



## Session 2

### CONTINUING MEDICAL EDUCATION

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Abstracts presented in this meeting are published as part of *Antiviral Therapy* Volume 9 Issue 4.  
The page number given for each abstract is correct for Issue 4. The correct citation for an abstract is:  
Authors. Title. *Antiviral Therapy* 2004 9:S Page number.



# ANTIVIRAL THERAPY

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**SESSION 2**  
**Mechanisms of HIV Drug Resistance**



**ABSTRACT 19***Antiviral Therapy* 2004; **9**:S25.**Resistance to HIV-1 entry inhibitors may occur by multiple molecular mechanisms***CJ Petropoulos<sup>1</sup>, W Huang<sup>1</sup>, J Toma<sup>1</sup>, S Fransen<sup>1</sup>, S Bonhoeffer<sup>2</sup> and JM Whitcomb<sup>1</sup>*

1 ViroLogic, Inc., South San Francisco, Calif., USA; and 2 ETH Zurich, Zurich, Switzerland

**BACKGROUND:** HIV-1 entry inhibitors represent a diverse new class of antiretroviral agents. Virus entry is a multi-step process involving several virus envelope proteins (gp120, gp41) and host cell receptors (CD4, CCR5, CXCR4). The cascade of protein–protein interactions and conformational changes that mediate virus entry represent novel targets that are functionally distinct from conventional enzymatic targets, i.e. reverse transcriptase and protease. Consequently, one might presume that resistance to entry inhibitors may differ significantly from that of protease and reverse transcriptase inhibitors, and may emerge via alternative mechanisms depending on the specific molecular interaction that is targeted.

**METHODS:** Phenotypic drug susceptibility testing was performed using an envelope pseudo-virus infectivity assay. Drug susceptibility was plotted as percent inhibition vs  $\log_{10}$  drug concentration and defined based on the  $IC_{50}$  and percent inhibition at the highest drug concentration (max % inhibition).

**RESULTS:** We have formulated a model that describes different molecular mechanisms of entry inhibitor escape. Susceptibility to fusion inhibitors is best described by changes in the  $IC_{50}$ . Resistant viruses display log-sigmoid inhibition curves with increased  $IC_{50}$  compared to susceptible viruses, analogous to protease and reverse transcriptase inhibitor resistance. The ability to inhibit 100% of virus replication at high concentrations of fusion inhibitors is consistent with a competitive mechanism of inhibition and escape. In contrast, resistance to inhibitors that block receptor or co-receptor attachment (competitively or allosterically) is often associated with a decreased max % inhibition (plateau) that reflects an uninhibited fraction. The inability to inhibit 100% of virus replication at high drug concentrations is consistent with a non-competitive mechanism of inhibition, that is, binding in the presence of drug. To support this model we will present susceptibility curves

generated using envelope proteins derived from naturally occurring viruses that exhibit variation in max % inhibition as well as viruses that have been selected by exposure to entry inhibitors.

**CONCLUSION:** Resistance to entry inhibitors can occur by multiple molecular mechanisms that may be dependent on the mode of inhibition. Our preliminary data suggest that escape from inhibitors that block receptor or co-receptor binding may occur by acquiring the ability to bind and utilize receptor–inhibitor complexes.

**ABSTRACT 20***Antiviral Therapy* 2004; **9**:S26.**Mutations in HIV-1 RNase H domain confer high-level resistance to nucleoside reverse transcriptase inhibitors and provide novel insights into the mechanism of nucleotide excision-mediated drug resistance***GN Nikolenko<sup>1</sup>, S Palmer<sup>1</sup>, F Maldarelli<sup>1</sup>, JW Mellors<sup>2</sup>, JM Coffin<sup>1</sup> and VK Pathak<sup>1</sup>*<sup>1</sup> HIV Drug Resistance Program, NCI-Frederick, Frederick, Md.; and <sup>2</sup> University of Pittsburgh, Pittsburgh, Pa., USA

**BACKGROUND:** Understanding the mechanisms of drug resistance is critical for developing more effective antiretroviral agents and successful management of therapy. We recently observed that 3'-azido-3'-deoxythymidine (AZT) increased the frequency of reverse transcriptase (RT) template switching and recombination. Based on this observation and our previously described dynamic copy-choice mechanism for retroviral recombination, we now propose a novel mechanism for nucleoside reverse transcriptase inhibitor (NRTI)-mediated abrogation of HIV-1 replication. We postulate an equilibrium between NRTI incorporation, excision of NRTI, resumption of DNA synthesis, and RNase H activity; degradation of the RNA template by RNase H before resumption of DNA synthesis, leads to dissociation of the template and primer strands, termination of reverse transcription and abrogation of HIV-1 replication. In these studies, we tested a prediction of our model that mutations reducing the rate of RNA degradation will confer NRTI resistance by increasing the time period for excision of incorporated NRTIs from terminated primers.

**METHODS:** We determined the sensitivity to AZT, 2,3-didehydro-2,3-dideoxythymidine (d4T), 2',3'-dideoxyinosine (ddI), 2',3'-dideoxy-3'-thiacytidine (3TC), and efavirenz of wild-type RT, RT containing a cluster of thymidine analogue-associated mutations (TAMs) and two RNase H mutations (H539N and D549N). The sensitivities to antiretroviral agents were determined using a single cycle of infection and the firefly luciferase reporter gene.

**RESULTS:** The D549N mutation increased resistance to AZT and d4T 10- and 2.6-fold, respectively, similar

to the increase in resistance observed by the TAMs. Even more dramatically, the H539N substitution increased resistance to AZT and d4T by 180- and 10-fold, respectively, which was nine- and four-fold higher relative to the TAMs. The RNase H mutations resulted in a modest increase in resistance to ddI but did not alter the sensitivity to 3TC or efavirenz. Interestingly, one clone isolated from a patient (Genbank No. 13095143) possessed the D549N substitution in association with other TAMs, suggesting that it may have contributed to drug resistance.

**CONCLUSIONS:** These results support our proposed mechanism for NRTI-mediated abrogation of HIV-1 replication and indicate that mutations in that RNase H domain can confer a high level of resistance to AZT and d4T. Our results strongly suggest that mutations in RNase H could be selected during antiviral therapy and significantly contribute to drug resistance either alone or in combination with NRTI resistance mutations in RT.

**ABSTRACT 21***Antiviral Therapy* 2004; **9**:S27.**Misincorporation of thymidine analogues can increase drug susceptibility***B Marchand and M Götte*

McGill University, Lady Davis Institute-Jewish General Hospital, Montreal, Canada

**BACKGROUND:** HIV-1 reverse transcriptase (RT) is an error-prone enzyme, which frequently incorporates mismatched nucleotides. In view of the fact that G:T mispairs are the easiest to form, we asked whether thymidine analogues may likewise be incorporated opposite template G. Such a scenario may affect susceptibility to these drugs in the context of both wild-type HIV-1 and resistant mutant variants. Thymidine analogue mutations (TAMs) can increase the rates of phosphorolytic excision of incorporated zidovudine-monophosphate (AZT-MP)- and stavudine-monophosphate (d4T-MP); however, it remains to be seen whether misincorporated chain-terminators are removed with similar efficiency.

**METHODS:** We have purified wild-type HIV-1 RT and TAMs-containing mutant enzymes in order to study the efficiency of both incorporation and excision of mismatched AZT-MP and d4T-MP in gel-based assays.

**RESULTS:** Steady-state kinetic analyses revealed that wild-type HIV-1 RT is able to incorporate thymidine-analogues opposite template G with similar high efficiency as seen with the natural counterpart dTMP. In contrast, mismatch formation was hardly observed with lamivudine-MP (3TC-MP) or other non-thymidine analogues. We next studied whether misincorporated AZT-MP or d4T-MP can be excised in the presence of ATP, which can act as a pyrophosphate donor. We found that the wild-type enzyme was neither capable of removing AZT-MP, nor d4T-MP, while TAMs-containing mutant enzymes were able to remove AZT-MP. Moreover, our data also show that the M184V mutation, which has previously been associated with AZT resensitization effects, can diminish the efficiency of excision of G-mispaired AZT-MP when introduced in a background of TAMs. Most importantly, we found that the removal of mispaired d4T-MP was completely blocked, although different enzymes containing mutations at positions 41, 67, 70, 210, 215 and 219

clearly facilitated the excision of d4T-MP opposite the correct template base.

**CONCLUSIONS:** Our biochemical data suggest that thymidine analogue RT inhibitors can be misincorporated, provided that the concentration of the nucleoside triphosphate is sufficiently high. The finding that TAMs-containing mutant enzymes are unable to remove mispaired d4T-MP may help to explain why the increase in phenotypic resistance to d4T is only minimal when measured in cell-based assays.

**ABSTRACT 22***Antiviral Therapy* 2004; 9:S28.**Effects of mutations associated with suppression of zidovudine resistance in HIV-1 reverse transcriptase on removal of tenofovir from blocked primer/template***N Hassani Espili<sup>1</sup>, T Bergroth<sup>1</sup>, A Pavlova<sup>1</sup>, X-W Shao<sup>2</sup>, P Mc Kenna<sup>3</sup>, A Sönnnerborg<sup>1</sup>, J Lennerstrand<sup>1</sup>*

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**BACKGROUND:** It has been reported that the M184V mutation in HIV-1 reverse transcriptase (RT) interferes with ATP mediated excision of zidovudine (AZT)-MP. However, depending on the type of RT assay used, there have been contradictory results regarding the effects of the M184V mutation on excision. We have now investigated the effect of known AZT suppression mutations (M184V and Y181C) for tenofovir unblocking, in a background of multi-nucleoside resistance mutations.

**METHODS:** Site directed RT mutants were constructed carrying: M41L, D67N, K70R, L210W, T215Y (TAM mutant) and M41L, T69S-SG, L210W, T215Y (69S-SG+TAM mutant). The M184V mutation was added to wild-type, TAM mutant and the 69S-SG+TAM mutant, respectively. Also, Y181C was added to the 69S-SG+TAM mutant. Tenofovir resistance was studied by recombinant virus assay (Antivirogram<sup>®</sup>, Virco, Belgium). The ATP mediated excision of tenofovir was studied for the RT mutants, purified in the form of p66/p51 heterodimers, with a non-radioactive RT assay (Cavidi Tech, Sweden), using a long hetero-polymeric DNA template and low concentrations of dNTP.

**RESULTS:** The mutants carrying the 69S-SG insertion were analysed with the recombinant virus assay. The relative increase in IC<sub>50</sub> for tenofovir compared to wild-type virus was: 69S-SG+TAM, 17.8; 69S-SG+TAM+184V, 3.2; and 69S-SG+TAM+181C, 7.5. In studies of purified RT mutants in the RT assay with ATP added, the level of tenofovir-DP IC<sub>50</sub> relative to wild type RT were as follows: 184V, 0.5; TAM, 2.8; TAM+184V, 1.5; 69S-SG+TAM, 10.2; 69S-SG

+TAM+184V, 2.8; and 69S-SG+TAM+181C, 2.5. When conducting the RT assay without ATP, the mutants behaved similar to wild-type enzyme (1 ±0.4), with exception for 69S-SG+TAM, which showed a non-ATP dependent 2.6 times decreased tenofovir incorporation rate. Furthermore, inhibition by dNTP complementary to the next nucleotide position on primer blocked tenofovir and ddA-MP template was studied for the 69S-SG+TAM mutant. Removal of tenofovir was found to be somewhat less sensitive to inhibition by the next dNTP than was removal of ddA-MP.

**CONCLUSIONS:** The M184V mutation seems to reduce the ATP mediated excision of incorporated tenofovir both in a background of wild-type and multiple thymidine analogue-associated mutations. Furthermore, significant suppression of tenofovir resistance was found for both M184V and Y181C in the presence of T69S-SG insertion together with thymidine analogue-associated mutations. Thus, our biochemical data is consistent with cell culture data. This indicates that there would possibly be a benefit with tenofovir therapy, combined with therapy rendering suppression mutations, and these findings should therefore be taken into account in genotyping interpretations.

**ABSTRACT 23***Antiviral Therapy* 2004; **9**:S29.**Kinetic mechanism of HIV-1 reverse transcriptase catalysed AZT excision***N Sluis-Cremer and MA Parniak*

Division of Infectious Diseases, Viral Diseases Unit, University of Pittsburgh School of Medicine, Pittsburgh, Pa., USA

**BACKGROUND:** The phenotypic mechanism of HIV-1 resistance to 3'-azido-2',3'-dideoxythymidine (AZT) involves reverse transcriptase (RT)-catalysed phosphorolytic excision of chain-terminating AZT. To further define the kinetic events involved in the AZT phosphorolytic reaction we have compared the ability of wild-type (wt) and AZT-resistant RT to incorporate and excise phosphorothioate analogues of AZT-triphosphate (AZTTP). The stereo selectivity of an enzyme toward the Rp and Sp isomers of phosphorothioate analogues of dNTP has long been established as a useful tool in probing detailed interactions between the metal ions and nucleotides at the enzyme active site.

**METHODS:** Pre-steady-state kinetic parameters for the incorporation and excision of AZT triphosphate (AZTTP) and the Rp and Sp diastereomers of AZTTP $\alpha$ S were determined for wt and D67N/K70R/T215F/K219Q and M41L/L210W/T215Y mutant enzymes.

**RESULTS:** The wt and AZT-resistant enzymes incorporated AZTTP and Sp-AZTTP $\alpha$ S with equivalent catalytic efficiencies, but were significantly less efficient (~12-fold) in incorporating Rp-AZTTP $\alpha$ S. The decreased catalytic efficiency of incorporation of the Rp-analog by wt and AZT-resistant RT was due to decreased nucleotide affinity ( $K_d$ ) and decreased rate of incorporation ( $k_{pol}$ ). However, none of the enzymes exhibited a significant phosphorothioate elemental effect [defined as  $k_{pol}(AZTTP)/k_{pol}(AZTTP\alpha S)$ ] for the incorporation of these analogues. In contrast to nucleotide incorporation, the mutant RTs were significantly more efficient than wt RT in the phosphorolytic excision of the different analogues of AZT. However, RT was much less efficient in excising both the Rp and Sp isomers of AZTMP $\alpha$ S than in excising AZTMP. Indeed, large phosphorothioate elemental effects (>10) were determined for the excision of Rp and Sp AZTMP $\alpha$ S by wt and AZT-resistant RT.

**CONCLUSION:** The absence of any observed phosphorothioate elemental effects in RT-catalysed incorporation of AZTTP and AZTTP $\alpha$ S is consistent with the hypothesis that the chemistry step is not rate-limiting during nucleotide incorporation. However, the large phosphorothioate elemental effects observed for the excision of both Rp and Sp isomers of AZTTP $\alpha$ S by wt and mutant RT indicates that the rate-limiting step of the phosphorolytic reaction is the chemistry step. The difference in rate-limiting steps for RT catalysed DNA synthesis and phosphorolytic excision suggests that inhibitors selective for the excision process may be possible.

**ABSTRACT 24***Antiviral Therapy* 2004; **9**:S30.**Selective primer unblocking of carbovir, lamivudine, tenofovir and zidovudine by wild-type HIV reverse transcriptase with nucleotide and deoxynucleotide triphosphates***P Gerondelis, MR Underwood and ER Lanier*

Department of International Clinical Virology, GlaxoSmithKline, Research Triangle Park, NC, USA

**BACKGROUND:** Incorporation of nucleoside and nucleotide reverse transcriptase inhibitors (NRTI/NtRTIs) by HIV reverse transcriptase (RT) into primer-template results in chain-termination of DNA synthesis. One type of resistance to the NRTI class is the nucleophilic-excision primer unblocking (PUB) activity of RT, which may compromise the stability of chain termination. Studies of the PUB activity of RT have primarily focused on the purine nucleoside triphosphate (NTP), ATP, as the nucleophilic PUB-substrate. With the exception of ddAMP-terminated primer-template, NRTI/NtRTIs have not been evaluated for their sensitivity to the pyrimidine-NTPs, CTP and UTP. In addition, deoxynucleoside triphosphates (dNTPs) have not been generally studied as substrates for PUB.

**OBJECTIVE:** To evaluate the PUB of NRTI/NtRTIs by measuring sensitivity to the NTP- and dNTP-mediated PUB activity of wild-type (wt) RT.

**METHODS:** Wt RT PUB activity was evaluated with primer-templates chain-terminated by carbovir-MP, lamivudine-MP, tenofovir and zidovudine-MP. NTPs (ATP, CTP, GTP and UTP), and dNTPs (dATP, dCTP, dGTP and dTTP) were used as nucleophiles in the PUB reaction.

**RESULTS:** Carbovir-MP and zidovudine-MP terminated primer-templates were susceptible, while lamivudine was resistant to PUB with NTPs as the nucleophiles. Interestingly, the tenofovir-terminated primer-template was susceptible only to pyrimidine (i.e. CTP and UTP) NTP-based PUB. In addition, tenofovir-terminated primer-template was susceptible to dNTP-PUB, similar to that observed in studies of ddAMP-terminated primer-template (P Meyer *et al.*; *PNAS*,

1998). In contrast, the C-, G- and T-based NRTIs of this study were relatively resistant to dNTP-PUB.

**CONCLUSION:** These studies suggest that NRTIs/NtRTIs have different capacities to maintain chain-termination of wt RT catalysed DNA-dependent DNA polymerization under different experimental conditions, dependent in part upon the structure of the pyrophosphate donor. Generally, the PUB activity of wt RT appears to be dependent upon the nucleophile, the NRTI/NtRTI and the primer-template. Future studies of the PUB activity of both wt and resistant RT should take into consideration the selective PUB by nucleophiles other than ATP, as well as the dependence of this activity on specific primer-template sequence.

**ABSTRACT 25***Antiviral Therapy* 2004; **9**:S31.**The selective advantage of AZT-resistance mutations can be demonstrated *in vitro* but is suppressed at high levels of PP<sub>i</sub>***AJ Smith, PR Meyer, and WA Scott*

University of Miami School of Medicine, Miami, Fla., USA

**BACKGROUND:** *In vivo*, the growth of HIV-1 in the presence of chain-terminating drugs leads to the appearance of resistance mutations in reverse transcriptase (RT), such as the thymidine analogue mutations (TAMs). *In vitro*, TAMs are associated with increased RT-dependent removal of chain terminators. In this study, we wanted to define conditions where TAMs would provide an enzymatic advantage over WT. Experiments were carried out using immune cell extracts or mixtures of ATP and PP<sub>i</sub> to compare the excision by 67N/70R/215Y/219Q RT (RT<sup>AZT</sup>) and wild-type RT (RT<sup>WT</sup>).

**METHODS:** Cell extracts were prepared from purified primary CD4+ T cells. Removal of 2', 3'-dideoxyadenosine monophosphate (ddAMP) or AZTMP was assessed by incubating 3' [<sup>32</sup>P]-labelled ddAMP or AZTMP-terminated primer/template with either RT<sup>AZT</sup> or RT<sup>WT</sup> and cell extract or mixtures of ATP and PP<sub>i</sub>.

**RESULTS:** The rate of excision of [<sup>32</sup>P]ddAMP or [<sup>32</sup>P]AZTMP from terminated primer/templates by HIV-1 RT<sup>AZT</sup> was 3–6-fold greater than RT<sup>WT</sup> when the acceptor substrates were provided from an extract of unstimulated CD4+ T cells (PP<sub>i</sub>, 8 μM). In contrast, no difference was observed in excision rates for RT<sup>WT</sup> and RT<sup>AZT</sup> when the acceptor substrates were provided by an extract of highly stimulated CD4+ T cells (PP<sub>i</sub>, 79 μM). To more specifically define the ATP/PP<sub>i</sub> conditions required for an excision advantage with the mutant enzyme, mixtures of ATP and PP<sub>i</sub> were used as the acceptor substrates. In a mixture of 2.9 mM ATP and submicromolar concentrations of PP<sub>i</sub>, excision of the drugs by RT<sup>AZT</sup> occurred at about 10 times the rate of RT<sup>WT</sup>. This difference between enzymes diminished as the PP<sub>i</sub> levels were increased; no difference was observed when PP<sub>i</sub> was greater than 35 μM.

**CONCLUSIONS:** The most significant biochemical alteration in RT<sup>AZT</sup> in comparison with RT<sup>WT</sup> is enhanced excision activity, yet this difference is not

manifested in a setting where the PP<sub>i</sub>-dependent reaction is greater than 35 μM. This suggests that the selection of AZT resistance occurs when PP<sub>i</sub> concentrations are low, such as in unstimulated CD4+ T cells or macrophages, or under conditions that restrict the ability of PP<sub>i</sub> to participate in the excision reaction at the site of reverse transcription.

Supported by NIH grant AI-39973 and American Heart Association Predoctoral Fellowship 0215087B.

**ABSTRACT 26***Antiviral Therapy* 2004; **9**:S32.**Bisphosphonate inhibitors of nucleoside reverse transcriptase inhibitor excision***MA Parniak<sup>1</sup>, S McBurney<sup>1</sup>, E Oldfield<sup>2</sup> and JW Mellors<sup>1</sup>*

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ATP and/or  $PP_i$  for binding to RT. BPH may represent a new class of antiviral adjuvant agents with potential clinical utility for the treatment of NRTI-resistant HIV infection.

This work was supported in part by NIH AI52010 and AI60452 (to MAP).

While nucleoside reverse transcriptase inhibitors (NRTI) are highly effective anti-HIV agents, viral resistance limits their therapeutic longevity. Resistance to 3'-azido, 3'-deoxythymidine (AZT) arises from phospholytic excision of the incorporated NRTI, which enables continued viral DNA synthesis. This excision is catalysed by reverse transcriptase (RT) and is enhanced by specific mutations in RT termed TAMs, or thymidine analogue mutations. Most highly drug experienced patients are infected with TAM-HIV and it is increasingly apparent that TAMs impact on resistance to other NRTI as well. Specific inhibitors of phospholytic excision could be very useful therapeutically by preventing the removal of incorporated NRTI, thereby restoring activity of NRTI such as AZT against TAM-containing HIV-1. Optimally, such inhibitors should not reduce incorporation of the NRTI during reverse transcription, which would antagonize their antiretroviral activity. We have identified certain bisphosphonates (BPH) as selective inhibitors of NRTI excision. BPH-218 inhibits RT-catalysed ATP-dependent and  $PP_i$ -dependent excision of AZTMP *in vitro* ( $IC_{50} \approx 2 \mu M$ ), but has minimal potency against RT-catalysed DNA synthesis ( $IC_{50} > 50 \mu M$ ), showing that BPH-218 is selective for inhibition of phosphorolysis. BPH-218 alone is only weakly active against replication of TAM-HIV ( $EC_{50} \approx 50 \mu M$ ). However, when BPH-218 and AZT are added in combination, significant enhancement of antiviral activity is noted. As an example, AZT is only marginally active against TAM-HIV ( $EC_{50} \approx 3.5 \mu M$  compared to  $0.3 \mu M$  against wild-type virus). In the presence of  $50 \mu M$  BPH-218, the antiviral activity of AZT against TAM-HIV is dramatically increased ( $EC_{50} \approx 0.05 \mu M$ ), suggesting that BPH-218 restores activity of AZT against this virus. BPH-218 also enhances activity of AZT against wild-type HIV-1, implying that NRTI excision is a factor in replication of wild-type virus in the presence of NRTI. Molecular modeling suggests that BPH-218 may compete with

**ABSTRACT 27**

*Antiviral Therapy* 2004; **9**:S33.

**Impaired rescue of chain-terminated DNA synthesis associated with the L74V mutation in HIV-1 reverse transcriptase**

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**OBJECTIVE:** The M184V, K65R, and L74V mutations in the RT of HIV-1 share a number of characteristics that include: 1. similar discriminatory mechanisms in regard to incorporation of relevant NRTI inhibitors of reverse transcription; 2. all result in diminished RT processivity and viral replicative capacity; 3. they are associated with diminished error rates during reverse transcription as measured in biochemical assays. In addition, both M184V and K65R have been shown to cause a reduction in the efficiency of the excision reaction associated with RT. Our study was performed to assess whether L74V might also have this effect.

**METHODS:** Recombinant wt and L74V-containing RTs were chromatographically purified as previously described. The rescue of chain-terminated DNA synthesis was studied at a single template position using an assay in which a pre-hybridized duplex of PPT-57 template and PPT-18 primer was incubated with either wt or mutated RT in a buffer containing 10  $\mu$ M dCTP and 10  $\mu$ M zidovudine triphosphate (ZDV-TP). The excision of the ZDV-terminated primer was initiated by adding a mix containing 3.5 mM ATP, 100  $\mu$ M dTTP, 10  $\mu$ M dGTP and 100  $\mu$ M ddATP. DNA synthesis was monitored in time course experiments.

**RESULTS:** We found that the presence of the L74V mutation in RT caused an approximate 50% reduction in the efficiency of excision of ZDV-monophosphate from newly synthesized viral DNA. In addition, ATP-dependent unblocking of the ZDV-terminated primer and the continuation of DNA synthesis were compromised when the L74V mutation was present. Furthermore, wt enzyme was able to unblock 50% of the ZDV-terminated primer after  $\approx$ 35 minutes in this reaction, whereas L74V-containing RT required  $\approx$ 85 min to accomplish this task. Studies were also performed with recombinant RTs containing K65R or M184V,

and, in general, it appears that the effect of M184V on excision exceeds that of L74V and K65R, which behave similarly to each other in this regard.

**CONCLUSION:** These findings add to the evidence that K65R, L74V and M184V should be regarded as a group with regard to shared mechanisms of resistance to NRTIs and their consequences on RT enzymatic function.

**ABSTRACT 28***Antiviral Therapy* 2004; **9**:S34**Thermodynamic analysis of capravirine binding to HIV reverse transcriptase and inhibition of DNA polymerase and RNase H activities***S Rajendran, JQ Hang, Y Yang, Y Li, S Tsing, J Barnett, N Cammack, A Ahene and K Klumpp*

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Structurally diverse non-nucleoside reverse transcriptase inhibitors (NNRTI) can bind to a defined NNRTI binding pocket on the polymerase domain of HIV reverse transcriptase (HIV-RT). At present, the relationship between HIV-RT binding affinity and inhibitory potency of NNRTIs is unclear. Capravirine (S-1153) was used as a prototype NNRTI to determine the thermodynamic parameters of the binding interaction with HIV-RT. Capravirine binding to HIV-RT was enthalpy driven at temperatures above 21°C and entropy driven at temperatures below 12°C. The binding was associated with potent inhibition of DNA polymerase activity and partial inhibition of polymerase independent RNase H activity through long-range inhibition at the RNase H domain. Binding affinity of capravirine to the HIV-RT Y181C point mutant protein was reduced six-fold and associated with a significant loss in binding enthalpy (4.4 kcal/mol), which was partly compensated by an entropy gain. Binding to the K103N point mutant protein was increased 2.4-fold as compared to wild-type. The double mutant K103N/Y181C showed an intermediate loss of binding enthalpy (3.5 kcal/mol), but a highly reduced binding affinity (16.4-fold), due to lower entropy compensation as compared to Y181C. The thermodynamic binding parameters for capravirine binding were consistent with different protein interactions in wild-type and mutant proteins. The inhibitory potency of capravirine on RNase H and polymerase activity was highest with wild-type protein and reduced with mutant proteins. However, there was no direct correlation between the effect of resistance mutations on protein binding, polymerase and RNase H inhibition. The molecular understanding of the relationship between NNRTI binding and enzyme inhibition will improve prediction of inhibitory potencies from *in silico* binding models.

**ABSTRACT 29***Antiviral Therapy* 2004; **9**:S35.

**Novel mechanisms involved in non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance of both the subtype D HIV-1 isolate and reverse transcriptase derived from a drug-naive Ugandan**

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**BACKGROUND:** Recent studies have shown that ARV treatment of non-subtype B HIV-1 infections can select for novel drug resistance mutations or substitutions considered 'rare' in drug-resistant subtype B isolates. We have recently identified a highly NNRTI-resistant subtype D HIV-1 isolate (D14-UG) from a drug-naive Ugandan. NNRTI-resistant mechanism(s) was examined using mutated subtype D virus and recombinant RT.

**METHODS:** HIV-1 isolates derived from HIV infected, treatment-naive Ugandan infants were propagated and tested for sensitivity to antiretroviral drugs. To investigate the possible sites responsible for resistance to NNRTIs, the reverse transcriptase (RT) coding region was sequenced and compared to that of NNRTI-resistant and sensitive subtype A, B and D HIV-1 isolates. Subtype D RTs containing substitution mutations at the putative NNRTI-resistant sites were then cloned (1) into the NL4-3 vector to test for NNRTI sensitivity with the Phenosense assay and (2) into RT expression vectors to study the mechanisms of this resistance.

**RESULTS:** D14-UG displayed high-level resistance to nevirapine in PBMC (>2000-fold) and in MT4 cultures (~800-fold), but weaker resistance to delavirdine (~13-fold) and efavirenz (~8-fold) in MT4 cultures. D14-UG did not contain the classic amino acid substitutions conferring NNRTI resistance (e.g. Y181C, K103N, G190A) but did have sites associated with drug resistance, I135L, T139V, and V245T. Phenosense assays confirmed that I135L and/or V245T mutations might

confer resistance to nevirapine. Preliminary analyses of heterodimeric RT (p66/p51) consisting of every NNRTI-resistant D14-UG/sensitive D subunit combination suggests that the L135 and V139 residues are enacting their effect through p51 and T245 through p66.

**CONCLUSION:** Native sequences responsible for this NNRTI resistance (L135, V139, and T245) were extremely rare in any HIV-1 subtype but nonetheless stable considering wild-type fitness. Since nevirapine is ARV of choice for blocking perinatal transmission and for combination therapy in Uganda, any NNRTI resistance in the drug-naive population is quite alarming.

**ABSTRACT 30**

*Antiviral Therapy* 2004; **9**:S36.

**Structural studies of benzophenone/HIV-1 RT complexes: insights into the potency of the next generation NNRTIs against WT and mutant HIV-1**

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**BACKGROUND:** Approximately 250 analogues of a benzophenone scaffold have been synthesized and assayed for anti-HIV activity. Lead compounds were selected based on approximately equivalent potency against WT, Y181C and K103N mutants. Optimized compounds maintain activity against many NNRTI-resistant strains (e.g. the  $IC_{50}$  for 678248 is <10 nM against 22 of 24 mutant viruses including nine double mutants). We have used crystallographic structures of RT-inhibitor complexes to aid in the design of compounds to define the binding mode of prototype compounds. A compound from this series, 678248X, is the active agent from the prodrug 695634G now in clinical trials.

**METHODS:** WT and site-directed mutant RTs were produced in *E. coli* and purified by ion-exchange procedures. RT/inhibitor complex crystals were obtained by co-crystallization and X-ray data collected at synchrotron sources. Data were indexed and integrated with DENZO, and data were merged with SCALEPACK. The molecular orientation and position in the unit cell were determined using rigid-body refinement with CNS. The structures were refined with CNS using positional, simulated annealing and individual B-factor refinement with bulk solvent correction and anisotropic B-factor scaling. Model rebuilding was done using O.

**RESULTS:** We have determined 14 structures of WT, K103N, L100I, and V106A/Y181C HIV-1 RT with various benzophenone NNRTIs, the best to 2.1 Å resolution. Novel features in the binding of these NNRTIs to RT include an intramolecular hydrogen bond in the inhibitor. Tyr181 is in a 'down' position which is unusual for such high affinity compounds. Also, there

are minimal inhibitor interactions with Tyr181. The inhibitors extend out of the NNRTI pocket in the region of Pro236. There are subtle changes in inhibitor conformation as a result of different mutations.

**CONCLUSIONS:** The potency of the benzophenones against WT and mutant RTs is accomplished by a wide range of contacts with the protein as well as the stabilizing effect of hydrogen bond formation within the compound itself. The minimal contacts with Tyr181 and the relative flexibility of these compounds that allow subtle rearrangements are factors that contribute to the remarkable resilience of the benzophenone series to such a wide range of resistance mutations.

**ABSTRACT 31***Antiviral Therapy* 2004; **9**:S37.**Dinucleoside polyphosphates are novel inhibitors of HIV-1 reverse transcriptase with increased potency against enzymes containing AZT-resistance mutations***P Meyer, A Smith, S Matsuura and W Scott*

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**BACKGROUND:** One of the greatest challenges facing prolonged antiretroviral therapy is the development of drug resistance. AZT resistance mutations, 41L, 67N, 70R, 210W, T215F/Y, 219Q, alone, or in combination with a dipeptide insertion in HIV-1 reverse transcriptase (RT) confer resistance to most nucleoside analogues in common use today. It is therefore important to devise strategies to prevent and/or suppress the appearance of AZT resistance mutations.

AZT resistance is associated with increased nucleotide-dependent removal of chain-terminators from blocked DNA ends. The products of this reaction are an unblocked primer terminus and a dinucleoside polyphosphate. It is quite possible that dinucleoside polyphosphates could serve as substrates and/or inhibitors of HIV-1 RT. We therefore prepared several homodimeric dinucleoside tetraphosphates containing dideoxynucleosides (ddNp<sub>4</sub>ddN) and tested their ability to inhibit DNA synthesis by WT or mutant HIV-1 RT.

**METHODS:** Incorporation of radioactive deoxynucleotide into M13 P/T by HIV-1 RT was measured in the absence or presence of varying concentrations of either ddNTP or ddNp<sub>4</sub>ddN.

**RESULTS:** DNA polymerization in the presence of either ddTTP or ddTp<sub>4</sub>ddT led to a concentration-dependent decrease in incorporated dNTPs due to incorporation of the inhibitor resulting in chain-termination. WT RT was over 70-fold less sensitive to inhibition by ddTp<sub>4</sub>ddT (IC<sub>50</sub>~10 µM) than by ddTTP (IC<sub>50</sub>~140 nM). However, RT containing the 67N/70R/215Y/219Q mutations was only fivefold less sensitive to inhibition by ddTp<sub>4</sub>ddT (IC<sub>50</sub>~0.9 µM) than to ddTTP (IC<sub>50</sub>~0.2 µM) and RT containing the 41L/69S-AG/210W/211K/214F/215Y mutations was equally sensitive to inhibition by ddTp<sub>4</sub>ddT (IC<sub>50</sub>~0.35 µM) and ddTTP (IC<sub>50</sub>~0.32 µM).

**CONCLUSION:** AZT resistance mutations, alone or in combination with a 69-dipeptide insertion, conferred an increased susceptibility to inhibition by dinucleoside polyphosphates containing chain-terminating nucleoside analogues. Dinucleoside polyphosphates served as substrates for DNA polymerization by HIV-1 RT and were incorporated much more readily by HIV-1 RT containing AZT resistance mutations. These results are very encouraging for development of novel, specific inhibitors of HIV-1 RT with increased efficacy against AZT-resistant RT which could be used to suppress the appearance of AZT-resistance mutations.

This work was supported by amfAR fellowship: 70567-31-RF, American Heart Predoctoral Fellowship 0215087B and NIH grant AI 39973.

**ABSTRACT 32**

*Antiviral Therapy* 2004; **9**:S38.

**Natural resistance of HIV-2 to zidovudine**

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**BACKGROUND:** Zidovudine is widely used to treat persons infected with HIV-1, and is also used to treat persons infected with HIV-2. Recent studies suggest differences in the responses to zidovudine treatment between HIV-1- and HIV-2-infected persons.

**METHODS:** We evaluated the antiviral activity of zidovudine on HIV-2 using multiple approaches including *in vitro* selection experiments, analysis of growth kinetics in the presence of zidovudine and phenotypic testing (MT-4/MTT assay). A total of five wild-type (WT) HIV-2 viruses, including three clinical isolates (GB122HU, CDC77618, and CDC310319) and two laboratory-adapted strains (ROD and CBL-20/H9) were used in the analysis. For comparison, four control WT HIV-1 strains (HXB2, CC/H9, IIB/H9, and LAI) and one HIV-1 mutant carrying the 215S mutation (HXB2<sub>S215</sub>) were evaluated in parallel.

**RESULTS:** All five HIV-1 strains acquired zidovudine resistance mutations after three to six passages with zidovudine or an increase in the concentration of zidovudine of four- to 32-fold. Among these viruses, the fastest selection of resistance was seen in HXB2<sub>S215</sub>, which acquired S215Y (one nucleotide change only) at passage 3 after only 17 days in culture. In contrast, none of the five HIV-2 viruses that naturally have S215 acquired S215Y or any other RT mutation during 10 passages with zidovudine (1024-fold increase in zidovudine concentration). In the presence of zidovudine+didanosine, both zidovudine and didanosine resistance mutations were selected in HIV-1, while only didanosine resistance mutations (K65R and M184I) were selected in HIV-2. All HIV-2 viruses replicated efficiently in a concentration of zidovudine that was 2800-fold higher than the EC<sub>50</sub> value for HIV-1, and were about 200-fold less sensitive to zidovudine than HIV-1. In contrast, HIV-2 and HIV-1 were equally susceptible to didanosine, a finding consistent with the selection of K65R and M184I in HIV-2 during passages with zidovudine+didanosine.

**CONCLUSION:** Our results demonstrate that the activity of zidovudine on HIV-2 is lower than previously thought. The poor antiviral activity of zidovudine and the fact that most non-nucleoside RT inhibitors are not effective against HIV-2 emphasize the need for novel antiretroviral drugs for HIV-2.

**ABSTRACT 33***Antiviral Therapy* 2004; **9**:S39.**Potential reversion pathways of Tyr 215 to Thr in HIV-1 reverse transcriptases having a dipeptide insertion between codons 69 and 70: consequences for zidovudine and stavudine resistance***T Matamoros<sup>1</sup>, S Franco<sup>2</sup>,  
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**BACKGROUND:** Dipeptide insertions between codons 69–70 in HIV-1 reverse transcriptase (RT) are usually associated with thymidine analogue resistance mutations, such as T215Y. Previously, we characterized a multidrug-resistant RT, which contained a Ser-Ser insertion and several drug-resistance mutations including T215Y. This enzyme (SS RT) had reduced susceptibility to zidovudine, stavudine and other nucleoside analogues [Mas *et al.* (2000) *EMBO Journal* **19**:5752]. Zidovudine and stavudine resistance was facilitated by the RT's increased ability to unblock thymidine analogue-terminated primers through a ribonucleotide-mediated reaction. The insertion alone was not sufficient to confer unblocking activity to wild-type BH10 RT, but its removal from the SS RT produced a significant reduction of this activity [Mas *et al.* (2002) *Journal of Molecular Biology* **323**:181]. Now, we have analysed the effects of residues at codon 215 on ATP- and pyrophosphate (PPi)-mediated removal of zidovudine- and stavudine-monophosphate by SS and BH10 RTs.

**METHODS:** Recombinant SS RTs with substitutions Y215T, Y215S or Y215N, and a BH10 RT containing the Ser-Ser insertion plus T215Y were obtained. We determined their chain terminator removal activity and their ability to form stable ternary complexes in the presence of the next complementary dNTP. The effects of mutations were also assessed in phenotypic assays with recombinant HIV-1.

**RESULTS:** Substituting Thr, Ser or Asn for Tyr-215 in the SS RT led to zidovudine and stavudine resensitiza-

tion in phenotypic assays, through the loss of ATP-mediated removal activity. Substituting Tyr-215 had a larger effect than deleting the dipeptide insertion. The presence of both the insertion and the mutation T215Y in the wild-type BH10 RT conferred significant ATP-mediated removal activity, and moderate resistance to zidovudine in phenotypic assays. However, resistance levels and unblocking activities were lower than those observed with SS RT. Both Tyr-215 and the insertion affect RT-DNA/DNA-dNTP ternary complex formation, an effect not detected in the presence of foscarnet.

**CONCLUSIONS:** Tyr-215 plays a critical role in ATP-dependent nucleotide excision, when a Ser-Ser insertion is present in the viral RT. Our data reveal that one-nucleotide changes at position 215 are sufficient to abrogate thymidine analogue resistance in isolates carrying the Ser-Ser insertion, thereby facilitating zidovudine and stavudine sensitization.

This work was supported in part by grants from FIPSE (36207/01) and FIS through Red Temática Cooperativa de Investigación en SIDA (Red G03/173) and projects 01/0067-01 and -02.

**ABSTRACT 34***Antiviral Therapy* 2004; **9**:S40.**The HIV-1 RT mutation H208Y combined with T215Y causes hypersusceptibility to efavirenz***SA Clark<sup>1</sup>, NS Shulman<sup>2</sup>, R Bosch<sup>3</sup> and JW Mellors<sup>1</sup>*

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**BACKGROUND:** Hypersusceptibility (HS) to non-nucleoside reverse transcriptase inhibitors (NNRTI) improves virological response to NNRTI-containing regimens. NNRTI HS is associated with nucleoside RT inhibitor (NRTI) mutations, especially those conferring resistance to 3'azidothymidine. Recent CART and logistic regression analyses of a large genotype-phenotype dataset by Shulman *et al.* (11th CROI) showed that the NRTI mutations most strongly associated with NNRTI HS are T215Y, H208Y and V118I. We then sought to determine the phenotypic effects of these mutations alone and in combination on the susceptibility of infectious molecular clones to efavirenz.

**METHODS:** Mutations were introduced into the RT gene of the HIV-1<sub>LAI</sub> infectious clone by PCR-based mutagenesis. The following mutants were produced: V118I alone, H208Y alone, T215Y alone, H208Y+T215Y, V118I+T215Y, D67N+K70R+T215Y+K219Q, D67N+K70R+T215F+K219Q, and M41L+L210W+T215Y. Virus was produced by transfection of MT-2 cells and susceptibility to efavirenz was determined using a single cycle replication assay in P4R5 cells. The fold change (FC) in drug susceptibility was calculated compared with wild type HIV-1<sub>LAI</sub>. The cutoff used for NNRTI HS was a FC in efavirenz susceptibility of less than 0.4.

**RESULTS:** The H208Y+T215Y mutant showed marked HS to efavirenz in four consecutive assays (average FC of 0.24), whereas mutants with H208Y alone or T215Y alone were not HS (average FC of 0.69 and 0.87, respectively). The V118I+T215Y showed variable susceptibility to efavirenz (average FC of 0.35 to 0.93) in five consecutive assays with a mean FC of 0.60. The D67N+K70R+T215Y or F+K219Q mutants were not HS to efavirenz (average FC of 0.85 and 0.58, respectively) but the M41L+L210W+T215Y mutant was HS to efavirenz (average FC of 0.28).

**CONCLUSIONS:** NNRTI HS to efavirenz can be demonstrated *in vitro* with as few as two codon changes in RT: H208Y + T215Y. This will facilitate investigation of the biochemical and structural basis of hypersusceptibility to NNRTIs.

**ABSTRACT 35***Antiviral Therapy* 2004; **9**:S41.**Effect of cell cycle arrest on HIV susceptibility to RT inhibitors***S Wurtzer, AJ Hance and F Clavel*

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**BACKGROUND:** *In vivo*, most of potential HIV target cells are non-dividing. HIV drug susceptibility, however, is generally evaluated in actively dividing primary or tumour cells. Because reverse transcription can be affected by cellular activation, differentiation and division, we have examined the effect of cell cycle arrest on HIV susceptibility to nucleoside analogues and NNRTIs.

**METHODS:** P4 indicator cells were treated with etoposide, a topoisomerase II inhibitor, or with aphidicolin, a polymerase  $\alpha$  inhibitor. The DNA content of treated cells was examined by FACS. Drug susceptibility was evaluated using a single-cycle Tat-dependent  $\beta$ -galactosidase colourimetric assay or a real-time PCR-based viral DNA synthesis assay.

**RESULTS:** Cell cycle arrest in S/G2 was seen in >90% of cells treated with 1  $\mu$ M etoposide and in >70% of cells treated with 1  $\mu$ M aphidicolin. HIV infectivity was modestly increased (two- to threefold) by S/G2 arrest. In contrast, S/G2 arrest induced a remarkable decrease in HIV susceptibility to AZT, with an exponential relationship between the proportion of cells in S/G2 and the fold increase in AZT  $IC_{50}$  ( $r^2=0.62$  for etoposide and  $=0.95$  for aphidicolin). A similar increase in  $IC_{50}$  was seen using a real-time PCR viral DNA elongation assay, indicating that an effect on Tat-dependent transcription was not involved. The increase in  $IC_{50}$  seen in S/G2 arrested cells was strongest for AZT (42-fold, mean of five experiments) but more modest for other drugs: d4T=tenfold, 3TC=sevenfold, nevirapine= threefold. In RT mutants, the increase in AZT  $IC_{50}$  (relative to that in dividing cells) was 24-fold with 41L-215Y, 36-fold with 151M, but only fivefold with 184V and sevenfold for 181C. Since the two latter mutations are known to impair pyrophosphorolysis of AZT-terminated DNA, improved primer rescue could account for the effect of cell cycle arrest on AZT susceptibility.

**CONCLUSION:** Cell cycle arrest in S/G2 has marked effects on HIV susceptibility to nucleoside analogues, notably AZT. These results emphasize the importance

of cellular proliferation in HIV drug susceptibility and underscore the need to evaluate HIV resistance in natural target cells under physiological conditions of activation and proliferation.

**ABSTRACT 36***Antiviral Therapy* 2004; **9**:S42.**Novel HIV drug resistance mechanism leading to protease inhibitor (PI) resistance in response to a high genetic barrier PI *in vitro****M Nijhuis*<sup>1</sup>, *NM van Maarseveen*<sup>1</sup>, *P Schipper*<sup>1</sup>, *IW Goedegebuure*<sup>1</sup>, *G Heilek-Snyder*<sup>2</sup>, *N Cammack*<sup>2</sup> and *CAB Boucher*<sup>1</sup>

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**BACKGROUND:** HIV drug resistance mechanisms described so far involve mutations in the viral target gene of the drug. The concept of high genetic barrier (HGB) drugs is based on the insight that the variation in the viral quasispecies is limited to one or two mutations. During HGB therapy the virus requires far more target gene mutations than are normally present in the quasispecies making resistance escape difficult. Nevertheless, one can hypothesize that HIV, like other microorganisms could exploit alternative resistance mechanisms. To investigate the potential of alternative drug resistance mechanisms, we have performed *in vitro* selection experiments using a HGB PI.

**METHODS:** To identify novel drug resistance pathways, multiple *in vitro* selection experiments were performed. Therefore, HIV-1 HXB2 was cultured in SupT1 cells in the presence of increasing levels of the HGB PI Ro-033-4649. Genotypic and phenotypic drug susceptibility analysis was performed.

**RESULTS:** After more than a year in the presence of increasing drug concentrations we were able to select a viral population displaying six- to eightfold resistance to the HGB drug Ro-033-4649 and to other PI. Interestingly, the viral population harboured no mutations in the viral protease. Sequence analysis of the viral gag gene demonstrated several nucleotide mixtures [1126, (matrix), 1460, (capsid) and 2180 (p6gag)]. Two nucleotide changes were observed at position 2095 and 2099. Interestingly, repeat experiments also demonstrated nucleotide changes in the same regions, nucleotide: 2093, 2098 or 2099. These changes are located in the ribosomal frameshift site, responsible for the production of the pol gene products. In addition, the same region codes for the transframe protein (TFP) and the p7/p1, p7/TFP and TFP/p6pol protease cleavage sites.

**CONCLUSION:** *In vitro* selection experiments using the HGB protease inhibitor Ro-033-4649 resulted in the selection of a viral population displaying six- to eightfold PI resistance without mutations in the viral protease. This clearly demonstrates that a novel alternative drug resistance mechanism was identified. Interestingly, reproducible nucleotide changes in the region coding for the ribosomal frameshift site, the TFP and protease cleavage sites were observed that may be responsible for the reduced drug susceptibility.

**ABSTRACT 37***Antiviral Therapy* 2004; **9**:S43.**‘Wide open’ 1.3 Å structure of the multidrug-resistant HIV-1 protease represents a novel drug target***LC Kovari<sup>1</sup>, JF Vickrey<sup>1</sup>, P Martin<sup>1</sup>, G Proteasa<sup>1</sup>, E Hales<sup>1</sup>, K Kondapalli<sup>1</sup>, Y Jimenez<sup>1</sup>, J Martinez<sup>2</sup>, R MacArthur<sup>2</sup>, Z Wawrzak<sup>3</sup>, MA Winters<sup>4</sup> and TC Merigan<sup>4</sup>*

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**BACKGROUND:** The goal of this study is to examine structural changes in highly mutated multidrug-resistant (MDR) HIV-1 protease variants and use the crystallographic information to design new inhibitors.

**METHODS:** The HIV-1 variants contain codon mutations at positions 10, 36, 46, 54, 63, 71, 82, 84 and 90 that confer resistance to protease inhibitors, and the crystal structures of the MDR proteases were solved to high resolution. We screened a random peptide library to identify heptapeptides binding with high affinity to the MDR HIV-1 protease variants.

**RESULTS:** Major differences between the wild-type and a mutant solved to 1.3 Å include a structural change initiated by the M36V mutation and amplified by additional mutations in the flaps of the protease, resulting in a ‘wide open’ structure that represents an opening that is 8 Å wider than to the ‘open’ structure of wild-type protease. A second structural change is triggered by the L90M mutation that results in reshaping the 23–32 segment equally important for inhibitor binding. A third key structural change of the protease is due to the mutations from longer to shorter amino acid side chains at positions 82 and 84. The overall result of the mutations is an active site expansion and reduced inhibitor binding. The high resolution crystal structure of a MDR HIV-1 protease variant reveals a scaffold containing about 100 water molecules that holds the active site cavity ‘wide open’. We initiated phage display experiments with a random peptide library and have identified several heptapeptides that bind with high affinity to the multidrug-resistant

HIV-1 protease variants. The tightly binding heptapeptides represent lead compounds for the synthesis of peptidomimetic inhibitors.

**CONCLUSION:** A series of HIV-1 MDR protease crystal structures reveal a domino effect of structural changes with multiple mutations altering the shape of the highly mutated HIV-1 protease. The crystal structures of the ‘wide open’ MDR HIV-1 protease represent a new ensemble of targets for the design of novel protease inhibitors. This work was supported by NIH, amfAR and the Michigan Life Sciences Corridor (GM62990, 106457-34-RGGN and #1798).

**ABSTRACT 38**

*Antiviral Therapy* 2004; **9**:S44.

**Susceptibility to saquinavir and atazanavir in highly protease inhibitor (PI) resistant HIV-1 is caused by lopinavir-induced drug resistance mutation L76V**

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**BACKGROUND:** Samples from seven patients were susceptible to saquinavir and atazanavir despite resistance to all other PI. Genotypically, all samples were expected to be resistant to all PI, indicating a new resensitization mechanism in highly PI-resistant viruses.

**METHODS:** Genotypic and phenotypic drug resistance were determined by sequencing (Viroseq) and by a recombinant virus assay, respectively. Sequence alignment has been performed to identify unique mutational patterns. The geno2pheno database and 246 samples from the BMS-045 study were screened for mutations identified in the first seven samples. Previous samples and clinical follow-up data were analysed.

**RESULTS:** All seven samples had 5–10 drug resistance associated mutations. All samples had mutations at codon 82 and mutation L76V, which has only been reported to be selected by lopinavir *in vitro*. Three of five patients with available drug histories had been treated with lopinavir, one with lopinavir and indinavir, and one with amprenavir. L76V was not present in any other sample of the geno2pheno database, but in 11 patients from the BMS-045 study, nine of them developed L76V during lopinavir treatment. Phenotypic resistance of L76V samples to atazanavir and saquinavir ranged from 0.4- to 52-fold and 0.3- to 19-fold (median 2.3- and 1.7-fold), respectively. Only two samples exhibited >10-fold resistance to atazanavir (51-fold) and saquinavir (19-fold) despite L76V. They carried additional 10 and 11 other mutations including V32I and V77I, which were not present in other L76V harbouring viruses. Clinical response data of three patients, who had been treated with saquinavir or atazanavir containing regimen, showed viral load decreases of >2.2 log, >2.3 log and 3.1 log within 12 weeks, receiving one, three and three active drugs, respectively.

**CONCLUSION:** L76V was not seen previously in clinical samples and seems to revert resistance to saquinavir and atazanavir in viruses with high level PI resistance. The mutation was mainly selected by lopinavir and was detected in 7% of patients in the lopinavir arm of BMS 045 study. Resensitization was highly clinically relevant in all three cases with follow-up. Therefore, the presence of L76V provides unexpected salvage therapy options. Further studies are needed to investigate high level resistance to atazanavir and saquinavir despite the presence of L76V.

**ABSTRACT 39***Antiviral Therapy* 2004; **9**:S45.**Resistance-related polymorphisms in HIV-1 non-B subtype protease influence the resistance pathway and amplify resistance to protease inhibitors***SH Qari, D Pieniazek and W Heneine*

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**BACKGROUND:** The protease (PR) in wild-type (WT) HIV-1 non-B subtypes has frequently occurring polymorphisms at multiple codons (10, 20, 36, 71 and 77) involved in the resistance to PR inhibitors (PIs). We evaluated the impact of such polymorphisms on the evolution of PI resistance *in vitro*.

**METHODS:** An HIV-1 subtype F (HIV-1<sub>F</sub>) and an HIV-1<sub>CRF02</sub> (HIV-1<sub>CRF02</sub>) having PI resistance-related polymorphisms L10V-K20R-M36I and L10V-M36I-V77I, respectively, were identified in two drug-naive African patients. PR were cloned from these viruses, two WT subtype B viruses (HIV-1<sub>B</sub>) and seven site-directed mutants from the HIV-1<sub>CRF02</sub> PR in which codons 10V, 36I and 77I were serially changed to L10, M36 or V77. Recombinant viruses were generated from the cloned PR and then passaged sequentially in indinavir (IDV). The kinetics, pathways, and levels of resistance to IDV and other PIs were compared.

**RESULTS:** All 11 baseline viruses replicated efficiently and all had WT susceptibility to PIs. IDV-selected mutations emerged faster in HIV-1<sub>F</sub> (44 days) and HIV-1<sub>CRF02</sub> (44 days) compared to HIV-1<sub>B</sub> (53 and 57 days). Distinct patterns of mutations were selected in HIV-1<sub>CRF02</sub> (M46I, I84A, N88S and M89T) and HIV-1<sub>F</sub> (G48V, I54T, A71V and V82T) and included many unique mutations (M89T, I54T, I84A, N88S). In contrast to the parental HIV-1<sub>CRF02</sub> virus, V82F was the primary mutation selected in all the HIV-1<sub>CRF02</sub>-derived mutants indicating that L10V, M36I and V77I influenced the genetic pathway of IDV resistance in this virus. The levels of resistance to PIs conferred by V82F in these mutants were substantially increased in the presence of V77I or L10V.

**CONCLUSIONS:** Diverse combinations of known and novel mutations confer resistance to PI in HIV-1<sub>F</sub> and

HIV-1<sub>CRF02</sub> isolates indicating the need for improved algorithms for interpreting resistance test results. The L10V, M36I and V77I affect the genetic pathway of resistance in HIV-1<sub>CRF02</sub> likely by modulating the fitness and resistance of the selected mutants. Both V77I and L10V amplify PI resistance and thus may play a role in the rapid emergence of high levels of clinical PI resistance.

**ABSTRACT 40***Antiviral Therapy* 2004; **9**:S46.**Predictors of selection of K65R: tenofovir use and lack of TAMs***L. Valer, L. Martín-Carbonero, A. Corral, C. de Mendoza and V. Soriano*

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**BACKGROUND:** Mutation K65R at the HIV-1 RT gene reduces the susceptibility to tenofovir (TDF) and in less extent affect the activity of other nucleoside analogues. Data about the rate of K65R and its association with other nucleoside analogue resistance mutations are scarce.

**METHODS:** The presence of K65R was examined in a large database of genotypic drug resistance reports collected over the last 5 years from drug-naive ( $n=216$ ) and treatment-experienced ( $n=1630$ ) HIV+ patients in Spain.

**RESULTS:** A total of 53 specimens showed K65R after testing plasma samples from 1846 different individuals (overall rate, 2.9%). None of drug-naive individuals showed K65R. The prevalence of K65R increased over time in pre-treated patients: 1/156 (0.6%) in 1999, 3/464 (0.6%) in 2000, 0/167 (0%) in 2001, 11/366 (3%) in 2002, 29/399 (7.3%) in 2003 and 9/78 (11.5%) during the first trimester of 2004. While K65R was identified mainly in subjects failing TDF-based combinations, in 10 individuals it appeared without exposure to TDF. These subjects were receiving d4T+ddI (4), ABC+3TC (2), d4T+3TC (2), d4T+ABC (1) and ddC (1). The remaining 43 specimens carrying K65R were collected from subjects failing different TDF-based combinations: TDF+ddI (32), TDF+ABC (6), TDF+3TC (4) and TDF alone (1).

The presence of TAMs was significantly lower in patients with K65R with respect to the rest. Accordingly, the rate of zero, one and two TAMs was 4.6%, 5.6% and 2.3%, respectively. Moreover, only K70R and/or K219E were seen along with K65R. No patients with  $\geq 3$  TAMs had K65R. In the multivariate analysis, the presence of K65R was inversely associated with the number of TAMs (OR=0.54; 95% CI=0.24–0.54) and the presence of T215Y/F (OR=0.09; 95% CI=0.01–0.9), while it was positively associated with the presence of Q151M (OR=4.82; 95% CI=1.76–13.22) and the total number of nucleoside analogue resistance mutations excluding TAMs

(OR=1.59; 95% CI=1.25–2.02). M184V accompanied K65R in 24 cases (45%).

**CONCLUSION:** The selection of K65R is significantly associated with the use of TDF. However, other nucleoside combinations including d4T, ddI and/or ABC, may favour its selection as well, although more rarely. Reciprocal exclusion of K65R and TAMs may reflect that they represent divergent and antagonistic pathways driving to nucleoside analogue resistance. The frequent selection of M184V along with K65R result in a novel multi-nucleoside resistance genotype.

**ABSTRACT 41***Antiviral Therapy* 2004; **9**:S47.***In vitro* antiviral interactions among tenofovir, abacavir, lamivudine and didanosine***CL Tremblay*<sup>1,2</sup>, *F Giguel*<sup>1</sup>, *H Dong*<sup>3</sup>, *TC Chou*<sup>3</sup> and *MS Hirsch*<sup>1</sup>

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**OBJECTIVES:** Triple-nucleoside regimens containing tenofovir have been associated with an unexpected high virological failure rate in several clinical trials. Viral isolates obtained from patients with failing regimens often contain K65R and/or M184V mutations. Other combinations with decreased clinical efficacy such as zidovudine plus stavudine have been shown to have *in vitro* antagonistic interactions resulting from competition for phosphorylation pathways. We evaluated whether high clinical failure rates associated with the emergence of resistance mutations could be attributed to unfavorable drug interactions (antagonism) lead to diminished potency of these regimens.

**METHODS:** Using peripheral blood mononuclear cells infected with an HIV-1 clinical isolate, 14aPre, we evaluated antiviral interactions between tenofovir, abacavir, didanosine and lamivudine. Single drugs or combinations of drugs were added to each well, using a fixed ratio among drugs and serial dilutions. Cultures were maintained for 4 days. HIV-1 p24 antigen was measured in supernatant fluid harvested at the end of culture. Results were analysed using the median-effect principle and are expressed as combination indices (CI) with CI values <0.9=synergy, between 0.9 and 1.1=nearly additive effects and >1.1=antagonism.

**RESULTS:** The mean 50% inhibitory concentrations for tenofovir, abacavir, didanosine and lamivudine were 0.66 µM, 0.42 µM, 1.67 µM and 0.13 µM, respectively. No drug toxicity was observed at the highest concentrations used. Drug interactions ranged from synergy to additivity for all combinations tested with CI values ranging from 0.70–0.95 at 50% inhibitory concentrations to 0.62–0.79 at 95% inhibitory concentrations.

**CONCLUSIONS:** Utilizing a clinical wild-type HIV-1 isolate, *in vitro* antiviral drug interactions between tenofovir, abacavir, didanosine and lamivudine are favourable, and cannot explain the diminished efficacy observed in the clinical setting. Other mechanisms such as the low genetic barrier for the emergence of the K65R and M184V mutations need to be explored.

**ABSTRACT 42***Antiviral Therapy* 2004; **9**:S48.**Prevalence of HIV-1 GAG cleavage site mutations in patients failing protease inhibitors in the GART Study (CPCRA 046)***JD Baxter<sup>1</sup>, RE Leduc<sup>2</sup>, A DuChene<sup>2</sup>, H Leong<sup>3</sup>, MR Furtado<sup>4</sup>, OV Petruskenskaya<sup>4</sup> and the CPCRA 046 Study team for the Terry Bein Community Programs for Clinical Research on AIDS*

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**BACKGROUND:** GAG protease cleavage site (CS) mutations have been associated with enhanced phenotypic resistance to protease inhibitors and increased viral fitness of HIV-1. To better understand the prevalence of HIV-1 GAG CS mutations in patients failing protease inhibitors, plasma from the GART study was examined. The GART study was one of the early trials demonstrating the utility of genotyping for patients failing therapy.

**METHODS:** Stored HIV-1 RNA samples from 148 patients at study entry were tested using Celera Diagnostics ViroSeq<sup>®</sup> HIV Genotyping System v2.0 with reagent and software modifications to sequence and examine the GAG region codons 350–488. Prevalence of GAG cleavage site mutations and insertions were determined.

**RESULTS:** Patients were failing combination therapy most commonly with indinavir (79/148) or nelfinavir (47/148). Baseline median HIV-1 RNA was 27100 copies/ml (range 5200–347400). Approximately 75% of patients had major protease mutations, most frequently 30N, 46I/L, 82A/F/T, and 90M. The median number of GAG mutations per patient was seven (range 2–16). The commonly identified GAG mutations occurred in the GAG reading frame (RF) p2/NC CS at codons 372–376 and 380 (134/148), NC/p1 CS at codon 431 (42/148) and p1/p6 CS at codons 449, 451 and 453 (69/148). In the GAG-POL RF commonly identified gag mutations were in TPF/p6 and p6/PR CS at codons 3, 7 and 52–54 (146/148). Insertions, which varied in length from 1–14 amino acids, were detected in 40% of the 148 samples (59/148) and were found frequently in the p6 region: in GAG RF codons

454–461 (20/148) and in GAG-POL RF codons 22–27 (25/148). Analysis of the amino acid sequences of the insertions revealed the appearance of additional GAG CS and some cellular factor binding sites, such as a proline-rich motif PTAP known to recruit TSG101. Further, we analysed the association of gag CS mutations and insertions with major protease mutations. Mutation A431V (NC/p1 CS) was associated with protease mutations 46I/L ( $P < 0.0001$ ) and 82A/F/T ( $P < 0.0001$ ).

**CONCLUSION:** GAG cleavage site mutations and insertions were common in HIV-1 from patients failing protease inhibitor therapy. This analysis demonstrates associations between the presence of GAG cleavage site mutations and recognized protease mutations.

**ABSTRACT 43***Antiviral Therapy* 2004; **9**:S49.**No evidence for stavudine resistance due to nevirapine-selected mutation Y181C in HIV-1 reverse transcriptase in a large genotype/phenotype database***K Korn, B Schmidt and H Walter*

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**BACKGROUND:** Two recent publications (Blanca *et al.*, *JBC* 2003, Baldanti *et al.*, *AIDS* 2003) claim that the mutations Y181C/I in HIV-1 reverse transcriptase (RT), which cause high-level resistance to the non-nucleoside RT inhibitor nevirapine also confer cross-resistance to the nucleoside analogue stavudine. In one of these papers, a Y181C clone is described to show a more than eightfold reduced stavudine susceptibility compared to wild-type virus in a recombinant virus assay. This would have significant implications for drug resistance interpretation and for treatment strategies. Especially, there would be a major concern for programs aimed at increasing the availability of antiretroviral therapy in resource-poor countries, since such programs heavily rely on low-cost production of combination drugs containing both stavudine and nevirapine.

**METHODS:** Since these results were very surprising to us, we scanned our database of more than 500 samples from HIV-1-infected patients containing RT sequences and corresponding phenotypic data from recombinant virus assays for stavudine to determine if a similar effect could be observed in our data.

**RESULTS:** From a total of 511 samples, 427 samples without Y181C showed a median resistance factor for stavudine of 1.8 (IQR 1.0–2.9) compared to a median resistance factor of 2.7 (IQR 1.3–5.4) for 84 samples with Y181C. This difference was due to the strong linkage of Y181C with known nucleoside analogue mutations. Three samples containing Y181C without any known nucleoside analogue resistance mutations had resistance factors of 1.0, 1.1, and 1.4. Of 59 isolates with a D4T resistance factor >8, only 16 had the Y181C mutation, whereas all had other mutational patterns previously associated with stavudine resistance (Q151M complex:  $n=20$ , insertions:  $n=4$ , multiple ‘thymidine analogue mutations’ with or without mutations at positions 75 or 118:  $n=35$ ).

**CONCLUSION:** In our data set, we cannot find any evidence for D4T resistance conferred by the Y181C mutation in HIV-1 RT. Therefore, we conclude that there is no reason to withhold stavudine from patients because of Y181C viruses, that interpretation systems for stavudine do not have to be changed and that there is no particular risk of failure with treatment regimens containing both stavudine and nevirapine.