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Rapid screening and identification of active ingredients in licorice extract interacting with V3 loop region of HIV-1 gp120 using ACE and CE-MS

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ABSTRACT

The binding of envelope protein gp120 to glycosphingolipids is very important during the human immunodeficiency virus entering into the host cell. This step occurs in the V3 loop region in particularly. The conserved core sequence of V3 loop in gp120 was named R15K. Anti-HIV drug targeting to R15K would avoid the drug-resistance caused by HIV-1 genetic diversity. Here, for the first time, affinity capillary electrophoresis (ACE) and capillary electrophoresis-mass spectrometry (CE-MS) were used for establishing a simple, rapid and effective method of screening the licorice extract for biological activity (anti-HIV), which avoided the complicated isolation and purification process. R15K, 3′-sialyllactose (the positive control), and D-galactose (the negative control) were used for the development and validation of ACE method. After the interaction between licorice extract and R15K was confirmed by ACE, the relative active ingredients were isolated by SPE and their structures were determined by CE-ESI-MS online. In this research, two mixtures from licorice extract were found to be active. Furthermore, glycyrrhizin and licorice saponin G2 were verified as the main ingredients that significantly interacted with R15K via CE-MS and LC–MS. The results of quantitative assays showed that the active mixture contained glycyrrhizin of 74.23% and licorice saponin G2 of 9.52%. Calculated by Scatchard analysis method, glycyrrhizin/R15K complex had the highest binding constant $(1.69 \pm 0.08) \times 10^7$ L/mol among 27 compounds isolated from licorice extract. The anti-HIV activity of glycyrrhizin was further confirmed by bioactive experiment of cellular level. This strategy might provide a high throughput screening and identifying platform for seeking HIV-1 inhibitors in natural products.

1. Introduction

HIV virions bind to particular cell surface receptors to gain access into target cells. The envelope protein of HIV (Env), which acts as indispensible role during the binding process, composed of a transmembrane (gp41) and an outer (gp120) subunit. [1–4]. Apart from these well-defined receptors and coreceptors, a variety of glycosphingolipids (GSL) involving galactosyl ceramide (GalCer) and a monoganglioside GM3 have been shown to regulate Env-mediated fusion [5–7]. It has been investigated that glycolipids GalCer and GM3 interacted with HIV gp120 through a specific portion of the V3 loop region (Fig. 1a) [8,9]. The fusion process cannot start without the V3 loop-binding step. RIQRGPGRAFVTIGK, a synthetic V3 loop peptide, corresponding to the glycolipid binding domain of the V3 loop of HIV-1 gp120, can be used for binding studies instead of the recombinant gp120 molecule. Gangliosides, the membrane constituents of all the types of cells, which could bind to R15K, consist of a double chain hydrophobic part (ceramide) within the membrane and a hydrophilic head called 3′-sialyllactose (structure is shown in Fig. 1b) [10]. 3′-Sialyllactose is located outside the cell membrane which could bind to R15K. The highly conserved sequences we chose for target, rather than other parts of the envelop proteins or the important enzymes of HIV-1, can avoid the problem of drug resistance owing to the high degree of HIV-1 genetic diversity, which attributed to a rapid rate of viral replication combined with the error-prone nature of reverse transcriptase (RT) and frequent recombination events [11].

Derived from the dried Radix or rhizome of *Glycyrrhiza uralensis* Fisch.ex DC, *Glycyrrhiza inflate* Batalin, *Glycyrrhiza glabra* L. is a kind of popular Chinese herbal medicine. The main bioactive

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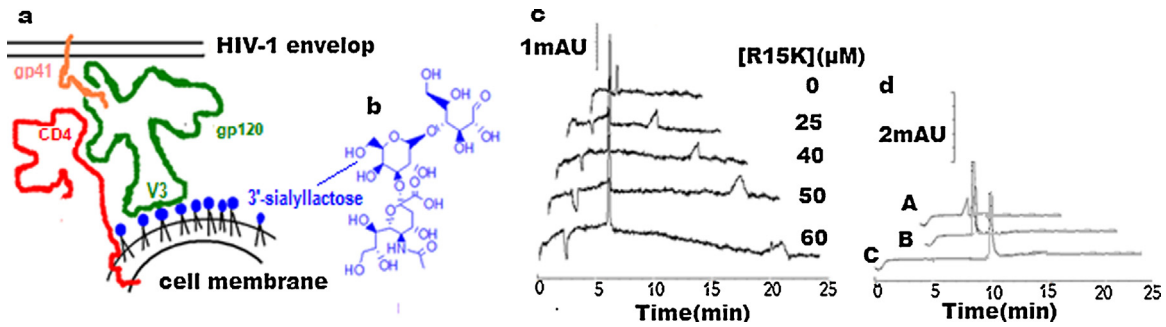

Fig. 1. (a) The binding of the V3 loop of gp120 to GM3 and (b) the structure of 3′-sialyllactose. 3′-sialyllactose is the hydrophilic head of GM3. The binding allows the fusion peptide of gp41 to penetrate the cellular membrane. Electropherograms of (c) positive control 3′-sialyllactose and (d) negative control D-galactose under different concentrations of R15K in the running buffer. The concentration of 3′-sialyllactose was 250 μM. (A) 250 μM D-galactose was injected into running buffer A. (B) 250 μM D-galactose with 0.1% DMSO was injected into running buffer A. (C) 250 μM D-galactose with 0.1% DMSO was injected into running buffer A with 60 μM R15K. The conditions used were as follows: Beckman P/ACE MDQ capillary electrophoresis system. Injection: 0.5 p.s.i. for 5 s. Applied voltage: 15 kV. Capillary: capillary of 30.2 cm (effective length 20 cm) 75 μm I.D. Running buffer: 30 mM Tris–acetic acid, pH 7.45. Observation by wavelength 214 nm.

constituents of licorice are triterpene saponins and various types of flavonoids. Licorice shows a variety of pharmacological activities, including antiviral, anti-inflammatory, antiulcer, antispasmodic, antioxidative, antiallergic, anticancer and memory-enhancing activities [12–14]. Glycyrrhizin (the major component of licorice) and its derivatives have been reported to inhibit infection of HIV, SARS, Hepatitis B and C, and influenza. Glycyrrhizin is a kind of glycoside, occurring as a mixture of calcium, sodium and potassium salts of glycyrrhizinic acid (also named glycyrrhizic acid). The bioactivity of glycyrrhizic may attribute to its interference with virus-cell binding [15–18]. To screen the potential HIV-1 entry inhibitors in natural products, we investigated the interactions between licorice extract and R15K.

The interactions between small ligands and biomolecules have been studied by means of nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), mass spectroscopy (MS), ultracentrifugation, fluorescence chromatography and capillary electrophoresis. Among the methods above, capillary electrophoresis (CE) achieves remarkable advantages including low sample consumption (nanogram), relatively short analysis time and high efficiency [19,20]. During the past decade, affinity capillary electrophoresis (ACE) has emerged as a rapid and sensitive technique for studying a variety of interactions including protein–protein, protein–DNA, protein–drug, protein–carbohydrate, peptide–peptide, peptide–carbohydrate and carbohydrate–drug [21,22]. In ACE, the binding constants could be determined from the variation of mobility shifts of a sample as a role of additive concentrations in the running buffer [23]. CE-ESI-MS allows the online structural determination of the active ingredients and characterization of complex formations. Here, the interaction between licorice extract and R15K was investigated by ACE for the first time. After the active ingredients were found, solid phase extraction (SPE) technique was applied to isolation and purification of the active ingredients. To identify the structures of the active ingredients, CE-ESI-MS and LC-ESI-MS were performed. The main ingredients of the active mixture in licorice were separated and the quantitative analyses of two main ingredients were performed by CE. The interactions between the 27 compounds isolated from licorice extract were also investigated by ACE. This method avoided the isolation process before drug screening and provided a rapid and convenient platform of seeking HIV-1 entry inhibitors in natural extracts.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade unless otherwise indicated. Tris base (ultrapure), ammonium acetate, acetic acid, boric acid and sodium dodecyl sulfate (SDS) were purchased from Beijing Chemical Reagent Factory (Beijing, China). The deionized water was derived from a Millipore Milli Q-Plus system (Massachusetts, USA). Methanol (HPLC grade) was purchased from Merck Corporation (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was obtained from Sinopharm Chemical Reagent (Shanghai, China). A total of 30 mM Tris–HAc, served as running buffer A, was prepared by dissolving 0.9108 g Tris in 250 mL deionized water and adjusted to pH 7.45 at 25 °C using diluted acetic acid. 50 mM boric acid buffer at pH 9 containing 40 mM SDS was applied as buffer B. All CE solutions were filtered through 0.45 μm membranes (Agilent, Waldbronn, Germany) before use.

The peptide, corresponding to the R15K, was synthesized by Chinese Peptide in Hangzhou (China), subsequently purified (purity ≥ 95.8%) and characterized by RP-HPLC and MS. The synthesized amino acid sequence R15K was Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys. 3′-Sialyllactose (the soluble GM3 carbohydrate head) was purchased from Carbosynth Company (Berkshire, United Kingdom). D-galactose was purchased from Acros Organics Company (New Jersey, USA). Chinese herbal medicine licorice and 27 compounds isolated from licorice extract (purity ≥ 95%) (structures shown in Table 1) were provided by professor Min Ye (Peking University). The stock solution of R15K, prepared by dissolving the R15K in 30 mM Tris–HAc (buffer A) to the concentration of 2 mM, was diluted to concentrations required by adding appropriate amounts of buffer A. 3′-Sialyllactose, D-galactose and 27 compounds isolated from licorice extract were dissolved in methanol (10 mM) as stock solution. The methanol was evaporated under nitrogen stream before being redissolved by adding appropriate buffer A.

2.2. Apparatus

The experiments were performed on a Beckman P/ACE™ MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a photodiode array detector as well as the 32 Karat™ software version 5.0 (Fullerton, CA, Beckman). The CE-ESI-MS detection was applied on the coupling of PA800 plus CE system (Beckman Coulter, Brea, CA, USA) and Agilent 6320 ion trap Mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The bare fused silica capillaries (365 μm O.D.; 75 μm I.D.), used for affinity capillary electrophoresis, were purchased from Yongnian Optical Fibers (Hebei, China). The bare fused-silica capillaries (365 μm O.D.; 50 μm I.D.), used for CE-MS, were obtained from Beckman Coulter (Brea, CA, USA).