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An analysis method for simultaneous screening of deoxyribonucleic acid-binding active compounds and investigating their mechanisms by ultra-fast liquid chromatography tandem mass spectrometry coupled with fluorescence detection technology

Cangman Zhang[a,1], Biao Ren[a,1], Zongtao Lin[b], Lingkun Tong[a], Hong Wang[a,*], Shizhong Chen[a,*]

[a] School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
[b] Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, TN 38163, USA

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ABSTRACT

DNA has been known as the cellular target for many cytotoxic anticancer agents over the years. Discovering DNA-binding compounds has become an active research area, while various DNA-binding mechanisms make the drug discovery even more difficult. In this article, we present a novel analysis method to rapidly identify specific DNA-binding compounds from *Pyrrosia lingua* (*Thunb.*) using DNA-dual-fluorescent probes, ethidium bromide and Hoechst 33258, with the technology of ultra-fast liquid chromatography-diode array detector-tandem mass spectrometry and dual-wavelength fluorescence detector (UFLC-DAD-MS^n-DFLD). Sixty-two compounds were identified, of which 22 were found to be active in DNA-binding. After investigation of their dose-response behaviors and structure–activity relationships, chlorogenic acids and flavonoid glycosides were found to be DNA-binders *via* both minor groove-binding and intercalation modes. The precision, reproducibility and stability of this method were validated by vitexin. The established system was sensitive, precise, and reliable to be used for both screening of DNA-binding compounds and investigating of their mechanisms.

1. Introduction

Interaction of small molecules with DNA has been studied for decades. The mode of DNA-drug interactions can be classified into two major categories, intercalation and groove binding due to the relative planar stacking of aromatic bases along the helix sugar phosphate backbone [1,2]. Some compounds take advantage of both modes by possessing intercalative unit as well as the groove-binding side chain. Intercalators, with fused bi/tricyclic ring structures [3], had been generally considered to be the result of planar ring stacking with base pairs and hydrophobic interaction [4]. Groove binders were usually narrow curved shaped (crescent-shaped) and consists of flat hydrophobic aromatic rings, creating a snug fit between the rather hydrophobic sugar walls in the minor groove [5,6]. They were stabilized by forming hydrogen bonds to bases, typically to N_3 of adenine and O_2 of thymine [7]. Groove binding typically results in structural alternations in DNA grooves, while intercalation results in a substantial change in DNA structure. It is generally believed that these DNA-binding compounds can affect the replication, transcription and repair processes [8]. Thus understanding the mode of DNA-drug interactions is helpful to screen DNA interfering drugs *in vitro*.

Various techniques were used to screening DNA-binding compounds. They included X-ray diffraction [9], viscosity measurement [10], nuclear magnetic resonance (NMR) [11] and spectrometric methods including UV spectra, fluorescence spectra and circular dichroism [7,10]. Other techniques were electrochemical measurements including cyclic and differential pulse voltammetry, DNA melting studies [12], and electrospray ionization mass spectrometry (ESI-MS)[13]. Among these methods, spectrophotometric ones are common and convincing [11]. Traditional pharmacological methods for the discovery of DNA-binding compounds from

* Corresponding authors at: School of Pharmaceutical Sciences, Peking University, No. 38 Xueyuan Road, Beijing 100191, China. Tel.: +86 010 82802723; fax: +86 010 82802723.
E-mail addresses: zcm899@163.com (C. Zhang), hw9505@bjmu.edu.cn (H. Wang), chenshizhong66@163.com (S. Chen).
[1] These authors contributed equally to this work.

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natural products and distinguish intercalation and groove binding modes are of high cost, long cycle, more intensive labor which can sometimes result in false positives or lose active ingredients during the pretreatment process [14]. Recently developed liquid chromatography–mass spectrometry-fluorescence detection techniques make it possible to simultaneously identify multiple active ingredients from the traditional Chinese medicines (TCMs) without purification and activity assay of individual compound [15,16]. However, single fluorescent probe was used to screen active compounds and determine their mode of action with DNA which may lead to one-side conclusion or losing active ingredients.

In this study, two fluorescent probes have been adopted. Ethidium bromide (EB), a standard intercalator, has been extensively studied on its binding to DNA. Hoechst 33258 (H258) is a well-known groove binding agent [5,8,14]. The fluorescence intensity of EB, H258 or DNA separately is very weak, but the complexes of DNA-EB and DNA-H258 will show a remarkable increase in fluorescence intensity (24-fold and 140-fold increase for DNA-EB and DNA-H258, respectively) [3,17]. Previous report suggested that the complexes' fluorescence can be quenched by the addition of a second molecule [7]. Which means an intercalator will lead to a decrease of fluorescence intensity of DNA-EB complex [11]. And this is the same for DNA-H258 complex with the presence of a minor-groove binder. Taking advantage of both EB and H258 would allow us to design dual-fluorescent probes for rapid screening of DNA intercalators and minor-groove binders, literatures showed that dry leaves of *Pyrrosia lingua* (*Thunb.*) contained organic acids, flavonoids, xanthones, triterpenoids, steroids and volatile oil, which possesses a lot of physiological activities including antiviral, bacteriostatic, anti-inflammatory, diuretic, immunity enhancement and kidney protection activities [18]. Furthermore, *Pyrrosia petiolosa* had DNA protective effect by hydroxyl radical-induced DNA strand scission assay [19]. In order to further understand the activities of *P. lingua* (*Thunb.*), an established online screening system were used for fast separation, identification, activity evaluation and mechanism differentiation of DNA-binding compounds from *P. lingua* (*Thunb.*).

2. Experimental

2.1. Materials and reagents

14 Reference drugs, including quinic acid, protocatechuic acid, 3-CQA (3-caffeoylquinic acid), 4-CQA, 5-CQA, 4,5-diCQA, 3,5-diCQA, mangiferin, cynaroside, rutin, vitexin, isovitexin, orientin, and isoorientin, were obtained from the National Institute for the Control of Pharmaceutical (Beijing, China). Highly polymerized DNA from fish sperm (Sigma Code D-3159), H258 (Sigma Code R2264, purity 98.0%) and EB (Sigma Code E8751, purity 98.0%) were purchased from Sigma Aldrich (St. Louis, MS).

HPLC grade methanol and acetonitrile (Merck, Darmstadt, Germany), analytical grade formic acid (FA) from Tianjin Guangfu fine chemical Research institute, water purified by a Milli-Q system obtained from Millipore (Milford, MA, USA) were used for the preparation of the mobile phase. Analytical grade methanol (Beijing Chemical Works, Beijing, China) was used for sample preparation.

2.2. Sample preparation

The dried leaves of *P. lingua* (*Thunb.*) were crushed into powder (40 meshes). 1.0 g was extracted with 50 mL of methanol: water (50:50, v/v) in an ultrasonic oscillation (100 W, 40 kHz) for 50 min at room temperature. The extracted solution was filtered and dried under reduced pressure. The obtained residue was dissolved in 10 mL of 50% methanol. The solution was filtered through a membrane (0.22 μm) prior to injection (1 μL) into the UFLC-MS system.

2.3. Online detection of solutions

Stock solutions of EB and H258 were prepared by directly dissolving the dyes in deionized water, and then stored at −4 °C protected from light. The DNA solution was prepared in Tris–HCl buffer. The purity of the DNA was evaluated by monitoring the absorption spectrum and the ratio of the absorbance at 260 and 280 nm. The DNA solution gave a ratio of A260/A280 > 1.8, suggesting that the protein has been isolated from DNA and its purity has fulfilled the experimental requirement [20,21]. 0.05 mol/L Tris–HCl buffer (pH 7.4) was used to control the pH of the reaction system.

2.4. Apparatus of UFLC-MS^n-DNA-DFLD system

The chromatographic analysis was performed on a Shimadzu UFLC system (Kyoto, Japan) composing of two LC-$20AD_{XR}$ pumps, CTO-20AC column oven, DGU-$20A_5$ degasser, SPD-M20A, and SIL-$20AC_{XR}$ auto injector. The UFLC system was coupled with ion-trap time-of flight (IT-TOF) mass spectrometer (Shimadzu MS-IT-TOF, Kyoto, Japan) by an ESI interface. And Formula Predictor software (version 3, Shimadzu, Kyoto, Japan).

The fluorescence detection was carried out using a Shimadzu RF-20Axs fluorescence detector (Kyoto, Japan). The DNA and probes solutions were pumped by two additional LC-20AD pumps, respectively. Offline fluorescent experimental data were obtained from a Cary Eclipse fluorescence spectrophotometer (Agilent, Australia).

2.4.1. UFLC conditions

The column was Kromasil 100–2.5 C_{18} (100 mm × 2.1 mm i.d., 2.5 μm). The mobile phase was composed of A (water containing 0.05% FA) and B (MeOH containing 0.05% FA); detecting wavelengths were set at 326 nm and 254 nm. Flow rate was 0.4 mL/min. To achieve better separation, we use gradient conditions for column temperature: 0–15 min, 47 °C; 15–20 min, 47–50 °C; 20–50 min, 50–47 °C and the mobile phase gradient were: 0–10 min, 6–10% (B); 10–13 min, 10–13% (B); 13–17 min, 13–18% (B); 17–30 min, 18–19% (B); 30–50 min, 19–21% (B).

2.4.2. ESI-IT–TOF-MS analysis

LCMS-IT-TOF System (Shimadzu Corp., Japan) was used as multi-stage mass spectrometer. Optimized MS operating conditions were as follows: scan spectra range is *m*/*z* 100 to *m*/*z* 800 in negative ion mode, ESI source voltage was −3.5 kV, nitrogen flow rate of 1.5 L/min, and the curved desolvation line (CDL) and heat block temperature was set at 200 °C. The capillary voltage, CDL voltage and detector voltage were fixed at 4.5 kV, 10 V and 1.7 kV, respectively.

2.5. Design of on-line UFLC-MS^n-Dual-Probes-FLD detection

The screening system (Fig. 1) was composed by two main sections, namely, UFLC-DAD-MS^n and DNA-dual-wavelength-FLD. The DNA and probes solutions were introduced at a constant flow rate and then reacted in the warmed reactor. Thereupon, the fluorescence intensities (baselines) of DNA-EB and DNA-H258 complexes could be detected by DFLD. While the crude extraction was isolated by the column in the UFLC system, the LC fingerprint was obtained by the DAD detector. Then eluents were divided into two streams, one of which was introduced into the MS detector to obtain MS^n data; while the other stream was pumped into the reactor. Once an active ingredient encountered either DNA-EB or DNA-H258, the corresponding baseline would reduce, and thus, a negative peak