Development of a Novel Screen for Protease Inhibitors

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We have developed a novel plasmid-based, quantitative, in vitro screen to test the protease-inhibiting activities of existing and newly discovered agents.

Since the onset of the AIDS epidemic, a number of antiretroviral drugs have been developed for the treatment of human immunodeficiency virus type 1 (HIV-1) infection. While the initial target for therapy was the viral reverse transcriptase, inhibitors targeting the viral protease (PR) enzyme have become a mainstay of antiretroviral therapy. Although use of these compounds in multidrug regimens has dramatically reduced viral load as well as morbidity and mortality, their long-term benefit in HIV-1-infected patients has been limited by the emergence of drug-resistant viral strains. The high rate of mutation of HIV-1 coupled with incomplete viral suppression and widespread use of this class of drugs will continue to contribute to this problem. For this reason, it is essential that new drugs targeting PR, as well as new viral targets, be developed.

Here we report on the development of an in vitro screen to test the potential PR-inhibiting activities of different agents. Our system utilizes a responder plasmid transcribing several recognition sites of the HIV-1 PR linked to a reporter gene encoding a protein with very low basal activity. In the presence of active viral PR the polyprotein encoded by the responder plasmid is cleaved at the PR recognition sites, resulting in a marked increase in activity of the reporter gene-encoded protein. In the absence of active PR or in the presence of both PR and viral PR inhibitors, the reporter gene-encoded protein activity is the same as that at basal levels. Because the reporter gene in this system is luciferase, there is a quantitative relationship between PR-inhibitory activity and reporter gene-encoded protein activity. While other systems that can be utilized to screen for PR-inhibitory activity have been developed, our system is novel in that it does not use infectious material and shows direct quantification of PR activity in a single, eukaryotic cell culture assay (4, 6, 7, 8).

Several plasmids were constructed and utilized in the development of this assay. A 216-nucleotide (nt) fragment of DNA corresponding to amino acids 349 to 421 of the Gag protein sequence from the HXB2c (Los Alamos) molecular clone of HIV-1 was amplified. This sequence contains three cleavage sites of the HIV-1 PR: p24CA-p2, p2-p7NC, and the recognition site p2. The first two cleavage sites correspond to sites of proteolytic cleavage by the viral PR during the normal maturation process, while the p2 cleavage site produces a further product of partial proteolysis. The specific primers used to amplify were P1 (5'-GAA GAT CTA GCA TGT CAG GGA GTA GGA GGA-3') (sense) and P2 (5'-AGG GTC ACC GTG TGC CCT TCT TTG CCA CA-3') (antisense), where the bases corresponding to nt 1830 to 1852 (P1) and nt 1970 to 1987 (P2) in the HXB2 viral DNA sequence (Los Alamos) are underlined and the BglII site (P1) and BstEII site (P2) are in boldface. After amplification, the DNA fragment was digested with BglII and BstEII and inserted in frame and upstream of the coding sequence of the luciferase gene derived from Photinus pyralis in the BglII- and BstEII-restricted pSP-Luc+NF fusion vector (Promega, Madison, Wis.), producing the pPC-Luciferase plasmid. To obtain the pEGFP-PC-Luciferase plasmid (Fig. 1), the fragment of DNA corresponding to the cleavage sites of the HIV-1 PR and the luciferase protein in the pPC-Luc plasmid were digested with BglII and XhoI and inserted in the corresponding sites in frame and downstream of the enhanced green fluorescent protein (EGFP) sequence in the pEGFP-C3 plasmid (Clontech, Palo Alto, Calif.). The resulting responder plasmid, pEGFP-PC-Luciferase, encodes a single polyprotein consisting of EGFP, the recognition sites of the PR, and luciferase under the control of the cytomegalovirus immediate-early promoter. Plasmid pRL-TK vector (Promega), Fig. 1 containing the luciferase gene derived from Renilla reniformis driven by the thymidine kinase promoter, was used in cotransfection experiments to normalize for transfection efficiency. The luciferases expressed from the responder plasmid (luciferase-1) and the control plasmid (luciferase-2) are active in different buffers, allowing their individual activities to be measured in a single sample. In addition, each of the luciferase genes is driven by a different promoter to avoid promoter competition and/or interference. The plasmids LTRPR, LTR2XPR, and LTR4XPR, expressing the monomer, dimer, and tetramer, respectively, of the viral PR under the control of the HIV-1 long terminal repeat (Figure 1), were obtained from Arrigo et al. All transfection experiments were performed in HeLa-Tat cells, which provide the Tat protein of HIV-1 in trans. HeLa-Tat cells were obtained from the American Type Culture Collection (Manassas, Va.) and kept in Dulbecco modified Eagle medium complete medium (Life Technologies, Gaithersburg, Md.) containing 10% fetal calf serum (Biofluid, Rockville, Md.), 2 mM glutamine (Gibco-BRL), 240 U of penicillin per ml, and 120 μg of streptomycin per ml. For transfection experiments HeLa-Tat cells were seeded at a con-
centration of $10^5$ cells/well in six-well plates (Costar, Cambridge, Mass.) the day before transfection. The next day cells were transfected using the Profection calcium phosphate transfection system (Promega) according to the manufacturer's instructions. A total of 6 μg of DNA was used, as follows: 0.5 μg of the normalizing plasmid pRL-TK, 1 μg of the responder plasmid pEGFP-PC-Luciferase, and 4.5 μg of plasmid LTRPR, LTR2XPR, or LTR4XPR. On days 1, 3, and 4 after transfection, cells were lysed with passive lysis buffer (Promega) and analyzed for luciferase-1 activity from the reporter gene construct pEGFP-PC-Luciferase and subsequently for luciferase-2 activity derived from the internal control vector pRL-TK using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. In all of the experiments luciferase-1 activity derived from the responder plasmid was normalized to the amount of luciferase-2 activity derived from the pRL-TK vector. Each experiment included duplicate wells and was repeated five times.

Recovery of luciferase-1 activity from the pEGFP-PC-Luciferase responder plasmid at day 1 posttransfection was the
same in the presence or absence of PR expression and therefore represents basal responder plasmid activity (data not shown). However, at day 3 posttransfection (and day 4 [data not shown]), an increase in luciferase activity was evident in the presence of all of the PR-expressing plasmids (Fig. 2). Furthermore, the magnitude of the increase was related to the number of copies of the PR genes. Cotransfection of the responder plasmid expressing either a single copy (LTRPR), two copies (LTRX2PR), or four copies (LTRX4PR) of the PR gene resulted in an increase in luciferase activity of 359, 463, and 646%, respectively, over that detected in the absence of any PR. The enhanced activity seen with multiple copies of the PR gene is consistent with previous observations (1). Furthermore, these data suggest that the enzymatic activity of luciferase encoded by the responder plasmid is partially blocked in the context of the chimeric polyprotein and that the PR expressed by any of the PR-expressing plasmids acts on the cleavage site of the PR and results in the release of a more active luciferase.

In order to prove the direct role of PR activity in luciferase modulation, we next determined whether our system could be utilized to screen drugs for PR-inhibiting activity. PR inhibitors should block cleavage of the reporter plasmid-encoded polyprotein, resulting in only basal levels of luciferase activity. We tested our screening system using two known PR inhibitors, Ritonavir and Nelfinavir (a gift from Agouron Pharmaceuticals, Inc., La Jolla, Calif.). HeLa-Tat cells were plated at a confluence of 2.5 \times 10^4 cells/well in 24-well plates (Costar) the day before transfection. These experiments have been scaled down compared to those previously performed in six-well plates, to allow for the use of less DNA and fewer cells. The experiments could be scaled down further to 96-well plates, to allow for the use of even smaller amounts of DNA and fewer cells, making the use of this system for large-scale screening of drugs more feasible. The day of transfection, cells were washed and concentrations of drug spanning known active concentrations were added to the cells (5). The cells were transfected in the presence of the drugs, as described above, with a total of 1.5 \mu g of DNA, maintaining a ratio of responder plasmid to PR plasmid of 1:4.5. In these experiments only the LTRX4PR plasmid was used, as it was found to have the greatest PR activity in previous experiments. One day after transfection, cell lysates were assayed for both luciferase-1 and luciferase-2 activities. As can be seen in Fig. 3, as the drug concentration was decreased there was a corresponding increase in luciferase activity, consistent with PR activity being blocked at the higher drug concentrations and active at the lower concentrations. It was also seen that at the highest concentrations of Nelfinavir and Ritonavir, the luciferase activity in the presence of LTRX4PR was maintained at levels equivalent to those seen in the absence of PR (data not shown). These results indicate that luciferase activity is proportional to PR activity. Repeated experiments carried out with each of the drugs generated similar dose-response curves. Experiments were performed to determine the effects of different ratios of responder plasmid to PR plasmid DNA on the 50% inhibitory concentrations of the PR inhibitors. We were able to demonstrate that the 50% inhibitory concentrations of the PR inhibitors tested reach a plateau at the ratio utilized in this report (1:4.5) and remains at the plateau at ratios with higher PR plasmid DNA input (data not shown). This plateau is likely due to the fact that the PR plasmid saturates the responder plasmid substrate. Of note, in both experiments levels of luciferase activity are increased in the presence of 0.001 and 0.003 \mu M Ritonavir and Nelfinavir, respectively, over the luciferase activity levels seen in the complete absence of drug. This phenomenon is present in all experiments with Ritonavir but in only about 50% of those with Nelfinavir. It is possible that the PR inhibitors may, at a very low concentration, be activating the PR. It has been shown that some drugs which inhibit protein activity at high concentrations can cause an increase in activity at lower concentrations (3, 9).

In conclusion, we have developed a novel assay that can be used to identify new PR-inhibitory drugs. Alternative methods for measuring PR activity have been developed (4, 6, 7, 8). The system described here allows for the rapid and reproducible quantification of luciferase activity that is directly proportional to PR activity. A cell culture system is used, but no infectious virus is necessary. While this system provides a means to screen for PR inhibitors, it has other potential applications. Many other pathogens also encode a PR whose activity is essential to their replication, and the system described here could be utilized to screen for potential inhibitors of these PRs. This could easily be accomplished by replacing the HIV-1 PR gene within the PR plasmids with a PR gene from another virus and changing the PR recognition sites present in the luciferase responder.
plasmid. For example, one such target is the serine PR of hepatitis C virus, for which the cleavage site is known (2).

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