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A fast and robust ^{19}F NMR-based method for finding new HIV-1 protease inhibitors

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Abstract—The human immunodeficiency virus (HIV) which encodes, among other indispensable enzymes, an aspartic protease that is essential for viral maturation and replication. Numerous inhibitors of the protease have been developed. However, the eventual resistance of HIV-1 to these drugs implies a continuous battle to develop new inhibitors. Proposed herein is a robust, fast, and reliable method employing ^{19}F NMR for the evaluation of the inhibitory activity of new compounds against HIV-1 protease.

HIV-1 protease encoded by retroviruses is required for the processing of *gag* and *pol* polyprotein precursors,[1–3] hence it is essential for the production of infectious viral particles. In vitro inhibition of this enzyme results in the production of progeny virions that are immature and non-infectious,[4,5] suggesting its potential as a therapeutic target for AIDS. There are two kinds of inhibitors against this enzyme: inhibitors of the active site[6,7] and dimerization inhibitors.[8–10] While compounds in the latter class have already been approved by FDA for use as AIDS drugs, the second class is very promising as well.

The constant search for new inhibitors is greatly facilitated by any advances that allow the evaluation of the inhibitory activity of new compounds. This need for new inhibitory assays is especially true in the case of screening of extracts from natural products. Usually, these very complex extracts contain compounds that, due to their colored or fluorescent characteristics, interfere with the most commonly used assays, based on UV or fluorescence spectroscopy.

A common problem of currently available assays is the appearance of false positives caused by the precipitation of the substrate, due to interactions among some of the compounds in the assayed system. This is a general problem for all those methods that are based exclusively on the appearance of the reaction product. Described herein is a method that employs ^{19}F NMR to screen potential HIV-1 protease inhibitors by following the simultaneous appearance and disappearance of fluorine signals that correspond to a fluorine-containing, peptidic HIV-1 protease substrate and its fluorinated reaction product, respectively.

The method is based on the seminal paper by Dalvit et al.,[11] who first proposed an approximation of this kind. While a similar work has been carried out for caspases,[12,13] this is the first time it is used for an enzyme with the medical relevance of HIV-1 protease.

The method comprises incubation of purified HIV-1 protease with a substrate that contains at least one fluorine atom, followed by monitoring of the resulting hydrolysis reaction by ^{19}F NMR spectroscopy. Signals corresponding to the substrate and hydrolyzed product can then be identified by their different chemical shifts (Scheme 1).

HIV-1 protease containing five mutations: Q7K, L33I, and L63I to minimize the autoproteolysis,[14,15] as well as C67A and C95A to prevent cysteine-thiol oxidation,[16] was produced in *Escherichia coli* using the expression vector pET11 and host bacterium strain Rosetta(DE3)pLysS. Cells were grown in LB media at 37 °C and, when the cell density reached an optical density of ca 0.8 at 600 nm, protein expression was induced with

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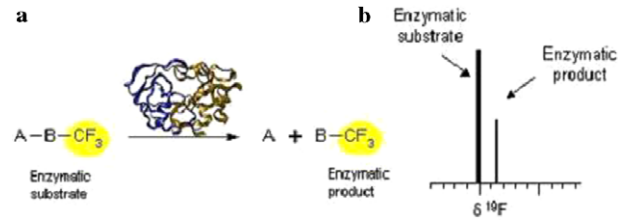

Scheme 1. (a) Enzymatic reaction of HIV-1 protease in the presence of a fluorinated substrate; (b) a model ^{19}F NMR spectrum corresponding to partial enzymatic reaction.

2 mM IPTG at 37 °C for 3 h. After isolation of inclusion bodies,[17] the extract was loaded onto a Superdex 75 column.[18] The peak fractions containing pure protease were pooled. In order to fold the protein, the pure fraction was dialyzed for 12–14 h at 4 °C with two changes of 3 L of 10 mM sodium acetate, pH 4.8, containing 1 mM DTT and 30 mM NaCl. The protein was stored at −80 °C. The concentration of purified protein was determined spectrophotometrically.

HIV-1 protease cleaves peptide bonds by typical aspartyl protease mechanism, which requires a molecule of water in the lysis process.[19] The most highly conserved cleavage site consists of a pentapeptide (Ser/Thr)-X-Y-(Tyr/Phe)-Pro, whereby substrate cleavage occurs between the aromatic residue Tyr or Phe and the Pro residue.[20]

We chose the sequence SQNYP, which represents the p17–p24 junction in the HIV-1 gag polyprotein. While there is no homology among polyprotein substrates outside of the consensus pentapeptide, P'_2 and P'_3 sites generally contain hydrophobic residues, hence Val was used for these sites in the peptide test. The peptides were prepared as its *N*-acetyl carboxamide derivative and subsequently fluorinated. In order to synthesize the substrate, peptide elongations on solid phase were carried out on a 100 μmol scale of Fmoc-AM-p-MBHA resin (0.3 mmol/g). Fmoc/^{t}Bu chemistry and TBTU and DIEA coupling reagents were used. An additional coupling was required to complete incorporation of the amino acid after the proline residue. In this case total coupling was determined by a DeClercq test.[21]

The trifluoromethyl group was chosen because it provides high sensitivity, due to the presence of three isochronous (i.e., having the same chemical shift) fluorine nuclei.

In this substrate (**1**), the CF_3 group was introduced in N-terminal obtaining an *N*-trifluoroacetylated peptide (Fig. 1a). Acetylation of N-terminal function was performed on solid phase using ethyl trifluoroacetate ester. Final protected amide peptides were cleaved from the resin using a cleavage cocktail of TFA:H_2O:triisopropylsilane. The combined solutions were evaporated to dryness under reduced pressure and lyophilized. Peptide purity was calculated by HPLC RP-C_{18} and peptide identity confirmed by MALDI-TOF spectrometry and amino acid analysis.

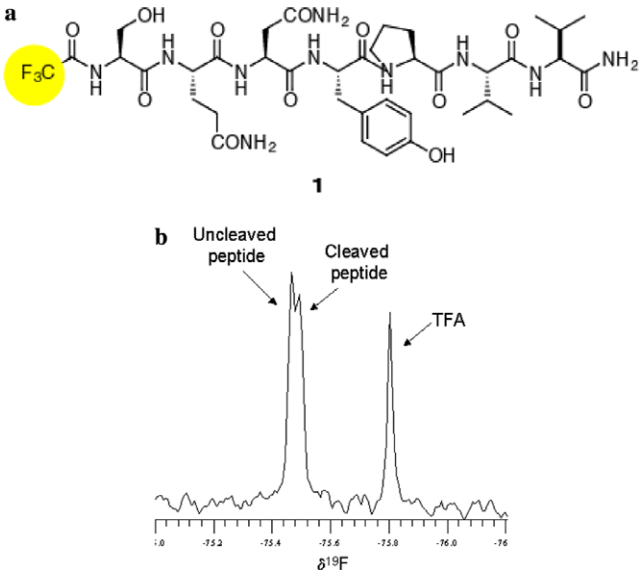

Figure 1. Substrate 1 and ^{19}F NMR spectra of its enzymatic reaction. (a) Fluorinated peptidyl substrate **1**. (b) ^{19}F NMR spectrum recorded for the substrate **1** after incubation with purified HIV-1 protease.

The peptide was treated with HIV-1 protease. The enzymatic assay was performed with 700 μL of sample volume at 30 °C. The reaction buffer contained 30 nM of HIV-1 protease, 200 μM of substrate, 50 mM 2-(*N*-morpholino)-ethanesulfonic acid, 200 mM NaCl, and 1 mM EDTA, pH 6.0. The substrate was dissolved in hexadeuterated dimethylsulfoxide before addition to the buffer. The reaction was stopped after 30 min by the addition of 20 μL of HCl.

The result was analyzed by ^{19}F NMR as shown (Fig. 1b). ^{19}F NMR spectra were obtained at 25 °C with a Varian 400 NMR spectrometer.[22]

As the CF_3 group in the substrate **1** is far from the cleaved site, the signals for the substrate and product in the ^{19}F NMR spectrum overlapped. We thus decided to prepare an alternative substrate.

The sequence SQNFP, in which Phe was substituted for Tyr, was chosen because it also occurs in the consensus cleavage sequence. Analogously to substrate **1**, Val was also used for the P'_2 and P'_3 sites, and the peptide was prepared as its *N*-acetyl carboxamide derivative. For substrate **2**, the CF_3 group was introduced during peptide synthesis via a chemically protected Fmoc-4-(trifluoromethyl)-L-phenylalanine (Fluka, Chemika) during the peptide synthesis (Fig. 2a). Acetylation of N-terminal function was performed on solid phase and a standard protocol of Ac_2O and DIEA in DMF was used. This peptide was also treated with the HIV-1 protease, as above, and the resulting product was analyzed by ^{19}F NMR, revealing two different peaks (Fig. 2b).

HPLC–MS of the hydrolysis product was performed to determine if the substrate had been cleaved in the proper site. An HPLC–MS chromatogram of the product from the incubation of substrate **2** with purified recombinant HIV-1 protease for 30 min is shown in Figure 3. The two

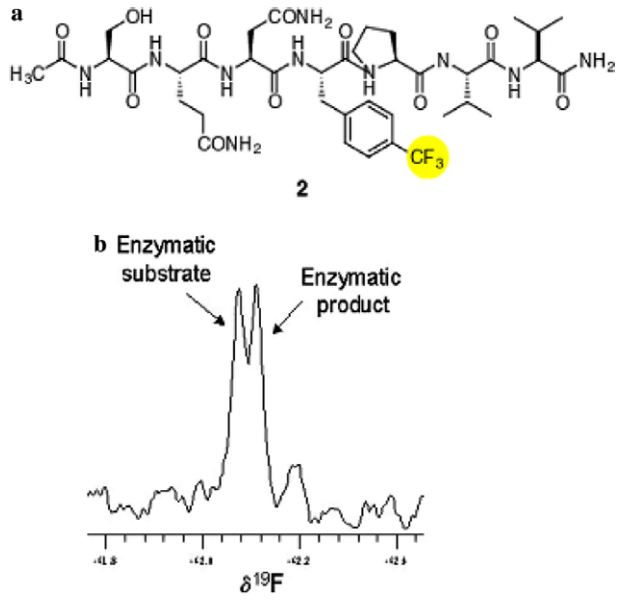

Figure 2. Substrate **2** and ^{19}F NMR spectra of its enzymatic reaction. (a) Fluorinated peptidyl substrate **2**. (b) ^{19}F NMR spectra recorded for the substrate **2** after incubation with purified HIV-1 protease.

peaks observed, with retention times of 7.72 and 8.78 min, correspond to the expected enzymatic product Ac-Ser-Gln-Asn-(4-CF_3)-Phe-OH and the uncleaved peptide, respectively. The other proteolysis fragment can be detected by HPLC, but its extinction coefficient is comparatively small and it typically is not detected under the conditions normally employed. The results were confirmed by mass spectrometry.

Inhibition assays: The rapid mutation of HIV to form drug-resistance strains, through the development of HIV-1 proteases that are inert to currently administered active site inhibitors,[23] has led to the development of an alternative inhibitory mechanism for AIDS treatment: 'dimerization inhibition.'[24] HIV-1 protease is a homodimeric protein composed of two identical subunits of 99 amino acids, whereby the catalytic site as well as the substrate-binding pocket are only formed when the protease is in its dimeric form.[25] Thus, molecules that interfere with the stability of the dimer have been shown to diminish the activity of the enzyme.

We tested the screening method with an active site inhibitor and a dimerization inhibitor to determine if it were capable of detecting compounds that function by both mechanisms.

The active site inhibitor **3** (Fig. 4a) is a hydrophilic tripeptide with a K_i value of ca. 3 mM. It has been studied by X-ray crystallography.[26] The dimerization inhibitor **4** (Fig. 4b), a tripeptide that contains an N-terminal palmitoyl group, has a K_i value of ca. 11 nM.[27]

For the synthesis of inhibitors, Barlos resin was used (nominal loading, 1.5 mmol/g) and the loading was

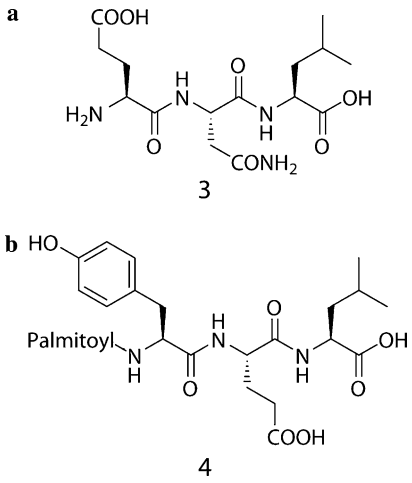

Figure 4. Synthetic inhibitors of HIV-1 protease. (a) Active site inhibitor, inhibitor **3**. (b) Dimerization inhibitor, inhibitor **4**.

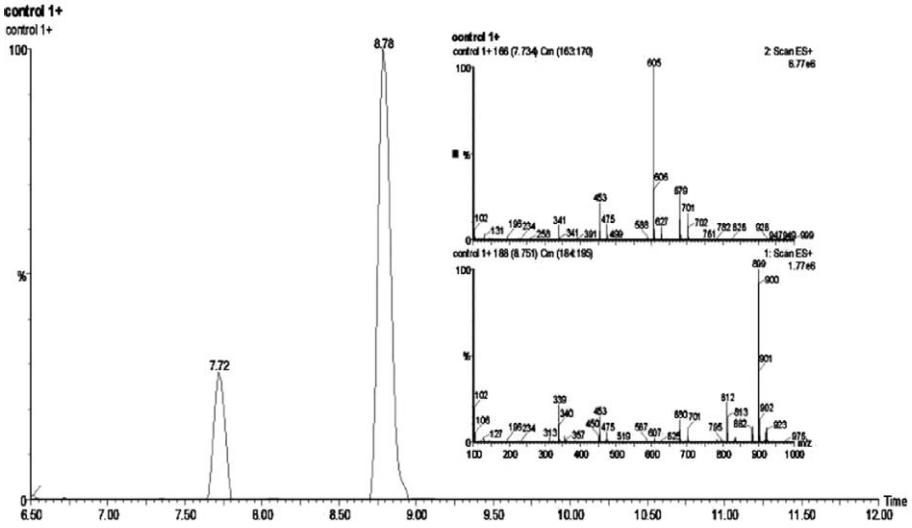

Figure 3. HPLC–MS chromatogram of the partial hydrolysis of substrate **2** by HIV-1 protease. Inset: ES+/MS spectra for the uncleaved peptide (top) and the product (bottom).

reduced to 0.75–0.9 mmol/g after partial incorporation of the first amino acid. Syntheses were carried out on a 100 μmol scale using Fmoc/^{t}Bu chemistry as above. For peptide **4**, the palmitoyl group was incorporated as for other amino acids using palmitic acid.

In order to study the inhibition, substrate **2** (200 μM) was subjected to the proteolysis in the presence (400 μM) and absence of each inhibitor. The resulting ^{19}F NMR spectrum (Fig. 5) indicated that the test method rapidly and reliably identifies both classes of HIV-1 protease inhibitors. In the case of inhibitor **3**, some time course of inhibitor work was carried out (Fig. 6) to prove that the apparent reduction in the amount of cleaved peptide is truly a reduction in the presence of **3**. Thus, the method allows detecting inhibitors from nanomolar to millimolar range.

Finally, we sought to determine if the method was amenable to complex mixtures of natural products. For these tests, we chose mixtures of products from Traditional Chinese Medicine (TCM), the therapeutic efficacy of which has been confirmed for several therapeutic areas by clinical studies.[28–30] TCM plants were obtained from Herbasin (Shenyang, China). 30 g of dried plant material was washed with double-distilled (dd) H_2O and ground. The resulting powder was extracted with 100 mL dd H_2O under reflux for 5 h using a Soxhlet apparatus. The aqueous extracts (20 extracts) were then freeze-dried under vacuum. Aqueous extracts of common TCM natural products were thus screened for their potential HIV-1 protease inhibitory activity. The final concentrations of the TCMs in the assay buffer were 100 μg/mL.

Among the extracts tested, *Cortex cinnamoni* (Jinjinapi in Chinese; TCM10) was found to have a potent inhibitory activity against HIV-protease, whereas, *Rhizoma coptidis* (Huanglian in Chinese; TCM16) had no inhibitory activity (Fig. 7). Data show that this methodology can be applied to screen complex mixtures of natural compounds. The fractionation and monitoring of the extracted molecules with inhibitory activity are currently

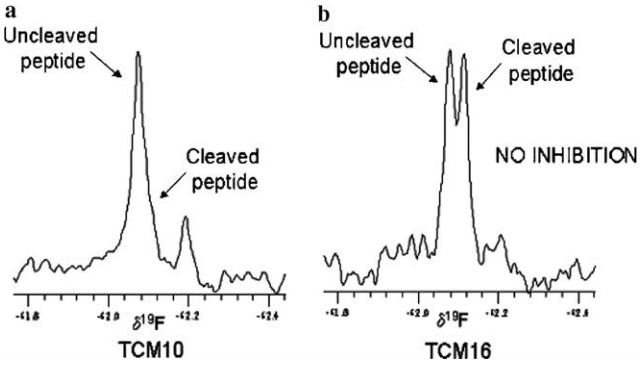

Figure 7. ^{19}F NMR spectra corresponding to the substrate **2** (a) in the presence of Traditional Chinese Medicine extract **10** (b) in the presence of Traditional Chinese Medicine extract **16**.

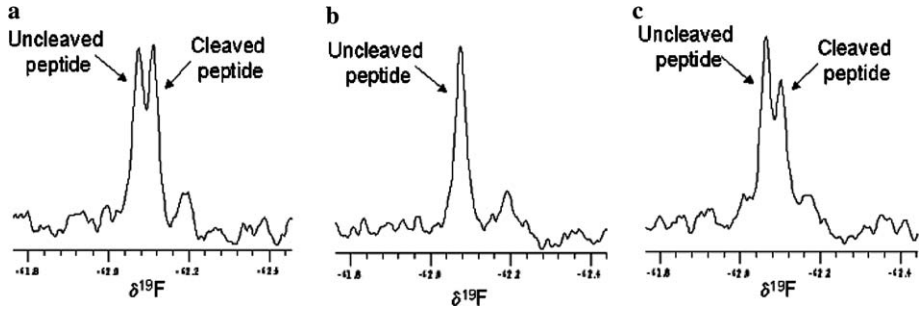

Figure 5. ^{19}F NMR spectra corresponding to the substrate **2** (a) in the absence of inhibitor; (b) in the presence of dimerization inhibitor, inhibitor **4**; (c) in the presence of active site inhibitor, inhibitor **3**.

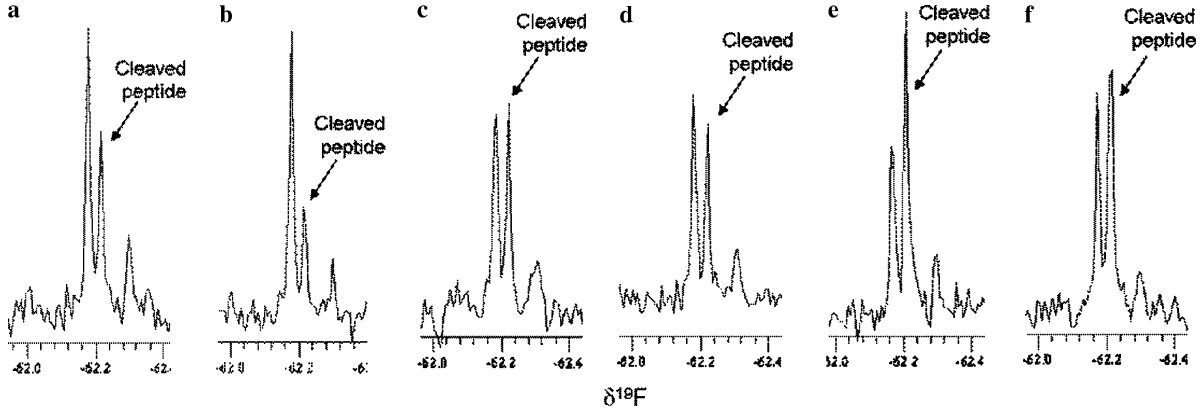

Figure 6. ^{19}F NMR spectra corresponding to the substrate **2** after incubation with purified HIV-1 protease for (a) 15 min in the absence of inhibitor; (b) 15 min in the presence of inhibitor **3** (c) 30 min in the absence of inhibitor; (d) 30 min in the presence of inhibitor **3**; (e) 1 h in the absence of inhibitor (f) 1 h in the presence of inhibitor **3**.

being carried out in our laboratory and fall out of the scope of this article.

NMR-based screening has emerged as a powerful tool for identification of molecules that interact with targets of interest. Here, we have prepared two new substrates of HIV-1 protease, which contain a CF_3 group. They were used as enzymatic substrate in the biochemical assays, the results of which were monitored by ^{19}F NMR. Using these substrates, we have developed a ^{19}F NMR methodology to identify inhibitory activity for the HIV-1 protease in a fast, reliable, and sensitive way, and without interferences common to other methodologies. The method permits the screening of complex natural product extracts as well as synthetic inhibitors, which may interact at the active site or destabilize the HIV-1 protease dimer. Thus, this new methodology facilitates the ever-lasting need to find new inhibitors for the HIV-1 protease.

Acknowledgments

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17. The cells were centrifuged and the resulting pellet was suspended in five volumes of lysis buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, and 100 mM NaCl. Cells were lysed by sonication and clarified by centrifugation at 20,000*g* for 30 min. The insoluble HIV protease was washed by resuspension with lysis buffer containing 0.1% Triton X-100, and subsequently, centrifuged for 30 min. This step was repeated twice. The final pellet of inclusion bodies was dissolved in 10 mL of 50 mM Tris–HCl, pH 8.0, containing 7 M guanidine–HCl, 5 mM DTT, 1 mM EDTA, and 100 mM NaCl.
18. The extract was loaded onto a Superdex 75 column equilibrated with 50 mM Tris–HCl, pH 8.0, 6 M urea, 5 mM DTT, 1 mM EDTA, and 100 mM NaCl. The purity of the protein was examined by Tris–tricine gel electrophoresis (15% acrylamide gel).
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