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Short communication

Study on the interaction between HIV reverse transcriptase and its non-nucleoside inhibitor nevirapine by capillary electrophoresis

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ABSTRACT

The HIV reverse transcriptase (RT) is an important antiviral target for the chemotherapy of AIDS because of its key role in virus replication. Nevirapine is a first generation of non-nucleoside reverse transcriptase inhibitors (NNRTIs), which is usually used for the therapy of AIDS. In this study, a high-performance analytical method based on capillary electrophoresis (CE) to investigate interactions between HIV RT and nevirapine was developed. Samples containing HIV RT and nevirapine at various ratios were incubated at 37 °C for 45 min and then separated by CE with Tris–acetate buffer at pH 7.3 containing 0.15% SDS. Both qualitative and quantitative characterizations of the binding were determined by CE for the first time. The binding constants of the interactions between HIV RT and nevirapine were calculated as $(3.25 \pm 0.16) \times 10^4$ and $(1.25 \pm 0.07) \times 10^2\ M^{-1}$ by Scatchard analysis. HIV RT and nevirapine have two binding sites. The presented methodology should be generally applicable to study the interactions between HIV RT and nevirapine quantitatively and qualitatively.

1. Introduction

Reverse transcriptase (RT) is a key enzyme in the replication cycle of the human immunodeficiency virus type 1 (HIV-1), catalyzing the conversion of virally encoded RNA into proviral DNA [1]. This essential step in the retroviral life cycle is targeted by a variety of drugs in clinical use to combat AIDS (acquired immune deficiency syndrome). RT is a heterodimeric enzyme with subunits of 66 and 51 kDa. The p66 subunit consists of fingers, palm and thumb domains (DNA polymerase subdomain), as well as connection and ribonuclease H (RNase H) domains [2]. The p51 subunit contains only the polymerase domain [3]. The relative arrangement of the two subunits is different in p66 and p51. The active sites of the enzyme are in the p66 subunit. In detail, the palm contains residues which are critical for polymerase catalytic activity and the fingers domain is relative to the processivity, which resembles many DNA and RNA polymerases [4].

Current anti-AIDS therapy is based on drugs that belong either to the class of nucleoside/nucleotide (NRTIs/NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease or entry inhibitors. NNRTIs are a structurally diverse group of compounds which interact with a specific allosteric non-substrate binding pocket site of HIV-1 RT (non-nucleoside inhibitor binding pocket, NNIBP), leading to a non-competitive inhibition of the enzyme [5]. These inhibitors are highly specific for HIV-1 RT and lock it into an inactive conformation by fitting into an allosteric site which is approximately 10 Å from the polymerase active site, causing a displacement of the catalytic aspartate residues. Furthermore, they show lower cellular toxicity than NRTIs [2]. The non-nucleoside binding pocket only exists in the structures of RT complexed to a NNRTI, with its formation probably being induced by the proximity of the inhibitor. In unliganded RT, the p66 thumb subdomain is folded into the DNA-binding cleft and lies over the palm subdomain, nearly touching the fingers subdomain in a "thumb down" configuration. As a consequence, the DNA-binding cleft is closed. Moreover, the conformational change effected by NNRTI binding reduces the catalytic efficiency of the enzyme [6]. Nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2′,3′-e][1,4]diazepin-6-one) is a first generation NNRTIs, which is usually included in the generic fixed-dose combinations recommended by WHO [7]. Fig. 1 shows the structure of nevirapine. Like most of the NNRTIs, nevirapine displays a butterfly-like conformation, which is verified from the crystalline structure of the pure compound [8] and some complexes with the HIV-1 reverse transcriptase [9–11]. Several researchers suggest that this conformation is related to the degree of affinity of the drug and to the probability of appearance of viral resistance [12,13].

Many methods have been developed and applied to study the interactions between ligands and receptors, such as equilibrium dialysis, ultrafiltration, spectroscopic methods, and chromato-

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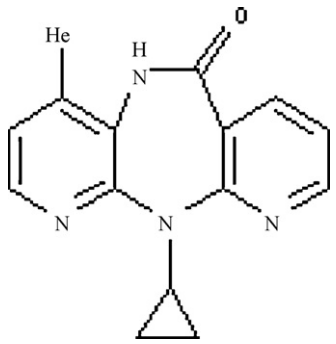

Fig. 1. Structure of nevirapine.

graphic methods [14]. Among these powerful techniques, CE has been used in a wide range of binding studies, such as protein–protein, protein–DNA, protein–drug, protein–sugar, DNA–peptide, peptide–drug, peptide–peptide, antibody–antigen, and peptide–carbohydrate [14,15]. CE separations are based on the principles of the electrically driven flow of ions in solution. The selectivity of CE can be manipulated by the alteration of electrolyte properties such as pH, ionic strength, and electrolyte composition, or by the incorporation of electrolyte additives [16]. CE offers some advantages in the binding studies, such as easy automation, low requirement of sample volume, simple instrument, short analysis time, high separation efficiencies and sample throughput. Additionally, the CE-based binding assays can be performed at near physiological conditions [17]. CE is able to study the interaction of individual components in a mixture as well as to determine binding parameters in a single step. This is a unique feature as compared with other techniques that were used for the study of noncovalent interactions [15]. CE is the most appropriate method for both the assay of strong binding systems (the complex will not dissociate during the required separation time period) and the assay of weak binding systems [18]. There have been many studies on the interactions between nevirapine and HIV RT of their binding site simulation by computational chemistry in the allosteric nonsubstrate binding pocket site [2,19–21], but there is no literature reported on the apparent binding constants of them. Here, CE was first utilized to investigate the interactions between nevirapine and HIV RT quantitatively and qualitatively.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade unless otherwise indicated. HIV reverse transcriptase was purchased from Ambion Inc (TX, USA). Nevirapine was from Desano Chemical Pharmaceutical Co., Ltd (Shanghai, China). Ultrapure water was obtained in a Milli-Q system from Millipore (Bedford, MA, USA). Both tris base (ultrapure) and acetic acid were from Beijing Chemical Reagent Factory (Beijing, China). Sodium dodecyl sulfate (SDS, ultrapure) was purchased from Invitrogen™ life technolofes (Carlsbad, CA, USA).

2.2. Instrumentation

All experiments were performed on a Beckman P/ACETM MDQ system (Beckman Coulter, Fullerton, CA, USA) equipped with a photodiode array detector as well as the 32 KaratTM software version 5.0 (Beckman). Uncoated fused-silica capillary tube (Yongnian Optical Fibre, Hebei, China) with an internal diameter of 75 μm was used. The total and effective lengths of the capillary were 30.2 and 20 cm, respectively.

2.3. Electrophoretic conditions

Before using, the new capillaries were rinsed with 0.1 mol/L NaOH solution for 20 min, and subsequently with deionized water for 5 min. To study the system after incubation, the temperatures of the cartridge and sample chamber were kept at 15 and 4 °C, respectively. Samples were injected using the pressure injection mode at 0.5 psi for 5 s (1 psi = 6894.76 Pa). The applied voltage was +12 kV. 30 mM Tris–HAc (pH 7.3) buffer with 0.15% SDS was served as running buffer. The capillary was washed between runs with the NaOH, H_2O and running buffer for 1, 1 and 2 min at 20 psi, respectively. Each concentration was analyzed twice.

2.4. Sample preparation

Nevirapine was dissolved in 30 mM Tris–HAc buffer (pH 7.3) at the concentration of 4 mM as stocking solution. To investigate the standard curve, the stocking solution was diluted to a series of concentrations. To investigate the interactions between HIV RT and nevirapine, different concentrations of HIV RT were added to 0.16 mM nevirapine to form complex. The mixtures of HIV RT and nevirapine were incubated for 45 min at 37 °C before CE analysis.

2.5. Quantitative model for the binding study

In the binding studies, the binding constant and the stoichiometry are important parameters to be determined. Scatchard analysis is a common way to linearize the binding data, as expressed in Eq. (1):

$$\frac{r}{C_f} = -Kr + nK \quad (1)$$

where r is the ratio of the concentrations of the bound ligand (or receptor) to the total receptor (or ligand) and C_f is the unbound ligand (or receptor) concentration. K is the apparent binding constant and n is the number of binding sites [22]. In this study, r is the concentration ratio of the bound nevirapine to the total HIV RT and C_f is the free nevirapine concentration.

3. Results and discussion

3.1. Optimization of the CE conditions

No significant change was observed in the peak heights due to complex formation in 45 and 60 min, indicating that the interaction reached equilibrium in solution within 45 min. All samples were, therefore, incubated at 37 °C for at least 45 min before CE analysis. In our experiment, electrophoretic conditions were optimized, in particular with the choice of buffer type, buffer concentration, buffer pH value, applied voltage, and the length of the capillary. First, we investigated the effect of phosphate buffer and Tris–HAc. Using phosphate buffer, the reproducibility was not well. Tris–HAc provided the better peak shape and reproducibility. Thus, Tris–HAc solution was selected as the running buffer. Different concentrations of the Tris–HAc buffer were tested and we selected 30 mM Tris–HAc as the running buffer ascribing its suitable migration time and better peak shape. pH was another important factor for evaluating the interactions in CE. In general, the binding constant was determined in physiological condition. Thus pH 7.3 Tris–HAc solution was selected. The HIV reverse transcriptase was stored in 50% glycerol. In the running buffer described above, glycerol and nevirapine cannot be baseline separated. So additives should be used to reach baseline separation. Different types and concentrations of additives were tested, such as glycerol, Triton X-100 and SDS, and finally 0.15% SDS was chosen. In order to obtain short migration times, the length of the capillary should be chosen, and finally

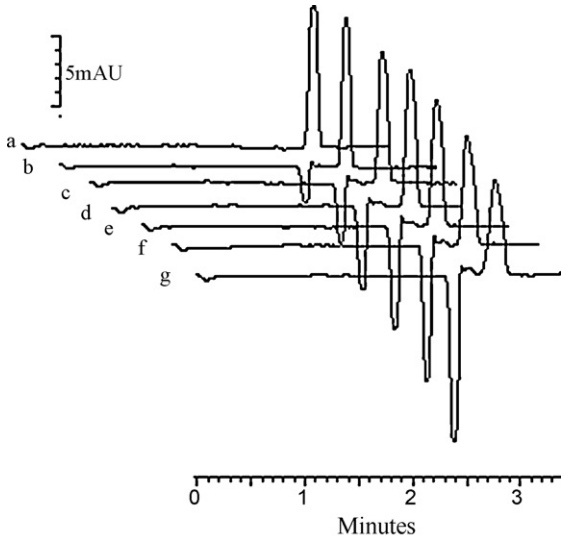

Fig. 2. Electropherograms of HIV RT–nevirapine interactions. The concentration of nevirapine was fixed at 0.16 mM in each sample. HIV RT:nevirapine: (A) 0:1; (B) 0.3:1; (C) 0.6:1; (D) 0.9:1; (E) 1.3:1; (F) 1.9:1; (G) 2.5:1. Peaks 1, 2 and 3 represent glycerol, HIV RT and nevirapine, respectively. The conditions used were as follows: Beckman P/ACE MDQ CE system with DAD, 214 nm; injection: 0.5 psi for 5 s; applied voltage: +12 kV; capillary: capillary of 30.2 cm (effective length 20 cm) × 75 μm i.d.; running buffer: 30 mM Tris–acetate, pH 7.3, 0.15% SDS.

20 cm effective length was chosen. To reach high separation efficiency, the applied voltage and the cartridge temperature were set at +12 kV and 15 °C, respectively. Under the CE conditions employed in this paper, the baseline and peak shape were good enough for the quantification.

3.2. Determination of the interaction between HIV RT and nevirapine

Nevirapine solutions with the concentrations range from 0.05 to 0.28 mM were injected into the capillary column to get the calibration plot. The peak height of each sample was proportional to its concentration. The RSD of repeated injection of 0.16 mM nevirapine solutions was 4.91% ($n = 4$) for peak height and 0.59% for migration time, respectively. The calibration plot of nevirapine solutions was peak height = $72238C_{\text{nevirapine}} - 27$ ($n = 6$) with a correlations coefficient of 0.9960. Fig. 2 shows the electropherograms of the interaction between HIV RT and nevirapine. When the ratio between HIV RT and nevirapine was 0.3:1, no interaction could be observed because no peak height decreasing was observed. With increasing HIV RT ratios in the sample, the peak height of nevirapine decreased consecutively and regularly, which means that it is not occasional or due to other effects, but rather, the result of the interaction. While at the same time, the peak areas of nevirapine are constant and the peak broadens. The peak heights of each sample can represent the free concentrations of nevirapine [23]. In our experiment the peaks of HIV RT cannot be seen. Because the enzyme was stored in glycerol, the negative peak was the peak of glycerol. They can represent the amounts of enzyme added in nevirapine solutions. The binding constant is an important quantitative parameter for characterizing the interaction. The peak heights of nevirapine in each sample were determined and free concentrations were calculated from the calibration curve to obtain the values of r. Fig. 3 shows the Scatchard plot based on Eq. (1), the calculating method is following a previous report [24]. We can see from Fig. 3 that there are two binding sites in the HIV RT for nevirapine. One was strong binding and the other was weak binding. From the two slopes of the Scatchard plot, K was calculated to

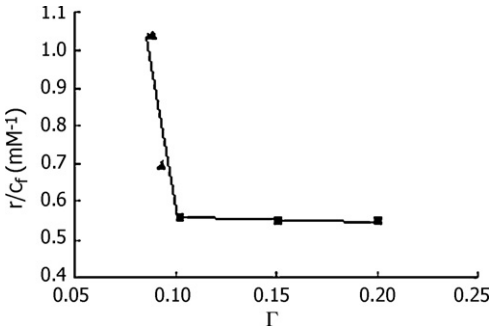

Fig. 3. Scatchard plot for the interaction between HIV RT and nevirapine. The CE conditions used were specified in caption of Fig. 2.

be $(3.25 \pm 0.16) \times 10^4$ and $(1.25 \pm 0.07) \times 10^2$ M^{-1}. From the results we can suppose that the strong binding site is the allosteric non-substrate binding pocket site [5] that is frequently studied by the computational chemistry, while the weak binding may be locates in some non-active site of the HIV RT. The results indicated that CE provides a highly efficient, fast and quantitative method for studying interactions between HIV RT and nevirapine.

4. Conclusions

The CE method described here has potential for the evaluation of interactions between HIV RT and nevirapine under aqueous conditions. The CE approach is a highly efficient, fast, quantitative method for the study of biomolecular interactions, and the approach also avoids the interference when protein is immobilized on a plate. Each run was completed within 3.5 min, and the binding constants and binding sites could be determined. There are two binding sites in the HIV RT for nevirapine. One was strong binding and the other was weak binding. The binding constants of the interactions between HIV RT and nevirapine were calculated as $(3.25 \pm 0.16) \times 10^4$ and $(1.25 \pm 0.07) \times 10^2$ M^{-1} by Scatchard analysis.

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