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Nucleotide substitution patterns can predict the requirements for drug-resistance of HIV-1 proteins

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Abstract

The enzyme reverse transcriptase (RT) plays a fundamental role in the replication of the human immunodeficiency virus type 1 (HIV-1) and several antiviral agents that target this key enzyme have been developed. Unfortunately, treatment of patients with RT inhibitors results in the appearance of drug-resistant variants with specific mutations in the RT protein. We hypothesized that if 'difficult' resistance mutations (e.g. transversions/double-hits) are consistently observed at certain positions, it is likely that 'easier' nucleotide substitutions (transitions/single-hits) at that codon do not result in a drug-resistant and/or active RT enzyme. In this study, we examined codon changes involved in RT drug resistance against nucleoside and non-nucleoside inhibitors and listed all easier substitutions, which apparently were not selected, either due to reduced enzyme RT activity or lack of drug resistance. These predictions on the requirements for resistance were confirmed by published mutational data on RT variants. We also propose that differences in mutation type can explain the order of appearance of substitutions in case multiple amino acid changes are required for optimal fitness. Differences in mutation pattern have been reported for drug-resistant HIV-1 variants selected in tissue culture compared with variants found in treated patients. In contrast to the in vivo situation, a relatively small population size is handled in in vitro tissue culture systems and this may limit the chances of creating a resistance mutation. Indeed, inspection of the codon changes indicates that the in vitro culture system is more strongly biased towards the relatively easy nucleotide substitutions. These results suggest that the nucleotide substitution pattern can provide important information on RT drug resistance.

Keywords: Drug resistance; Reverse transcriptase; HIV-1; Mutation bias; Transition/transversion

1. Introduction

During treatment with an antiviral drug, spontaneously generated human immunodeficiency virus type 1 (HIV-1) mutants with drug-resistance properties will increase in frequency and ultimately become fixed in the population. Two factors are primarily responsible for this enormous genetic flexibility of the HIV-1 virus: (i) the high virus titer in infected individuals combined with a high replication rate of ~ 2 days and (ii) the error-proneness of the HIV-1 reverse transcriptase

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(RT) enzyme. (i) Recent data demonstrate that the long asymptomatic period is not one of viral latency, but rather one of highly efficient virus replication, which is somewhat masked by the rapid turnover of both the HIV-1 virus and infected cells (Ho et al., 1995; Wei et al., 1995). It is estimated that virions are produced at a rate of 10^8 or more daily. (ii) Replication of a retroviral genome is intrinsically error-prone. Retroviral replication involves three replicative steps: RNA → dsDNA by the viral RT enzyme, DNA → DNA by the very accurate host replication complex, and DNA → RNA by cellular transcription. The first and third step occur in the absence of proofreading, as both the viral RT polymerase and the cellular RNA polymerase are devoid of 3′-exonucleolytic activity and both reactions display about the same accuracy ($\sim 10^{-4}$ per base copied (Anderson and Menninger, 1986; Roberts et al., 1989)). Although it is possible for mutations to be introduced into the viral genome during any of these replication steps, much attention has focused on the fidelity of DNA synthesis by the RT enzyme (Bebenek and Kunkel, 1993).

Evolution of drug-resistant HIV-1 variants is a multi-step process. The first step consists of the generation of variation; that is, of suitable genetic variants that can serve as the material that will be exposed to phenotypic selection, which forms the second stage. The first step of evolution is completely independent of the actual selection process. The probability that a particular mutation will become fixed in a population depends on (i) the rate of mutation at that particular site, or in case the mutation preexists, its initial frequency, (ii) its selective advantage, and (iii) the effective population size (Li and Graur, 1991). Although the fixation probability of a certain genotype may depend on aspects of population structure, population size is a constant factor when we analyze the pattern of drug-resistance development within one HIV-infected individual. The most important factor in determining the fixation of drug-resistant mutations is the selection intensity (which is determined by functional constraints on the RT protein), i.e. induction of drug resistance and maintenance of catalytic activity (these combined enzyme properties will be termed 'fitness'). Even though phenotypic selection criteria will eventually shape the virus population, base substitution rates will largely determine the set of mutants available for the subsequent selection process. Given the high population size of HIV-1 in an infected individual, combined with the rapid replication kinetics and the high error rate during retroviral replication, it can be calculated that all variant genotypes with single base substitutions will be available at any point in time for an appropriate response to the drug selection pressure (Coffin, 1995; Najera et al., 1995). On the other hand, it is likely that complex nucleotide substitutions (e.g. double-hit codon changes) are not available in the initial virus pool. Furthermore, a natural HIV-1 population may contain insufficient variability to satisfy the demand of drugs like AZT that require multiple amino acid changes in the RT protein for optimal fitness.

To address the question of how specific amino acid substitutions can confer drug resistance, detailed structure–function analyses should be performed with both wild-type and mutant RT enzymes. In this study, however, we propose that some of the requirements for drug resistance can be predicted by inspection of the type of nucleotide substitution. The type of substitutions can be divided into transitions (substitutions between purines (A and G) or between pyrimidines (C and T)) and transversions (substitutions between a purine and a pyrimidine). We analyzed literature data on the type of nucleotide substitution (e.g. transition versus transversion, single-hit versus double-hit) that cause drug resistance in tissue culture and in patients undergoing therapy. We hypothesize that if a 'difficult' nucleotide substitution (transversion) is consistently found in drug-resistant viruses, it is likely that all 'easy' substitutions (transition) at that codon will not result in a fit RT enzyme that is both functionally active and drug-resistant. The validity of this hypothesis was demonstrated for those amino acids in the RT protein for which detailed mutational analysis is available.

2. Results and discussion

2.1. Informative codon changes in HIV-1 drug resistance

The pattern of spontaneous nucleotide substitutions has been studied for cellular pseudogenes that are generally believed not to be subject to selective constraints since they are devoid of function (Gojobori et al., 1982; Li et al., 1984). In this system, it was found that transitions occur more often than transversions. A similar mutational spectrum was observed in naturally occurring HIV sequences (Myers et al., 1995), cell-free RT reactions (Ji et al., 1994; Ji and Loeb, 1994), infection experiments in tissue culture (Berkhout and Klaver, 1995; Klaver and Berkhout, 1994) and SIV infections of macaques (Johnson et al., 1991; Pelletier et al., 1995). Based on this spectrum of mutations, we analyzed mutations in the HIV-1 RT gene that confer resistance to antiviral drugs as relatively 'easy' (transitions, type 1) or 'difficult' (transversions, type 2).

All mutations in the RT gene that are currently known to cause resistance to nucleoside analogs are listed in Table 1 (Mellors et al., 1995; adapted from the original with the kind permission of International Antiviral News). Table 1 presents the following information: (i) the drugs used in the selection for resistance, (ii) the amino acid and (iii) corresponding codon change involved, (iv) whether this mutation was observed in tissue culture infections (in vitro) or (v) in drug-treated patients (in vivo), (vi) the type of substitution observed, (vii) a list of all substitutions at that codon position that are easier to generate and (viii) the potential intermediates in case of double-hits. Let us first discuss some of the cases where easier substitutions are not selected. For instance, AZT treatment consistently selects for resistant RT forms with the Met41Leu substitution. Interestingly, whereas the Leu-variant is obtained in two codon forms (ATG → TTG/CTG, both transversions), alternative codon changes that are easier to generate (Ile, Val and Thr; transitions) were never observed. This means that, although other amino acids are possible through easier nucleotide substitutions, the enzyme needs the Leu at position 41 to maintain optimal fitness in the presence of the drug. Structural properties may specify the requirements for this position because amino acid 41 is part of an α-helix in the RT structure (Kohlstaedt et al., 1992), and both the wild-type Met and variant Leu residues are similar with respect to their capacity to facilitate the α-helix conformation. In fact, Met and Leu are good α-helix formers ($P_\alpha = 1.20$ and 1.34, respectively) compared with the alternative residues Ile, Val and Thr ($P_\alpha = 1.00$, 1.14 and 0.82, respectively; (Chou and Fasman, 1978)). Similarly, the drug ddI selects for the Leu74Val mutation (transversion), suggesting that a Leu74Ser mutation (transition) is unable to either confer drug resistance or maintain enzyme function.

This type of codon analysis is not restricted to nucleoside drug resistance in the RT protein. A similar analysis was performed for the RT mutations that confer resistance towards non-nucleoside drugs (Table 2, adapted from the original (Mellors et al., 1995) with the kind permission of International Antiviral News) and for resistance markers in the HIV-1 protease enzyme (data not shown). For instance, a common mutation seen in the HIV-1 RT protein upon treatment with non-nucleoside inhibitors is the Leu100Ile change (TTA → ATA, transversion). Although a transition (Ser) is possible, such a mutation has never been detected, suggesting that it creates a less fit RT enzyme. Interestingly, the Ser-variant has been tested as part of a mutational analysis (Boyer et al., 1994). Consistent with our prediction, this mutant was less than 5% active compared to wild-type RT. Thus, it seems that RT enzyme requirements do restrict the variation allowed at position 100.

Detailed mutational analysis has also been reported for RT position 190, which is involved in resistance to the non-nucleoside RT drug nevirapine (Balzarini et al., 1994a; Chao et al., 1995; Kleim et al., 1994; Wei et al., 1995). A transversion (GGA → GCA, Gly → Ala) is observed, suggesting that easier codon changes do not encode a functional and/or resistant RT enzyme (Arg and Glu, both transitions). Interestingly, resistance properties cannot explain this biased evolution

Table 1
Characteristics of RT resistance mutations against nucleoside drugs [a]

Drug	Amino acid substitution	Codon change	Observed		Type of substitution [b]	Easier substitutions	Possible intermediates
			In vitro	In vivo			
AZT	Met41Leu	ATG-TTG	nd [c]	Yes	2	Ile(1), Val(1), Thr(1)	–
	Met41Leu	ATG-CTG	nd	Yes	2	Ile(1), Val(1), Thr(1)	–
	Asp67Asn	GAC-AAC	Yes	Yes	1	–	–
	Lys70Arg	AAA-AGA	Yes	Yes	1	–	–
	Thr215Phe	ACC-TTC	nd	Yes	2.1	Ile(1), Ala(1), Ser(2), Pro(2), Asn(2),	Ile (ATC:1), Ser (TCC:2)
	Thr215Tyr	ACC-TAC	Yes	Yes	2.2	Ile(1), Ala(1), Ser(2), Pro(2), Asn(2), Val(1.1), Met(1.2), Asp(1.2), Gly(1.2), Leu(2.1), Phe(2.1)	Asn (AAC:2), Ser (TCC:2)
	Lys219Glu	AAA-GAA	Yes	No	1	–	–
	Lys219Gln	AAA-CAA	No	Yes	2	Glu(1)	–
ddI	Leu74Val	TTA-GTA	No	Yes	2	Ser(1)	–
	Val75Thr	GTA-ACA	Yes	Yes	1.1	Ile(1), Ala(1), Glu(2), Gly(2), Leu(2)	Ile (ATA:1), Ala(GCA:1)
	Met184Val	ATG-GTG	Yes	Yes	1	–	–
ddC	Lys65Arg	AAA-AGA	Yes	Yes	1	–	–
	Thr69Asp	ACT-GAT	No	Yes	1.2	Ile(1),Ala(1), Ser(2), Pro(2), Arg(2), Asn(2), Val(1.1)	Ala (GCT:1), Asn (AAT:2)
D4T	Ile50Thr	ATT-ACT	Yes	nd	1	–	–
	Val75Thr	GTA-ACA	Yes	Yes	1.1	Ile(1), Ala(1), Glu(2), Gly(2), Leu(2)	Ile (ATA:1), Ala(GCA:1)
3TC	Met184Ile	ATG-ATA	Yes	Yes	1	–	–
	Met184Val	ATG-GTG	Yes	Yes	1	–	–

[a] Adapted from the original with the kind permission of International Antiviral News (Mellors et al., 1995).
[b] Type 1 = transition, Type 2 = transversion.
[c] nd = not determined.

Table 2
Characteristics of RT resistance mutations against non-nucleoside drugs [a]

Drug	Amino acid substitution	Codon change	Observed		Type of substitution [b]	Easier substitutions
			In vitro	In vivo		
Nevirapine	Ala98Gly	GCA-GGA	No	Yes	2	Val(1), Thr(1), Ala(1)
	Leu100Ile	TTA-ATA	No	Yes	2	Ser(1)
	Lys103Asn	AAA-AAC	No	Yes	2	Glu(1), Arg(1)
	Val106Ala	GTA-GCA	Yes	Yes	1	–
	Val108Ile	GTA-ATA	No	Yes	1	–
	Tyr181Cys	TAT-TGT	Yes	Yes	1	–
	Tyr181Ile	TAT-ATT	Yes	Yes	2.2	Tyr(1), Cys(1), His(1), Asp(2), Asn(2) [d], Phe(2), Ser(2), Arg(1.1), Trp(1.2), Gln(1.2), Leu(1.2), Pro(1.2), Gly(2.1)
	Tyr188Cys	TAT-TGT	No	Yes	1	–
	Gly190Ser	GGC-AGC	nd [c]	Yes	1	–
	Gly190Ala	GGA-GCA	No	Yes	2	Arg(1), Glu(1)
R82150	Leu100Ile	TTA-ATA	Yes	nd	2	Ser(1)
R82913	Leu100Ile	TTA-ATA	Yes	nd	2	Ser(1)
	Lys103Asn	AAA-AAC	Yes	nd	2	Arg(1), Glu(1)
	Val106Ala	GTA-GCA	Yes	nd	1	–
	Glu138Lys	GAG-AAG	Yes	nd	1	–
	Tyr181Cys	TAT-TGT	Yes	nd	1	–
	Tyr188His	TAT-CAT	Yes	nd	1	–
	Tyr188Leu	TAT-TTA	No	Yes	2.2	Tyr(1), Cys(1), His(1), Asp(2), Asn(2), Phe(2), Stop(2), Ser(2), Arg(1.1), Trp(1.2), Gln(1.2), Leu(1.2), Pro(1.2), Gly(2.1)
L'697 593	Lys103Asn	AAA-AAC	Yes	nd	2	Arg(1), Glu(1)
	Tyr181Cys	TAT-TGT	Yes	nd	1	–
L'697 661	Ala98Gly	GCA-GGA	No	Yes	2	Val(1), Thr(1), Ala(1)
	Leu100Ile	TTA-ATA	Yes	No	2	Ser(1)
	Lys101Glu	AAA-GAA	No	Yes	1	–
	Lys103Asn	AAA-AAC	Yes	Yes	2	Arg(1), Glu(1)
	Lys103Gln	AAA-CAA	No	Yes	2	Arg(1), Glu(1)
	Val108Ile	GTA-GCA	Yes	Yes	1	–
	Val179Asp	GTT-GAT	No	Yes	2	Ile(1), Ala(1)
	Val179Glu	GTT-GAG	No	Yes	2.2	Ile(1), Ala(1), Leu(2), Phe(2), Gly(2), Asp(2), Val(2), Thr(1.1), Ser(1.2), Asn(1.2), Met(1.2), Pro(2.1)
	Tyr181Cys	TAT-TGT	Yes	Yes	1	–
U-90152	Pro236Leu	CCT-CTT	Yes	nd	1	–

Table 2
Characteristics of RT resistance mutations against non-nucleoside drugs [a]

Drug	Amino acid substitution	Codon change	Observed		Type of substitution [b]	Easier substitutions
			In vitro	In vivo		
U-87201	Lys101Glu	AAA-GAA	No	Yes	1	–
	Lys103Asn	AAA-AAC	No	Yes	2	Arg(1), Glu(1)
	Tyr181Cys	TAT-TGT	No	Yes	1	–
	Tyr188His	TAT-CAT	No	Yes	1	–
	Glu233Val	GAA-GTA	No	Yes	2	Lys(1), Gly(1)
	Pro236Leu	CCT-CTT	Yes	No	1	–
	Lys238Thr	AAA-ACA	No	Yes	1	Arg(1), Glu(1)
U-88204	Leu100Ile	TTA-ATA	Yes	nd	2	Ser(1)
	Val106Ala	GTA-GCA	Yes	nd	1	–
	Tyr181Cys	TAT-TGT	Yes	nd	1	–
	Tyr181Ile	TAT-ATT	Yes	Yes	2.2	Tyr(1), Cys(1), His(1), Asp(2), Asn(2), Phe(2), Ser(2), Arg(1.1), Trp(1.2), Gln(1.2), Leu(1.2), Pro(1.2), Gly(2.1)
α-APA	Tyr181Cys	TAT-TGT	Yes	nd	1	–
S-2720	Gly190Glu	GGA-GAA	Yes	nd	1	–
TSAO	Glu138Lys	GAG-AAG	Yes	nd	1	–

[a] Adapted from the original with the kind permission of International Antiviral News (Mellors et al., 1995).
[b] Type 1 = transition, Type 2 = transversion.
[c] nd = not determined.
[d] Potential intermediates are underlined.

since several 190-variants were constructed and shown to confer drug resistance. These RT variants, however, could easily be distinguished on the basis of polymerase activity. Of several 190-variants tested, only the 190-Ser and 190-Ala variants demonstrated > 50% polymerase activity (290% and 75–110%, respectively, as a percentage of the wild-type activity (Chao et al., 1995; Kleim et al., 1994)). Among the inactive RT enzymes were the 190-Arg and 190-Glu variants (2–8% and 4% active, respectively), which is consistent with our prediction based on nucleotide substitution patterns. Furthermore, analysis of the codons involved does also provide a simple explanation for the inability to select for the 190-Ser variant, which is both active and resistant. The route from the wild-type 190-Gly codon to a Ser requires at least a 2-hit substitution (GGA → AGT/AGC, transition and transversion, type 1.2), which is less likely to occur than the 1-hit route towards the 190-Ala variant. Interestingly, one nevirapine-treated patient was recently identified with the 190-Ser codon (Wei et al., 1995). It was demonstrated that the initial virus population contained the Gly-190 codon GGC instead of GGA, which facilitates a Gly190Ser substitution by means of a single transition (GGC → AGC). This example underscores the necessity to obtain pre-treatment HIV-1 sequences of every individual patient, and that one cannot rely on consensus HIV-1 genotypes.

Some amino acid changes responsible for RT drug resistance are caused by multiple mutations or 'double-hits' within one codon. In general, the frequency of a double substitution is expected to be equal to the product of the frequency of a single substitution if the two mutational events are fully independent. Misincorporation of one nucleotide, however, may affect extension of the mismatched primer and may increase additional misincorporations at the adjacent positions. Nevertheless, double substitutions should occur much less frequently than the single ones. For instance, AZT treatment results in selection of variants at codon 215 (Thr215Phe or Thr215Tyr) that require two substitutions each (ACC → TTC and ACC → TAC, types 2.1 and 2.2, respectively). A large number of easier mutations can be listed for 2-hit substitutions and all 1-hit and 2-hit mutations easier than Thr215Phe are listed in Fig. 1A and summarized in Table 1 (Mellors et al., 1995). If an alternative amino acid can be generated by multiple routes, only the easiest possibility is listed. For instance, Fig. 1A shows that a Thr215Ile substitution can be made either through a single transition (type 1) or a double-transition (type 1.1), but only the former route is listed in Table 1. Thus, there are 5 amino acid changes (Ile, Ala, Ser, Pro, Asn) that are easier to generate than Thr215Phe, yet none of these substitutions have been detected in vivo or in vitro. Ten codon substitutions are easier to generate than Thr215Tyr but apparently not selected (Ile, Ala, Ser, Pro, Asn, Val, Met, Leu, Asp, Gly). These combined data strongly suggest that only an aromatic residue at position 215 can provide the AZT-resistant phenotype. The third possible aromatic residue (215Trp) has never been observed, which can also be explained on the basis of mutation rates since a difficult triple mutation is required (ACC → TGG, type 2.2.2). Although both 215Tyr and 215Phe variants have been observed in patients, the two routes also differ in their mutational probability (type 2.2 versus 2.1). In fact, the observation that the more difficult 2.2 pattern is frequently selected indicates that the 215Tyr enzyme should have a higher fitness than the 215Phe variant.

The conclusions derived at in this theoretical exercise are fully consistent with experimental data from a recent mutational study of codon-215 variants (Lacey and Larder, 1994). Of all mutants tested, RT enzyme activity varied from 30% (Pro) to 100% (Thr(wt), Ala, Ser). No AZT resistance was observed for the wild-type RT enzyme and a large set of variants (Pro, Arg, Ser, Leu, Ala, Ile), low level resistance was observed for the Trp variant, and high level resistance for the Phe and Tyr variants. As predicted by our analysis, the Tyr mutant was found to be slightly more AZT-resistant than the Phe variant (Lacey and Larder, 1994). For codons that differ by more than one nucleotide from the initial sequence, we need to consider several evolutionary pathways that can lead to the observed changes (Fig. 1B). Besides fitness criteria for the intermediate RT forms, mutational bias can also determine which route is

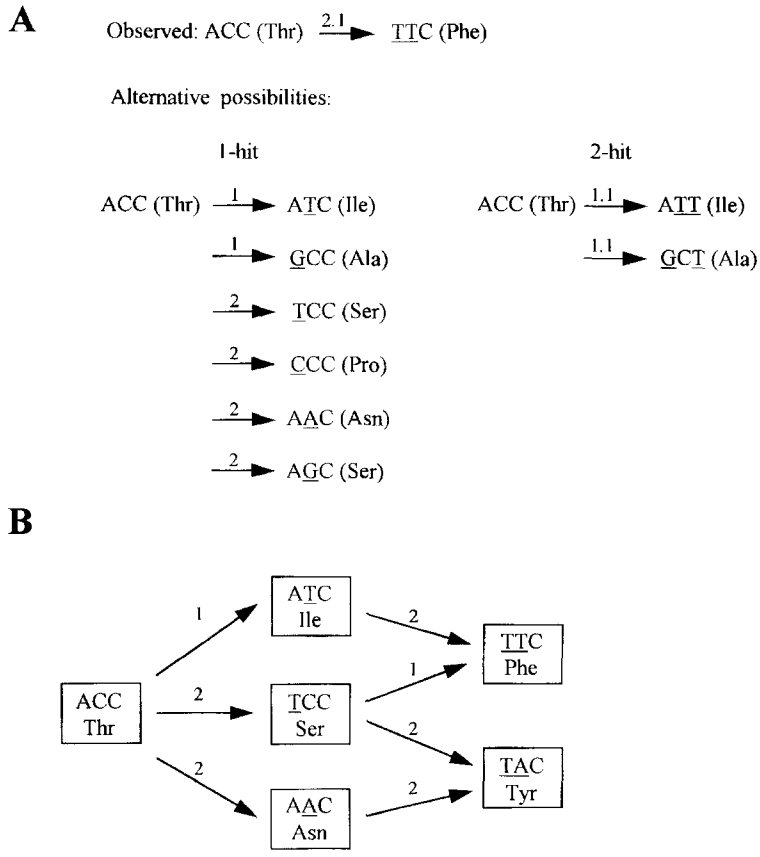

Fig. 1. (A) Alternative codon mutations that are easier to acquire than the observed Thr215Phe mutation (ACC→TTC, type 2.1). Listed are all 1-hit and 2-hit routes that should occur more frequently based on the mutational bias of HIV-1 replication. The mutated nucleotides are marked by underlining. Three silent substitutions are not included: ACT, ACA, ACG. (B) Schematic representation of the Thr215Phe/Tyr pathways.

preferred. It is obvious that every substitution pattern needs its own detailed discussion. In order to allow researchers to address such questions, we listed the possible intermediates for every double-hit scenario for HIV-1 RT drug resistance (Tables 1 and 2).

2.2. Mutation bias can explain the order of appearance of drug resistance mutations

Resistance to AZT comprises a sequence of events that appears to involve at least five separate amino acids in the HIV-1 RT domain (Kellam et al., 1992; Larder, 1994). The order of appearance of drug-resistance markers is similar among different individuals: Lys70Arg is commonly reported to occur first. Secondly, a new subpopulation with Thr215Phe/Tyr and subsequently Met41Leu is observed, followed by the appearance of variants with a combination of several of these markers, possibly through genetic recombination (Kellam and Larder, 1995). Finally, Asp67Asn and Lys219Gln changes are frequently detected in samples of AZT-treated patients, but usually in combination with some of the initial substitutions. A natural HIV-1 popula-

tion contains insufficient variability to satisfy the demand of drugs like AZT that require multiple amino acid changes in the RT protein for optimal resistance. Therefore, we propose that mutation rate is one of the factors that determine the order of appearance of the five AZT-resistance mutations. For instance, resistance properties alone cannot explain why Lys70Arg precedes the Thr215Phe/Tyr substitution (8-fold and 16-fold resistance, respectively), but the transition (type 1) that generates Lys70Arg is easier to accomplish than the double-hit leading to Thr215Phe/Tyr (type 2.1/2.2). Consistent with this idea, the Lys70Arg variant was recently reported to pre-exist in some untreated patients (Najera et al., 1995). That other relatively easy mutations (Asp67Asn, type 1; Lys219Glu/Gln, type 1/2) appear only after a prolonged period of drug treatment may suggest that their contribution to drug resistance is small. Alternatively, these 'late' mutations may represent compensatory amino acid changes that are needed either to restore optimal RT enzyme function or to obtain optimal drug resistance in the initial 41-70-215 mutant. Indeed, there is some evidence that the Asp67Asn mutation is beneficial only in the context of the Lys70Arg mutation. Whereas introduction of Asp67Asn provides increased AZT resistance in combination with Lys70Arg (70Arg-215Tyr → 67Asn-70Arg-215Tyr, 6-fold → 31-fold resistance; 41Leu-70Arg-215Tyr → 41Leu-67Asn-70Arg-215 Tyr, 34-fold → 179-fold resistance), a reduction in resistance was observed in HIV-1 variants lacking the 70Arg substitution (41Leu-215Tyr → 41Leu-67Asn-215Tyr, 64-fold → 43-fold resistance; Larder, 1994).

There are two interesting cases where HIV-1 variants with easier mutations are transiently detected soon after initiation of drug treatment in vivo, followed by outgrowth of a fitter variant that was more difficult to generate by mutation. First, individuals treated with the nucleoside analog 3TC develop resistance within 2 weeks, at which time point a stable Met184Val substitution (ATG → GTG, transition) can be detected (Schuurman et al., 1995). Multiple patient samples taken at earlier times (~ 1 week) demonstrated another variant at this position (Met184Ile, ATG → ATA, transition). Both RT variants have been demonstrated to provide the drug-resistant phenotype (Boucher et al., 1993; Gao et al., 1993; Schinazi et al., 1993; Tisdale et al., 1993). Inspection of the codons indicates that the 184Ile variant is not an intermediate in the generation of the 184Val variant, but rather an independent pathway towards 3TC-resistance. Thus, 184Ile seems to be generated at a higher frequency than 184Val, but 184Ile is lost in the subsequent competition with the 184Val mutant. The initial appearance of the 184Ile variant suggests that the frequency of G → A transitions at this codon position is higher than that of A → G transitions. Furthermore, eventual outgrowth of the 184Val variant is consistent with the observation that this RT variant provides better enzyme properties than the 184Ile variant (Back, Boucher and Berkhout, unpublished data).

A second interesting pattern of virus evolution was observed at RT codon 181 in some patients treated with the non-nucleoside RT drug nevirapine (Wei et al., 1995). Initially, the substitution Tyr181Cys was observed (TAT → TGT; type 1), but at later timepoints the viral population consisted exclusively of the Tyr181Ile mutant, which involves a difficult double transversion (TAT → ATT, type 2.2). Inspection of the codons involved indicates that the Tyr181Cys variant is not an intermediate from the wild-type RT to the Tyr181Ile mutant. Again, these results suggest a correlation between the order of appearance of HIV-1 variants and the corresponding mutation rates. Furthermore, eventual outgrowth of the difficult 181Ile variant predicts that this RT protein is either a better enzyme or more drug-resistant than the 181Cys variant. Optimal fitness of the 181Ile variant was convincingly demonstrated in an in vitro selection experiment with nevirapine starting with a 181Cys (codon TGT) construct that was able to convert to 181Ile by a 2-hit mutation (TGT → ATT, type 2.2, Balzarini et al., 1994b).

If multiple pathways can lead to resistance, the route with the smallest number and/or easiest type of substitutions will be preferentially used. In

more complex selection schemes that are more demanding on the RT enzyme, for instance in combination therapies, there may be a limited number of routes that lead to resistance, and the initial step may in fact represent a difficult substitution. Combination therapy with AZT and ddC provides such a situation (Shirasaka et al., 1995). Interestingly, some patients did not develop the AZT- or ddC-resistance mutations that are typically observed during monotherapy, but instead selected five new substitutions during combination therapy. The first mutation that became fixed is a difficult double transversion at codon 151 (CAG → ATG, type 2.2). The fact that it took 16 months for this mutation to develop may reflect the difficulty of generating this type of mutation. Therefore, we would predict that this amino acid substitution (Gln151Met) is essential for the multi-drug resistance phenotype, and that no easy 1-hit substitutions can provide this phenotype. Indeed, experiments with mutant HIV-1 clones confirmed that the individual Gln151Met change can confer resistance to nucleoside analogs (Shirasaka et al., 1995). Analysis of the alternative codon mutations that are easier to produce, yet are not observed in vivo, predicts that a large number of amino acids (Arg(1), Lys(2), Asp(2), Leu(2), Pro(2), His(2), Trp(1.1), Ser(1.2), Tyr(1.2), Gly(2.1)) at position 151 will not produce an active and/or resistant RT protein.

2.3. Mutation rates can limit the potential of in vitro drug-selection experiments

It has been calculated that an HIV-1 infected patient can harbour 10^6–10^8 genetically distinct HIV-1 genomes (Ho et al., 1995; Wei et al., 1995). This makes it likely that some drug-resistant mutations are present even in the absence of drug therapy (Coffin, 1995; Najera et al., 1995). This is in contrast to the relatively small HIV-1 population sizes that are usually handled in in vitro tissue culture systems. Within such a population of finite size, a smaller number of mutants are generated which may limit the possibilities of generating drug-resistant variants. Other limitations apply to the culture system. Whereas patients contain a highly variable HIV-1 population, or quasispecies, and drug treatment usually lasts for a prolonged period of time, in vitro experiments are usually performed with HIV-1 molecular clones and generally last for only a couple of weeks. These combined arguments indicate that appearance of drug resistance in vitro may be controlled more strictly by the chance of creating the resistance mutation than by phenotypic selection. This may result in different resistance patterns in vitro versus in vivo. To test this, we compared the ratio of transitions (type 1) to transversions (type 2) in all in vitro and in vivo selection experiments reported so far. We calculated this ratio for RT mutations in response to either nucleoside or non-nucleoside RT drugs, and protease resistance mutations (Fig. 2). It is obvious that the relatively easy transitions are highly favoured in the in vitro experiments for all three types of drug (86%, 64%, 64%, respectively). This is particularly striking if we realize that a random mutation scenario would predict only 33% transitions, since each nucleotide is subject to two transversions as opposed to one transition. The contribution of transitions in the development of drug resistance in vivo is significantly reduced for all three data sets (60%, 40%, 50%, respectively). Thus the in vivo selection system has the tendency to generate more difficult substitutions. In other words, in vitro selection may yield RT/protease variants with sub-optimal fitness compared with variants observed in vivo.

The difference in the pattern of resistance development in in vivo versus in vitro systems is also evident when we analyze the response to individual drugs. For instance, nevirapine resistance is mediated by two changes detected both in vitro and in vivo (Val106Ala, GTA → GCA and Tyr181Cys, TAT → TGT, both type 1 transitions), but more difficult type 2 transversions and double-hits can be observed in vivo (Table 1, Mellors et al., 1995). Similarly, the U-87201 non-nucleoside drug triggers outgrowth of one particular variant in vitro (Pro236Leu, CCT → CTT, type 1), whereas combinations of more difficult substitutions have been selected in vivo.

Perhaps most intriguing, individual amino acid positions in the RT protein have been shown to respond differently to treatment with the same

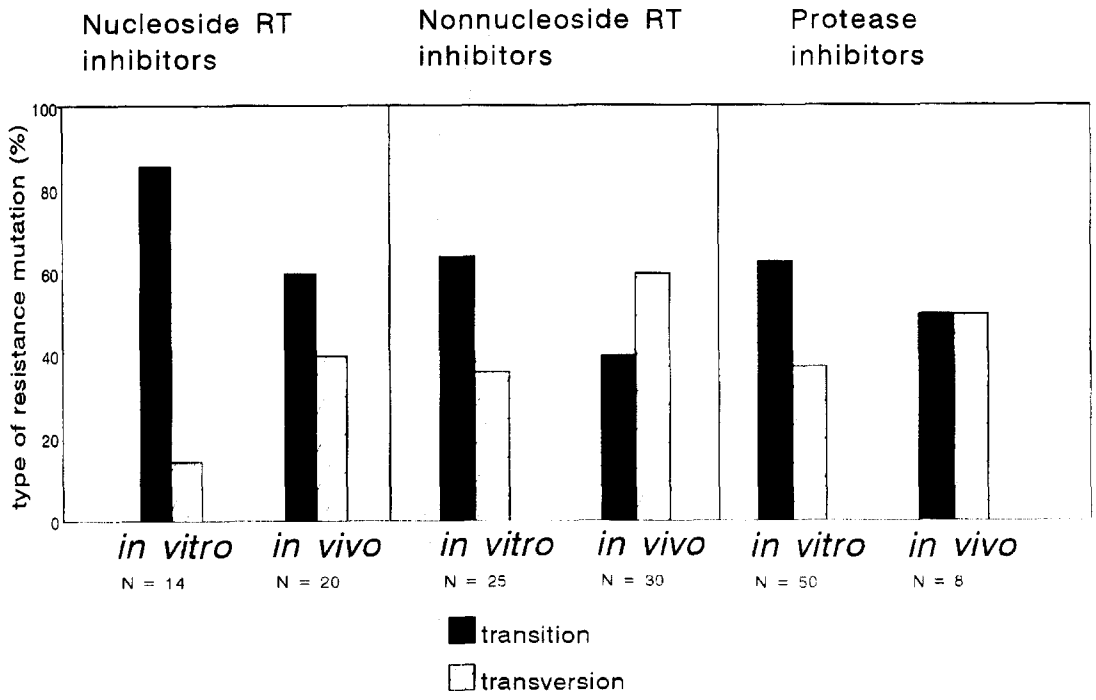

Fig. 2. Analysis of the transition/transversion ratio of drug-resistance mutations obtained in tissue culture experiments (in vitro) and in drug-treated HIV-infected individuals (in vivo). Mutational data were derived from the International Antiviral News HIV resistance mutations Table (Mellors et al., 1995 (protease inhibitors), Tables 1 (nucleoside RT inhibitors) and 2 (non-nucleoside RT inhibitors)). The number of entries (N) is indicated for each individual dataset.

drug in tissue culture systems versus natural infections (Tables 1 and 2, Mellors et al., 1995). It can be argued that such differences do reflect subtle differences in either the mechanism of RT or the RT–drug interaction in different cellular environments (Cinatl et al., 1994). Alternatively, specific drug responses may simply reflect differences in virus load and mutation probabilities in the two systems. Indeed, we could identify several cases where arduous mutations are exclusively found in drug-treated patients, whereas easier substitutions are observed in tissue culture infections. For instance, the AZT-resistance mutation Lys219Gln (type 2, transversion) is frequently observed in vivo, but in vitro an easier type 1 transition (Lys219Glu) was selected (Larder et al., 1991). Selection of the more difficult pathway in vivo strongly suggests that the 219Gln enzyme is fitter (more resistant/active) than the 219Glu variant. Other examples are provided by the non-nucleoside drugs nevirapine and R82913 (Balzarini et al., 1994b; Mellors et al., 1993; Wei et al., 1995). Nevirapine treatment can select for a double transversion in vivo (Tyr181Ile, TAT→ATT, type 2.2), whereas a relatively simple transition is observed at that same position in vitro (Tyr181Cys, TAT→TGT, type 1). Similarly, whereas R82913 treatment generates a double transversion in vivo (Tyr188Leu, TAT→TTA, type 2.2), a type 1 transition is observed in vitro (Tyr188His, TAT→CAT). Thus, some of the observed differences in the selection of drug-resistance markers in in vivo and in vitro systems can be explained on the basis of mutational bias. Other have suggested that structural constraints within the viral RNA genome may also influence the development of drug-resistant mutations (Schinazi et al., 1994).

Finally, this type of analysis was facilitated in part by the periodical publication of all HIV-1 resistance mutations in the journal International Antiviral News (Mellors et al., 1995). These updates are very useful for all investigators in the field and future editions could be expanded with substitution analyses as performed in this study. In this manuscript, we demonstrate that such an analysis can provide valuable information on the requirements for drug resistance. This knowledge should guide future mutagenesis experiments with the aim of building a detailed picture of the RT enzyme and its interaction with antiviral drugs. Therefore, we advocate that all original research manuscripts should not only list the amino acid changes responsible for drug resistance, but also the corresponding codon changes.

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References

Anderson, R.P. and Menninger, J.R. (1986) The accuracy of RNA synthesis. In: T.B.L. Kirkwood, R.F. Rosenberger and D.J. Galas (Eds), Accuracy in Molecular Processes, pp. 159–189. Chapman and Hall, London.

Balzarini, J., Karlsson, A., Meichsner, C., Paessens, A., Riess, G., De Clerq, E. and Kleim, J.-P. (1994a) Resistance pattern of human immunodeficiency virus type 1 reverse transcriptase to quinoxiline S-2720. J. Virol. 68, 7986–7992.

Balzarini, J., Karlsson, A., Sardana, V.V., Emini, E.A., Camarasa, M.-J. and de Clerq, E. (1994b) Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K) RT HIV-1 mutants or select for highly resistant (Y181C→C181I) RT HIV-1 mutants. Proc. Natl. Acad. Sci. USA 91, 6599–6603.

Bebenek, K. and Kunkel, T.A. (1993) The fidelity of retroviral reverse transcriptases. In: A.M. Skalka and S.P. Goff (Eds), Reverse Transcriptase. Cold Spring Harbor Laboratory, New York.

Berkhout, B. and Klaver, B. (1995) Revertants and pseudo-revertants of HIV-1 viruses mutated in the LTR promoter region. J. Gen. Virol. 76, 845–853.

Boucher, C.A.B., Cammack, N., Schipper, P., Schuurman, R., Rouse, P., Wainberg, M.A. and Cameron, J.M. (1993) High-level resistance to (−) enantiomeric 2′-deoxy-3′-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 37, 2231–2234.

Boyer, P.L., Ferris, A.L., Clark, P., Whitmer, J., Frank, P., Tantillo, C., Arnold, E. and Hughes, S.H. (1994) Mutational analysis of the fingers and palm subdomains of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase. J. Mol. Biol. 243, 472–483.

Chao, S.-F., Chan, V.L., Juranka, P., Kaplan, A.H., Swanstrom, R. and Hutchison III, C.A. (1995) Mutational sensitivity patterns define critical residues in the palm subdomain of the reverse transcriptase of human immunodeficiency virus type 1. Nucleic Acids Res. 5, 803–810.

Chou, P.Y. and Fasman, G.D. (1978) Prediction of secondary structure of proteins from their amino acid sequence. In: A. Meister (Ed), Advances in Enzymology and Related Areas of Molecular Biology, pp. 45–148. John Wiley and Sons, New York.

Cinatl Jr., J., Cinatl, J., Rabenau, H., Doerr, H.W. and Weber, B. (1994) Failure of antiviral therapy: role of viral and cellular factors. Intervirolgy 37, 307–314.

Coffin, J.M. (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 267, 483–489.

Gao, Q., Gu, Z., Parniak, M.A., Cameron, J., Cammack, N., Boucher, C. and Wainberg, M.A. (1993) The same mutation that encodes low-level human immunodefiency virus type 1 resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. 37, 1390–1392.

Gojobori, T., Li, W.-H. and Graur, D. (1982) Patterns of nucleotide substitution in pseudogenes and functional genes. J. Mol. Evol. 18, 360–369.

Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M. and Markowitz, M. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373, 123–126.

Ji, J. and Loeb, L.A. (1994) Fidelity of HIV-1 reverse transcriptase copying a hypervariable region of the HIV-1 env gene. Virology 199, 323–330.

Ji, J., Hoffmann, J.-S. and Loeb, L. (1994) Mutagenicity and pausing of HIV reverse transcriptase during HIV plus-strand DNA synthesis. Nucleic Acids Res. 22, 47–52.

Johnson, P.R., Hamm, T.E., Goldstein, S., Kitov, S. and Hirsch, V.M. (1991) The genetic fate of molecularly cloned simian immunodeficiency virus in experimentally infected macaques. Virology 185, 217–228.

Kellam, P. and Larder, B.A. (1995) Retroviral recombination can lead to linkage of reverse transcriptase mutations that confer increased zidovudine resistance. J. Virol. 69, 669–674.

Kellam, P., Boucher, C.A.B. and Larder, B.A. (1992) Fifth mutation in human immunodeficiency virus type 1 reverse

transcriptase contributes to the development of high-level resistance to zidovudine. Proc. Natl. Acad. Sci. USA 89, 1934–1938.

Klaver, B. and Berkhout, B. (1994) Evolution of a disrupted TAR RNA hairpin structure in the HIV-1 virus. EMBO J. 13, 2650–2659.

Kleim, J.-P., Bender, R., Billhardt, U.-M., Meichsner, C., Riess, G., Rosner, M., Winkler, I. and Paessens, A. (1994) Mutational analysis of residue 190 of human immunodeficiency virus type 1 reverse transcriptase. Virology 200, 696–701.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Crystal structure at 3.5A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256, 1783–1790.

Lacey, S.F. and Larder, B.A. (1994) Mutagenic study of codons 74 and 215 of the human immunodeficiency virus type 1 reverse transcriptase, which are significant for nucleoside analog resistance. J. Virol. 68, 3421–3424.

Larder, B.A. (1994) Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. J. Gen. Virol. 75, 951–957.

Larder, B.A., Coates, K.E. and Kemp, S.D. (1991) Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. J. Virol. 65, 5232–5236.

Li, W.-H. and Graur, D. (1991) In: D. Graur (Ed), Fundamentals of Molecular Evolution, pp. 20–41. Sinauer Associates, Inc., Sunderland, MA.

Li, W.-H., Wu, C.-I. and Luo, C.-C. (1984) Nonrandomness of point mutation as reflected in nucleotide substitutions in pseudogenes and its evolutionary implications. J. Mol. Evol. 211, 58–71.

Mellors, J.W., Im, G.J., Tramontano, E., Winkler, S.R., Medina, D.J., Dutschman, G.E., Bazmi, H.Z., Piras, G., Gonzalez, C.J. and Cheng, Y.C. (1993) A single conservative amino acid substitution in the reverse transcriptase of human immunodeficiency virus-1 confers resistance to (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk]11,4]benzodiazepin-2(1H)-thione(TIBO R82150). Mol. Pharmacol. 43, 11–16.

Mellors, J.W., Larder, B.A. and Schinazi, R.F. (1995) Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance. Int. Antiviral News 3, 8–13.

Myers, G., Wain-Hobson, S., Henderson, L.E., Korber, B., Jeang, K.-T. and Pavlakis, G.N. (1995) In: G.N. Pavlakis (Ed), Human Retroviruses and AIDS. A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Los Alamos National Laboratory, Los Alamos, NM.

Najera, I., Holguin, A., Quinones-Mateu, M.E., Munoz-Fernandez, M.A., Najera, R., Lopez-Galindez, C. and Domingo, E. (1995) Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. J. Virol. 69, 23–63.

Pelletier, E., Saurin, W., Cheynier, R., Letvin, N.L. and Wain-Hobson, S. (1995) The tempo and mode of SIV quasispecies development in vivo calls for massive viral replication and clearance. Virology 208, 644–652.

Roberts, J.D., Preston, B.D., Johnston, L.A., Soni, A., Loeb, L.A. and Kunkel, T.A. (1989) Fidelity of two retroviral reverse transcriptases during DNA-dependent DNA synthesis in vitro. Mol. Cell. Biol. 9, 469–476.

Schinazi, R.F., Lloyd, R.M., Nguyen, M., Cannon, D.L., McMillan, A., Ilksoy, N., Chu, C.K., Liotta, D.C., Bazmi, H.Z. and Mellors, J.W. (1993) Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob. Agents Chemother. 37, 875–881.

Schinazi, R.F., Lloyd, R.M., Ramanathan, C.S. and Taylor, E.W. (1994) Antiviral drug resistance mutations in human immunodefiency virus type 1 reverse transcriptase occur in specific RNA structural regions. Antimicrob. Agents Chemother. 38, 268–274.

Schuurman, R., Nijhuis, M., van Leeuwen, R., Schipper, P., de Jong, D., Collis, P., Danner, S.A., Mulder, J., Loveday, C., Christopherson, C., Kwok, S., Sninsky, J. and Boucher, C.A.B. (1995) Rapid changes in human immunodeficiency virus (HIV-1) RNA load and appearance of drug resistant virus populations in individuals treated with 3TC (lamivudine). J. Infect. Dis. 171, 1411–1419.

Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W.-Y., Kojima, E., Alcaide, M.L., Chokekijchai, S., Roy, B.M., Arnold, E., Yarchoan, R. and Mitsuya, H. (1995) Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA 92, 2398–2402.

Tisdale, M., Kemp, S.D., Parry, N.R. and Larder, B.A. (1993) Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. USA 90, 5653–5656.

Wei, X., Ghoosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H., Saag, M.S. and Shaw, G.M. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373, 117–122.