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Previews

An 'Inside-the-Box' Approach to Drug Resistance

Drug resistance is a growing problem in medicine that demands creative solutions. In this issue, Schiffer and colleagues describe their novel approach to pinpointing hot spots of resistance, which might lead to new anti-HIV therapeutics [1].

The introduction of HIV protease inhibitors (PIs) nearly ten years ago revolutionized the treatment of HIV disease. As the basis for combination regimens constituting highly active antiretroviral therapy (HAART), PIs turned HIV infection from a death sentence to a manageable chronic disease that, under optimal circumstances, can be held in check indefinitely. Thousands of individuals suffering from the ravages of AIDS were able to recover immune competence and return to normal lives.

Despite the above unequivocal success, a dilemma remains. Because HIV is a retrovirus with a high rate of replication, it exists as a quasispecies or swarm of viral variants in pseudoequilibrium, where potential drug-resistant mutants are likely to preexist prior to therapy. As the role of HIV protease in the viral replication cycle became recognized, early researchers speculated that resistance to PIs might be impossible since cleavage of at least nine different nonhomologous substrates by the protease is required for the formation of mature, infectious viral particles. Supposedly, the enzyme would be unable to accommodate mutations decreasing the affinity of the active-site inhibitors without seriously compromising its ability to cleave at least one of its many substrates. Contrary to those expectations, virtually all patients in initial studies with PIs used as monotherapy experienced drug failure with the stepwise accumulation of multiple mutations in the HIV protease gene [2, 3]. Indeed, HIV drug resistance has become sufficiently widespread, even in the era of HAART, that it supports an entire industry in HIV resistance diagnostics. Most resistance occurs within an individual patient as a result of an incompletely suppressive regimen and/or poor adherence to the regimen (setting up a Darwinian system). However, drug-resistant HIV can also be transmitted. Because HIV infection cannot be cured, there is a consequent need for more drugs that either prevent/delay resistance or remain effective in patients in whom the virus has become resistant to current regimens.

The traditional approach to combating drug resistance includes the characterization and targeting of discrete mutants, enabling the identification of new compounds with the proper complementarity to the mutant protein [4, 5]. However, more than 20% of the 99 amino acids constituting HIV protease have been known to mutate and contribute in combination to resistance to protease inhibitors. Furthermore, key mutable amino acids such as valine 82 can change to smaller (alanine), larger (phenylalanine), or polar (threonine or serine) amino acids. The resulting number of discrete mutants is astronomical, and traditional methods are not equipped to deal with the vast number of new targets. To deal with this complexity, King et al., on page 1333–1338 of this issue of *Chemistry & Biology*, illustrate an "outside-the-box" scientific strategy for analyzing resistance to HIV protease inhibitors [1]. Combining protein X-ray crystallography and a mathematical analysis using structural "envelopes", the authors provide simple visual tools that prominently pinpoint regions of focus for future research. Crystal structures of HIV protease with six of the nine substrates (the remaining three are under continuing investigation) were generated. A mathematical union operation upon the three-dimensional coordinates of the six substrates was carried out to generate an "envelope." This provides a representation of the active site volume that must always be made available by the enzyme. Analogously, an envelope of known drugs is also created. Notably, the two envelopes are similar but not identical. Therein lies the importance of this new work. The three-dimensional regions in which individual inhibitors protrude beyond the substrate envelopes are almost perfect indicators of the locations of drug-resistance mutations reported for those drugs. The implication for future drug design of new-generation HIV protease inhibitors is clear: stay "inside-the-box"!

The principle of this inside-the-box analysis can be extended to other targets. The serine protease of the hepatitus C virus (HCV) also has a finite set of substrate cleavage sites, and the substrate envelope for this enzyme could facilitate current design efforts. In a less straightforward extension, it is possible to envision an envelope that would reflect an extremely high number of discrete oligomer substrates enveloped by the HCV polymerase as an approximation to its transcription of a 9.6 kilobase strand of RNA. An example of application to drug targets in higher organisms may be found in the peptide binding site of protein kinases. In contrast to the conserved ATP-site, the peptide substrate binding site is more variable between kinases and more accepting of a large variety of peptide substrate sequences. In general, any protein with multiple substrates might be subjected to this envelope analysis. In the case of infectious organisms, the envelope has importance for the consideration of drug resistance. For human drug targets, the importance might be more for understanding specificity or mechanism of action, rather than resistance. With the modern improvements in protein X-ray crystallographic techniques, such daunting experimental and analytical tasks become imaginable.

Returning to drug-resistant HIV infection, the harsh reality is unfortunately more complex than simple mathematically calculated envelopes. HIV can accumulate mutations rapidly, and viral strains containing as many as 10 or more resistance mutations are not uncommon. These multiple mutant proteins have the potential of exhibiting atomic shifts both in the protein and inhibitor that would cause any calculated envelope to undergo

some deformation. Further confounding the envelope analysis are the mutations that appear in the substrates themselves. Although not yet present in the majority of resistant strains, mutations near one or more of the *gag* cleavage sites allow more "extreme" mutations within the protease protein that would otherwise cripple viral replication. Such substrate mutations are likely to impact the shape of the substrate envelope. A refinement of the envelope analysis reported here will need to incorporate these additional factors.

Finally, while the approach outlined by King et al. is likely to enable the identification of new PIs to which HIV has more difficulty (or requires more genetic steps) in becoming resistant, a complete strategy for minimizing resistance also requires an understanding of conditions (e.g., regimen adherence, drug pharmacokinetics, and viral fitness) that define selective pressure in the host. Recent results indicate that pharmacokinetically boosted [6] regimens of existing PIs can erect a substantial barrier to resistance in vivo [6, 7], illustrating the fact that drug discovery is a multi-faceted effort and that all available tools are needed for defeating a plague such as HIV. The approach of King et al. is a welcome addition to the tool chest.

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Selected Reading

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Catching Proteases in Action with Microarrays

Proteases regulate many essential functions in biology, yet their precise roles are only beginning to be unraveled. In this issue, two related papers describe a novel method to dissect specific protease activities from complex mixtures [1, 2].

Proteolysis provides an irreversible means to activate or terminate signaling events in complex biological settings by destruction of proteins. With greater than 500 putative protease genes in the typical mammalian genome, understanding their precise roles in biology is daunting [3]. The generation of protease-deficient mice and small-molecule inhibitors has revealed essential functions for proteases in many areas of biology, ranging from cell cycle control [4] to antigen presentation [5] and extracellular matrix remodeling [6]. Functional redundancy within protease subfamilies and limited methods for identifying substrates in vivo complicate analysis. The majority of proteases are synthesized as zymogens and are activated only in specific subcellular compartments or upon stimulation. Furthermore, many proteases have endogenous inhibitors that attenuate their destructive capacity. As proteolysis is regulated posttranslationally, the evaluation of mRNA or polypeptide levels do not necessarily reflect their activity. To address the functional activity of proteases, two papers in this issue from Harris, et al. and Winssinger, et al. describe microarray technologies to identify proteases and their substrates from complex mixtures with peptide-nucleic acid (PNA)-encoded libraries of activity-based probes [1] and fluorogenic peptide substrates [2], respectively (Figure 1).

By and large, the characterization of protease substrates has been determined with purified proteins or peptide libraries in vitro, which then suggests potential substrates in vivo. The development of positional-scanning fluorogenic-substrate libraries has allowed the characterization of preferred amino acids at the site of proteolysis and has been used to determine the substrate specificities for several protease families in a high-throughput manner [7–9]. Using these positional-scanning substrate libraries, Harris, et al. evaluated the proteolytic activities in dust mite extracts that may be responsible for allergies [1]. By including class-specific protease inhibitors, they showed that the predominant proteolytic activities in dust mite extracts that cleaved P1 basic residues were attributed to cysteine proteases, whereas serine proteases were responsible for cleaving substrates with P1 proline residues. Although these positional-scanning peptide libraries revealed the overall proteolytic activities in dust mite extracts, the specific protease(s) responsible for these activities from complex mixtures remained to be identified.

In order to retrieve the cysteine-protease(s) from the dust mite extracts, Harris et al. turned to mechanism-based probes that irreversibly label active enzymes [1].