MS/MS provides a high-throughput platform for screening bioactive compounds from the crude nature extracts. By taking the advantage of the screening system, salvianolic acid A and C in extract of Salvia miltiorrhiza were discovered as the new mTOR inhibitors.

1. Introduction

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, belongs to phosphatidylinositol-3 kinase (PI3K) related protein kinases (PIKK) family [1,2]. It forms two structurally and functionally distinct multiprotein complexes called mTORC1 and mTORC2. mTOR regulates cellular metabolism, growth, and proliferation. Dysregulated mTOR is often in the onset and progression of diseases, such as diabetes, cancer, neurological diseases and inflammations [3,4]. Therefore mTOR is becoming a target of drug discovery for developing potential molecularly targeted therapeutic agents [5,6]. The most established mTOR inhibitors are so-called rapalogs (rapamycin and its analogs), which show antitumor responses in clinical trials against various tumor types [7–10]. However, they could potentially activate the survival pathway PI3K/Akt that may lead to treatment failure [9]. By contrast, the second generation of mTOR inhibitors, which compete with ATP in the catalytic site, would inhibit all of the kinase-dependent functions of mTOR without activating the survival pathway. So far, several mTOR inhibitors have already entered the clinical trials [11]. These achievements excite great passion to discover more mTOR inhibitors for developing potential anticancer agents with a better efficacy and selectivity.

Currently, a commercially available assay kit based on time-resolved fluorescence resonance energy transfer (TR-FRET) is frequently used for screening of mTOR inhibitors. Other methods based on fluorescence immunoassay or chemiluminescence ELISA have been reported recently [12–14]. However, radioactive ATP or phosphorylation-specific antibodies are required in the methods, and they are expensive while only semi-quantitative data can be obtained. Therefore, there is a current and increasing demand for simple, robust, nonradiactive assay methods for discovering mTOR inhibitors.

Natural products and their derivatives have long been used as the most productive sources of new drug discovery because of their great diversity of the chemical structures and better drug-like

* Corresponding author. Tel.: +86 21 54925385; fax: +86 21 54925481.
E-mail address: jingwu.kang@sioc.ac.cn (J. Kang).

http://dx.doi.org/10.1016/j.chroma.2015.02.033
0021-9673/

properties compared to the synthetic compounds. About 60% and 75% of anti-cancer and anti-infection drugs originated from natural sources [15]. Recently, a number of inhibitors towards PI3K/Akt/mTOR have been found in natural products, and some of them exhibit potent anticancer activities [16]. However, in the past decade, research into the natural products has declined in the pharmaceutical industry [17–19]. This probably is because almost all the high-throughput screening techniques require the pure compounds, while purification of natural compounds is a time-consuming and laborious process. Therefore, methods used for directly screening unfractionated natural product extracts will be greatly helpful to renew the interest of utilizing natural products for drug discovery.

Capillary electrophoresis (CE) represents a promising tool for such a purpose [20–27]. This is because CE-based method can integrate the biochemical assay into a separation process to avoid the interference from the complex sample matrix. The separation and detection of specific substrate peptide and product peptide could be achieved by CE-based method [28,29]. Moreover, CE-based screening method has several other advantages, such as minute requirement of reagents and test compounds, automation, and short analysis time [30–33]. To date, several CE methods have been reported for studying kinase inhibition or screening of kinase inhibitors [14,21,34–36]. Few papers dealing with evaluation of the inhibitors towards the PI3K/Akt/mTOR signaling pathway has been reported as well [14].

In our previous work, we proposed a strategy for screening and identification of PKA inhibitors in crude natural compound extracts [21]. We describe here our further effort to extend the strategy to discover mTOR inhibitors in natural compound extracts. mTORC1, 5-carboxyfluorescein labeled substrate peptide, a known mTOR inhibitor AZD8055, as well as a small natural product library consisting of 18 natural compound extracts were used to establish the screening system. By taking the advantage of the strategy, salvianolic acid A and C in extract of *Salvia miltiorrhiza* were identified as the new mTOR inhibitors.

2. Experimental

2.1. Reagents and chemicals

mTORC1 (mTOR/Raptor/MLST8 human, 0.27 mg/mL), adenosine 5′-triphosphate disodium salt (ATP), Tween 20, dithiothreitol (DTT) and N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid (Hepes), sodium fluorescein, and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (Steinheim, Germany). $MnCl_2{\cdot}4H_2O$, ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), and NaOH were obtained from Aladdin Reagent (Shanghai, China). (5-(2,4-bis((S)-3-methylmorpholino)pyrido[2,3-d]pyrimidin-7-yl)-2-methoxyphenyl)methanol (AZD8055) was purchased from Selleckchem Chemicals (Houston, TX, USA). 5-Carboxyfluorescein-labeled peptide (5-FAM-Ser-Thr-Thr-Pro-Gly-Gly-Thr-Leu-Phe-Ser-Thr-Thr-Pro-Gly) was synthesized by GL Biochem (Shanghai, China) and further purified by reversed-phase HPLC. The peptide was denoted F-4EBP1, which is named after eukaryotic initiation factor 4E-binding protein-1 (4EBP1), the downstream substrate protein of mTORC1 in the translation control pathway [37]. The peptide was designed according to the fragment of 4EBP1 in amino acid sequence from residues 35 to 48 [38]. Its phosphorylated product was denoted pF-4EBP1.

The CE running buffer was composed of 100 mM Hepes buffer (pH was adjusted to 7.5 with NaOH solution). The mTOR/Raptor/MLST8 solution (18 μg/mL) was prepared with 50 mM Hepes buffer (pH 7.5) containing 1 mM EGTA, 3 mM $MnCl_2$, 10 mM $MgCl_2$, 2 mM DTT and 0.01% (v/v) Tween 20. The substrate solution was prepared by mixing a certain amount of F-4EBP1, ATP and sodium fluorescein in 50 mM Hepes buffer (pH 7.5) to give the final concentrations of each component: 4 μM F-4EBP1, 400 μM ATP and 1×10^{-7} sodium fluorescein. All solutions were freshly prepared in each day.

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

Sample	Inhibition (%)	Sample	Inhibition (%)
Semen coicis	0	*Corydalis tuber*	0
Orange shell	0	*Panax pseudoginseng*	0
Peach kernel	0	*Liquoric root*	0
Thunder God Vine	0	*Radix ginseng rubra*	0
Radix astragali	0	*Bidentate achyranthes*	0
Radix et rhizoma rhei	0	*Rhizoma coptidis*	0
Radix ophiopogonis	0	*Notoginseng triterpenes*	0
Acorus gramineus	0	*Radix salviae miltiorrhizae*	100
Platycodon grandiflorus	0	AZD8055	94
Radix angelicae sinensis	0		

The concentration of AZD8055 was 125 nM, the concentration of natural product extracts were 0.125 mg/mL.

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

2.2. Instrumentation

2.2.1. Capillary electrophoresis

All CE experiments were carried out on a P/ACE MDQ CE system equipped with laser-induced fluorescence (LIF) detection (Beckman Coulter, CA, USA). 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).

Before use, a new capillary was pretreated by 0.1 M NaOH for 30 min, followed by flushing with 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 0.1 M NaOH, deionized water, and the running buffer at pressure of 0.21 MPa for 1 min each. The assays were conducted using the short-end injection technique, that is, samples were injected by a pressure of 1379 Pa for 5 s from the outlet of the capillary to pursue a faster separation. The capillary length from the outlet to the detection window was 10.5 cm. A voltage of −15 kV was applied to separate pF-4EBP1 from F-4EBP1 and the sodium fluorescein (internal standard). The temperature of the capillary cartridge was set at 25 °C.

2.2.2. HPLC–FLD–MS/MS

The enzyme reaction was monitored by HPLC equipped with dual detectors: fluorescence detection (FLD) and LCQ-Fleet ion-trap mass spectrometer (Thermo Scientific, CA, USA). The separations were carried out on an Agilent ZORBAX Eclipse XDB-C18 reversed-phase column (2.1 mm × 150 mm, 3.5 μm, 80 Å), which was protected with a guard column. The column temperature was maintained at 30 °C. The FLD was set with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Water containing 0.1% (v/v) HCOOH and acetonitrile containing 0.1% (v/v) HCOOH were used as solvent A and B, respectively. A gradient elution program was applied as follows: 20–40% B over 20 min, 40–100% B over 5 min, and finally keeping 100% B constant for 5 min; flow rate, 0.3 mL/min. The sample volume