HIV protease inhibitors, mutations, viral load

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INTRODUCTION

One of DNA's main functions is to provide the instructions for making proteins. Proteins are so versatile that they are the primary structural elements of all living things including viruses. With the exception of a few peculiar RNAs, all enzymes are protein. Enzymes are the biological molecules that get things done by acting on other molecules called substrates.

One of the largest classes of enzymes are the proteases. On the surface, proteases perform one of the simplest of biological reactions: they clip proteins into smaller proteins or peptides. Peptides are just short pieces of proteins. The point at which a peptide is elevated to the exalted status of a protein is arbitrary. Proteases that cleave primarily proteins are called proteinases, whereas proteases that cleave primarily peptides are called peptidases. For our purposes HIV protease can properly be called a proteinase since its primary substrate is the Gag-Pol polyprotein coded for by the HIV proviral DNA.

The 20 or so amino acids are linked together through amide bonds to make all the proteins and peptides that exist. The amide bonds of proteins have been given the special name of peptide bonds to signify that they belong to proteins and peptides. Peptide bonds are very strong chemical bonds and are difficult to break. Nonetheless, under the right conditions proteases can easily break peptide bonds. An every day example is the very active cysteine protease called chymopapain that comes from the papaya plant and is the active ingredient in meat tenderizer. Chymopapain breaks the peptide bonds of meat before it is cooked. The aspartyl protease pepsin in your stomach and the serine proteases chymotrypsin and trypsin in your small intestine digest the proteins you eat. It turns out that proteases are far more complex and interesting than this simple picture implies. The vast majority of proteases are involved in the processing and regulation of other proteins including other enzymes.

Proteases are globular proteins with an indentation or cavity called the active site. Substrates fit into the active site of the protease where the enzyme catalytically breaks the specific peptide bonds to be cleaved. A large number of proteins act as specific inhibitors of proteases, regulating their activities. There are also many tens of thousands of small synthetic inhibitors designed in laboratories around the world to stick tightly to the active sites of many different proteases. The synthetic inhibitors are used most often as research tools and occasionally as potential therapeutic agents.

Proteases have been divided into four main classes according to the active site features that are common to each group: serine, cysteine, aspartyl, and metallo. These designations have nothing to do with the substrates the proteases cleave. HIV protease belongs to the aspartyl family of proteases. Examples of human aspartyl proteases are

pepsin (a digestive enzyme in the stomach), cathepsin D (located within lysosomes inside cells), and renin (one of the proteases that regulates blood pressure).

HIV PROTEASE

Everything that is known about HIV has been constructed from in vitro experiments. It is important to recognize that all of the published statements concerning HIV's presence and activity are extrapolations from the in vitro studies and have not been directly observed in humans.

HIV, like all retroviruses, contains genes that code for structural proteins, envelop proteins and enzymes. During translation the individual proteins are all stuck together end-to-end via peptide bonds to form the Gag-Pol polyprotein. It appears that the envelop proteins are severed from the polyprotein by a host protease and not by HIV protease. The viral structural proteins (coded for by the gag region of the gene) and the viral enzymes (including HIV protease, coded for by the pol region of the gene) are connected by peptide bonds that must be processed (cleaved) by HIV protease.

HIV protease doesn't clip every peptide bond in sight; it is very particular about the sites of cleavage. Of the hundreds of possibilities, there are only eight specific peptide bonds of the Gag-Pol polyprotein that HIV protease must cleave for the virus to replicate and mature properly into infectious particles (1) .

The production of infectious HIV particles is dependent on proper assembly of structural proteins into the core particle. The initial steps in assembly involve the association of the Gag and Gag-Pol precursor proteins with the inner face of the membrane of the infected cell, followed by interaction of the precursors with each other. The membrane-based association of the precursor proteins precedes cleavage of the precursors by HIV protease. HIV protease is part of the larger Gag-Pol polyprotein and is only functional as a dimer. This arrangement allows the precursor proteins to arrive at the membrane in a coordinated manner and is largely successful in preventing premature activation of HIV protease. Once bound to the membrane, HIV protease cleaves the individual precursor proteins in an ordered, sequential manner (2-6) . If all goes well, budding and release of the mature, infectious virus particle occurs, leading to a new cycle of infection and viral replication.

Disrupting the proteolytic processing of HIV precursor proteins is an excellent strategy for blocking the production of infectious virus. Incomplete processing of the precursors by HIV protease still leads to budding but the viral particle produced is not infectious. That is why in the presence of inhibitor infected cells are still capable of producing viral particles, but the virus produced is defective and not infectious (2,7) . The central question is will inhibiting HIV be of therapeutic benefit?

INHIBITOR-RESISTANT MUTANTS: A MIRAGE

Numerous in vitro experiments have demonstrated that impaired proteolytic activity, due either to the presence of protease inhibitors (2,7-9) or deleterious mutations of HIV protease (10-15) , results in noninfectious HIV particles. As a consequence of these studies and several human clinical trials (16-19) , a number of HIV protease inhibitors have recently been approved for clinical use. The disappointing clinical efficacy of these inhibitors during the early trials led to the widespread belief that the HIV protease

develops resistance to the inhibitors by mutating to less susceptible forms of the enzyme (17-25) .

To date, however, all of the inhibitor-resistant mutant proteases "identified" in clinical samples were obtained from traces of inactive HIV proviral DNA in a few cells of some people. These inhibitor-resistant mutant proteases have never been seen in viable, infectious virus. So far, only special laboratory conditions are capable of producing viral particles containing inhibitor-resistant mutant HIV protease. Even here, the viability and infectivity of these mutant particles relative to the so-called wild-type virus have not been reported (19,20,26,27) .

The inability to find inhibitor-resistant HIV protease in infectious virus is readily explained. The minimum viable catalytic efficiency of HIV protease was found to be 2% of the wild-type activity (11,12) . The catalytic efficiencies of the mutant enzymes, however, are many orders of magnitude below the 2% level (28,29) . The reason for the extremely low levels of activity is that both the inhibitors and the substrates bind to the same catalytic site of HIV protease. Since the wild-type HIV protease has evolved to the optimal level of activity, virtually all alterations to the enzyme's structure that affect catalytic efficiency are lethal to the virus. Mutations of the protease that reduce inhibitor binding result in an even more profound reduction in catalytic activity. This is because the overall catalytic efficiency of a mutant HIV protease is given by the product of the relative efficiencies of the mutant enzyme with respect to the wild-type for all eight obligatory cleavages (28) . These eight cleavages can be thought of as an eight-number combination lock. Not only does HIV protease have to make all eight cleavages, but the enzyme must do it in the right order. Therefore, even in the absence of inhibitors, the inhibitor-resistant mutant HIV proteases do not lead to viable, infectious virus. That's not the end of the bad news for mutant HIV. As with the wild-type enzyme, the eight sequential cleavages force the inhibitor-resistant proteases to be exponentially sensitive to inhibitors, which more than compensates for their weaker binding (28) .

THERAPEUTIC DOSES TOO HIGH

The extremely high doses of protease inhibitors that are being prescribed to patients in the hope of preventing the appearance of inhibitor-resistant mutants are unwarranted. The daily dose of around 1-2 grams of HIV protease inhibitors is well over an order of magnitude above what is needed to render HIV noninfectious and, therefore, nonpathogenic if it were true that HIV is pathogenic.

The toxicities of the protease inhibitors (so severe that 35-50% of patients cannot tolerate them) are well documented (30) . The high doses of the inhibitors pose long-term risks to patients as well. The oral doses of protease inhibitors currently administered to patients are at minimum 50 times that needed to completely inhibit the intestinal aspartyl protease cathepsin D (calculation based on the Roche inhibitor Saquinavir; the Abbott inhibitor Ritonivar is 1000 times more potent against cathepsin D than Saquinavir). The inhibition of cathepsin D in the intestines of patients my have clinical consequences since mice deficient in this enzyme (generated by gene targeting) develop normally during the first two weeks, stop thriving in the third week and die in a state of anorexia at day 26 ± 1 (31) . The fact that diarrhea is a common problem with all the protease inhibitors may be a warning of problems ahead. In light of the immune compromised state of AIDS patients, it is also important to note that the cathepsin D-minus mice suffered massive destruction

of the thymus and spleen with fulminant loss of T and B cells. The maintenance of high blood levels of protease inhibitors makes their potential effects on the lymphocytes more than an academic concern.

Since all the protease inhibitors were approved under the FDA's accelerated approval process, the long-term safety of these agents is unknown (30) . This is especially disturbing in view of the disclaimer attached to each of the HIV protease inhibitors approved by the FDA. The Merck entry is typical:

> Crixivan is not a cure for HIV or AIDS. People taking Crixivan may still develop infections or other conditions associated with HIV. Because of this, it is very important for you to remain under the care of a doctor. It is not yet known whether taking Crixivan will extend your life or reduce your chances of getting other illnesses associated with HIV. Information about how well the drug works is available from clinical studies up to 24 weeks.

Such a notice does little to inspire confidence either to prescribe or take the protease inhibitors, especially when faced with the known and potential toxicities of these compounds.

Finally, the specificity of the HIV protease inhibitors is not absolute and it is impossible to determine the toxic effects of new inhibitors just by looking at them. For example, the Abbott HIV protease inhibitor unexpectedly inhibits a subset of the cytochrome P450 enzymes the liver uses to detoxify drugs, while high levels of the Merck compound cause kidney stones (30,32) and severe hepatitis (33) . The FDA recently discovered 83 patients who contracted diabetes or hyperglycemia, high blood sugar, or had those diseases suddenly worsen after they began taking protease inhibitors. (The Associated Press, 11 June 1997)

INFECTIOUS VIRUS IS THE THING

There is also a problem with the surrogate marker used to evaluate the clinical efficacy of the protease inhibitors. The so-called viral-load test is claimed to measure pieces of HIV viral RNA (termed copy number), but the assay gives no indication as to the viability or infectivity of the viral particles presumed to be present in a patient's blood plasma. Even before antiretroviral therapy, 99.9% of the virus detected by the viral-load test was found to be noninfectious in the sole individual examined (34) . (The 99.9% figure agrees with the known level of defective variants of the HIV genome (35,36) .) And after 2 days of protease inhibitor-cocktail therapy none of the over 500,000 viral particles per ml in that patient's blood plasma was infectious (34) . Typically, in inhibitor naive patients, as well as in cell cultures, only about 1 in 100,000 viral particles is infectious (35,37-40) . The viral-load test, then, is measuring overwhelmingly noninfectious virus. The significance of all this noninfectious virus has not been adequately addressed.

To further complicate matters, protease inhibitor therapy leads to defective, noninfectious viral particles (2,7-9) that are more stable than wild-type virus, thus adding to the already erroneously high measure of "viral-load." Confounding the issue even further, in 1993, Ho et al. reported 12 AIDS patients, including 8 who had AIDS "risk factors," who were totally HIV-free: "Specific antibody assays, viral cultures, and polymerase chain

reaction (PCR) techniques" for HIV were all negative (41) . In short, no one has shown that the viral-load assay has anything to do with infectious virus. In fact, the most that can be claimed for the viral-load test is that at best it is detecting viral debris.

SUMMARY
The degree to which the mutant proteases are resistant to inhibitors is meaningful only in the context where the viability and infectivity of the mutant viruses are also quantitated. The general misunderstanding regarding the significance of the inhibitor-resistant mutants can be attributed in large part to the common practice of testing the mutant proteases against a single substrate without taking into consideration the eight sequential cleavages necessary for viable maturation of HIV (42) . It is extremely unlikely that mutations of the enzyme, substantial enough to protect the protease against inhibition, will at the same time leave virtually unimpaired its proteolytic activity towards all eight cleavages. None of the inhibitor-resistant mutant HIV proteases reported so far (even in the absence of inhibitors) has come anywhere near the minimum level of overall catalytic activity necessary for infectious, viable virus. The conclusion of this analysis is that inhibitor-resistant mutant HIV proteases are very unlikely to contribute to viral viability *in vivo*. Therefore, traumatizing patients with the specter of drug-resistant mutants of HIV resulting from a failure to adhere to a Draconian regimen of medication is unjustified. It is important to emphasize that the protease inhibitors are toxic compounds with a growing list of serious and life-threatening consequences. Finally, the deceptively named "viral-load" test does not measure infectious virus and should not be used to indicate the presence of HIV in the blood plasma of patients.

REFERENCES

1. Darke, P. L., Nutt, R. F., Brady, S. F., Garsky, V. M., Ciccarone, T. M., Leu, C. T., Lumma, P. K., Freidinger, R. M., Veber, D. F., and Sigal, I. S. (1988) *Biochemical and Biophysical Research Communications* **156**(1)**,** 297-303
2. Kageyama, S., Hoekzema, D. T., Murakawa, Y., Kojima, E., Shirasaka, T., Kempf, D. J., Norbeck, D. W., Erickson, J., and Mitsuya, H. (1994) *AIDS Research and Human Retroviruses* **10**(6)**,** 735-743
3. Gowda, S., Stein, B., and Engleman , E. (1989) *Journal of Biological Chemistry* **264,** 8459-8462
4. Kaplan, A. H., and Swanstrom, R. (1991) *Proceedings of the National Academy of Sciences (USA)* **88,** 4528-4532
5. Mervis, R. J., Ahmad, N., Lillehoj, E. P., Raum, M. G., Salazar, F. H. R., Chan, H. W., and Venkatesan, S. (1988) *Journal of Virology* **62,** 3993-4002
6. Pettit, S. C., Moody, M. D., Wehbie, R. S., Kaplan, A. H., Nantermet, P. V., Klein, C. A., and Swanstrom, R. (1994) *Journal of Virology* **68**(12)**,** 8017-8027
7. Kaplan, A. H., Zack, J. A., Knigge, M., Paul, D. A., Kempf, D. J., Norbeck, D. W., and Swanstrom, R. (1993) *Journal of Virology* **67**(7)**,** 4050-4055
8. Craig, J. C., Duncan, I. B., Hockley, D., Grief, C., Roberts, N. A., and Mills, J. S. (1991) *Antiviral Research* **16,** 295-305
9. Ashorn, P., McQuade, T. J., Thaisrivongs, S., Tomasselli, A. G., Tarpley, W. G., and Moss, B. (1990) *Proceedings of the National Academy of Sciences (USA)* **87,** 7472-7476

10. Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., and Sigal, I. S. (1988) *Proceedings of the National Academy of Sciences (USA)* **85,** 4686-4690
11. Babé, L. M., Rosé, J., and Craik, C. S. (1995) *Proceedings of the National Academy of Sciences (USA)* **92,** 10069-10073
12. Rosé, J., Babé, L. M., and Craik, C. S. (1995) *Journal of Virology* **69**(5)**,** 2751-2758
13. Konvalinka, J., Heuser, A.-M., Hruskova-Heidingsfeldova, O., Vogt, V. M., Sedlacek, J., Strop, P., and Kräusslich, H.-G. (1995) *European Journal of Biochemistry FEBS* **228,** 191-198
14. Mergener, K., Fäcke, M., Welker, R., Brinkmann, V., Gelderblom, H. R., and Kräusslich, H. G. (1992) *Virology* **186,** 25-39
15. Peng, C., Ho, B. K., Chang, T. W., and Chang, N. T. (1989) *Journal of Virology* **63,** 2550-2556
16. Pollard, R. B. (1994) *Pharmacotherapy* **14**(6 Pt 2)**,** 21S-29S
17. Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H.-M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., Ho, D. D., Boucher, C. A. B., Leonard, J. M., Norbeck, D. W., and Kempf, D. J. (1996) *Nature Medicine* **2**(7)**,** 760-766
18. Schapiro, J. M., Winters, M. A., Stewart, F., Efron, B., Norris, J., Kozal, M. J., and Merigan, T. C. (1996) *Annals of Internal Medicine* **124**(12)**,** 1039-1050
19. Jacobsen, H., Hänggi, M., Ott, M., Duncan, I. B., Owen, S., Andreoni, M., Vella, S., and Mous, J. (1996) *Journal of Infectious Diseases* **173,** 1379-1387
20. Condra, J. H., Schlelf, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and A., E. E. (1995) *Nature* **374,** 569-571
21. Pazhanisamy, S., Stuver, C. M., Cullinan, A. B., Margolin, N., Rao, B. G., and Livingston, D. J. (1996) *The Journal of Biological Chemistry* **271**(30)**,** 17979-17985
22. Rose, R. E., Gong, Y.-F., Greytok, J. A., Bechtold, C. M., Terry, B. J., Robinson, B. S., Alam, M., Colonno, R. J., and Lin, P.-F. (1996) *Proceedings of the National Academy of Sciences (USA)* **93,** 1648-1653
23. Moutouh, L., Corbeil, J., and Richman, D. D. (1996) *Proceedings of the National Academy of Sciences (USA)* **93,** 6106-6111
24. Bartlett, J. G. (1996) *Annals of Internal Medicine* **124**(12)**,** 1086-1087
25. Carpenter, C. C. J., Fischl, M. A., Hammer, S. M., Hirsch, M. S., Jacobsen, D. M., Katzenstein, D. A., Montaner, J. S. G., Richman, D. D., Saag, M. S., Schooley, R. T., Thompson, M. A., Vella, S., Yeni, P. G., and Volberding, P. A. (1996) *Journal of the American Medical Association* **276**(2)**,** 146-154
26. Kozal, M. J., Shah, N., Shen, N., Yang, R., Fucini, R., Merigan, T. C., Richman, D. D., Morris, D., Hubbell, E., Chee, M., and Gingeras, T. R. (1996) *Nature Medicine* **2**(7)**,** 753-759
27. Lech, W. J., Wang, G., Yang, Y. L., Chee, Y., Dorman, K., McCrae, D., Lazzeroni, L. C., Erickson, J. W., Sinsheimer, J. S., and Kaplan, A. H. (1996) *Journal of Virology* **70**(3)**,** 2038-2043
28. Rasnick, D. (1997) *The Journal of Biological Chemistry* **272**(10)**,** 6348-6353
29. Lin, Y., Lin, X., Hong, L., Foundling, S., Heinrikson, R. L., Thaisrivongs, S., Leelamanit, W., Raterman, D., Shah, M., Dunn, B. M., and Tang, J. (1995) *Biochemistry* **34**(4)**,** 1143-1152
30. Deeks, S. G., Smith, M., Holodniy, M., and Kahn, J. O. (1997) *Journal of the American Medical Association* **277**(2)**,** 145-153

31. Saftig, P., Hetman, M., Schmahl, W., Weber, K., Heine, L., Mossmann, H., Köster, A., Hess, B., Evers, M., von Figura, K., and Peter, C. (1995) *EMBO Journal* **14**(15)**,** 3599-3608
32. Daudon, M., Estépa, L., Viard, J. P., Joly, D., and Jungers, P. (1997) *The Lancet* **349,** 1294-1295
33. Brau, N., Leaf, H. L., Wieczorek, R. L., and Margolis, D. M. (1997) *The Lancet* **349,** 924-925
34. Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M., and Ho, D. D. (1996) *Science* **271**(5255)**,** 1582-1586
35. Piatak, M., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K.-C., Hahn, B. H., Shaw, G. M., and Lifson, J. D. (1993) *Science* **259,** 1749-1754
36. Sheppard, H. W., Ascher, M. S., and Krowka, J. F. (1993) *Nature* **364**(6435)**,** 291-292
37. Cao, Y., Quin, L., Zhang, L., Safrit, J., and Ho, D. D. (1995) *N Engl J Med* **332**(4)**,** 201-208
38. McKeating, J. A., and Moore, J. P. (1991) *Nature* **349**(6311)**,** 660
39. Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L., Conley, S. R., Moore, J. P., Raina, J. L., Renz, H., Gelderblom, H. R., and Nara, P. L. (1992) *Virology* **189**(2)**,** 695-714
40. Bourinbaiar, A. S. (1991) *Nature* **349**(6305)**,** 111
41. Ho, D. D., Cao, Y., Zhu, T. Z., Farthing, C., Wang, N., Gu, G., Schooley, R. T., and Daar, E. S. (1993) *NEJM* **328,** 380-385
42. Gulnik, S. V., Suvorov, L. I., Liu, B., Yu, B., Anderson, B., Mitsuiya, H., and Erickson, J. W. (1995) *Biochemistry* **34**(29)**,** 9282-9287