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# In vitro selection of mutations in human immunodeficiency virus type 1 reverse transcriptase that confer resistance to capravirine, a novel nonnucleoside reverse transcriptase inhibitor

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**Abstract**

Capravirine (CPV; formerly AG1549 and S-1153) is a novel, nonnucleoside reverse transcriptase inhibitor (NNRTI) of human immunodeficiency virus type 1 (HIV-1) that has demonstrated potent in vitro antiviral activity against several HIV-1 laboratory strains and clinical isolates with $EC_{50}$ values ranging from 0.7 to 10.3 nM. In this study, we evaluated the resistance and cross-resistance profiles of CPV through selection of resistant HIV-1 variants from in vitro serial passage of HIV-1 NL4-3 and HIV-1 IIIB and by performing susceptibility assays on HIV-1 variants constructed to contain CPV-specific amino acid substitutions in reverse transcriptase (RT). Results demonstrate that HIV-1 variants selected at increasing CPV concentrations contained multiple substitutions in diverse patterns including L100I, Y181C, G190E and/or L234I in various combinations with K101R/E, K103T, V106A/I, V108I, E138K, T139K, A158T, V179D/I/G, Y188D, V189I, G190A, F227C, W229R, L234F, M230I/L and P236H/T. Interestingly, HIV-1 variants constructed to contain the T215Y zidovudine (AZT)-resistance associated substitution with CPV-resistance associated substitutions V106A, Y181C, F227C, F227L, L234I or V106A/F227L demonstrated 2.4–5.4-fold increased susceptibility to CPV. Results also demonstrate that the CPV-resistance associated substitutions Y181C, F227C, F227L and L234I reverse the phenotypic resistance to AZT conferred by the T215Y substitution.


*Keywords:* Capravirine; Nonnucleoside reverse transcriptase inhibitor; HIV; Resistance

## 1. Introduction

Currently, four classes of antiretroviral drugs have been approved for the treatment of HIV infection. These include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and entry inhibitors. Highly active antiretroviral therapy (HAART), defined by combinations of $\geq$3 drugs from $\geq$2 classes, has significantly reduced morbidity and mortality related to human immunodeficiency virus type 1 (HIV-1) infection (Yeni et al., 2004). Despite the success of such potent treatment regimens, some patients experience a loss of viral suppression and the subsequent emergence of resistant variants (Hirsch et al., 2003). Currently, there are three NNRTIs (nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV)) approved for treatment of HIV infection. Although a valuable component of HAART, virologic failure to the currently approved NNRTIs is often associated with the rapid selection of HIV variants with single amino acid substitutions in RT that confer high levels of resistance and the generation of cross-resistance to the entire class. Consequently, the sequential use of NNRTIs is not possible, and patients have but one chance to successfully respond to their initial NNRTI-based therapy. There thus remains a pressing need for the development of new NNRTIs with novel resistance profiles, as well as improved potency, safety and tolerability, to be used as first line therapy and/or in the treatment of patients following the failure of an initial NNRTI-containing regimen.

Capravirine (CPV; formerly AG1549 and S-1153) is a novel, potent NNRTI ($IC_{50}$ of 0.45 μM) (Fujiwara et al., 1998) dis-

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covered by Shionogi & Co., Ltd. from a screening program designed to identify compounds with antiviral activity against both wild-type and NNRTI-resistant HIV-1 variants. In vitro, CPV has demonstrated potent antiviral activity against several HIV-1 laboratory strains and clinical isolates with $EC_{50}$ values ranging from 0.7 to 10.3 nM (Fujiwara et al., 1998, 1999). In addition, CPV has demonstrated potent activity against virus resistant to NRTIs, as well as strains constructed to contain RT substitutions known to confer broad cross-resistance to other NNRTIs (e.g., L100I, K103N, V106A, Y181C, Y188C, G190A, F227L and P236L) (Fujiwara et al., 1998, 1999). Previous studies have identified CPV-resistant HIV-1 variants selected by in vitro serial passage of HIV-1 IIIB in M8166 cells in increasing concentrations of CPV. Two HIV variants characterized following 31 days (nine passages) of culture contained the substitutions V106A/F227L and K103T/V106A/L234I, and exhibited 26-fold and >500-fold reductions in susceptibility to CPV, respectively (Fujiwara et al., 1998, 1999). To further evaluate the in vitro resistance profile of CPV, resistant HIV-1 variants were selected following in vitro serial passage of HIV-1 NL4-3 and HIV-1 IIIB in escalating concentrations of CPV and the number and type of mutations required for resistance were evaluated at each compound concentration. In addition, susceptibility assays were performed with HIV-1 variants constructed to contain the specific amino acid substitutions in RT that arose under CPV selection. Results demonstrate that HIV-1 variants selected at increasing CPV concentrations contained multiple substitutions in diverse patterns.

## 2. Materials and methods

### 2.1. Compounds

CPV, NVP, DLV, EFV, lamivudine (3TC) and stavudine (d4T) were synthesized at Shionogi & Co. Ltd. (Osaka, Japan). Nelfinavir (NFV) was synthesized at Pfizer Global Research and Development (San Diego, CA). Zidovudine (AZT) was purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2. Cells and virus

The HeLa-CD4 cell line was kindly provided by Shionogi & Co., Ltd.. The T-cell line, M8166, is a previously described subclone of C8166 (Clapham et al., 1987). HIV-1 IIIB and HIV-1 NL4-3 were obtained from S. Harada (Kumamoto University, Kumamoto, Japan) and A. Adachi (Tokushima University, Tokushima, Japan).

### 2.3. Isolation of HIV-1 variants in increasing compound concentrations

M8166 cells were initially infected with HIV-1 NL4-3 or HIV-1 IIIB at a multiplicity of infection (m.o.i) of 0.01–0.1. From $3 \times 10^5$ to $4 \times 10^5$ infected cells were subsequently resuspended into each well of a 12-well tissue culture plate (in triplicate) in 3 ml of medium containing the appropriate dilution of test compound. If no cytopathic effect was observed, infected cells were passaged every 3–4 days at a 1:5 ratio. If cytopathic effect was observed, 0.5 ml of supernatant from infected cell cultures were resuspended with $2 \times 10^5$ uninfected M8166 cells in 3 ml fresh medium containing a five-fold higher concentration of the test compound. Culture supernatants were harvested and cells were stored as a pellet at −80 °C for subsequent analysis of HIV-1 proviral DNA.

### 2.4. RT DNA sequence determination

The nucleotide sequence of HIV-1 proviral DNA was determined at Operon Biotechnologies (Tokyo, Japan). PCR-amplified RT genes were sequenced with primer 3 (5′-AACTCAAGATTTCTGGGAAG) or primer 14 (5′-AGTGTAGCATGACAAAAATC) on an ABI 377 DNA Sequencer using Dye Terminator Cycle Sequencing. The nucleotide sequences of two adjacent regions, 285–750 (primer 3) or ≥750 (primer 14) of reverse transcriptase (RT) were analyzed by Shionogi & Co. Ltd. using Geneworks software with nucleotide mixtures evaluated by examining chromatograms. Nucleotide alignments and translations were performed using the DNAstar programs SeqMan, EditSeq and MegAlign. Reverse transcriptase amino acid substitutions were reported if they occurred at an overall frequency of ≥5% in the isolates evaluated, at any residue in the NNRTI-binding pocket (irrespective of frequency) (Balzarini, 1999), or at any residue previously reported to be associated with NNRTI resistance (irrespective of frequency) (Hammond et al., 1999; Schinazi et al., 2000).

### 2.5. Construction of recombinant HIV-1 variants by site-directed mutagenesis

In vitro site-directed mutagenesis was performed on the RT-coding region of the infectious HIV-1 pNL4-3 plasmid. The RT region of the pNL4-3 plasmid was PCR amplified using mutagenic primers. The amplification product containing the desired mutations was then subcloned into the infectious pNL4-3 plasmid. The plasmids were subsequently transfected into SW480 cells to generate infectious virus. Supernatants were harvested 2–3 days after transfection and were stored as cell-free culture supernatants at −80 °C.

### 2.6. Susceptibility assays

HeLa-CD4 cells containing an HIV-1 LTR driven β-galactosidase reporter gene (Isaka et al., 1999), were added at $2.5 \times 10^4$ cells per well into 96-well plates containing five-fold dilutions of test compounds. After a 1-h incubation, cells were infected with either HIV-1 NL4-3 or site-directed variants of HIV-1 NL4-3, at a concentration standardized to produce 10 000 CPM of RT activity (Sato et al., 1995). After 3 days of infection, the supernatants were removed and β-galactosidase activities in the cells were assayed by the Luminescent β-galactosidase Detection Kit II (Clontech, Palo Alto, CA) or the Reporter Assay Kit-β gal (Toyobo, Japan). Percent inhibition is expressed as the reduction in β-galactosidase activity

Table 1
Genotypic changes identified in RT gene regions from HIV-1 isolated following serial passage in increasing concentrations of CPV

| HIV-1 strain | Fold $>EC_{90}$[a] | Concentration (nM) | Number of isolates | Number of amino acid substitutions[b] | | | Amino acid | *n* |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 1 | >1 | | |
| NL4-3 | 1 | 14 | 3 | 0 | 3 | 0 | Y181C | 3 |
| | 7 | 70 | 14 | 0 | 11 | 3 | Y181C | 7 |
| | | | | | | | L234I | 3 |
| | | | | | | | L100I | 1 |
| | | | | | | | Y181C, L234I | 1 |
| | | | | | | | W229R, L234I | 1 |
| | | | | | | | V106A, Y181C, Y188D | 1 |
| | 34 | 350 | 5 | 0 | 2 | 3 | Y181C | 1 |
| | | | | | | | L234I | 1 |
| | | | | | | | L100I, Y181C | 1 |
| | | | | | | | L100I, Y181C, F227C | 1 |
| | | | | | | | L100I, Y181C, M230L | 1 |
| | 176 | 1800 | 9 | 0 | 0 | 9 | Y181C, L234I | 4 |
| | | | | | | | V106I, Y181C | 2 |
| | | | | | | | Y181C, F227C | 2 |
| | | | | | | | L100I, Y181C | 1 |
| | 882 | 9000 | 9 | 0 | 0 | 9 | Y181C, L234I | 2 |
| | | | | | | | Y181C, F227C | 1 |
| | | | | | | | F227C, M230I | 1 |
| | | | | | | | L100I, V179I, L234F | 1 |
| | | | | | | | K101R, V106I, Y181C | 1 |
| | | | | | | | V106I, A158T, Y181C, L234I | 1 |
| | | | | | | | V108I, F227C, M230I | 1 |
| | | | | | | | Y181C, F227C, L234I, P236T | 1 |
| IIIB | 5 | 70 | 10 | 1 | 7 | 3 | G190E | 4 |
| | | | | | | | L100I | 1 |
| | | | | | | | Y181C | 1 |
| | | | | | | | L234I | 1 |
| | | | | | | | L100I, Y181C | 2 |
| | | | | | | | V106A, G190E, W229R | 1 |
| | 24 | 350 | 15 | 0 | 10 | 5 | G190E | 7 |
| | | | | | | | Y181C | 2 |
| | | | | | | | L100I | 1 |
| | | | | | | | V106A, L234I | 1 |
| | | | | | | | L100I, K103T, Y181C | 1 |
| | | | | | | | L100I, Y181C, L234F | 1 |
| | | | | | | | L100I, A158T, Y181C | 1 |
| | | | | | | | K101E, Y181C, G190A | 1 |
| | 122 | 1800 | 26 | 0 | 8 | 18 | G190E | 8 |
| | | | | | | | Y181C, L234I | 2 |
| | | | | | | | L100I, Y181C | 1 |
| | | | | | | | L100I, F227C | 1 |
| | | | | | | | L100I, L234I | 1 |
| | | | | | | | V106A, L234I | 1 |
| | | | | | | | V179G, G190E | 1 |
| | | | | | | | Y181C, G190E | 1 |
| | | | | | | | F227C, M230I | 1 |
| | | | | | | | L100I, A158T, Y181C | 2 |
| | | | | | | | L100I, K103T, Y181C | 1 |
| | | | | | | | L100I, Y181C, G190E | 1 |
| | | | | | | | L100I, Y181C, Y318F | 1 |
| | | | | | | | K103T, Y181C, L234I | 1 |
| | | | | | | | T139K, V189I, G190E | 1 |
| | | | | | | | A158T, Y181C, L234I | 1 |
| | | | | | | | V179D, Y181C, L234I | 1 |
| | 612 | 9000 | 8 | 0 | 1 | 7 | G190E | 1 |
| | | | | | | | E138K, G190E | 2 |
| | | | | | | | V189I, G190E | 2 |
| | | | | | | | L100I, V106A | 1 |
| | | | | | | | L100I, K101E, A158T, Y181C, L234I, P236H | 1 |
| | | | | | | | L100I, Y181C, G190A, F227C | 1 |

[a] $EC_{90}$ = concentration of compound that inhibited HIV-1 NL4-3 or HIV-1 IIIB replication by 90% in M8166; CPV $EC_{90}$ against NL4-3 and IIIB: 10.2 and 14.7 nM, respectively.

[b] Reverse transcriptase amino acid substitutions were reported if they occurred at an overall frequency of ≥5% in CPV-selected isolates, at any residue in the NNRTI-binding pocket (Balzarini, 1999) or at any residue previously reported to be associated with NNRTI resistance (Hammond et al., 1999; Schinazi et al., 2000).

Table 2
In vitro susceptibility of CPV-resistant HIV-1 NL4-3 variants to NNRTIs[a]

| HIV-1 variant | CPV | | NVP | | DLV | | EFV | |
|---|---|---|---|---|---|---|---|---|
| | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] |
| NL4-3 (wild-type) | 13 ± 0.003 | | 490 ± 0.18 | | 290 ± 0.10 | | 10 ± 0.005 | |
| L100I | 65 ± 0.008 | 5 | 1900 ± 0.22 | 4 | 19100 ± 2.6 | 66 | 112 ± 0.068 | 11 |
| K101E[c] | 69 ± 0.017 | 5 | 4900 ± 2.2 | 10 | 1300 ± 0.33 | 4 | 67 ± 0.010 | 7 |
| K103T[d] | 33 ± 0.007 | 3 | 3800 ± 2.1 | 8 | 2900 ± 0.6 | 10 | 12 ± 0.003 | 1 |
| V106A[c] | 84 ± 0.055 | 7 | 48100 ± 10.7 | 98 | 2500 ± 1.7 | 9 | 19 ± 0.013 | 2 |
| V106I[c] | 28 ± 0.014 | 2 | 1000 ± 0.14 | 2 | 710 ± 0.14 | 2 | 14 ± 0.002 | 1 |
| E138K[c] | 116 ± 0.060 | 9 | 1800 ± 0.84 | 4 | 1200 ± 0.43 | 4 | 42 ± 0.021 | 4 |
| V179D[c] | 47 ± 0.018 | 4 | 790 ± 0.39 | 2 | 2100 ± 1.4 | 7 | 29 ± 0.016 | 3 |
| Y181C | 164 ± 0.028 | 13 | 88100 ± 27.1 | 179 | 18100 ± 6.3 | 62 | 16 ± 0.005 | 2 |
| G190A[c] | 15 ± 0.003 | 1 | 50900 ± 3.8 | 103 | 70 ± 0.02 | 0.2 | 66 ± 0.011 | 7 |
| G190E[e] | 4460 ± 1.9 | 345 | 188000 | >381 | 8200 ± 3.6 | 28 | 47600 ± 3.2 | 4734 |
| F227C[c] | 246 ± 0.064 | 19 | 8100 ± 3.5 | 16 | 770 ± 0.16 | 3 | 44 ± 0.023 | 4 |
| F227L[d] | 27 ± 0.004 | 2 | 2200 ± 0.19 | 5 | 10 ± 0.004 | 0.04 | 5 ± 0.002 | 0.5 |
| M230L[c] | 268 ± 0.036 | 21 | 10300 ± 1.5 | 21 | 12600 ± 7.6 | 43 | 95 ± 0.019 | 9 |
| L234I | 219 ± 0.051 | 17 | 230 ± 0.11 | 0.5 | 460 ± 0.20 | 2 | 14 ± 0.002 | 1 |
| Y318F[c] | 172 ± 0.039 | 13 | 1300 ± 0.29 | 3 | 5600 ± 0.58 | 19 | 17 ± 0.001 | 2 |
| L100I V106A | 1400 ± 0.48 | 108 | 168000 ± 62.0 | 342 | 132000 | 454 | 316 ± 0.051 | 31 |
| L100I Y181C | 689 ± 0.35 | 53 | 82300 ± 35.1 | 167 | 118000 ± 13.8 | 405 | 133 ± 0.068 | 13 |
| L100I G190A[c] | 149 ± 0.064 | 11 | 10300 ± 1.3 | 21 | 4300 ± 0.57 | 15 | 2160 ± 0.15 | 215 |
| L100I F227C | 3370 ± 0.61 | 260 | 52700 ± 4.3 | 107 | 128000 | 441 | 1680 ± 0.14 | 167 |
| L100I L234I | 721 ± 0.17 | 56 | 870 ± 0.39 | 2 | 16900 ± 1.5 | 58 | 368 ± 0.019 | 37 |
| V106A Y181C[c] | 69 ± 0.010 | 5 | 289000 | >586 | 156000 ± 19.8 | 538 | 55 ± 0.011 | 6 |
| V106A F227L[d] | 4860 ± 0.35 | 376 | 361000 | >732 | 930 ± 0.16 | 3 | 97 ± 0.002 | 10 |
| V106I Y181C | 1080 ± 0.18 | 84 | 166000 ± 57.3 | 336 | 23900 ± 3.8 | 82 | 43 ± 0.015 | 4 |
| Y181C F227C | 27000 ± 7.2 | 2090 | 289000 | >586 | 131000 ± 4.6 | 451 | 210 ± 0.045 | 21 |
| Y181C L234I | 4800 ± 1.7 | 371 | 22800 ± 9.5 | 46 | 92200 | 317 | 46 ± 0.015 | 5 |
| V106I Y181C L234I[c] | 26400 ± 9.2 | 2044 | 49300 ± 16.4 | 100 | 131000 ± 18.9 | 450 | 137 ± 0.061 | 14 |

[a] Inhibition of virus replication was determined by measuring β-galactosidase activity 3 days after infection of HeLa-CD4 cells with either wild-type (NL4-3) or HIV-1 strains constructed to contain specific amino acid substitution(s). Results represent the mean ± standard deviation (3–25 experiments).

[b] Fold change is the ratio of the $EC_{90}$ value obtained for mutant HIV-1 as compared to the $EC_{90}$ value for wild-type HIV-1.

[c] Individual amino acid substitution(s) identified in in vitro selected, capravirine-resistant HIV-1 variants containing multiple genotypic changes (Table 1).

[d] Amino acid substitutions identified in CPV-resistant, HIV-1 IIIB variants selected in vitro (Fujiwara et al., 1998, 1999).

[e] Represents a chimaeric NL4-3 virus containing the HIV-1 IIIB RT gene with G190E.

in compound-treated samples relative to infected, compound-free controls. The fold-change in compound susceptibility is expressed as the ratio of the $EC_{90}$ value obtained for mutant HIV-1 as compared to the $EC_{90}$ value for wild-type HIV-1 NL4-3.

## 3. Results

### 3.1. Selection of CPV-resistant HIV-1 variants in vitro

To characterize in vitro HIV-1 resistance to CPV, HIV-1 NL4-3 and HIV-1 IIIB variants were selected following serial passage in the presence of increasing concentrations of compound (Table 1). The number of HIV-1 variants that contained zero, one or more than one NNRTI-resistance associated amino acid substitution(s) at each passage are summarized (Table 1). As results indicate, HIV-1 NL4-3 and HIV-1 IIIB variants selected at lower CPV concentrations ($\leq$7- and $\leq$24-fold above the $EC_{90}$, respectively) contained predominately single amino acid substitutions. HIV-1 NL4-3 and HIV-1 IIIB variants selected at higher CPV concentrations ($\geq$34- and $\geq$122-fold above the $EC_{90}$, respectively), however, predominately contained more than one substitution.

The frequencies of specific genotypic changes in the RT gene from the CPV-resistant HIV-1 variants isolated at each passage are also summarized in Table 1. As results indicate, the most predominant single amino acid substitutions were L100I, Y181C, G190E and a novel substitution, L234I. The G190E substitution was identified in HIV-1 IIIB variants but was not observed in any of the HIV-1 NL4-3 variants. This is consistent with the failure to grow an HIV-1 NL4-3 variant constructed to contain the G190E substitution (data not shown). The genotypic changes in HIV-1 variants selected in increasing CPV concentrations were diverse and appeared in several combinations of double amino acid substitutions. The most frequently observed double amino acid substitutions in HIV-1 IIIB or NL4-3 variants included Y181C/L234I, L100I/Y181C, F227C/M230I and Y181C/F227C. Additional amino acid substitutions observed in HIV-1 NL4-3 or HIV-1 IIIB variants that contained multiple genotypic changes included L100I, Y181C, G190E and/or L234I in various combinations with K101R/E, K103T, V106A/I, E138K, T139K, A158T, V179D/I/G, Y188D, V189I, G190A, F227C, W229R, L234F, M230I/L and P236H/T.

### 3.2. In vitro susceptibility of CPV-resistant HIV-1 variants to NNRTIs

The in vitro resistance and cross-resistance profiles of CPV were evaluated by examining the susceptibility of HIV-1 strains constructed to contain single or multiple CPV-resistance associated amino acid substitutions that had been detected in isolates derived from serial passage of HIV-1 in the presence of increasing concentrations of CPV (Tables 2–4). CPV exhibited potent antiviral activity against HIV-1 variants containing single amino acid substitutions associated with CPV resistance with $EC_{90}$ values of 268 nM or less sufficient to inhibit 14 of 15 strains but demonstrated diminished antiviral activity against variants containing $\geq$2 amino acid substitutions (Table 2). Varying levels of susceptibility to CPV were likewise observed (Table 2). Specifically, little to no reductions in susceptibility ($\leq$4-fold) were observed for variants containing the K103T, V106I, V179D, G190A and F227L substitutions, moderate reductions in susceptibility (>4 to $\leq$10-fold) were observed for variants containing the L100I, K101E, V106A, E138K and V106A/Y181C substitutions and high levels of

Table 3
In vitro susceptibility of CPV-resistant HIV-1 NL4-3 variants to NRTIs

| Virus | AZT | | 3TC | | d4T | |
|---|---|---|---|---|---|---|
| | $EC_{90}$ (nM)[a], mean ± S.D. | Fold change[b] | $EC_{90}$ (nM)[a], mean ± S.D. | Fold change[b] | $EC_{90}$ (nM)[a], mean ± S.D. | Fold change[b] |
| NL4-3 (wild-type) | 535 ± 0.364 | | 1000 ± 0.60 | | 2400 ± 1.12 | |
| K101E[c] | 408 | 0.8 | 432 | 0.4 | ND | |
| V106A[c] | 97 ± 0.0613 | 0.2 | 1150 ± 0.16 | 1 | 339 ± 0.151 | 0.1 |
| V106I[c] | 657 ± 0.379 | 1 | 830 ± 0.535 | 0.8 | 2360 ± 0.83 | 1 |
| Y181C | 420 ± 0.321 | 0.8 | 590 ± 0.111 | 0.6 | 1740 ± 1.03 | 0.7 |
| F227C[c] | 53 ± 0.030 | 0.1 | 2710 ± 0.39 | 3 | 586 ± 0.145 | 0.2 |
| F227L[d] | 416 ± 0.337 | 0.8 | 1920 ± 1.2 | 2 | 2030 ± 0.94 | 0.8 |
| L234I | 357 ± 0.261 | 0.7 | 1950 ± 0.95 | 2 | 7350 ± 5.21 | 3 |
| L100I L234I | 213 | 0.4 | 279 | 0.3 | ND | |
| V106A Y181C[c] | 235 ± 0.186 | 0.4 | 829 ± 0.615 | 0.8 | 1860 ± 0.85 | 0.8 |
| V106A F227L[d] | 485 ± 0.040 | 0.9 | 1350 ± 0.79 | 1 | 2380 ± 0.60 | 1 |
| V106I Y181C | 219 ± 0.102 | 0.4 | 831 ± 0.515 | 0.8 | 1940 ± 0.92 | 0.8 |
| Y181C F227C | 107 ± 0.011 | 0.2 | 5330 ± 1.12 | 5 | 2370 ± 0.39 | 1 |
| Y181C L234I | 71 ± 0.010 | 0.1 | 1240 ± 0.57 | 1 | 1190 | 0.5 |

[a] Inhibition of virus replication was determined by measuring β-galactosidase activity three days after infection of HeLa-CD4 cells with either wild-type (NL4-3) or HIV-1 strains constructed to contain specific amino acid substitution(s). Results represent the mean ± standard deviation (3–15 experiments) or individual values (1 experiment); ND = not determined.

[b] Fold change is the ratio of the $EC_{90}$ value obtained for mutant HIV-1 as compared to the $EC_{90}$ value for wild-type HIV-1.

[c] Individual amino acid substitution(s) identified in in vitro selected, CPV-resistant HIV-1 variants containing multiple genotypic changes (Table 1).

[d] Amino acid substitutions identified in CPV-resistant, HIV-1 IIIB variants selected in vitro (Fujiwara et al., 1998, 1999).

Table 4
In vitro susceptibility of NRTI-resistant and multidrug-resistant site-directed HIV-1 NL4-3 variants to CPV and other RTIs[a]

| NRTI resistance substitutions | NNRTI resistance substitutions | CPV | | AZT | | NVP | | DLV | | EFV | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] | $EC_{90}$ (nM), mean ± S.D. | Fold change[b] |
| – | – | 13 ± 0.003 | | 535 ± 0.36 | | 493 ± 0.182 | | 291 ± 0.099 | | 10 ± 0.005 | |
| T215Y | – | 6 ± 0.001 | 0.5 | 4230 ± 4.1 | 8 | 390 ± 0.045 | 0.8 | 75 ± 0.035 | 0.3 | 5 ± 0.001 | 0.5 |
| – | K103N | 11 ± 0.003 | 0.8 | 1120 ± 0.69 | 2 | 50200 ± 4.1 | 102 | 32300 ± 15.1 | 111 | 666 ± 0.389 | 66 |
| T215Y | K103N | 3 ± 0.0004 | 0.2 | 9130 ± 4.4 | 17 | 29100 ± 8.7 | 59 | 4700 ± 1.8 | 16 | 278 ± 0.034 | 28 |
| – | V106A | 84 ± 0.055 | 7 | 97 ± 0.061 | 0.2 | 48100 ± 10.7 | 98 | 2500 ± 1.7 | 9 | 19 ± 0.013 | 2 |
| T215Y | V106A | 35 ± 0.008 | 3 | 6850 ± 3.8 | 13 | 41600 ± 6.8 | 84 | 781 ± 0.194 | 3 | 14 ± 0.002 | 1 |
| – | Y181C | 164 ± 0.028 | 13 | 420 ± 0.32 | 0.8 | 88100 ± 27.1 | 179 | 18100 ± 6.3 | 62 | 16 ± 0.005 | 2 |
| T215Y | Y181C | 52 ± 0.003 | 4 | 361 ± 0.038 | 0.7 | 47000 ± 6.2 | 95 | 3530 ± 0.78 | 12 | 10 ± 0.0005 | 1 |
| – | F227C | 246 ± 0.064 | 19 | 53 ± 0.030 | 0.1 | 8060 ± 3.5 | 16 | 774 ± 0.162 | 3 | 44 ± 0.023 | 4 |
| T215Y | F227C | 47 ± 0.019 | 4 | 913 ± 0.84 | 2 | 2030 ± 0.64 | 4 | 224 ± 0.121 | 0.8 | 14 ± 0.002 | 1 |
| – | F227L | 27 ± 0.004 | 2 | 416 ± 0.34 | 0.8 | 2220 ± 0.19 | 5 | 12 ± 0.004 | 0.04 | 5 ± 0.002 | 0.5 |
| T215Y | F227L | 5 ± 0.002 | 0.4 | 314 ± 0.23 | 0.6 | 913 ± 0.273 | 2 | 4 ± 0.003 | 0.01 | 3 ± 0.001 | 0.3 |
| – | L234I | 219 ± 0.051 | 17 | 357 ± 0.26 | 0.7 | 229 ± 0.110 | 0.5 | 456 ± 0.204 | 2 | 14 ± 0.002 | 1 |
| T215Y | L234I | 53 ± 0.010 | 4 | 252 ± 0.14 | 0.5 | 95 ± 0.018 | 0.2 | 136 ± 0.016 | 0.5 | 8 ± 0.003 | 0.8 |
| – | V106A F227L | 4860 ± 0.35 | 376 | 484 ± 0.040 | 0.9 | >361 | >732 | 932 ± 0.163 | 3 | 97 ± 0.002 | 10 |
| T215Y | V106A F227L | 931 ± 0.21 | 72 | 9140 ± 4.1 | 17 | >361 | >732 | 206 ± 0.043 | 0.7 | 70 ± 0.007 | 7 |

[a] Inhibition of virus replication was determined by measuring β-galactosidase activity three days after infection of HeLa-CD4 cells with either wild-type NL4-3 (no substitutions) or HIV-1 strains constructed to contain specific amino acid substitution(s); results represent the mean ± standard deviation (3–25 experiments).
[b] Fold change is the ratio of the $EC_{90}$ value obtained for mutant HIV-1 as compared to the $EC_{90}$ value for wild-type HIV-1.

resistance (>10-fold) were observed for variants containing the Y181C, G190E, F227C, L234I, M230L and Y318F substitutions. High levels of resistance were also observed for variants containing various patterns of two or more substitutions.

A comparable range of susceptibilities was also observed for CPV-resistant HIV-1 variants to other NNRTIs (Table 2). Specifically, no to moderate reductions in susceptibility (≤10-fold) were observed to one or more of the NNRTIs tested for HIV-1 variants containing K103T, K101E, V106A/I, E138K, V179D, Y181C, G190A, F227C/L, L234I, Y318F, Y181C/L234I, V106I/A/Y181C, V106A/F227L and L100I/L234I substitutions. High levels of resistance (>10-fold) to all NNRTIs (e.g., DLV, NVP, EFV) were observed for HIV-1 variants containing the G190E substitution and variants containing various patterns of two substitutions (Table 2). Interestingly, L234I was identified as a novel, CPV-resistance-associated substitution. An HIV-1 variant constructed to contain the L234I substitution demonstrated 17-fold reduction in susceptibility to CPV, but retained full susceptibility to NVP, DLV and EFV (Table 2). Similar results were obtained in experiments conducted using MT-4 cells (data not shown).

### *3.3. In vitro susceptibility of CPV-resistant HIV-1 variants to NRTIs*

In a similar manner, the susceptibility of HIV-1 strains constructed to contain single or multiple CPV-resistance associated amino acid substitutions to the NRTIs 3TC, AZT and d4T were evaluated (Table 3). With the exception of a Y181C/F227C HIV-1 variant that demonstrated five-fold reduced susceptibility to 3TC, no significant reductions in susceptibility to the NRTIs AZT, 3TC and d4T, were observed for all CPV-resistant HIV-1 variants evaluated. Similar results were obtained in experiments conducted using MT-4 cells (data not shown). Interestingly, the CPV-resistance associated substitutions V106A, F227C, L100I/L234I, Y181C/F227C and Y181C/L234I were found to confer 3–10-fold hypersusceptibility to NRTIs including AZT, 3TC and d4T (Table 3).

We also evaluated the interaction of the NRTI mutation T215Y in combination with CPV resistance associated mutations or mutations associated with resistance to other NNRTIs (e.g., K103N) in in vitro susceptibility assays. HIV-1 variants constructed to contain the T215Y AZT-resistance associated substitution in combination with CPV-resistance associated substitutions V106A, Y181C, F227C, F227L, L234I or V106A/F227L or the NNRTI-resistance associated substitution K103N, demonstrated 2.4–5.4-fold increased susceptibility to CPV and at least one other NNRTI (Table 4). Results presented in Table 4 also demonstrate that the CPV-resistance associated substitutions Y181C, F227C, F227L and L234I reverse the phenotypic resistance to AZT conferred by the T215Y substitution. Specifically, the T215Y substitution alone confers an 8.0-fold increase in the AZT $EC_{90}$ relative to wild type HIV-1, but in the presence of Y181C, F227C, F227L or L234I, the fold-change in AZT $EC_{90}$ relative to wild type HIV-1 is 0.7, 2.0, 0.6 or 0.5, respectively.

## 4. Discussion

Findings from a previous study (Fujiwara et al., 1999) indicate that resistance to CPV is slower to emerge in vitro relative to NVP resistance, and that two combinations of substitutions, V106A/F227L and K103T/V106A/L234I, associated with HIV-1 variants isolated following 31 days of serial passage conferred 26-fold and >500-fold reductions in susceptibility to CPV, respectively. The goals of the present study were to further characterize the in vitro resistance profile of CPV by comprehensively identifying the genotypes associated with in vitro resistance to CPV with two different viral strains, and by assessing the resistance and cross-resistance of HIV-1 variants constructed to contain CPV-resistance associated substitutions.

In the present study, HIV-1 variants selected at increasing CPV concentrations were found to contain multiple substitutions in diverse patterns including L100I, Y181C, G190E and/or L234I in various combinations with up to 20 different amino acid substitutions. These results are consistent with what has thus far been observed in vivo in CPV clinical studies. Virus isolated from HIV-infected patients treated with CPV monotherapy (Potts et al., 1999) or combination therapy (Hammond et al., 2004) contained one, two or three new NNRTI-resistance associated substitutions at various positions in reverse transcriptase (101, 103, 106, 108, 181, 188, 190 and/or 227). In this study, HIV-1 variants constructed to contain CPV-resistance associated substitutions selected in vitro were found to display low- to high-levels of resistance to CPV and the other approved NNRTIs evaluated, but remained susceptible (and in some cases hypersusceptible) to the NRTIs AZT, d4T and 3TC.

The crystal structure of CPV complexed with HIV-1 RT has been solved (Ren et al., 2000a) and provides important insights into the in vitro resistance profiles reported here. As reported by Ren et al., CPV forms three hydrogen bonds with the protein main chain at residues 101, 103 and 236 of the p66 subunit of RT. The hydrogen bonds to residues 103 and 236 are direct, whereas the hydrogen bond with residue 101 is mediated via a water molecule. This is in contrast to other currently approved NNRTIs, which form zero (NVP) (Ren et al., 2000a), one (EFV) (Ren et al., 2000b) or two (DLV) (Ren et al., 2000a) main chain hydrogen bonds within the binding pocket of RT. Presumably, mutations involving substitutions at amino acid side chains would be predicted to have little influence on inhibitor (CPV)-main chain binding interactions. Results from the in vitro resistance studies are consistent with this prediction. Specifically, mutations at each of the residues involved in forming main chain hydrogen bonds between CPV and the NNRTI binding pocket arose during in vitro selection (e.g., K101E/R, K103T, P236H/T) but were not isolated as single mutants. In addition, HIV variants containing either the K103T or K101E substitution demonstrated only two- and five-fold reductions in susceptibility to CPV, respectively. CPV has also been shown to form an extensive network of hydrophobic main and side chain interactions with many other amino acids, most of which were identified in these selection experiments (e.g., 101, 102, 103, 106, 181, 188, 190, 227, 229, 235, etc.).

Our studies also identified a mutation (L234I) that has not been associated with in vitro or in vivo resistance to the currently approved NNRTIs and appears to be uniquely associated with CPV resistance. Although an HIV-1 variant constructed to contain L234I had a 17.5-fold reduction in susceptibility to CPV, this HIV-1 variant was fully susceptible to other NNRTIs tested, e.g., NVP, DLV and EFV. Modeling studies derived from the co-crystal structure of CPV bound to RT provide a likely explanation for the loss of susceptibility of an HIV-1 variant that contains L234I (Ren et al., 2000a). Specifically, an isoleucine substitution at position 234 is predicted to result in unfavorable contacts between CPV and this amino acid.

An additional finding from the current study is that CPV does not select for the K103N mutation. K103N is strongly selected by all of the currently approved NNRTIs (Bacheler et al., 2000; Deeks, 2001; Demeter et al., 2000; Hanna et al., 2000; Joly et al., 2000; Richman et al., 1994), and has been shown to confer high level resistance and broad class cross-resistance. The appearance of HIV-1 variants containing K103N is also associated with virologic failure to NNRTI treatment (Bacheler et al., 2001; Shulman et al., 2000). Modeling studies derived from the co-crystal structure of CPV bound to RT suggest that CPV is capable of forming a hydrogen bond with the asparagine at position 103, thus providing a possible explanation for why K103N is not selected for by CPV. This structural data is also supported by experimental data that shows that an HIV-1 variant with K103N retains full susceptibility to CPV. These data are also consistent with what has thus far been observed in vivo in CPV clinical studies. One patient treated with CPV monotherapy developed a K103T substitution (Potts et al., 1999), but no CPV-treated patients have developed the K103N substitution while receiving CPV therapy.

The NNRTI-associated substitutions L100I and Y181C have previously been reported to reverse the phenotypic resistance observed with AZT-resistance substitutions (Byrnes et al., 1994; Larder, 1992). Results from this study demonstrate that the CPV-resistance associated substitutions Y181C, F227C, F227L and L234I also reverse the phenotypic resistance to AZT conferred by T215Y. In addition, the presence of the T215Y substitution reduces the level of CPV resistance conferred by the V106A, Y181C, F227C, F227L, L234I or V106A/F227L mutations. Although the clinical relevance of these results are not known, these results indicate that interactions between CPV and NRTI mutations may enhance antiviral susceptibility. While the in vitro data suggests a promising role for CPV in NNRTI-experienced patients, CPV failed to meet its primary outcome in two Phase 2 clinical trials (Pesano et al., 2005; Hawley et al., 2006), and no further development of CPV is planned.

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## References

Bacheler, L.T., Anton, E.D., Kudish, P., Baker, D., Bunville, J., Krakowski, K., Bolling, L., Aujay, M., Wang, X.V., Ellis, D., Becker, M.F., Lasut, A.L., George, H., Spaulding, D.R., Hollis, G., Abremski, K., 2000. Human immunodeficiency virus type 1 mutations selected in patients failing efavirenz combination therapy. Antimicrob. Agents Chemother. 44, 2475–2484.

Bacheler, L., Jeffrey, S., Hanna, B., D'Aquila, R., Wallace, L., Logue, K., Cordova, B., Hertogs, K., Larder, B., Buckery, R., Baker, D., Gallagher, K., Scarnati, H., Tritch, R., Rizzo, D., 2001. Genotypic correlates of phenotypic resistance to efavirenze in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. J. Virol. 75, 4999–5008.

Balzarini, J., 1999. Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. Biochem. Pharmacol. 58, 1–27.

Byrnes, V.W., Emini, E.A., Schleif, W.A., Condra, J.H., Schneider, C.L., Long, W.J., Wolfgang, J.A., Graham, D.J., Gotlib, L., Schlabach, A.J., Wolanski, B.S., Blahy, O.M., Quintero, J.C., Rhodes, A., Roth, E., Titus, D.L., Sardana, V.V., 1994. Susceptibilities of human immunodeficiency virus type 1 enzyme and viral variants expressing multiple resistance-engendering amino acid substitutions to reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 38, 1404–1407.

Clapham, P.R., Weiss, R.A., Dalgleish, A.G., Exley, M., Whitby, D., Hogg, N., 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. Virology 158, 44–51.

Deeks, S.G., 2001. International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. J. AIDS 26 (Suppl. 1), S25–S33.

Demeter, L.M., Shafer, R.W., Meehan, P.M., Holden-Wiltse, J., Fischl, M.A., Freimuth, W.W., Para, M.F., Reichman, R.C., 2000. Delavirdine susceptibilities and associated reverse transcriptase mutations in human immunodeficiency virus type 1 isolates from patients in a phase I/II trial of delavirdine monotherapy (ACTG 260). Antimicrob. Agents Chemother. 44, 794–797.

Fujiwara, T., Sato, A., el-Farrash, M., Miki, S., Abe, K., Isaka, Y., Kodama, M., Wu, Y., Chen, L.B., Harada, H., Sugimoto, H., Hatanaka, M., Hinuma, Y., 1998. S-1153 inhibits replication of known drug-resistant strains of human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 42, 1340–1345.

Fujiwara, T., Sato, A., Patick, A.K., Potts, K.E., 1999. In vitro antiviral activity and resistance profile of AG1549 (S-1153), a new non-nucleoside inhibitor of HIV-1 reverse transcriptase. Int. Antiviral News 7, 18–20.

Hammond, J., Pesano, R., Hawley, P., Patick, A.K., 2004. Analysis of time of failure genotype and phenotype from NNRTI-experienced patients treated with capravirine. Thirteenth International HIV Drug Resistance Workshop, Costa Adeje (Abstract 15).

Hammond, J., Calef, C., Larder, B., Schinazi, R., Mellors, J.W., 1999. Mutations in retroviral genes associated with drug resistance. In: Kuiken, C.L., Foley, B., Hahn, B., Korber, B., McCutchan, F., Marx, P.A., Mellors, J.W., Mullins, J.I., Sodroski, J., Wolinksy, S. (Eds.), Human Retroviruses and AIDS. A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Theoretical Biology and Biophysics Group. Los Alamos National Laboratory, Los Alamos, NM, pp. 562–591.

Hanna, G.J., Johnson, V.A., Kuritzkes, D.R., Richman, D.D., Brown, A.J., Savara, A.B., Hazelwood, J.D., D'Aquila, R.T., 2000. Patterns of resistance mutations selected by treatment of human immunodeficiency virus type 1 infection with zidovudine, didanosine, and nevirapine. J. Infect. Dis. 181, 904–911.

Hawley, P., Hammond, J., Ryan, R.J., Tressler, R.L., Raber, S.R., Hodges, M., 2006. Final 48 week safety, tolerability and efficacy of capravirine + lopinavir/ritonavir and 2 NRTIs in treatment experienced patients. In: Proceedings of the 13th Conference on Retroviruses and Opportunistic Infections, Colorado (Abstract J-130).

Hirsch, M.S., Brun-Vezinet, F., Clotet, B., Conway, B., Kuritzkes, D.R., D'Aquila, R.T., Demeter, L.M., Hammer, S.M., Johnson, V.A., Loveday,

C., Mellors, J.W., Jacobson, D.M., Richman, D.D., 2003. Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society—USA Panel. Clin. Infect. Dis. 37, 113–128.

Isaka, Y., Sato, A., Miki, S., Kawauchi, S., Sakaida, H., Hor, T., Uchiyama, T., Adachi, A., Hayami, M., Fujiwara, T., Yoshie, O., 1999. Small amino acid changes in the V3 loop of human immunodeficiency virus type 2 determines the coreceptor usage for CXCR4 and CCR5. Virology 264, 237–243.

Joly, V., Moroni, M., Concia, E., Lazzarin, A., Hirschel, B., Jost, J., Chiodo, F., Bentwich, Z., Love, W.C., Hawkins, D.A., Wilkins, E.G., Gatell, A.J., Vetter, N., Greenwald, C., Freimuth, W.W., de Cian, W., 2000. Delavirdine in combination with zidovudine in treatment of human immunodeficiency virus type 1-infected patients: evaluation of efficacy and emergence of viral resistance in a randomized, comparative phase III trial. The M/3331/0013B Study Group. Antimicrob. Agents Chemother. 44, 3155–3157.

Larder, B.A., 1992. 3′-Azido-3′deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. Antimicrob. Agents Chemother. 36, 2664–2669.

Pesano, R., Piraino, S., Hawley, P., Hammond, J., Tressler, R.L., Ryan, R.J., Nickens, D., Ruiz, R., 2005. 24-week safety, tolerability, and efficacy of capravirine as add-on therapy to nelfinavir and 2 nucleoside reverse transcriptase inhibitors in patients failing a nonnucleoside reverse transcriptase inhibitor-based regimen. In: Proceedings of the Twelfth Conference on Retroviruses and Opportunistic Infections, Boston (Abstract 555).

Potts, K.E., Fujiwara, T., Sato, A., Cao, J., Jackson, R.L., Isaacson, J., Maldonado, O., Atkinson, B., Wang, B., Nash-Alexander, T.C., Patick, A.K., 1999. Resistance profile of AG1549, a novel non-nucleoside reverse transcriptase inhibitor. Antivir. Ther. 4, S10.

Ren, J., Nichols, C., Bird, L.E., Fujiwara, T., Sugimoto, H., Stuart, D.I., Stammers, D.K., 2000a. Binding of the second generation non-nucleoside inhibitor S-1153 to HIV-1 reverse transcriptase involves extensive main chain hydrogen bonding. J. Biol. Chem. 275, 14316–14320.

Ren, J., Milton, J., Weaver, K.L., Short, S.A., Stuart, D.I., Stammers, D.K., 2000b. Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. Struct. Fold. Des. 8, 1089–1094.

Richman, D.D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S.A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., Shih, C., Myers, M., Griffin, J., 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. 68, 1660–1666.

Sato, A., Kodama, M., Abe, K., Miki, S., Nishimura, M., Suyama, A., Ogata, M., Toyoda, T., Sugimoto, H., Yoshie, O., Fujiwara, T., 1995. A simple and rapid method for preliminary evaluation of in vivo efficacy of anti-HIV compounds in mice. Antiviral Res. 27, 151–163.

Schinazi, R.F., Larder, B.A., Mellors, J.W., 2000. Mutations in retroviral genes associated with drug resistance: 2000–2001 update. Int. Antiviral News 8, 65–91.

Shulman, N.S., Zolopa, A.R., Passaro, D.J., Murlidharan, U., Israelski, D.M., Brosgart, C.L., Miller, M.D., Van Doren, S., Shafer, R.W., Katzenstein, D.A., 2000. Efavirenz- and adefovir dipivoxil-based salvage therapy in highly treatment-experienced patients: clinical and genotypic predictors of virologic response. J. AIDS 23, 221–226.

Yeni, P.G., Hammer, S.M., Hirsch, M.S., Saag, M.S., Schechter, M.S., Carpenter, C.C.J., Fischl, M.A., Gatell, J.M., Gazzard, B.G., Jacobsen, D.M., Katzenstein, D.A., Montaner, J.S.G., Richman, D.D., Schooley, R.T., Thompson, M.A., Vella, S., Volberding, P.A., 2004. Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society—USA Panel. JAMA 292, 251–265.