Session 2 Abstracts

CONTINUING MEDICAL EDUCATION

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CONTENTS

page	session	title and author	abstract
S31	**Session 2: Mechanisms of HIV Drug Resistance**		
S33		Mechanisms involved in zidovudine hypersusceptibility in the presence of foscarnet resistance-conferring mutations *M Götte*	28
S34		HIV-1 reverse transcriptase mutations that suppress zidovudine resistance also increase *in vitro* susceptibility to tenofovir, but not stavudine *NT Parkin*	29
S35		The Δ67 complex of mutations enhances the ability of HIV-1 reverse transcriptase to excise zidovudine, stavudine and PMPA from blocked primers *SH Hughes*	30
S36		The 3′-azido group is not the primary structural determinant for the excision phenotype correlated with HIV-1 resistance to AZT *N Sluis-Cremer*	31
S37		Evolution of amino acid 215 in HIV-1 reverse transcriptase in response to intermittent drug selection *C Chappey*	32
S38		Molecular mechanisms of resistance to tenofovir by HIV-1 reverse transcriptase dontaining a di-serine insertion after residue 69 and multiple thymidine analogue-associated mutations *MD Miller*	33
S39		Drug resistance and viral fitness at the molecular level: the case of tenofovir *B Canard*	34
S40		Molecular mechanism for the mutual exclusion of K65R and L74V substitutions in HIV-1 reverse transcriptase-mediated dideoxynucleoside resistance *J Deval*	35
S41		Mechanism of anti-HIV activity of dioxolane nucleosides against lamivudine-resistant HIV-1 reverse transcriptase-molecular modelling approach *CK Chu*	36
S42		Prevalence and quantitative phenotypic resistance patterns of specific nucleoside analogue mutation combinations and of mutations 44 and 118 in reverse transcriptase in a large dataset of recent HIV-1 clinical isolates *M Van Houtte*	37

S43	Removal of chain-terminating nucleoside analogues by HIV-1 reverse transcriptase utilizing intracellular substrates *AJ Smith*	38
S44	Colinearity of reverse transcriptase inhibitor resistance mutations detected by population-based sequencing *MJ Gonzales*	39
S45	Identification of the minimal conserved structure of the HIV reverse transcriptase under the presence and absence of drug pressure *F Ceccherini-Silberstein*	40
S46	Identification of a clinical reverse transcriptase backbone that improves replication of a non-nucleoside reverse transcriptase inhibitor-resistant mutant by increasing the rate of polymerization *LM Demeter*	41
S47	Non-nucleoside reverse transcriptase inhibitor hypersusceptibility can be demonstrated in multicycle phenotype assays and in inhibition assays of purified HIV-1 reverse transcriptases *NS Shulman*	42
S48	Genetic correlates of phenotypic hypersusceptibility to efavirenz among 446 baseline isolates from five ACTG studies *NS Shulman*	43
S49	Rare 1 and 2 amino acid insertions in the non-nucleoside reverse transcriptase inhibitor (NNRTI) binding pocket of HIV-1 reverse transcriptase affect NNRTI susceptibility *MA Winters*	44
S50	Crystal structure of a multidrug-resistant HIV-1 protease clinical isolate reveals an expanded active site cavity and represents a novel target for the design of protease inhibitors *LC Kovari*	45
S51	Structural correlates of broad-spectrum activity for a resistance-repellent HIV protease inhibitor *AM Silva*	46
S52	Co-evolution of the nucleocapsid-p1 cleavage site with the V82A mutation in HIV-1 protease preserves substrate recognition *CA Schiffer*	47
S53	Preliminary characterization of a newly described protease substrate cleft mutation at position 23 *RW Shafer*	48
S54	Emergence of a novel lopinavir resistance mutation at codon 47 correlates with ARV utilization *RM Kagan*	49

S55	Parameters driving the selection of nelfinavir-resistant HIV-1 variants *F Mammano*	50
S56	I84A and I84C mutations in protease confer high-level resistance to protease inhibitors and impair replication capacity *H Mo*	51
S57	The HIV-1 protease mutation K55R is associated with the presence of the M46I/L mutation *E Morgan*	52
S58	Nucleic acid differences between HIV-1 non-B and B reverse transcriptase and protease sequences at drug resistance positions *R Kantor*	53
S59	Search for polymorphic sites in R5 tropic HIV-1 Env and enfuvirtide drug susceptibility in baseline isolates from TORO 1 & TORO 2 *C Su*	54
S60	Subgroup analysis of baseline susceptibility and early virological response to enfuvirtide in the combined TORO studies *P Sista*	55

SESSION 2
Mechanisms of HIV Drug Resistance

ABSTRACT 28

Mechanisms involved in zidovudine hypersusceptibility in the presence of foscarnet resistance-conferring mutations

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BACKGROUND: Phosphonoformate (PFA, foscarnet) binds at or in close proximity to the nucleotide binding site of HIV-1 reverse transcriptase (RT) and inhibits DNA synthesis presumably through interference with the exchange of pyrophosphate. Like other PFA-resistance conferring mutations, E89K is distal from the dNTP binding site and interacts with the template strand. This mutation is also associated with increased susceptibility to zidovudine (ZDV). We hypothesized that this effect might be attributable to alterations with regard to the precise alignment of ATP, that acts as a pyrophosphate donor and the primer/template substrate.

METHODS: To address this problem, we developed novel site-specific footprinting techniques that allowed us to monitor the position of RT on its template at single nucleotide resolution. We used different sources of hydroxyl radicals that promote site-specific cleavage on the bound template, and compared the cleavage patterns between wild-type RT and the mutant enzyme under different reaction conditions.

RESULTS: Wild-type RT promotes cleavage at positions –8 and –18 in the absence of the incoming dNTP, when using a substrate that contained a ZDV-terminated primer. This is the configuration that allows excision of the incorporated ZDV-monophosphate. The presence of the next dNTP forces the enzyme to translocate a single position further downstream, as evidenced by cleavage at positions –7 and –17. The presence of PFA diminishes the translocation of RT, which provides a novel mechanism for drug action. The E89K mutation appears to alter the relative position of RT. The mutant promotes cleavage at positions –9 and –19 in the absence of the incoming dNTP. Excision of ZDV cannot occur in this configuration, which helps to explain earlier findings that pointed toward diminished rates of primer unblocking associated with resistance to PFA. Moreover, relatively high concentrations of the next nucleotide are required to force the translocation of RT.

CONCLUSIONS: Enzymes containing PFA resistance conferring mutations alter the precise positioning of RT on its nucleic acid substrate. Such displacement diminishes the unblocking of ZDV-terminated primer strands. The high concentrations of dNTPs that are required to force translocation also helps to explain the diminished rates of DNA synthesis. These parameters may directly correlate with ZDV resensitization effects and diminished viral replication fitness associated with viruses that contain the E89K mutation.

ABSTRACT 29

HIV-1 reverse transcriptase mutations that suppress zidovudine resistance also increase *in vitro* susceptibility to tenofovir, but not stavudine

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BACKGROUND: Susceptibility of HIV-1 to zidovudine is increased by at least five mutations in reverse transcriptase (RT): K65R, L74V, L100I, Y181C and M184V. In some cases these ‘suppressive mutations’ restore susceptibility to zidovudine despite of the presence of resistance mutations such as K70R or T215F/Y. In general, susceptibility to stavudine and tenofovir is modulated by RT resistance mutations in a manner that qualitatively parallels that of zidovudine. Thus, we evaluated the effect of suppressive mutations on susceptibility to stavudine and tenofovir.

METHODS: A clinical sample database of over 16 000 matched genotypes and phenotypes was queried for samples containing T215F or Y without, or with one or more, suppressive mutations (K65R, L74I and V, L100I, Y181C, I, and V, and M184I and V; different variants at each position were grouped together). Samples containing multi-nucleoside RT inhibitor (NRTI) resistance mutations (T69ins or Q151M) were excluded, as were samples with mixtures at positions that were part of the query. NRTI fold change (FC) in IC_{50} vs NL4-3 reference was compared between groups of viruses using the Mann-Whitney non-parametric test.

RESULTS: Median zidovudine FC for T215Y/F samples with no suppressive mutations (n=966), L74I/V alone (that is, no other suppressive mutations, n=87), L100I alone (n=54), Y181I/C/V alone (n=283) or M184I/V alone (n=1423) was 145-, 102-, 78-, 75- and 12-fold, respectively (P<0.05 for each suppressive mutation group vs no suppressive mutations). The corresponding FC values for tenofovir were 2.8-, 2.2-, 2.1-, 2.4- and 1.3-fold, respectively (all P<0.05), and for stavudine were 2.4-, 3.3-, 2.5-, 2.8- and 1.8-fold, respectively (all P<0.05 except L100I). The number of samples with K65R and T215F/Y was too low to provide meaningful comparisons. Combinations of two or more suppressive mutations were generally additive in suppressing zidovudine and tenofovir resistance. For example, median zidovudine and tenofovir FC for samples with L100I+M184IV (n=28) were 3.8- and 0.8-fold, and for Y181I/C/V+M184I/V (n=174) were 10- and 1.1-fold, respectively. Lower FC in groups with suppressive mutations could not be explained by fewer thymidine analogue mutations (median number 3 to 4 for all groups). As expected, FC for didanosine, zalcitabine (ddC) and abacavir was higher in groups containing L74I/V and/or M184I/V, and was not significantly affected by L100I or Y181C/I/V.

CONCLUSIONS: M184I/V increases susceptibility to zidovudine, tenofovir and stavudine. Other suppressive mutations in RT affect tenofovir and zidovudine, but not stavudine. Susceptibility to stavudine decreased in the presence of L74I/V and Y181I/C/V. Additive effects were observed when suppressive mutations were present together. Combined mutations were capable of re-sensitizing tenofovir (FC<1.4) and zidovudine (FC<2.5) in the presence of multiple thymidine analogue mutations. Since genotype interpretation algorithms do not account for the effects of most suppressive mutations, these observations provide an explanation for phenotype/genotype discordance for zidovudine and tenofovir.

ABSTRACT 30

The Δ67 complex of mutations enhances the ability of HIV-1 reverse transcriptase to excise zidovudine, stavudine and PMPA from blocked primers

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BACKGROUND: Most patients that receive anti-HIV-1 therapy are treated with drug combinations. Many patients are treated with multiple nucleoside analogues (NRTIs). Viruses that are resistant to multiple NRTIs are an increasing problem. Insertions in the fingers of reverse transcriptase (RT) (most often between amino acids 69 and 70), in combination with T215F/Y, causes enhanced excision of a number of NRTIs. The Δ67 complex (41L/Δ67/T69G/K70R/L74I/K103N/T215Y/K219Q) has been reported to cause resistance to a number of NRTIs and non-nucleoside inhibitors. We asked whether RT carrying the Δ67 complex mutations could efficiently excise NRTIs.

METHODS: We purified recombinant wild-type HIV-1 RT, RT carrying the Δ67 complex mutations and RT carrying the classical zidovudine (AZT) resistance mutations. These RTs were used to investigate the mechanism(s) of NRTI resistance of the Δ67 complex RT.

RESULTS: The Δ67 complex RT was able to excise AZT, stavudine and PMPA more efficiently than either wild-type RT or AZT-resistant RT. Both the Δ67 complex and the AZT-resistant RT were able to excise PMPA much more efficiently than ddA. In addition, the Δ67 complex RT was able to excise AZT at much lower concentrations of ATP than AZT-resistant RT.

CONCLUSIONS: It would appear that PMPA is relatively susceptible to excision by RTs that carry the classical AZT resistance mutations. PMPA excision is enhanced by RTs carrying the mutations in the Δ67 complex. The ability of RT carrying the Δ67 complex mutations to efficiently excise AZT at low ATP concentrations might provide an advantage for the virus in quiescent cells, where ATP levels are expected to be low. The data suggest that one or more of the Δ67, T69G, L74I or K103N mutations contribute to the ability of the mutant RT to excise AZT at low ATP concentrations.

ABSTRACT 31

The 3′-azido group is not the primary structural determinant for the excision phenotype correlated with HIV-1 resistance to AZT

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BACKGROUND: The phenotypic mechanism of HIV-1 resistance to 3′-azido-2′,3′-dideoxythymidie (AZT) has been proposed to involve the removal or excision of the incorporated chain-terminating AZT molecule by a reverse transcriptase (RT)-mediated enzymatic reaction termed phosphorolysis. Previous studies have indicated that primers terminated with AZT-monophosphate (AZT-MP) are better substrates for this reaction than those terminated with 2′-3′-dideoxynucleoside monophosphate (2′,3′-ddNMP) analogs that lack a 3′-azido moiety. This has lead to the suggestion that the 3′-azido group may be a major structural determinant for maintaining the primer terminus in the appropriate site to allow phosphorolytic excision by AZT-resistant RT. We tested this possibility by carrying out detailed biochemical and virological evaluations of the incorporation, phosphorolytic excision and antiviral properties of a panel of 3′-azido-2′,3′-ddN compounds including 3′-azido-2′,3′-ddA, 3′-azido-2′,3′-ddC, 3′-azido-2′,3′-ddG, 3′-azido-2′,3′-ddU and AZT.

METHODS: Steady-state and pre-steady-state kinetic parameters for the incorporation and ATP-mediated excision of the 3′-azido-2′,3′-ddNTPs were determined for purified wild-type (wt) RT and D67N/K70R/T215F/K219Q AZT-resistant RT. Antiviral activities of the 3′-azido-2′,3′-ddN nucleosides were evaluated in MT2 cells using WT or D67N/K70R/T215Y/K219Q AZT-resistant virus.

RESULTS: Each of the 3′-azido-2′,3′-ddNTPs was an excellent substrate for DNA-dependent single nucleotide incorporation reactions catalysed by wt and AZT-resistant RT. The relative catalytic efficiencies of incorporation (k_{pol}/K_d) were 3′-azido-2′,3′-ddA > AZT > 3′-azido-2′,3′-ddC, with no differences noted between wt and AZT-resistant RT. In contrast to the incorporation data, significant differences in the rates of ATP-mediated phosphorolytic excision of the various 3′-azido-2′,3′-ddNMP were noted. The relative rates of ATP-mediated phosphorolysis by wt and AZT-resistant RT (in the absence of dNTP) were 3′-azido-2′,3′-ddA>AZT>3′-azido-2′,3′-ddC>3′-azido-2′,3′-ddG. However, under reaction conditions that enabled multiple rounds of 3′-azido-2′,3′-ddNMP incorporation and excision, the AZT-resistant RT enzyme was 15-fold more efficient at forming full length DNA products in the presence of AZTTP than the wt enzyme. In contrast, only a threefold difference between mutant and wt RT was noted for reactions in the presence of 3-azido-2′,3′-ddCTP, and no differences between the two enzymes were noted for reactions in the presence of 3′-azido-2′,3′-ddATP and 3′-azido-2′,3′-ddGTP. The antiviral activities of the various 3′-azido-2′,3′-ddN were consistent with the enzymatic data, in that AZT-resistant virus was 10-fold resistant to AZT and fourfold resistant to 3′-azido-2′,3′-ddC, but was not cross-resistant to either 3′-azido-2′,3′-ddA or 3′-azido-2′,3′-ddG.

CONCLUSIONS: AZT resistance mutations do not confer significant cross resistance of RT or virus to other nucleosides having a 3′-azido group. Furthermore, the presence of a 3′-azido group on the 3′-terminal nucleotide of the primer does not enhance phosphorolytic excision by AZT-resistant RT *in vitro*, suggesting that other structural factors must play a role in defining the specificity of the excision phenotype arising from mutations correlated with AZT-resistance.

ABSTRACT 32

Evolution of amino acid 215 in HIV-1 reverse transcriptase in response to intermittent drug selection

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BACKGROUND: HIV-1 reverse transcriptase (RT) amino acid position 215 is monomorphic for threonine (T) in the 'wild-type' virus population. Thymidine analogues such as zidovudine and stavudine select for drug resistant variants containing tyrosine (215Y) or phenylalanine (215F). Two nucleotide changes are required to substitute T by Y or F. Intermediate alleles containing single 'forward' mutations encoding for 215N/S/I are drug-sensitive. 215N/S/I variants can also appear as the result of single revertant mutations of T215Y/F when drug pressure is interrupted. Variants 215D/V/C/H/L generated by single revertant mutations of T215Y/F preserve the two 'forward' nucleotide changes from 215T. Our objective is to evaluate the emergence of T215 variants in (a) a large sequence database compiled from HIV-1 isolates submitted for routine antiretroviral drug resistance testing and (b) a small cohort of HIV-1-infected patients that participated in a structured treatment interruption study.

METHODS: A database of matched genotypes and phenotypes from over 1000 clinical longitudinal samples gathered from 1999 to 2003 was queried for samples containing T215Y or F (not mixed) at the first time point. Correlation between the presence of T215Y or F mutation at the first and the 215-mutant at the second time point was tested using logistic regression. Mean replication capacity (RC) of each 215 substitution was calculated from protease wild-type samples. Ten longitudinal plasma samples were part of a supervised structured treatment interruption study (Deeks *et al.*, *New England Journal of Medicine* 2001).

RESULTS: Fifty-five longitudinal pairs of viruses were identified with T215Y (*n*=44) or T215F (*n*=11) at the first time point. Twenty-two of these viruses (20 T215Y and 2 T215F) showed complete replacement by the wild-type 215T virus at the second time point. Five viruses showed complete replacement by revertant mutants: 215Y by S, D, and C, 215F by S and V. Two of these five viruses showed a complete reversion to phenotypic susceptibility to all drugs and loss of drug-selected mutations at other positions in RT and protease, strongly suggesting an outgrowth of archival drug-sensitive viruses carrying a revertant codon at position 215. Twenty-eight viruses (21 T215Y and 7 T215F) showed complex mixtures of position 215. Mean RC was high for 215T, S, D, C (100, 120, 102, 98 respectively), and lower for 215V, L, I (62, 61, 40 respectively).

CONCLUSIONS: T215 revertants are common in HIV-1 patients. T215Y and F revert preferably to 215-codons that are one nucleotide different (Y215S/D/C, F215S/V). The relationship between the variant at the first time point and at the second time point supports the model that the RC of the various variants determines their relative presence in the archived virus pool and their subsequent emergence in the absence of selective pressure.

ABSTRACT 33

Molecular mechanisms of resistance to tenofovir by HIV-1 reverse transcriptase containing a di-serine insertion after residue 69 and multiple thymidine analogue-associated mutations

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BACKGROUND: The insertion of two amino acids after residue 69 of HIV-1 reverse transcriptase (RT) is a rare mutation that may develop in viruses containing multiple thymidine analogue-associated mutations (TAMs) and confers high-level resistance to all currently approved chain-terminating nucleoside and nucleotide RT inhibitors (NRTIs). Decreased incorporation and increased excision are two known mechanisms of NRTI resistance. Excision for many NRTIs may be inhibited by binding of the nucleotide complementary to the next position in the template (next nt) to form a stable dead-end complex where the chain-terminator is protected from excision. The mechanism of resistance to tenofovir for RT with an insertion mutation and multiple TAMs was examined in this study.

METHODS: A patient-derived HIV-1 virus (FS-SSS) was obtained that contained the SS insertion after residue 69 in a background of additional resistance mutations M41L, T69S, L74V, L210W and T215Y. The insertion and T69S were reverted by site-directed mutagenesis in a second virus (FS) that retained the other resistance mutations. *In vitro* drug susceptibility was determined by the PhenoSense assay. Incorporation of tenofovir was examined by measuring steady-state kinetic constants for wild-type and mutant RTs. ATP-mediated excision with or without the next nt was measured. Molecular models and molecular dynamics simulations were produced using Sybyl software.

RESULTS: The multiple TAM-containing FS virus exhibited a large reduction in zidovudine susceptibility and smaller reductions in susceptibility to other NRTIs including tenofovir; the FS-SSS virus showed greater reductions in susceptibility to all NRTIs including tenofovir. The relative binding/incorporation of tenofovir diphosphate was slightly decreased for FS-SSS RT (2.8-fold) compared to wild-type, but not significantly for FS RT (1.7-fold). However, significant ATP-mediated excision of tenofovir was detected for both mutant RTs with FS-SSS > FS > wild-type, and excision rates of 3.5, 1.8 and 0.8% per min, respectively. The presence of physiological concentrations of the next nt inhibited tenofovir excision by wild-type, slightly inhibited excision by FS, whereas excision by FS-SSS remained high (12-fold greater than wild-type). Computer modelling shows that the insertion mutation could generate a more flexible β3-β4 fingers loop domain that would enhance excision by facilitating dissociation of the ATP-mediated excision product and/or destabilizing the protective next nt complex.

CONCLUSIONS: Increased ATP-mediated excision of incorporated tenofovir without efficient inhibition by the next nucleotide appears to be the primary mechanism of tenofovir resistance for HIV-1 RT with T69 insertion mutations and multiple TAMs. Decreased binding/incorporation of tenofovir also makes a minor contribution to tenofovir resistance. The same TAMs without the insertion mutation showed detectable, but lower levels of excision and greater inhibition by the next nucleotide. Increased flexibility of the β3-β4 loop by the insertion mutation may be the basis for the high-level and broad NRTI cross resistance caused by the T69 insertion mutations.

ABSTRACT 34

Drug resistance and viral fitness at the molecular level: the case of tenofovir

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BACKGROUND AND METHODS: The selection of highly resistant HIV-1 mutant by tenofovir disoproxil fumarate (TDF), the most recently approved antiretroviral drug, is unfrequent. An integrated study of resistance to tenofovir and its active metabolites was performed using infected cell, biochemical assays, and 3D-structure modelization to determine resistance mechanisms and their impact on viral fitness.

RESULTS: K65R, the most significant tenofovir resistance variant in reverse transcriptase (RT) to date shows only a limited resistance to the active intracellular metabolite of tenofovir, TFV-DP (4.4-fold resistance), as judged by pre-steady state analysis. This resistance is due to a decrease in k_{pol}(TFV-DP): from 7 s^{-1} for wild-type RT to 0.32 s^{-1} for K65R RT. On the other hand, M184V RT, the lamivudine (3TC)-resistant mutant, displays a 2.5-fold increase in susceptibility to TFV-DP. When the two mutations are combined, the susceptibility of K65R/M184V RT to TFV-DP is similar to that of wild-type RT. Molecular modelling studies show that the methyl group of tenofovir makes a favourable Van der Waals interaction with valine 184, explaining TFV-DP resensitization with M184V. Cell culture susceptibility data corroborate the observation of resensitization as the K65R/M184V HIV double mutant shows only 1.7-fold reduced susceptibility to tenofovir. Interestingly, the decrease in resistance to tenofovir with the double mutant is associated with a fivefold decrease in the affinity of K65R/M184V RT for the natural nucleotide substrate dATP. The incorporation of all four natural nucleotides was thus investigated. We observed that each of them was poorly incorporated by K65R/M184V RT, with a 27% global incorporation efficiency as compared to wild-type RT. The M184V substitution was found to contribute to the diminished binding of the natural nucleotides to RT, whereas K65R affects catalysis generally. *In vitro* replication capacity assays using recombinant HIV also show that K65R/M184V is less fit than wild-type HIV, in agreement with our enzymatic data.

CONCLUSIONS: Our data describe at the molecular level both how a resistant virus is unable to resist to two drugs simultaneously, and for the first time, how viral fitness of a resistant virus is directly linked to its decreased ability to use natural nucleotide substrates. All together, these data predict a benefit for the combination of tenofovir DF with 3TC, as well as open new avenues in how to drive resistant virus to reduced viral fitness.

ABSTRACT 35

Molecular mechanism for the mutual exclusion of K65R and L74V substitutions in HIV-1 reverse transcriptase-mediated dideoxynucleoside resistance

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BACKGROUND: Either K65R or L74V in human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) confer resistance to 2′,3′-dideoxyinosine (ddI) *in vivo*. The two substitutions never occur together, while L74V is frequently found in patients receiving ddI and K65R is not.

METHODS: This integrated study involves *in vitro* infection assays with recombinant viruses, combined to a molecular and structural approach based on enzymatic assays with purified RTs. We investigated drug resistance and replication capacity with both methods.

RESULTS: Recombinant viruses carrying K65R and K65R/L74V display the same resistance level to ddI (about 9.5-fold) relative to wild-type. Consistent with this result, purified HIV-1 RT carrying K65R RT or K65R/L74V substitutions exhibits an eightfold resistance to ddATP. Resistance is due to a selective decrease of the catalytic rate constant k_{pol}: 22-fold (from 7.2 to 0.33 s^{-1}) for K65R RT and 84-fold (from 7.2 to 0.086 s^{-1}) for K65R/L74V RT. However, the K65R/L74V virus replication capacity (viral fitness) is severely impaired relative to that of wild-type virus. This loss of viral fitness is due to a poor ability of K65R/L74V RT to use natural nucleotides relative to wild-type RT: 15% that of wild-type RT for dATP, 36% for dGTP, 50% for dTTP, and 25% for dCTP. The order of incorporation efficiency is wild-type RT>L74V RT>K65R RT>K65R/L74V RT. Processivity of DNA synthesis, however, remains unaffected.

CONCLUSION: These results explain why the two mutations K65R and L74V do not combine in the clinic, and give a mechanism for a decreased viral fitness at the molecular level. This study gives rational support to the benefit in combining mutations that impair viral replication.

ABSTRACT 36

Mechanism of anti-HIV activity of dioxolane nucleosides against lamivudine-resistant HIV-1 reverse transcriptase-molecular modelling approach

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BACKGROUND: The development of viral resistance to lamivudine (3TC) prompted the discovery of nucleosides with activity against HIV isolates containing the 3TC resistance mutation. Since the finding that β–D-dioxolane-2,6-diaminopurine (DAPD or amdoxovir) is active against zidovudine (AZT)- as well as 3TC-resistant mutants, several nucleosides with a dioxolane moiety have been synthesized. Among the series of dioxolane nucleosides, the thymidine and 5-fluorocytidine analogues showed potent anti-HIV activity against 3TC-resistant mutant reverse transcriptase. Thus, it is of great interest to understand the role of dioxolane moiety in the anti-HIV activity against 3TC-resistant mutant.

METHODS: Various D-dioxolane nucleoside triphosphates and 3TC triphosphate were docked into the active site of wild-type as well as 3TC-resistant reverse transcriptase, and the resulting complexes were energy-minimized. The M184V mutation imposes steric hindrances to the incoming nucleoside triphosphates as well as the nearby primer chain. Therefore, if the nucleoside triphosphate–reverse transcriptase complex cannot provide enough conformational flexibility to escape from the steric hindrance, it results in an abortive binding state.

RESULTS: Our molecular modeling studies indicated that D-dioxolane nucleoside triphosphates–reverse transcriptase complexes, unlike 3TC triphosphate–reverse transcriptase complex, are not sterically hindered by the bulky side chain of Val184, and maintain the favourable binding modes through the interaction of 3′-oxygen with active site residues such as Arg72 or Tyr115. However, the way in which each complexes resolve the steric hindrance of Val184 with the primer strand without deforming the binding mode, was quite different depending upon the heterocyclic bases attached: slight rotation of Val184 into Tyr183 in dioxolane-T, conformational change in sugar moiety of the primer residue (from 3′-exo to 3′-endo) in 5-F-dioxoloane-C and rotation of Val184 into the nucleoside triphosphate binding site with concurrent conformational change in the dioxolane moiety (from 3′-endo to 3′-exo) in dioxolane G.

CONCLUSION: The molecular modeling studies show that the dioxolane moiety of D-dioxolane nucleosides enables the nucleoside triphosphate to strongly bind to the active site of 3TC-resistant mutant reverse transcriptase without steric hindrance with Val184. It is noteworthy that, depending upon the attached heterocyclic moiety, the binding modes of each dioxolane nucleoside triphosphate is different. This work would lead to the discovery of additional dioxolane with improved activity against 184V mutants (supported by NIH AI32351,AI25899 and Veterans Affairs).

ABSTRACT 37

Prevalence and quantitative phenotypic resistance patterns of specific nucleoside analogue mutation combinations and of mutations 44 and 118 in reverse transcriptase in a large dataset of recent HIV-1 clinical isolates

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BACKGROUND: Two independent pathways to resistance to nucleoside analogue reverse transcriptase inhibitors (NRTIs) associated with mutations at positions 41, 210, and 215 or 67, 70, and 219 (NAMs) of HIV-1 reverse transcriptase have been suggested. Furthermore, mutations at positions 44 and 118 in a NAM background are known to confer low-level lamivudine (3TC) resistance. We examined prevalence and phenotypic resistance of specific NAM combinations and of mutations 44 and 118 in recent clinical isolates.

METHODS: Over 31 400 viral patient isolates submitted for routine testing between January 2001 and February 2003 were analysed for presence of 41L, 44D/A, 67N, 70R, 118I, 210W, 215Y, 215F, 219Q/E, 219N, 219R and 184V/I. Prevalence analyses utilized a subset (*n*=23 616) of isolates without mutations 65, 69, 151 or 333. Phenotypic resistance profiles were obtained from Virco's large matched genotype/phenotype dataset.

RESULTS: 38.4% of isolates harboured ≥1 NAMs (mean 2.7). Mutation 184 was present in 58.7% of NAM-containing isolates, with similar prevalence in each NAM combination. Only 17/64 possible NAM combinations occurred at frequency >1% among NAM-containing isolates. Mean fold-change for zidovudine (AZT) in isolates with ≥3 NAMs (50% of NAM-containing isolates) ranged 3.1–35 and 6.15–45 in isolates with and without 184, respectively. Combinations in the 41-210-215 pathway were most common: 41L 215Y (12.4%), 41L 210W 215Y (11.8%), 41L 67N 210W 215Y (8.11%). Combinations 67N 70R, 67N 70R 219Q/E and 67N 70R 215F 219Q/E were present at 2.5, 8.7 and 3.0% respectively. Two-, three-, and four-NAM combinations in the 41-210-215 pathway had higher mean resistance to each of the NRTIs (for example, range mean fold-change for AZT: 4.0–30.6) compared to corresponding NAM combinations in the 67-70-219 pathway (range mean fold-change for AZT: 2.02–17.7). For each specific NAM combination studied, mutation 184 was associated with increased dalcitabine (ddC), didanosine (ddI) and abacavir (ABC) resistance and decreased AZT, stavudine (d4T) and tenofovir (TDF) resistance. Alleles 219R and 219N appeared at high frequency in five-NAM combinations: 41L 67N 210W 215Y 219**N** and 41L 67N 210W 215Y 219**R** in 4.1 and 1.6% of isolates, respectively, whereas 41L 67N 210W 215Y 219**Q/E** appeared in 0.4% of isolates. 219N and R appeared at low frequency in four-NAM combinations (0.32 and 0.15%), but were virtually absent from two-, three- and six-NAM combinations, and rarely found in combinations with mutation 70. Mutations 44 and 118 strongly associated with the 41-210-215 pathway and were associated with low-level 3TC resistance. 67-70-219 pathway isolates never harboured 44 and 118 simultaneously, some harboured either 44 or 118.

CONCLUSIONS: Associations among mutations causing resistance to NRTIs vary, as do levels of NRTI resistance associated with specific NAM combinations. The predominance of isolates belonging to the 41-210-215 pathway with higher levels of NRTI resistance may influence response to NRTI-containing therapy in treatment-experienced patients.

ABSTRACT 38

Removal of chain-terminating nucleoside analogues by HIV-1 reverse transcriptase utilizing intracellular substrates

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BACKGROUND: HIV-1 replication is inhibited by the incorporation of chain-terminating nucleotide analogues during proviral DNA synthesis. HIV-1 reverse transcriptase (RT) can remove these chain-terminating residues from blocked DNA chains through a reaction related to pyrophosphorolysis. Our laboratory and others have demonstrated that the removal of a chain terminator from a blocked DNA chain by HIV-1 RT occurs *in vitro* and that the substrate acceptor for this excision reaction can be pyrophosphate (PP_i) or any nucleoside di- or triphosphate. However, even though resistance mutations that confer enhanced excision activity are commonly selected during zidovudine (AZT) therapy, the intracellular acceptor(s) for this transfer reaction is unknown. Experiments were carried out to identify compounds present in cell extracts that could serve as substrate acceptors for the excision reaction.

METHODS: Cell extracts were prepared from purified primary CD4, CD8 and CD14 cells. Removal of 2′, 3′-dideoxyadenosine monophosphate (ddAMP) was assessed by incubating 3′ [^{32}P]-labelled ddAMP-terminated primer/template with mutant HIV-1 RT and cell extract. Low molecular weight labelled products generated from the removal reaction were separated by gel electrophoresis.

RESULTS: The major ^{32}P-labelled products formed after incubation with H9 (T lymphoid cell line) cell extract were identified as Ap_4ddA, ddATP and Gp_4ddA produced from the removal and transfer of ddAMP to ATP, PP_i and GTP, respectively. Similar results were obtained with extracts from unstimulated primary human T cells, monocytes, and monocyte-derived macrophages (MDMs). In each case, the predominant substrate acceptor for the transfer reaction was ATP, although transfer to PP_i and GTP could also be observed (estimated intracellular concentrations: ATP, 1.6–2.3 mM and PP_i, 10–28 µM). In contrast, the PP_i-dependent reaction predominated when extracts from activated CD4 and CD8 T cells were used as the source of substrate acceptors (estimated intracellular concentrations: ATP, 2.7–2.9 and PP_i, 55–85 µM).

CONCLUSIONS: The main substrate acceptors recovered from cells were ATP, PP_i and GTP. In the presence of cell extract, mutant HIV-1 RT can transfer ddAMP from the DNA primer terminus to one of these acceptor metabolites, generating Ap_4ddA (ATP), ddATP (PP_i) or Gp_4ddA (GTP). For unstimulated T cells, monocytes and MDMs, ATP was the predominant substrate acceptor utilized for ddAMP removal. Upon activation of T cells, PP_i levels increased three- to eight-fold and became the predominant acceptor substrate for the removal reaction. In light of previous results from our laboratory and others that the nucleotide-dependent excision reaction but not the PP_i-dependent excision reaction is enhanced in RT containing the AZT-resistance mutations, the *in vivo* selection of AZT-resistance mutations requires further explanation. It is possible that selection occurs specifically in a cell subpopulation or a subcellular compartment containing low levels of PP_i.

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ABSTRACT 39

Colinearity of reverse transcriptase inhibitor resistance mutations detected by population-based sequencing

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BACKGROUND: High-level resistance to each of the available reverse transcriptase inhibitors (RTIs) is often detected by the direct sequencing of uncloned PCR products (population-based sequencing). It is not known, however, how often the mutations observed by population-based sequencing are colinear (present in the same viral genome). We sought to determine the relative frequency with which clinical multi-RTI-resistant HIV-1 isolates consist of clones containing each of the mutations present in the population-based sequence rather than of mixtures of clones containing different subsets of the mutations present in the population-based sequence.

METHODS: We selected multi-RTI-resistant isolates from 25 heavily treated individuals in northern California. To determine whether the mutations in these isolates were colinear, we sequenced a mean of 2.8 molecular clones per isolate (71 clones, RT positions 24–310). Each clone was aligned to the population-based sequence to determine the proportion of mutations that were shared between the clones and the population-based sequences at drug resistance and non-drug resistance positions.

RESULTS: The 25 population-based sequences contained a mean of 5.7 nucleoside RTI (NRTI)-resistance mutations, 1.2 non-NRTI (NNRTI)-resistance mutations and 11.3 differences from consensus B at non-RTI-resistance positions. The 71 clones contained a mean of 5.3 NRTI-resistance mutations, 1.0 NNRTI-resistance mutations and 10.2 differences at non-RTI-resistant mutations. Sequences of the clones closely resembled the population-based sequences: 31 (51%) clones had each of the RTI mutations present in the population-based sequence, 25 (35%) had all but one RTI mutation, 4 (6%) had all but two RTI mutations, 3 (4%) had all but three RTI mutations, and 3 (4%) had all but four RTI mutations. The population-based sequence contained a mixture of wild-type and mutant nucleotides at 41/54 (76%) of the positions containing mutations that were not observed in at least one clone.

CONCLUSIONS: In this study multi-RTI-resistant isolates obtained from heavily treated patients generally consisted of viruses containing either all or nearly all of the mutations detected by population-based sequencing. This suggests that most RTI-resistance mutations are colinear. The potential benefit of mega-HAART in this population is likely to derive from the decreased replication that is often found in viruses containing multiple RTI-resistance mutations rather than from the effects of different drugs acting on different virus subpopulations.

ABSTRACT 40

Identification of the minimal conserved structure of the HIV reverse transcriptase under the presence and absence of drug pressure

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BACKGROUND: Several polymorphisms and drug-related variability occur in HIV-1 reverse transcriptase (RT). However, little is known regarding its minimal conserved structure. The extent of RT conservation in vivo in the absence and presence of pharmacological pressure has been studied in a large cohort of patients.

METHODS: Sequences of the first 320 amino acids (aa) of RT obtained from plasma of 463 drug-naive patients, and of 802 patients treated with an average of >3 highly active antiretroviral therapy (HAART) regimens, were compared and analysed. Consensus B was used as a reference strain for the definition of mutations. Amino acid positions with variability <1% were considered invariant.

RESULTS: In naive patients, the RT protein sequence showed no variation in 230 out of 320 RT aa sequence (72% overall conservation). Isolated and pairs of invariant residues were scattered throughout the sequence, while others were clustered to form regions (from 4 up to 25 consecutive invariant residues). The eight longest invariant regions were: I (T7-P19), II (W71-K82), III (T107-S117, containing the D110 catalytic site aa), IV (F124-S134), V (N147-P157), VI (Y181-E194, containing the catalytic active site aa D185 and D186), VII (K220-I244) and VIII (W252-Y271). Regarding variable residues, 41 aa were mutated in ≥5% of patients, of which 16 were highly variable (substituted in >25% of patients). Frequency of mutations associated with drug resistance was always <1%. In treated patients, despite the appearance of mutations primarily associated with drug resistance, aa invariance was still maintained in 204 out of 320 aa (64% of conservation versus 72% in naive patients). The long conserved areas I, III, IV, V and VIII, were preserved in drug-treated patients, with the minor exceptions K11, V108, F116, Q151 (variability in ≤3% of patients). Two other large conserved areas (II and VI) shrunk to smaller and fragmented domains (W71-K73, R78-K82, D185-L187 containing the catalytic active-site aa, and S191-L193), while the largest conserved region (VII) was cut into isolated residues with two smaller conserved areas (W229-D237 and W239-I244). In addition, a trend of RT structure conservation was also suggested by the finding of a high ratio of synonymous to non-synonymous aa substitutions (ds/dn>7) in RT nucleotide sequences of selected patients, of whom both pre- and post-therapy sequences were available.

CONCLUSIONS: Even in drug-treated patients, HIV-1 RT requires the preservation of at least two-thirds of aa (some with still unknown function), and of large areas of its tertiary structure in order to maintain a stable and functional structure. Future HIV RT inhibitors may be designed to target with these invariant domains.

ABSTRACT 41

Identification of a clinical reverse transcriptase backbone that improves replication of a non-nucleoside reverse transcriptase inhibitor-resistant mutant by increasing the rate of polymerization

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BACKGROUND: We previously identified a clinical reverse transcriptase (RT) sequence containing P236L (8-26), which, when placed into NL4-3, resulted in virus with improved replication kinetics compared to NL4-3 that contained P236L in an NL4-3 RT background (Dykes *et al., Virology* 2001; **285**:193.). We demonstrated that P236L/NL4-3 RT slows steady-state rates of both polymerase-independent and polymerase–dependent modes of RNase H cleavage (Gerondelis *et al., Journal of Virology* 1999; **73**:5803.). We also recently found that P236L/NL4-3 RT slows the turnover rate (kss) during single nucleotide incorporation with a DNA:DNA primer:template, under pre-steady state conditions (Domaoal *et al. 10th CROI* #612). For wild-type RT, kss reflects the rate of product dissociation, and is normally the rate-limiting step in polymerization. No reductions were seen in k_{pol} (the maximal rate of nucleotide incorporation) or K_d (affinity) for dGTP by P236L RT. We wanted to determine whether improvements in either RNase H or polymerase function accounted for the partial improvement in P236L/8-26 replication relative to P236L/NL4-3.

METHODS: Wild-type NL4-3, P236L/NL4-3 and P236L/8-26 RTs were expressed in *E. coli* and purified. Polymerase-dependent RNase H activity was assayed using a 5′-end-^{32}P-labelled 41 nucleotide (nt) RNA annealed to a shorter DNA, with the DNA 3′-end recessed relative to the RNA 5′-end. Polymerase-independent RNase H activity was assayed using the same RNA hybridized to a longer DNA, with the RNA 5′-end recessed relative to the DNA 3′-end. RNase H reactions were carried out in the absence of dNTPs and RT input was normalized for steady-state polymerization specific activity on a homopolymeric RNA:DNA template:primer. kss during single nucleotide incorporation of dGTP was measured under burst conditions (substrate excess) with a DNA:DNA template:primer, using a KinTek quench-flow apparatus.

RESULTS: Polymerase-dependent and polymerase-independent RNase H cleavages by P236L/NL4-3 and P236L/8-26 RTs were similarly slowed relative to wild-type RT. The turnover rate for dGTP incorporation (kss+sd) was 0.16 ±0.018/sec for wild-type, 0.04 ±0.018/sec for P236L/NL4-3 and 0.21 ±0.018/sec for P236L/8-26 RTs.

CONCLUSIONS: A clinical RT backbone that partially compensates for the replication defect of P236L also improves its reduced turnover rate (kss) during polymerization. We postulate that this significantly contributes to the ability of this clinical RT sequence to compensate for P236L's replication defect. These studies demonstrate that pre-steady state kinetics can identify the underlying biochemical mechanisms leading to modulation of drug resistance mutations by clinical RT backbones. In addition, these studies indicate that the polymerization abnormalities identified for P236L/NL4-3 RT contribute significantly to the reduction in replication efficiency conferred by this mutant in cell culture. We believe these abnormalities account for the infrequent occurrence of this mutant during delavirdine therapy, despite its high level of drug resistance, and that such studies can be used to better understand resistance patterns during clinical failure of other non-nucleoside RT inhibitors.

ABSTRACT 42

Non-nucleoside reverse transcriptase inhibitor hypersusceptibility can be demonstrated in multicycle phenotype assays and in inhibition assays of purified HIV-1 reverse transcriptases

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BACKGROUND: Nucleoside resistance has been shown to enhance susceptibility to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class. This NNRTI hypersusceptibility (HS), defined as a fold change in susceptibility of <0.4 compared to wild-type, has been associated with improved outcomes to NNRTI therapy in several retrospective studies. To date, NNRTI hypersusceptibility has only been reported in the PhenoSense HIV® single-cycle replication phenotypic assay (ViroLogic) and not yet been reported in multicycle phenotypic assays or in cell-free enzymatic systems.

METHODS: Ten recombinant HIV-1 cloned viruses derived from different patients were evaluated for their susceptibilities to nevirapine (NVP) and efavirenz (EFV) using the PhenoSense assay and the ACTG PBMC phenotypic method. Clones were selected on the basis of multiple nucleoside resistance mutations based on their genotypic nucleoside resistance patterns. In addition, *E. Coli*-expressed purified recombinant reverse transcriptases (RT) were generated from four of the cloned viruses as well as the wild-type NL4-3. The EC_{50} values of RT activity inhibition using a digoxigenin-labelled nucleotide incorporation assay were determined and compared with the wild-type NL4-3. Different p66 and p51 RT subunit compositions were analyzed to determine their relative contributions to NNRTI HS.

RESULTS: In the PhenoSense assay, 6 of 10 clones were HS to EFV and 4 of 10 were HS to NVP. In the PBMC assay, four of the six EFV HS and all four of the NVP HS were also HS. The four clones not HS to EFV and six not HS to NVP were also not HS in the PBMC method. The fold change values between the assays were well correlated with r^2 for EFV=0.98, NVP=0.97, *n*=10, and r^2 for EFV=0.79, NVP=0.77 among the eight clones with fold change values of <2.5. HS was also detected in the expressed RTs derived from three HS clones when compared with the wild-type in the RT activity inhibition assay. The p51–p66 heterodimers and p66–p66 homodimers derived from individual clones were inhibited similarly. The p66 from the HS clones when coupled with wild-type p51 were HS, but p51 from HS clones with wild-type p66 were not HS.

CONCLUSIONS: NNRTI HS is not an assay-dependent phenomenon, but exists in multicycle replication and cell-free assays as well as single-cycle phenotypic assays. As would be expected by the location of the NNRTI binding pocket, NNRTI HS is determined by the active p66 RT subunit.

ABSTRACT 43

Genetic correlates of phenotypic hypersusceptibility to efavirenz among 446 baseline isolates from five ACTG studies

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BACKGROUND: Increased phenotypic susceptibility (hypersusceptibility, HS) to non-nucleoside reverse transcriptase inhibitors (NNRTIs) is observed in ~30% of viral isolates with NRTI-resistance mutations and has been associated with improved virological response to NNRTI-based therapy in several studies. Although preliminary analyses have shown an association between zidovudine resistance mutations and NNRTI HS, the genetic basis for NNRTI HS has not been thoroughly defined. We sought to identify specific mutations in RT associated with efavirenz (EFV) HS. Elucidating the genetic basis for EFV HS will improve interpretation of genotypes to optimize use of EFV-containing treatment regimens.

METHODS: Paired baseline genotypes (VGI or ABI) and phenotypes (ViroLogic) were obtained from 446 subjects entering one of five ACTG studies: 290, 359, 364, 370 or 398. All subjects were NRTI-experienced but NNRTI-naive at study entry. Fisher's exact tests and recursive partitioning (CART) were used to identify RT mutations associated with EFV HS and evaluate their relative importance.

RESULTS: Of the 446 isolates, 153 (34%) demonstrated HS to EFV, defined by an IC_{50} <0.4-fold that of a wild-type reference virus. In univariate analyses, EFV HS was significantly associated (P<0.001) with the following NRTI resistance mutations: M41L, E44D, D67N, mutations/inserts at 69, V118I, H208Y, L210W, and T215Y/F, but not associated with K70R, L74V, Q151M, M184V, or K219Q/E/N. Two polymorphisms in the NNRTI binding site of RT, K103R and V179I, were significantly associated with EFV HS (P=0.052 and 0.028, respectively) but other substitutions in this region were absent in the HS group including A98G, L100I, K101E, and V106A. CART analysis was performed including mutations at 41, 67, 69ins, 210, 211 and 215 as variables. The first split selected was T215Y/F, present in 76% (117/153) of HS vs 37% of non-HS isolates. Further branching in the partitioning also found the L210W and D67N mutations were important. In a subgroup of 54 viruses containing D67N, L210W and T215Y/F, 39 (72%) were HS to EFV. A second CART analysis, expanded to include 29 codons, again selected T215F/Y at the first split, but found H208Y as the next branch. Of 26 isolates containing both T215Y/F and H208Y, 22 (85%) were HS to EFV. In comparing 215Y to 215F, EFV HS was present in 94/177 (53%) of isolates with 215Y and 24/55 (44%) with 215F.

CONCLUSION: Univariate and CART analyses of 446 genotype/phenotype pairs identified key mutations in RT associated with EFV HS. Mutation at codon 215 (Y>F) is most discriminatory but other mutations contribute to the HS phenotype including 67N, 208Y and 210W. In addition, polymorphisms in the NNRTI-binding region (K103R and V179I) appear to be associated with EFV HS. These results can be used to identify the biochemical and structural basis for EFV HS and to predict its presence in clinical samples.

ABSTRACT 44

Rare 1 and 2 amino acid insertions in the non-nucleoside reverse transcriptase inhibitor (NNRTI) binding pocket of HIV-1 reverse transcriptase affect NNRTI susceptibility

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BACKGROUND: Inserts in the β3–β4 region of the reverse transcriptase (RT) gene of HIV-1 have been shown to affect susceptibility to nucleoside RT inhibitors (NRTIs). Recently, an insert in the non-NRTI (NNRTI) binding pocket region of the RT gene from a patient was reported (Rosenbaum *et al.*, *Antiviral Therapy* 2000; **5**(**Suppl. 3**):29). We have identified and studied two patient-derived HIV-1 strains with NNRTI binding pocket inserts that display different genotypic features.

METHODS: Physician-requested population-based genotypes were obtained from plasma samples submitted to Quest Diagnostics. After RT-PCR amplification, RT genes from strains under investigation were transferred into an NL4-3-based vector. The recombinant clones were transfected into C8166 cells to generate virus stocks. *In vitro* susceptibility assays were performed using titered virus stocks in SupT1 cells. Homology modeling of RT gene structures was performed using the program MODELER, and was based on published RT-efavirenz (EFV) and RT-nevirapine (NVP) crystal structures. Changes in molecular recognition between genotypically-matched insert-containing and non-insert-containing strains were calculated using the programs HBPLUS and BONDSGL.

RESULTS: Two separate strains with inserts near codon 100 of the RT gene were identified from genotypes collected from 70 000 patient samples over the last 4–5 years. Both strains were identified in late 2002. Sample A had a strain possessing a single amino acid insertion, along with the drug resistance mutations K103N, V179D/E, M184V and P225P/H. Sample B had a two amino acid insert along with the drug resistance mutations M184V, G190E and the Q151M complex. Recombinant viral clones from both samples showed 1000- to 10 000-fold reduced susceptibility to EFV and 50- to 1000-fold reduced susceptibility to NVP. Specific clones from Patient A that were genotypically matched except for the presence of the insert showed that the insert-containing clone was 50-times less susceptible to EFV compared to the non-insert containing clone, indicating a contribution of the insert to EFV resistance. Site-directed mutants created with only the 1 or 2 amino acid insertion did not produce viral particles after transfection into cell lines. Molecular modelling studies of the patient-derived insert-containing strains showed conformation changes in the NNTRI binding that resulted in altered van der Waals interactions with the EFV and NVP. A larger number of van der Waals contacts were lost between the RT and EFV compared to NVP.

CONCLUSIONS: HIV strains possessing 1 or 2 amino acid inserts in the NNRTI binding pocket can be found in patients failing antiretroviral therapy and contribute to reduced susceptibility to NNRTI. The presence of these insertions appears to be dependent on other mutations and/or polymorphisms in the RT gene. The inserts affect molecular interactions between the NNRTI binding pocket and NNRTI. Further monitoring of treated patients will determine if these insertions are a new, emerging mechanism of resistance.

ABSTRACT 45

Crystal structure of a multidrug-resistant HIV-1 protease clinical isolate reveals an expanded active site cavity and represents a novel target for the design of protease inhibitors

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BACKGROUND: The goal of this work is to investigate the structural basis of HIV-1 protease multidrug resistance. The hypothesis tested is that HIV protease multidrug resistance is associated with the expansion of the protease active site cavity.

METHODS: We expressed, purified and crystallized a multidrug-resistant (MDR) HIV-1 protease variant (patient isolate 769). Isolate 769 is representative of the end-stage MDR form of the virus. This isolate contains the following amino acid changes relative to the wild-type NL4-3: L10I, M36V, M46L, I54V, I62V, L63P, A71V, V82A, I84V and L90M. The IC_{50} ratio of the 769 variant to the IC_{50} value of the NL4-3 wild-type is: 43 for saquinavir, 47 for nelfinavir, 14 for amprenavir, 80 for atazanavir, >100 for DG-3, >100 for palinavir and >100 for GS3333.

RESULTS: We solved the crystal structure of the HIV-1 protease MDR isolate 769 to 1.8 Å resolution. The 1.6 Å crystal structure of the 769 MDR protease complexed with the tripeptide EDF was also solved. When the 769 crystal structure is compared to the wild-type crystal structure (3PHV), there is an expansion of the active site cavity as we proposed earlier based on modelling studies. The active site cavity expands at positions 82 and 84. The active site expansion is due to mutations from larger to shorter side chains, e.g. V82A and I84V. A second observation is that the 'flaps' of the protease stay open wider compared to the wild-type. The distance between residues I50 and I150 of the two 'flaps' increases from 4 Å in the wild-type to 12 Å in the MDR variant. The crystal structure of the MDR protease – EDF complex reveals a new binding mode of the small molecule. The ligand binds to the 'open' MDR protease interacting with residues 28, 47, 48, 49 and 80 of a single monomer.

CONCLUSION: The crystal structure of an HIV-1 protease MDR clinical isolate reveals an expanded active site cavity and provides a structural basis for protease inhibitor resistance. The crystal structure of the complex represents a novel binding mode where the peptide binds only to one side of the active site cavity and the 'flaps' of the protease stay wide open. This crystal structure will provide structural insight to develop a new class of inhibitors against the 'open' form of the HIV-1 protease. All the licensed inhibitors were designed based on the structure of the 'closed' form of the HIV-1 protease.

ABSTRACT 46

Structural correlates of broad-spectrum activity for a resistance-repellent HIV protease inhibitor

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BACKGROUND: Cross resistance is a serious problem for long-term antiretroviral therapy. In an effort to design broad-spectrum HIV protease inhibitors (PIs), we analysed the structures of mutant HIV proteases (PR) derived from highly cross-resistant HIV strains in complexes with several highly potent PIs.

METHODS: X-ray crystallography and biochemical methods were used to compare the effects of several drug resistance protease mutants on the binding of a resistant-repellant PI (rrPI), UIC-94003; a structurally-related but resistant-susceptible analogue, amprenavir (APV); and, a structurally-unrelated, resistant-susceptible PI, saquinavir (SQV). For this study we used wild-type PR, an I84V mutant, (M1) and two mutants that showed average increases in Ki and IC_{50} values of over two orders of magnitude for all FDA-approved PIs. These mutants contained four substitutions in one case, including I50V (M2), and 14 substitutions in the other case (M3).

RESULTS: Crystallographic analysis indicates that UIC-94003 and APV bind in a nearly identical manner to both wild-type and mutant enzymes, with the exception of additional hydrogen bonds made by the rrPI with a structurally-conserved region of the enzyme. The I50V and I147V mutations in M2 create a larger hydrophobic S2′ cavity that results in weaker van der Waals interactions between the protein and inhibitor. In addition, weak electron density for the characteristic flap water that typically anchors carbonyl groups of most inhibitors is observed for M2 and M3. In the M2 and M3 mutant complexes, the intersubunit salt bridge between R8-D129 is absent. The flap water and the salt bridge are both a strong features of all known wild-type/inhibitor complexes, is broken. Comparison of the M3 and wild-type complexes with SQV shows that a critical hydrogen bond between the inhibitor and the D30 carboxyl group is lost.

CONCLUSIONS: Our analysis has led to the discovery of a conserved substructure of the active site of HIV PR that is important for the broad-spectrum activity of rrPIs. The mode of binding of UIC-94003 and APV are rigidly conserved in both the wild-type and mutant enzymes, but APV lacks key interactions with the conserved substructure. In SQV, critical interactions with the wild-type enzyme are lost due to mutations. Mutations involved in drug resistance not only affect the interactions between inhibitor and enzyme, but also can alter intrinsic properties of the enzyme, such as active site solvation and dimer stability. Our analysis reveals that structural flexibility of PIs is not a necessary factor in their ability to exhibit broad-spectrum activity.

ABSTRACT 47

Co-evolution of the nucleocapsid-p1 cleavage site with the V82A mutation in HIV-1 protease preserves substrate recognition

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BACKGROUND: The Nucleocapsid-p1 (NC-p1) cleavage site is the last, slowest and, therefore, rate determining site to be processed in the HIV-1 Gag by the viral protease. The sequence of the cleavage site is also the most unusual of the viral substrate sites, R-Q-A-N-*-F-L-G-K, with an asparagine at P1 (the only substrate site with a hydrophilic residue at P1) and an alanine at P2 (the only substrate site not to have a branched amino acid at P2). A co-evolution of this site to R-Q-V-N-*-F-L-G-K has been observed in patient sequences when the drug-resistant V82A mutation occurs in HIV-1 protease. Previous studies have shown that this mutation in the NC-p1 substrate site increases the rate of its cleavage by more than twofold over the wild-type. In this study we have solved the crystal structures of both the wild-type HIV-1 protease and the V82A HIV-1 protease in complex with their respective NC-p1 substrates and may be able to provide a structural explanation for the change in substrate sequence.

METHODS: The NC-p1 wild-type substrate, R-Q-**A**-N-*-F-L-G-K, and the AP2V mutant substrate, R-Q-**V**-N-*-F-L-G-K, were crystallized with the wild-type and V82A protease variants, respectively (referred to as ^{WT}NC-p1$_{WT}$ and ^{AP2V}NC-p1$_{V82A}$ complexes). Diffraction data were collected, processed and refined, using standard crystallographic techniques. The two structures were compared graphically with each other and with other substrate complexes previously determined in our laboratory.

RESULTS: The structures of ^{WT}NC-p1$_{WT}$ and ^{AP2V}NC-p1$_{V82A}$ complexes were refined to 2.2 Å (R=21.0%; R_{free}=24.2%) and 2.0 Å (R=19.5%; R_{free}=23.1%), respectively. In the ^{WT}NC-p1$_{WT}$ complex the conformation of the backbone of the substrate peptide rearranges relative to previous substrate complexes we have solved so that the P1′-Phe contacts V82, while the P2-Ala does not make extensive contact with the residues in the S2 pocket. In the ^{AP2V}NC-p1$_{V82A}$ complex the P1′-Phe no longer contacts A82, however P2-Val makes extensive contact with V32, I47 and I84 of the S2 pocket, and the substrate peptide backbone is aligned with the conformation of other substrate complexes.

CONCLUSIONS: The NC-p1 substrate peptide appears to have an unusual fit in the active site cavity of HIV-1 protease compared with the substrate peptides whose complexes have been solved which may account for the its slow cleavage rate. The backbone is rearranged and the P1′-Phe is in an unusual conformation contacting V82. This is most likely due to the P2-Ala that is unable to fill the S2 pocket of the active site effectively. When the drug-resistant V82A mutation occurs, this likely further destabilizes the complex as the P1′-Phe contact is lost. P2-Val compensates for this protease mutation, as has been reflected in previously measured kinetics, by filling the S2 pocket and stabilizing the substrate's conformation in the active site.

ABSTRACT 48

Preliminary characterization of a newly described protease substrate cleft mutation at position 23

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BACKGROUND: L23I/V is a recently recognized mutation associated with protease inhibitor (PI) therapy (*Journal of Virology* 2003; **77**:4836–4837). However, the risks for developing this mutation and the effect of this mutation on drug susceptibility have not been studied.

METHODS: We analysed the frequency of L23I/V in 1004 untreated patients and 1240 patients receiving one or more PIs to determine whether this mutation was associated with particular PI regimens. We determined the susceptibility and replication capacity of recombinant infectious molecular clones with this mutation using the PhenoSense Assay™ (ViroLogic, Calif., USA). Recombinant infectious clones were created by ligating amplified fragments containing the 3′ part of *gag* and complete protease gene into a *gag/pro*-deleted pNL43 vector (pNLPFB, provided by T Imamichi, NIAID). Two pairs of clones were derived from clinical isolates that contained a mixture of L and I at position 23. Each of the pairs was isogenic except for the presence or absence of the L23I mutation.

RESULTS: L23I/V occurred in 0/1004 untreated patients and in 18/1240 (1.5%) of patients receiving one or more PIs (*P*=0.0005; L23I in 16 patients and L23V in two patients). The median duration of PI therapy was 161 weeks (range 48–316). Three patients developed L23I during monotherapy with saquinavir, nelfinavir, or amprenavir; 15 patients received 2–4 PIs before L23I was detected. L23I was present at two or more time points in eight patients. All but three of the 18 isolates contained multiple other PI-resistance mutations. Seven recombinant infectious molecular clones encompassing the 3′ part of *gag* and complete protease gene were created from five clinical isolates containing L23I. One clone containing L23I in combination with the polymorphism V82I had 6.7-fold resistance to nelfinavir but to be susceptible to each of the other PIs. This clone had a replication capacity of 10%. The two pairs of clones that differed only at position 23 each had other drug resistance mutations. One pair had V82A, I84V and L90M with multiple accessory mutations; the other had I84V and L90M with multiple accessory mutations. In both of these pairs of clones, L23I was associated with increased replication capacity (6 to 39% and 35 to 59%) and high-level resistance to each of the PIs. Studies of site-directed mutants with and without L23I are pending.

CONCLUSION: L23I is a rare substrate cleft mutation that occurs in about 1% of patients receiving multiple PIs. By itself, L23I appears to be associated with nelfinavir resistance and decreased replication capacity. In combination with other mutations, L23I appears to be associated with multi-PI resistance and increased replication capacity.

ABSTRACT 49

Emergence of a novel lopinavir resistance mutation at codon 47 correlates with ARV utilization

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OBJECTIVES: Multiple primary protease mutations are required to produce high-level resistance to lopinavir (LPV). These mutations may be selected through antiretroviral therapy (ARV) with other protease inhibitors (PIs), leading to LPV cross resistance. We identified a novel I47A LPV-resistant variant and found that the emergence of mutations at codon 47 is highly correlated with increasing clinical LPV utilization.

METHODS: Genotypes were obtained by DNA sequencing of clinical samples and mutational frequencies were obtained from the Quest Diagnostics HIV-1 sequence database. Quarterly ARV prescription data was obtained from Scott Levin Source™ Prescription Audit. Phenotypes were determined by the Antivirogram™ assay. Changes in binding energy were calculated from PR-LPV complexes built by amino acid substitutions in the wild-type complex with subsequent energy minimization of the ligand and binding site residues.

RESULTS: We identified the I47A mutation in 40/47 797 clinical specimens genotyped after LPV became available in late 2000. None of 26 459 samples genotyped prior to October 2000 had the I47A mutation (χ^2=20.6 P<0.0001). Phenotypic data obtained for three I47A mutants showed high levels of LPV resistance (86.2×, >91.5× and >102.5×) that was not predicted by genotypic analysis. Molecular modeling and energy calculations for these variants showed binding energy changes (ΔE_{bind}) for LPV of 3.0, 3.1 and 2.8 kcal/mol, respectively, consistent with high levels of LPV resistance (>100×). I47V variants had smaller ΔE_{bind} changes (2.0, 1.9 and 0.93 kcal/mol, respectively). I47V is associated with resistance to amprenavir (APV) but not LPV. I47V frequency has increased 4× from 2000 to 2002. Spearman correlations between APV or LPV prescription utilization and I47V frequencies were not significant for APV (r_s=0.33, P=0.23) but were highly significant for LPV (r_s=0.93, P=0.0001). I47V results from an A→G nucleotide change, whereas I47A requires two changes (ATA→GCA). A single substitution is required for I47V→I47A (GTA→GCA). We identified five I47A mutants in specimens with prior sequences containing I47V, suggesting that the I47A variant emerges via a two-step pathway. In the protease-LPV model, the Ile side chain of residue 47 is positioned close to the LPV dimethoxyphenyl aromatic ring and VDW interactions may contribute to binding. A V47 model shows similar positioning with slightly decreased VDW interaction, however these interactions are lost for the smaller A47.

CONCLUSIONS: We have identified a second pathway to high-level LPV resistance that does not result from cross resistance to other PIs. Increased usage of LPV correlates with increased frequencies of the I47V mutation leading to the stepwise emergence of the LPV-resistant I47A variant. Surveillance of emerging resistance in large clinical databases may be facilitated by structural phenotypic methods that enable rapid computational determinations of the significance of newly identified mutational patterns.

ABSTRACT 50

Parameters driving the selection of nelfinavir-resistant HIV-1 variants

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BACKGROUND: Resistance to nelfinavir most often begins with the selection of D30N mutation in the protease. In a percentage of patients, however, the alternative L90M mutation is found. Mutants carrying both these mutations are only rarely found *in vivo*. Here, we investigated the parameters that determine the emergence of mutants following the D30N or the L90M pathways. To this end, a series of mutant clones were compared for infectivity in the absence of drug, resistance to nelfinavir and replicative advantage as a function of drug concentration.

METHODS: Replication-competent viral clones that carry single or combined mutations in the protease were constructed by site-directed mutagenesis. The drug-free infectivity of virions was measured in a single-cycle assay and expressed as percentage of the parental (pNL4.3) wild-type clone. Nelfinavir resistance was calculated as the ratio of IC_{50} and IC_{90} values of the mutant clones to those of wild-type virus. Replicative advantage curves were drawn by comparing the infectivity of mutant clones and of wild-type virus at several drug concentrations (from 0 nM to 2500 nM).

RESULTS: Both D30N and L90M mutations confer a selective advantage for replication in the presence of low nelfinavir concentrations. The advantage displayed by D30N mutant was mostly due to resistance, while the advantage of L90M mutant reflects preservation of infectivity coupled with a minimal reduction in susceptibility. At higher nelfinavir concentrations, viruses harbouring D30N coupled to additional mutations of this evolutionary pathway could reach higher levels of resistance and display a higher selective advantage compared to mutants of the L90M series. A mutant carrying both D30N and L90M was characterized by a dramatic loss of infectivity, which explains the rare appearance of this combination *in vivo*. Interestingly, the loss of infectivity could be efficiently rescued by the addition of compensatory mutations in the protease or by the L63P polymorphism.

CONCLUSIONS: Different biological properties account for the replicative advantage of D30N and L90M mutants in the presence of nelfinavir. The higher prevalence of D30N mutation in patients receiving nelfinavir reflects the higher level of resistance that can be attained by viruses engaged in the D30N pathway. In addition, restoration of infectivity of D30N-L90M double mutant by a common polymorphism reinforces the notion that the genetic context of the virus may substantially influence the impact of resistance mutations.

ABSTRACT 51

I84A and I84C mutations in protease confer high-level resistance to protease inhibitors and impair replication capacity

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BACKGROUND: The I84V mutation in HIV protease (PR) is associated with resistance to the PR inhibitor (PI) class. The I84A variant has been observed following ritonavir (RTV)/saquinavir (SQV) therapy and *in vitro* passage with the experimental PI BILA 1906 BS. However, the clinical relevance of I84A or other I84 mutations is not well characterized.

OBJECTIVE: To evaluate the effect of novel substitutions at position 84 on PI susceptibility and replication capacity (RC).

METHODS: Phenotypic susceptibility to PIs [RTV, indinavir (IDV), amprenavir (AMP), SQV, nelfinavir (NFV) and lopinavir (LPV)] and RC was evaluated using PhenoSense HIV™. Molecular modelling was performed using Insight-II™.

RESULTS: The I84C or I84A mutations were each identified in eight separate isolates. Several secondary mutations, as well as p7/p1 or p1/p6 cleavage site mutations were also observed. I54V and/or I47V were observed in 1/8 I84A, 0/8 I84C and 6/12 I84V isolates chosen for comparison. Compared to I84V isolates lacking I54V or I47V, I84C isolates displayed substantially higher (22- to 36-fold) resistance to NFV and SQV, slightly higher resistance to RTV, IDV and AMP (three- to sevenfold), but similar susceptibility to LPV (1.6-fold). I84A isolates exhibited >60-fold median resistance to all PIs except LPV. In contrast, I84V isolates with I54V and/or I47V were especially resistant to LPV (median IC_{50} 80-fold). The median fold IC_{50} values for the six PIs tested were 22-fold for I84C mutants, 76-fold for I84A mutants, 2.9-fold for I84V mutants without I54V or I47V, and 36.5-fold for I84V mutants with I54V and/or I47V. Greater than 10-fold resistance was observed 64, 96, 14 and 78% of the time with the above four groups of isolates, respectively. RC was impaired in all mutant isolates tested: median RC for I84A, C and V were 6.4, 11 and 28%, respectively (differences between groups not significant). The A431V p7/p1 cleavage site mutation was very common in I84A mutants and I84V (with I54V and/or I47V) isolates and uncommon in I84V mutants lacking I54V and I47V. In one patient from whom longitudinal data were available, the I84V mutant was an apparent precursor to I84A. Structure modelling of the mutant proteases indicated that I84V, I84C and I84A mutations all create unoccupied volume in the active site, with I84A introducing the most greatest change from the wild-type structure. Mutation of the *gag* p1-p7 and p1/p6 substrates reduced the unoccupied volume created by I84A.

CONCLUSION: In general, I84V mutants without other major mutations display relatively modest resistance to PIs. Increased resistance is accomplished by addition of other major mutations (common pathway) or (less commonly) by selection of I84C or I84A. Molecular modelling provides useful insight into the mechanism of resistance mutations on drug susceptibility and replicative capacity.

ABSTRACT 52

The HIV-1 protease mutation K55R is associated with the presence of the M46I/L mutation

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BACKGROUND: The association of specific HIV-1 protease resistance mutations is well documented; for example, D30N and N88D, V32I and I47V. Additional associations between resistance mutations and either natural polymorphisms or substitutions not considered as being key to resistance may exist. To identify additional associations, analysis was performed comparing the incidence of non-consensus substitutions at one position with non-consensus substitutions at the other 98 amino acid positions in protease.

METHODS: Protease genotypes from 803 protease inhibitor (PI)-experienced subjects that had failed at least one PI were available for analysis. Non-HXB2 consensus substitutions at each of the 99 residues of the protease gene were identified. The incidence of a non-consensus substitution occuring at amino acid X was compared to the incidence of a non-consensus substitutions at each of the other amino acid positions. A total of 9604 comparisons were made. The co-association between pairs of non-consensus substitutions was analysed, except when the incidence was low (<1%). Statistical significance was tested using the χ^2 test.

RESULTS: 39/99 positions had <1% non-consensus substitutions in the 803 samples of which 13/39 positions had no non-consensus substitutions (L5, W6, R8, P9, D25, T26, G27, A28, D29, G40, P44, G52, Y59). Analysis of association between non-consensus substitutions at the remaining positions confirmed previously described associations between protease resistance mutations and identified additional associations between natural polymorphisms; for example, I47*+V32*, D30*+N88*, V82*+I54*, M36*+E35*, M46*+L10*, I54*+L10*, M46*+L63*, G73*+L90*, M36*+K20*, L90*+A71*. In addition a novel association between K55* and M46* (P<0.001) was identified and, to a lesser extent, an association between K55* and V82* (P<0.001) and K55* with I54* (P<0.001). A non-consensus substitution at position K55* was present in 49/803 (6.1%) of the clinical isolates. The most common non-consensus substitution was K55R (30/49, 61%) or K55k/r (16/49, 33%). K55R was only rarely detected in PI-naive subjects (1/422 isolates). Of the subjects with the K55R substitution, the majority also had the M46I/L substitution (29/30, 97%; M46I n=18, M46L n=11). Of note, non-consensus substitutions at positions M46*, V82* or I54* were not associated with K55* indicating that K55R may develop after these mutations or only in a specific sub-set of viruses containing these mutations.

CONCLUSION: Analysis of the associations between non-consensus substitutions across all 99 protease amino acids identified additional associations not previously described including K55R being associated with either M46I or L, or V82A/S/T or I54V. K55R was only rarely detected in PI-naive subjects suggesting that this mutation could represent an accessory resistance mutation.

ABSTRACT 53

Nucleic acid differences between HIV-1 non-B and B reverse transcriptase and protease sequences at drug resistance positions

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BACKGROUND: HIV-1 subtypes are phylogenetically distinct due to non-synonymous and synonymous nucleic acid (NA) differences leading to dominant amino acid (AA) coding. Conservation and variation of codon usage among divergent subtypes may contribute to different mechanisms and pathways in the evolution of drug resistance.

METHODS: RT and protease codon usage was examined at non-polymorphic drug resistance positions in a large dataset of sequences from non-B and B-infected, untreated and subsequently in treated persons. Significant differences (P<0.001) in the prevalence of NA patterns at resistance positions among non-B and B sequences, were determined by Fisher's exact test.

RESULTS: Data were available from 289 persons infected with subtype A (72% untreated), 769 with C (55% untreated), 223 with D (64% untreated), 148 with F (57% untreated), 318 with G (43% untreated), 297 with CRF01_AE (47% untreated) and 343 with CRF02_AG (83% untreated). Synonymous NA codon differences were identified at 18 RT and seven protease drug resistance loci in sequences from non-B-infected untreated persons. AA at these positions in sequences from non-B-infected treated persons were similar to those seen in subtype B, except RT position V106 in subtype C. Of sequences from 548 untreated subtype C-infected persons, 79% (431/548) had GTG/valine, and 17% (93/548) GTA/valine, while in subtype B, 95% (1580/1671) had GTA/valine at RT position 106. Among sequences from non-nucleoside RT inhibitor (NNRTI)-treated subtype C-infected persons, 16% (22/142) had ATG/methionine and 1% (2/142) had GCG/alanine, while in subtype B 1% (4/450) had ATG /methionine and 4% (17/450) had GCA/alanine at that position. Of the 22 subtype C-infected persons with V106M, 19 (86%) were exposed to efavirenz and 3 (14%) to nevirapine. All isolates harboured other NNRTI-related mutations (G190A/S>K103N>K101E/P>F227L>Y188C/H>A98G=V179D>181C>M230L). Other RT valine residues (positions 75, 118, 179) harboured mainly codons GTT/GTA in sequences from B or C infected untreated persons, with non-synonymous G to A transitions to ATT/ATA, coding for isoleucine in sequences from treated persons. In other non-B subtypes only 2% (21/1043) of sequences from untreated persons had GTG/valine and 0.5% (1/186) from treated persons had ATG/methionine.

CONCLUSION: There are characteristic, subtype-specific baseline synonymous NA differences at RT and protease drug resistance positions, which rarely result in different non-synonymous substitutions with drug therapy. However, as described by Brenner *et al.*, V106M uniquely predominates in sequences from subtype C-infected persons after efavirenz, and to a lesser extent, as shown here, after nevirapine exposure. The predominant use of GTG/valine and a G to A transition select ATG/methionine at V106 preferentially in subtype C. This may be a consequence of G to A hypermutation, seen also at other RT valine drug resistance positions. This unique codon use may contribute to a lower threshold for NNRTI resistance in subtype C.

ABSTRACT 54

Search for polymorphic sites in R5 tropic HIV-1 Env and enfuvirtide drug susceptibility in baseline isolates from TORO 1 and TORO 2

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BACKGROUND: Whilst it is evident that the principal determinants for reduced enfuvirtide (ENF) susceptibility reside in HIV-1 gp41, the possibility remains that other determinants within baseline (BL) HIV-1 envelope amino acid sequences may also influence ENF susceptibility for virus from fusion inhibitor-naive patients. We explored such relationships by further mining the TORO 1 and TORO 2 clinical trial databases using univariate and multivariate statistical methods.

METHODS: Paired BL genotype and phenotype data were analysed for 377 R5 tropic recombinants from patients receiving ENF and an optimized background regimen. HIV-1 envelope (complete gp160) amino acid sequences and ENF susceptibility were generated using the GeneSeq™ and PhenoSense™ HIV Entry assays. The relationship of genotype (JRCSF reference) and phenotype at each individual gp160 position was explored using analysis of variance (ANOVA) models. The joint relationship between the entire gp160 sequence and ENF susceptibility was studied using cluster analysis and regression tree modelling.

RESULTS: The fold change in IC_{50} was approximately log-normally distributed with a geometric mean (GM) of 1.58 (range 0.04-37.71) and a standard deviation of 2.64. ANOVA identified 'important' amino acids ($P<0.05$) in both gp41 (for example, G3GT, N42S and V69L) and gp120 (for example, T50I and L444M) that were all associated with >1.5-fold change in susceptibility from the GM. To explore differences among gp41 genotypes, cluster analysis was used to identify two clusters of 365 (Cluster 1) and 12 (Cluster 2) recombinants. Recombinants in Cluster 2 were identified as non-B subtypes. Cluster 2 (GM=0.78, range 0.04-4.44) showed a higher ENF susceptibility ($P=0.01$) than Cluster 1 (GM=1.61, range 0.05–37.71); Cluster 1 had a GM and range similar to the complete dataset. Recombinants in Cluster 2 had higher frequencies of the polymorphisms N42S, E151A, N305D and T130T, which were associated with increased ENF susceptibility. Additionally, the clusters were compared using week 24 efficacy data; patients in Cluster 2 experienced a larger drop in viral load than patients in Cluster 1. To further explore the genotype and phenotype relationship, binary regression tree models were constructed. The optimized tree contained five terminal nodes. Tree branch points occurred at gp41 positions 3ins (insertion at 3), 24 and 42, which also appeared in the 'important' amino acid set obtained by ANOVA.

CONCLUSIONS: Statistical analyses have revealed an association between polymorphic sites in HIV Env and baseline variability of ENF susceptibility in HIV-1 R5 tropic recombinants. Cluster analysis of gp41 viral sequences identified combinations of polymorphisms that were associated with higher ENF susceptibility in the 12 non-B R5 recombinants and a regression tree model illustrated amino acid interactions associated with ENF susceptibility. The significance of these associations is under study.

ABSTRACT 55

Subgroup analysis of baseline susceptibility and early virological response to enfuvirtide in the combined TORO studies

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BACKGROUND: In the TORO 1 & 2 Phase III clinical studies, similar virological suppression occurred across a range of baseline (BL) viral susceptibility to enfuvirtide (ENF) through 24 weeks (Greenberg *et al, 10th CROI*). Viruses from patients who met virological failure (VF) had a 21-fold geometric mean decrease in susceptibility to ENF from BL to VF and exhibited concomitant genetic changes in gp41 amino acids (aa) 36–45. To minimize the confounding effects of the optimized background (OB) regimen, we have further examined the relationship between ENF BL IC_{50} and early virological response for patients with a genotypic sensitivity score (GSS) of 0 or 1. We also determined the correlation between BL GSS and decreases in susceptibility to ENF at VF.

METHODS: TORO 1 & 2 are randomized, open-label, controlled, multicentre, Phase III studies of patients receiving 90 mg twice daily of ENF by subcutaneous injection in combination with an OB regimen. The intent-to-treat population included 661 patients randomized to ENF+OB. Resistance data were generated using the ViroLogic GeneSeq for GSS and the experimental PhenoSense Entry Assay for ENF susceptibility. Correlation between baseline ENF susceptibility and virological response was assessed using non-parametric linear correlation and multiple linear regression analyses.

RESULTS: Of the 612 patients with BL viral phenotype and complete virological response data, 98 had BL GSS=0 and 167 had a GSS =1. There was no correlation with BL ENF IC_{50} and $\log_{10}$ plasma HIV-1 RNA change from BL to week 4 in both subgroups (r=0.15, *P*=0.13; and r=0.06, *P*=0.47, respectively). Similar results were obtained for both subgroups for virological response at week 2, or nadir, and also after adjustment for prognostic factors. Covariance analyses of virological response by BL ENF susceptibility category defined by the geometric mean (GM) IC_{50} ±1 or 2 SD yielded no significant relationships between BL ENF viral susceptibility and virological response (*P*>0.10 for all analyses across both subgroups and all virological response metrics). In an analysis of the relationship between BL GSS and GM decrease in susceptibility to ENF at VF, we found that patients with lower BL GSS had significantly greater decreases in susceptibility to ENF than those with higher GSS (r=–0.21, *P*=0.003).

CONCLUSION: No significant association between BL susceptibility to ENF and early virological response in patients with the lowest GSS was observed, suggesting that other factors may be influencing virological response. The inverse correlation seen between BL GSS and reduced susceptibility to ENF at VF suggests that the antiviral activity of ENF is preserved when combined with additional active agents in the optimized background