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Study on the interactions between anti-HIV-1 active compounds with *trans*-activation response RNA by affinity capillary electrophoresis

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Abstract

The study on the interactions between two anti-human immunodeficiency virus type 1 (anti-HIV-1) active compounds with *trans*-activation response (TAR) RNA by affinity capillary electrophoresis (ACE) with UV absorbance detection is presented. The results showed that the novel active molecules could interact with TAR RNA and inhibit the reproduce process of HIV-1. The binding constants were estimated by the change of migration time of the analytes through the change of concentrations of TAR RNA in the buffer solution. The yielded binding constants of 8.87×10^3 M^{-1} for active compound C_3 and 8.42×10^3 M^{-1} for MC_3 at 20.0 °C, 0.626×10^3 M^{-1} and 0.644×10^3 M^{-1} at 37.0 °C, respectively. The thermodynamic parameters ΔH and ΔS were obtained and shown that both hydrophobic and electrostatic interaction played roles in the binding processes. The results showed that the presented method was an easy and simple method to evaluate the interaction of small molecules with some bioactive materials.

Keywords: Binding constants; Capillary electrophoresis; Thermodynamic parameters; TAR RNA

1. Introduction

Non-covalent molecular interactions widely exist in nature and the characterization of such specific interactions is the focus of much biochemical research [1,2]. Different methods have been developed and applied in the study of the interactions between small ligands and biomacromolecules. Equilibrium dialysis, ultrafiltration, and ultracentrifugation are the most widely used because of their simplicity and general applicability to many different systems in vitro and ex vivo. However, these methods have some limitations such as the time needed to reach equilibrium, large amount of sample, Donnan effects, and difficulty in the control of some experimental variables [3,4]. Some spectroscopic methods, such as fluorescence, Nuclear magnetic resonance (NMR), circular dichroism (CD), and optical rotatory dispersion (ORD) have been applied in the study. These techniques can be monitored the three-dimensional protein structure and thus elucidate some complementary structural and conformational variations of a protein molecule resulting from ligand attachment [5,6]. These spectroscopy approaches were successful mainly for high-affinity interaction [3]. In addition, some chromatographic methods, including affinity chromatography and high-performance size-exclusion chromatography, have been used for a long time for the determination of binding parameters [7,8]. The progress in chromatographic technology has led to the development of highly automated systems yielding high resolution on small columns, allowing shorter analysis times, consuming less chemicals and avoiding the use of radiolabeled ligands. Since the interaction between macromolecules is consisted of different processes, which would

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cause the entropy change and electrochemical responses, many techniques have been tried in this study [2,3,9–12].

Among these powerful techniques, capillary electrophoresis (CE) is a versatile microanalytical tool that has gained much attention because of many advantageous features, such as low sample consumption, rapid analysis speed, ease of automation and high efficiency [12–14]. Various affinity interactions, such as drug–protein, protein–protein, protein–nucleic acid, protein–carbohydrate, peptide–antibiotic, enzyme–cofactors, lectin–sugar, antigen–antibody and cyclodextrins (CDs)–enantiomer have been investigated by CE recent year [2,3,11–13]. Bjerrum's group has devoted to the implementation of CE as a tool for analysis in inorganic system, one example is the analysis of the association between various cobalt(III) coordination compounds and the anions perchlorate and sulphate using capillary electrophoresis [15]. The stability constants of metal ions Ca^{2+} and Na^{+} binding to high affinity sites of calcium-containing proteins were studied by CE [16].

Several modes of CE are available to measure binding constants, e.g. affinity capillary electrophoresis (ACE), Hummel–Dreyer method (HD), vacancy affinity capillary electrophoresis (VACE), vacancy peak method (VP), frontal analysis (FA) and frontal analysis continuous capillary electrophoresis (FACCE) [2,12,17,18]. As pointed out by Busch et al. [17] the various methods are complementary rather than competitive. Measurements in the ACE and VACE mode are based on changes in electrophoretic mobility of the small molecule due to complexation, while the amount of bound compound is measured with the HD method and the free sample concentration is measured with the VP, FACCE, and FA [12,17,18]. VACE was first described as a new method for measuring association constants of vancomycin and *N*-Ac-D-alanyl-D-alanine by Busch et al. [1]. The concept of a partial-filling technique in ACE was evaluated using two model systems: vancomycin and carbonic anhydrase B (CAB) by Gomez's group [19]. Another example was that FA was applied to 12 low molecular weight compounds including eight drug substances displaying a range of different properties with respect to binding affinity, binding location, structure, lipophilicity, charge at physiological pH, and electrophoretic mobility [12].

It is one of the most critical and exciting challenges of this century to discover potential antiviral drug against human immumodeficiency virus type-1 (HIV-1). It has been demonstrated that the binding of *trans*-activator (Tat) protein to the *trans*-activation response region of mRNA (TAR RNA) plays a key role in the replication of HIV-1 [20–22]. Therefore, the molecule that disturbs this RNA-protein interaction might work as an anti-HIV drug and be employed for acquired immunodeficiency syndrome (AIDS) treatment. In theoretical consideration, aromatic heterocycle [23] and biscationic diphenylfuran derivatives [24] have been studied as potential pharmaceuticals. Analogs of amino acids, nucleotides and aminoglycosides, which could interfere the binding of Tat to TAR RNA were also studied [25–27].

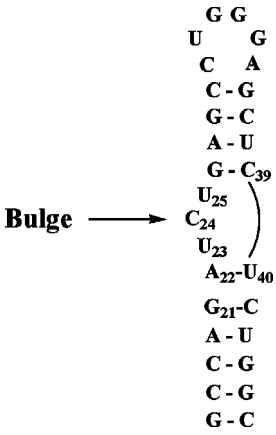

Fig. 1. The sequence of TAR RNA used in this study.

Two novel β-carboline alkaloids C_3 and MC_3, which have been demonstrated to have anti-HIV activity by binding to the "bulge" of TAR RNA (Fig. 1) specifically and inhibiting the Tat/TAR complexation [28], were developed in our group. To solve the limitations of potential compounds and amount of TAR RNA, a sensitive and low sample consumption CE method was developed in this work. The binding constants were estimated by the changes in the migration time of the analytes based on the different additions of TAR RNA in running buffers. The results were agreed with our previous data satisfactorily. Thermodynamic parameters were determined according to van't Hoff plots, which were applied for the discussion of the binding mechanism.

2. Experimental

2.1. Apparatus

Capillary electrophoresis was performed on a Beckman P/ACE 5000 system (Fullerton, CA, USA). The uncoated fused-silica capillary tubing 57 cm × 50 μm i.d., with a length of 50 cm to the detector (Beckman), was used. The capillary chamber temperature (±0.1 °C) was controlled by forced liquid cooling. A run voltage of +15 kV in the normal polarity mode was applied. UV detection was performed at 214 nm.

2.2. Chemicals

TAR RNA model sequence (MW = 9188.4, Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Two active compounds, 3-(*N*-propylamido-aminocarbonyl)-β-carboline (C_3, MW = 268) and 3-(*N*-propylamido-aminocarbonyl)-β-methylcarboline (MC_3, MW = 282) were designed and synthesized in the National Research Laboratories of Natural and Biomimetic Drugs (Health Science Center, Peking University). Other reagents were of analytical grade without further purification. Deionized water was used throughout.

2.3. Solution and sample preparation

The stock solutions of C_3 and MC_3 were prepared by accurately weighing definite powder of compounds and dissolving them in 1 cm^3 phosphate buffer saline (PBS, pH = 7.4), respectively, and then stored at −18 °C. The concentrations of them were 5.60×10^{-3} and 6.38×10^{-3} M, respectively. The concentration of TAR RNA stock solution was 7.62×10^{-3} M, which was determined from UV spectrophotometer at 257 nm. The running buffer in the CE experiments was 0.05 M sodium phosphate, pH 8.0. The investigated compounds and TAR RNA solutions in 0.05 M sodium phosphate buffer (pH 8.0) were prepared by diluting the definite volume of the stock solutions to the constant volume with buffer solution. The constant concentrations of C_3 and MC_3 in the sample were 5.60×10^{-5} and 9.57×10^{-5} M, respectively. Dimethyl sulfoxide (DMSO) was served as an electroosmotic flow (EOF) marker and was added to the sample at a concentration of 0.01% (v/v). The varying concentrations of TAR RNA in the running buffer were prepared from 0 to 31.8 μM. The buffer solutions were stored at 4 °C, being filtered through 0.22 μm cellulose acetate membrane filters (Shanghai Xingya Resin Co. Ltd., Shanghai, China) and sonicated for 10 min to remove air from solution prior to use.

2.4. Procedures for CE experiments

New capillary was conditioned for 60 min with 0.2 M NaOH, 30 min with water, and 10 min with running buffer. Between measurements, the capillary was flushed for 2 min with 0.2 M NaOH, 1 min with water, and 2 min with running buffer. The sample containing two active compounds and DMSO was introduced into the capillary by low-pressure injection (0.5 psi for 10 s). The electrophoresis was carried out using a phosphate buffer and differently accurate concentrations of TAR RNA (0–31.8 μM). All separations were in triplicate. Relative standard deviation (R.S.D.) of migration time was calculated from a series of three experiments carried out with the same sample within 1 day.

3. Results and discussion

3.1. Determination of the binding constants

The binding constants (K_b) are the most fundamentally important parameters for biologically active molecules [1,2]. The principle of this method for the determination of binding constants (K_b) is to exploit the changes in the migration time of the complexed sample in the background electrolyte containing the complexation agent (ligand). In order to be able to apply CE for this purpose the following conditions must be satisfied: (i) the sample must exhibit a migration time change upon complexation; (ii) the time needed to reach the equilibrium must be much faster than the running time during the electrophoretic experiment and (iii) sufficient concentrations of both the sample and the ligand must be available in the system [10]. After adding TAR RNA to background solution (BGS), the active compounds can interact with TAR RNA, the equilibrium represents by the following equation assumed that a 1:1 complex is formed:

$$M + R \overset{K_b}{\rightleftharpoons} MR \tag{1}$$

$$K_b = \frac{[MR]}{[M][R]} \tag{2}$$

where M, R and MR are the small molecule, TAR RNA and the complex, respectively. K_b is the binding constant for Eq. (1). As pointed out by Yang et al. [29], the capacity factor, k, which is used as an indicator to describe the relationship between retention time and mass distribution equilibrium in chromatography, can be utilized here to describe the migration behaviors of the active compounds in CE. Moreover, the addition of TAR RNA in the BGS is applied as a pseudo-stationary phase. From the definition of k, the following equation can be obtained:

$$k = K\frac{V_s}{V_m} = \phi\frac{[M]_s}{[M]_m} \tag{3}$$

where K is the concentration ratio of the analyte in the stationary phase and the mobile phase, (V_s/V_m) is called as phase ratio, ϕ, and s indicate stationary phase and m as mobile phase.

After adding TAR RNA to the BGS, part of the active compounds will bind to TAR RNA, resulting in the decrease of $[M]_m$ and the change of K. So $[M]_m$ can be described as the following equation:

$$[M]_m = [M]_{m0} - [MR]_m \tag{4}$$

where $[M]_{m0}$ is the concentration of the compounds without TAR RNA addition. Rearranging Eq. (2) and adding it to Eq. (4), the following equation can be obtained:

$$[M]_m = \frac{[M]_{m0}}{1 + K_b[R]_m} \tag{5}$$

Add Eq. (5) to Eq. (3),

$$k = \frac{\phi[M]_s}{[M]_{m0}}(1 + K_b[R]) \tag{6}$$

As defined in chromatography, the capacity factor k is equal to $(t - t_0)/t_0$, where t and t_0 are the migration times of the sample and neutral marker, respectively. With the linear relationship of k versus the concentration of TAR RNA ([R]), the binding constant is calculated from the slope, a, and intercept, b, consequently,

$$K_b = \frac{a}{b} \tag{7}$$

The migration times of each analyte (t) and DMSO (t_0) were measured in five running buffer solutions containing different concentrations of TAR RNA from 0 to 31.8 μM. R.S.D. data of migration times were calculated and summarized in Table 1, where t_1, t_2 and t_0 represented the migration

Table 1
R.S.D. data of migration times of C_3, MC_3 and DMSO at two temperatures

Temperature (°C)	[*R*] ($\times 10^{-6}$ M)	t_1 (min)	R.S.D. (%)	t_2 (min)	R.S.D. (%)	t_0 (min)	R.S.D. (%)
20.0	0	6.804	1.0	6.870	0.84	8.980	0.34
	2.540	6.811	0.80	6.880	0.65	8.993	1.0
	6.350	7.257	0.58	7.345	0.42	9.790	0.82
	19.05	7.263	0.93	7.367	0.84	10.106	0.77
	31.75	7.278	0.84	7.414	0.87	10.534	1.0
37.0	0	4.863	1.2	4.916	1.1	6.184	1.6
	2.540	4.638	1.3	4.692	1.3	5.902	1.2
	6.350	4.670	1.5	4.725	1.5	5.949	1.1
	12.70	4.694	1.5	4.748	1.5	5.985	1.1
	19.05	4.719	1.3	4.773	1.3	6.021	1.5

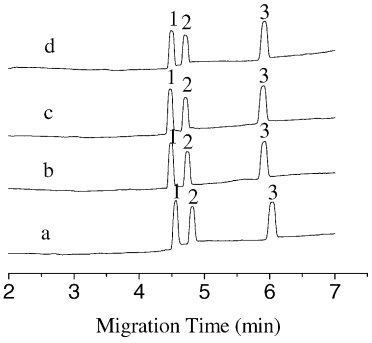

Fig. 2. The migration time shift of the compounds vs. TAR concentration change at 37.0 °C; conditions: running buffer 0.05 M phosphate solution (pH 8.0), applied voltage of 15 kV, pressure injection for 10 s at 0.5 psi, wavelength at 214 nm. The peaks 1, 2, 3 represent C_3, MC_3, DMSO, respectively, and a, b, c represent TAR concentration of 0, 2.54×10^{-6}, 6.35×10^{-6}, 12.7×10^{-6} M, respectively. The constant concentrations of C_3 and MC_3 in the sample were 5.60×10^{-5} and 9.57×10^{-5} M, respectively. DMSO was added to the sample at a concentration of 0.01% (v/v).

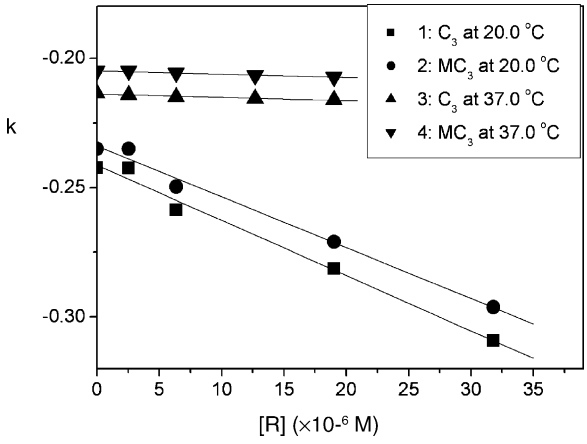

Fig. 3. Relationships between *k* with the concentration of TAR RNA.

time of C_3, MC_3 and DMSO, respectively. The values of R.S.D. at 37.0 °C were larger than them at 20.0 °C because of the instability of the higher capillary temperature. A set of electropherograms of migration time shift of the compounds versus TAR concentration change were shown in Fig. 2. The binding constants of two compounds with TAR RNA at two temperatures, 20.0 °C and 37.0 °C were obtained (Table 2) according to Eq. (7), respectively (Fig. 3).

The results showed that the active compounds would interact with TAR RNA in background solutions. The binding constants were about 10^2–10^3 M^{-1} level. The binding constant of the two active compounds were almost same compared with each other at one temperature. It demonstrated that the interactions between the active compounds and TAR RNA were both weak and similar due to the similar chemical structures. However, the binding constant values were smaller in 37 °C than that in 20 °C. The results showed that the dissociation of the complexation were more easy and rapid at the higher temperature. In addition, this method had more advantages including low sample consumption, short analysis time and simple data processing and calculation than the previous one based on the free molecules concentrations. Most importantly, it might also study multiple samples simultaneously one injected, provided they had sufficiently different migration times. However, care should be taken to ensure that the components of a mixture were not interacting either with each other.

Table 2
Binding constants ($\times 10^3$ M^{-1}) of two active compounds with TAR RNA at two temperatures

Compound	*T* (°C)							
	20.0				37.0			
	a ($\times 10^3$)	b	K_b	r^2	a ($\times 10^3$)	b	K_b	r^2
C_3	−2.14	−0.241	8.87	0.98	−0.134	−0.214	0.626	0.96
MC_3	−1.97	−0.234	8.42	0.98	−0.132	−0.205	0.644	0.98

3.2. Thermodynamic studies of the interactions

Small molecules are bound to macromolecule by four binding modes: H-bonds, van der Waals, electrostatic and hydrophobic interactions. The thermodynamic parameters, enthalpy change (ΔH) and entropy (ΔS) of reaction, are important for confirming binding mode. The ΔH and ΔS of binding reactions are evaluated based on the variation of capacity factor *k* with temperature. If ΔH does not vary significantly over the temperature range studied, as indicated

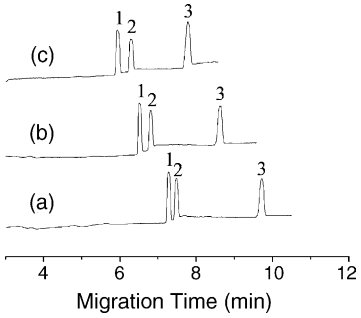

Fig. 4. Electrophoregrams at three temperatures; the peaks 1, 2, 3 represent C_3, MC_3, DMSO, respectively, and a, b, c represent 17.0 °C, 21.0 °C, 25.0 °C, respectively. The constant concentration of TAR RNA in running buffer was 31.8 μM.

by the van't Hoff equation [30,31]

$$\ln k = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} + \ln \phi \quad (8)$$

where R is the gas constant and ϕ is the phase ratio as mentioned above, the enthalpic and the entropic contributions to the Gibbs free energy can be determined. ΔH represents the measure of energy exchange in a system and ΔS represents the chaos of a system. The enthalpy change is calculated from the slope of the van't Hoff plot. The free energy change is estimated from the following relationship:

$$\Delta G = -RT \ln K \quad (9)$$

where K is the binding constant at the corresponding temperature. Because of the unknown value of ϕ, the entropy change must be determined from the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (10)$$

Keeping a constant concentration of TAR RNA in running buffer, 31.8 μM, the migration times of the two active compounds were obtained under five different temperatures from 17.0 °C to 37.0 °C. The results showed that the higher temperature would cause the decrease of the migration time of the samples, which meant that temperature affected the equilibriums taking place in the capillary (Fig. 4). The experimental data was found that $\ln|k|$ was linear with the reciprocal of absolute temperature. The explain $\ln|k|$ was that the shorter migration times of C_3 and MC_3 (t) than DMSO (t_0) (shown in Fig. 4), which was resulted from the positive charge of the analytes in the neutral separation systems, caused the values of $k < 0$. $\ln|k|$ versus $1/T$ plots and equations were shown in Fig. 5. The thermodynamic parameters ΔH, ΔG and ΔS were measured according to Eq. (8)–(10),

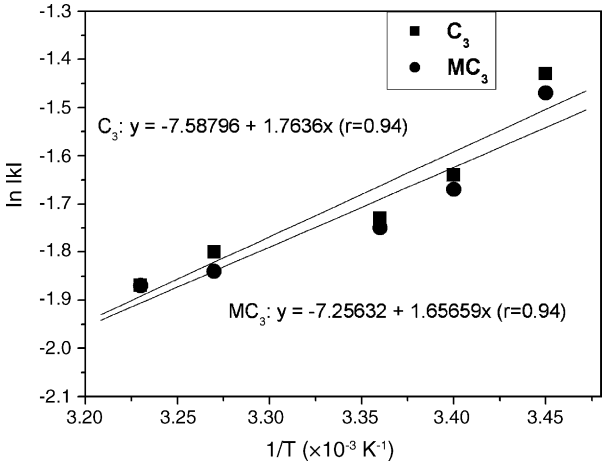

Fig. 5. van't Hoff plots of $\ln|k|$ vs. $1/T$.

respectively, the values of which were summarized in Table 3.

As shown in Table 3, ΔH values were negative under the experimental conditions, demonstrating that the complexation reactions were exothermic processes. The negative values of ΔG meant that the binding processes were spontaneous. The sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction were characterized [32,33]. From the point of view of water structure, positive entropy was frequently taken as the evidence for hydrophobic interaction, while it was also showed that positive entropy and negative enthalpy might be a manifestation of electrostatic interactions between ionic species in aqueous solution. Consequently, it was not possible to account for the thermodynamic parameters of these anti-HIV-1 active compounds and TAR RNA complex on the basis of a single intermolecular force model. It was more likely that hydrophobic, electrostatic interactions were both involved in the binding processes.

4. Conclusions

The ACE method described here had potential as an evaluation of non-covalent interactions between small molecules and biomacromolecules and a medium-throughput screen for determining the binding characteristics of drugs under aqueous conditions simulating the in vivo environment. With the addition of the definite concentration receptor to the background solutions, it was possible to subsequently determine the binding constants of a wide range of compounds by

Table 3
Thermodynamic parameters of interactions between C_3 and MC_3 with TAR RNA

Compound	K_b/(20.0 °C) ($\times 10^3$ M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
C_3	8.87	−14.66	−22.14	25.53
MC_3	8.42	−13.77	−22.02	28.16

evaluation of migration time. Furthermore, it would be utilized to study multiple components of a mixture simultaneously if they had different migration times and no interactions between each other. However, when the interaction was very weak, the reproducibility of the measurement would be worse because the complexation would be difficult to achieve equilibrium effectively under the rapid separation. In addition, the thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS), which were measured according to van't Hoff plots, indicated that both hydrophobic and electrostatic forces played roles in the binding processes.

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