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N348I in HIV-1 Reverse Transcriptase Counteracts the Synergy Between Zidovudine and Nevirapine

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Abstract

The efficacy of regimens that include both zidovudine and nevirapine can be explained by the synergistic interactions between these drugs. N348I in HIV-1 reverse transcriptase (RT) confers decreased susceptibility to zidovudine and nevirapine. Here we demonstrate that N348I reverses the synergistic inhibition of HIV-1 by zidovudine and nevirapine. Also, the efficiency of zidovudine-monophosphate excision in the presence of nevirapine is greater for N348I HIV-1 RT compared to the wild-type enzyme. These data help explain the frequent selection of N348I in regimens that contain zidovudine and nevirapine, and suggest that the selection of N348I should be monitored in resource-limited settings where these drugs are routinely used.

Keywords

HIV-1 drug resistance; reverse transcriptase inhibitors; antiretroviral therapy; N348I; connection subdomain; C-terminal domain; combination therapy; zidovudine; nevirapine

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Introduction

Zidovudine (ZDV) and nevirapine (NVP) target the HIV-1 reverse transcriptase (RT) by distinct mechanisms of action and belong to the nucleoside reverse transcriptase inhibitor (NRTI) and nonnucleoside reverse transcriptase inhibitor (NNRTI) classes, respectively. The combination of ZDV and NVP has been recommended for antiretroviral therapy to delay the emergence of drug-resistant strains and the onset of disease progression. Potency of the ZDV and NVP combination can be ascribed to synergistic inhibition of HIV-1 replication and the selection of mutations in the RT that are distinct to each drug class and that demonstrate antagonism.^{1,2} These include the thymidine analogue mutations (TAMs) and NNRTI mutations located in the N-terminal RT region from codons 1 – 240.

N348I, located in the connection subdomain of the HIV-1 RT, has been associated with ZDV and NVP treatment.³ N348I confers decreased susceptibility to ZDV, NVP and efavirenz and potentiates RT inhibitor resistance when combined with TAMs and key NNRTI resistance mutations in clade B and non-subtype B strains.^{3–6} N348I is highly prevalent in RT inhibitor-treated compared to drug-naïve individuals and normally appears prior to TAMs³, providing a genetic pathway for the selection of TAMs and mutations that are known to antagonize ZDV resistance.⁷ While increases in viral load similar to that seen with each of the individual TAMs have been observed, the role of N348I in virological failure remains unclear.³

Synergistic inhibition of HIV-1 by NVP and ZDV is mediated by NNRTIs blocking basal phosphorolysis in wild-type (WT) RT, and the removal of ZDV-terminated primers by RT containing TAMs, leading to ZDV resensitization.^{8,9} Apart from TAMs, N348I also confers decreased susceptibility to ZDV; however, resistance to ZDV is mediated by a ribonuclease H (RNase H) dependent mechanism.^{3,10–12} N348I decreases formation of RNase H secondary cleavage products favoring the RT to bind to the template/primer (T/P) in a polymerase-dependent mode (with the polymerase active site at the 3' end of the primer) leading to increased excision of ZDV-monophosphate (ZDV-MP). Conversely, NNRTIs promote binding of RT in an RNase H competent mode (with the RNase H active site positioned to cleave the RNA/DNA strand), thereby enhancing RNase H cleavage leading to inhibition of ZDV-MP excision on a RNA/DNA duplex and increased susceptibility to ZDV.^{10,13} Given the opposing actions of N348I and NNRTIs on RNase H cleavage and ZDV resistance we investigated whether N348I counteracts synergistic inhibition of HIV-1 conferred by ZDV and NVP.

Methods

Drugs and Reagents

RT inhibitors ZDV and NVP were obtained from the AIDS Research and Reference Reagent Program. 3'-azido-3'-deoxythymidine (ZDV-TP) was purchased from TriLink Biotechnologies. All other nucleotides were purchased from GE Healthcare.

Plasmids, mutagenesis and virus production

The NL4.3-derived HIV-1 molecular clone, pDRNL, was mutated by site-directed mutagenesis as previously described³ to generate HIV_{N348I}, harboring N348I. All HIV-1 constructs were verified by nucleotide sequencing. HIV-1 was generated by transfection of 293T cells, concentrated by ultracentrifugation and the viral titer determined in TZM-bl cells by staining for β -galactosidase activity as previously described.³

Expression, purification and characterization of recombinant HIV-1 RT

Recombinant HIV-1_{LAI} RT containing N348I (RT_{N348I}) was expressed in *E.coli*, purified by metal chelate affinity chromatography and characterized as published.³

Drug susceptibility assays

Assays were performed in TZM-bl cells as previously described³ except with the following modifications. Assays were performed in the presence of ZDV, NVP or combinations of ZDV and NVP at fixed ratios. Cells were infected with ~250 blue foci-forming units of virus and at 48 h post-infection luciferase activity in cell lysates was measured as described¹⁴ and the 50% effective concentration (EC₅₀) determined as published.³

Synergism analysis

Evaluation of synergistic drug interactions was determined using the CalcuSyn software (Biosoft, Cambridge, UK) according to instructions for fixed ratio combinations. The analysis is based on the median effect principle. Combination index (CI) values at effective doses of 50% (ED₅₀), 75% (ED₇₅) and 90% (ED₉₀) were calculated from five (for HIV_{WT}) and 3 (for HIV_{N348I}) independent assays with $r = 0.93$.

Biochemical Assays

WT (RT_{WT}) and N348I RT (RT_{N348I}) susceptibility to NVP was examined using an RNA/DNA T/P (sequence in Figure 1A) as described previously.⁷ Primer rescue assays were performed using a 5'-³²P- radiolabelled 26-nucleotide DNA primer (pr26) chain terminated with ZDV-MP (pr26+ZDV) annealed to a 35-nucleotide RNA (T-RNA) template (Fig 1A) as reported previously.⁷ RNase H cleavage of a 5'-³²P-labelled 35-nucleotide RNA template annealed to a 26-nucleotide ZDV-MP terminated primer was performed as described.³ The effect of NVP on RNase H cleavage was performed in the presence of 1 μ M of drug.

Statistical analyses

Statistical significance of differences between drug EC₅₀ values, fold-resistance or CI values was determined by using the Wilcoxon Rank-Sum test.

Results

To determine whether N348I decreases susceptibility to ZDV and NVP combinations we evaluated the capacity of HIV_{N348I} to replicate in the presence of fixed ratios of ZDV and NVP compared to WT (HIV_{WT}) (Table 1). ZDV and NVP combinations at fixed ratios of 1:1, 1:10 and 10:1 resulted in significant increases in the EC₅₀ values for HIV_{N348I} compared to HIV_{WT} of 5- ($p < 0.001$, $n = 7$), 6- ($p < 0.01$, $n = 6$) and 3-fold ($p < 0.01$, $n = 6$), respectively (Table 1). Consistent with previous studies, HIV_{N348I} demonstrated 2- ($p < 0.05$, $n = 17$) and 7-fold ($p < 0.003$, $n = 13$) decreased susceptibility to ZDV and NVP, respectively compared to HIV_{WT}.³

We next investigated whether decreased susceptibility to ZDV and NVP combinations was due to reversal of their synergistic activity against HIV-1. As previously reported¹, the combination of ZDV and NVP tested at a 1:1 fixed ratio was strongly synergistic for HIV_{WT} inhibition at the ED₅₀ (CI = mean \pm SEM; 0.3 ± 0.1), ED₇₅ (CI = 0.3 ± 0.1) and ED₉₀ (CI = 0.3 ± 0.04) levels. In contrast, N348I dramatically reversed the synergistic activity of ZDV and NVP (at a 1:1 fixed ratio) with slight antagonism observed at the ED₅₀ (CI = 1.1 ± 0.1), additivity at the ED₇₅ (CI = 1.0 ± 0.2), and moderate synergism at the ED₉₀ (CI = 0.7 ± 0.2) levels. Taken together, these data demonstrate that in addition to conferring decreased susceptibility to each drug alone, N348I confers decreased susceptibility to the combination

of ZDV and NVP and reverses the strong synergism normally seen with this drug combination against WT virus.

Our previous studies demonstrate that N348I confers more efficient ZDV-MP excision and DNA polymerization of recombinant HIV-1 RT in the presence of ZDV-TP and ATP on an RNA but not a DNA template.³ We investigated whether N348I improves DNA polymerization efficiency of recombinant RT in the presence of NVP under steady-state conditions. RT_{WT} or RT_{N348I} was incubated for 5 min at 37°C in the presence of 20 nM T/P, 1 μM dNTP and variable concentrations of NVP. The short incubation time ensured that the reaction was in the linear phase and that there was no substrate depletion. RT_{N348I} was more efficient in DNA polymerization in the presence of NVP (50% inhibitory concentration ± standard deviation, 3.5 ± 0.5 μM, n=3) compared to RT_{WT} (0.8 ± 0.1 μM) (data not shown). Next we determined the ZDV-MP excision activity of RT_{N348I} compared to RT_{WT}. RT_{N348I} showed greater ATP mediated excision of ZDV-MP and rescue of DNA polymerization compared to RT_{WT} in the absence and presence of 1, 2.5, 5 and 10 μM of NVP (Figures 1B and 1C). Notably the excision rate of RT_{N348I} in the presence of 2.5 μM NVP was similar to the RT_{WT} in the absence of drug (Figure 1C). These data show that N348I enables RT to unblock a ZDV-MP terminated primer and resume DNA polymerization even in the presence of high concentrations of NVP.

We next evaluated the ability of N348I to counteract increased RNase H cleavage activity mediated by NVP on a 5'-³²P-labelled RNA/DNA duplex with a ZDV-MP terminated primer. NVP increased the efficiency of RNase H cleavage by RT_{WT} resulting in an accumulation of a 19-nucleotide single-stranded RNA template corresponding to the 10-nucleotide excision incompetent RNA/DNA duplex (Figures 1D and 1E). Cleavage to the 10-nucleotide duplex was more efficient with RT_{N348I} when the reaction was performed in the presence of NVP with the efficiency of cleavage similar to RT_{WT} in the absence of NVP. These data show that N348I counteracts the accelerated RNase H cleavage by HIV-1 RT in both the absence and presence of NVP.

Discussion

ZDV and NVP synergistically inhibit HIV-1 and this is mediated by the ability of NVP to block ZDV-MP excision on DNA/DNA and RNA/DNA template primers.^{1,9,10} Here we show that N348I abrogates synergistic inhibition of HIV-1 by ZDV and NVP. N348I conferred decreased susceptibility to ZDV:NVP combinations at different drug ratios compared to WT. In addition, recombinant RT containing N348I also showed greater efficiency in the rescue of DNA polymerization from a ZDV-MP terminated primer in the presence of NVP compared to WT. The mechanism by which N348I reverses the synergistic inhibition of HIV-1 by ZDV and NVP can be ascribed to decreased RNase H cleavage activity of RT_{N348I} allowing greater preservation of the RNA/DNA duplex relative to RT_{WT} subjected to similar conditions (i.e. RT_{N348I} + NVP compared to RT_{WT} + NVP). Notably, the rate of excision of RT_{N348I} in the presence of NVP was equivalent to RT_{WT} in the absence of drug (Figure 1E). Preservation of the RNA/DNA duplex to >12 nucleotides provides more time for ZDV-MP excision to occur consistent with previous reports.^{10,13}

N348I is observed early in therapy before the selection of TAMs and is highly associated with NVP and ZDV therapy.³ N348I is associated with the NNRTI mutations K103N, Y181C/I and G190A/S and the lamivudine mutations M184V/I.^{3,15} These mutations are known to suppress ZDV resistance mediated by TAMs by directly antagonizing the ATP-mediated ZDV-MP excision reaction.^{2,7} In contrast, N348I compensates for the antagonism of ZDV resistance conferred by Y181C and M184V on RNA/DNA template/primers by an indirect RNase H-dependent mechanism. This involves the reduction of secondary RNase H

cleavage products allowing more time for the excision reaction to take place. Therefore, N348I overcomes the suppression of ZDV resistance mediated by Y181C and NVP by similar RNase H-dependent mechanisms, resulting in decreased susceptibility to the combination of ZDV and NVP.

The World Health Organisation recommends that first line antiretroviral therapy in resource-constrained settings comprises a NNRTI with two NRTIs, one of which should be ZDV or tenofovir and that stavudine should be phased out due to its toxicities.¹⁶ The roll out of tenofovir could be hampered due to its cost.¹⁷ Accordingly, treatment with lamivudine, stavudine and NVP, that are widely used in Africa¹⁸, are likely to be maintained or could be replaced with a combination that includes ZDV, NVP and lamivudine. Of note, both of these treatments have been associated with the selection of N348I.^{3,19}

We propose that N348I, due to its ability to counteract the synergistic inhibition of HIV by ZDV and NVP, would allow for the selection of mutations that confer higher levels of resistance e.g. Y181C and TAMs. In contrast, Y181C, which confers NVP resistance and hypersusceptibility to ZDV, in the absence of N348I, would make it relatively more difficult to select TAMs. The role of N348I in providing a genetic pathway for the selection of TAMs, and the Y181C and M184V mutations that antagonize TAMs⁷ may have implications in the efficacy of recommended first line therapies in resource-constrained settings.⁶

Acknowledgments

S-H.Y, B.D.H, J.R, N.S-C and G.T conceived and designed the experiments, S-H.Y and J.R performed the experiments, S-H.Y, B.D.H, J.R, N.S-C and G.T analyzed the data, G.T performed statistical analyses and S-H.Y, N.S-C and G.T wrote the paper. We thank Yanille Scott for experimental assistance. We thank the NIH AIDS Research & Reference Reagent Program for providing ZDV, NVP and the TZM-bl cell line that was contributed by Dr John C. Kappes, Dr Xiaoyun We and Tranzyme Inc.

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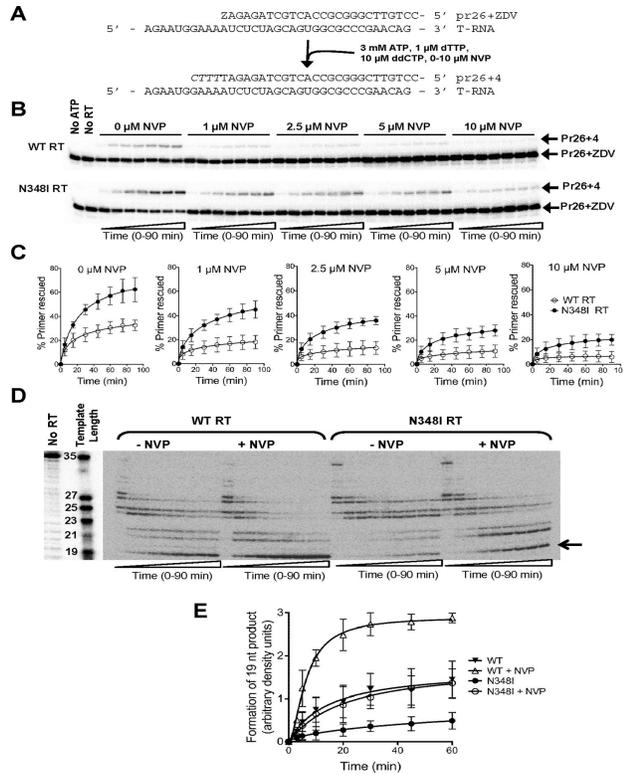


Figure 1.

ATP mediated rescue of DNA polymerization by WT and N348I mutant RT from a ZDV-MP terminated primer in the absence and presence of NVP, (A) ZDV-MP (Z) terminated DNA primer (pr26+ZDV) annealed to a 35 nucleotide RNA template (T-RNA) and the expected product following ZDV-MP excision and DNA polymerization. Italicized CTTT's represent incorporated nucleotides following unblocking of the ZDV-MP (Z) terminated DNA primer and its replacement with a T (B) Representative denaturing polyacrylamide gel showing time course of ZDV-MP excision in the absence and presence of NVP for WT RT and N348I RT. Pr26+ZDV and Pr26+4 denote primer and extended product, respectively. Lanes 1 and 2 are control reactions performed in the absence of ATP and RT, respectively. (C) Isotherms for ATP-mediated excision of ZDV-MP by WT and N348I RT in the absence and presence of NVP from four independent assays. Error bars denote standard deviation. (D) Representative denaturing polyacrylamide gel showing time course of WT and N348I RT mediated RNase H degradation of RNA/DNA T/P in the absence (-NVP) and presence (+NVP) of NVP. Template length of 19 nucleotides corresponds to the original 10-nucleotide RNA/DNA duplex (present prior to electrophoresis) as denoted by the arrow. (E) Isotherms for RNase H degradation by WT and N348I RT in the absence and presence of NVP from two independent assays. Error bars denote standard deviation.

Table 1

N348I decreases susceptibility to ZDV, NVP and combinations of ZDV and NVP

ZDV:NVP ^a	EC ₅₀ ± SE (μM) ^b		Fold Change ^c
	HIV _{WT}	HIV _{N348I}	
1:0	0.13 ± 0.02	0.20 ± 0.02	2
0:1	0.11 ± 0.01	0.73 ± 0.05	7
1:1	0.06 ± 0.008	0.29 ± 0.044	5
1:10	0.01 ± 0.001	0.06 ± 0.13	6
10:1	0.12 ± 0.02	0.33 ± 0.05	3

^aDrug susceptibility assays performed in the presence of ZDV, NVP or fixed ratios of both ZDV and NVP. A range of drug concentrations were used to achieve between 0 – 100% inhibition of HIV-1 replication for each drug alone and for the drug combinations at fixed ratios. For EC₅₀ values derived from combination assays, the combined drug concentration is expressed e.g. 0.06 μM of a 1:1 ZDV:NVP combination comprises 0.03 μM of both ZDV and NVP.

^bMean 50% effective concentration (EC₅₀) and standard error (SE) values from drug susceptibility assays from at least n=6 independent assays.

^cFold changes in susceptibility of mutant compared to WT which were all statistically significant (p<0.05) using the Wilcoxon rank-sum test.