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EMERGENCE OF MUTANT HIV REVERSE TRANSCRIPTASE CONFERRING RESISTANCE TO AZT

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1. INTRODUCTION

The likelihood of emergence of drug resistance is a function of mutation rate and the number of replicative events. The mutation rate of HIV is presumed to be at least as high as any virus with a single stranded RNA genome. The number of replicative events in a host infected with HIV are incalculably high as a result of years of persistent replication that is poorly restricted by immune surveillance. Viral chemotherapy in humans has selected for drug resistant variants of influenza A virus, herpes simplex virus, varicella zoster virus, cytomegalovirus and rhinovirus.[1] The prospect of drug resistant AZT therefore did not appear as a surprise to many.

2. THE PHENOTYPE OF REDUCED SUSCEPTIBILITY

2.1. *The Initial Report*

In the initial study of HIV susceptibility to AZT (zidovudine),[2] isolation of HIV was attempted with 186 peripheral blood mononuclear cell specimens from subjects at different stages of infection who had received therapy with AZT for various periods. A total of 46 isolates were obtained from 33 individuals. The use of MT2 lymphoblastoid cells for the isolation procedure permitted the preparation of large stocks of virus that could be frozen in aliquots and titrated. Susceptibility to AZT with these low-passage clinical isolates of HIV could not be tested reliably with the described assays of inhibition of cytopathology or production of p24 antigen.[3] This dilemma was resolved by the use of a CD4-expressing HeLa cell line (obtained from B. Chesebro, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Mont.) that readily permitted plaque reduction assays with all isolates tested.[4] In addition, this system permitted assay of a wide range of compounds, including nucleosides, interferons, and recombinant soluble CD4.

Study of these original isolates permitted several conclusions: (1) Isolates from subjects not treated with AZT display a narrow range of susceptibility to AZT, with

TABLE I
Sensitivity of HIV isolates to zidovudine, by duration of therapy

Duration of therapy (mo)	No. of isolates	IC_{50} (μM)		
		Mean	Median	Range
0	18	0.03	0.03	0.01–0.05
1–5	6	0.03	0.03	0.1–5.5
6–11	8	1.0	0.6	0.06–4.0
12–17	8	1.0	0.07	0.04–6.0
$\geqslant 18$	3	3.0	2.0	0.1–6.0

NOTE. IC_{50} values were determined directly from plots of percentage plaque reduction vs. zidovudine concentration (log_{10}). All isolates obtained during therapy and 15 of 18 isolates obtained in the absence of therapy were from patients with AIDS-related complex or AIDS. Reprinted with permission from *Science*.[2] Copyright 1989 by the AAAS.

the 50% inhibitory concentration (IC_{50}) ranging from 0.01 to 0.05 μM (Table I). (2) This narrow range of susceptibility is seen with isolates from subjects at all stages of HIV infection from asymptomatic through advanced AIDS. (3) This narrow range of susceptibility is observed with isolates displaying both cytopathic and non-cytopathic phenotypes *in vitro*. (4) Isolates from patients with AIDS or advanced AIDS-related complex display no detectable reduction in susceptibility during the first 6 months of AZT treatment; almost all isolates from such individuals display some reduction in susceptibility after 6 months of therapy (Table I). (5) Sequential isolates from individual patients receiving AZT therapy may display progressive, step-wise increases in resistance. (6) Several isolates with > 100-fold increases in the IC_{50} of AZT have been identified.

An additional observation suggested by the original study was the presence of a "shoulder" on the syncytial focus inhibition curve that was consistent with a more highly resistant subpopulation. Unfortunately the focus assay does not permit the retrieval of infectious virus from foci to clone phenotypically distinct virus. More recently additional inhibition curves even more suggestive of phenotypic mixtures have been observed and these virus stocks have been confirmed to be genotypic mixtures (see below).

2.2. *Other Assays and Reports of Reduced Susceptibility*

A distinct advantage of the syncytial focus assay in CD4-HeLa cells is that it generates a monotonic sigmoid curve that is highly reproducible when focus number is plotted against the log of the concentration of drug. The assay thus permits reproducible results, quantitative susceptibilities, easy detection of spurious single values and the detection of phenotypic mixtures. A disadvantage of the assay however is that it required high titer virus stocks which in practice can only be obtained from approximately one-third of specimens from seropositive individuals. Other assays have used the inhibition of production of supernatant reverse transcriptase or p24 antigen in the presence of various concentrations of drug. These assays have also documented the selection of isolates of HIV with reduced susceptibilities to AZT after prolonged therapy.[5,6]

2.3. *Cross Resistance to Other Antivirals*

With regard to resistance to other antiretroviral compounds, the 5 highly AZT resistant isolates have been shown to display cross-resistance to nucleosides containing a 3′-azido moiety including 3′-azido-2′, 3′-dideoxyuridine (AZdU), 3′-azido-2′, 3′-dideoxyguanosine (AZG) and 3′-azido-2′, 3′-dideoxyadenosine (AZA),[3] (Richman, unpublished observations). No cross resistance to other nucleosides, including several thymidine analogues, has yet been documented,[3] although this possibility is at least theoretically conceivable. In addition no cross resistance to other non-nucleoside reverse transcriptase inhibitors or of compounds acting at other sites of HIV replication have been documented.[3,7] These observations are encouraging with regard to the use of other nucleosides such as ddC or ddI and of drug combinations.

2.4. *Effect of Stage of Disease and Drug Dose on AZT Resistance*

An extension of the original studies to a total of 97 isolates from 73 individuals has provided information regarding the effects of disease stage and drug dose on the rates of emergence of resistance.[8] For baseline data, 42 isolates were examined from subjects who denied any history of prior AZT therapy. Forty-one of these displayed IC_{50} values to AZT of $< 0.045\,\mu M$. One displayed an IC_{50} of $0.17\,\mu M$. Upon investigation this individual had developed macrocytosis and had measureable serum levels of AZT on multiple stored serum specimens obtained prior to the development of the drug resistant isolate.

Susceptibilities to zidovudine were determined in 55 isolates from 31 patients receiving zidovudine.[8] Patients with late-stage HIV infection (AIDS or advanced ARC) developed resistance significantly sooner than those with early stage disease ($p = 0.002$). By 12 months after initiation of AZT therapy, an estimated 89% (95% confidence interval = 64%–99%) of persons with late-stage HIV infection have developed resistance, compared with 31% (95% confidence interval = 16%–56%) of those with early-stage infection (Figure 1). It is possible that the clinical significance of resistance could be a function of the degree of drug susceptibility. All six subjects who developed highly resistant virus were from among the 14 with late-stage HIV infection. For this population, the estimated proportion that develops highly resistant virus within 1 year after initiation of AZT is 33% (95% confidence interval = 16%–59%), while no high level resistance was documented in the first 18 months of therapy among earlier stage patients (Figure 1).

Lower initial CD4 lymphocyte counts were also predictive of increased likelihood of the emergence of resistant isolates ($p = 0.004$). The estimated rates of resistance at 1 year were 89%, 41% and 27% for baseline CD4 cell counts > 100, 100–400, and > 400 CD4 cells/mm^3 (95% confidence intervals = 63%–99%, 18%–75%, and 11%–59%, respectively).

Development of resistance occurred somewhat sooner among individuals assigned to higher daily doses of AZT (1200–1500 mg) than those assigned to lower doses (500–600 mg), although this difference did not attain statistical significance ($p = 0.18$ without controlling for stage and $p = 0.06$ after controlling for stage). Baseline positivity (> 37 pg/ml) for serum HIV p24 antigen, which occurred in five of the late-stage subjects and none of the early-stage subjects, was not significantly correlated with the development of resistance ($p = 0.2$). These results are encouraging based upon the studies and recommendations that a 500 mg daily dose of AZT is indicated.[9–11]

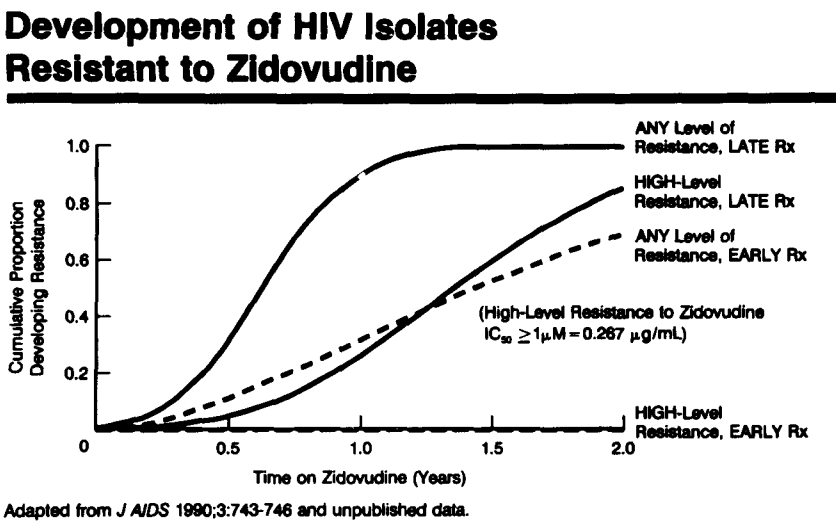

FIGURE 1 Estimated cumulative proportion of persons developing resistant isolates as a function of time since initiation of AZT by stage of HIV infection. Patients with late stage infection had less than 200 CD4 lymphocytes and HIV related symptoms. Patients with early stage disease had 200 to 500 CD4 lymphocytes and mild or no symptons. Any level resistance is defined as an IC_{50} of $> 1.0\,\mu M$. No high level resistance was seen in the early stage patients during this interval. With permission from Richman *et al.* 1990.[8]

2.5. *Enzymologic Basis of Resistance*

The antiviral effect of AZT is conferred by the triphosphate that is generated by anabolic phosphorylation by host cell thymidine kinase and other enzymes.[12] AZT triphosphate inhibts the reverse transcriptase of HIV in cell free enzyme assays and also acts as a terminator of DNA chain elongation because the 3′-azido group prevents the formation of 3′, 5′-phosphodiester bonds. It was not surprising therefore when mutations in the gene for the resistant viral reverse transcriptase were documented (see below). What remains puzzling, however, has been the inability to demonstrate an enzymologic difference in the mutant reverse transcriptase.[2] Utilizing enzyme extracted either from AZT-sensitive and resistant virions or prepared from enzyme expressed in *Escherichia coli* after molecular cloning from these viruses, no difference in inhibition by AZT triphosphate has been demonstrated in cell free enzyme assays. Because the genetics are definitive, these observations would suggest that cell free enzyme assays do not reflect the mechanism of inhibition of AZT triphosphate upon the transcription complex in the cell.

2.6. *Clinical Significance of AZT Resistance*

The clinical importance of AZT resistant HIV has been difficult to document. There are several reasons for this difficulty. First the development of resistance is not abrupt. It occurs slowly and progressively. It is possible that the significance of different levels of susceptibility could be quite different clinically. These different levels appear over

periods of months to years, and recent observations with assays for the presence of mutations in clinical specimens suggests that mixtures of viruses with different resistance genotypes may be circulating simultaneously in the same individual. Moreover clinical endpoints in HIV disease are often neither clearcut nor the immediate consequence of a change in virus replication, but rather the result of an opportunistic consequence of immunosuppression. Nevertheless it seems probable to many that replication *in vitro* in the presence of concentrations of AZT that are clinically unattainable will prove to be important.

One approach that may prove informative is the selection of AZT resistant mutants of feline immunodeficiency virus (FIV).[13] AZT resistant mutants of HIV have been difficult to select in cell culture.[14] In contrast, resistant mutants of FIV have been readily selectable, perhaps because growth and plaquing is possible in a cat monolayer cell culture system. The resistant isolates have cross-resistance to other compound identical to the resistant isolates of HIV.[3] The mutant and parental strains of FIV may provide information regarding both drug susceptibility *in vivo* and virulence.

3. GENETICS OF AZT RESISTANCE

3.1. *Mutations Associated with Resistant Isolates*

The mechanism of resistance to AZT is attributable not suprisingly to mutations in the viral reverse transcriptase. Sequencing the reverse transcriptase gene of 5 pairs of isolates that displayed more than 100-fold reductions in susceptibility during the course of therapy documented multiple mutations, four of which appeared common.[15] When these 4 mutations at codons 67, 70, 215 and 219 were inserted by site directed mutagenesis into the susceptible, infectious molecular clone pHXB2, a greater than 100 fold reduction in AZT susceptibility resulted. Sequential isolates from the same individual that displayed progressive, step-wise increments in resistance were associated with the sequential cumulative acquisition of these 4 mutations.[15] Cumulative mutations thus contribute additively or synergistically to stepwise reductions in susceptibility. Mutations at the four identified codons are among the most important, but almost certainly not the exclusive, contributors to the resistance phenotype.

Several investigators have developed assays utilizing sequence amplification methodologies to assay for these point mutations. Boucher and colleagues utilizing primer pairs for the polymerase chain reaction (PCR) that include the wild type or mutant sequence for codon 215 demonstrated the appearance of mutant sequence at codon 215 in asymptomatic patients receiving chronic therapy with AZT.[16] Mutations gradually appeared over a two year period with therapy and correlated with the phenotype of resistance. Mitsuya and colleagues have discriminated wild type or mutant sequences with restriction endonucleases after PCR of the reverse transcriptase gene to identify the appearance of mutations at codon 215 with therapy (H. Mitsuya, personal communication). López-Galíndez *et al.* have utilized patterns of susceptibility to ribonuclease A to discriminate wild type or mutant sequence at codon 215.[17] Although isolates from California and Spain had very distinctive digestion patterns, the pattern characteristic of a mutation at codon 215 occurred only in patients from either area receiving chronic AZT therapy.

Richman *et al.*[18] have utilized a PCR technique with primer pairs that generate a 535 base pair sequence that spans all 4 codons of interest in the reverse transcriptase. Four pairs of oligonucleotide probes that are specific for wild type mutant sequence

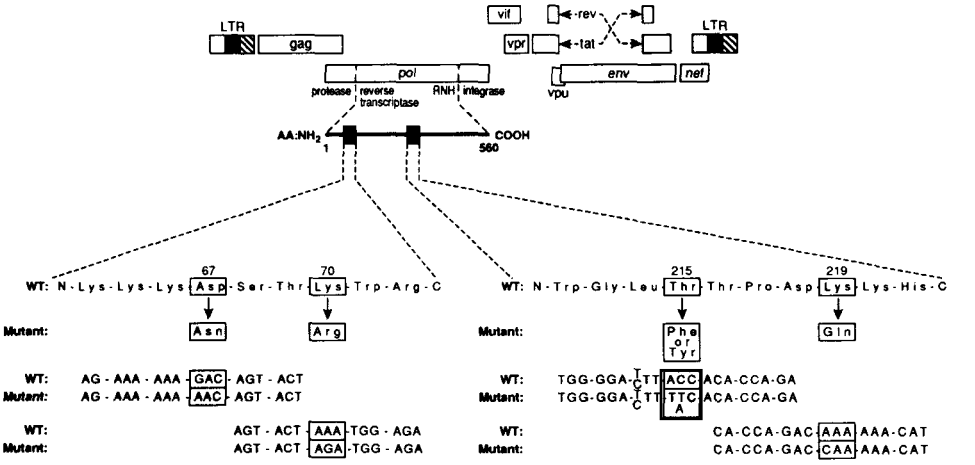

FIGURE 2 Location and sequence of oligonucleotides used to detect mutations in the reverse transcriptase gene. With permission from Richman *et al.*, 1991.[18]

at each of the codons are then used on to characterize the sequence at each codon utilizing as target either patient peripheral blood mononuclear cells or virus isolates on which AZT susceptibility has been performed (Figure 2). The results generated with this assay are summarized below.

Gingeras *et al.* have utilized an isothermal, single cycle amplification technique,[19] termed the self-sustained sequence replication (3SR) system, that can discriminate wild type or mutant sequence at each of the four codons. Because this amplification method generates a predominance of one strand of single stranded RNA, direct sequencing is possible, thus permitting a number of additional issues to be investigated.[20]

3.2. *Time Course and Sequence of Appearance of Mutations*

366 independent specimens (virus stocks or peripheral blood mononuclear cells) from 168 individuals have been genotypically characterized utilizing the PCR method.[18] 67 specimens have been obtained prior to AZT therapy, all of which have displayed wild type genotype. Sixty specimens obtained from individuals receiving AZT therapy have contained a mutant sequence at a single codon: 5 at codon 67, 23 at codon 70, 31 at codon 215 and 1 at codon 219. Thus a mutation may first appear at any codon but the selection for some mutations occurs far more readily than for others.

The cumulative proportion of individuals with a mutations at each codon has also been determined. These overall rates do not indicate the sequence of appearance of mutations in all individual, as is demonstrated by the data already regarding the first mutation to develop. In addition because of the number of specimens tested, the 95% confidence limited of the values for each curve are relatively wide. The proportion of specimens with mutations at 1 year were 33% for codon 67 (95% CI: 20%–51%) 59% for codon 70 (95% CI: 44%–75%), 63% for codon 215 (95% CI: 48%–78%), and 13% for codon 219 (95% CI: 1%–31%).

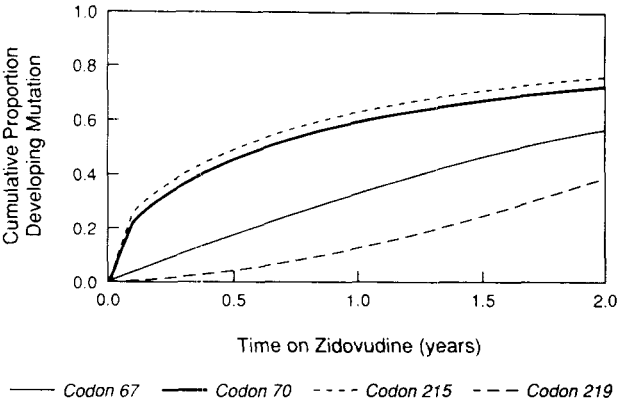

FIGURE 3 Cumulative proportion of individuals with a mutation at each of the four codons of the HIV reverse transcriptase after the initiation of zidovudine. With permission from Richman *et al.*, 1991.[18]

3.3. *Correlation of Genotype with Phenotype*

Highly resistant virus contains mutant sequence in the codons under consideration and when these mutations were placed in an infectious DNA construct, the resulting virus was converted from sensitive to highly resistant. Nevertheless it has not been clearly established to what extent each mutation contributed to reduced AZT susceptibility and what proportion of AZT resistance can be attributed to these four codons. All isolates obtained prior to AZT therapy were previously shown to have an IC_{50} of $< 0.045\,\mu M$.[2,8] Isolates with a single mutation at codons 70 or 215 were examined for drug susceptibility. Most isolates with a single mutation had a susceptibility that fell within the range of sensitive isolates, although the susceptibilities ranged over a more than tenfold range for isolates with mutations at codons 70 or 215. This observation suggests that a mutation may have a variable impact in a different genetic context or that additional mutations, not at one of the 4 codons, also contribute to reduce susceptibility. This hypothesis is supported by the observation in a patient whose isolate with a single mutation at codon 215 changed from sensitive ($IC_{50} = 0.02\,\mu M$) to resistant ($IC_{50} = 0.17\,\mu M$) over a two month period.[18]

The results of logistic regression analyses have shown that phenotypic resistance is statistically related to sites 215 ($p = 0.003$), site 70 ($p = 0.006$), site 219 ($p = 0.04$), and marginally associated with site 67 ($p = 0.084$).[18] However, the best fitting model includes the total number of mutant sites among sites 70, 215, and 219 ($p < 0.0001$). In fact, when all three sites were mutant, the probability of resistance was 100%; if two of the three sites were mutant, the probability of resistance was 90%; if one of the three sites was mutant, the probability of resistance was 45%; and if none of the sites were mutant, the probability of resistance was 0% (Figure 3). The results of the linear regression analyses on the continuous log transformed IC_{50} were consistent with those from the logistic regression analyses. The most predictive model includes the total number of mutant sites among 70, 215, and 219 (Figure 4). Mutation information from site 67 did not add any additional information, and adjusting for time on study had no effect on the estimated in either analysis.

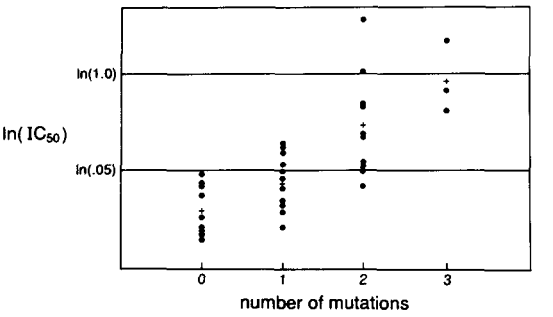

FIGURE 4 Relation of susceptibility of isolates of HIV to number of mutations at codons 70, 215 and 219 of the reverse transcriptase gene. The natural log of the 50% inhibitory concentration (IC_{50}) of virus isolates is indicated with the number of mutations at codons 70, 215 and 219. All values are graphed for virus stocks on which both zidovudine susceptibility testing and genotyic analysis were performed. Sequential specimens from the same patient with the same value were excluded. Values for individual virus stocks are depicted with ●. The cutoff for sensitive virus has been defined as 0.05 μM. The geometric means (+) of each set of values was 0.02 μM for 0 mutations, 0.04 μM for 1 mutation, 0.21 μM for 2 mutations and 0.88 μM for 3 mutations. With permission from Richman *et al.* 1991.[18]

3.4. *Evidence for the Existence of Genotypic Mixtures in Patients on Therapy*

The suggestion of mixed populations of virus with different AZT susceptibilities has been confirmed with genotypic analysis. Boucher *et al.*[16] documented several individuals with the simultaneous presence of both wild type and mutant sequence at codon 215. López-Galíndez *et al.*[17] documented by sequencing of clones the simultaneous presence in the same individuals of both the phenylalanine and tyrosine mutations at codon 215. Richman *et al.*[18] have demonstrated mixtures at each of the codons, occasionally several simultaneously, during the transition from pure wild type to pure mutant sequences in that individual.

3.5. *Selection with a Subpopulation With Passage* In Vitro

A small proportion of virus isolates have been documented to change susceptibility pattern or genotype with passage *in vitro* in the absence of drug.[18,20] This phenomenon occurs more frequently if a mixed population is present in the original mononuclear cell specimen. Shifts to either more or less sensitive populations have been documented.

3.6. *Non Reactivity with Either Wild Type or Mutant Probes*

The PCR assay described by Richman *et al.*[18] did not fail to amplify and detect at least one codon in any of the 67 clinical samples obtained from seropositive individuals prior to AZT therapy. With zidovudine therapy, however, 222 in 237 peripheral blood cell or virus stock specimens failed to react with either wild type or mutant probe for 1 or more of the codons. Preliminary sequencing studies suggests that the appearance of additional mutations either with the codon in question or in adjacent codons accounts for this diminished probe affinity. This problem will presumably affect most such assays with this highly mutable virus. It is important to appreciate that a mutation in the reverse transcriptase could affect drug susceptibility, have a neutral effect on drug susceptibility or confound assays to detect other specific sequences.

4. CONCLUSIONS

The development of reduced susceptibility to zidovudine *in vitro* has been described by several laboratories using isolates from patients receiving prolonged drug therapy. The likelihood of developing resistance increases with advanced disease stage and with lower CD4 lymphocyte counts but appears not to be related to drug doses with daily doses of zidovudine at 500 mg or more. Mutations at 4 codons of the reverse transcriptase have been associated with the resistance phenotype. Assays to detect these mutations directly in the peripheral blood mononuclear cells of treated patients have confirmed the development of these mutations in association with reducd susceptibility in epidemiologically unrelated study populations in the Netherlands, Spain, California.

Several approaches to reduce the rate of emergence of drug resistance clinically are under investigation. Alternating regimes, for example of AZT and ddC[21–23] would be expected to ameliorate drug toxicity with two drugs with non-overlapping toxicities. An alternating regimen would also be predicted to at least double the interval before resistance developed, because the selective pressure of therapy with any one drug is present only one half of the time. AZT and ddC in combination are also in clinical trial.[24] Combination chemotherapy, by analogy with bacteria and tumor cells, would be predicted to select for resistance with a probability that is the product of the probabilities of each drug alone. These hypotheses are currently under investigation.

Assuming that the importance of drug resistance will be documented, all future *in vitro* and *in vivo* evaluation of candidate drugs must contend with the prospect of drug resistance. Studies of candidate drugs *in vitro* must include an assessment of *in vitro* selection for resistance, of cross-resistance with other compounds and of drug interactions with regard to synergy, antagonism and toxicity. The evaluation of drugs in the clinic must be designed with the prospect that the future of chemotherapy for HIV, as for tuberculosis and most malignancies, will be with combination regimens.

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