

Prevention of viral drug resistance by novel combination therapy

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A new form of antiviral clinical therapy is proposed in which three different drugs are administered against three different targets on the same virus-coded protein. If the physiological functions of the three different target sites are not independent of each other, then a mutation conferring drug resistance at one site may alter the physiological functions at the other sites and further drug resistance may not arise. The adenovirus proteinase, with its two cofactors that act synergistically on enzyme activity, may be a good model system within which to test the efficacy of this form of combination therapy.

Keywords Adenovirus, antiviral, cofactor, combination therapy, HIV, proteinase, resistance

Introduction

Virus-coded proteinases are attractive targets for antiviral therapy. These enzymes are essential for the synthesis of infectious virus. Among the medically important viruses known to encode proteinases are poliovirus, rhinovirus, hepatitis A and C viruses, influenza virus, herpes virus, HIV and adenovirus [1].

The effectiveness of proteinase inhibitors in acting as antiviral agents depends not only upon the specificity of the inhibitors for the proteinase but also upon the clinical strategy used to administer the inhibitors. The latter is very much dependent upon successfully overcoming the problem of resistance [2]. A single proteinase inhibitor is not very effective in treating AIDS as resistance arises rapidly, sometimes within 2 weeks. A more effective treatment is the use of combination therapy where two or more drugs are administered [3••,4••]. Presumably this is because the probability of generating resistance against multiple drugs in a single provirus is much lower than that of generating resistance against a single drug. It has been estimated that since the beginning of the AIDS epidemic, 10^{16} to 10^{17} mutants of HIV have arisen [5••], so unfortunately it is only a matter of time before multidrug-resistant mutants arise [6].

Here we consider two forms of triple combination therapy. One form involves three different drugs directed against three different targets on three independent virus-coded proteins,

eg, the reverse transcriptase, proteinase and integrase of HIV. The new form involves three different drugs acting on three functionally dependent sites on the same virus-coded protein. The latter form is designed to reduce the probability of a virus arising that is resistant to all three drugs to less than the reciprocal of the total number of variant viruses present in an individual. We also describe a viral system, human adenovirus, that may be useful in assessing experimentally the efficacy of this new form of combination therapy [7••].

Resistance and the cost of resistance

Calculation of the variant population size

The variant population size is the maximum number of mutant proviral genomes per infected person. If the probability of forming a drug-resistant provirus is less than the reciprocal of the variant population size, then drug-resistant virus is not likely to arise. On the other hand, if the probability of forming a drug-resistant provirus is higher than the reciprocal of the variant population size, then drug-resistant virus will probably arise. The variant population size can be calculated as follows: the average person has roughly 5×10^{11} CD4+ T-lymphocytes. If they all were to become infected, which has never been observed, and assuming the mutation rate of HIV-1 is 3×10^{-5} mutations/base pair/generation and the number of base pairs per genome is 10,000; then, the number of variant HIV-1 proviruses in that individual would be $(5 \times 10^{11}) \times (3 \times 10^{-5}) \times (10,000)$ or $\sim 10^{11}$ [5••]. Thus, the maximum variant population size, the population out of which drug-resistant virus arises, is 10^{11} .

One way to calculate the probability of multiple resistance mutations

Pelletier and Wain-Hobson state that the probability of multiple resistance mutations occurring can be calculated by simply multiplying together the individual mutation rates. Thus, the frequency of a genome containing three mutations, each arising at a rate of 3×10^{-5} /base pair/generation, will be $(3 \times 10^{-5})^3$ or $\sim 3 \times 10^{-14}$, which is much smaller than the reciprocal of the variant population size, 10^{11} . This analysis assumes that resistance to each drug can arise as the result of a single point mutation.

Another way to calculate the probability of multiple resistance mutations

Coffin [8•] disagrees with the analysis described by Pelletier and Wain-Hobson because it ignores the history of the virus population. Mutations in a viral quasi-species accumulate with each replication cycle until their rate of appearance is balanced by counter-selection, reverse mutation, or both. He calculates the frequency of a viral genome containing three mutations as follows: the intrinsic mutation rate of HIV-1 is 3×10^{-5} mutations/base pair/cycle. From the original starting virus, in 13 months there will be 1000 cycles of virus replication. Therefore, the frequency of mutation will be $\sim 1000 \times (3 \times 10^{-5})$ or 3×10^{-2} mutations/base pair. Mutants with combined resistance to three drugs will be present at $(3 \times 10^{-2})^3$ or about 3×10^{-5} . Thus, in 13 months, $(3 \times 10^{-5}) \times 10^{11} = 3 \times 10^6$ or more than one million variant proviruses will emerge resistant to all three drugs.

Cost of resistance

According to Coffin, there are only two ways that combined drug treatment will not be made ineffective by drug-resistant mutants. One way is if an individual is treated soon after infection when a limited distribution of viral genotypes is present. Here the variant population size from which a resistant virus can arise is significantly decreased. Another way that combination therapy can work is if the mutations that lead to resistance are not neutral, but confer some associated disadvantage or 'cost' to the virus. In this case, the equilibrium frequency of each mutation will be the mutation rate divided by the cost of the mutation. For example, a 10% cost would give an equilibrium frequency of $(3 \times 10^5)/0.1 = 3 \times 10^4$ mutations/base pair. The probability of acquiring the required three mutations would be $(3 \times 10^4)^3 \approx 3 \times 10^{11}$. This means that only three variant proviruses, $(3 \times 10^{11}) \times 10^{11}$ (the population size), would be present per person per year. Since most virions and infected cells, and hence most variants, are destroyed by innate and acquired immunity [9,10], it is theoretically possible to circumvent the problem of drug resistance.

How to increase the cost of resistance

If the utility of triple-combination therapy is ultimately dependent upon the cost to the virus, how can one design a clinical therapy with high cost? The cost is probably negligible if the three different drugs are directed against three functionally independent but essential proteins, eg, inhibitors against the reverse transcriptase, the proteinase and the integrase of HIV. The cost is probably negligible because mutation to resistance in one protein will not affect the functions of the other proteins. However, if there are three different drugs acting on three different regions of the same enzyme, and the physiological functions of the three regions are not independent of each other, then theoretically the cost is proportional to the functional dependency of the three regions and could become prohibitive.

Adenovirus as a model system

The adenovirus proteinase

The adenovirus proteinase (AVP) presents an ideal enzyme system within which to test the hypothesis that the probability of a virus becoming resistant to three different inhibitors directed against three functionally dependent sites on the same virus-coded protein is much lower than for three different inhibitors directed against three functionally independent virus-coded proteins. AVP utilizes at least three geographically distinct sites for its optimal activity: an active site and two cofactor-binding sites. These sites have been characterized biochemically. The crystal structure of the enzyme with one of the cofactors bound is known at a resolution of 2.6 Å [11••] and revealed that AVP is a new type of cysteine proteinase. The amino acid residues involved in catalysis are in a configuration similar to those in the canonical cysteine proteinase, papain, although AVP is not related to papain. Another benefit of the adenovirus system is that a wide range of animals can be infected by different strains of adenovirus, including mice, chickens and monkeys, so that once effective inhibitors of the enzyme are found they can be tested as antiviral agents in several animal systems.

AVP is required late in infection. After formation of empty capsids, the viral DNA, along with core components including AVP, enters the capsids to form immature virions. AVP then becomes activated. About 70 AVP molecules [12] cleave multiple copies of six virion precursor proteins 3200 times, thereby rendering a virus particle infectious [13,14]. The gene

for AVP was identified [15], cloned, expressed and the recombinant 204-amino acid protein purified [16,17•,18•, 19,20•] and patented [101,102]. An extremely sensitive *in vitro* and *in vivo* assay for the proteinase was developed using the fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine [17•,21] and the rhodamine-based substrates have also been patented [103•,104]. Purified recombinant AVP exhibited no proteinase activity [17•,18•] and eventually, cofactors necessary for its activity were discovered. One cofactor identified was the viral DNA itself [17•] and the second was the 11-amino acid peptide, pVlc, from the C-terminus of the precursor to virion protein VI [17•,18•]. The amino acid sequence of pVlc was shown to be GVQSLKRRRCF.

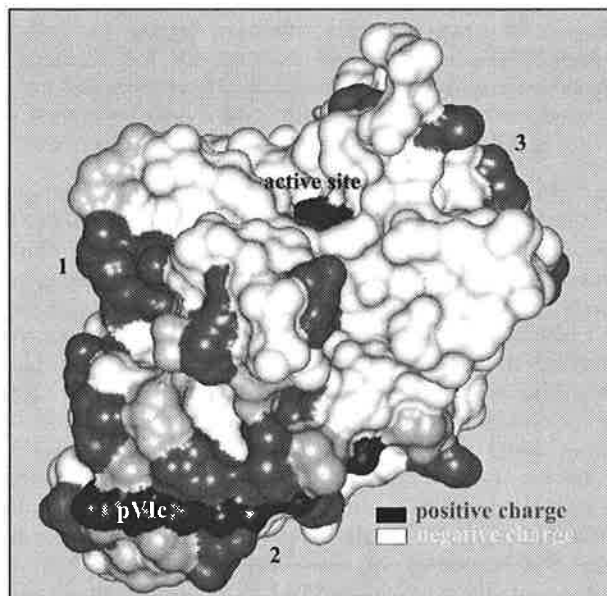
Functions of the cofactors

The cofactors affect the macroscopic kinetic constants of the interaction of AVP with the rhodamine-based fluorogenic substrates [20•]. AVP alone has only a small amount of activity. By incubating Ad2 DNA with AVP, the Michaelis constant (K_m) increases 2-fold and the catalytic rate constant (k_{cat}) 3-fold. By incubating pVlc with AVP, the K_m increases 2-fold and the k_{cat} increases 350-fold. With all three components together, AVP plus Ad2 DNA plus pVlc, the K_m increases 2-fold and the k_{cat} increases 6000-fold relative to those with AVP alone. Thus, the cofactors increase proteinase activity by increasing the k_{cat} , not decreasing the K_m .

Three drug target sites

The three sites on AVP against which drugs will be designed are the active site, the pVlc binding site, and the DNA binding site. The structure of the AVP-pVlc complex [11••] is shown in Figure 1. There are extensive interactions between pVlc and AVP through 26 hydrogen bonds, four ionic bonds and a disulfide bond. The exact location of the DNA binding site is unknown, but the surface of the AVP-pVlc complex has four main clusters of positive charge, which are all potential DNA binding sites.

Figure 1. Surface map of the AVP-pVlc complex.



The active site and the 11-amino acid cofactor, pVlc, are labeled. The three positive charge clusters, potential DNA binding sites, are numbered. The fourth positive charge cluster is in the lower right region but on the face of the molecule opposite to the one shown here.

Methods to decrease fidelity of adenovirus DNA replication

For adenovirus to be useful as a model system for studying drug resistance, the fidelity of DNA replication must be lowered so that resistance to drugs can arise quickly. Adenovirus utilizes a virus-coded DNA polymerase for DNA replication and it is probably much less error-prone than the reverse transcriptase of a retrovirus. However, adenovirus DNA replication can be made more error-prone, possibly even as error-prone as reverse transcription. For experiments with cells in culture, nucleoside analogs that are mutagenic can be used, and alternatively, for tests in animals, viruses expressing a mutant form of the adenovirus DNA polymerase in which its proof reading function, its 3' → 5' exonuclease activity [22], is inactivated can be used. The inability to proofread should greatly increase the error rate in DNA replication.

Conclusion

A debate is taking place on how best to use antiviral agents to treat AIDS [4••,5••,8•]. It has been estimated that 10^{16} to 10^{17} variants have been produced since the beginning of the AIDS epidemic [5••]. This is thought to be due to the high error rate of the reverse transcriptase (0.3 to 2 mutations/genome/replication [5••,23]) and the massive amounts of virus that are produced daily in infected individuals. Thus, it is not surprising that HIV mutants with partial or complete resistance to any drug have been found [2]. On the other hand, resistance may not be a consequence of a single base change. Full resistance *in vivo* to the reverse transcriptase inhibitor zidovudine (AZT; Glaxo Wellcome plc) seems to require four or five sequential base changes. This could be a consequence of the large proportion of virions and infected cells, and hence variants being destroyed by innate and acquired immunity [9,10]. Such complications make it difficult to calculate mutation frequencies. Wain-Hobson and Pelletier [4••] describe the objective of multidrug therapy as being the reduction of the probability of a virus variant arising (that is resistant to all the drugs) to less than the reciprocal of the effective population size of productively infected cells. However, the magnitude of the variant population size is presently unknown, because most virions and infected cells, and hence most variants, are destroyed by innate and acquired immunity. Regardless of theoretical calculations and empirical observations, the clinical success of combination therapy is wonderfully obvious.

The new form of combination therapy described here is based upon the expectation that there will be a cost to the virus in generating resistance to three inhibitors. The cost arises if the mutations to resistance are not independent. From the biochemical and structural biology studies of the adenovirus proteinase presented here, it is clear that the active site and the two cofactor binding sites are not independent of each other. pVlc, which exerts a powerful control over the rate of catalysis, binds quite far from the active-site residues involved in catalysis. The active site nucleophile Cys¹²² is located 32 Å from Cys¹⁰⁴, which forms a disulfide with pVlc. Yet, the binding of pVlc increases the rate of substrate hydrolysis 350-fold. Similarly, the binding of DNA to the enzyme increases the rate of substrate hydrolysis. Thus, it is reasonable to expect that a mutation in one site that renders the enzyme resistant to a drug may

impair the physiological functions in the other sites. And the cost associated with this mutation may be high enough to prevent resistance from arising to three different drugs against three different sites on the same protein.

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