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# Screening of Inhibitors of HIV-1 Protease Using an *Escherichia coli* Cell Assay

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**To evaluate the available peptidic and pseudopeptidic inhibitors of HIV protease for their possible *in vivo* activity, a screening test using *Escherichia coli* was established. *E. coli* cells carrying the plasmid pET9c-PR containing the gene for HIV-1 protease under the control of a T7-promotor are grown in the absence and in the presence of inhibitors. The action of the toxic protease produced by the cells is counteracted by the inhibitors. Provided sufficient membrane permeability of the inhibitors exists, this results in accelerated cell growth. From the peptides known to be active in an *in-vitro* enzyme test, most compounds inhibit HIV protease only to a limited degree in this test. However, two short peptides (Ac-Ser-Tyr-Glu-Leu and Lys-Ile-Ser-Tyr-Asp-Tyr) protect cell growth to an considerabe extent, thus indicating that they reach the *E. coli* cytosol and there block HIV protease. Two pseudopeptides known to be very potent in the enzyme test (SDZ PRI 053 and CIBA 61755) also inhibit HIV-1 protease strongly in this cell growth test.** 

Among the present approaches to develop anti-HIV drugs those directed against HIV-protease (PR) activity seem to be most promising. The main reasons are: firstly, the inhibition of PR abolishes infectivity of the HI-virions (1,2) even if the PR activity is not completely silenced (3) and, secondly, inhibitors of the viral PR do not strongly affect the activity of cellular aspartic proteases. This should be especially true for such PR inhibitors which exploit the dimeric structure of PR (4,5) by interfering with dimer formation or stability. This target of intervention is present only in retroviral aspartic proteases with their unique composite active sites. Since the main contribution to dimer stability has been shown to derive from the 'interface' part of PR consisting of the four terminal amino acid segments, synthetic peptides with similar amino acid sequence-possibly competing with the subunit segments-have been investigated (6-8). Such peptides show inhibition of the enzymatic activity of PR in the low $\mu$M range and below.

Peptides are usually of restricted value for therapeutic purposes because of their limited ability to penetrate cell membranes (and from other reasons). The cell permeability of peptides and pseudopeptides should therefore be evaluated prior to more expensive and time consuming tests. We modified a published test (7-9) to screen peptides known to inhibit PR (6) for their activity in living cells. HIV-1 PR is a toxic protein when expressed in *E. coli* since many cellular proteins are cleaved by PR. If cells are transformed with a plasmid, which encodes PR under the control of a (induced or leaky) promotor, cell growth is arrested. Although most of the recombinant PR forms inclusion bodies, some of the PR seems to fold properly yielding functional-and toxic-PR. Our test uses *E. coli* cells which carry the plasmid pET9c-PR coding for PR. Such cells produce small amounts of protease and grow only slowly. They show improved growth rates, however, if a potent inhibitor is added to the medium and if this inhibitor is able to penetrate the cell wall and the inner and outer membranes of *E. coli.*

## MATERIALS AND METHODS

*Materials.* All organic compounds were purchased from Sigma-Aldrich Chemie, D-89555 Steinheim. PR was obtained by bacterial expression in *E. coli* (6, 10). The peptides were purchased from Neosystem (Strasbourg, France) or were synthesized in our laboratory (6).

*Enzyme inhibition assay.* HIV-1 PR inhibition was measured using recombinant HIV-1 PR and the nonapeptide substrate H-Lys-Ala-Arg-Val-Nle*(p-nitro-Phe)-Glu-Ala-Nle-$NH_2$ (10). The spectrophotometric determination was carried out as described in (6) at pH 4.7.

*Cell assay. Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI, USA) was used as host organism. The plasmid pET9c-PR

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Abbreviations used: HIV, human immunodeficiency virus; *E. coli, Escherichia coli;* PR, HIV-1 protease.

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contains cDNA coding for the HIV-1 protease under the control of the T7-promotor. The resistance against ampicillin was replaced by the resistance against kanamycin. The plasmid pET9c-PR was transformed in competent *E. coli* BL21(DE3)pLysS and transformants selected on a LB-plate with 50 mg/l kanamycin and 34 mg/l chloramphenicol. As most of the peptides are relatively insoluble in water, they were dissolved in DMSO and diluted to a final concentration of 1% DMSO. This concentration was found to be not toxic for the bacterial strain used here (data not shown). In some cases, the low solubility of the compounds did not allow concentrations above 10 $\mu$M in the media. In these cases stock solutions of 1 mM were used. The final concentrations were 0 (zero), 20 and 60 $\mu$M IPTG for the induction of expression. The mean generation times in the absence of inhibitors were detected as 75, 120 and 240 min in precence of zero, 20 and 60 $\mu$M IPTG.

Fifty microliters of overnight culture (optical density between 0.9 and 1.1) were diluted into 440 $\mu$l fresh LB-medium containing 50 mg/ml kanamycin and 34 mg/ml chloramphenicol. 5 $\mu$l inhibitor-solution (or pure DMSO for the reference value) and 5 $\mu$l IPTG-solution (0, 2 or 6 mM in water) were added and the samples were shaken at 37°C in 3 ml vessels. After 60 to 90 minutes, 100 $\mu$l of these cultures were transferred into wells of an ELISA plate and the optical density was determined at 490 nm in an ELISA reader. All measurements were performed in triplicate for up to 6 hours after starting the fermentation.

The obtained optical densities were fitted to the equation $y=\exp(A \times t) \times B$ (y: absorption at 490nm, t: time in minutes) using the software 'GraFit 3.01'(Erithacus Software Ltd.). The calculation of the generation time follows the equations: $T=\ln(2)/A$. The ratio of the generation times of cells grown with an inhibitor and cells without inhibitor under the same conditions and made from the same overnight culture gives the relative change in growth.

**TABLE 1**

Relative Growth Improvement of *E. coli* Cultures by Peptidic and Pseudopeptidic Inhibitors of HIV-1 Protease

| Compound | $M_r$ | $IC_{50}$** [$\mu$M] | Conc. [$\mu$M] | R.G.I.*** |
|---|---|---|---|---|
| Ac-YDL | 467 | 34 | 100 | 8% |
| Ac-YEL | 467 | 34 | 500 | 6% |
| YEL | 467 | 34 | 100 | 8% |
| Ac-SYEL | 552 | 55 | 100 | 31% |
| ISYEL | 624 | 4.5 | 100 | 6% |
| Ac-ISYEL | 665 | 3.6 | 500 | 0% |
| TVSYEL | 711 | 12 | 100 | 10% |
| KISYDY | 787 | 2.5 | 20 | 28% |
| KKISYDY | 916 | 9 | 100 | 0% |
| Pam-TVSYEL | 949 | 0.5 | 10 | 7%* |
| Pam-TISYDW | 1020 | 0.22 | 20 | 4% |
| Pepstatin A | 686 | 0.1 | 100 | 8% |
| SDZ PRI 053 | 722 | n.d. | 3 | 24%* |
| CIBA 61755 | 660 | 0.004 | 10 | 58% |
| | | | 15 | 110% |

*Note.* We list the synthetic peptides and pseudopeptides, their molecular mass, the $IC_{50}$ values as determined in the *in-vitro* enzyme assay and the relative growth improvement at the respective concentrations in the assay employing *E. coli* cells. Ac-, acetyl-; Pam-, palmitoyl-; *, insoluble at concentrations >10 mM; **, as determined by the enzyme test; ***, the relative growth improvement ("R.G.I.") in % was determined by measuring the turbidity at 490 nm in comparison to the untreated cells.

## RESULTS AND DISCUSSION

As references, two pseudopeptidic protease inhibitors-SDZ PRI 053 (12) and CIBA 61755 (13)-developed by Sandoz and Ciba-Geigy, respectively, were used. They showed effective protection of the cells against the toxicity of PR at low concentrations, resulting in high growth rates (Table 1). Pepstatin A, the standard inhibitor of aspartic proteases, was only of moderate activity, confirming results of Sedlácek et al. (10).

In this cell test, protection comparable with that of pepstatin A was detected with most of our peptides although pepstatin A is a much better PR inhibitor in the enzyme test with an $IC_{50}$ constant of about 0.1. A stronger improvement of the growth of PR containing cells is found with some of our other compounds. Especially two peptides, the acidic acetyl-Ser-Tyr-Glu-Leu-OH and the neutral H-Lys-Ile-Ser-Tyr-Asp-Tyr-OH, are much better than pepstatin A with relative growth values comparable to those of the pseudopeptides. The relative growth inhibition ("R.G.I.", Table I) of these peptides was of the same order of magnitude as that of the SDZ and CIBA pseudopeptides, although the $IC_{50}$ values of the pseudopeptides in the *in-vitro* test were much lower. This indicates that lower inhibition activity may be compensated by higher uptake of the peptides by the cells. This holds true also for the contrary: the palmitoylated peptides were quite efficient in the enzyme assay (in comparison to the short peptides) but did not block *E. coli* growth to the same extent. We surmise that they may not reach the cytoplasma because the fatty acid moiety inserts into the membranes and traps the peptide at the surface of the outer membrane or in the periplasm. By the addition of anionic phospholipids, improved membrane insertion of such peptides could possibly be mediated as described by Leenhouts et al. (16).

Tests with the peptide H-Lys-Ile-Ser-Tyr-Asp-Tyr-OH and HIV-1 infected human T lymphocytes (HUT-78) showed limited antiviral activity (Gürtler, L. et al., to be published). Thus, the inexpensive *E. coli* test supplies a method to select *in-vivo* active PR inhibitors in a fast and simple way. Although the cell entry mechanism into *E. coli* may differ in some aspects from the uptake of peptides by human lymphocytes, it is likely that substances, which are effective in this *E. coli* test, also possess antiviral activity in the HIV replication inhibition assay in lymphocytes.

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