



Session 1 Abstracts

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PLENARY ABSTRACT 1

Mechanisms of HIV-1 diversity

B Korber

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HIV has evolved many ways to vary, particularly in the envelope gene where immune escape for neutralizing antibody responses and features of cellular tropism are determined. The rate of base substitution is extraordinarily high, and modelling the evolution of these changes enables phylogenetic reconstructions of HIV viruses. But HIV has other mechanisms of variation that can result in immune evasion including recombination, shifting glycosylation patterns, insertions and deletions, and action at a distance through conformational change. Each of these mechanisms of diversity will be reviewed in the context of what can be learned from the database, with particular focus on the immunological impact of HIV variation, studies where recombination was not seen (surprising in the context of some of the recent studies documenting high levels of recombination), and patterns of glycosylation site variation. New tools available at the Los Alamos HIV database will be discussed.

SESSION 1
Resistance to New Antiretroviral Agents

ABSTRACT 2**Elvucitabine: potent antiviral activity demonstrated in multidrug-resistant HIV infection**

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BACKGROUND: Elvucitabine (ELV) is an L-nucleoside analogue with potent *in vitro* anti-HIV activity, particularly against strains with resistance mutations to numerous other drugs, including nucleosides, all non-nucleosides (NNRTI) and HIV protease inhibitors (PI). ELV exhibits reduced potency against strains harbouring the M184V mutation [lamivudine (3TC)-resistant], but the inhibitory concentration (IC_{50}) remains within the range of achievable plasma concentrations. This multicentre study was designed to assess the anti-HIV activity of ELV in an otherwise unchanged combination of highly active antiretroviral therapy (HAART) in individuals with the M184V mutation.

METHODS: Adults receiving triple, 3TC-containing HAART therapy whose genotype included the M184V mutation were eligible; full genotypic profiles were available for each subject. Subjects had HIV RNA levels of 1000–30000 copies/ml and CD4 counts >200 cells/ μ l. Minimum baseline safety parameters were required, together with stable clinical disease and no active AIDS-defining conditions. Subjects were randomized to receive blinded ELV 50 or 100 mg/day or continued 3TC 300 mg/day in a ratio of 25:25:10, while continuing the other two agents of their HAART regimen. Subjects were treated for 28 days; HIV RNA and safety determinations were measured weekly. Open-label ELV was available for subjects who wished to continue ELV after the 4 weeks.

RESULTS: Fifty-nine subjects were randomized, 56 initiated treatment and 46 completed 28 days of therapy. Mean age was 43.5 years (range 21–65), mean CD4 count was 471 cells/ μ l (range 120–960) and mean plasma HIV RNA level was 10300 copies/ml (range 120–75000; Roche Amplicor 1.5[®] assay). Prior antiretroviral therapies included all commercially available agents. HIV genotypes (HIV Genosure[™]) revealed \geq one primary nucleoside resistance mutation (in addition to the required M184V) and primary resistance mutations to either the entire class of NNRTIs or PIs

in all subjects. Potent anti-HIV activity was observed in both ELV groups: mean declines at 28 days of -0.67 and $-0.78 \log_{10}$ copies/ml in the 50 and 100 mg groups, respectively, as compared with an increase of $+0.01 \log_{10}$ copies/ml in the 3TC group ($P < 0.0001$ for both comparisons). Baseline genotypes were not clearly correlated with antiviral activity. Myelosuppression was the only study-related adverse event observed and was generally associated with the ELV 100 mg dose group.

CONCLUSIONS: ELV demonstrated potent anti-HIV activity in patients with multidrug-resistant HIV, comparable or superior to other potential 'salvage' therapies, with a convenient single daily oral dose. Doses of 50 and 100 mg/day were similarly potent; the safety profile of 50 mg daily was more desirable than 100 mg. Further study with doses up to 50 mg daily is warranted to identify the optimum dose for long-term clinical development of ELV in this population with limited treatment options.

ABSTRACT 3**Antiviral activity of SPD754 against clinical isolates of HIV-1 resistant to other nucleoside reverse transcriptase inhibitors***RC Bethell¹, N Parkin² and Y Lie²*

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BACKGROUND: SPD754 is a heterocyclic cytidine analogue with anti-HIV activity against a range of wild-type viruses and viruses resistant to other nucleoside reverse transcriptase inhibitors (NRTIs). In order to further characterize the resistance profile of SPD754 antiviral activity has been assessed against a panel of 215 clinical isolates of HIV-1 with genotypes associated with resistance to NRTIs.

METHODS: All viruses were selected from the Virologic library of clinical isolates. Groups of viruses were selected with defined mutation patterns at codons 41, 67, 70, 184, 210, 215 and 219 of reverse transcriptase, with at least 10 different viruses per group. Viruses with 69 insertion mutations, Q151M, or mutations at positions 65, 69, 74 or 75 were excluded. The antiviral activity of zidovudine (AZT), lamivudine (3TC), didanosine (ddI), abacavir, tenofovir and SPD754 against these viruses was assessed using the PhenosenseTM assay.

RESULTS: Viruses with no resistance-associated RT mutations had median fold-change (FC) to SPD754 of 0.9 (range 0.7–1.1, $n=20$). Viruses with mutations at codons 67 and 70 of RT remained sensitive to SPD754 (median FC 1.0 vs 5.5 for AZT, $n=10$). Addition of mutations at codons 219, 215 and 41 progressively decreased the sensitivity of HIV-1 to SPD754, with viruses containing mutations at 41, 67, 70, 215 and 219 having a median FC of 1.3 (range 0.9–1.9, $n=15$). The same group of viruses showed 108-fold reduced sensitivity to AZT, and 3.2-, 3.0-, 1.3- and 2.5-fold resistance to 3TC, abacavir, ddI and tenofovir, respectively. Mutations at codons 41 and 215 of RT were associated with 1.2-fold reduced sensitivity to SPD754 (range 0.7–1.7 vs 33 for AZT, $n=20$). Addition of mutations at codons 210, 67 and 219 progressively decreased the sensitivity of HIV-1 to SPD754, with viruses containing mutations at 41, 67, 210, 215 and 219 having median FC of 1.8 (range 1.2–2.6, $n=15$). The same group of viruses showed 438-fold reduced

sensitivity to AZT, and 4.8-, 4.5-, 1.4- and 3.6-fold resistance to 3TC, abacavir, ddI and tenofovir, respectively. Pairwise comparison of separate groups of viruses with different background genotypes (wild-type, 41 and 215, and 67, 70 and 219) with and without mutations at codon 184 showed that in all three cases, the addition 184 was associated with a 1.8-fold reduction in sensitivity to SPD754. Pairwise regression analysis of log-transformed FC values showed a high degree of cross resistance with abacavir and ddI ($r^2=0.79$ and 0.74 , respectively), but a substantially lower level of cross resistance with AZT and tenofovir (r^2 approximately 0.5).

CONCLUSION: The presence of up to five mutations at codons 41, 67, 70, 210, 215 and 219 of RT confers no more than a twofold reduction in median sensitivity to SPD754. Mutation at codon 184 of RT is associated with a 1.8-fold reduction in sensitivity to SPD754.

ABSTRACT 4**Synthesis and anti-HIV activity of enantiomerically pure D-FDOC**

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The potential of 1',3'-dioxolanyl nucleosides as anti-HIV drugs has been recognized for some time. In the mid-1990s, we both enantiomers of 2',3'-dideoxy-5-fluoro-oxacytidine (FDOC) were synthesized. Although both enantiomers exhibited good potency against HIV, they both appeared to be too toxic to be used clinically. Subsequent to these initial studies, we discovered that the sample of the less toxic D-enantiomer that was tested actually contained 3–5% of its significantly more toxic L-counterpart. This then raised the interesting question as to whether the observed toxicity was inherent to D-FDOC or resulted from the presence of small quantities of its more toxic enantiomer. To answer this question, we used preparative chiral chromatography to obtain several grams of optically pure D- and L-FDOC, respectively. With these two enantiomers in hand, we could, for the first time, unambiguously evaluate their anti-HIV activity, cytotoxicity and resistance profile. The results of these studies indicated that D-FDOC not only showed excellent potency (EC_{50} and EC_{90} values in primary human lymphocytes infected with HIV-1_{LAI} are 0.04 μ M and 0.26 μ M, respectively) and low toxicity (>100 μ M in uninfected primary human lymphocytes), but also exhibited no cross resistance to lamivudine, zidovudine or nevirapine. In addition, in primary mouse bone marrow cells, D-FDOC showed no increase in lactic acid production even at 300 μ M. In contrast, treatment with either L-FDOC and zalcitabine resulted in a >300-fold increase in lactic acid production relative to untreated control. Furthermore, in HepG2 cells (5-day assay), D-FDOC displayed no toxicity when tested up to 100 μ M, whereas its L-counterpart demonstrated significant toxicity at 1.4 μ M. While these data, taken in aggregate, clearly indicate that our original toxicity determinations were compromised by the presence of small quantities of the toxic L-enantiomer, they also suggest that clinical evaluations of D-FDOC should only be performed with materials that contain very little, if any, of the L-enantiomer. Herein, we describe a syn-

thetic approach that employs a tandem kinetic resolution/chiral salt crystallization protocol for preparing the D-enantiomer of FDOC in high enantiopurity. In addition, we report conditions that allow for the racemization and recycling of the unwanted butyrate ester of the L-enantiomer of FDOC.

ABSTRACT 5

***In vitro* induction of HIV variants with reduced susceptibility to elvucitabine (ACH-126,443, β -L-Fd4C)**

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BACKGROUND: Elvucitabine is an L nucleoside analogue designed to improve on anti-HBV and anti-HIV potency of lamivudine while maintaining the improved safety profile with respect to mitochondrial toxicity over D-nucleoside analogues. *In vitro*, elvucitabine has demonstrated potent activity against a wide range of HIV-1 variants, including all clinic subtypes and mutant strains carrying common mutations resistant to nucleosides, non-nucleosides and protease inhibitors. This study was designed to evaluate *in vitro* resistance induction with elvucitabine.

METHODS: CEM-SS cells were infected with HIV-1_{LAI} and were passaged at increasing concentrations of elvucitabine. The phenotypes and genotypes of viruses collected during each passage were determined at the end of induction. The mutations were back-cloned into the wild-type HIV-1_{LAI} backbone and the genotypes and phenotypes associated with the mutants were identified.

RESULTS: After 159 days encompassing seven passages in elvucitabine concentrations ranging from 0.05 to 2.0 μ M, variants of HIV_{LAI} with reduced susceptibility were isolated. The EC₅₀^{LAI} of the resultant variant was 0.2 μ M, ~10-fold increase over the wild-type. Genotypic sequencing of the variant isolated from each sequential passage revealed that two mutations in the reverse transcriptase (RT) had emerged simultaneously, M184I and D237E. The mutation at the 184 locus has been described after exposure to lamivudine, but is usually rapidly replaced in the presence of that drug with M184V. No 'switch' to M184V could be detected in the presence of elvucitabine. The contribution of the two mutations to viral resistance was confirmed after they were introduced into the wild-type backbone and the resulting virus was tested for its susceptibility to elvucitabine. Preliminary cross resistance study indicated that the mutant was cross resistant to lamivudine, as expected, but not to other nucleoside inhibitors tested. The role of each mutation in the gen-

eration of elvucitabine resistance was examined with respect to growth fitness and resistance after they were introduced back into the wild type backbone individually. A computational model was developed to explain the observed phenomenon that the mutation at 184 confers only moderate resistance to elvucitabine but total resistance to lamivudine.

CONCLUSION: An *in vitro* resistance induction study was performed with elvucitabine. The resulting variant with reduced susceptibility carried mutations at amino acid 184 (M to I) and 237 (D to E). The D237E mutation has not been described previously and its role in the generation of resistance variant is under investigation. The double mutation conferred moderate resistance to elvucitabine (approximately 10-fold shift in EC₅₀). A computational model is proposed to explain the phenomenon.

ABSTRACT 6

Antiviral activity of the nucleoside Reverset following single oral doses in HIV-1-infected patients

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BACKGROUND: Reverset (RVT, β -D-2',3'-dideoxy-2',3'-dideoxy-5-fluorocytidine) is a nucleoside analogue that retains activity against lamivudine- and zidovudine-resistant HIV-1 *in vitro*. It is being developed under a US IND for the treatment of HIV-1 in nucleoside reverse transcriptase inhibitor (NRTI)-experienced patients.

METHODS: RVT was administered as a single oral dose to antiretroviral treatment-naive HIV-1-infected males (six per cohort) at doses of 0, 10, 25 or 50 mg as buffered solutions or 0, 50, 100 or 200 mg as enteric-coated tablets. Blood samples, obtained over a 48 h period for pharmacokinetic analysis, were analysed for HIV RNA levels using quantitative real-time RT-PCR.

RESULTS: Viral loads dropped significantly over 48 h with an average reduction of $0.4 \pm 0.2 \log_{10}$ for all dose levels. The antiviral response over the 48 h was not dose-dependent. At the 10 mg dose, a $0.42 \pm 0.2 \log_{10}$ was observed ($P=0.005$), while at 100 mg a similar effect of 0.44 ± 0.17 were noted ($P=0.0007$). The dose-independent significant antiviral response was also observed at the 24 h time-point, with an average reduction of 0.11 ± 0.11 ($P=0.03$). The 12 h time-point was not significantly different from the baseline. The mean plasma C_{\max} values ranged from 1 to 8 μ M. A maximal effect of viral inhibition was obtained at the lowest C_{\max} (0.87 mM), which is equivalent to the *in vitro* EC_{90}^{\max} value for wild-type virus. All available viral strains were sequenced in the reverse transcriptase gene before ($n=18$ strains) and after ($n=18$ strains) the treatment schedule. Wild-type viral genotype was found in all but one subject who showed at baseline the following genotype: L41+N103+C181+W210+D210, suggesting past exposure to zidovudine (possibly in another host) and non-nucleoside analogues. This subject received the 10 mg, placebo and 25 mg treatment

schedule, and showed a 0.61, -0.05 and 0.43 \log_{10} drop, respectively. The viral genotype for all subjects remained unchanged at the end of the treatment schedule.

CONCLUSIONS: RVT reduced the HIV-1 viral load after a single dose by a mean of 0.4 \log_{10} for all doses tested. One subject infected with a mutant virus responded as well as subjects infected with wild-type virus.

ABSTRACT 7**Characterization of baseline and treatment-emergent resistance mutations following 1 year of therapy on an entirely once a day regimen including emtricitabine**

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BACKGROUND: While highly active antiretroviral therapy has significantly impacted the treatment of HIV infection, complexity of administration and toxicity issues can lead to suboptimal adherence and the development of resistance mutations. Emtricitabine (FTC) is a new once-daily nucleoside analogue reverse transcriptase inhibitor (NRTI) with potent activity against HIV. In study FTC-301 we evaluated the safety and antiviral activity of a simple, non-thymidine, once-daily regimen as first-line treatment for therapy-naive HIV-infected patients.

METHODS: 571 antiretroviral-naive patients with plasma HIV-1 RNA (VL) >5000 copies/ml were randomized in a 1:1 ratio to receive 200 mg FTC once daily or stavudine (d4T) twice daily in combination with once-daily didanosine (ddI) and once-daily efavirenz (EFV). VL was measured at baseline and every 4 weeks to week 48. Virological failure (VF) was defined as never achieving <400 copies/ml or rebound >400 copies/ml on two consecutive measurements. The incidence of VF was compared between treatment arms using a two-sided exact test. Patients who experienced VF had sequence analysis performed on the HIV RNA isolated from plasma at baseline and at the time of VF.

RESULTS: Virological failure occurred in 6% (17) of patients in the FTC arm and 15% (41) of patients in the d4T arm ($P=0.0014$). Genotypic data were obtained for 57/58 VF (16/17 FTC, 41/41 d4T) at baseline and at time of VF. In these 57 patients experiencing VF, the incidence of mutations was 69% in the FTC arm and 85% in the d4T arm. Development of non-nucleoside reverse transcriptase inhibitors (NNRTI) mutations accounted for the majority of mutations in both subgroups of VF, 63% FTC and 85% d4T. Development of the M184V mutation

occurred only in FTC failures, 31%, while development of thymidine analogue-associated mutations (TAMs) occurred only in d4T failures, 15%. One VF in the FTC arm (6%) and three VF in the d4T arm (7%) developed ddI-associated mutations. In these antiretroviral-naive patients who failed therapy, the prevalence of mutations at baseline was relatively high; 38% FTC and 32% d4T. NNRTI-associated mutations (31% FTC, 22% d4T) and TAMs (19% FTC and 15% d4T) were the most prevalent baseline mutations in each group. No FTC (M184I/V) or ddI (L74V/K65R) mutations were present in either cohort at baseline. The incidence of VF remained statistically higher in the d4T arm after censoring patients with baseline TAMs (d4T arm), $P=0.0132$, or baseline K103N (FTC arm) and K103N/TAMs (d4T arm), $P=0.0182$.

CONCLUSIONS: These results demonstrate that a regimen containing once daily FTC was statistically superior to twice-daily d4T, in a background of once-daily ddI plus EFV, with a significantly lower rate of VF with fewer mutations, even after adjusting for the prevalence of baseline mutations.

ABSTRACT 8

Antiviral activity of TMC125, a potent next-generation non-nucleoside reverse transcriptase inhibitor (NNRTI), against >5000 recombinant clinical isolates exhibiting a wide range of NNRTI resistance

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BACKGROUND: TMC125 is a potent next-generation non-nucleoside reverse transcriptase inhibitor (NNRTI), active against wild-type as well as NNRTI-resistant HIV-1. *In vitro* selection experiments have demonstrated an increased genetic barrier to the development of resistance to the compound. TMC125 also showed *in vivo* antiviral activity in patients with documented phenotypic NNRTI resistance in a 7-day Phase IIa trial. In the present study, we determined the antiviral activity of TMC125 in more than 5000 clinical isolates submitted for phenotypic resistance testing in 1999–2000 (panel A) and 2001–2003 (panel B). The antiviral activity of TMC125 on these isolates was compared to the currently approved NNRTIs.

METHODS: Recombinant clinical isolates were constructed according to the Antivirogram[®] method. Phenotypic and genotypic analyses were performed by the Antivirogram[®] and *VirtualPhenotype*[™] assays, respectively. Data analysis was performed using SAS and Spotfire DecisionSite software.

RESULTS: The prevalence of mutations at 15 NNRTI resistance-associated positions (98, 100, 101, 103, 106, 108, 179, 181, 188, 190, 225, 230, 236, 238 and 318) was compared between panels A ($n=2065$) and B ($n=3545$). A relative increase in frequency of mutations K101P, K103S, V106A, V179I and Y188L was observed in panel B. No significant change was observed for V106M, a recently described NNRTI resistance mutation. TMC125 inhibited 91% of all samples ($n=5610$) with an $EC_{50} < 10$ nM, while efavirenz only inhibited 67% at 10 nM. The number of samples resistant to at least one of the three current NNRTIs (defined as a fold change in $EC_{50} > 10$) was 1050 (51%) and 1580 (45%) for panels A and B,

respectively. Most (79%) of these 2630 samples were resistant to efavirenz and 69% were resistant to all current NNRTIs. At 10 nM, TMC125 inhibited 80% of the samples resistant to at least one NNRTI and 76% of the samples resistant to all current NNRTIs. The corresponding percentages for efavirenz were 29% and 9%, respectively. In addition, TMC125 inhibited 78% of the EFV-resistant subset ($n=2066$), at 10 nM. The median EC_{50} of TMC125 for samples containing V106M, which was observed together with at least one other NNRTI resistance mutation, was 1.6 nM ($n=11$). Sixty-three percent of the samples harbouring four NNRTI resistance mutations still had an EC_{50} below 10 nM for TMC125, whereas 70% of the samples with only two mutations had an EC_{50} above 10 nM for efavirenz.

CONCLUSIONS: TMC125 is a potent next-generation NNRTI, with activity against most of recently circulating strains of HIV, including samples that are resistant to all marketed NNRTIs. The antiviral activity against class-associated NNRTI resistance together with the increased genetic barrier to development of resistance, are unique features of TMC125.

ABSTRACT 9

Characterization of resistance before and after short-term therapy with TMC125 in patients with documented non-nucleoside reverse transcriptase inhibitor resistance

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BACKGROUND: TMC125-C207 was an open-label Phase IIa study to evaluate the antiviral activity, safety and tolerability of TMC125 in patients with documented non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance. Sixteen HIV-1-positive subjects on a failing antiretroviral regimen (viral loads above 2000 HIV RNA copies/ml), consisting of two NRTIs and an NNRTI, and with phenotypically confirmed resistance to efavirenz, were enrolled. They received TMC125 (900 mg twice daily) for 7 days as a substitution for the failing NNRTI; NRTI therapy remained unchanged. TMC125 was highly active in patients infected with NNRTI-resistant HIV-1, as demonstrated by a median viral load drop of 0.89 log RNA copies/ml from baseline to day 8, and was well tolerated. In the present study, comparative phenotypic and genotypic resistance data from screening, baseline and end of therapy have been analysed.

METHODS: Drug susceptibility profiles were determined using the Antivirogram[®] assay and mutational patterns were determined using VirtualPhenotype[™]. Both resistance determinations were performed on plasma samples taken at screening (within 49 days prior to treatment start), baseline (day 1) and end of therapy (day 8) time-points.

RESULTS: The population in this study had a wide range of mutations associated with resistance to NNRTIs, including changes at positions 98, 100, 101, 103, 108, 179, 181, 188, 190, 225 and 238. The median number of NNRTI mutations was two (range 1–4), at both screening and baseline. One patient acquired a partial NNRTI mutation (Y181C/Y) between screening and baseline (in the absence of TMC125). Between baseline and end of therapy, three patients acquired additional changes in the RT gene: these were all mutant/wild-type mixtures (K101Q/K, K103N/K and

V189V/I). The appearance of these partial mutations was not associated with an increase in fold resistance for TMC125 or any of the current NNRTIs. At baseline, there was no correlation between the fold resistance values of nevirapine or efavirenz with TMC125. The median (range) fold resistance values at baseline for nevirapine, efavirenz and TMC125 were 128 (58–136), 116 (5–820) and 2.2 (0.5–8.5), respectively. At the end of therapy, the median (range) fold resistance values for nevirapine, efavirenz and TMC125 were 120 (46–146), 103 (4–974) and 2.6 (0.8–11.6), respectively. Neither NNRTI fold resistance values at baseline nor the presence of mutations associated with NNRTI resistance at baseline were predictive for response in this group of patients.

CONCLUSIONS: TMC125 is effective in suppressing resistant HIV strains from patients failing on an NNRTI-containing regimen and with phenotypic evidence of resistance. No evidence has been found that TMC125 selected for increased resistance during 7 days of treatment. By overcoming class-associated NNRTI resistance, TMC125 is considered to be a next-generation NNRTI.

ABSTRACT 10**The identification of active site mutations that confer resistance to structurally diverse inhibitors of HIV-1 integrase strand transfer supports a general mechanism of phosphotransferase inhibition**

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Inhibitors of the integrase strand transfer reaction have been shown to be effective inhibitors of integration and HIV-1 replication *in vitro* and *in vivo*. The diketone S-1360 and the naphthyridine L-870810 are the first compounds in this novel class to enter into clinical studies in HIV-1-infected patients. In the presence of human serum, S-1360 and L-870810 inhibit HIV-1 replication in cell culture with IC₉₅s of 12000 and 100 nM, respectively. Although structurally distinct, the compounds have identical mechanisms of action and compete for binding to the same site. In an attempt to understand the potential for cross resistance within this new class of agents we have used a variety of diketones and naphthyridines related to S-1360 and L-870810, respectively, to select resistant HIV-1 variants *in vitro*. Mutations in integrase were identified upon sequencing each of the resistant virus populations. The observed mutations were introduced into an isogenic virus background and the recombinant viruses were then used to evaluate their respective susceptibility to a panel of integrase inhibitors from each structural class. All of the inhibitors we evaluated selected for similar but not identical mutations in integrase. In each case the mutations were localized to the integrase active site proximal to the residues that coordinate the catalytic metal ions (D64, D116 and E152). Although similar mutations were selected with a variety of structurally diverse compounds, marked differences were observed in the susceptibility profiles of these inhibitors both within and between each structural class. Some compounds including S-1360 were affected by a wide range of different mutations, while other inhibitors, including L-870810, displayed an overlapping but more restricted resistance profile. In the course of this extensive evaluation we also identified compounds that exhibited apparently discrete resistance profiles and integrase inhibitors that were effective against all of the resistant variants we have identified to date. These analyses demonstrate it is possible to identify

integrase inhibitors with distinct resistance profiles, however there appears to be a significant potential for cross resistance between many such compounds (such as, S-1360 and L-870810) despite their apparent differences in structure. Localization of the critical determinants for resistance to the integrase active site is consistent with biochemical studies that demonstrate these inhibitors function by sequestering the active site metals in integrase and the observation that similar compounds have been identified which inhibit mechanistically-related metal-dependent phosphotransferases such as HIV-1 RNase H.

ABSTRACT 11

Mappicine inhibitors of HIV-1 reverse transcriptase-associated ribonuclease H*MM Hossain¹, W Zhang², D Curran³ and MA Parniak¹*

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INTRODUCTION: Current therapeutics for treatment of HIV infection are directed at two viral targets, protease and the DNA polymerase activity of reverse transcriptase (RT). Viral resistance to these therapeutics is an increasingly serious problem, thus identification of drugs directed at new HIV targets is essential. The ribonuclease H (RNase H) activity of RT resides in a subdomain that is spatially distinct from the RT DNA polymerase active site. The viral RNase is absolutely essential for retroviral replication and thus presents a logical target for antiviral intervention. However, while numerous inhibitors of HIV-1 RT DNA polymerase activity have been identified (including 10 drugs in current clinical use), very few inhibitors of RNase H have been identified, and none are in pre-clinical development. We have developed a fluorescence-based assay for HIV RT RNase H that enables high-throughput screening for inhibitors of this potential target. Using this screening assay, we have discovered that certain analogues of mappicine are potent inhibitors of HIV-1 RT-associated RNase H.

OBJECTIVE: To characterize the antiviral properties of mappicine analogue inhibitors of HIV-1 RT-associated RNase H.

RESULTS: Initial screening of a small library of 110 mappicine analogues using a novel fluorescence-based high-throughput screening assay for HIV RT RNase H resulted in the identification of two compounds with reasonable antiviral potency, but unfortunately with significant cytotoxicity. A larger library of 560 mappicine analogues was prepared by fluorine-tagged combinatorial synthesis and screened, leading to the identification of 55 additional inhibitors. Of these, mappicine 756 was among the most potent ($IC_{50} \approx 2 \mu M$ against RT-associated RNase H *in vitro*). Mappicine 756 showed very good antiviral activity against the wild-type HIV-1 III_B strain ($EC_{50} \approx 2.5 \mu M$) and had

virtually no cytotoxicity ($CI_{50} > 100 \mu M$) as assessed in several cell lines. Importantly, mappicine 756 retained full antiviral potency against several drug-resistant HIV-1 strains, including virus with high-level resistance to nevirapine, delavirdine and efavirenz, the three clinically approved non-nucleoside RT inhibitors. HIV strains resistant to mappicine 756 developed upon serial passage of the virus in the presence of increasing concentrations of the drug, leading to virus with 80-fold resistance to the drug. Several mutations were found in the virus resistant to mappicine 756; these mutations localized only in the segment of the RT gene corresponding to the RNase H subdomain.

CONCLUSION: The good antiviral activity in the absence of significant cytotoxicity suggest that mappicine analogues may represent an interesting new class of antiretroviral agents, those targeting RT-associated RNase H. Structural variants of mappicines are readily prepared by combinatorial methods, and it is therefore expected that improvements in antiviral potency may be attained.

ABSTRACT 12

Characterization of the impact of genotype, phenotype, and inhibitory quotient on antiviral activity of tipranavir in highly treatment-experienced patients

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BACKGROUND: Tipranavir (TPV), the first non-peptidic protease inhibitor (NPPI), has demonstrated robust antiviral activity against multiple protease inhibitor (PI)-resistant HIV-1, both *in vitro* and in clinical studies. A large Phase IIB study of TPV/r has allowed characterization of the impact of genotype, phenotype and inhibitory quotient (IQ) on antiviral activity of TPV in highly treatment-experienced (HTE) patients.

METHODS: BI 1182.52 was a multicentre, international, randomized, blinded trial of three twice-daily doses of TPV/r (500 mg/100 mg, 500 mg/200 mg and 750 mg/200 mg). Entry criteria included experience with the three main classes of antiretroviral, including at least two PIs and ≥ 1 primary PI mutation. The primary end-points were viral load (VL) reduction after 2 weeks functional monotherapy and safety at 4 weeks. Genotype was measured using the Visible Genetics Trugene[®] 4.0 assay; and phenotype was measured using the VIRCO Antivirogram[™] assay. IQ was calculated using the trough plasma TPV concentration at 14 days, divided by the protein-adjusted viral IC_{50} for each patient (protein adjustment factor was 3.75).

RESULTS: Two-hundred-and-sixteen patients were randomized and evenly distributed across arms with regard to baseline viral load, CD4 cell count, demo-

graphics and prior treatment experience. The median fold-change in susceptibility relative to wild-type (WT) for TPV and available PIs at baseline was 1.1, 76.5, 8.7, 7.0, 12.2, 36.8 and 94.2 for TPV, lopinavir, amprenavir, saquinavir, indinavir, nelfinavir and ritonavir, respectively. This study has identified four universal PI-associated mutations (UPAMs; mutations at codons 33, 82, 84 and 90) that are associated with class cross resistance. Three of these mutations (33, 82 and 84) are in the protease active site, such that 16 to 20 compensatory mutations may be required for ≥ 2 UPAMs to coexist. Patients with 0–2 UPAMs had a -0.97 to $-1.32 \log_{10}$ change in VL, compared with $-0.32 \log_{10}$ in patients with 3 UPAMs. The relationship of UPAMs to phenotypic susceptibility was evaluated. In the presence of 0–2 UPAMs, the median fold-change in TPV IC_{50} was 0.9, 1.0 and 1.3, compared with 2.2-fold in patients with 3 UPAMs. Across all three arms of the study, patients whose isolates had ≤ 1 and >1 - to twofold change in TPV IC_{50} relative to WT demonstrated a -1.23 and $-1.24 \log_{10}$ change in VL, respectively, at 2 weeks. A $-0.21 \log_{10}$ response was seen in patients whose isolates had >2 - to fourfold change in TPV IC_{50} , suggesting a phenotypic cut off of approximately twofold WT IC_{50} (VIRCO assay). Similarly, the median VL responses after 2 weeks for IQs ≤ 5 , >5 – 25 , >25 – 50 , >50 – 100 , >100 – 150 and >150 were -0.19 , -0.35 , -0.82 , -1.31 , -0.96 and $-1.23 \log_{10}$, respectively. This result suggests that there is an apparent IQ breakpoint of roughly 50 in HTE patients, below which there is a decrease in antiviral response.

CONCLUSION: This analysis has determined that a good response to TPV is maintained in the presence of <3 UPAMs, IC_{50} $<$ twofold WT, and IQ >50 . Importantly, over two-thirds of patients, even in this HTE population, met these criteria. Considering that most HIV-1 isolates remain fully susceptible to TPV until a large number of protease gene mutations (>15) are present, this high IQ suggests that TPV/r will provide an important option for the majority of HTE HIV-1-positive patients.

ABSTRACT 13**Characterization of treatment-emergent resistance mutations in two Phase II studies of tipranavir***D Hall, S McCallister, D Neubacher, M Kraft and DL Mayers*

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BACKGROUND: Tipranavir (TPV) is the first non-peptidic protease inhibitor (NPPI). Phase II clinical trials have demonstrated a sustained viral load response for up to 80 weeks of treatment in single and multiple-protease inhibitor (PI)-experienced patients. Furthermore, these studies have demonstrated that as many as 16 to 20 protease gene mutations may be required for reduced susceptibility to TPV. This analysis investigates treatment-emergent mutations seen in two Phase II trials in treatment-experienced HIV-1-positive adults.

METHODS: In two Phase II, open-label, randomized trials (BI 1182.4 and BI 1182.2), 91 HIV-1-positive adults with single- or multiple-PI failure received various doses of TPV/ritonavir (TPV/r). Fifty single PI-experienced patients received two new nucleoside reverse transcriptase inhibitors (NRTIs) plus either low-dose or high-dose TPV/r twice daily; and 41 multiple PI-experienced, non-NRTI (NNRTI)-naive patients received low-dose or high-dose TPV/r plus efavirenz (EFV) and one new NRTI. Genotypic testing was performed at baseline and at follow-up.

RESULTS: On-treatment genotypes were available for 24 patients from BI 1182.4 and 39 from BI 1182.2. The mean number of protease gene mutations at study entry was 10 in BI 1182.4 and 12 in BI 1182.2. The most frequent treatment-emergent protease mutations were L33I/F/V, V82L/T and I84V, occurring in 11, 9 and 9 isolates, respectively. Recent studies have shown that >2 universal PI-associated mutations (UPAMs; defined as any mutation at codons 33, 82, 84 or 90) may be required for reduced susceptibility to TPV at clinically relevant doses and reduced antiviral activity; 1 or 2 UPAMs may be sufficient for reduced susceptibility to available PIs. Three of five patients in BI 1182.2 who developed reduced susceptibility to TPV during treatment had 2 UPAMs at baseline, and went on to accumulate a third; one had 3 UPAMs at baseline. Other treatment-emergent mutations at codons associated with protease resistance that occurred in

three or more patients across the two studies include L10I/V (5), K20M/L/T (3), M46I (3), I54V (4) and L63A/D/T (3). Mutations occurring in three or more patients out of a total 63, at codons not generally associated with resistance, were I13V (3), K55R/Q (3), H69Y (3) and T74A (3).

CONCLUSION: Analysis of HIV-1 viral isolates from patients enrolled in studies of treatment-experienced patients has identified several mutations that emerged during TPV/r treatment of patients with a mean of approximately 10 baseline protease gene mutations. The most frequent mutations seen in this study at codons 33, 82 and 84 are UPAMs located in the protease active site. The presence of ≥ 2 of these mutations, therefore, appears to require the accumulation of 16-20 other protease mutations, and may have important implications for the fitness of TPV-resistant HIV-1. Few patients in these studies accumulated >2 UPAMs.

ABSTRACT 14

Characterization of HIV-1 showing decreased susceptibility to tipranavir and their inhibition by tipranavir-containing drug mixtures

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BACKGROUND: Tipranavir (TPV) is a non-peptidic protease inhibitor that maintains potent activity against a broad range of multiple protease inhibitor resistant human immunodeficiency virus (HIV-1) isolates. The genotypic changes responsible for the reduced susceptibility to TPV and their effect on cross resistance to other PIs are only partially understood. Here we present our characterization of TPV-resistant viruses at the genotypic and phenotypic levels including their *in vitro* susceptibility to inhibition by mixtures of the protease inhibitors TPV:amprenavir (APV) or TPV:lopinavir (LPV).

METHODS: Recombinant viruses were reconstructed to represent HIV-1 selected to grow in culture in the presence of TPV or representing clinical isolates from patients undergoing therapy with TPV. Viruses contained between 2–17 mutations in the protease gene and in some cases an additional mutation in the CA/P2 p55^{gag} cleavage site. Viruses were studied in antiviral assays to determine their susceptibility to protease inhibitors and in a Jurkat-LTR luciferase reporter cell line to determine their replication capacity. Susceptibility to inhibition by TPV containing drug mixtures was determined using constant ratio combination experiments and the calculation of combination indices (CI). Using this model, CI values <0.9=synergy, CI between 0.9 and 1.1=additive effects (no drug interaction) and CI>1.1=antagonism.

RESULTS: *In vitro* selected TPV-resistant viruses contain up to 10 mutations in the protease gene, including L33F, V82L and I84V. Introduction of these mutations into viral molecular clones by site-directed mutagenesis conferred up to 69-fold resistance to TPV and two- to 118-fold decreased susceptibility to other protease inhibitors in addition to decreasing viral replication capacity. A CA/P2 cleavage site mutation observed during *in vitro* selection did not directly contribute to TPV resistance. The following two clinical protease

genotypes were found to confer five- and sevenfold decreased susceptibility to TPV, respectively: V3I/L10V/I15V/L19I/M36L/S37N/R41K/M46I/K55R/Q61N/I64V/I72V/T74S/V82T/I84V/I85V/I93L and V3I/L10V/L33I/E35D/M36I/S37N/I54V/Q58E/I62V/L63P/A71V/V82L/L90M/I93L/C95V. Drug mixture experiments using these clinical isolate-derived viruses or wild-type HIV-1 showed that combinations of TPV:APV and TPV:LPV displayed predominantly additive effects against wild-type virus replication and somewhat lower CIs against protease mutant viruses. However, only in a few instances was the deviation from the additive effect outside the inter-experimental variability.

CONCLUSIONS: Resistance to TPV involves multiple mutations in the protease gene and leads to a reduced sensitivity to most other PIs and to a decreased replication capacity of viruses. TPV, however, maintains mostly additive effects on TPV-resistant or wild-type virus when used in combination with the protease inhibitors APV and LPV.

ABSTRACT 15

TMC114, a potent next-generation protease inhibitor: characterization of antiviral activity in multiple protease inhibitor-experienced patients participating in a Phase IIa study

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BACKGROUND: TMC114 is a potent next-generation protease inhibitor (PI), active against wild-type as well as PI-resistant HIV. The study TMC114-C207 was a placebo-controlled Phase IIa trial to evaluate the antiviral activity, safety and tolerability of TMC114 over 14 days treatment. Fifty multiple PI-experienced subjects (range 2–4 PIs) on a failing nucleoside reverse transcriptase inhibitor (NRTI)- and PI-containing regimen (HIV-1 RNA >2000 copies/ml) were enrolled. They received TMC114 with low-dose ritonavir (TMC114/RTV) at one of three doses (300/100 mg twice daily, 600/100 mg twice daily or 900/100 mg q.d.) as a substitution for their current PI or remained on their current regimen (control group) for 14 days. Afterwards, all patients switched to an investigator-selected highly active antiretroviral therapy (HAART) regimen. Overall, the median change in plasma HIV-1 RNA for the three TMC114/RTV groups at day 14 was $-1.35 \log_{10}$ compared to $+0.02 \log_{10}$ for the control group. No significant difference was observed between the three TMC114/RTV treatment arms. In this study, phenotypic and genotypic resistance data from screening, baseline and end of therapy were analysed.

METHODS: Phenotypic analysis was conducted using the Antivirogram[®] assay and genotypic analysis using the *VirtualPhenotype*[™]. Both determinations were performed on plasma samples taken at screening (within 28 days prior to treatment start), baseline (day 1) and end of therapy (day 15) time-points.

RESULTS: Subjects in this study had a broad range of protease mutations at baseline. The median number of total protease gene mutations was 15 (range 8–26) and the median number of PI resistance-associated mutations was 6 (range 1–11), with a median number of primary PI mutations of 3 (range 0–5) (including

D30N, M46I/L, G48V, I50V/L, V82A/F/T/S, I84V or L90M). More than 80% of subjects had more than one primary PI mutation. All primary PI mutations, except I50L and V82S, were present at baseline in at least one sample. Phenotyping of the baseline samples showed that 46% of the subjects were resistant to all currently approved PIs and only 27% of the subjects were sensitive to two or more PIs (cut-offs as defined by the Antivirogram[®]). In the treatment arms, the median fold change in EC₅₀ as compared to wild-type for TMC114 was 1.8 (range 0.3 to >21) at baseline and 1.5 (range 0.3–13.1) at end of treatment. There was no correlation between TMC114 susceptibility at baseline and virological outcome at day 14. Many genotypic changes were observed between screening, baseline and end of treatment. No mutation pattern could be associated with virological response to TMC114.

CONCLUSIONS: This study demonstrates the potent antiviral activity of TMC114, a next-generation PI, in multiple PI-experienced patients over 14 days. No mutation patterns influencing the response to treatment with TMC114 could be detected in this study.

ABSTRACT 16

TMC114 binds within the substrate envelope of HIV-1 protease, which could account for its efficacy against multi-protease inhibitor-resistant virus

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INTRODUCTION: HIV-1 protease is the target of very potent antiviral drugs for the treatment of HIV-1 infection. All current protease inhibitors (PIs) are the successful result of structure-based drug design. Unfortunately, as the viral reverse transcriptase is highly error prone, and under the selective pressure of drug therapy, many viable drug-resistant variants of HIV-1 protease have emerged. These PI-resistance mutations occur mostly at positions in the protease that will compromise inhibitor binding whilst retaining substrate specificity. We have determined from crystal structures of substrate complexes with HIV protease that the current PIs protrude beyond the substrate envelope, this may explain why resistance mutations constrain inhibitor binding. TMC114 is a next generation PI: recent virological and clinical results indicate that it is effective against known multi-PI-resistant variants of HIV-1. Furthermore, *in vitro* selection of TMC114-resistant variants from wild-type HIV-1 has proven difficult. In the present study we determined and compared the high-resolution crystal structure and thermodynamics of TMC114 or substrate binding to wild type HIV-1 protease.

METHODS: TMC114 was crystallized in complex with wild-type HIV-1 protease, and X-ray diffraction data was collected, processed and refined, using standard crystallographic techniques. The structure of the inhibitor complex was compared graphically with substrate complexes previously determined in our laboratory. The thermodynamics of inhibitor binding was determined using isothermal titration calorimetry (ITC) at 25°C and compared with other inhibitors binding.

RESULTS: The structure of TMC114 in complex with wild-type protease was determined to 1.93Å (P2₁2₁2₁; R=19.7; R_f=22.2). In addition, the binding constant determined by ITC showed that the interaction of the

inhibitor with the enzyme is very tight ($K_d=10^{-12}$ M) and is extremely enthalpically driven. When the structure was compared with the substrate complexes of HIV-1 protease, it was found that TMC114 occupies a volume that is contained within the substrate envelope, unlike most of the currently approved PIs.

CONCLUSIONS: Many drug-resistant variants of HIV protease evolve to maintain substrate recognition while compromising inhibitor binding, especially when the inhibitors extend beyond the substrate envelope. The fact that TMC114 fits well within the substrate envelope, associated with its tight binding to the enzyme, therefore, could account for why TMC114 remains active against most multi-PI-resistant variants. Hence, a mutation that affects TMC114 binding will likely cause a dramatic change in the ability of HIV-1 protease to recognize its substrates. This may also explain why selection of TMC114-resistant virus *in vitro* has proven difficult, as this might require changes beyond the protease gene, most probably in the cleavage sites. These results support our previous hypothesis that inhibitors that fit within the substrate envelope of HIV-1 protease may be more effective and less susceptible to drug resistance mutations.

ABSTRACT 17

Antiviral activity of TMC114, a potent next-generation protease inhibitor, against >4000 recent recombinant clinical isolates exhibiting a wide range of (protease inhibitor) resistance profiles

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INTRODUCTION: TMC114 is a potent, next-generation protease inhibitor (PI), active against wild-type as well as PI-resistant HIV. Recently, TMC114 showed *in vivo* antiviral efficacy in a 2-week Phase IIa trial in multiple PI-experienced patients. In order to assess the performance of TMC114 against currently circulating strains of HIV, the compound was tested against >4000 clinical isolates submitted for phenotypic resistance testing. The antiviral activity of TMC114 on these isolates was compared to the currently approved PIs: indinavir, ritonavir, nelfinavir, saquinavir, amprenavir and lopinavir.

METHODS: Recombinant clinical isolates were constructed according to the Antivirogram[®] method. Phenotypic and genotypic analyses were performed by the Antivirogram[®] and *VirtualPhenotype*[™] assays, respectively. Data analysis was performed using SAS and Spotfire DecisionSite software.

RESULTS: From the 4024 tested recombinant clinical isolates, 1666 (41%) were resistant to at least one of the currently approved PIs, defined as a change in EC₅₀ >fourfold as compared to wild-type. The median fold change in EC₅₀ against these 1666 resistant isolates for TMC114 was 1.1, corresponding to an EC₅₀ of 3.5 nM. Eighty percent of these PI-resistant isolates were still susceptible (defined as fold change in EC₅₀ <4) to TMC114. For the remaining 20% isolates, the median fold change in EC₅₀ for TMC114 was 10, thus showing that the compound can inhibit 90% of the 1666 PI-resistant isolates with a fold change ≤10. A subgroup of 1501 isolates, for which data for all six approved PIs were available, was used to determine the influence of the number of PIs with a fold change >4 on the activity of TMC114. Among these PI-resistant isolates, 67% were resistant to 4 or more PIs, with 31% resistant to all 6 approved PIs, 23% to 5, and 13% to 4.

The median fold change in EC₅₀ for TMC114 was <4 for each of these subgroups, which illustrates the activity of TMC114 against PI-resistant isolates. A genotype was available for 498 of the 1666 PI-resistant isolates. The number of primary mutations (D30N, M46I/L, G48V, I50V/L, V82A/F/T/S, I84V or L90M) was determined for each of these isolates. One percent had no primary mutation, 23% had 1, 41% had 2, 31% had 3 and 4% had 4 primary mutations. The median fold change in EC₅₀ for TMC114 was <4 for each of these subgroups.

CONCLUSIONS: TMC114 is a potent, next-generation PI with activity against a wide range of PI-resistant recombinant clinical isolates. This activity, defined by a median fold change of <4, extended to isolates resistant to all currently-approved PIs and also to isolates carrying up to four primary PI mutations.

ABSTRACT 18***In vitro* cross resistance profile of RO033-4649 against a panel of multiply-substituted protease inhibitor-resistant viruses: role of common protease resistance mutations***G Heilek-Snyder¹, A Kohli¹, N Cammack¹ and N Parkin²*¹ Roche, Palo Alto, Calif.; and ² Virologic, Inc., South San Francisco, Calif., USA

BACKGROUND: RO033-4649 is a potent, highly selective HIV-1 protease inhibitor (PI) with activity against PI-resistant viruses and a promising pharmacokinetic profile.

METHODS: Susceptibility to RO033-4649 and to amprenavir, indinavir, nelfinavir, saquinavir and lopinavir was tested in single cycle (PhenoSense™) and live HIV-1 virus assays. The fold change (FC) in IC₅₀ from NL4-3 reference was calculated and analysed using correlation matrices to investigate relationships between protease mutations and FC, either as a continuous or dichotomous variable. FC values between groups of viruses were compared using the Mann-Whitney non-parametric test. Virus isolates were passaged in MT-4 cells in the presence of RO033-4649 and the emergence of resistant populations was analysed by sequencing of the outgrowing virus isolates.

RESULTS: In a panel of 49 clinical isolates 39 and 28 samples showed high-level resistance (FC>20) to ≥3 or ≥4 PIs, respectively. The number of samples with FC<5 and between 5 and 20 were 11 and 30 for RO033-4649, 11 and 26 for amprenavir, 0 and 17 for indinavir, 0 and 5 for nelfinavir, 3 and 2 for saquinavir, and 0 and 12 for lopinavir. Isolates with RO033-4649 FC>20 (*n*=9) carried on average 14 protease mutations, with 2.67 primary mutations (primary mutations at positions 30, 46, 48, 50, 82, 84, 90). There was a significant positive correlation between RO033-4649 FC and the presence of mutations at positions 10, 33, 46, 54, 54, 71 and 73. For example, isolates containing mutations at position 73 (C, S or T) had a median FC of 17 compared to 7.6 for isolates lacking such mutations (*P*=0.003). A negative effect was observed in the five samples with V82F or T: compared to samples without a mutation at position 82, median FC was lower (3.9 vs 13.5, *P*=0.035). The two isolates with an

I50V mutation also had low RO033-4649 FC (median threefold). Cross resistance between pairs of PIs showed the highest degree of correlation with nelfinavir (*r*²=0.63) and lowest with amprenavir (*r*²=0.15). Both HXB2 and a clinical isolate G34 (M46L, G48V, I62V, L63P, T74S, V77I, V82A) were used in passaging studies at increasing drug concentrations. No mutation arose in the wild-type HXB2 culture after 11 passages, and in the clinical isolate, V32I and later I54V appeared under increasing drug pressure (120 nM and 480 nM, respectively).

CONCLUSIONS: (1) An average of 14 mutations are required before RO033-4649 demonstrates a loss of susceptibility greater than 20-fold. (2) In a background of multiple (10–18) resistance mutations and polymorphisms, clinical samples carrying G73C, S or T show reduced susceptibility to RO033-4649. (3) In a background of seven pre-existing resistance mutations, V32I and I54V arose under drug pressure, confirming the requirement of multiple mutations to confer reduced susceptibility to RO033-4649.

ABSTRACT 19**HIV clinical isolates containing mutations representative of those selected after first-line failure with unboosted GW433908 remain sensitive to other protease inhibitors***L Ross¹, N Parkin², C Chappey², M Tisdale³ and R Elston³*

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BACKGROUND: GW433908 (908) is an investigational protease inhibitor (PI) with demonstrated antiviral efficacy, durability and tolerability in antiretroviral (ART)-naive and -experienced subjects. Although no protease resistance-associated mutations were selected during 48 weeks of treatment with ritonavir-boosted 908 QD (SOLO trial, $n=322$) protease mutations were detected infrequently with unboosted 908 twice-daily (NEAT trial, $n=166$). In the NEAT trial, the predominant protease mutations selected were the V32I+I47V ($n=4$) and I54L or M ($n=4$) mutations, consistent with an amprenavir-like resistance profile for unboosted 908. In one subject, the I54L was replaced with the L33F+I50V mutations at a later time-point, after continued treatment with unboosted 908 in the presence of detectable viral load. To understand the potential cross resistance to other PIs, clinical isolates containing the V32I+I47V, I54L/M or I50V mutations were selected from a database of patient samples and susceptibility to all marketed PIs was assessed.

METHODS: Approximately 16 000 HIV clinical samples from the ViroLogic database, with matching genotypes and phenotypes, were examined to identify samples with the following primary mutational pathways: V32I+I47V, I54L/M or I50V. Samples with mixtures at these specific amino acids were excluded, as were samples that also contained certain primary protease mutations (D30N, G48V, V82A/T/S/F, I84V or L90M) that would indicate prior exposure to PIs. The presence of the primary protease mutation M46I/L was included because this mutation had been observed with the V32I+I47V ($n=1$) or I54L/M ($n=1$) mutations in NEAT. The median fold-change in susceptibility was calculated for samples with the specified mutation(s).

RESULTS: Fifty clinical isolates were identified: V32I+I47V ($n=12$), I54L/M ($n=13/n=6$) and I50V

($n=19$). The primary mutation M46I/L had a high frequency for all mutational pathways (67% with V32I+I47V, 37% with I54L/M and 74% with I50V). Certain secondary mutations were also present at an elevated incidence of >25%, including L63P for the V32I+I47V mutations; L10V, M36I, L63P and V77I for the I54L/M mutations; and L33F, L63P, A71V, V77I and V82I for the I50V mutation. For the viruses identified, and in the presence of these additional protease mutations, viruses with V32I+I47V, I54L/M and I50V, had median fold-changes to amprenavir of 3.4-, 3.6- and 20-fold, respectively. For V32I+I47V, I54L/M and I50V, the median fold-change for saquinavir (0.5, 1.0 and 1.2, respectively) and indinavir (2.5, 1.3 and 1.0, respectively) were below the assay cut-off. The median fold-changes were also below the clinical cut-off for lopinavir (2.9, 1.5 and 8.2, respectively). With respect to nelfinavir, samples were sensitive or had low-level resistance, with fold-changes of 3.0, 3.6 and 2.2. Low-level cross resistance was also seen for ritonavir, with fold-changes of 3.4, 2.9 and 6.8.

CONCLUSION: Clinical isolates with mutational patterns similar to those selected by unboosted 908 remain sensitive to most other PIs, suggesting that viruses present after treatment failure of an unboosted 908 regimen will respond to second-line PI-containing therapy.

ABSTRACT 20

Antiviral activity of P-1946, a novel anti-HIV protease inhibitor

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BACKGROUND: The use of protease inhibitors (PIs) in the treatment of HIV infection has led to significant improvement in AIDS-related morbidity and mortality. Unfortunately, the rapid emergence of HIV strains resistant to currently available antiretroviral drugs threatens to make obsolete the current therapeutic approaches. Therefore, novel PIs with distinct resistance profiles are needed to help resolve this dilemma.

METHODS: PIs derived from an amino acid were prepared using a simple, straightforward synthesis scheme developed in our laboratory. Antiviral activity (EC_{50}) of the candidate compounds was determined for wild-type and mutant viral strains, using MT-4 as the cell line. The assay was based on the inhibition of HIV-induced cytopathic effect, measuring cell viability by MTT colorimetric assay. The threshold for phenotypic resistance to the test compound was defined as fold resistance ($EC_{50\text{test}}/EC_{50\text{wt}}$) > 4.0.

RESULTS: A novel family of amino acid derivatives was readily obtained in few synthetic steps using classic chemistry. The five compounds (P-1933, P-1935, P-1939, P-1946, P-1999) with the highest antiviral activity against the wild-type HIV-1 NL4.3 virus (EC_{50} < 400 nM) also remained active against two typical strains carrying mutations 48/90 (saquinavir-resistant strain) and 10/46/63/82/84 (strain 4596). Compound P-1946 was selected as a prototype of the family and further characterized. P-1946 displayed good antiviral activity against wild-type strain NL4.3 (EC_{50} = 150 nM). Cytotoxicity ($CCIC_{50}$) of P-1946 was 40 μ M, in the same range as currently marketed PIs. In order to define the resistance profile of P-1946, we analysed the antiviral activity in the presence of protease mutations at amino acids 10, 46, 48, 63, 82, 84 and 90, using HIV strains obtained from the NIH. In addition to saquinavir-resistant and 4596 strains, P-1946 was active (fold resistance < 4) in the presence of combined mutations 82/84, which are associated with

resistance to several PIs, including cyclic urea. Eight additional viruses were used to better define the phenotypic resistance profile of P-1946 using the PhenoSense assay (ViroLogic, Inc.). The compound remained fully active in the presence of mutation at position 30 or 50, which are associated with resistance to nelfinavir and amprenavir, respectively. Significant resistance to P-1946 required the presence of at least six mutations in the protease gene.

CONCLUSION: P-1946 is an amino acid derivative typical of a new family of PIs. Its antiviral activity profile makes it a good lead compound for the development of new potent agents that would offer therapeutic alternatives for individuals carrying isolates resistant to current PIs.

ABSTRACT 21

Baseline and on-treatment gp41 genotype and susceptibility to enfuvirtide (ENF) and T-1249 in a 10-day study of T-1249 in patients failing an ENF-containing regimen (T1249-102)

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BACKGROUND: T-1249 is a second-generation fusion inhibitor that has shown potent *in vitro* antiviral activity against most HIV isolates resistant to enfuvirtide (ENF). Study T1249-102 evaluated the short-term antiretroviral activity of T-1249 in patients failing a regimen containing ENF. Here we present the baseline and day 11 results of genotypic and phenotypic testing, and their correlation to treatment responses, for the first 25 patients included in the study's planned interim analysis.

METHODS: Patients with two viral loads >5000 copies/ml while dosing on an ENF-containing regimen discontinued ENF and added 192 mg/day of T-1249 subcutaneously to the unchanged background regimen for 10 days. The intent-to-treat population included patients with amplifiable plasma virus at baseline (BL) that demonstrated ENF-resistance mutations and/or decreased phenotypic susceptibility to ENF. Resistance data were generated on Env amplified from patient plasma samples using the novel GeneSeq™ and PhenoSense™ Entry Assays. Fold changes in ENF and T-1249 IC₅₀ were calculated in relation to reference strains tested in parallel with the patient samples (FCIC₅₀).

RESULTS: Plasma virus from 24 of 25 patients (98%) who entered T1249-102 exhibited ENF resistance-associated substitutions in gp41 amino acids 36–45 at BL. ENF IC₅₀ were available for viral envelopes from 23 (96%) patients at BL. Geometric mean (GM) BL ENF FCIC₅₀ was 150.1 (range 1.7–2041.6) and T-1249 FCIC₅₀ was 1.8 (range 0.14–12.6). For those patients with paired samples at BL in their ENF parent study and at BL for the T1249-102 study (*n*=13), there was a GM increase of 70.6- and 1.8-fold in FCIC₅₀ for ENF and T-1249, respectively. On day 11, 22 (92%) patients had both genotype and phenotype available

for paired analysis. In four patients, there was a >four-fold increase in T-1249 FCIC₅₀; plasma virus from these patients demonstrated genotypic substitutions in amino acid 36–45. Virological response was not associated with viral tropism, baseline HIV RNA or baseline FCIC₅₀ to ENF or T-1249, but was associated with length of time receiving a failing ENF-containing regimen and with day 11 fold change from BL in T-1249 FCIC₅₀.

CONCLUSIONS: T-1249 retains antiviral activity in most patients experiencing viral replication in the presence of isolates with reduced susceptibility to ENF and/or changes in the target region of ENF. Treatment emergent amino acid substitutions in gp41 and reduced susceptibility to T-1249 were identified in some patients.

ABSTRACT 22

Determinants of susceptibility to enfuvirtide map to gp41 in enfuvirtide-naïve HIV-1

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BACKGROUND: Enfuvirtide (ENF, formerly T-20) is the first fusion inhibitor to demonstrate efficacy in controlled Phase III trials. Fusion inhibitor (FI)-naïve HIV-1 isolates exhibit a range of susceptibility to ENF *in vitro*, and several studies have suggested that HIV-1 envelope co-receptor tropism or affinity may contribute to this range. Co-receptor tropism and affinity are conferred by the gp120 subunit of the HIV-1 envelope glycoprotein, thus, those studies imply that determinants of susceptibility to ENF lie within gp120. Using chimeric envelope constructs and site-directed mutagenesis, we examined the envelope gp120 and gp41 subunits as loci for determinants of ENV susceptibility.

METHODS: Sensitivity of HIV-1 isolates to ENF was determined in a cMAGI infectious centre assay. HIV-1 env genes from R5- and X4-tropic isolates exhibiting a range of ENF sensitivities were cloned into an expression vector and served as the starting point for gp120-gp41 chimeric envelope constructs. Co-receptor tropism and sensitivity of envelope clones and chimeras to ENF were determined from Env-deficient reporter viruses pseudotyped with these envelopes following cotransfection of envelope expression vectors and an env-deficient NL4-3-based reporter virus construct into 293T cells. The pseudotyped reporter viruses produced were evaluated on U87 cells expressing CD4 and either CCR5 or CXCR4. Sequences of all cloned and chimeric envelope constructs were determined by dideoxy sequencing chemistries using a Beckman Coulter CEQ 2000XL system and DNASTar software.

RESULTS: Full-length functional envelopes were cloned from five R5-tropic and two X4 tropic clade B primary virus isolates of HIV-1. The cloned envelopes exhibited ENF IC₅₀s ranging 0.04–12.6 µg/ml in the U87-based pseudotyped reporter virus assay. The cloned envelopes retained the same tropism characteristics noted with the parental virus isolates. The gp120/gp41 chimeric envelopes exhibited tropism specificity of the gp120 parental virus as expected. On

the other hand, the major determinants of ENF sensitivity tracked with the gp41 donor. Chimeric env constructs exchanging either the N-terminal ectodomain (containing HR1) or the C-terminal ectodomain through the end of gp41 (containing HR2) suggested that both regions can contribute to baseline susceptibility to ENF. In support of this assertion, we found that ENF sensitivity can be modulated by SDM of gp41 amino acids 45 (within HR1) and 135 (within HR2) with amino acids observed in rare FI-naïve isolates or those more commonly found.

CONCLUSIONS: Previous studies have demonstrated that the HR1 region of the HIV-1 gp41 is the target for ENF. In addition, results from Phase III clinical studies of ENF have shown that this same region is the primary locus for development of ENF resistance. Our current results suggest that gp41 also contains the major determinants for baseline sensitivity to ENF of clade B FI-naïve virus.

ABSTRACT 23

Sensitivity of Env-gene recombinant viruses derived from antiretroviral drug-sensitive and -resistant HIV-1 clinical isolates to the novel CCR5 antagonist, UK-427,857

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UK-427,857 is a novel small molecule CCR5 antagonist that is currently being developed for the treatment of HIV infection. Its antiviral activity was evaluated against 200 clinically-derived R5 HIV-1 isolates using the ViroLogic PhenoSense HIV Entry inhibitor susceptibility assay. A panel of recombinant viruses was prepared using HIV-1 gp160 envelope genes derived from 100 clinical isolates lacking known 'drug-selected' mutations in either protease (PR) or reverse transcriptase (RT) ('drug-sensitive' isolates) and 100 clinical isolates with one or more drug selected PR or RT mutations ('drug-resistant' isolates). The virus panel comprised 160 clade B and 40 isolates from other clades.

UK-427,857 inhibited all 200 recombinant viruses tested with a geometric mean IC_{50} of 1.6 nM (range 0.3–8.9 nM). The geometric mean IC_{50} s derived from drug-sensitive and drug-resistant isolates were 1.3 nM (range 0.3–3 nM) and 2.1 nM (range 0.7–8.9 nM), respectively. The difference between these two groups (1.6-fold) is statistically significant ($P < 0.05$) but less than the expected assay-to-assay variation. There was no difference in sensitivity between the clade B and non-clade B isolates, with geometric mean IC_{50} of 1.6 nM (range 0.5–6.9 nM) and 1.6 nM (range 0.3–8.9 nM), respectively, consistent with a previous study demonstrating broad cross-clade activity of UK-427,857 against primary isolates grown in mitogen-activated peripheral blood lymphocytes.

These data provide further evidence that UK-427,857 is a potent antiviral compound with broad activity against recombinant viruses derived from a large number of clinically-relevant isolates and diverse clades. The compound inhibited CCR5-mediated infection of Env-recombinant viruses derived from antiretroviral

drug-resistant R5 clinical isolates, suggesting that viruses selected *in vivo* during HIV drug treatment retain sensitivity to UK-427,857. The study supports the continued development of this compound for the treatment of HIV-infected individuals.

ABSTRACT 24**ADS-J1, a non-peptidic low molecular weight HIV fusion inhibitor targeting gp41, with no cross-drug resistance with peptidic HIV fusion inhibitors T-20 and C-34, and HIV binding inhibitors***M Armand-Ugón¹, A Gutiérrez¹, S Jiang², B Clotet¹ and JA Esté¹*

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BACKGROUND: ADS-J1 is a low molecular weight compound selected for its ability to interfere with the association of the N- and C-terminal heptad repeat regions of HIV-1 gp41 envelope glycoprotein. Since ADS-J1 is a polysulfonic acid compound, it is interesting to know whether it, like other polyanionic anti-HIV compounds, blocks the binding of HIV-1 to CD4 cells through electrostatic interactions and to test the anti-HIV activity of ADS-J1 against HIV strains that have been made resistant to polyanionic HIV binding inhibitors.

METHODS: Evaluation of anti-HIV activity in MT-4 cell culture against wild-type and drug-resistant HIV-1 strains. Cell culture selection of HIV drug resistance to known inhibitors of gp41-dependent fusion (T-20 and C-34). Flow cytometry evaluation of drug interaction with HIV co-receptors.

RESULTS: Here, we show that ADS-J1 was active against T-20- and C-34-resistant HIV-1 isolates with similar potency to the wild-type HIV-1 NL4-3 strain (EC_{50} 0.6, 0.3 and 0.4 $\mu\text{g/ml}$, respectively). ADS-J1 (10 $\mu\text{g/ml}$) could not block the binding of an HIV strain that was made resistant to AR177, a negatively charged oligonucleotide that blocks HIV binding, and is cross-resistant (>100-fold) to dextran sulfate (DS), a known polysulfonic HIV binding inhibitor. However, ADS-J1 blocked AR177-resistant virus fusion and replication (EC_{50} 1.5 $\mu\text{g/ml}$), suggesting that ADS-J1 has a mechanism of action different from the polyanionic HIV binding inhibitor AR177.

CONCLUSION: If the activity of polyanionic compounds on HIV binding is 'bypassed' by selection of resistance, compounds such as ADS-J1 may exclusive-

ly act on gp41-dependent fusion. Our results support the hypothesis that ADS-J1 binds to a hydrophobic cavity region within gp41 for preventing fusion-active gp41 core formation. ADS-J1 may serve as a lead low molecular weight compound to develop new anti-HIV agents.

ABSTRACTS 25

Human β -defensins inhibit HIV-1 replication *in vitro*

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BACKGROUND: Mechanisms underlying the infrequent transmission of HIV-1 through the oral mucosa are incompletely understood. Here we describe the anti-HIV-1 activity of human β -defensins (hBDs), small cationic innate defence molecules that provide a first line of protection at mucosal surfaces.

METHODS: Recombinant hBD-1, -2, and -3 were generated and used to evaluate their anti-HIV-1 activity and cytotoxicity in PBMC, CEM X4/R5 and Ghost X4/R5 cells. Real-time RT-PCR was used to quantify hBD mRNA expression in normal human oral epithelial cells. Flow cytometric and confocal microscopy analysis was used to determine the effect of hBDs over CCR5 and CXCR4 co-receptors. Finally, the potential interaction between hBDs, cell membrane and viral particles was analysed by immunogold transmission electron microscopy.

RESULTS: HIV-1 induced expression of hBD-2 and -3 mRNA, but not hBD-1, four- to-78-fold above baseline, in normal human oral epithelial cells. HIV-1 failed to infect normal human oral epithelial cell monolayers, even after 5 days of exposure. While recombinant hBD-1 had no antiviral activity, both rhBD-2 and -3 showed a concentration-dependent inhibition of HIV-1 replication without cellular toxicity. This antiretroviral effect was greater against the CXCR4-tropic than against CCR5-tropic HIV-1 isolates. HBD-2 and -3 bound to virions and induced a direct and irreversible effect on virion infectivity, with electron microscopy confirming direct binding of hBDs to viral particle. In addition, hBD-2 and -3, but not hBD-1, induced down-modulation by internalization of the HIV-1 co-receptor CXCR4 (but not CCR5) in peripheral blood mononuclear cells and T-lymphocytic cells, as shown by confocal microscopy and flow cytometric.

CONCLUSION: This study shows for the first time that (i) HIV-1 induces hBD expression in human oral epithelial cells; and (ii) hBDs block HIV-1 replication

via a direct interaction with virions and through modulation of the CXCR4 co-receptor. These properties may be exploited as new strategies for mucosal protection against HIV-1 transmission.

ABSTRACT 26

***In vitro* resistance development of human immunodeficiency virus type 1 towards mannose-specific plant lectins**

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BACKGROUND: Mannose-specific plant lectins from the *Amaryllidaceae* family, such as *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA), have previously been shown to exhibit pronounced anti-HIV activity (Balzarini *et al.*, 1991). There is strong evidence that these drugs target the heavily glycosylated envelope glycoprotein gp120. Therefore, the aim of this study was to select for resistance against GNA and HHA, to determine the phenotype and the associated genotypic changes in the HIV envelope gene.

METHODS: HIV-1 was subjected to sub-cultivations in the presence of dose-escalating concentrations of GNA and HHA. Virus isolates, recovered in the presence of different concentrations of the respective drugs, were investigated for their phenotypic susceptibility towards a subset of mannose-specific plant lectins, and a variety of viral entry and reverse transcriptase inhibitors. Their gp120 and gp41 gene sequences were also determined.

RESULTS: Many sub-cultivation steps were required to obtain virus strains that could grow in the presence of drug concentrations that were at least 100-fold higher than the initial EC₅₀ (50% effective concentration) values. The mutations found in the different virus isolates were predominantly related to N-glycosylation sites and at the S or T residues that are part of the N-glycosylation motif. One potential O-glycosylation site in gp120 was also affected. The degree of resistance of HIV-1 to the plant lectins was correlated with an increasing number of mutated glycosylation sites in gp120. The susceptibility of the GNA- and HHA-resistant virus strains towards other viral entry and RT inhibitors was fully preserved.

CONCLUSION: Resistance development of HIV-1 against the mannose-specific plant lectins GNA and HHA is associated with a unique spectrum of resistance mutations in the gp120 envelope gene, which has not been previously observed with any of the other known viral entry inhibitors.

ABSTRACT 27

Viral resistance against a candidate HIV microbicide

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BACKGROUND: Sexual transmission accounts for greater than 90% of worldwide HIV infection. Moreover, the incidence and prevalence of HIV infection in women has been increasing. Vaginal microbicides provide a female-controlled strategy to prevent HIV transmission in women. We have evaluated an HIV inactivating agent, 2-hydroxypropyl-beta-cyclodextrin (BCD), as a potential microbicide. Significantly, BCD recently has been proven as an effective microbicide in a mouse model for intravaginal HIV-1 transmission and is used extensively for other purposes in individuals.

METHODS: First, we evaluated the efficacy of virus neutralization *in vitro*, using a single cycle replication assay with HIV or SIV in the presence or absence of BCD. Based on encouraging *in vitro* data, we administered BCD intravaginally in rhesus macaques, followed by inoculation with large doses of highly pathogenic SIV. Control animals were treated with gel alone or nothing before inoculation with the same dose of SIV. The animals have been evaluated for infection by RT-PCR of gag sequences in their plasma and nested PCR of gag sequences in their PBMC. These animals continue to be monitored for antiviral humoral and cellular immune responses. In addition, we selected different strains of HIV-1 *in vitro* in the presence of escalating doses of BCD.

RESULTS: BCD was successful in neutralizing infection of cells with both X4- and R5-tropic HIV as well as SIV *in vitro*. In fact, we could not see outgrowth of virus after 30 days post-exposure. Our *in vivo* data indicated that intravaginal pretreatment with BCD significantly reduced SIV mucosal transmission (1/6 animals infected) relative to untreated control animals (8/10 animals infected). Currently we are performing repeated challenges with BCD and SIV in the uninfected animals to assess whether they continue to be protected from infection. In addition, we are determining whether or not the uninfected BCD-treated animals

have antiviral immune responses as compared to the controls. HIV that was selected with BCD *in vitro* was shown to be resistant to complete inactivation by BCD. Sequences of these viruses show mutations as compared to the same virus without BCD exposure.

CONCLUSIONS: Should BCD continue to prevent SIV transmission and not perturb mucosal tissues in this model, its current approved use in humans suggests it would be an important candidate for use as an anti-HIV microbicide. Nonetheless, the possibility of drug resistance against microbicides should be carefully evaluated before such drugs are administered to humans. Systemic antiviral therapy to manage HIV infection indicates that combinatorial approaches have significant benefits over monotherapy. Thus, we are also evaluating new models in which to examine the efficacy of BCD in combination with other potential microbicides.