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In vitro synthesis of enzymatically active HIV-1 protease for rapid phenotypic resistance profiling

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Abstract

Background: Given the expanding antiretroviral therapy, inexpensive and fast HIV drug resistance assays are urgently needed. In this view, we have developed a novel phenotypic resistance test for HIV-1 protease inhibitors (PIs) based on recombinant expression of patient-derived HIV PR in *Escherichia coli* and subsequent enzymatic testing in a fluorescent readout.
Objectives: To facilitate and expedite the test procedure, we have introduced coupled in vitro transcription/translation using a commercially available technology called RTS for producing enzymatically active HIV-1 protease (PR).
Study design: We expressed one wild type PR and one highly resistant mutant starting from molecular clones as well as three patient-derived PRs. The amplified PR gene was either ligated into an expression vector or directly used as a template for the in vitro transcription/translation reaction. Enzymatic susceptibility data derived from in vitro expressed PRs were correlated to the respective results from *E. coli* expression and genotypic evaluation.
Results: All tested enzymes were obtained in sufficient quantities for complete resistance profiling to five PIs. The PRs required no purification prior to the enzymatic assay. Inhibition constants and enzymatic resistance factors compared well to corresponding data from PRs expressed in parallel in *E. coli*. Enzymatic resistance was in good agreement with the respective PR genotype.
Conclusion: The presented in vitro transcription/translation system represents a novel approach for HIV PR expression starting from molecular clones or patient samples. Coupled with the enzyme-kinetic PR assay recently developed in our group it allows to sensitively quantify resistance to PIs. The test system is significantly less laborious and faster than currently available phenotypic drug resistance assays.
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1. Introduction

Antiretroviral therapy – particularly modern regimens, including simultaneous administration of three or four drugs – lowered the mortality and improved the quality of life of HIV-infected individuals (Palella et al., 1998). At present, reverse transcriptase (RT) and protease are the main target enzymes for antiretroviral therapy. Resistance of these enzymes to the respective inhibitors is the most important factor limiting their efficacy (Richman, 2001). It is associated with mutations leading to amino acid exchanges in the protein. Depending on their localisation, these amino acid substitutions either directly affect the substrate binding or alter the conformation of the protein molecule. Drug resistance is particularly frequent in HIV because of its high mutation rate and long-term therapy. Due to the clinical significance of HIV resistance, many assays have been developed to detect and quantify it.

Genotypic assays determine the individual mutation pattern of the patient's virus population. These results are interpreted using extensive databases including well-characterized resistance mutations coupled with respective

Abbreviations: HIV-1, Human Immunodeficiency Virus Type 1; PI, protease inhibitor; RTS, Rapid Translation System; PR, protease; *E. coli*, Escherichia coli; RT, reverse transcriptase; IC, inhibitory concentration; K_i, inhibition constant; APV, amprenavir; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; WT, wild type; TBS, tris-buffered saline; NP-40, Nonidet P-40; DMSO, dimethylsulfoxide

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phenotypic and epidemiologic data. However, no standardized criteria are available on how to quantitatively interpret the sequence data, particularly in the presence of complex mutation patterns (Hanna and D'Aquila, 2001). As a consequence, significantly different resistance data can result from PR and RT sequences when investigated with various algorithms (Sturmer et al., 2003). Phenotypic assays measure the inhibitor's effect on HIV replication or on the activity of the target enzyme. All commercially available phenotypic tests follow the same concept: starting from reference clones or patient samples, RT and PR gene are amplified by PCR and cloned into HIV-1 vectors. These plasmids contain indicator genes to quantify viral replication (Petropoulos et al., 2000). There is a considerable risk of selection due to different replication characteristics of the constructs (Kusumi et al., 1992; von Briesen et al., 1999). Even without selection resistant subpopulations can be reliably detected only if present in a proportion >20% (Petropoulos et al., 2000; Hanna and D'Aquila, 2001). The inhibitory potency of a given drug is quantified by its concentration resulting in 50% (IC50) and 90% (IC90) inhibition of the virus replication or enzymatic activity. In enzymatic assays the inhibition constant K_i is a more significant parameter since it is enzyme-inherent and independent of the particular test conditions (Hoffmann et al., 2003). The target enzyme is either isolated from patient samples (Heneine et al., 1995) or recombinantly expressed (von der Helm et al., 1994; Gehringer et al., 2003). As the indications for resistance testing have been expanded during the past few years (EuroGuidelines Group for HIV Resistance, 2001), there is an increasing need for sensitive assays (Harrigan and Cote, 2000). Another aspect of growing interest is the detection of resistant quasispecies, even if they constitute only a minority in the patient's virus population (Van Laethem et al., 1999). In this view, we have recently developed a fluorescence test for enzymatic evaluation of HIV PR in 96-well format (Hoffmann et al., 2003). It allows K_i determination for the therapeutically used PIs amprenavir (APV), indinavir (IDV), nelfinavir (NFV), ritonavir (RTV), and saquinavir (SQV), and thereby offers a very accurate approach to drug resistance testing. The PR assay takes only 3–4 h including data analysis. Since the conventional expression of HIV PR in *E. coli* requires at least 3–4 days, we have sought faster alternatives. Coupled in vitro transcription/translation is a promising approach for HIV PR as it is a very small protein consisting of 99 amino acids and requiring no posttranslational modifications.

2. Materials and methods

2.1. Sample preparation

The tested patient samples originated from different sources within and outside of the University Hospital and had been sent to the Max von Pettenkofer Institute for genotyping. In vitro expression of patient-derived HIV PR was validated in terms of resistance testing by comparing the enzymatic data to the respective genotypic results. RT reaction and first PCR round were carried out conjointly for both methods, improving their comparability. RNA was extracted from 400 μl EDTA plasma using the High Pure Viral Nucleic Acid Kit (Cat. No. 1858 874, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instruction. PR and RT gene were reverse transcribed with the Superscript Reverse Transcriptase Kit (Cat. No. 18064-014, Invitrogen, Eggenstein, Germany) and the primer Geno 2 5′-GCYTGCCAATAI-TCYRTCCACC. The c-DNA was then amplified employing the primers Geno1 5′-GGCTGTTGGAAATGTGGAARRGA, Geno2, and AmpliTaq Gold polymerase (Cat. No. 4311818, Applied Biosystems, Weiterstadt, Germany). The resulting amplificate was used both for genotyping by sequencing and in vitro PR expression. Wild type (MvP-8999 WT) and mutant A (MvP-899/R1.5 μM) (Eberle et al., 1995) had been cloned in the expression vector pBD_2 (Broeker, 1986) and were directly amplified in the second PCR round.

2.2. Large-scale in vitro protein expression

In the nested PCR the PR sequence was amplified with the primers p3 GATCTGGCCTTCCTACAAGGGAAG and p4 GTCTACCAACGTGAAATTTAAAA. The amplificate was digested with NotI and BglII (Cat. Nos. 1014714 and 1175068, Roche Diagnostics), and ligated into the vector pIVEX2.4c (Cat. No. 3018 008, Roche Diagnostics), which had been restricted with NotI and BamHI (Cat. Nos. 1014714 and 0798975, Roche Diagnostics). The ligation product was transformed into *E. coli* TG1 cells (Cat. No. 200123, Stratagene, Heidelberg, Germany) (Dower et al., 1988), the resulting clones were selected and expanded in 10 ml LB/ampicillin (100 μg/ml) (Cat. No. 01503-2, Biomol, Hamburg, Germany) over night. The next day, 90 ml LB/ampicillin (100 μg/ml) was added to increase the amount of construct for plasmid preparation. Plasmid DNA was purified with Nucleobond PC 100 (Cat. No. 740573, Macherey und Nagel, Dueren, Germany) and 15 μg DNA was used per RTS 500 *E. coli* Circular Template Kit reaction (Cat. No. 3018 008, Roche Diagnostics). Protease expression was done in 1 ml total volume following the manufacturer's recommendation.

2.3. Small-scale HIV PR expression using linear template

To avoid cloning, PCR amplificate was employed as a template for in vitro transcription/translation. As with the large-scale expression, cloned reference strains or patient-derived PR sequences served as starting material. Two PCR amplification rounds were conducted with the RTS *E. coli* Linear Template Kit, His-tag (Cat. No. 3186237, Roche Diagnostics) following the manufacturer's instructions. The first round introduced an overlap region to the HIV

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