Mutational patterns associated with the 69 insertion complex in multidrug-resistant HIV-1 reverse transcriptase that confer increased excision activity and high-level resistance to zidovudine

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) strains having dipeptide insertions in the fingers subdomain and other drug resistance-related mutations scattered throughout their reverse transcriptase (RT)-coding region show high-level resistance to zidovudine (AZT) and other nucleoside analogues. Those phenotypic effects have been correlated with their increased ATP-dependent phosphorolytic activity on chain-terminated primers. Mutations T69S and T215Y and a dipeptide insertion (*i.e.* Ser-Ser) between positions 69 and 70 are required to achieve low-level resistance to thymidine analogues. However, additional amino acid substitutions are necessary to achieve the high-level phenotypic resistance to AZT shown by clinical HIV isolates carrying a dipeptide insertion in their RT-coding region. In order to identify those mutations that contribute to resistance in the sequence context of an insertion-containing RT derived from an HIV clinical isolate (designated as SS RT), we expressed and purified a series of chimeric enzymes containing portions of the wild-type or SS RT sequences. ATP-mediated excision activity measurements using AZT- and stavudine (d4T)-terminated primers and phenotypic assays showed that molecular determinants of high-level resistance to AZT were located in the fingers subdomain of the polymerase. Further studies, using recombinant RTs obtained by site-directed mutagenesis, revealed that M41L, A62V and in a lesser extent K70R, were the key mutations that together with T69S, T215Y and the dipeptide insertion conferred high-levels of ATP-dependent phosphorolytic activity on AZT- and d4T-terminated primers. Excision activity correlated well with AZT susceptibility measurements, and was consistent with phenotypic resistance to d4T. Structural analysis of the location of the implicated amino acid substitutions revealed a coordinated effect of M41L and A62V on the positioning of the β3-β4 hairpin-loop, which plays a key role in the resistance mechanism.

Keywords: HIV; reverse transcriptase; drug resistance; thymidine analogues; zidovudine

INTRODUCTION

The human immunodeficiency virus (HIV) reverse transcriptase (RT) plays an essential role in the replication of the viral genome. It converts the viral (+) RNA genome into a double-stranded DNA that integrates into the host genome.[1,2] The HIV-1 RT is an asymmetric heterodimer, composed of two structurally related subunits of 66 and 51 kDa (designated as p66 and p51, respectively). It is a multifunctional enzyme with RNA- and DNA-dependent DNA polymerase, RNase H, strand transfer, and strand displacement activities. Current antiretroviral regimens include RT inhibitors targeting the DNA polymerase activity of the enzyme.[3,4] Approved RT inhibitors can be divided into two groups: nucleoside analogues [mainly represented by 3´-azido-3´-deoxythymidine (AZT, zidovudine), 2´,3´-didehydro-2´,3´-dideoxythymidine (d4T, stavudine), and 2´,3´-dideoxy-3´-thiacytidine (3TC, lamivudine)] and nonnucleoside inhibitors (nevirapine, delavirdine and efavirenz).

Nucleoside analogues are pro-drugs that need to be phosphorylated to their active 5´-triphosphate form in order to compete with their natural dNTP counterpart for incorporation into DNA. These compounds are chain terminators, which after incorporation into the growing DNA chain are not further elongated due to the lack of a hydroxyl group in the 3´ position of their ribose moiety. Mutations conferring nucleoside analogue resistance appear in the RT-coding regions of virus isolated from treated patients. HIV-1 RT becomes resistant to nucleoside analogues by (i) improving discrimination against the RT inhibitors, or (ii) by increasing the RT´s ability to remove 3´-terminal chain-terminating inhibitors from blocked DNA primers, through phosphorolysis mediated by ATP or pyrophosphate (reviews[5,6]). The substitution of Val for Met-184 that leads to reduced incorporation of lamivudine-triphosphate is an example of the first type of mechanism.[7] On the other hand, ATP-mediated removal of chain-terminating nucleotide analogues arises as the major AZT resistance mechanism in virus harboring the RT substitutions M41L, D67N, K70R, L210W, T215F/Y and K219E/Q.[8] In the presence of ATP, AZT- and d4T-monophosphates are efficiently removed by RTs bearing substitutions D67N/K70R, M41L/T215Y, D67N/K70R/T215F/K219Q or M41L/D67N/K70R/T215Y, in assays carried out with heteropolymeric template-primers.[8-13] Although excision rates can be strongly influenced by the template-primer sequence,[14] available data indicates that other inhibitors, such as 2´,3´-dideoxyadenine (ddA), carbovir monophosphate (the product of abacavir metabolism) or tenofovir are excised at a lower rate than the thymidine analogues, while cytidine analogues [2´,3´-dideoxycytidine (ddC)- and 3TC-monophosphates] are poor substrates of the reaction.[15,16]

Excision reactions can be inhibited by the next complementary dNTP, due to the formation of a stable "dead-end complex" formed by the RT, the DNA-DNA duplex containing the blocked DNA primer, and the incoming dNTP.[8,17] Removal of AZT-monophosphate is much less sensitive to inhibition by the next complementary dNTP, due to the higher "dead-end complex" stability in the presence of d4T- and ddA-terminated primers.[9,16,18]

The extensive use of antiretroviral therapy and multiple combination treatments has favored the emergence of novel patterns of mutations conferring multidrug-resistance. Among them, RTs containing dipeptide insertions (typically, Ser-Ser, Ser-Gly or Ser-Ala) between codons 69 and 70 together with substitutions T69S, T215Y and other drug resistance mutations have been identified in heavily-treated patients[19,20] (reviews[21,22]), with a prevalence of around 1 %.[23,24]

Virus isolates harboring a dipeptide insertion (*i.e.* Ser-Ser) in their RT-coding region show high-level resistance to AZT and moderate levels of resistance to other nucleoside RT inhibitors, such as d4T, ddC and 2′,3′-dideoxyinosine (ddI, didanosine).[21,23-26] Previously, we demonstrated that the insertion by itself did not confer the resistant phenotype.[25] Removal of the Ser-Ser insertion in the sequence context of an HIV-1 RT bearing the nucleoside analogue resistance-related mutations M41L, A62V, T69S, K70R, V118I, M184I, L210W, T215Y and G333E, produced a 5.5-fold reduction in the viral susceptibility to AZT. However, the obtained recombinant HIV lacking the dipeptide insertion in the RT still retained high-level resistance to the inhibitor.[25] Phenotypic resistance correlated with an increased ATP-dependent phosphorolytic activity that allowed efficient removal of the 3′-terminal nucleotide from AZT-terminated primers.

Further studies showed that mutations T69S and T215Y together with the dipeptide insertion were required to attain significant levels of ATP-dependent phosphorolytic activity on primers terminated with thymidine analogues.[27,28] Increased excision activity correlated with detectable but low-level resistance to AZT and d4T, as obtained in phenotypic assays carried out with the HIV-1 strains bearing those mutations.[26,27] Recombinant HIV variants containing the BH10_SSSY RT (whose sequence is given in Figure 1) showed 4- to 6.5-fold and 5.5- to 8.7-fold decreased susceptibility to AZT and d4T, respectively.[26,27] Available data suggest that other AZT resistance mutations (*i.e.* M41L, K70R or L210W), which are frequently found in insertion-containing RTs from viral isolates obtained from heavily-treated patients, could be responsible for the high-level AZT resistance found in those viral isolates. Those mutations contributing to increase phenotypic AZT resistance were identified by studying their effects on the ATP-mediated

excision on AZT- and d4T-terminated primers using recombinant RTs, as well as on drug susceptibility in assays carried out with HIV constructs containing different combinations of mutations in their RT-coding region.

RESULTS

ATP-mediated excision activity of chimeric recombinant RTs

The recombinant SS RT contains a Ser-Ser insertion between residues 69 and 70, as well as 44 additional mutations scattered throughout the entire RT coding region. Twenty-nine of those mutations are located within the DNA polymerase domain[25] (Figure 1). Our previous studies revealed that this enzyme had a high ATP-dependent phosphorolytic activity on AZT- and d4T-terminated primers, while the activity displayed by a wild-type HIV-1 RT (strain BH10) was almost negligible. These differences were not observed in assays carried out in the presence of 200 μM pyrophosphate.[16] Interestingly, the presence of both T69SSS and T215Y in a wild-type BH10 sequence context conferred low, albeit significant ATP-mediated excision activity.[27] To identify the additional mutations responsible for the high-level resistance to thymidine analogues shown by HIV clones bearing the SS RT sequence, we obtained chimeric RTs containing sequence portions of the SS and BH10 RTs, with or without T69SSS and T215Y (Figure 1), that we tested in enzymatic assays as well as in phenotypic assays using recombinant HIV-1 containing the corresponding RT-coding sequences.

We tested the ability of those enzymes to rescue AZT- and d4T-terminated primers using the template-primer shown in Figure 2(a). These experiments were carried out in two steps. First, the HIV-1 RT was incubated with the template-primer in the presence of AZT triphosphate (AZTTP) or d4T triphosphate (d4TTP) depending on the experiment. Then, unblocking and extension reactions leading to the accumulation of a 38-nucleotide product were carried out by adding dNTPs in the presence of ATP 3.2 mM. These reactions can be inhibited by the next complementary dNTP (dATP in our assay conditions). Therefore, time courses were carried out in the presence of low dATP concentrations (*i.e.* 1 μM). Non-complementary dNTPs were supplied at 100 μM to facilitate full extension of primers after removal of chain-terminating nucleotides. As shown in Figure 2(a), the ATP-dependent phosphorolytic activity of the SS RT was very high in comparison with the BH10 RT. Under our assay conditions, after a 5-min incubation at 37ºC,

more than 70% of the AZT-monophosphate had been removed by the excision activity of the multidrug-resistant RT.

The time courses of ATP-mediated AZT removal reactions catalyzed by chimeric RTs are given in Figure 2(b). The SS/BH_L2 RT was as efficient as the SS RT in excising AZT-monophosphate from blocked DNA primers. In contrast, the SS/BH_L1 RT showed a 5-fold reduced ATP-dependent phosphorolytic activity in comparison with the SS RT. The BH10_SSSY RT, an enzyme which differs from the wild-type in having the insertion plus the two additional mutations T69S and T215Y showed similar excision rates in comparison with SS/BH_L1 RT. Chimeric enzymes containing T215Y and other mutations found within residues 136-560, such as L210W, showed decreased removal activity in comparison with BH10_SSSY, even when the dipeptide insertion was present.

The results obtained using d4T-terminated primers as substrates were similar to those obtained with AZT-terminated primers (Figure 2(c)), although differences between the SS RT and the chimeric SS/BH_L2 RT and the other enzymes were more pronounced. Altogether, these results suggest that several mutations in the vicinity of the insertion are critical for the increased excision activity shown by the SS RT.

As previously shown, excision reactions can be inhibited by the next complementary nucleotide.[8] AZT removal reactions catalyzed by all of the tested enzymes were highly resistant to dNTP inhibition (IC_{50} > 0.4 mM), while unblocking of d4T-terminated primers was >50-fold more sensitive to inhibition by the next complementary dNTP (Table 1). All of the studied RTs showed similar IC_{50} values, although AZT and d4T removal by the SS/BH10_L1 RT was slightly more sensitive to dNTP inhibition.

Phenotypic drug susceptibility of recombinant HIV-1 variants with chimeric RTs

Wild-type BH10 RT, the SS RT and all of the four chimeric RTs were introduced in an infectious HIV-1 clone using a recombinant virus assay. Following transfection, virus was cultured and production of p24 antigen was monitored. All viruses recovered from transfections, except the one carrying the chimeric SS/BH_L4 RT were able to infect and replicate in SupT1 cells. Recombinant HIV-1 clones were assayed to measure the level of resistance to nucleoside RT inhibitors in clinical use. The virus containing the SS RT showed high-level resistance to

AZT, d4T and 3TC, moderate resistance to ddI, and low-level resistance to ddC (Table 2). Interestingly, the chimeric SS/BH_L2 RT conferred high-level resistance to AZT, while the other two chimeric RTs had a small influence on AZT susceptibility. Recombinant virus having the SS/BH_L2 RT showed the highest levels of d4T and ddI resistance, among those containing chimeric RTs. These results are in good agreement with available data supporting the correlation between phenotypic drug susceptibility in cell culture and ATP-dependent phosphorolytic activity as measured in enzymatic assays, since thymidine analogues and ddA (the product of ddI metabolism) are good substrates of the excision reaction.[9] As expected, the high-level resistance to 3TC maps within the C-terminal region of SS RT and can be attributed to the presence of M184I. In fact, reversion of Ile-184 to wild-type Met in SS RT leads to virus showing low-level resistance to 3TC (M.A. Martínez, unpublished observations).

These data were in good agreement with the results of the biochemical assays and suggested that mutations located in the N-terminal region of the SS RT were responsible for the high and moderate levels of resistance found with AZT and d4T, respectively, in viral isolates containing RTs bearing insertions in their fingers subdomain.

Key mutations of the 69 insertion complex of the multinucleoside-resistant RT and their effects on thymidine analogue susceptibility

The N-terminal sequence of the SS RT found in SS/BH_L2 contains nine additional mutations apart from T69S and T215Y, and the two-serine insertion. Those mutations are M41L, K43E, A62V, K70R, K104R, V108I, V118I, D123E and I135T. The statistical analysis of HIV sequences having dipeptide insertions in their RT-coding regions revealed that M41L, A62V and K70R were relatively frequent, while the other mutations appeared only occasionally.[22] Therefore, we studied the effect of those mutations (alone or in different combinations) when appearing in a wild-type BH10 sequence context containing the insertion as well as additional mutations T69S and T215Y (sequences given in Figure 1).

The time courses for AZT-monophosphate removal in the presence of ATP showed that by themselves all of three mutations increased the ATP-dependent phosphorolytic activity of BH10_SSSY (Figure 3(a)). This effect was more pronounced with M41L and A62V than with K70R. The combination of M41L and K70R did not produce a significant increase in the reaction rate in comparison with M41L alone. However, when M41L and A62V were combined, the ATP-

mediated excision reaction rate was significantly increased, while adding up all three mutations (as in mutant SSSY_41L/62V/70R) rendered an RT that showed AZT excision rates that were not distinguishable from those obtained with the SS RT.

The relative effects of the studied mutations in ATP-mediated excision assays carried out with d4T-terminated primers were similar to those obtained with AZT-terminated primers (Figure 3(b)). The presence of the three mutations M41L, A62V and K70R within the sequence background of the BH10_SSSY RT produced a 5-fold increase in the excision rate, although in this case the phosphorolytic activity obtained with mutant SSSY_41L/62V/70R was about 1.5 times lower than with the SS RT. As occurred with AZT-terminated primers, M41L and A62V had the largest influence on the d4T removal rate in the presence of ATP.

Further similarities between the mutant SS/BH_L2 and SSSY_41L/62V/70R RTs and the original SS RT were observed in primer unblocking inhibition assays. All of the enzymes were highly resistant to inhibition by the next complementary dNTP in AZT-monophosphate removal assays (IC_{50} > 0.4 mM). In addition, the mutant SSSY_41L/62V/70R RT showed an IC_{50} for the next complementary dNTP of 2.8 ± 0.3 μM, in ATP-mediated excision reactions using DNA primers blocked with d4T. The obtained IC_{50} value was similar to the one determined for the chimeric SS/BH_L2 RT, but somewhat lower than the one obtained with the SS RT (Table 1).

Available evidence suggests that excision-proficient RTs have a low-affinity binding site for ATP.[31] Further proof of the similar properties shown by the mutant SSSY_41L/62V/70R RT and the multinucleoside-resistant SS RT was obtained by performing ATP-mediated excision assays in the presence of different concentrations of ATP. As shown in Figure 4, the SS RT and its derivatives SS/BH_L2 and SSSY_41L/62V/70R showed similar excision patterns in the presence of 0.4 and 3.2 mM ATP. Furthermore, the excision rates at 3.2 mM ATP were 3.0 – 3.5 higher than at 0.4 mM for all of the three tested enzymes, suggesting that there were only minor differences in their ATP binding affinity.

AZT susceptibility assays carried out with recombinant virus containing mutations M41L, A62V and/or K70R in the sequence background of the BH10_SSSY RT were roughly in agreement with the biochemical data (Figure 5). The largest inhibitory effects (>100-fold inhibition) were observed with HIV-1 clones containing the SSSY_41L/62V/70R RT, followed by the variant containing mutations M41L and A62V. In addition, the amino acid substitutions M41L and A62V had a larger effect on resistance than K70R, although differences were relatively small.

For d4T susceptibility, differences were relatively small in comparison with AZT. However, the mutant SSSY_41L/62V/70R RT was among the ones exerting a largest effect on drug resistance.

DISCUSSION

The increasing complexity of the antiretroviral regimens and the larger number of drugs involved in current therapies has facilitated the emergence of unusual mutational patterns in the HIV-1 RT. Among them, insertions of two amino acids in the β3-β4 hairpin-loop-coding region of the polymerase appear to have a significant impact on thymidine analogue resistance (reviews[21,22]). The ATP-dependent phosphorolytic activity of insertion-containing RTs derived from clinical isolates was very high when tested on primers terminated with AZT or d4T.[25] Phenotypic resistance correlated with the increased ATP-mediated excision activity shown by those enzymes. The excision activity was largely dependent on the presence of Tyr at position 215.[27] However, the contribution of other mutations, including thymidine analogue resistance mutations had not been studied in detail.

The results reported in this work show that in addition to the insertion and Tyr-215, other amino acid substitutions such as M41L, A62V and K70R contribute to increase the excision rate of AZT- and d4T-monophosphate in the presence of physiological concentrations of ATP. In comparison with the heavily-mutated SS RT, the recombinant HIV-1 RT containing the Ser-Ser insertion as well as mutations M41L, A62V, T69S, K70R and T215Y showed similar ATP-mediated excision rates on primers terminated with AZT or d4T. Furthermore, their sensitivity to inhibition by the next complementary dNTP was also similar, and both enzymes showed a similar behavior at different concentrations of ATP. In comparison with K70R, the amino acid substitutions M41L and A62V produced larger increases in the excision rate. An additive effect was observed for combinations of two or three of those mutations (*i.e.* M41L and A62V), although this effect was very small when K70R was present.

Chemical foot-printing techniques have allowed the distinction between pre- and post-translocation complexes in HIV-1 RT.[18] In the pre-translocation complex, the 3' end of the chain-terminated primer is located in the nucleotide binding site (N site), in a position that would be accessible for excision. In the "dead-end complex", the N site is occupied by the incoming dNTP (*i.e.* the next complementary dNTP), while the blocked primer is shifted one position, forming the so-called post-translocation complex. The crystallographic structures of HIV-1 RT with pre- and

post-translocation AZT-monophosphate-terminated primers[32] were consistent with molecular modeling studies suggesting that the chain-terminating RT inhibitor should be located in the N site (pre-translocation complex N) to be accessible to the pyrophosphate donor, and therefore, allowing the excision reaction to proceed.[28]

The ternary complex of HIV-1 RT, a DNA/DNA template-primer and an incoming dNTP[33] and the pre-translocation complex N may represent the crystal structures before and after the catalytic step of DNA polymerization. Their comparison shows that the pyrophosphate release after nucleotide incorporation results in a loss of contacts between the incoming substrate and the side-chains of Lys-65 and Arg-72.[32] Those residues help to maintain the fingers subdomain in a closed conformation in the ternary complex. The excision reaction is the polymerase reaction run in reverse. Nucleoside diphosphates, nucleoside triphosphates and inorganic pyrophosphate are all potential acceptors of the excision reaction.[34] However, available evidence suggests that ATP is the relevant pyrophosphate donor *in vivo*.[8,10,16] Tyr-215 could play a role in facilitating the interaction with ATP,[10] while other residues of the 69 insertion complex would be required to achieve the proper orientation of the β- and γ-phosphate groups of the ribonucleotide, to attack the phosphodiester bond close to the 3' end of the blocked primer.

Unfortunately, a crystallographic structure for the insertion-containing HIV-1 RT is still not available and concerns on the accuracy of a model based on the three-dimensional structure of the ternary complex of HIV-1 RT are justified by the high number of potential conformations acceptable for a β3-β4 hairpin-loop containing two additional serine residues, and the relatively large number of amino acid changes found in the fingers subdomain of the polymerase. However, mapping the location of residues whose effects in excision have been demonstrated in this work on the crystal structure of an HIV-1 RT ternary complex (Figure 6(a)) reveals that the conformation of the β3-β4 hairpin-loop is a major structural determinant of the efficiency of the excision reaction. In addition to the insertion and the amino acid substitutions involving positions of the tip of the β3-β4 hairpin-loop (*i.e.* T69S, K70R), conformational changes affecting the side-chain of Lys-73 as a result of an increase in the side-chain volume of the residue at position 62 (*i.e.* A62V) and a decrease in the length of the side-chain at position 41 (*i.e.* M41L) are likely to affect the hydrogen bond interaction between the ε-NH group of Arg-72 and the oxygen atom bridging phosphorous α and β of the incoming dNTP (Figure 6(b)). Arg-72 is a highly conserved residue among retroviral polymerases. Ala substitution for Arg-72 causes a drastic reduction in the catalytic rate constant of nucleotide incorporation (k_{pol}) without having a significant effect on the

affinity for dNTP (K_d).[35] These data are consistent with the decreased pyrophosphorolytic activity of the mutant enzyme,[36] and suggest a role for Arg-72 in pyrophosphate binding/removal.[37,38]

The ATP-mediated excision rates obtained with AZT-terminated template-primers were broadly in agreement with the results of the phenotypic assays, with both M41L and A62V rendering the largest increases in the IC_{50} for the inhibitor. These results were consistent with phenotypic data reported by other groups, since significant levels of phenotypic AZT resistance were also observed with recombinant HIV-1 strains bearing the Ser-Ser insertion and mutations M41L, T69S, and T215Y.[11,39] Unlike M41L, K70R or T215Y, A62V is not a canonical mutation for AZT resistance. Although A62V by itself, does not confer significant resistance to zidovudine or stavudine in phenotypic assays, it is commonly found associated with multidrug-resistant mutations such as V75I, F77L, F116Y and Q151M.[40,41] In this sequence context, A62V improves viral fitness without producing large effects on the IC_{50} values for zidovudine, didanosine or zalcitabine.[41] In agreement with our findings, Larder *et al*.[42] have shown that introducing A62V in a recombinant virus containing the Ser-Ser insertion and mutations T69S, L210W and T215Y produced a >10-fold increase in the IC_{50} for AZT, while others have shown high levels of phenotypic AZT resistance using clinical isolates carrying A62V in combination with T69SSS, K70R and T215Y.[39]

Although, in the sequence context of an insertion-containing RT, the mutation L210W could have an effect on drug resistance, the magnitude of this effect is not clear. Thus, recombinant HIV-1 strains containing a Ser-Ser insertion and mutations T69S, L210W and T215Y showed high-level resistance to AZT in phenotypic assays.[42] However, other studies have shown that the contribution of L210W to the ATP-dependent phosphorolytic activity of mutant RTs bearing the insertion and mutations M41L, T69S and T215Y was relatively small, as determined in excision assays carried out with ddA-terminated primers.[43] In agreement with this observation, the chimeric RT bearing a wild-type sequence at positions 136-560 (SS/BH_L1) was found to be susceptible to thymidine analogues, while showing negligible ATP-dependent phosphorolytic activity on both AZT- and d4T-terminated primers (Figure 2). The data obtained with those chimeric RTs also indicated that the contribution of mutations in the thumb and connection subdomains of the polymerase, or in the RNase H domain was not significant. Although mutations impairing RNase H activity are likely to enhance thymidine analogue resistance by increasing nucleotide excision,[44] none of the mutations found in the RNase H domain of the SS RT was expected to diminish RNase H activity.

Drug susceptibility data obtained with virus containing the chimeric RT SS/BH_L3 (Table 2) suggested that major determinants of resistance to didanosine and cytidine analogues were located within positions 136-560 of the viral RT. These results were consistent with the presence of the lamivudine resistance mutation M184I (reviews[3,4]), as well as with our previously reported data showing that primers terminated with cytidine analogues were poor substrates of the excision reaction.[16]

The chimeric enzyme SS/BH_L2 and the mutants SSSY_41L and SSSY_41L/62V/70R also showed significant levels of resistance to stavudine in phenotypic assays, although the correlation between drug susceptibility and ATP-dependent phosphorolytic activity was not as good as in the case of zidovudine. Although d4T-terminated primers are good substrates of the excision reaction, they form "dead-end complexes" at lower dNTP concentrations than AZT-terminated primers (Table 1). *In vivo*, thymidine analogue removal will then depend on the intracellular concentrations of dNTPs (review[45]). The lack of cross-reactivity between AZT and d4T observed in phenotypic assays[46] could be explained by the relatively high nucleotide concentrations found in the replicating cells used in phenotypic assays, and the higher sensitivity to dNTP inhibition of the d4T excision reaction. However, the relevance of the ATP-dependent phosphorolytic reaction *in vivo* is supported by clinical studies that demonstrated that specific AZT-resistance mutations (*i.e.* M41L, D67N, T215Y, etc.) arise during the antiretroviral treatment with d4T.[47-50]

Recently published work supports the existence of two distinct mutational patterns that confer resistance to AZT, based on the distribution of thymidine analogue resistance mutations in patients treated with nucleoside analogues. The first pattern (designated as TAM-1) involves the association of M41L, L210W and T215Y, while excluding K70R. The second (TAM-2) includes mutations D67N, K70R and K219E/Q, and sometimes T215F.[51-54] Drug susceptibility studies and clinical data from HIV-infected patients carrying variants bearing different mutational patterns have revealed that TAM-1 combinations are responsible for a more extensive cross-resistance to antiretroviral drugs, while conferring higher levels of AZT resistance. Dipeptide insertions in the fingers subdomain of the RT are commonly associated with the TAM-1 pattern, in patients failing to the antiretroviral treatment.[55] In addition, D67N is rarely found in insertion-containing RTs.[22,27] The identification of M41L and T215Y as key mutations of the 69 insertion complex supports the observed correlation, while providing a biochemical explanation for the effects observed *in vivo*. Our work demonstrates that A62V, a mutation which is not typically associated with thymidine analogue resistance, is an important part of the 69 insertion complex, since it produces a

significant increase in the ATP-dependent excision activity of primers terminated with thymidine analogues, when associated with the insertion and T215Y. Based on the available crystal structures of HIV-1 RT, a direct interaction between A62V and other mutations found in the 69 insertion complex is not likely. However, coordinated effects of A62V with M41L on the conformation of β3-β4 hairpin loop and specifically on the side-chain of Arg-72, are probably affecting the excision rate, which appears to be responsible for the high-level zidovudine resistance exhibited by insertion-containing RTs derived from clinical isolates of heavily-treated patients.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis

The expression vectors pRT6 and pT51H were used to obtain the 66- and 51-kDa subunits of the HIV-1 RT, respectively.[56,57] Several plasmids derived from pRT6 and pT51H and containing the nucleotide sequences encoding for the 66-kDa and 51-kDa subunits of wild-type BH10 RT, SS RT, and mutants BH10_SSSY and T69SSS were available from previous studies.[25,27] The vector used for the expression of the p66 subunit of the chimeric RT, designated as SS/BH_L1, was obtained after cloning the NcoI – EcoRV insert containing the SS RT sequence into the corresponding sites of pRT6 (encoding for the BH10 RT sequence). For SS/BH_L2, the vector was a 3.2 Kbp fragment obtained after digestion of plasmid pRT6 (encoding for the SS RT) with EcoRV and HindIII, while the insert was a 1.3 Kbp-fragment derived from the pRT6 vector used in the expression of BH10_SSSY, obtained after cleavage with both EcoRV and HindIII.[27] Expression vectors for SS/BH_L3 and SS/BH_L4 were obtained after cloning EcoRV – HindIII inserts encoding for the SS RT into vectors derived from the cleavage of the pRT6 plasmids used in the expression of wild-type BH10 RT and T69SSS, respectively.[25] Plasmid DNA containing the appropriate RT coding region was digested with MscI and KpnI to obtain a fragment of 1208 bp (extending from position 25 to 427 of p51), which was then cloned at the appropriate sites of pT51H to generate the corresponding expression vectors for the p51 subunit. In all cases, the integrity of the chimeric RTs was confirmed by DNA sequencing.

Site-directed mutagenesis was carried out with the QuikChange™ site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. Individual mutations M41L, A62V and K70R were introduced in the plasmid pRT6 carrying the nucleotide sequence

encoding the 66-kDa subunit of the mutant BH10_SSSY RT[27] to generate mutant enzymes designated as SSSY_41L, SSSY_62V and SSSY_70R, respectively (see Figure 1). Mutant SSSY_41L/70R was obtained after introducing mutations M41L and K70R in the plasmid pRT6 carrying the nucleotide sequence encoding for the BH10_SSSY RT. Mutants SSSY_41L/62V and SSSY_41L/62V/70R were obtained after introducing the mutation A62V in the plasmids containing the sequences of the SSSY_41L and SSSY_41L/70R RTs, respectively. The mutagenic primers used were: 5'-GTAGAAATTTGTACAGAACTGGAAAAGGAAGGGAA-3' and 5'-TTCCCTTCCTTTTCCAGTTCTGTACAAATTTCTAC-3' for SSSY_41L, 5'-CAATACTCCAGTATTTGTCATAAAGAAAAAAGACTC-3' and 5'-GAGTCTTTTTTCTTTATGACAAATACTGGAGTATTG-3' for SSSY_62V, 5'-GACTCGAGTAGTTCGAGATGGAGAAAATTAGTA-3' and 5'-TACTAATTTTCTCCATCTCGAACTACTCGAGTC-3' for SSSY_70R. The introduced mutations were confirmed by DNA sequencing, and the appropriate MscI – KpnI inserts were then cloned in plasmids derived from pT51H,[25,56] for expression of the p51 subunits of the SS and BH10 RTs.

Protein expression and purification

RTs were purified as previously described,[25,27,56] after independent expression of their subunits (p66 and p51). All of the RTs were purified as p66/p51 heterodimers, and the mutations were introduced in both subunits of the enzyme. The 51-kDa polypeptide was obtained with an extension of 14 amino acids at its N-terminal end, which includes 6 consecutive histidine residues to facilitate its purification by metal chelate affinity chromatography, as previously described.[56]

Nucleotides and template-primers

Stock solutions (100 mM) of dNTP and ATP, and [γ-^{32}P]ATP were obtained from GE Healthcare. AZTTP and d4TTP were purchased from Moravek Biochemicals and Sierra Bioresearch, respectively. Before use, nucleoside-triphosphates were treated with inorganic pyrophosphatase to remove traces of pyrophosphate, as described.[16]

Polyacrylamide gel-purified DNA oligonucleotides 25PGA (5´-TGGTAGGGCTATACATTCTTGCAGG-3´) and D38 (5´-

GGGTCCTTTCTTACCTGCAAGAATGTATAGCCCTACCA-3′) were obtained from Life Technologies. The oligonucleotide 25PGA was labeled at its 5'-terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs), and then annealed to D38 in a solution containing 150 mM NaCl and 150 mM magnesium acetate, as described.[58] The template-primer molar ratio was adjusted to 1:1.

Chain terminator excision assays

RT-catalyzed DNA rescue reactions were performed with D38/25PGA DNA duplexes by following a previously described procedure.[16] Briefly, the phosphorylated template-primer (30 nM) was preincubated at 37°C for 10 min in the presence of the corresponding RT at 15-20 nM active enzyme concentration, in 50 mM Hepes buffer (pH 7.0), containing 15 mM NaCl, 15 mM magnesium acetate, 130 mM potassium acetate, 1 mM dithiothreitol, and 5 % (w/v) polyethylene glycol. RT active site concentrations were determined as previously described.[57] Rescue reactions were initiated by adding an equal amount of preincubation buffer AZTTP or d4TTP at a final concentration of 25 µM. After incubating the samples at 37°C for 30 min, the rescue reactions were initiated by adding a mixture of all dNTPs at a final concentration of 100 µM, in the presence of ATP at 0.4 or 3.2 mM depending on the assay. Because the next complementary dNTP (dATP, in our assay conditions) has an inhibitory effect on the rescue reaction, time courses of the unblocking and extension reactions were carried out in the presence of 1 µM dATP.

The inhibitory effect of dATP was determined after measuring the amount of rescued primer in reactions carried out for 0-15 min in the presence of different concentrations of dATP. In these experiments, incubation times were within the linear range of the corresponding time course.

Reactions were stopped by adding an equal amount of 10 mM EDTA in 90 % (v/v) formamide containing 3 mg/ml xylene cyanol FF and 3 mg/ml bromophenol blue. Products were resolved on a denaturing 20 % polyacrylamide / 8 M urea gel, and primer rescue was quantified by phosphorimaging with a BAS 1500 scanner (Fuji), using the program Tina version 2.09 (Raytest Isotopenmessgerate Gmbh, Staubenhardt, Germany).

Recombinant virus and drug susceptibility tests

These assays were performed as previously described.[25,59] Briefly, full-length RT-coding sequence DNA was amplified from plasmids carrying the different RTs using primers IN5 (5´-AATTTTCCCATTAGTCCTATTGAAACTGTACCA-3´) and IN3 (5´-TCTATTCCATCYAAAAATAGTACTTTCCTGATTCC-3´). The PCR products were then cotransfected with an RT-deleted HXB2-D clone in SupT1 cells.[60] Culture supernatants were harvested when the HIV-1 p24 antigen concentration surpassed 20 ng/ml. Progeny virus was propagated and titrated in MT-4 cells. The nucleotide sequence of the RT-coding region of the progeny virus was checked for possible reversions or undesired mutations. The SupT1 and MT-4 cells and the deleted HXB2-D clone were obtained from the AIDS Reagent Program (Medical Research Council). HIV-1 drug susceptibility profiles were obtained after infecting 35000 MT-4 cells with 100 50% tissue culture infective doses of virus, at a multiplicity of infection of 0.003, by exposing the HIV-1-infected cultures to various concentrations of each drug (5-fold dilutions). After MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was determined using a tetrazolium-based colorimetric method.[25,61]

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LEGENDS TO FIGURES

Figure 1. Amino acid sequence differences within residues 1-350 of wild-type BH10 and SS RTs and mutants analyzed in this study. Residues that are identical to those found in the wild-type BH10 RT are indicated by dots. Underlined amino acid residues are those related to drug resistance.

Figure 2. Excision efficiencies of chimeric RTs on primers terminated with thymidine analogues. (a) Rescue DNA polymerization reactions were carried out with a heteropolymeric template-primer whose sequence is given above. First, the inhibitor (AZTTP or d4TTP) was incorporated at position +1 of the 25-nucleotide primer (P) (lane P) to generate a 26-nucleotide product (T) (lane 0). Excision of AZT-monophosphate and further extension of the primer in the presence of ATP (3.2 mM) and a mixture of dNTPs leads to the formation of a fully-extended product of 38 nucleotides. The next complementary dNTP (dATP, under the assay conditions) can inhibit the rescue reaction and is supplied at a relatively low concentration. Lanes 1 to 9 correspond to aliquots removed 2, 4, 6, 8, 10, 12, 15, 20 and 30 minutes after addition of ATP. Time courses of primer rescue reactions carried out with primers terminated with AZT-monophosphate or d4T-monophosphate are shown in panels (b) and (c), respectively. Active enzyme concentration in these assays was 15-30 nM. Represented values were obtained from two to three independent experiments. Standard deviations were <20% in these assays.

Figure 3. Effect of mutations on rescue DNA polymerization reactions initiated from primers terminated with thymidine analogues. Time courses of primer rescue reactions carried out with primers terminated with AZT-monophosphate (a) or d4T-monophosphate (b) in the presence of 3.2 mM ATP. In these assays, all of the dNTPs were supplied at 100 µM, except for dATP whose concentration was 1 µM. Active enzyme concentration in these assays was 24 nM. Represented values were obtained from two to four independent experiments. Standard deviations were <20% in these assays.

Figure 4. Time courses of the excision reactions catalyzed by the SS RT and mutants SS/BH_L2 and SSSY_41L/62V/70R, in the presence of different concentrations of ATP. Rescue DNA polymerization reactions were carried out in the presence of 100 µM of each dNTP, except dATP whose concentration was 1 µM. Active enzyme concentration in these assays was 24 nM. Samples loaded in the gel shown above correspond to the unextended 25-nucleotide primer and to aliquots removed 0, 2, 6, 10, 15 and 30 min after addition of the ATP at the indicated

concentration. The time courses of the rescue reactions carried out in the presence of 3.2 mM and 0.4 mM ATP are shown below. Represented values were obtained from two to four independent experiments. Standard deviations were <20% in these assays.

Figure 5. Drug susceptibility measurements obtained with recombinant HIV-1 clones containing RTs bearing an insertion and different combinations of drug resistance-related mutations. Represented values are the fold-increase in drug susceptibility of the indicated mutant viruses compared to those obtained with recombinant HIV containing the BH10 RT sequence. Drug susceptibility values for AZT, d4T and ritonavir are shown using black, grey and white bars, respectively.

Figure 6. Location of relevant positions of the 69 insertion complex in the crystallographic structure of HIV-1 RT. (a) Ribbon representation of the p66 (blue) subunit of the RT complexed with a DNA/DNA template primer (template shown in red, and primer in yellow) and dTTP (orange), showing the location of residues 41, 62, 69, 70 and 215. (b) Met-41 and Ala-62 are located in the vicinity of Lys-73, a conserved residue in the β3-β4 hairpin loop of the RT. Arg-72 is part of the dNTP binding site and its side chain makes a hydrogen bond with the oxygen atom bridging phosphorous α and β in the incoming nucleotide. Dots are used to represent the Van der Waals surfaces of the indicated amino acids.

ABBREVIATIONS

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Abbreviations used: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AZT, 3´-azido-3´-deoxythymidine; d4T, 2´,3´-didehydro-2´,3´-dideoxythymidine; 3TC, 2´,3´-dideoxy-3´-thiacytidine; ddA, 2´,3´-dideoxyadenosine; ddC, 2´,3´-dideoxycytidine; ddI, 2´,3´-dideoxyinosine; AZTTP, AZT-triphosphate; d4TTP, d4T-triphosphate; IC_{50}, 50% inhibitory concentration.

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Table 1. Ability of the next complementary dNTP to inhibit ATP-dependent primer rescue by wild-type and mutant RTs.

RTs	IC_{50} for the next dNTP (μM)	
	AZT-terminated primer	d4T-terminated primer
BH10	N.D.[a]	N.D.
SS	>400	7.5 ± 1.6
SS/BH_L1	305 ± 42	2.5 ± 0.3
SS/BH_L2	>400	3.1 ± 0.4
SS/BH_L3	N.D.	N.D.
SS/BH_L4	>400	N.D.
BH10_SSSY	>400 [b]	N.D.

Assays were carried out in the presence of 3.2 mM ATP. All dNTPs in these assays were supplied at 100 μM, except for dATP whose concentration ranged from 1 to 800 μM, depending on the assay. Active enzyme concentrations in these assays were in the range of 10 – 20 nM, and the concentration of the template-primer was 30 nM. Samples were incubated for 0 – 15 min depending on the assay. In all cases, incubation times were within the linear range of the corresponding time-course. The percent inhibition was plotted against the concentration of dATP, and the data were fitted to a hyperbola to obtain the IC_{50} for each enzyme. Reported values were obtained from 2 – 3 experiments and are given in means ± standard deviations.

[a] N.D., not determined due to the low efficiency of the excision reaction.

[b] Data were taken from Matamoros *et al.*[27]

Table 2. Susceptibility of HIV-1 constructs to nucleoside RT inhibitors.

RTs	IC_{50} (μM)				
	AZT	d4T	ddC	3TC	ddI
BH10	2.9×10^{-3}	0.095	0.89	1.35	0.71
SS	>3.74 (1289x)	1.98 (20.8x)	2.76 (3.1x)	>21.8 (>16x)	6.11 (8.6x)
SS/BH_L1	7.4×10^{-3} (2.6x)	0.069 (0.7x)	0.15 (0.17x)	0.3 (0.2x)	0.22 (0.3x)
SS/BH_L2	1.45 (500x)	0.67 (7.1x)	1.12 (1.3x)	3.4 (2.5x)	3.67 (5.2x)
SS/BH_L3	5.1×10^{-3} (1.8x)	0.23 (2.4x)	2.26 (2.5x)	>21.8 (>16x)	2.92 (4.1x)

IC_{50} values represent the mean of 2-5 tests, each one performed in sextuplicate. The fold increase in IC_{50} relative to wild-type HXB2 virus control carrying the RT sequence of BH10 is shown in parentheses. Biologically relevant cut-off values of the drug susceptibility test were associated to increases in the relative IC_{50}s of >4-fold for AZT, >3-fold for d4T, >3.5-fold for ddC and ddI, and >4.5-fold for 3TC.[29] High-level resistance is generally associated with >20-fold increases in the relative IC_{50}s.[30]

Figure 1

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Figure 2

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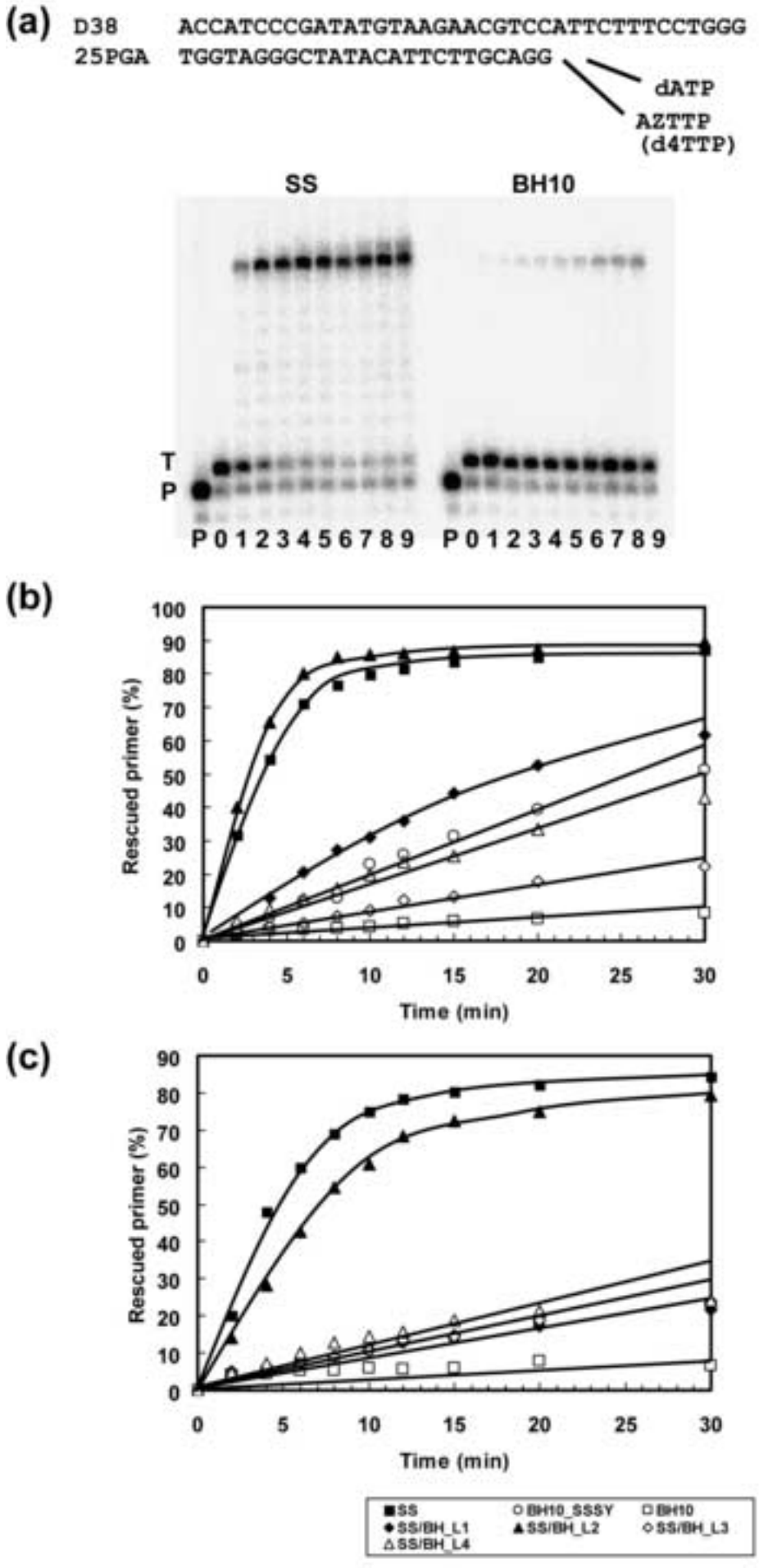

Figure 3

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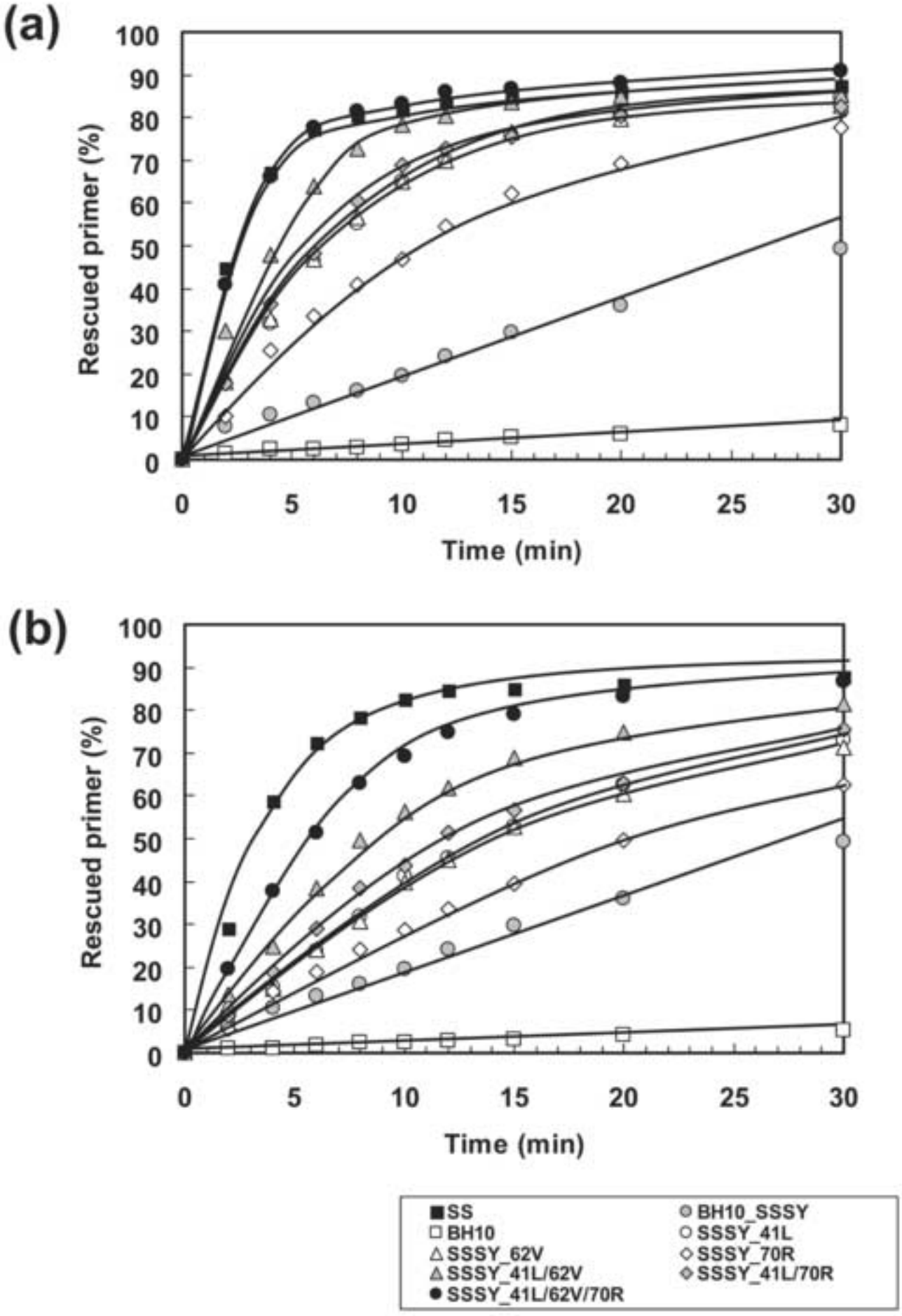

Figure 4

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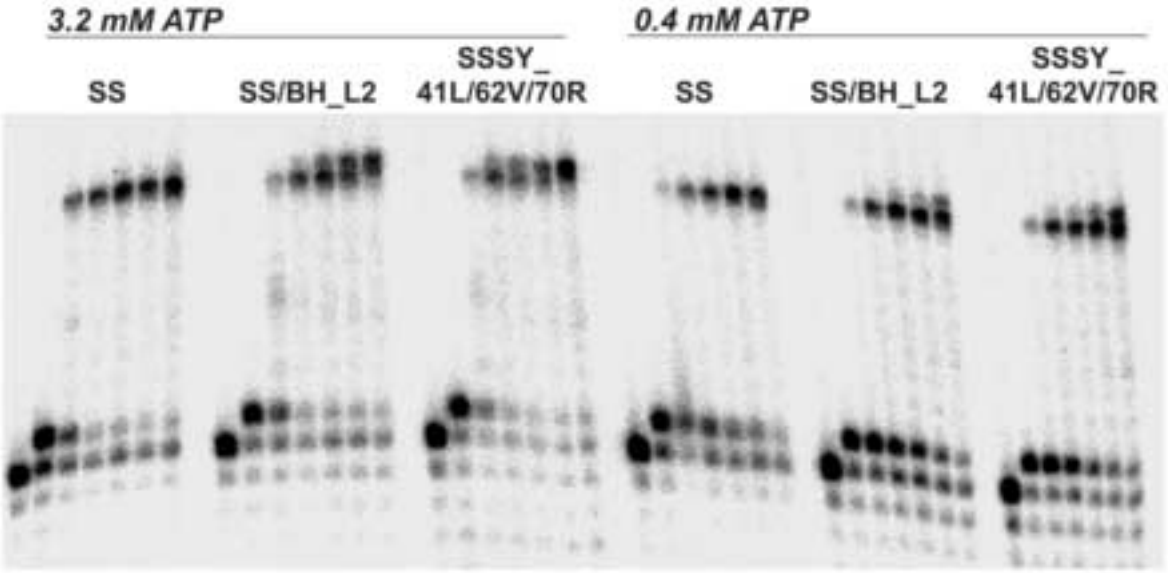

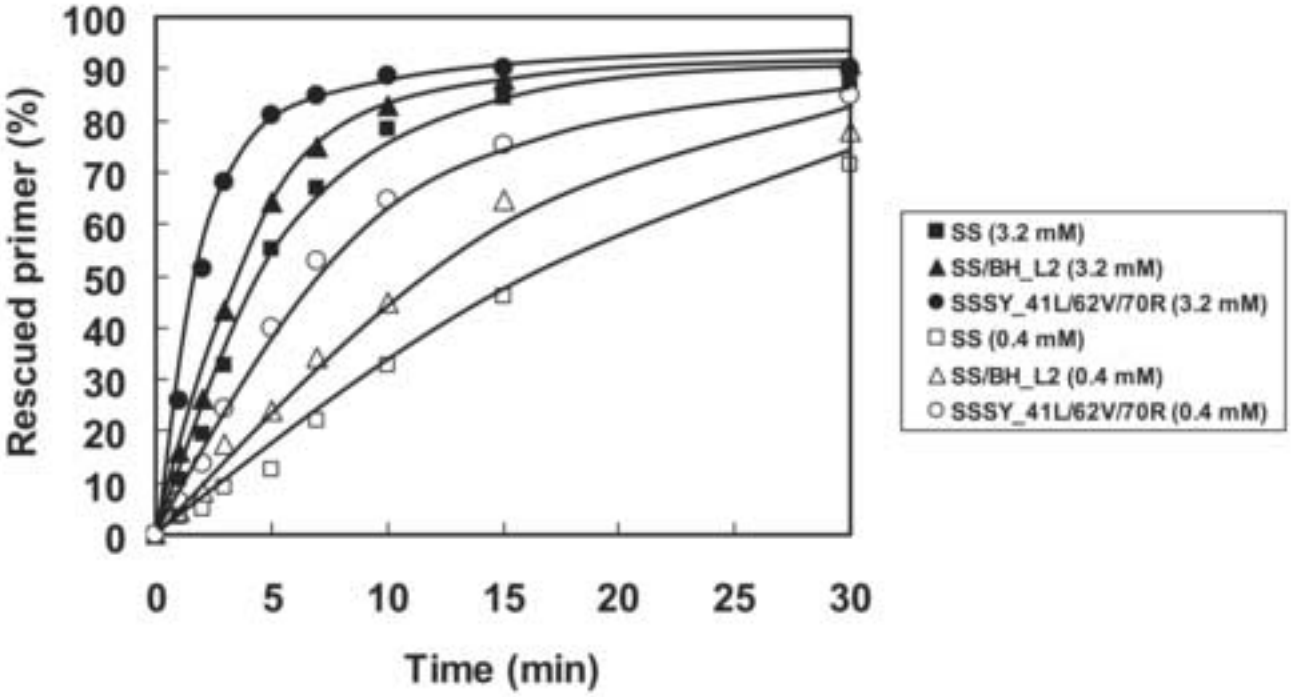

Figure 5

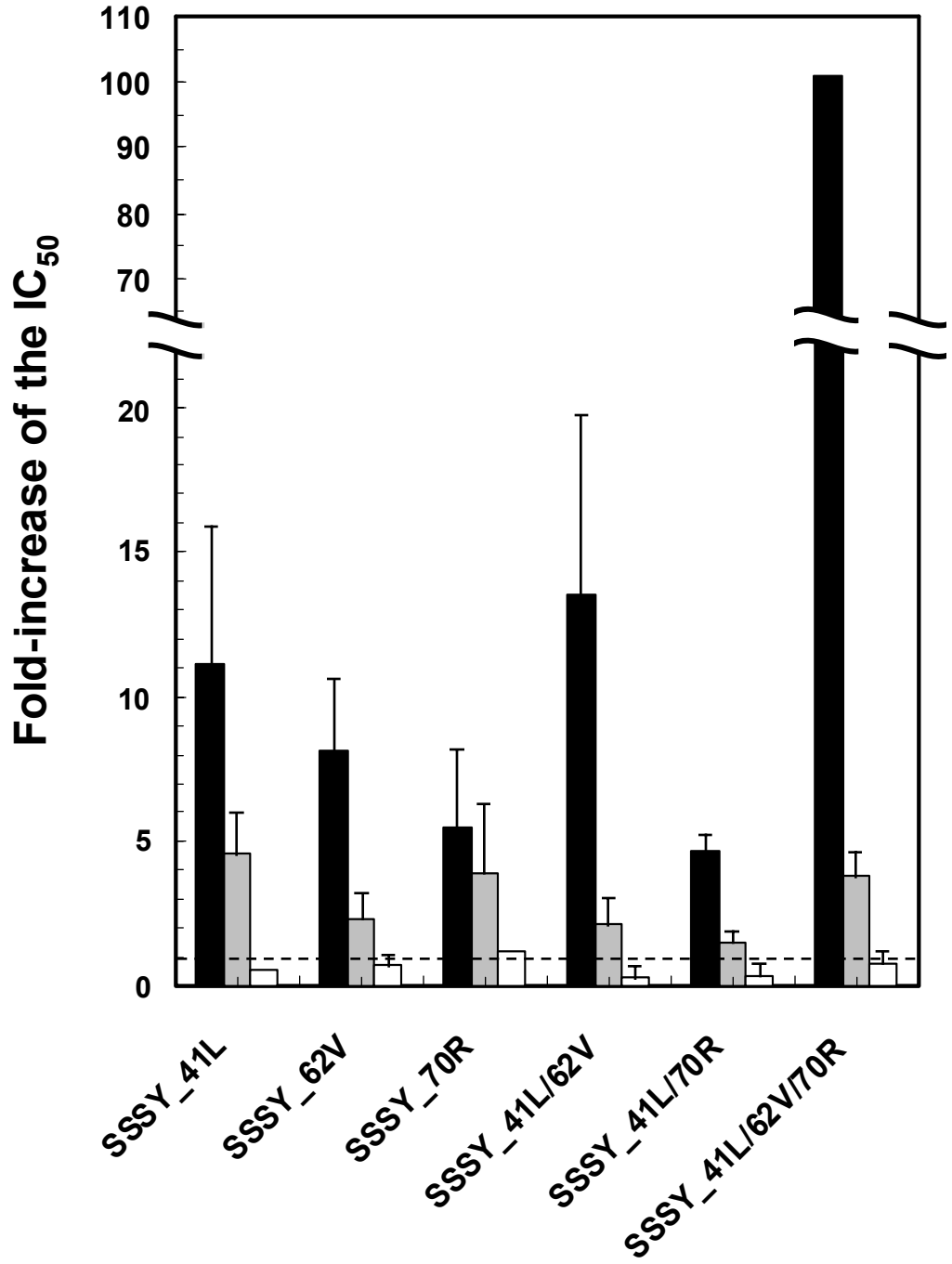
Fold-increase of the IC50
110
100
90
80
70
20
15
10
5
0
SSSY_41L
SSSY_62V
SSSY_70R
SSSY_41L/62V
SSSY_41L/70R
SSSY_41L/62V/70R

Figure 6

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