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Connection subdomain mutations in HIV-1 subtype-C treatment-experienced patients enhance NRTI and NNRTI drug resistance

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ABSTRACT

Mutations in the connection subdomain (CN) and RNase H domain (RH) of HIV-1 reverse transcriptase (RT) from subtype B-infected patients enhance nucleoside and nonnucleoside RT inhibitor (NRTI and NNRTI) resistance by affecting the balance between polymerization and RNase H activity. To determine whether CN mutations in subtype C influence drug sensitivity, single genome sequencing was performed on Brazilian subtype C-infected patients failing RTI therapy. CN mutations identified were similar to subtype B, including A376S, A400T, Q334D, G335D, N348I, and A371V, and increased AZT resistance in the presence of thymidine analog mutations. CN mutations also enhanced NNRTI resistance in the presence of classical NNRTI mutations: etravirine resistance was enhanced 6- to 11-fold in the presence of L100I/K103N/Y181C. These results indicate that selection of CN mutations in treatment-experienced patients also occurs in subtype-C-infected patients and are likely to provide valuable information in predicting clinical RTI resistance.

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Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of the global AIDS pandemic and is subdivided into four major groups: M (Main), O (Outlier), N (Non-M/O), and the recently described group P (Plantier et al., 2009). Group M is responsible for >95% of all HIV infections worldwide and is comprised of nine different subtypes: A, B, C, D, F, G, H, J, and K (Robertson et al., 2000), in addition to 49 intersubtype recombinants designated circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/). HIV-1 epidemiological surveys show that subtype C accounts for 48% of infections globally and is found mainly in southern Africa and India (Hemelaar et al., 2011). Subtype B is found predominantly in the western world, and despite accounting for only 11% of HIV-1 infections, this subtype remains the major model subtype from which data is collected to develop antiviral drugs, identify drug resistance mutations and determine drug treatment regimens.

Sequence diversity within subtype B ranges from 10–30 %, while sequence diversity within subtype C ranges from 5–15 % (Gaschen et al., 2002). These inherent differences are due to naturally occurring nucleotide polymorphisms which can impact not only drug resistance and viral fitness, but also potential vaccine success. In general, the HIV-1 subtypes have been shown to respond similarly to standard drug therapy and acquire similar drug resistance mutations, suggesting that in most cases analyses compiled with subtype B can be extended to other subtypes (Alexander et al., 2002; Frater et al., 2002; Geretti et al., 2009; Kantor et al., 2005; Palmer et al., 1998; Pillay et al., 2002). However, numerous reports have also shown that intersubtype diversity can influence the effectiveness of antiviral therapy [reviewed in (Santos and Soares, 2010)]. Genetic differences between the HIV-1 subtypes can alter the response to certain antiviral drugs, the pathway and speed of acquiring resistance, the drug mutational pattern, the level of drug resistance, and the overall viral replicative capacity (Abecasis et al., 2005; Brenner et al., 2003; Brenner et al., 2006; Caride et al., 2001; Grossman et al., 2004; Holguin et al., 2006; Lai et al., 2010; Martinez-Cajas et al., 2009; Palmer et al., 1998; Soares et al., 2007). Therefore, it is

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important to understand the emergence of drug resistance in non-B subtypes to develop different drug strategies and better aid those patients not responding to therapy.

Reverse transcriptase (RT) is composed of the polymerase domain (POL) which includes the fingers, palm, thumb, and connection subdomains (CN) as well as the RNase H domain (RH) (di Marzo Veronese et al., 1986; Lowe et al., 1988), with RT drugs targeting POL to block DNA polymerization during reverse transcription. Analysis of patient drug resistance is usually confined to the first 300 amino acids of POL (Johnson et al., 2010; Martinez-Cajas et al., 2008), leaving the C-terminal domain of RT, which includes the CN and RH, largely understudied for drug resistance mutations. We have previously reported that drug resistance mutations C-terminal to POL can also significantly increase resistance to both nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs, respectively) (Delviks-Frankenberry et al., 2010; Nikolenko et al., 2007; Nikolenko et al., 2010). Analysis of RT C-terminal domains from seven treatment-experienced subtype B-infected patients showed that the patient-derived CNs increased AZT resistance by as much as a 500-fold in the context of TAMs (Nikolenko et al., 2007). Eight novel mutations, E312Q, G335C/D, N348I, A360I/V, V365I, and A376S, were identified within the CN of RT that significantly contributed to AZT resistance, and these mutations were further found to decrease RNase H activity, leading to decreased susceptibility to AZT (Delviks-Frankenberry et al., 2008; Nikolenko et al., 2007; Nikolenko et al., 2005).

We further analyzed 450 subtype B isolates from the Stanford University HIV Drug Resistance Database and showed that 9 CN mutations (I326V, R358K, G359S, A360T, A360V, K366R, A371V, K390R and A400T) and 6 RH mutations (I506L, K512R, K527N, K530R, and Q457K) were positively associated with NRTI or AZT monotherapy exposure (Santos et al., 2008). The recent OPTIMA trial also identified CN mutations Y318F, G333D/E, G335D, N348I, V365I, A371V and A376S as positively associated with treatment experience in a 345 subtype-B-infected patient cohort (Dau et al., 2010). Associations of these and other CN mutations with drug exposure and TAMs has also been confirmed through other subtype-B-infected patient cohorts and in vitro studies (Cane et al., 2007; Dau et al., 2010; Ehteshami et al., 2008; Gupta et al., 2010; Hachiya et al., 2008a; Lengruber et al., 2011; Michels et al., 2010; Price et al., 2010; von Wyl et al., 2010a; von Wyl et al., 2010b; Waters et al., 2009; Yap et al., 2007) Recently, CN mutation A360V was shown to be selected in subtype-B-infected patients receiving AZT monotherapy (Brehm et al., 2012b).

The contribution of RT C-terminal domain mutations in non-B subtypes remains to be determined. We have previously reported that CN mutation A400T in CRF01_AE is an important determinant for AZT resistance in CRF01_AE patients containing TAMs (Delviks-Frankenberry et al., 2009). Recent data from the Lampang Cohort of CRF01_AE patients failing d4T, 3TC and NVP therapy showed that CN mutations N348I and E399D were associated with treatment failure (Saeng-aroon et al., 2010), and in vitro studies for CRF01_AE viruses containing N348I and A371V in the presence of TAMs also showed enhanced NRTI drug resistance (Tanuma et al., 2010). Additionally, a cohort of treatment-naïve patients from Mali, West Africa, 71% of whom where CRF02_AG, showed a high prevelance for CN polymorhisms A371V (63%), G335D (76%) and E399D (11%) (Haidara et al., 2010). Furthermore, 10% of treatment naïve subtype C-infected patients from Southern Brazil were shown to already contain CN mutations (for example, T369I and A376S) (Santos et al., 2011), suggesting that drug therapy may already be compromised in patients harboring CN polymorphisms associated with drug treatment. Together, these data suggest that other HIV-1 subtypes and CRFs may also contain CN mutations that influence drug resistance. Therefore, the aim of this paper was to determine whether subtype-C-infected patients also acquire CN mutations in response to drug therapy, whether the CN mutations differ from those reported from subtype B, and whether the CN mutations contribute to enhanced drug resistance in the background of a subtype C genotype.

Results

Characterization of RT mutations present in subtype C-infected patients

Since subtype C accounts for the majority of HIV-1 infections worldwide, we sought to determine whether mutations in the CN of subtype C-infected patients would also influence RTI drug resistance. Fig. 1A shows schematically the RT region of the four treatment-experienced patients analyzed in this study. We included a subtype B treatment-experienced patient (P1) from Brazil for comparison to the two treatment-experienced Brazilian subtype C-infected patients, P2 and P5. Patient P3, a B/C recombinant was also included in the analysis as B/C recombinants are prevalent in southern Brazil due to the high proportions of patients infected with subtype B and/or subtype C isolates in that population (Soares et al., 2005; Soares et al., 2003). SimPlot analysis of the RT region (data not shown) confirmed that P1 is

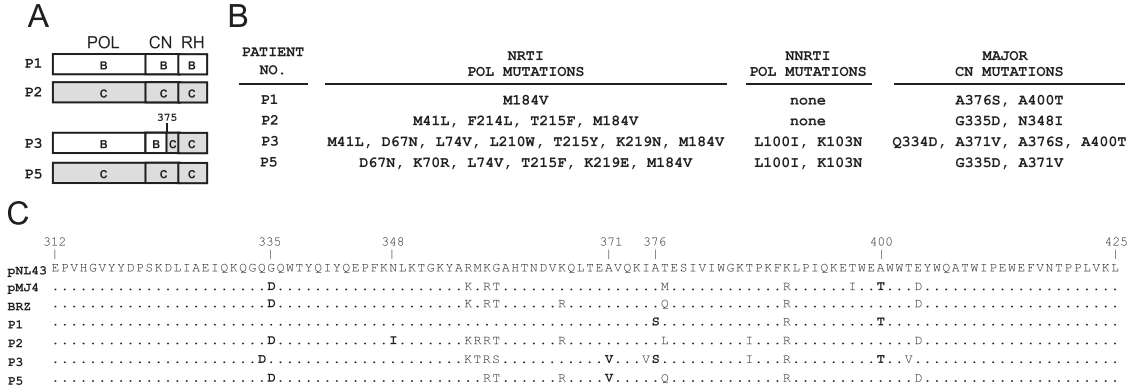

Fig. 1. Patient genotypic profiles. (A) The polymerase domain (POL), connection subdomain (CN) and RNase H domain (RH) of the four RTI treatment-experienced patients (P1, P2, P3, and P5) compared in this study are shown. P1 is subtype B (white boxes), P2 and P5 are subtype C (gray boxes), and P3 contains a chimeric RT with the 5′ half of RT up to amino acid 375 subtype B, and the remainder subtype C. (B) Major NRTI, NNRTI, and CN mutations for each patient identified by single genome sequencing. (C) Amino acid alignment of the HIV-1 RT CN (amino acids 312–425) for each patient, for subtype C reference strain MJ4 from Cameroon and for the Brazilian subtype C consensus sequence (BRZ; consensus of Brazilian subtype C's AF286228, AY727522, AY727523, AY727524, AY727525). Subtype B pNL43 serves as the reference strain for alignment. CN mutations previously identified to be associated with drug resistance in subtype B are indicated in bold.

subtype B, P2 and P5 are subtype C, and P3 contains a chimeric RT (Fig. 1A) with the POL and the CN up to amino acid 375 from subtype B and the remainder of the CN and RH from subtype C.

The major POL and CN mutations associated with each patient are shown in Fig. 1B. All patients received AZT and 3TC treatment, and the inclusion of 3TC resulted in the selection of the M184V mutation. P3 and P5 also received EFV treatment, which was associated with the selection of the NNRTI POL mutations L100I and K103N. P1 and P2 did not receive any NNRTIs, however they were treated with protease inhibitor NFV, with P2 also receiving ATV/R treatment. Standard TAMs (M41L, D67N, L210W, F214L, T215F/Y, K219N/E), were observed in POL, with P1 lacking NRTI TAMs. Alignment of the CN amino acid sequence (Fig. 1C) showed that each patient had a unique pattern of CN mutations. Interestingly, the CN mutations observed in the subtype-C-infected treatment-experienced patients were similar to those previously identified in subtype B: P2, G335D and N348I; P3, Q334D, A371V, A376S, and A400T; and P5, G335D and A371V. Subtype B patient P1 also contained previously identified CN mutations A376S and A400T. Thus, similar CN mutations as subtype B are associated with RTI therapy of subtype C-infected patients.

We further analyzed the prevalence of these CN mutations in subtype-C naïve and treatment-experienced patients from the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) (Table 1). Mutation Q334D, G335D, N348I, and A371V were found to be significantly associated with subtype-C-infected treatment-experienced patients. Database analysis showed A400T to be a common polymorphism in subtype C, while N348I (15%) and A371V (14%) were positively associated with RTI treatment. Interestingly, Q334D and G335D were negatively associated with treatment (Q334D: 11% naïve vs. 3.5% treated, G335D: 81% naive vs. 56% treated).

RT and CN from subtype-C and subtype-B exhibit similar levels of AZT resistance

To test for drug resistance, vectors were created by inserting the POL, CN and RH from wild-type subtype C into pHL[B-WT] to create pHL[C-WT] (Fig. 2A). These two basic vectors (referred to as B-WT and C-WT) were used to create additional chimeric POL, CN, and/or RH vectors containing subtype B, subtype C and/or patient RT sequences. Any reference to "wild type" refers to the prototype subtype strain pNL4-3 for subtype B and MJ4 for subtype C.

Inherent levels of AZT resistance were first tested for wild-type subtype C in the absence (C-WT) or presence (C-TAMs) of TAMs (D67N, K70R, T215F/Y and K219Q). No significant differences in the 50% effective concentration [EC_{50}] for AZT were observed between B-WT (EC_{50}=0.05 μM; 1×) and C-WT (EC_{50}=0.06 μM; 1×) (Fig. 2B). Slight increases in the AZT EC_{50} were observed between subtype B (18×) and C (28×) in the presence of TAMs, however this is likely due to the fact that the subtype B TAMs combination contained mutation T215Y instead of T215F. We have previously shown that the difference in AZT resistance between TAMs combination D67N, K70R, T215Y and K219Q versus D67N, K70R, T215F and K219Q in a subtype B background is ~1.6-fold (Nikolenko et al., 2007). Thus the 28-fold increase in resistance for C-TAMs versus the 18-fold increase in resistance for B-TAMs is likely due to the T215F amino acid change (resulting increase also 1.6-fold).

We further tested the wild-type subtype C CN in the background of subtype B to test for any inherent AZT resistance associated with the subtype C wild-type CN (Fig. 2C). The addition of the subtype-C CN to B-WT did not change the baseline AZT resistance either in the absence (EC_{50}=0.04 μM; 1×) or presence of TAMs (EC_{50}=0.92 μM; 18×). Therefore, subtype-B and subtype-C RTs have similar baseline levels of AZT resistance and no inherent polymorphisms in the MJ4 subtype-C CN alter that resistance.

RT from subtype-C treatment-experienced patients P2, P3 and P5 exhibit enhanced AZT resistance.

To test the levels of drug resistance associated with each patient, vectors were created in which the POL, CN and RH from

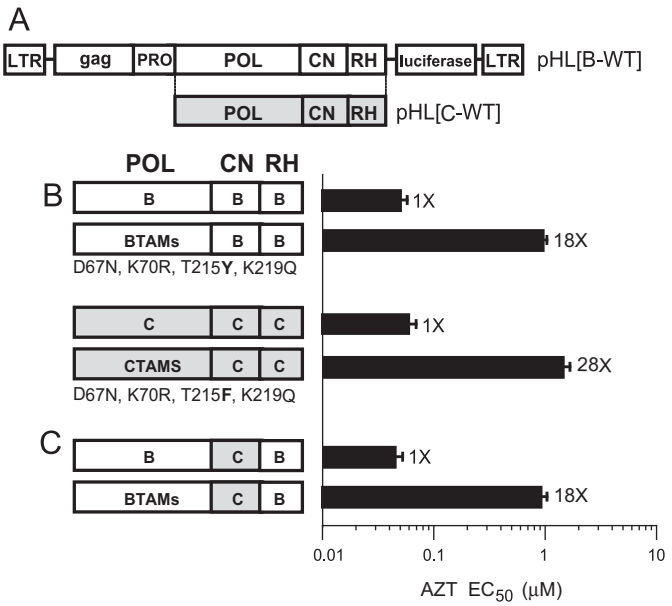

Fig. 2. AZT resistance associated with the wild-type subtype C RT and CN. (A) Schematic representation of the subtype B HIV-1 luciferase-expressing vector used for antiviral drug resistance and for generating chimeric viruses. pHL[B-WT], white boxes and pHL[C-WT], gray boxes. (B) AZT resistance associated with subtype B and subtype C RTs in the presence or absence of TAMs. (C) AZT resistance associated with the subtype C CN in the background of subtype B in the presence or absence of TAMs. Error bars represent the standard error of the mean of at least two replicates from three independent experiments. Increases in drug resistance are indicated relative to wild-type subtype B (one-fold, defined as 1×). LTR, long terminal repeat, PRO, protease. POL, CN and RH are the polymerase domain, connection subdomain and RNase H domain of RT, respectively.

Table 1
Prevalence of CN mutations in subtype C-infected patients in Stanford University HIV Drug Resistance Database.

Mutation	Treatment naïve			RTI treated			Chi-Square/ Fisher's
	Total no. of CN mutations	Total no. of CN sequences	% CN mutations	Total no. of CN mutations	Total no. of CN sequences	% CN mutations	
Q334D	164	1465	11	5	142	4	0.0024
G335D	1067	1322	81	57	102	56	0.0070
N348I	2	1191	0.2	9	62	15	<0.0001
A371V	35	1131	3	8	56	14	0.0006
A376S	50	1110	5	2	57	4	1.0000
A400T	607	993	61	20	30	67	0.5751

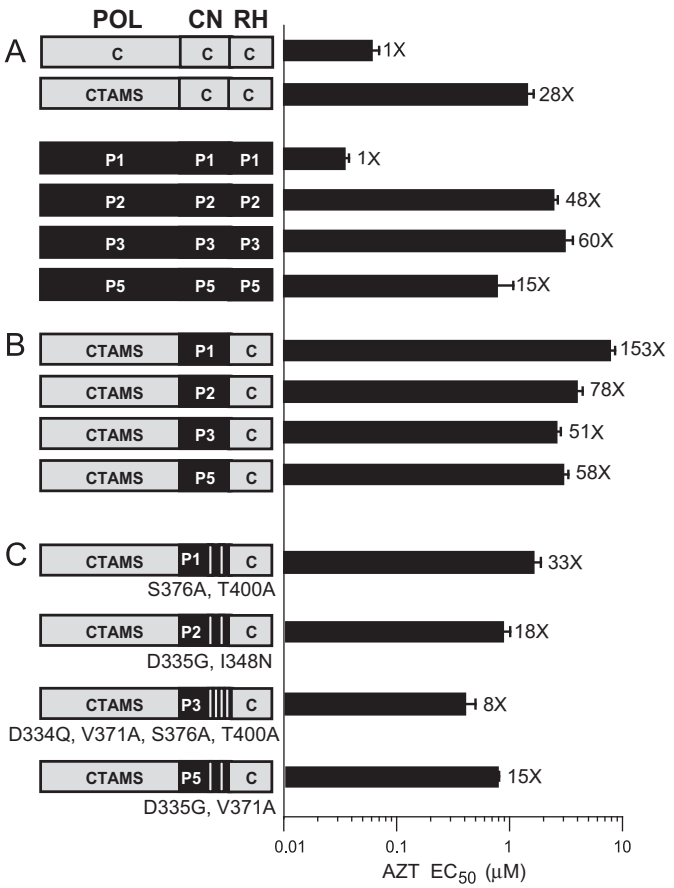

Fig. 3. AZT resistance associated with patient subtype C CNs. (A) AZT resistance associated with the entire RT isolated from three subtype-C-infected patients (P2, P3, P5) and one subtype B patient (P1) (black boxes). Wild-type subtype C AZT resistance in the presence or absence of TAMs serves as a reference (gray boxes). (B) AZT resistance associated with each patient's CN in the presence of a subtype C TAMs-containing POL. (C) AZT resistance levels associated with revertant mutations (indicated as thin gray lines with amino acid position below labeled with an "x") for each patient's CN in the presence of TAMs. Error bars represent the standard error of the mean of at least two replicates from three independent experiments.

each patient were subcloned into pHL[B-WT]. Fig. 3A shows the overall level of AZT resistance associated with the entire POL, CN and RH from each patient in reference to C-WT . For P1, the level of AZT resistance was 1× (EC_{50}=0.03 μM), likely due to the absence of TAMs in this patient's POL. P2 and P3 exhibited higher levels of AZT resistance (48× and 60×, respectively), while P5 exhibited similar levels of resistance (15×) to the subtype-C TAMs background (28×). The overall AZT resistance levels are a reflection of the unique combinations and interactions of mutations present in the POL, CN and RH of each patient.

Increase in AZT resistance associated with subtype C-infected treatment-experienced patients is localized to the CN

Since mutations in POL, especially L74V and M184V, can counteract AZT resistance levels (Boyer et al., 2002; Frankel et al., 2005; Gotte et al., 2000; Miranda et al., 2005), additional vectors were created in which the patient CNs were subcloned into pHL[C-TAMs]. This way the level of resistance associated with each patient CN could be evaluated in the context of the same POL genotype. We have previously shown that subtype-B and CRF01_AE mutations in the CN can increase AZT resistance above the TAMs background (Delviks-Frankenberry et al., 2009; Nikolenko et al., 2007). As shown in Fig. 3B, for patients P1, P2, P3 and P5, each CN was associated with increases in AZT resistance up to 153×, 78×, 51×, and 58×, respectively. This increase in resistance was above the C-TAMs background (28×), and shows that the mutations present in the subtype-C-infected patient CNs (P2, P3, P5) as well as subtype-B-infected patient CN (P1) exhibit enhanced resistance to AZT. Interestingly, patient P1 which showed no increase in AZT resistance with its entire RT, exhibited a 153× increase in AZT resistance associated with its CN. This result likely shows the power of the M184V mutation in POL to counteract AZT resistance, as previously reported (Boyer et al., 2002; Gotte et al., 2000; Nikolenko et al., 2007). However, the resistance profile for patient P1 is likely more complex and cannot be explained by the effect of M184V alone, as patients P2, P3 and P5 also contained M184V and exhibited increases in AZT resistance (Fig. 3B).

To verify whether the CN mutations were responsible for the increase in AZT resistance, CN revertant mutants were generated in which key amino acids previously associated with increased AZT resistance were replaced with amino acids not associated with resistance. These revertant mutants were generated for each patient's CN and tested for AZT resistance (Fig. 3C). The revertant mutants reduced AZT resistance levels on average 4- to 6-fold, back to levels similar to the C-TAMs background (28×). Fold changes in AZT resistance dropped from 153× to 33× for P1, 78× to 18× for P2, 51× to 8× for P3, and 58× to 15× for P5, suggesting that these CN mutations in the subtype C context do contribute to AZT resistance.

Amino acid T400 in subtype C contributes to AZT resistance

The role of the A400T polymorphism in subtype C was also investigated, as it was previously shown that this same CN polymorphism in CRF01_AE played a significant role in AZT resistance; the T400A revertant mutant reduced AZT resistance 11-fold in wild-type CRF01_AE (Delviks-Frankenberry et al., 2009). Fig. 4A shows that changing the T400 to alanine in the context of C-TAMs, reduced the level of AZT resistance 2.8-fold. When the subtype C CN was cloned into B-TAMs, converting the T400 to alanine resulted in a 1.8-fold reduction in AZT resistance (Fig. 4B), similar to that seen in the background of C-TAMs . No change was observed in a wild-type B background. Overall, this data suggests a small role for T400 in contributing to the overall drug resistance associated with C-TAMs . This was in agreement

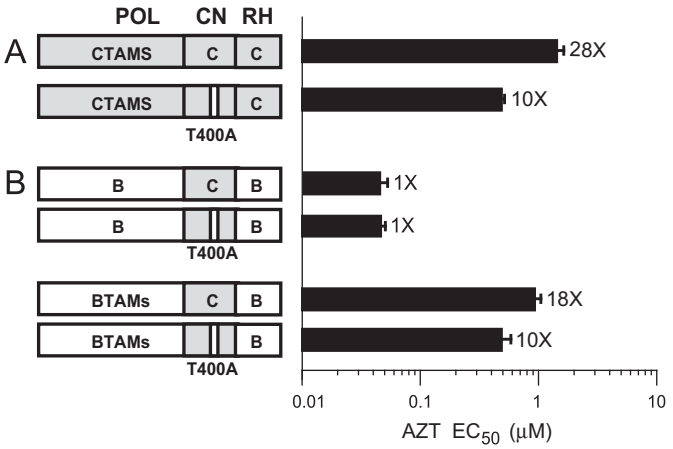

Fig. 4. AZT resistance associated with amino acid 400 in subtype C RT. Level of resistance associated with amino acid conversion T400A in (A) subtype C RT containing TAMs or (B) the subtype C CN in the background of wild-type or TAMs-containing subtype B. Increases in drug resistance are indicated relative to wild-type subtype B (one-fold, defined as 1×). Error bars represent the standard error of the mean of at least two replicates from three independent experiments.

	POL	CN	RH	NRTI AZT	d4T	3TC	TDF	NNRTI EFV	NVP	DLV	ETR
A	B	B	B	1	1	1	1	1	1	1	1
	C	C	C	1	1.1	0.9	0.8	0.9	0.6	1.1	0.8
	BTAMS	B	B	18	2.2	2.6	2.0	0.6	0.5	0.8	0.8
	CTAMS	C	C	28#	1.4	2.3	1.8	0.4	0.4	1.3#	0.4#
B	P1	P1	P1	1	0.8	>29	0.6	1.9	3.4	5.5	3.9
	P2	P2	P2	48	1.3	>29	2.7	0.5	1.3	1	0.4
	P3	P3	P3	60	1.8	>29	3.0	>6	>357	>6.5	>12
	P5	P5	P5	15	1.2	>29	1.3	>6	>71	>6.6	>12
C	CTAMS	C	C	28	1.4	2.3	1.8	0.4	0.4	1.3	0.4
	CTAMS	P1	C	153*	2.4*	1.7	5.2*	2.1*	0.3	2.6*	1.3*
	CTAMS	P2	C	78*	1.0	1.3	2.9*	0.7*	0.6	1.4	0.9*
	CTAMS	P3	C	51*	1.1	1.5	2.7	0.7	0.6	0.9	0.2
	CTAMS	P5	C	58*	1.8	7.2	1.7	0.9*	1.0	1.7	0.7

Fig. 5. NRTI and NNRTI resistance associated with patient-derived subtype C RTs and CNs. Shown are the NRTI and NNRTI drug resistance levels for (A) reference subtype-B and subtype-C RTs in the presence or absence of TAMs, (B) patient-derived subtype-C RTs and (C) CNs in the presence of a subtype C RT containing TAMs. Increases in drug resistance are indicated relative to wild-type subtype B (one-fold, defined as 1 ×). *, Significant fold increases (*t*-test: $P<0.05$) in drug resistance compared to subtype C-TAMs control. #, Significant fold increases in drug resistance compared to B-TAMs control.

with the observation that A400T has a similar prevalence in treatment-naïve and RTI treated subtype C-infected patients, 61% and 67%, respectively (Table 1).

Subtype-C CN mutations enhance resistance to other NRTIs and NNRTIs in the context of TAMs

The HIV-1 vectors containing subtype-C CNs were also tested for increases in drug resistance to NRTIs d4T, 3TC, and TDF, as well as NNRTIs EFV, NVP, DLV, and ETR. Fig. 5 summarizes the levels of drug resistance with all fold changes normalized to B-WT for reference (see Supplementary Fig. 1 for detailed EC_{50} data). For the entire RT (Fig. 5A), subtype B and subtype C in the presence or absence of TAMs had similar levels of drug resistance. The increase in AZT resistance associated with C-TAMs (28 ×) versus B-TAMs (18 ×) was statistically significant ($P=0.04$), as was resistance to DLV (0.8 × to 1.3 ×) and ETR (0.8 × to 0.4 ×) ($P=0.04$ and 0.02, respectively). The entire RTs obtained from subtype-C-infected patients (Fig. 5B) showed variable levels of resistance depending upon the NRTI and/or NNRTI drug mutation combinations present in their respective POL in addition to effects of CN and/or RH mutations. Fig. 5C shows the levels of drug resistance associated with each patient's CN in the presence of C-TAMs so that they can be evaluated in the context of the same POL genotype. P1, P2, P3, and P5 all showed significant increases in AZT drug resistance ($P<0.003$) compared to C-TAMs (data described previously in Fig. 3B). Even though P2 and P3 contain N348I and A369V, CN mutations previously shown to potently increase AZT resistance in a WT pol background (2–5 fold and 9 fold, respectively) (Gupta et al., 2010; Hachiya et al., 2008a; Hachiya et al., 2009; Lengruber et al., 2011; Yap et al., 2007), their resistance levels were still lower than P1 in the presence of TAMs. This suggests that it is hard to predict the phenotype based upon individual CN mutations and how they will interact with other mutations. Patient P1 also showed significant increases in d4T resistance, and P1 and P2 showed significant increases in TDF resistance ($P<0.05$). Interestingly, even in the presence of TAMs, small significant increases in NNRTI drug resistance were seen with P1, P2 and P5 for EFV ($P<0.04$), P1 for DLV ($P=0.04$), and P1 and P2 for ETR ($P<0.03$). Thus, CN mutations in a subtype-C genetic background also increased NNRTI resistance above the TAMs background.

CNs from subtype C enhance NNRTI drug resistance in the context of classical NNRTI resistance mutations

Since TAMs are not normally selected in response to NNRTIs, NNRTI resistance associated with each patient's CN was further evaluated by constructing vectors in which the patient's CN was paired with a subtype-C POL domain containing mutations that increase NNRTI resistance, specifically either L100I, K103N, Y181C, G190A, or L100I+K103N+Y181C (Fig. 6). These vectors were tested for NVP, DLV, EFV or ETR resistance compared to C-WT (see Supplementary Fig. 2 for detailed EC_{50} data). The mutations present in each patient's CN significantly enhanced resistance to NVP, DLV, and EFV in the context of these classical NNRTI resistance mutations. G190A is not normally associated with DLV resistance (Huang et al., 2003), so only small increases in resistance were observed with the addition of CNs from P2, P3 and P5 (2–3 ×); however P1 did exhibit a modest 6 × increase above reference background (C-G190A, 1.4 ×). For the most recently approved potent NNRTI ETR, the patient CNs were still able to enhance resistance in the background of single POL mutants, except for K103N which is not a resistance mutation for ETR (Johnson et al., 2010). P1 CN however was able to increase resistance 3-fold over the K103N background for ETR. Even more impressive are the results with the triple POL mutant containing L100I+K103N+Y181C: all four patients' CNs could still enhance resistance 6 to 25-fold above the background ETR resistance (40 ×). Overall, these results indicate that CN mutations from subtype-C-infected patients can contribute to both NRTI and NNRTI resistance.

Discussion

The role of CN mutations in contributing to overall virological failure remains under debate, and additional genotypic and phenotypic resistance studies with larger patient cohorts are needed (Brehm et al., 2012a; Brehm et al., 2011; Gupta et al., 2011; Hachiya et al., 2009; von Wyl et al., 2010a). In this report we examined the contribution of CN mutations to drug resistance from subtype-C-infected patients failing drug therapy. We confirm that the subtype-C-infected patients (P2, P3, P5), just like the subtype-B-infected patient (P1), acquired mutations in the CN of RT that can influence RTI resistance. The mutations observed with our subtype-C cohort, A376S, A400T, Q334D, G335D, N348I, and A371V, were similar to those previously reported for subtype B. No new CN mutations were identified for subtype C; however, even with the limited cohort size, six of the commonly recognized CN mutations were present amongst these patients. Stanford University HIV Drug Resistance Database analysis confirmed that three of the identified CN mutations, G335E, N348I and A371V were positively associated with subtype-C treatment-experienced patients. In agreement with these results, Brehm et al. recently reported that N348I was significantly increased in subtype-C-infected patients failing therapy, and was associated with enhanced resistance to EFV, NVP, ETR and AZT (Brehm et al., 2012a).

Overall, there was no inherent difference in resistance levels between wild-type subtype B and wild-type subtype C, indicating that their RTs have similar sensitivities to RT inhibitors. This is in agreement with in vitro biochemical data in which both subtype-B and -C RTs have been shown to have similar RT activity, drug susceptibility and RNase H cleavage (Xu et al., 2010). We also did

	POL CN RH	NVP	DLV	EFV	ETR
A	C C C	1*	1*	1*	1*
B	L100I C C	8#	291#	18#	1.4#
	L100I P1 C	**68**	**3352**	**91**	**6.6**
	L100I P2 C	**22**	**824**	**37**	**2.8**
	L100I P3 C	**28**	**521**	**64**	**3.0**
	L100I P5 C	**25**	**881**	**66**	**2.8**
C	K103N C C	85#	374#	38#	1.2#
	K103N P1 C	**2552**	**5558**	**>145**	**3.9**
	K103N P2 C	**252**	627	**63**	1.2
	K103N P3 C	**289**	674	**65**	1.2
	K103N P5 C	**201**	**802**	**70**	0.6
D	Y181C C C	81#	314#	1.6#	1.5#
	Y181C P1 C	**4894**	**4300**	**13**	**9.3**
	Y181C P2 C	**716**	497	**3.5**	2.6
	Y181C P3 C	**751**	**446**	**3.8**	1.6
	Y181C P5 C	**802**	**959**	**5**	**7.3**
E	G190A C C	80#	1.4#	16#	0.7#
	G190A P1 C	**4232**	**>8**	**>73**	**4.9**
	G190A P2 C	**370**	2.4	**39**	**1.4**
	G190A P3 C	**606**	2.5	**43**	1.4
	G190A P5 C	**897**	3.5	**54**	**1.9**
F	100/103/181 C C	335#	4379#	>636 #	40#
	100/103/181 P1 C	**>10,909**	**12344**	**>636**	**1018**
	100/103/181 P2 C	**4047**	**7967**	**>636**	**227**
	100/103/181 P3 C	**3070**	**8655**	**>636**	**352**
	100/103/181 P5 C	**2072**	**10036**	**>636**	**421**

Fig. 6. NNRTI drug resistance associated with patient-derived CNs from subtype C. (A) Increases in NNRTI drug resistance are reported relative to the wild-type subtype C for each NNRTI (*, one-fold, defined as 1 ×). NVP, DLV, EFV and ETR drug resistance levels for each patient's CN in the background of a NNRTI-containing POL are indicated: (B) L100I, (C) K103N, (D) Y181C, (E) G190A, and (F) L100I+K103N+Y181C. Significant increases in drug resistance ($P < 0.05$) for each patient's CN relative to the baseline vector containing the NNRTI POL mutation(s) with a wildtype subtype C CN (#), are shown bold for each drug.

not observe a difference in RNase H cleavage activity between wild-type subtype B and C (data not shown).

As observed with subtype B, the CN mutations in subtype C increased RTI drug resistance levels. In POL backgrounds containing similar TAMs or classical NNRTI mutations, the CN from each patient in general increased AZT and NNRTI resistance levels. The contribution of the identified CN mutations to drug resistance was further confirmed by reversion mutation experiments, which decreased the levels of AZT resistance up to six fold. Interestingly, the mutations in the patients' CNs were also able to increase resistance to ETR, in a POL containing three NNRTI resistance mutations. We have previously shown that CN mutations in subtype B exhibit dual resistance to NRTIs and NNRTIs especially in the context of a POL containing NNRTI mutations (Nikolenko et al., 2010). These results are also in agreement with Gupta et al. which showed enhanced ETR resistance with CN mutations T369I and N348I in the presence of specific NNRTI-containing POL domains (Gupta et al., 2011). Brehm et al. confirmed enhanced ETR resistance for N348I with NNRTI-containing POLs (Brehm et al., 2012a). Furthermore, CN mutation E399D was also found to be associated with ETR resistance (Poveda et al., 2008), and T386A CN mutation was selected for ETR resistance during in vitro selection studies (Vingerhoets et al., 2005). Recently, McCormick et al. showed that N348I increases resistance across different subtypes and increases ETV (~2 fold), NVP (~5 fold) and EFV (~3–5 fold) resistance (McCormick et al., 2011), Furthermore, it was shown that N348I may be selected in the presence of NVP or EFV drug therapy (Price et al., 2010). These studies highlight the importance of CN mutations in clinical NNRTI resistance.

Conclusions

Overall, these data show that subtype-C acquires mutations in the CN of RT that contribute to both NRTI and NNRTI resistance. With growing numbers of patients infected with subtype C receiving ART, it is becoming increasingly important to understand the baseline polymorphisms in the non-B subtypes that contribute to drug resistance, and the pattern and emergence of drug resistance mutations in response to therapy. Reports of drug resistance mutations and patterns in non-B subtypes (reviewed in (Martinez-Cajas et al., 2009; Martinez-Cajas et al., 2008)) still do not include mutations downstream of POL in RT. Our studies show that CN mutations can enhance RTI resistance in subtypes B and C, implying that perhaps CN mutations contribute to enhanced RTI resistance in all subtypes. Therefore, inclusion of CN mutations in resistance testing is likely to facilitate design of drug regimens that are specifically directed to the individual patients' viral genotypes. Furthermore, identification of unique CN mutations will also be useful for novel RT drug design and understanding of the biochemical mechanisms of RT resistance to antiviral drugs.

Material and methods

Patient samples

Plasma samples from seven treatment-experienced HIV-positive patients regularly followed at the HIV/AIDS Unit of Universidade Federal de Rio Grande, Southern Brazil, were collected. All patients signed a written consent to participate in the study, and the Institutional Ethics Committee (CEPAS) approved the study. Results from single genome sequencing showed three patients to contain no RTI mutations in the POL domain relative to wild-type subtype C (MJ4, GenBank AF321523), and therefore were excluded from these studies as their RT represented a wild-type genotype. The majority genotypes from the remaining four patients (P1, P2, P3, and P5) were used in this study. Viral loads for P1, P2, and P3 were 4789, 1925, and 153,453 copies/ml, respectively. Viral load for P5 was not determined. P1 received AZT, 3TC and NFV treatment. P2 received AZT, 3TC, NFV/TDF and 3TC+ATV/R treatment. P3 received AZT, 3TC, TDF and EFV treatment. P5 received AZT, 3TC and EFV treatment.

Plasmids, cloning, and mutagenesis

pHCMV-G expresses the G glycoprotein of vesicular stomatitis virus (VSV-G) (Yee et al., 1994). Construction of vector pHL[WT] was previously described (Nikolenko et al., 2007) and will be referred to as pHL[B-WT] in this paper to specify that it is a wild-type subtype B genotype. pHL[C-WT] contains the POL, CN, and

RH from wild-type subtype C subcloned into pHL[B-WT] (Fig. 2A). All three constructs express the firefly luciferase reporter gene and all of the HIV-1 proteins except Nef and Env. Vectors pHL[B-TAMs] and pHL[C-TAMs] were created by introducing NRTI resistance mutations D67N, K70R, T215Y/F, and K219Q into pHL[B-WT] and pHL[C-WT], respectively (Fig. 2B and C). Site-directed mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and the presence or absence of each mutation was verified by DNA sequencing.

Single genome sequencing

Viral RNA was extracted from patient plasma samples and used for single genome sequencing as previously described (Kearney et al., 2008). Primers were modified to specifically amplify the entire subtype C RT. Round 1 PCR primers were ExtC for 5′–AAAGCCAGGAATGGATGGCCC-3′ and ExtCrev 5′–CTCACTAGCCATTGCTCTCC-3′. Round 2 nested PCR primers were Msc1-1for 5′–CCCAAAAGTTAAACAATGGCCATTGAC-3′ and ClaRev 5′–CTTCTTGGGCCTTATCGATTCC-3′, which contained unique *Msc* I and *Cla* I restriction sites, respectively, to use for cloning into pHL[WT]. Positive PCR reactions were subcloned and sequenced and the majority genotype from each patient was used for cloning and drug resistance analysis.

Antiviral drugs

AZT and 2′,3′-didehydro-3′-dideoxythymidine (d4T) were obtained from Sigma-Aldrich . Inhibitors nevirapine (NVP), efavirenz (EFV) and etravirine (ETR) and tenofovir (TDF) were obtained from the NIH AIDS Research & Reference Reagent Program. Delavirdine (DLV) and 2′,3′-dideoxy-3′-thiacytidine (3TC) were purchased from Movarek Biochemicals.

Cells, transfection, virus production, and drug susceptibility assays

Human embryonic kidney 293T cells (American Type Culture Collection) were maintained at 5% CO_2 and 37 °C in Dulbecco's modified Eagle's medium (CellGro) supplemented with 10% FCS (HyClone), penicillin (50 U/ml; Gibco) and streptomycin (50 μg/ml; Gibco). To produce virus, 293T cells were transfected with each vector using calcium phosphate precipitation (Clontech) in the presence of pHCMV-G . Virus was harvested 48-h later, spun to remove cellular debris, filtered through a Millex GS 0.45 μm-pore-size filter (Nalgene), and concentrated 25-fold by ultracentrifugation. Single-cycle drug susceptibility assays were performed as previously described (Nikolenko et al., 2005). Final drug EC_{50} data represents the standard error of the mean of at least two replicates from three independent experiments.

Statistical analysis

Student's *t*-test was used to determine statistically significant differences for EC_{50} data (SIGMAPLOT 8.0 software). Chi-Square and Fisher's exact tests were performed using statistical tools freely available online (http://www.quantitativeskills.com/sisa/statistics/).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.09.021.

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