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A high throughput assay for inhibitors of HIV-1 protease

Screening of microbial metabolites

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A novel method for discovery of HIV-1 protease inhibitors in complex biological samples has been developed. The assay is based on two specific reagents: a recombinant protein constituted by a portion of the HIV-1 Gag polyprotein comprising the p17–p24 cleavage site, fused to *E. coli* β-galactosidase, and a monoclonal antibody which binds the fusion protein in the Gag region. Binding occurs only if the fusion protein has not been cleaved by the HIV-1 protease. The assay has been adapted for the screening of large numbers of samples in standard 96-well microtiter plates. Using this method about 12000 microbial fermentation broths have been tested and several HIV-1 protease inhibitory activities have been detected. One of these has been studied in detail.

Human immunodeficiency virus; Proteinase; Fusion protein; Immunological assay; Microbial alkaline protease inhibitor

1. INTRODUCTION

There is growing interest in developing specific inhibitors of the HIV-1 aspartyl protease as possible therapeutic agents in the treatment of AIDS [1,2]. Several different HIV-1 protease assays, based either on HPLC separations [3–7] or on chromophoric [8,9], fluorogenic [10–12] or radiolabeled [13–15] synthetic peptides have been developed in various laboratories to test a variety of compounds for inhibitory activity.

The 3D structure of the HIV-1 protease has recently been determined [16–18] and many investigators have made extensive use of the rational approach to design substrate analogs with specific inhibitory activity [19–23]. This has led to peptide derivatives which are potent inhibitors, but often suffer major drawbacks, e.g. scarce solubility in aqueous solutions, poor cell permeability, very rapid in vivo degradation, all factors causing serious limitations in bio-availability. As a consequence, there is at the present time a renewed interest in the screening of natural products for novel, possibly non-peptidic molecules which could display comparable inhibitory activity against HIV-1 protease and, at the same time, more interesting in vivo properties. In addition, in a random screening it is possible to discover inhibitors of dimer formation, a process which appears to be required for full HIV-1 protease activity [16–19,24].

With this in mind, we have used a biotechnology approach to develop an assay which is particularly suitable for the detection of HIV-1 protease inhibitors in large numbers of samples. The assay has been used to screen microbial fermentation broths and several inhibitory activities have been found.

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Abbreviations: HIV-1, human immunodeficiency virus type-1; HPLC, high performance liquid chromatography; IPTG, isopropyl-βD-thiogalactoside; PMSF, phenylmethylsulphonyl fluoride; β-ME, β-mercaptoethanol; TPEG, *p*-aminophenyl-βD-thiogalactoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethane-sulphonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PNPG, *p*-nitrophenyl-βD-galactoside; MAb, monoclonal antibody; α-MAPI, α-microbial alkaline protease inhibitor.

2. MATERIALS AND METHODS

2.1. *HIV-1 protease*

Recombinant *E. coli* expressing HIV-1 protease was kindly provided by Dr B. Harris [24]. For screening of fermentation broths the enzyme was routinely used as a crude preparation [24]. Briefly, after induction and harvesting, cells (50 g) were resuspended in 100 ml of 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.05% Triton X-100, sonicated and centrifuged at 10 000 × *g* for 20 min. HIV-1 protease activity in *E. coli* extracts was assayed by cleavage of a synthetic heptapeptide substrate and HPLC analysis of products (C. Tarnus, MMRDI, Strasbourg, personal communication), essentially as described [3]. A corresponding extract from *E. coli* cells lacking the HIV-1 protease expression vector was employed for controls.

2.2. *Expression and purification of gal-gag110*

The *Hin*dIII-*Hin*dIII fragment from bp 631 to 1258 of HIV-1 *gag* DNA [25] was subcloned into the unique *Hin*dIII site of pUC19 (Gibco BRL). From the resulting construct the *Pst*I-*Pst*I fragment containing 8 bp from the pUC19 polylinker (*Pst*I-*Hin*dIII) fused to the *Hin*dIII-*Pst*I fragment from bp 631 to 961 of HIV-1 DNA, was

isolated and inserted into the unique *Pst*I site of pUR292 [26]. This gave plasmid pGA22 in which the *gag* fragment is fused in frame to the end of the *lacZ* gene to encode a fusion protein. The structure of this 'gal-gag110' fusion protein is depicted in Fig. 1.

E. coli JM109(pGA22) was grown at 37°C in Luria Broth in the presence of 50 μg/ml ampicillin. At OD_{600} = 0.5 IPTG (1 mM) was added and after 2 h bacteria were harvested and resuspended in 1/50 volume of 50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA (Buffer A), containing 0.1 mM PMSF, 1 mM benzamidine-HCl, 0.1 M arginine and 1 mg/ml lysozyme. After 30 min at 4°C cells were disrupted by sonication and centrifuged for 10 min at 25 000 × g. The pellet was washed twice with Buffer A containing 70 mM β-mercaptoethanol (β-ME) and 0.05% Triton X-100 and then resuspended in 0.1 M Tris-HCl, pH 7.8, 0.1 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 70 mM β-ME (Buffer B), containing 8 M urea. After centrifugation (20 min at 50 000 × g), the supernatant was adjusted to 5 M urea by dilution with Buffer B, then extensively dialyzed against Buffer B without β-ME. Affinity chromatography on TPEG-Sepharose was performed essentially as described [27]. Protein purification was checked by SDS-PAGE using the PHAST-GEL system (Pharmacia, Sweden).

2.3. *Production of MAb 1G12*

BALB/c mice were immunized i.p. with 50 μg of gal-gag110 at days −60, −42, −30 and −18. At day −3 mice were boosted i.v. with the same protein. Spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells and hybridoma supernatants were screened by ELISA techniques for antibodies against gal-gag110 which did not bind β-galactosidase. Clone 1G12 was isolated and subcloned by limiting dilution technique. MAb 1G12 was identified as IgG_1 using the Mouse-Typer Sub-Isotyping Panel (Bio-Rad). A western Blot analysis confirmed that MAb 1G12 binds gal-gag110 with no detectable cross-reactivity with β-galactosidase.

2.4. *Immunoassay for detection of HIV-1 protease inhibitors*

After removal of microbial cells by centrifugation, 5 μl of fermentation broth were mixed in 96-well microtiter plates with 45 μl of 0.25 M MES/NaOH buffer, pH 6.0, 0.1 M NaCl, 0.6% w/v BSA, 0.025% v/v Tween 20, 1 mM sodium EDTA, 5 μg/ml leupeptin, 1 mM PMSF and 1% v/v *E. coli* extract containing recombinant HIV-1 protease (section 2.1). After 10 min of pre-incubation at room temperature to allow binding of a putative inhibitor to the viral enzyme, 25 μl of gal-gag110 fusion protein (50 μg/ml) were added to the wells and the microtiter plates incubated at 37°C for 40 min. 50 μl of reaction mixture were then transferred from each well into the corresponding well of a flat-bottom microtiter plate pre-coated with MAb 1G12.

These plates were coated by incubating 50 μl of MAb 1G12 (50 μg/ml in PBS) per well overnight at 25°C. Unreacted protein binding sites were blocked by incubation with 3% BSA in PBS (1 h, 25°C). Finally, wells were emptied, washed 4–5 times with PBS and pat-dried on paper towels. MAb 1G12-coated plates were usually used freshly made.

After addition of the reaction solutions, the MAb 1G12-coated plates were incubated for 2 h at 25°C to allow the selective binding of the uncleaved gal-gag110 to the antibody. The solutions were then discarded and the plates washed 4–5 times with PBS containing 0.05% Tween 20. To quantitate the amount of uncleaved gal-gag110, 240 μl/well of 1 mg/ml PNPG, a β-galactosidase-specific chromogenic substrate, in 50 mM sodium phosphate buffer, pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$ and 70 mM β-ME were added and the plates incubated for 1 h at 25°C. Finally, 60 μl/well of 1.5 M Na_2CO_3 were added to stop the β-galactosidase reaction and the absorbance at 405 nm was determined using a Titertek Multiscan microplate reader (Flow Labs).

3. RESULTS

3.1. *Production and characterization of gal-gag110*

In order to obtain an HIV-1 protease substrate which was specific and, at the same time, suitable for a simple solid-phase assay, a gene coding for a fusion protein was constructed. The resulting protein was engineered to contain the Ala^{100} to Ala^{210} fragment from HIV-1 Gag polyprotein (p55) fused to the carboxy-terminus of *E. coli* β-galactosidase (Fig. 1). The gag portion of this 'gal-gag110' fusion protein comprised the p17/p24 HIV-1 protease cleavage site Tyr^{132}–Pro^{133} [25], while the β-galactosidase portion provided an easily measurable enzymatic activity.

E. coli JM109 containing plasmid pGA22, in which the expression of the fusion gene was driven by the P_{lac} promoter, was induced with 1 mM IPTG during steady state growth. gal-gag110 protein accumulated in cells at concentrations that were estimated by Coomassie blue-stained SDS-PAGE to be as high as 40–50% of total protein content (not shown). After cell lysis and centrifugation most of the fusion protein was found in the precipitate, from which it was solubilized and quantitatively recovered with a denaturation-renaturation step. This procedure yielded a large amount of gal-gag110 (80–90% pure) which retained full β-galactosidase activity and was routinely used for HIV-1 protease assays of microbial fermentation broths. Pure gal-gag110 was obtained by affinity chromatography on TPEG-Sepharose, exploiting the binding properties of the β-galactosidase portion [27].

Fig. 2A shows the SDS-PAGE analysis of gal-gag110 fusion protein before and after incubation with recombinant HIV-1 protease. As incubation time increases the intact protein of about 130 kDa (lane b) is gradually replaced by the cleaved product (about 120 kDa, lanes d and e), as expected from the loss of an 80-amino acid fragment (Fig. 1). The cleavage reaction is strongly inhibited by pepstatin A (Fig. 2A, lane f), a known inhibitor of HIV-1 protease [28,29].

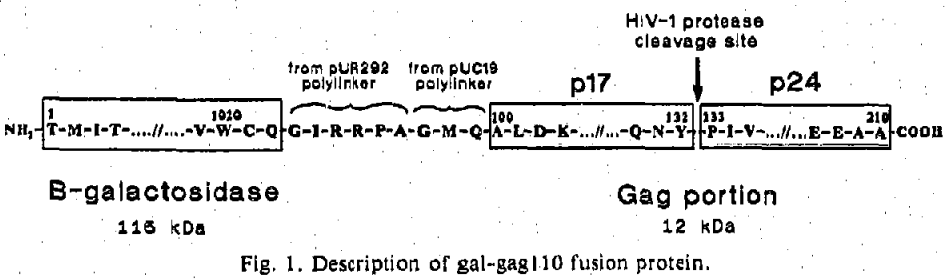

Fig. 1. Description of gal-gag110 fusion protein.

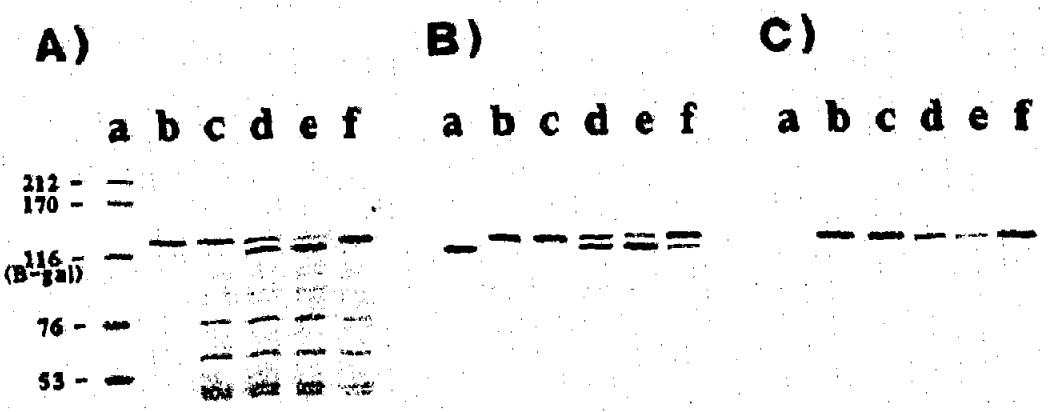

Fig. 2. Cleavage of gal-gag110 fusion protein by HIV-1 protease. (A) SDS-PAGE analysis. Renatured gal-gag110 (200 μg) was treated with 1 μl of crude HIV-1 protease preparation [24] in 100 μl of 0.2 M MES/NaOH buffer, pH 6.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 1 mM PMSF at 37°C. Lanes: (a) molecular mass standards, indicated in kDa; (b) gal-gag110, non-treated; (c) gal-gag110 treated for 60 min with a crude extract from *E. coli* cells lacking the HIV-1 protease expression vector (control); (d) and (e) gal-gag110 treated with HIV-1 protease for 30 and 60 min respectively; (f) same as (e), but in the presence of 0.5 mM pepstatin A (Calbiochem). SDS-PAGE was performed in a Phast System apparatus (Pharmacia), using 7.5% acrylamide gels, β-ME as reducing agent and Coomassie blue for staining. (B) Western Blot analysis of the same gel using an anti-β-galactosidase monoclonal antibody (Boehringer Mannheim, Germany) followed by anti-mouse AuroProbe BL (Janssen, Belgium). Lane order as in (A). (C) Western Blot analysis of the same gel using an anti-p24 polyclonal antibody (Biochrom, Germany) and anti-sheep AuroProbe BL. Lane order as in (A). Both immunoblots were treated with Intense BL silver enhancement kit (Janssen).

A Western Blot analysis with two different antibodies confirms the identity of the protein bands. As expected, an anti-β-galactosidase antibody recognizes both uncleaved and cleaved gal-gag110 (Fig. 2B), while an anti-p24 polyclonal antibody binds only to the uncleaved protein (Fig. 2C), since in the cleaved fusion the p24 portion has been cut off by HIV-1 protease.

3.2. *Characterization of MAb 1G12*

The gal-gag110 fusion protein was used as immunogen to produce monoclonal antibodies. MAb 1G12, was selected for its ability to recognize the intact antigen but not β-galactosidase, as indicated by both ELISA and Western Blot experiments (not shown).

When MAb 1G12 was analyzed with a Western Blot of a total HIV-1 lysate, containing all viral proteins, only the Gag precursor, p55, was recognized (Fig. 3). Neither p24 or p17 were bound by the antibody. This indicates that the epitope recognized by MAb 1G12 is indeed localized in the Gag portion of gal-gag110, but is destroyed upon cleavage by HIV-1 protease. This strongly suggests that the HIV-1 protease cleavage site is comprised in such an epitope region.

Experiments with MAb 1G12 immobilized on microtiter wells confirmed that the antibody binds only uncleaved and not HIV-1 protease-cleaved gal-gag110. In addition, its binding affinity was found to be suitable for an ELISA-type immunoassay, as indicated by the observation that the amount of bound antigen was not significantly reduced after repeated washing cycles.

3.3. *Screening of microbial metabolites for HIV-1 protease inhibitors*

Using recombinant HIV-1 protease, gal-gag110 fusion protein and MAb 1G12, a solid-phase immunoassay was devised to test microbial metabolites for HIV-1 protease inhibitors (see section 2 for description of the assay).

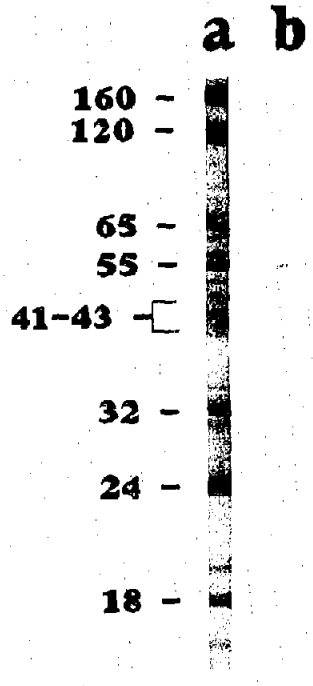

Fig. 3. Characterization of MAb 1G12 by Western Blot analysis of total HIV-1 proteins. Novapath Immunoblot strips (Bio-Rad, Richmond) were used. Lanes: (a) human serum (1:100 dil.) from an AIDS patient followed by anti-human AuroProbe BL (Janssen); (b) MAb 1G12 (1 μg/ml) and anti-mouse AuroProbe BL (Janssen). In both cases silver enhancement (IntenSE BL, Janssen) was performed.

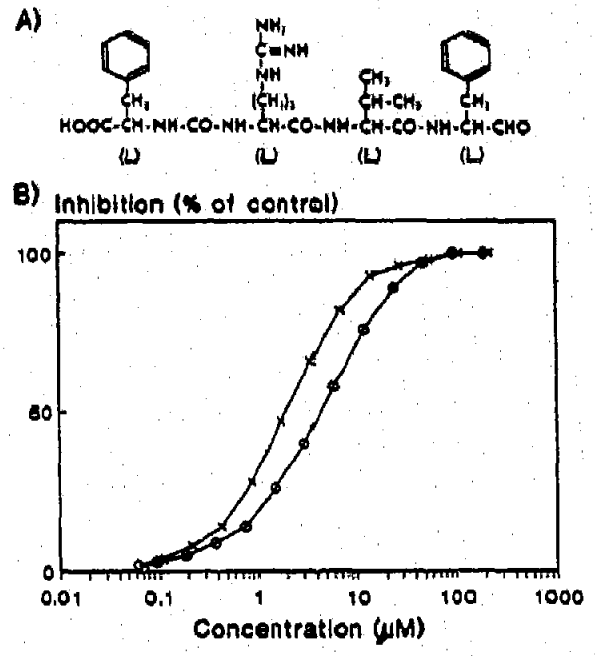

Fig. 4. (A) Chemical structure of α-MAPI [31]. (B) Dose-response plot showing the inhibition of cleavage of gal-gag110 by HIV-1 protease observed in the presence of the indicated concentrations of (x) α-MAPI or (o) pepstatin A (Calbiochem). The assay was performed essentially as described in section 2, in the presence of 5% DMSO. Determinations were from two separate experiments.

With this detection system about 12 000 fermentation broths were tested for the presence of HIV-1 protease inhibitors. These broths were derived mostly from Actinomycetes genera or fungal isolates. About 0.25% of them were found to contain significant and reproducible inhibitory activities against the viral enzyme. Among these activities one which showed good stability even after 1 h of incubation with horse or bovine serum was selected and the protease inhibitor was purified and characterized. The chemical structure determination allowed its identification as a peptide derivative of known structure, previously named α-microbial alkaline protease inhibitor, or α-MAPI (Fig. 4A) [30,31]. Fig. 4B shows the inhibition of the HIV-1 protease reaction observed at different concentrations of α-MAPI and pepstatin A. These two natural products display similar dose-response curves with 50% inhibition of substrate cleavage observed at 2.0 μM α-MAPI and 4.5 μM pepstatin A.

4. DISCUSSION

A novel solid-phase immunoassay has been developed to detect HIV-1 protease inhibitors in large numbers of biological samples. For such purpose the system described is fast and efficient and it has been used to screen about 12 000 fermentation broths. The most interesting aspect of this method is the ability to detect the presence of an HIV-1 protease inhibitor by the appearance of an enzymatic activity (i.e. β-galactosidase) instead of the disappearance of the protease activity. This provides an internal control: a positive response (colour) is obtained only when the protease reaction is inhibited. All other possible interfering activities (e.g. inhibition of MAb 1G12 binding, non-specific proteolysis of gal-gag110 or MAb 1G12, inhibition of β-galactosidase activity, etc.) would result in a negative response. Consequently, such an assay is particularly suitable for the screening of complex mixtures like microbial fermentation broths, where false positives are often a serious problem.

However, the detection system described here can be used to screen compounds from any origin for inhibitory activity on HIV-1 protease. The assay is also relatively unaffected by low concentrations (5–10%) of organic solvents like methanol, acetonitrile or DMSO, a feature particularly useful when it is used to monitor the purification of an inhibitory activity.

Using this detection system about 12 000 fermentation broths were screened and several positives were found. One of these inhibitory activities was purified and identified as α-MAPI [31]. This finding was largely unexpected since this compound, as expressed in its name, had been previously characterized as an inhibitor of alkaline proteases, with no activity on pepsin or other aspartic proteases [30]. Nevertheless, as shown in Fig. 4B, the inhibitory activity of α-MAPI on HIV-1 protease is comparable to pepstatin A, a characteristic inhibitor of aspartic proteases.

As shown in Fig. 4A, α-MAPI has a peptide-like structure with a C-terminal aldehyde group, like another natural product, tyrostatin, which has recently been reported to inhibit some acidic proteases [32]. In preliminary kinetic studies using purified HIV-1 protease and an HPLc assay based on a synthetic heptapeptide as substrate [3], α-MAPI was found to act as a non-competitive inhibitor (C. Tarnus, personal communication). These observations suggested the direct involvement of the C-terminal aldehyde group in the inhibition mechanism. Such a prediction was confirmed by the loss of inhibitory activity on HIV-1 protease observed after treating α-MAPI with permanganate (S. Stella, unpublished results) in conditions known to cause the selective oxidation of the aldehyde to carboxylic acid [31].

Studies are currently in progress to elucidate the mechanism of inhibition of HIV-1 protease by α-MAPI. At the same time other HIV-1 protease inhibitors found in microbial metabolites are being characterized, with the goal to find novel structures with improved in vitro and in vivo anti-HIV-1 properties.

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