Mass Spectrometric Characterization of HIV-1 Reverse Transcriptase Interactions with Non-nucleoside Reverse Transcriptase Inhibitors

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Note

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Non-nucleoside reverse transcriptase inhibitors (NNRTIs) of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) have been developed for the treatment of acquired immunodeficiency syndrome. HIV-1 RT binding to NNRTIs has been characterized by various biophysical techniques. However, these techniques are often hampered by the low water solubility of the inhibitors, such as the current promising diarylpyrimidine-based inhibitors rilpivirine and etravirine. Hence, a conventional and rapid method that requires small sample amounts is desirable for studying NNRTIs with low water solubility. Here we successfully applied a recently developed mass spectrometric technique under non-denaturing conditions to characterize the interactions between the heterodimeric HIV-1 RT enzyme and NNRTIs with different inhibitory activities. Our data demonstrate that mass spectrometry serves as a semi-quantitative indicator of NNRTI binding affinity for HIV-1 RT using low and small amounts of samples, offering a new high-throughput screening tool for identifying novel RT inhibitors as anti-HIV drugs.

Key words mass spectrometry; human immunodeficiency virus type 1; drug screening

RESULTS AND DISCUSSION

First, we assessed the heterodimeric state of HIV-1 RT using MS. The mass spectrum of HIV-1 RT exhibited two ion series corresponding to the molecular mass of the p51 (52632 Da) and p66 (65168 Da) subunits. Thus, as rilpivirine and etravirine. Hence, ITC and SPR are seldom applied to determine HIV-1 RT binding affinities of NNRTIs except for those with higher water solubility, such as nevirapine and efavirenz. Therefore, a conventional and rapid method that requires small sample amounts is desirable for characterizing the interactions between HIV-1 RT and NNRTIs with lower water solubility.

Recently, the use of mass spectrometry (MS) under non-denaturing conditions has become a powerful tool for analyzing non-covalently associated biomacromolecular complexes. Several reports have shown that MS can be used to characterize the binding of drugs to target enzymes in terms of stoichiometry, specificity, and stability. This method is advantageous because it requires small sample amounts. Because of its rapidity, sensitivity, and ability to directly determine binding stoichiometry, MS is potentially applicable as a superior high-throughput screening method for developing anti-HIV drugs.

In this study, we tested the applicability of MS to the characterization of HIV-1 RT binding to four NNRTIs (nevirapine, efavirenz, etravirine, and rilpivirine) with different inhibitory activities (Fig. 1).

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we confirmed that the ion series observed under the non-denaturing condition corresponds to the heterodimer composed of the p66 and p51 subunits.

Next, we attempted to characterize the interactions of the p66/p51 heterodimer of HIV-1 RT with NNRTIs. Figure 2 shows the spectral changes of HIV-1 RT upon addition of etravirine. In the MS analysis, intensities of the original ion peaks of the p66/p51 heterodimer were attenuated on titration with etravirine, with concomitant appearance of a new peak series. The molecular mass determined for this complex was 118296±32 Da, which corresponds to a 1:1:1 complex composed of p66, p51, and etravirine (with a calculated mass
of 435.3 Da). Furthermore, these data indicated that 10 µM etravirine was sufficient to form a clearly observable complex with 3.8 µM HIV-1 RT.

Based on these observations, we measured a series of MS spectra of HIV-1 RT in the presence of one of the other compounds at the same concentration. Consequently, a new peak series from the NNRTIs-HIV-1 RT complex was observed upon addition of rilpivirine with similar spectral changes to etravirine, whereas less pronounced and virtually no spectral changes were induced by efavirenz and nevirapine, respectively (Fig. 3).

It has been reported that the dissociation constants for the binding of HIV-1 RT to nevirapine, efavirenz, and rilpivirine are $4 \times 10^{-7}$ M, $17 \times 10^{-8}$ M, and $18 \times 10^{-9}$ M, respectively. Thus, the affinities of these NNRTIs are in agreement with the MS peak intensities of their complex with HIV-1 RT, indicating that relative affinities of the inhibitors to HIV-1 RT can be estimated by MS measurements (Fig. 4).

In addition, our previous NMR analysis revealed that the methyl $^{13}$C chemical shifts of methionine-230 of HIV-1 RT, which is in close proximity to the NNRTI binding pocket, can be used as an indicator of the efficacy of NNRTI. The NMR data suggest that etravirine and rilpivirine have comparable binding affinity for HIV-1 RT, consistent with the present MS results.

In the present study, we successfully applied MS to observe the interactions between HIV-1 RT and various NNRTIs under non-denaturing conditions. Our data demonstrate that MS serves as a semi-quantitative indicator of NNRTI binding affinity for HIV-1 RT using low concentrations and small amounts of samples. Therefore, MS could potentially be used as a new high-throughput screening technique to identify novel RT inhibitors for anti-HIV drug development.

**MATERIALS AND METHODS**

**NNRTIs** Powdered rilpivirine and etravirine were purchased from MedChem Express. Nevirapine and efavirenz were purchased from Sigma. A stock solution (0.2 mM) of each inhibitor was prepared in dimethyl sulfoxide (DMSO).

**Preparation of HIV-1 RT Protein** The recombinant plasmids containing the HIV-1 RT genes, pGEX3X for p66 and pET33B for p51, were previously constructed. Recombinant HIV-1 RT was expressed and purified as previously
described. Briefly, the heterodimeric HIV-1 RT protein was purified from cell lysates with sequential use of a diethylaminoethyl (DEAE) cellulose column (Whatman), phosphocel lulose P11 column (Whatman), Chelating Sepharose Fast Flow (GE Healthcare) charged with nickel sulfate, and RESOURCE S column (GE Healthcare). Finally, the HIV-1 RT protein was purified by a Superdex-200 (HiLoad 16/60) gel filtration using an FPLC column (GE Healthcare). All purification steps for HIV-1 RT were performed at 4°C with buffer containing protease inhibitors.

MS Measurements The purified HIV-1 RT sample was buffer-exchanged to 200 mM ammonium acetate, pH 7.6, by passing through a Bio-Spin 6 column (Bio-Rad). HIV-1 RT (3.8 µM) was incubated with each inhibitor in the presence of 5% DMSO, and the samples were analyzed by nanoflow electrospray using in-house made gold-coated glass capillaries (2–5 µL of sample was loaded for each analysis). Under a denaturing condition, 30% (v/v) formic acid was added to the sample solution. Spectra were recorded using a SYNPAT G2-Si HDMS mass spectrometer (Waters) in positive ionization mode at 1.33 kV with 150 V of sampling cone voltage and 5 V of trap and 0 V of transfer collision energy, and 5 mL/min trap gas flow. Spectra were calibrated using 1 µg/mL cesium iodide and analyzed using MassLynx (Waters) and IGOR Pro (Wave Metrics) software.

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Conflict of Interest The authors declare no conflict of interest.

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