Abstracts presented at the
XVII International HIV Drug Resistance Workshop:
Basic Principles & Clinical Implications
June 10–14 2008, Sitges, Spain
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Monogram Biosciences, USA

Deenan Pillay
University College London, UK

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## PROGRAMME

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<td>Welcome dinner: Melia Sitges</td>
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<td>John Mellors; University of Pittsburgh, USA</td>
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<td>8:45</td>
<td>Diversity-generating retroelements</td>
<td>Jeffery F Miller; UCLA School of Medicine, USA</td>
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<td><strong>RESISTANCE TO NEW ANTIMVIRAL AGENTS</strong></td>
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<td>Douglas Richman</td>
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<td>9:30</td>
<td>Potent HIV-1 entry inhibitors with a reserve of binding energy</td>
<td>Michael Kay; University of Utah School of Medicine, USA</td>
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<td>Resistance to CCR5mAb RoAb3952 is associated with a shift in binding</td>
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<td>10:00</td>
<td>Novel resistance profile of the potent nucleoside analogue</td>
<td>Jeffrey Meteer; University of Pittsburgh, USA</td>
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<td>10:15</td>
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<td>10:45</td>
<td>Hexadecyloxypropyl tenofovir (CMX157) has enhanced potency in vitro</td>
<td>Randall Lanier; Chimerix Inc., USA</td>
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<td>against NRTI-resistant HIV relative to tenofovir and a favourable</td>
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<td>11:00</td>
<td>In vitro cross-resistance profile, antiviral activity, safety and</td>
<td>Robert Murphy; Northwestern University Feinberg School</td>
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<td>pharmacokinetics in HIV-1-infected patients of IDX899, a novel HIV-1</td>
<td>of Medicine, USA</td>
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<td>NNRTI with high barrier to resistance</td>
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<td>Longitudinal analysis of resistance to the HIV-1 integrase inhibitor</td>
<td>Michael Miller; Merck &amp; Co., Inc., USA</td>
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<td>raltegravir: results from P005 a Phase II study in treatment-</td>
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<td>experienced patients</td>
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<td>Loss of raltegravir susceptibility in treated patients is conferred</td>
<td>Signe Fransen; Monogram Biosciences, USA</td>
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<td>HIV-1 Gag polymorphisms determine treatment response to bevirimat (PA-</td>
<td>Scott McCallister; Panacos Pharmaceuticals, USA</td>
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<td>12:00</td>
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Wednesday, June 11, 2008

Afternoon Sessions
13:30-16:00 Poster viewing session
Resistance to new antiretroviral agents and clinical implications of resistance

16:00-17:00 Themed discussion: Resistance and cross-resistance among HIV-1 integrase inhibitors
Victoria Johnson; University of Alabama at Birmingham School of Medicine, USA

Free evening to enjoy Sitges

Thursday, June 12, 2008

Morning Sessions
8:30 Introduction of plenary
Charles Boucher; Erasmus Medical Center Rotterdam and University Hospital Utrecht, the Netherlands

8:45 Cellular cofactors of HIV integrase as novel antiviral targets
Zeger Debyser; KU Leuven, Belgium

MECHANISMS OF HIV DRUG RESISTANCE
Chairs: Brendan Larder RDI, UK
Mark Wainberg McGill University AIDS centre, Canada

9:30 Mechanism by which the HIV integrase active-site mutation N155H confers resistance to raltegravir
Jay Grobler; Merck Research Laboratories, USA

9:45 Structural explanations to altered drug resistance pathways in HIV-1 non-clade B proteases
Rajintha Bandaranayake; University of Massachusetts Medical School, USA

10:00 How hepatitis C virus NS3-4A protease R155K/T strains can discriminate VX-950 and ITMN-191 but affect differentially SCH-503034
Jerome Courcambeck; GenoScience, France

10:15 BREAK

10:45 Structural basis for K65R function: tenofovir resistance, reduced nucleotide incorporation and excision antagonism
Kalyan Das; CABM & Rutgers University, USA

11:00 Zidovudine resistance related connection mutations in HIV-1 reverse transcriptase cause selective dissociation from RNase H competent complexes
Maryam Ehteshami; McGill University, Canada

11:15 Q509L in HIV-1 reverse transcriptase increases zidovudine resistance by promoting polymerase-competent versus RNase H competent binding on RNA/DNA template/primers with short duplex lengths
Jessica Brehm; University of Pittsburgh, USA

11:30 Evolution of reverse transcriptase connection domain mutations in patients on antiretroviral therapy
Viktor von Wyl; University Hospital Zurich, Division of Infectious Diseases and Hospital Epidemiology, Switzerland

11:45 Delayed chain-termination protects the hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase
Matthias Götze; McGill University, Canada

12:00 LUNCH
**Thursday, June 12, 2008**

**Afternoon Sessions**

13:30-16:00  **Poster viewing session**  
Epidemiology, HBV/HCV drug resistance, and mechanisms of HIV drug resistance

16:00-17:00  **Themed discussion:** In depth genetic analyses of virus populations  
Richard Haubrich; University of California, San Diego, USA

19:00-21:00  **Dinner:** Melia Sitges

**Friday, June 13, 2008**

**Morning Sessions**

**HIV PATHOGENESIS, FITNESS AND RESISTANCE**

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<td>Single cell analysis of HIV DNA from infected patients</td>
<td>Sarah Palmer; Karolinska Institute, Sweden</td>
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<td>8:45</td>
<td>Correlations between transmitted HIV-1 drug resistance mutations and the human leukocyte antigen alleles of therapy-naive HIV patients</td>
<td>Rolf Kaiser; University of Cologne, Germany</td>
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<td>9:00</td>
<td>HIV-1 recombination in patients infected with multiple HIV-1 variants from the same donor</td>
<td>Mary Kearney; National Cancer Institute, USA</td>
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<tr>
<td>9:15</td>
<td>The selection, transmission and persistence of drug-resistant HIV-1 in infants prophylaxed with single-dose nevirapine varies by the timing of infection</td>
<td>Ana Judith Blanco; Health Alliance International, USA</td>
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<tr>
<td>9:30</td>
<td>Intensification with efavirenz or lopinavir/ritonavir does not reduce residual HIV-1 viraemia in patients on standard antiretroviral therapy</td>
<td>Frank Maldarelli; HIV Drug Resistance Program, USA</td>
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**NEW RESISTANCE TECHNOLOGIES AND INTERPRETATIONS**

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<td>Dynamic HIV-1 escape from vicriviroc therapy <em>in vivo</em></td>
<td>Atho Tsibris; Massachusetts General Hospital, USA</td>
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<td>10:15</td>
<td>Response to vicriviroc in HIV-infected treatment-experienced individuals using an enhanced version of the Trofile HIV co-receptor tropism assay [Trofile (ES)]; reanalysis of ACTG 5211 results</td>
<td>Jacqueline Reeves; Monogram Biosciences, USA</td>
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<td>10:30</td>
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<td>11:00</td>
<td>Improved detection of X4 virus <em>By V3</em> genotyping: application to plasma RNA and proviral DNA</td>
<td>Richard Harrigan; BC Centre for Excellence in HIV/AIDS, Canada</td>
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<td>11:15</td>
<td>Improved genotypic prediction of HIV-1 coreceptor usage by incorporating V2 loop sequence variation</td>
<td>Alexander Thielen; Max-Planck Institute for Informatics, Germany</td>
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<td>11:30</td>
<td>Inferring viral tropism from genotype with massively parallel sequencing: qualitative and quantitative analysis</td>
<td>Martin Däumer; Institute of Immunology and Genetics, Germany</td>
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CLINICAL IMPLICATIONS OF RESISTANCE

Chairs: John Mellors
       Carlo-Federico Perno

11:45 Emergence of drug resistance after failure of first-line HAART is associated with intensity of virological monitoring: a systematic analysis of cohort and trial data specifically addressing the WHO public health approach to antiretroviral therapy

Ravindra Gupta; Royal Free and University College Medical School, University College London, UK

12:00 Low frequency K103N mutations are strongly associated with inadequate virological responses to non-nucleoside reverse transcriptase inhibitor based therapy

Gillian Hunt; National Institute for Communicable Diseases, South Africa

12:15 Determination of phenotypic clinical cut-offs for etravirine: pooled week 24 results of the DUET-1 and DUET-2 trials

Monika Peeters; Tibotec BVBA, Belgium

12:30 Biological and clinical cutoff analyses for etravirine in the PhenoSense™ HIV assay

Eoin Coakley; Monogram Biosciences, USA

12:45 LUNCH

Friday, June 13, 2008

Afternoon Sessions

13:30-16:00 Poster viewing session

HIV pathogenesis, fitness/resistance and new resistance technologies and interpretations

16:00-17:00 Themed discussion: Emerging epidemiology of drug resistance in the developing world

Deenan Pillay; University College London, UK

17:00 Closing remarks

20:00-23:00 Awards dinner: Cavas Codorniu
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<td>Resistance to new antiretroviral agents</td>
<td>Potent HIV-1 entry inhibitors with a reserve of binding energy against resistance mutations</td>
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<td>Resistance to CCR5mAb RoAb3952 is associated with a shift in binding from the extracellular domain 2 to the N terminus of CCR5</td>
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<td>Novel resistance profile of the potent nucleoside analogue reverse transcriptase inhibitor 3′-azido-2′,3′-dideoxyguanosine</td>
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<td>Longitudinal analysis of resistance to the HIV-1 integrase inhibitor raltegravir: results from P006 a Phase II study in treatment-experienced patients</td>
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<td>A8</td>
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<td>HIV-1 Gag polymorphisms determine treatment response to bevirimat (PA-457)</td>
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A58 A new class of tight binding non-nucleoside reverse transcriptase inhibitors inhibit the burst and steady-state phases of wild-type and Y188L HIV-1 reverse transcriptase activity
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PLENARY ABSTRACTS
ABSTRACT P1

Diversity-generating retroelements

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Host–parasite interactions are often driven by mechanisms that promote genetic variability. In the course of our studies on bacterial pathogenesis, we discovered a group of temperate bacteriophages that generate diversity in a gene that specifies tropism for receptor molecules on host Bordetella species, which cause respiratory infections in humans and other mammals. This microevolutionary adaptation is produced by a novel ‘diversity-generating retroelement’ (DGR) that combines the basic retroelement life-cycle of transcription, reverse transcription and integration with site-directed, adenine-specific mutagenesis. Central to this process is a reverse transcriptase-mediated exchange between two repeats, one serving as a donor template (TR) and the other as a recipient of variable sequence information (VR). Recent work has focused on the genetic basis of diversity-generation. The directionality of information transfer is determined by the initiation of mutagenic homing (IMH) sequence present at the 3’ end of VR. We have demonstrated that DGR function occurs through a TR-containing RNA intermediate by a unique target-primed reverse transcription mechanism that precisely regenerates target sequences. This non-proliferative, ‘copy and replace’ mechanism enables repeated rounds of protein diversification and optimization of ligand–receptor interactions. The potential utility of DGRs is illustrated by the identification of over 40 related elements in bacterial, phage and plasmid genomes. DGRs are present in human pathogens (Treponema, Legionella spp.), human commensals (Bacteroides, Bifidobacterium spp.), green sulfur bacteria (Chlorobium, Prosthecochloris spp.), cyanobacteria (Trichodesmium, Nostoc spp.), magnetotactic bacteria (Magnetospirillum spp.), and many other diverse species. DGRs comprise a new family of retroelements with the potential to confer powerful selective advantages to their host genomes.
ABSTRACT P2
Antiviral Therapy 2008; 13 Suppl 3: P4

Cellular cofactors of HIV integrase as novel antiviral targets

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BACKGROUND: Lentiviruses can insert their viral genome into the chromosome of a non-dividing cell. During this process the viral integrase is assisted by various cellular cofactors. Our group has identified and validated cellular cofactors of nuclear import and integration to develop novel targets for anti-HIV therapy. LEDGF/p75 was originally identified in our group as a binding partner of HIV-1 integrase and as a chromosomal tether. Its role during HIV replication was independently confirmed by RNA interference (RNAi) knockdown, mutagenesis and in mouse knockout cells. Truncation mutants of LEDGF/p75 lacking the chromosome attachment site strongly inhibit HIV replication by competition for interaction with integrase. Data suggest a role for LEDGF/p75 in targeting integration. As for nuclear import of HIV, no cellular cofactor has been identified unambiguously.

METHODS: Using cells overexpressing truncation mutants, we selected HIV strains that overcome the inhibition. Resistant strains were sequenced and the mutations studied in the context of viral clones and recombinant integrase. Virus phenotype and replication were studied in detail. Interaction between mutant integrase and LEDGF/p75 was determined by confocal microscopy, in vitro pull down, fluorescence cross-correlation spectroscopy and AlphaScreen. The mutations were compared with the reported crystal structure of the integrase core in complex with the integrase-binding domain of LEDGF/p75. Yeast two-hybrid analysis was used to identify cellular partners of LEDGF/p75. AlphaScreen is used for drug discovery.

RESULTS: Detection of integrase mutations in the resistant strains at key positions in the LEDGF/p75–integrase interface provides crucial evidence for the importance of LEDGF/p75 in HIV integration. The resistance mutations obtained corroborated in vitro results from alanine scanning of the integrase–LEDGF/p75 interface. Resistance selection occurs, but at the cost of a reduced affinity of integrase for LEDGF/p75 and impaired replication kinetics in human peripheral blood lymphocytes. Resistance mutations do not overlap with those observed in clinical trials with strand-transfer inhibitors. We identified JPO2 and POGz as cellular binding partners of LEDGF/p75. Counterscreens are established to identify small molecules inhibiting integrase interaction without cellular toxicity. Using yeast two-hybrid analysis we identified a novel cofactor of integrase mediating nuclear import of HIV.

CONCLUSION: Our results provide a striking example of the power of viral molecular evolution and biological relevance to the crystal structure of the LEDGF/p75 integrase interface. Demonstration of the exclusive role of LEDGF/p75 in HIV integration justifies our ongoing effort in developing small-molecule inhibitors targeting the interaction between integrase and LEDGF/p75. Cofactor-based anti-HIV therapy may become a new paradigm in antiviral research.
SESSION 1

Resistance to new antiretroviral agents
ABSTRACT 1

Antiviral Therapy 2008; 13 Suppl 3:A3

Potent HIV-1 entry inhibitors with a reserve of binding energy against resistance mutations

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BACKGROUND: Enfuvirtide is the first approved HIV-1 entry inhibitor and is particularly useful for ‘salvage therapy’ in patients resistant to highly active antiretroviral therapy. Unfortunately, resistance to enfuvirtide rapidly emerges in vitro and in vivo. During HIV-1 entry, the gp41 HR1 trimer ‘pocket’ region becomes transiently exposed and vulnerable to inhibition. This pocket is an attractive inhibitory target because it is extremely well-conserved and essential for viral entry.

METHODS: In contrast to natural peptides, D-peptides are promising therapeutic agents due to their insensitivity to degradation by proteases. Using mirror-image phage display and structure-assisted design, we have produced D-peptides that bind the gp41 pocket with high affinity and potently inhibit viral entry. To further improve binding and potency via avidity, we have designed trimeric D-peptides connected via flexible polyethylene glycol linkers that simultaneously bind all three pockets in the gp41 HR1 trimer.

RESULTS: Our lead trimeric D-peptide (PIE12-trimer) potently inhibits a diverse panel of primary isolates (clades A–G) with an average IC50 >60-fold better than enfuvirtide. Viral strains resistant to other HIV entry inhibitors (including enfuvirtide, C34 and 5-helix) remain sensitive to the D-peptide trimer. Interestingly, for our ultra-high affinity (subpM) inhibitors, potency appears to be determined by association rate rather than affinity due to limited time-window of exposure of the target during viral entry. We exploit this property to ‘over-engineer’ our inhibitors with a reserve of binding energy against affinity-perturbing resistance mutations. This ‘resistance capacitor’ also deprives HIV of an evolutionary pathway for the step-wise accumulation of subtle resistance mutations by uncoupling affinity and inhibitory potency. Initial passaging data show a superior resistance profile compared with other fusion inhibitors, such as enfuvirtide, C34 and earlier generation D-peptides.

CONCLUSIONS: Our D-peptide trimer is a broad and potent inhibitor of HIV entry that has been designed to prevent or delay the development of resistance. This inhibitor addresses limitations associated with enfuvirtide and is a strong candidate for the prevention and treatment of HIV/AIDS.

ACKNOWLEDGEMENTS: Supported by NIH grants GM082545 and AI076168.
ABSTRACT 2

Antiviral Therapy 2008; 13 Suppl 3:A4

Resistance to CCR5mAb RoAb3952 is associated with a shift in binding from the extracellular domain 2 to the N terminus of CCR5

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BACKGROUND: RoAb3952 is a de-immunized version of the previously published mouse anti-CCR5 monoclonal antibody (mAb) ROAb14. RoAb3952, which binds to the extracellular loop 2 (ECL2) of CCR5, has potent antiviral activity in vitro. To determine the pattern of viral resistance against RoAb3952, we passaged two HIV-1 isolates in human peripheral blood mononuclear cells (PBMCs) in the presence of RoAb3952.

METHODS: The CCR5-tropic HIV-1 isolates Bal and CC1/85 were passaged in vitro in the presence of increasing RoAb3952 concentrations. CD8-depleted PBMC and genetically diverse, high titre viruses were used to facilitate fast resistance development. The sensitivity of resistant and no drug control (NDC) viruses to RoAb3952 and other CCR5mAbs was measured in PBMCs and single-cycle assays. The env genes of these viruses were sequenced to identify mutations associated with resistance to RoAb3952.

RESULTS: Passaging of Bal and CC1/85 in the presence of increasing concentrations of RoAb3952 resulted in the selection of two highly RoAb3952-resistant viruses. The NDC viruses remained sensitive to RoAb3952. In the presence of another CCR5mAb (RoAb13) recognizing the N-terminal of CCR5, the phenotype was reversed; both the Bal_3952res and CC1/85_3952res virus were more sensitive to RoAb13 compared with their respective NDC virus, suggesting a shift in the binding ability of these Envs. Sequence analysis revealed that Bal_3952res differed from Bal_NDC in only two amino acid positions (K163N and S531A). K163N is located in the V2 region, whereas S531A is located in gp41. S531A was also detected in CC1/85_3952res, which had additional mutations throughout gp120. Furthermore, pseudotyped virus containing Envs of CC1/85_3952res and Bal_3952res, but not of Bal_NDC and CC1/85_NDC, were also resistant to RoAb3952 and the CCR5mAb 2D7.

CONCLUSIONS: Using high titred, genetically diverse viruses and CD8-depleted human PBMCs, we were able to select virus strains resistant to the CCR5mAb RoAb3952. Resistance to RoAb3952 was associated with a change in the binding properties of the virus; RoAb3952-sensitive virus still required binding to ECL2 of CCR5 for viral entry, whereas RoAb3952-resistant virus was capable of efficiently using the N-terminal loop of CCR5 for viral entry in the presence of an antibody that binds to CCR5 ECL2.
ABSTRACT 3

*Antiviral Therapy* 2008; 13 Suppl 3:A5

Novel resistance profile of the potent nucleoside analogue reverse transcriptase inhibitor 3'-azido-2',3'-dideoxyguanosine

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BACKGROUND: Base modifications of nucleoside reverse transcriptase inhibitors (NRTI) influence their incorporation, excision, antiretroviral activity and resistance profile. For example, 3'-azido-2',3'-dideoxyguanosine (AZG) exhibits potent activity against zidovudine (AZT)-resistant HIV-1 and AZG-5'-monophosphate is poorly excised from terminated primers by reverse transcriptase (RT) that readily excises AZT-5'-monophosphate. Mutations that confer resistance to AZG have not been identified. Here we selected AZG-resistant HIV-1 by serial *in vitro* passage.

METHODS: Wild-type HIV-1 was passaged in increasing concentrations of AZG in MT-2 and human peripheral blood mononuclear cells (PBMCs). Passaged virus was assayed for drug susceptibility to AZG and other NRTI in P4/R5 or PBMCs. The entire coding region of RT was amplified and sequenced from wild type and AZG-resistant virus to identify mutations that were selected.

RESULTS: After 90 passages in MT-2 cells, virus was 10.9-fold resistant to AZG (EC50=9.2 μM) compared with wild type (0.84 μM). AZG-resistant virus showed reduced susceptibility to lamivudine (14.3-fold), AZT (5.6-fold), didanosine (4.3-fold), stavudine (3.5-fold), abacavir (3.4-fold) and tenofovir (1.8-fold). Whole RT sequencing identified the following mutations compared with the starting wild-type virus: L74V, F77L, V106I, L214F and K476N. The K476N mutation is located in the RNase H primer grip of RT. After 70 weeks of AZG selection in PBMCs, virus was selected that encoded V75I, F77L and H221Y. Site-directed mutagenesis showed that the triple mutant exhibited 1.5-fold reduction in susceptibility to AZG.

CONCLUSIONS: AZG selects a novel combination of mutations in the polymerase and RNase H domains of RT that do not include classical thymidine analogue mutations (TAMs). These findings are consistent with the lack of cross-resistance of viruses containing TAMs to AZG and inefficient excision of AZG-5'-monophosphate by RT with TAMs. This study also provides further evidence that the primary determinant for the selection of TAMs is the base structure and not the 3'-azido group on the ribose sugar.
ABSTRACT 4

Antiviral Therapy 2008; 13 Suppl 3:A6

Hexadecyloxypropyl tenofovir (CMX157) has enhanced potency in vitro against NRTI-resistant HIV relative to tenofovir and a favourable preclinical profile

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BACKGROUND: Tenofovir disoproxil fumarate (tenofovir-DF) is one of the most widely used nucleoside/tide reverse transcriptase inhibitors (NRTIs). However, it loses activity against specific HIV mutants, including those with K65R, multiple thymidine analogue mutations (TAMs) or multi-NRTI resistant (MNR) mutations and has been associated with nephrotoxicity. Unlike tenofovir-DF and most prodrugs, hexadecyloxypropyl tenofovir (CMX157) is not efficiently cleaved to free tenofovir in the plasma in vivo. This should increase the levels of active tenofovir-diphosphate in target cells for HIV, thereby lowering the apparent IC50, and reduce the rate of secretion into the kidney.

METHODS: CMX157 IC50s were determined for a panel of 30 NRTI isolates with major NRTI mutations, including K65R with or without M184V, multiple TAM combinations with or without M184V, K70E in various combinations and MNR complexes including T69SSG and Q151M (PhenoSense™). A separate panel of 14 NRTI-resistant clinical isolates and wild-type isolates from subtypes A–G, O and HIV-2 were examined in plasma blood mononuclear cells (PBMCs). Additional studies determined activity in monocytic cells, the effect of human serum on activity, cytotoxicity in dividing and non-dividing cells, tenofovir-diphosphate levels in PBMCs and toxicity/toxicokinetics in rats.

RESULTS: IC50 for CMX157 ranged from 0.66 nM for L74V/M184V to 57 nM for T69SSG/M184V/L210W/T215Y in the PhenoSense™ assay (corresponding IC50 for tenofovir were 227 nM and 16,959 nM, respectively). CMX157 IC50s for M41L/L210W/T215Y averaged 6.3 nM without M184V and 2.2 nM with M184V (2,240 and 770 nM for tenofovir, respectively). Similar data were obtained in PBMCs. CMX157 IC50s against major HIV subtypes ranged from 0.2 nM (B) to 7.2 nM (O). No toxicity was observed in rats administered CMX157 up to 100 mg/kg/d for 7 days; the CMX157 Cmax was 1,200 nM on day 7 and the AUC0-inf was 8,050 ng*h/ml. Tenofovir-diphosphate levels were 33-fold higher in PHA/IL-2 stimulated human PBMCs after 24 h exposure to 1,000 nM CMX157 versus tenofovir in vitro (1.65 and 0.05 pM/10^6 cells, respectively).

CONCLUSIONS: CMX157 is substantially more potent than tenofovir in vitro, possibly due to higher active anabolite levels in target cells. Toxicology and toxicokinetic data reveal high systemic exposure with no indication of nephrotoxicity. CMX157 is being developed for treatment of HIV.
ABSTRACT 5
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In vitro cross-resistance profile, antiviral activity, safety and pharmacokinetics in HIV-1-infected patients of IDX899, a novel HIV-1 NNRTI with high barrier to resistance

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BACKGROUND: IDX899, a second generation non-nucleoside reverse transcriptase inhibitor (NNRTI), was studied in vitro to determine its cross-resistance potential versus other NNRTIs (etravirine, efavirenz and rilpivirine) and in a clinical study to assess its antiviral activity, safety and pharmacokinetics in HIV-1-infected patients naive to antiretroviral therapy.

METHODS: Wild-type HIV-1 was passaged under increasing drug pressure to generate drug-resistant mutant viruses. Resistance and cross-resistance profiles were determined (as EC50 fold-shifts versus wild type) for each viral supernatant. Thirty treatment-naive patients with HIV-1 RNA viral load ≥5,000 copies/ml and CD4+ T-cell count ≥200 cells/mm3 were enrolled and randomized (8:2) to receive IDX899 or placebo once a day for 7 days. IDX899 doses of 800, 400 and 200 mg by mouth daily were assessed. HIV-1 RNA levels were measured using Roche Cobas TaqMan® HIV-1 assay and IDX899 plasma levels were quantitated using a validated LC/MS-MS methodology.

RESULTS: In vitro selection experiments IDX899 resistance required more passages and mutations than efavirenz; IDX899 selected mutations at codons V90I, E138K, Y181C/I, S134I, I135R, G190E and M230L and exhibited less in vitro cross-resistance than efavirenz and TMC125. In the clinical study, the median changes in HIV-1 plasma RNA from baseline to day 8 were -1.95 log10 copies and median CD4+ T-cell count increased by 52.0 cells/µl in the 800 mg cohort. The median changes in HIV-1 plasma RNA from baseline to day 8 were +0.08 log10 copies and median CD4+ T-cell count decreased by 14 cells/µl in the placebo cohort. There were no treatment discontinuations, treatment emergent serious adverse events or dose-limiting toxicities. No discernable patterns in adverse events, laboratory abnormalities or ECG abnormalities were observed within or between treatment groups. Results from the 400 mg and 200 mg cohorts will be presented.

CONCLUSIONS: In a 7 day proof-of-concept study, IDX899 was well tolerated and demonstrated potent HIV-1 antiviral activity. These results, together with a favourable in vitro cross-resistance profile, support further evaluation of IDX899 in combination therapy to assess durability of antiviral response and long-term safety.
ABSTRACT 6

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Longitudinal analysis of resistance to the HIV-1 integrase inhibitor raltegravir: results from P005 a Phase II study in treatment-experienced patients

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BACKGROUND: Raltegravir (RAL) is the first licensed agent in a new class of antiretrovirals that inhibit integration. Understanding resistance and cross-resistance to these agents will become important in clinical practice but is still limited. Initial analysis from the Phase II trials of RAL and the mechanistically related integrase inhibitor elvitegravir suggest the potential for several resistance pathways, each involving multiple mutations with different effects on resistance and viral replication capacity. For RAL, two pathways characterized by signature mutations at either N155(H) or Q148(R/H/K) have been observed.

METHODS: We have followed up on these preliminary observations by performing a longitudinal analysis of resistance in patients with triple class resistant HIV-1 infection failing RAL in the Phase II study. Genotypic analysis was performed by population sequencing and identifying changes in the integrase coding region relative to the sequence prior to RAL treatment. Phenotypic analysis and evaluation of replication capacity was studied with site-directed mutants in a single-cycle infection assay.

RESULTS: Longitudinal genotyping and clonal analysis revealed a consistent preference for the Q148 pathway. Virus populations with mixed N155H and Q148H variants resolved to Q148H over time. Viruses with Q148 patterns, particularly G140S/Q148H, were generally stable while virus populations with N155H often switched to G140S/Q148H. In a subset of patients where the N155H viruses remained the majority population, additional mutations evolved over time. Although Q148H/R/K and N155H were observed to have similar effects on RAL susceptibility, Q148 variants with secondary mutations consistently displayed higher level resistance as compared with N155H variants with multiple mutations. The replication capacity of different combinations varied with Q148H/G140S being the least impaired.

CONCLUSIONS: These studies define four distinct evolutionary patterns for RAL resistance and provide evidence for ongoing selective pressure and a clear preference to evolve to pathways associated with higher level resistance. Whether differential effects on resistance and replication capacity associated with these patterns have consequences for clinical outcomes remains to be examined.
ABSTRACT 7
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Loss of raltegravir susceptibility in treated patients is conferred by multiple non-overlapping genetic pathways

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BACKGROUND: The HIV-1 integrase mutations N155H, Q148R/H/K and Y143R/C reduce susceptibility to the integrase inhibitor raltegravir and have been identified in patients failing raltegravir-containing treatment regimens. Whether these primary resistance mutations occur individually or in combination is not fully understood and the susceptibility of viruses containing secondary mutations only has not been well-characterized. To address these important questions we have explored the susceptibility and replication capacity (RC) of viruses containing primary and/or secondary mutations by analyzing individual clones isolated from raltegravir treated patients.

METHODS: Raltegravir susceptibility was characterized using the PhenoSense™ and GeneSeq™ HIV integrase assays. Samples included HIV-1 populations from patients enrolled in the BENCHMRK Phase III trials, molecularly cloned integrase sequences derived from 11 trial patients and a series of site-directed mutants (SDMs) containing the N155H, Q148R/H/K, E92Q and G140S/A mutations, separately or in combination.

RESULTS: In patients treated with raltegravir, mutation N155H was selected independently from mutations at position Q148. No virus population examined by clonal analysis harboured single variants containing mutations at both positions. Reduction in raltegravir susceptibility was associated with primary mutations and, in a small number of cases, secondary mutations alone. Clonal analysis revealed variants containing E92Q in combination with N155H but not with Q148 mutations. G140A/S was found in combination with Q148 mutations but not N155H. Individual variants containing E92Q in the absence of N155H were identified and displayed reduced raltegravir susceptibility. In general, secondary mutations enhanced resistance conferred by primary mutations. SDMs demonstrated that E92Q and N155H, in combination, reduced susceptibility to raltegravir much more than either mutation alone. Similar observations were found using SDMs containing combinations of G140A/S with Q148 mutations, except G140S and Q148K together, which interestingly, suppressed resistance conferred by Q148K alone. Both N155H and Q148 mutations reduced RC while the addition of secondary mutations improved or reduced RC depending on which primary mutation was present.

CONCLUSIONS: This is the first study that demonstrates the selection of distinct resistance pathways in raltegravir-treated patients using clonal analysis. Combinations of drug resistance mutations differentially affected raltegravir susceptibility and RC depending on the patterns of mutations that were selected.
ABSTRACT 8
Antiviral Therapy 2008; 13 Suppl 3:A10

HIV-1 Gag polymorphisms determine treatment response to bevirimat (PA-457)
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BACKGROUND: Bevirimat is a novel HIV-1 maturation inhibitor in Phase II development that targets the capsid SP-1 cleavage site of Gag. Despite optimal plasma concentrations, not all patients given bevirimat have a robust viral load reduction (VLR). The determinants of treatment response were unknown.

METHODS: In a study to assess the bevirimat trough level associated with an optimal treatment response, 44 heavily treatment-experienced patients were given bevirimat for 14 days as functional monotherapy in escalating dose groups. Baseline clinical and virological variables were assessed to establish the determinants of bevirimat response. Response was also correlated with a standard Gag amino acid sequence.

RESULTS: Nineteen of the 20 responder patients (≥0.5 log10 VLR) and 19/24 non-responder patients (<0.5 log10 VLR) had optimal bevirimat trough levels of at least 20 μg/ml. In this bimodal bevirimat treatment response distribution, the mean VLR was -1.26 or -0.05 log10 copies/ml for responder or non-responder patients, respectively. Non-responder patients had more frequent baseline Gag polymorphisms near the capsid SP-1 cleavage site than responders (7.3; 5.9; P(not significant)); Q369H, V370A and T371A/T371 deletion were more frequent in non-responders. Patients with any amino acid change at positions 369, 370 and 371 had mean VLR of -0.16, -0.24 and -0.32 log10, respectively. Patients with consensus amino acid at positions 369, 370 and 371 had mean VLR of -0.69, -0.79 and -0.73 log10, respectively. Patients without any change at 369, 370 or 371 had mean VLR of -1.08 log10. Twelve (92%) of the 13 patients with bevirimat trough >20 μg/ml and with consensus amino acid at 369, 370 or 371 had VLR >0.5 log10. Lower baseline CD4+ T-cell count was the only clinical variable significantly (P=0.01) associated with non-response. Analysis of Gag genotype in a separate database of 567 treatment-naive HIV positive patients showed that 60.2% had the clade B consensus amino acid at positions 369, 370 or 371.

CONCLUSIONS: Using a genotype assay, treatment response to bevirimat is associated with baseline amino acid polymorphisms at Gag positions 369, 370 or 371 on SP-1; lower baseline CD4+ T-cell count may be a surrogate for these Gag changes. The Gag data were confirmed by phenotypic assay and a new prospective clinical study to verify these findings is underway.
ABSTRACT 9
Antiviral Therapy 2008; 13 Suppl 3:A11

Resistance mutations in HIV-1 integrase selected with raltegravir or elvitegravir confer reduced susceptibility to a diverse panel of integrase inhibitors
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BACKGROUND: The first generation integrase inhibitors, raltegravir (RAL, MK-0518) and elvitegravir (ELV, GS-9137) show great promise for the treatment of HIV infection. However, as with all antiretroviral drugs, resistance to integrase inhibitors (INIs) is expected to emerge. Currently, little is known regarding resistance associated mutations (RAMS) to INIs and the degree of cross-resistance conferred by those mutations.

METHODS: We used in vitro selection (IVS) with MT4/IIIB to delineate the emergence of RAMS to raltegravir and elvitegravir, conducting eight parallel IVS experiments for each inhibitor. The resulting viruses were genotyped and phenotyped and mutants were generated by site-directed mutagenesis (SDMs) to assess the effect of selected RAMS on the susceptibility to a panel of INIs including raltegravir, elvitegravir, L-870,810, PYRAZ (a pyrimido-azepine), PYCA (a pyrido-carboxamide) and L-731,988.

RESULTS: Population genotyping of viruses selected with raltegravir highlighted one pathway towards resistance, that is, Q148R followed by E138K, G140A and V54I. One selected virus (genotype Q148R, E138K, G140A and V54I) showed greatly reduced susceptibility for both raltegravir and elvitegravir (>600-fold) and significantly reduced susceptibility to other INIs. An SDM carrying the Q148R mutation alone showed reduced susceptibility to raltegravir (22-fold) and elvitegravir (47-fold). Combined mutations Q148R and G140A further reduced susceptibility to raltegravir (>1,000-fold) and elvitegravir (260-fold). Resistant viruses selected with elvitegravir contained at least one of the following RAMS: Q148R, E92Q and T66I in addition to further mutations (H114Y, L74M, R20K, A128T, E138K and/or S230R). All selected viruses showed significantly reduced susceptibility to elvitegravir and other INIs with only moderately reduced susceptibility to raltegravir. The SDMs E92Q and T66I were significantly reduced in susceptibility to elvitegravir (57-fold and 35-fold, respectively) with small reductions in susceptibility to raltegravir.

CONCLUSIONS: The Q148R mutation is selected by both raltegravir and elvitegravir and conferred resistance to a diverse panel of INIs. Additionally, mutations selected with elvitegravir (E92Q and T66I) conferred significant resistance to many INIs with a small reduction in susceptibility to raltegravir. We suggest that Q148R, E92Q and T66I be considered as RAMS conferring resistance to a diverse panel of INIs.
ABSTRACT 10

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Virological and immunological outcomes in a cohort of patients failing integrase inhibitors

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BACKGROUND: Although integrase inhibitors are highly effective in the management of drug-resistant HIV, some patients fail to achieve durable viral suppression. The long-term consequences of integrase inhibitor failure have not been well defined.

METHODS: We identified 13 individuals who exhibited evidence of incomplete viral suppression on a regimen containing an integrase inhibitor. Genotypic and phenotypic resistance testing was performed at Monogram Biosciences.

RESULTS: Baseline CD4+ T-cell count and plasma HIV RNA levels were 66 cells/mm3 and 4.78 log10 copies/ml, respectively. Patients were followed for a median 12.7 months. Despite evidence of integrase inhibitor failure, patients appeared to have a persistent immunological benefit, with a median change in CD4+ T-cell count of +37 and +71 cells/mm3 at month 3 and month 6 of documented failure, respectively. Integrase inhibitor failure was often associated with the emergence of genotypic and phenotypic resistance, although three individuals with partial adherence lacked evidence of resistance. The G140S/Q148H pattern and T97A/Y143R patterns were each associated with high-level phenotypic resistance (>400-fold change in IC50). One individual harbouring a virus with an isolated N155H mutation (41-fold change in IC50) discontinued raltegravir while remaining on a stable background regimen. Plasma HIV RNA levels remained stable in the absence of raltegravir (suggesting limited residual antiviral activity), but subsequently increased 10-fold as genotypic/phenotypic evidence for raltegravir resistance waned (suggesting a significant fitness defect).

CONCLUSIONS: Although experience from clinical trials suggests that the majority of patients receiving the newer antiretroviral agents do well, there remains a subset of individuals for whom these drugs will not work because of pre-existing resistance or non-adherence. There may, however, be a residual clinical benefit despite lack of viral suppression. This benefit may be due in part to alterations in viral fitness, as suggested by changes after removal of raltegravir in a single individual. Notably, this latter observation is consistent with observations from the SIV-infected macaque model, where removal of integrase inhibitors in animals harbouring the N155H mutation was associated with initial stable viraemia followed by rapid increase in viraemia as the mutations waned.
ABSTRACT 11

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Virological evolution in HIV treatment-experienced patients with raltegravir-based salvage regimens

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BACKGROUND: Raltegravir (RAL) belongs to a new class of anti-HIV drugs that has been studied in multidrug failure situations mainly through clinical trials with excellent results. However, little is known about resistance to RAL in patients with persistent viraemia.

METHODS: All patients in multidrug failure who initiated RAL between November 2006 and September 2007 were prospectively followed for at least 24 weeks. The CD4+ T-cell count and pHIV-RNA values were collected at baseline and at weeks (W) 4, 12, 24, 36 and 48 after RAL introduction. HIV genotype was performed just before RAL introduction for each patient and later in case of virological failure.

RESULTS: Fifty patients were included. Prior antiretroviral drug therapy contained enfuvirtide (57%), darunavir (68%), etravirine (37%) and foscarnet (12%). Median baseline characteristics were as follows: CD4+ T-cell count=169 cells/mm³ (1–833); pHIV-RNA=15,136 copies/ml (339–724,436). Baseline mutation number was NRTI=7 (1–10), NNRTI=1 (0–4) and PI=13 (8–20). RAL was used in combination with enfuvirtide (33%), darunavir (84%), etravirine (74%), atazanavir (29%) and foscarnet (6%). The median follow-up was W36 (6–15). Thirty-four patients (68%) had virological success with pHIV-RNA <40 at W24. Eleven patients (22%) had low persistent viral load (40 < pHIV-RNA <400) and three (6%) had high persistent viraemia (pHIV-RNA >400). The viral load of the remaining two patients were between 40 and 400 copies/ml at W24, but one of them became undetectable at W28 and the other reached levels above 400 copies/ml. Among the patients in virological failure, genotype resistance testing was successfully performed in 13 patients and RAL resistance mutations were detected in four. G140S+Q148H were detected in two patients, N155H in one patient and a mutation switch from N155H to G140S+Q148H was observed in the last one. Three of these four patients had pHIV-RNA >400.

CONCLUSIONS: In our experience, the majority of patients in virological failure to RAL-based regimens showed low viraemia for a prolonged period without exacerbation. The presence of RAL-resistance mutations was associated to the highest viral replication rates. A longer follow-up of such patients will allow a better understanding of the evolution of RAL resistance profile.
Mutational patterns in the HIV-1 integrase related to virological failures on raltegravir-containing regimens

BACKGROUND: We aimed to study the in vivo viral genetic pathways for resistance to raltegravir (RAL), an HIV-1 integrase inhibitor (INI), in antiretroviral-experienced patients with absence of complete inhibition of HIV-1 replication on RAL-containing regimens.

METHODS: We set up a prospective study including antiretroviral-experienced patients receiving RAL and an optimized background antiretroviral regimen as salvage therapy. The virological and immunological response was studied at months (M) 0, 1, 3 and 6 after initiation of RAL. Genotypic resistance analysis was performed at baseline of RAL by sequencing analysis of the HIV-1 pol gene (protease, reverse transcriptase and integrase). The integrase was also sequenced at the time of virological failure (VF; that is, the absence of decrease of viral load <50 copies/ml at month 3 and 6 or rebound of viral load >50 copies/ml). We used a list of mutations previously reported to be related to in vivo or in vitro resistance to INI for the description of mutations emerging between baseline and VF.

RESULTS: We included 46 patients of the ANRS CO3 Aquitaine Cohort. At baseline, the median plasma viral load (pvl) was 4.43 log_{10} copies/ml and the median CD4+ T-cell count was 200 cells/μl. The proportion of patients with pvl <50 copies/ml was 48.8% at M3 (n=46) and 63.7% at M6 (n=35). Integrase sequences were obtained at baseline and at follow-up for 12 patients with VF. Four different patterns of mutations were observed: emergence of Q148H/R with secondary mutations V72I, L74M, G140A/S, E138A, K156N, K160N, V201I and T206S (five patients); emergence of N155S/H, in the following replaced by a pattern including L74M, T97A, Y143C/H/R, G163R, V151I, S230R (three patients); selection of S230N (one patient); and no selection of mutation but conservation of mutations from baseline to VF (V201I, E157Q+T206S and L74M with one patient each).

CONCLUSIONS: Complex and diverse genetic profiles can be associated to VF on RAL-containing regimens, including dynamics of replacement of mutational profiles. This genetic evolution deserves further molecular investigation in order to better characterize the resistance to RAL.
ABSTRACT 13
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Integrase inhibitor resistance involves complex interactions among primary and secondary resistance mutations: a novel mutation L68V/I associates with E92Q and increases resistance

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BACKGROUND: Elvitegravir (EVG, GS-9137) is a boosted once-daily (QD) integrase inhibitor (INI) being developed for antiretroviral therapy. Previously, we presented genotypic and phenotypic data on INI-resistant viruses from patients receiving EVG 125 mg QD in a Phase II study (GS-US-183-0105). Here, we studied the INI susceptibility and viral fitness of viruses containing different INI-resistance mutations.

METHODS: IN clones were isolated from patients treated with EVG in the Phase II study. The INI susceptibility (EVG and raltegravir [RAL]) and replication capacity (RC) of patient-derived IN clones and site-directed mutants (SDM) were measured using the PhenoSense HIV integrase assay. Relative fitness (1+s) of SDM viruses was also determined using a growth competition assay.

RESULTS: E92Q, Q148R/H/K and/or N155H mutations were each detected in 11/28 (39%) EVG virological failure patients. Two (7%) patients had both E92Q and Q148R as mixtures whereas four (14%) had both E92Q and N155H mutations as mixtures. By clonal analysis, E92Q+N155H but not E92Q+Q148R were found on the same viral genome. Compared with single mutants, E92Q+N155H further reduced INI susceptibility and conferred further reductions in RC and relative fitness. Six of 11 patients with E92Q also had an L68V/I IN mutation, which occurred only in the presence of E92Q (6/6). L68V caused low-level reduced susceptibility to EVG. Double mutant viruses with L68V/I+E92Q combined had further reduced EVG susceptibility versus E92Q alone (57- to 89-fold versus 33-fold). L68V+E92Q reduced susceptibility to RAL versus E92Q alone (12-fold versus 6-fold). L68VI alone, or in combination with E92Q, had no effect on relative fitness.

CONCLUSIONS: Evolution of INI resistance involves complex interactions among both primary and secondary drug-selected mutations. Evidence for mutual exclusion of the E92Q and Q148R mutations was demonstrated by clonal analysis. Viruses containing E92Q or N155H mutations had reduced relative fitness; their combination further reduced viral fitness and enhanced resistance to INIs. Novel secondary EVG-selected mutations, L68V and L68I, were shown to be strongly linked to E92Q and enhanced resistance to INI, but did not affect relative viral fitness. Acquisition of higher phenotypic drug resistance is a significant factor in the evolution of INI resistance pathways.
ABSTRACT 14

Antiviral Therapy 2008; 13 Suppl 3:A16

Pre-existing mutations in the U5 viral DNA end of the HIV-1 LTR do not affect response to the integrase inhibitor elvitegravir: data from Study GS-US-183-0105

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BACKGROUND: Integration of HIV-1 requires interaction between the viral DNA ends and the integrase. Sequence variation in the viral DNA termini could potentially affect antiviral activity of integrase inhibitors (INI). Deviations from the consensus sequence of the U5 end of the viral cDNA were defined in HIV-1-infected patients entering a Phase II study (GS-US-183-0105) of the INI elvitegravir (EVG, GS-9137) and their effect on INI susceptibility analyzed.

METHODS: Highly treatment-experienced patients were randomized to 20, 50 or 125 mg once-daily doses of ritonavir-boosted EVG, or a comparator protease inhibitor, plus optimized nucleoside reverse transcriptase inhibitors +/- T20. Mean plasma HIV-1 RNA at baseline ranged from 4.47–4.71 log10 copies/ml. A 250 base pair fragment of the U5-psi junction was reverse transcriptase-PCR amplified and sequenced from plasma viral RNA. A site-directed mutant of U5 was constructed in the upstream LTR of HXB2. Mutant viruses were phenotyped using a luciferase-based cell-viability assay.

RESULTS: The U5-psi junction was PCR amplified and sequenced from baseline samples of 50 randomly selected patients. The consensus of the U5 terminal 16 nucleotides was GAAAATCTCTAGCAGT-3′. No mutations were detected in the four highly-conserved terminal bases (CAGT). An ‘A’ insertion, designated ‘5A’, at positions 12–16 (AAAA to AAAAA) was observed in 5/50 patients (10%), four of whom received EVG-containing therapy for >24 weeks. All four patients showed a strong viral load decrease with <400 copies/ml by week 16. A 5A site-directed mutant virus showed minimal changes (<twofold) in EC50 for both EVG and raltegravir, compared with >10-fold changes associated with known INI resistance mutations in integrase. The U5-psi junction was also sequenced from 23 randomly selected patients at virological failure, 12 of these had matching baseline data. No changes in the U5 viral ends were observed in response to EVG therapy.

CONCLUSIONS: Among this highly treatment-experienced population, <10% of patients examined had sequence variation within the first 16 nucleotides of the U5 viral ends prior to treatment with EVG. The observed polymorphic changes did not appear to affect susceptibility or clinical response to EVG. Additionally, patients did not develop detectable changes in the U5 viral ends in cases of virological failure.
ABSTRACT 15

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Resistance pathway analysis of diketo acid-based integrase inhibitors allows mapping and prediction of chemical properties of integrase inhibitors

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BACKGROUND: Integrase inhibitor development has been hampered over the last decade by the lack of a crystal structure of the full length enzyme. Moreover, a functional pre-integration complex harbours next to an integrase tetramer or octamer, viral DNA and most probably LEDGF/p75, a cofactor of integration. Diketo acids (DKAs) bind integrase after 3′ processing of the viral DNA. Most integrase inhibitors harbour three structural components, the –CO-CO-CH2-CO- pharmacophore, a hydrophobic moiety and a fluorobenzyl side chain.

METHODS: Using the MOE modelling package, L-708,906, L-870,810, raltegravir, elvitegravir and S-1360 were complexed with the structure of an integrase catalytic core domain bound to the viral cDNA (based on the transposon 5-DNA complex structure).

RESULTS: Although resistance against DKA is mainly located in amino acids surrounding the catalytic core domain, the DKA hydrophobic group (which is oriented outside the surface upon MOE mapping) determines resistance in the β-barrel C-terminal part of integrase. As a consequence, DKAs lacking the hydrophobic group (for example, S-1360) do not select for resistance in the C-terminal part of integrase and are not cross-resistant for these mutations. These results also indicate that the C terminus of integrase folds towards the integrase catalytic core in the functional integrase complex. The LEDGF binding side can be mapped in the dimer integrase complex and is different from the DKA binding side. The DKA fluorobenzyl ligand, which is important for antiviral activity, binds into the groove in near proximity of the D64 and D116 suggesting that this ligand locks the complex. The 5′ viral DNA is localized near the Q148, which explains why mutations in Q148 alter affinity for 5′ viral DNA.

CONCLUSIONS: The current resistance mutations against DKAs allow mapping of the strand transfer inhibitors to a model of integrase and to better understand the organization of the functional integrase complex and the different binding sides of DKAs and LEDGF/p75. S-1360 lacks the hydrophobic moiety across from the DKA pharmacophore, produces a different resistance pattern and is not sensitive to mutations in the C-terminal part of integrase. Better understanding of the integrase viral DNA-inhibitor complex is highly important for the development of new generation integrase inhibitors.
ABSTRACT 16

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Small-molecule inhibitors of HIV-1 integrase

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BACKGROUND: HIV-1 integrase has recently been validated clinically as an important target for the treatment of HIV/AIDS. Raltegravir is the first marketed integrase inhibitor and there are also a number of integrase inhibitors in clinical trials. Here, we report novel compounds as integrase inhibitors.

METHODS: In single-cycle infection assays, MT4 cells were infected with a defective envelope (env-), luciferase-bearing NL-4.3 virus pseudotyped with HIV-1 env (HXBc2) or VSV-G env. In multicycle infection assays, the antiviral activity of the compounds was determined by a cytoprotection assay (MTT) using NL-4.3 in MT4 cells. The cytotoxicity of the compounds was tested in parallel using the same assay but without adding virus to the cells. The time-of-addition experiments were performed in MT4 cells infected with virus pseudotyped with HXBc2 env in single-cycle infection assays. The drug was added to the culture at the concentration of EC95 at different time intervals.

RESULTS: We reported previously some vitamin B6 derivatives as non-cytotoxic integrase inhibitors with activities against integrase enzyme and wild-type HIV-1 viruses in cellular assays. These derivatives displayed a different mechanism of action of integrase strand transfer inhibition by potentially binding to a novel site on integrase. A recent lead optimization effort has led to the discovery of new vitamin B6 derivatives, which show improved activities against integrase enzyme and viruses. These new derivatives demonstrate selectivity toward integrase enzyme over other enzymes (that is, >150-fold more specific for integrase versus reverse transcriptase). In the cell-based assays, these compounds display not only potent antiviral activity (EC50 <100 nM) but also selectivity against cellular integrase. A similar antiviral activity was observed when HIV-1 envelope was replaced with VSV-G suggesting that these compounds are post-entry inhibitors. Furthermore, the time-of-addition studies indicate that these compounds have a very similar inhibitory profile to that of raltegravir.

CONCLUSIONS: A novel series of vitamin B6-based integrase inhibitors has been developed. These compounds demonstrate potent inhibition against HIV-1 integrase strand transfer activity, antiviral activity and specificity against HIV-1 integrase in cellular assays.
ABSTRACT 17

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Raltegravir resistance mutations affect strongly the integrase activities and the replicative capacity of viruses harbouring such mutations

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BACKGROUND: Raltegravir (MK-0518) is a potent inhibitor of HIV integrase (IN) and is clinically effective against viruses resistant to other antiretroviral classes. However, it can select mutations in HIV IN gene. It was demonstrated that most of these mutations altered both 3′-processing and strand transfer activities of IN. The aim of this study was to evaluate in vitro the effect of some of these mutations on the replicative capacity (RC) of mutated viruses.

METHODS: The two most prevalent profiles of raltegravir mutations were studied (N155H and G140S+Q148H). The entire IN genes (288 amino acids) from plasma samples corresponding to baseline (before raltegravir treatment) and to failure to raltegravir with IN mutations were amplified. These PCR products were cloned into a plasmid pNL4-3 deleted for IN gene. Recombinant viruses were obtained after plasmid transfection. The production of recombinant viruses was measured using AgP24 quantification up to 22 days.

RESULTS: In vitro, there were strong differences of virus production comparing recombinant viruses harbouring baseline IN (wild type) and mutated IN with N155H or G140S+Q148H mutations. At days 5, 10 and 22, the Ag P24 quantification was 7.10^3 pg/ml versus 6.10^2 pg/ml, 2.10^5 pg/ml versus 10^4 pg/ml and 2.10^6 pg/ml versus 10^3 pg/ml for the wild type versus mutant viruses, respectively. Thus, the kinetics of replication and the total amount of produced viruses are strongly decreased in viruses with raltegravir resistance mutations. However, in vivo, patients harbouring viruses with these raltegravir mutations showed HIV viral load rebound after selection of raltegravir mutations raising the question of other genetic determinants located outside of the IN gene that could be needed to get failure and resistance to raltegravir.

CONCLUSIONS: These results show that recombinant viruses with raltegravir resistance mutations have a very low RC. This is in accordance with biochemical results showing that these mutated IN have both 3′-processing and strand transfer altered activities and with the high difficulty to select such mutations using in vitro passages under raltegravir progressive pressure. Discrepancy between in vitro low RC and in vivo high level of HIV viral load rebound after selection of raltegravir mutations raises the question of other genetic determinants located outside of the IN gene that could be needed to get failure and resistance to raltegravir.
ABSTRACT 18
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Virological response and resistance in multi-experienced patients treated with raltegravir

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BACKGROUND: Raltegravir is a novel and potent integrase inhibitor that showed very promising results in clinical trials. Data about the efficacy of raltegravir in uncontrolled studies, as well as the dynamics of resistance mutations, have yet to be defined. The objective was to study the potential correlations between virological response and the development of short-term resistance.

METHODS: HIV-1-infected treatment-experienced patients with triple-class resistant virus who received raltegravir plus optimized background therapy (OBT) were analyzed. Integrase genotyping together with CD4+ T-cell count and plasma HIV-1 RNA were assessed at baseline and at weeks 4–24.

RESULTS: Fifty-two patients received raltegravir plus OBT. At baseline, median HIV-1 RNA was 4.67 log copies/ml and median CD4+ T-cell count was 127 cells/mm3. At 7–19 days after raltegravir-based therapy, a very sharp decrease of HIV-1 RNA (median reduction -2.03 log copies/ml) was found. After 4 weeks, the median HIV-1 RNA reduction was -2.43 log copies/ml and 50% of patients reached <50 copies/ml. Median CD4+ T-cell count increase was 71 cells/mm3 (P=0.007). At week 12, <50 HIV-1 RNA copies/ml was achieved in 64% of available patients and maintained to the end of observation period. Baseline sequences showed only secondary integrase mutations, such as L74I (1.9%), T97A (1.9%), S119G (3.8%), M154I (17.3%), K156N (11.5%), V165I (20.1%), V201I (40.3%) and I203M (1.9%). Integrase sequences of 16 patients after 18–60 days of raltegravir-based therapy (median HIV-1 RNA 92 copies/ml) were successfully obtained. Of interest, 4/5 that did not reach <50 copies/ml at 12 weeks developed primary mutations, while the same occurred in only 1/11 that achieved <50 copies/ml (P=0.013). One patient added G140S+Q148Q mutations. Three patients presented the N155H mutation, all having the V165I secondary mutation at baseline, while one patient presented the Y143R mutation, having T97A at baseline. This suggests that different patterns of mutations conferring resistance to raltegravir can be related to selected polymorphisms present at baseline.

CONCLUSIONS: Raltegravir showed an extraordinary potent antiretroviral and immunological efficacy in the large majority of multi-experienced patients. In a minority of patients, a rapid resistance to raltegravir emerged and correlated with later virological outcome, thus confirming the importance of associating this new integrase inhibitor to other active drugs to maintain long-term efficacy.
ABSTRACT 19

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HIV DNA viral load evolution under a raltegravir-based therapy

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BACKGROUND: The recent antiretroviral drug class of integrase inhibitors, including raltegravir (RAL), is also expected to have a possible effect on HIV DNA viral load level. However, few data are available about HIV DNA level evolution during a RAL-based regimen.

METHODS: Eighteen highly experienced patients initiated a RAL-containing salvage regimen. Plasma and peripheral blood mononuclear cells samples were collected for all patients at baseline and at week 12 (W12) of RAL; HIV RNA and HIV DNA viral loads were assessed. HIV DNA quantification assessed total HIV DNA, integrated and unintegrated, using the real-time PCR procedure previously described with the NEC005, NEC131 primers and the MLC1 probe.

RESULTS: Among the 18 patients, baseline median HIV RNA level and CD4+ T-cell count were 4.05 log10 copies/ml (range 2.00–5.56) and 29 cells/mm3 (range 3–109), respectively. Median baseline HIV DNA viral load was 3.50 log10 copies/106 cells (range 2.12–4.78). At W12 of RAL-based regimen, eight of the patients exhibited an HIV viral load <40 copies/ml; among the 10 remaining patients median HIV viral load was 2.12 log10 copies/ml (range 1.67–4.65). Median HIV DNA level at W12 was 2.95 log10 copies/106 cells (range 2.39–4.42). Median HIV DNA viral load decrease between baseline and W12 was -0.48 log10 copies/ml (range -2.26–+0.39). The degree of HIV DNA decrease does not correlate with CD4+ T-cell count or HIV RNA level at baseline. However, the degree of HIV DNA decrease was found to be correlated with HIV DNA baseline level (R=0.28; P=0.03). The HIV DNA level/CD4+ T-cell count ratio decrease between baseline and W12 was correlated with the magnitude of the HIV RNA level reduction (R=0.35; P=0.01).

CONCLUSIONS: Our data showed no drastic effect of RAL on total HIV DNA level as only a median decrease of -0.48 log10/106 cells was observed at W12. However, when HIV DNA was reported to the proportion of CD4+ T-cell count, a correlation was found with the magnitude of HIV RNA decrease. Thus, these findings may suggest that newly CD4+ T-cells generated during treatment may integrate less of HIV DNA copies.
ABSTRACT 20

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Genotypic analysis of patients enrolled in Study AVX-201 and treated with apricitabine for 24 weeks

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BACKGROUND: Apricitabine is a novel cytidine analogue nucleoside reverse transcriptase inhibitor (NRTI). Apricitabine retains activity in the presence of the M184V mutation in HIV-1 reverse transcriptase (RT) alone or when accompanied by thymidine analogue mutations (TAMs – by either pathway) or other nucleoside analogue mutations (NAMs), such as L74V. AVX-201 is a 48-week study of apricitabine compared with lamivudine in patients failing therapy with M184V, with or without additional mutations. Genotypic analysis of patient isolates was performed at baseline, after 21 days of functional monotherapy with apricitabine and after 24 weeks of treatment with apricitabine together with an optimized background to search for any development of resistance to apricitabine.

METHODS: The approved Bayer Trugene® HIV-1 genotyping assay was used to sequence HIV-1 RT from plasma samples of patients enrolled in the AVX-201 study at screening, baseline, day 21 (after 21 days on functional monotherapy with apricitabine), week 12 and week 24. Background therapy was optimized on day 21 according to the screening genotype.

RESULTS: At baseline, the distribution of genotypes (n=50 in total, all M184V) was M184V alone, n=17; M184V plus the following: 74V, n=7; 1 TAM, n=3; 2 TAMs, n=3; 3 TAMs, n=14; 4 TAMs, n=6; 5 TAMs, n=4; 6 TAMs, n=1; and 41L/210W, n=13. No patients had K65R present at baseline. Evidence of non-NRTI resistance was common. At day 21, 38/38 genotypes obtained had M184V. At week 12, 10/14 had M184V, 2/14 had M184M/V and two had reverted to 184M. At week 24, 6/9 had M184V and three had reverted to 184M. Very few other changes were seen and no patients developed K65R. Individual genotypes will be presented.

CONCLUSIONS: No additional mutations developed in patients treated with apricitabine either during a 21-day period of functional monotherapy, nor up to 24 weeks in combination with an optimized background. The M184V mutation was maintained in the majority of patients where a genotype could be obtained. No development of K65R, L74V, TAMs or other NAMs was seen.
ABSTRACT 21

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In vitro selection and characterization of viruses resistant to RO-5028, a novel non-nucleoside reverse transcriptase inhibitor

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BACKGROUND: RO-5028 represents a new class of potent non-nucleoside reverse transcriptase inhibitors (NNRTIs). RO-5028 is broadly active against NNRTI-resistant HIV variants. Three independent sequential passaging experiments were performed to identify mutations selected by RO-5028, efavirenz and etravirine in vitro.

METHODS: The passaging experiments were performed with HIV HXB2 at a low multiplicity of infection in MT4 cells and increasing concentration of RO-5028, efavirenz and etravirine. Breakthrough viruses were characterized by pool genotypic and phenotypic analysis.

RESULTS: RO-5028 inhibited HIV-1 HXB2 replication in MT4 with an IC_{50} value of 1.3 nM. RO-5028 is broadly active against HIV-1 strains carrying prevalent point mutations associated with resistance to current NNRTIs. In three independent sequential passaging experiments, the concentration of RO-5028 was increased from 1 nM to 10 μM within 13 passaging steps. The pool sequencing of RO-5028 breakthrough viruses in passage 13 consisted of V106A plus F227L for experiment 1, V106V/I, V108V/I plus Y188L for experiment 2 and V106A, and V108I plus F227F/L for experiment 3. Our results confirmed the published resistance pathways described previously for efavirenz and etravirine. In isogenic mutant constructs carrying the mutations identified during the passaging, we determined that development of resistance to RO-5028 required in general multiple mutant positions within the NNRTI binding pocket to loose susceptibility to RO-5028. One exception was Y188L, a mutation requiring a two nucleotide change, which by itself can confer >100-fold shift in the IC_{50}. RO-5028 passage 13 resistant viruses exhibited cross-resistance to efavirenz. In contrast, the RO-5028 resistant viruses remained sensitive to inhibition by etravirine, suggesting a complementary mutant profile between these compounds.

CONCLUSIONS: Passaging of HIV-1 HXB2 in MT4 cells under increasing drug pressure of RO-5028 in three independent experiments has identified two diverse resistant pathways independent of those seen with efavirenz and etravirine. Notably RO-5028 passage 13 resistant viruses contain mutations that are less prevalent when compared with high prevalent mutations such as K103N, Y181C and G190A. RO-5028 is a potent candidate for the potential treatment of NNRTIs naive and pretreated patients.
Increased phenotypic susceptibility to etravirine in HIV-1 with nucleoside reverse transcriptase inhibitor resistance

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BACKGROUND: Increased susceptibility to the non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz, nevirapine and delavirdine associated with mutations selected by nucleoside reverse transcriptase inhibitors (NRTIs) has been previously reported and has been shown to have clinical benefit in some cases. The effects of NRTI resistance-associated mutations (NAMs) on susceptibility to etravirine, the most recently approved NNRTI, are unknown.

METHODS: The median fold change (MFC) in etravirine IC_{50} of groups of viruses containing various NAMs was determined using data derived from 2,056 samples submitted for routine resistance testing. NAMs were defined as M41L, K65R, D67N, T69X, K70E/R, L74I/V, V75A/M/S/T, Y115F, Q151M, M184I/V, L210W, T215F/Y and K219X (X=any non-wild-type amino acid). NNRTI mutations were defined as A98G, L100I, K101E/P, K103N/S, V106A/M, Y181X, Y188X, G190X, P225H, F227L, M230L and P236L.

RESULTS: Groups of viruses lacking NNRTI mutations containing various NAMs (often in combination with others) demonstrated varying degrees of increased etravirine susceptibility, with MFC ranging from 0.19 (K219N, n=9) to 0.67 (T69N, n=15). When present as the only NAM, T215Y, M41L, M184V and T69N were associated with MFC of 0.36 (n=6), 0.67 (n=17), 0.69 (n=118) and 0.85 (n=5), respectively. In combination with no more than two other NAMs, L74V, M184I, L210W and T215F/Y were associated with MFC<0.4. Increasing numbers of NAMs was associated with decreasing MFC, whether or not NNRTI mutations were present. For example, in the absence of NNRTI mutations, etravirine MFC was 0.89, 0.69, 0.60 and 0.35 with 0, 1, 2 or >2 NAMs; with one NNRTI mutation, MFC was 0.98, 1.0, 1.2, and 0.69, and with two NNRTI mutations it was 6.4, 4.1, 2.6 and 2.1.

CONCLUSIONS: Resistance to NRTIs is associated with increased etravirine susceptibility, causing hypersusceptibility when NNRTI resistance mutations are absent or few and reducing the level of resistance in combination with multiple NNRTI mutations. The clinical relevance of this phenomenon is unknown but deserves further study and may affect the derivation of genotype algorithms for prediction of reduced etravirine susceptibility.
ABSTRACT 23
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Nucleoside-associated mutations cause hypersusceptibility to etravirine

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BACKGROUND: Nucleoside associated mutations (NAMs) have been implicated in hypersusceptibility (HS) to first-generation non-nucleoside reverse transcriptase inhibitors (NNRTIs); HS has been associated with better clinical responses to NNRTIs. In vitro HS to etravirine was investigated.

METHODS: A panel of 29 HIV-1 recombinant clinical isolates with well-characterized HS to nevirapine and efavirenz was tested for etravirine phenotypic susceptibility (PhenoSense, Monogram Biosciences). The panel consisted of four groups: a) isolates with no mutations in RT (n=8), b) isolates with M184V alone (n=6), c) isolates with NAMs plus M184V (n=8) and d) isolates with NAMs (n=7). NAMs included amino acid changes at positions 41, 65, 67, 69, 70, 115, 118, 151, 210, 215 and/or 219. In addition, isolates carrying the K103N mutation (n=11), with or without NAMs and other NNRTI mutations, as well as 1,023 wild type routine clinical samples with no known nucleoside reverse transcriptase inhibitors-, NNRTI- or protease inhibitor-resistance mutations were tested. HS was defined as a fold change (FC) in EC50 ≤ 0.4.

RESULTS: The proportion of samples with HS to etravirine, efavirenz and nevirapine, respectively, in each group was a) 0%, 62.5% and 100%; b) 100%, 100% and 100%; c) 87.5%, 87.5% and 100%; and d) 100%, 71% and 87.5%. Median FC values to etravirine, efavirenz and nevirapine, respectively, in each group were a) 0.53, 0.4 and 0.3; b) 0.27, 0.25 and 0.25; c) 0.29, 0.27 and 0.26; and d) 0.26, 0.33 and 0.34. In addition, one sample with K103N and NAMs showed HS to etravirine (FC=0.24). However, none of 10 samples with K103N alone (n=4) or with K103N plus other NNRTI mutations but no NAMs (n=6) showed HS to etravirine. The median FC among samples with K103N was 1.18 (range 0.49–2.22) for etravirine in contrast to >100 for efavirenz and nevirapine. Among wild type samples, 2.8%, 3.1% and 9.0% showed HS to etravirine, efavirenz and nevirapine, respectively.

CONCLUSIONS: Among the HIV-1 isolates studied, HS to etravirine was mainly observed among those carrying NAMs and/or M184V. K103N-containing isolates did not exhibit HS to etravirine; nevertheless, FC values were below the PhenoSense clinical cut-off for etravirine (2.9). The potential effect of HS on response to etravirine deserves further investigation.
**ABSTRACT 24**

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An update of the list of NNRTI mutations associated with decreased virological response to etravirine: multivariate analyses on the pooled DUET-1 and DUET-2 clinical trial data

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BACKGROUND: Etravirine (TMC125) is a next generation non-nucleoside reverse transcriptase inhibitor (NNRTI), with activity against NNRTI-resistant HIV-1 and a high genetic barrier to development of resistance. Analyses of the pooled DUET-1 and DUET-2 Phase III clinical trials identified 13 etravirine resistance-associated mutations (RAMs; V90I, A98G, L100I, K101E/H/P, V106I, V179D/F, Y181C/I/V and G190A/S). The presence of three or more of these RAMs was associated with decreased virological response. In this study, additional statistical approaches were used to refine this list and improve the genotype/phenotype correlation.

METHODS: The effect of baseline resistance on virological response (≤50 copies/ml) to etravirine at week 24 was studied in patients not using enfuvirtide *de novo* and excluding those who discontinued for reasons other than virological failure (n=406). Multivariate analyses included logistic regression controlling for baseline viral load, darunavir fold change (FC) in EC₅₀ and nucleoside reverse transcriptase inhibitor sensitivity. Mutations were identified based on the association with decreased virological response and/or increased etravirine FC. Mutations in the reverse transcriptase (amino acids 1–400) were included in the final analysis if present in ≥5 patients.

RESULTS: The analyses confirmed the effect on response of the 13 etravirine RAMs identified previously and also identified K101H, E138A and V179T as associated with a decreased virological response and/or increased etravirine FC. The V179F/T, Y181V and G190S mutations were associated with the lowest virological response but were present in <5% of patients at baseline. Virological response decreased in subgroups with increasing numbers of baseline etravirine RAMs (77%, 61%, 56% and 38% for 0, 1, 2 and ≥3 RAMs, respectively). Relative weighting of the 16 etravirine RAMs improved the correlation between baseline etravirine FC and the number of etravirine RAMs.

CONCLUSIONS: A comprehensive analysis of baseline resistance data from DUET-1 and DUET-2 identified three additional mutations resulting in a list of 16 etravirine RAMs (V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V and G190A/S). Weighting these mutations improved the correlation between genotypic and phenotypic resistance interpretations. Decreased virological response was a function of the number of baseline etravirine RAMs with the largest effect observed in the subgroup of patients with three or more RAMs.
ABSTRACT 25

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Antiviral activity, safety and pharmacokinetics of IDX899, a novel HIV-1 non-nucleoside reverse transcriptase inhibitor with high barrier to resistance, in treatment-naive HIV-1-infected patients

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BACKGROUND: In vitro data indicate that IDX899, a second generation non-nucleoside reverse transcriptase inhibitor (NNRTI), has potent activity against wild-type and NNRTI-resistant HIV-1 with a higher genetic barrier to resistance than efavirenz. Favourable clinical safety and pharmacokinetics (PK) in healthy patients supported the current study which assessed antiviral activity, safety and PK in HIV-1-infected patients naive to antiretroviral therapy.

METHODS: Ten treatment-naive patients with HIV-1 RNA viral load \( \geq 5,000 \) copies/ml and a CD4+ T-cell count \( \geq 200 \) cells/mm\(^3\) were enrolled and randomized (8:2) to receive 800 mg of IDX899 or placebo once a day (QD) for 7 days. HIV-1 RNA levels were measured using the Roche Cobas TaqMan® HIV-1 assay and IDX899 plasma levels were quantitated using a validated LC/MS-MS methodology. As potent antiviral activity was demonstrated at 800 mg, sequential cohorts evaluating 400 mg, 200 mg or lower doses are being enrolled.

RESULTS: At the time of abstract submission, patients in the 800 mg cohort have completed dosing and all patients in the 400 mg cohort have initiated or completed therapy. The median changes in HIV-1 plasma RNA from baseline to day 8 were \(-1.95 \) log\(_{10}\) copies/ml and median CD4+ T-cell count increased by 52.0 cells/ml in the 800 mg cohort. The median changes in HIV-1 plasma RNA from baseline to day 8 were \(+0.08 \) log\(_{10}\) copies/ml and median CD4+ T-cell count decreased by 14 cells/µl in the placebo cohort. There were no treatment discontinuations, treatment emergent serious adverse events or dose-limiting toxicities. No discernable patterns in adverse events, laboratory abnormalities or ECG abnormalities were observed within or between treatment groups. Results from the 400 mg and 200 mg cohorts will be available for presentation.

CONCLUSIONS: IDX899, dosed at 800 mg QD for 7 days was well tolerated and demonstrated potent HIV-1 antiviral activity. Our results support further evaluation of IDX899 at lower doses as well as longer studies of combination therapy to assess durability of antiviral response and long-term safety.
BACKGROUND: IDX899 is an HIV-1 non-nucleoside reverse transcriptase inhibitor (NNRTI), which has demonstrated significant HIV-1 inhibition in treatment-naïve patients. Here we compare the *in vitro* resistance mutations elicited by IDX899, TMC125 (etravirine) and efavirenz (EFV) and profile the susceptibility of the resultant virus isolates to each of the three agents along with TMC278 (rilpivirine).

METHODS: Wild-type HIV-1 (BH10, subtype B) was passaged under increasing drug pressure to generate resistant mutant viruses. Viral supernatants were characterized by RT-PCR and direct population sequencing. Resistance and cross-resistance profiles were determined (as EC50 shifts) for each viral supernatant.

RESULTS: As reported previously (Richman et al., CROI 2008, poster #729), high level (>100-fold) resistance to efavirenz emerged rapidly, requiring just five to 13 passages and one to three mutations. In contrast, IDX899 and TMC125 resistance required more passages (25–30) and mutations (three to five). Selected mutations included: L100I, K103R, V179D, G190A, V35L and R83K (efavirenz); L100I, K103R, V179D/I, E138G, Y181C, K219R and R83K (TMC125); and V90I, E138K, Y181C/I, I134I, I135R, G190E and M230L (IDX899). All six efavirenz-selected virus isolates were highly (>100-fold) resistant to efavirenz; 4/6 isolates lost >10-fold susceptibility to TMC125 versus only 1/6 for both IDX899 and TMC278. For TMC125-selected isolates (containing up to five mutations), IDX899 retained good activity (< sixfold shift) against 9/10 isolates, compared with only 2/10 for efavirenz, 3/10 for TMC125 and 4/10 for TMC278. For IDX899-selected viruses, 6/8 showed a > sixfold loss of susceptibility to both IDX899 and TMC278, versus 7/8 for TMC125, and 5/8 for efavirenz. Efavirenz retained the best overall activity against the IDX899-selected isolates, whereas the TMC compounds were often less active than IDX899. Overall, of the 24 NNRTI-selected virus pools tested, 71% were <10-fold cross-resistant to IDX899 versus 25%, 38% and 67% for efavirenz, TMC125 and TMC278, respectively. Moreover, the average EC50 shift for IDX899 was five times less than that of TMC125.

CONCLUSIONS: IDX899 has previously been shown to be active against most of the resistance mutations produced by first-generation NNRTIs. The present study of NNRTI-selected mutants suggests that IDX899 has less *in vitro* cross-resistance than efavirenz and TMC125.
ABSTRACT 27

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Preclinical profile of R7180, a prodrug of the novel NNRTI RO-5028, with high antiviral potency against wild-type and NNRTI-resistant viruses

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BACKGROUND: A new series of non-nucleoside reverse transcriptase inhibitors (NNRTIs) was optimized with regards to antiviral potency against wild-type and NNRTI-resistant viruses. In addition, a delivery strategy was devised to allow increased oral bioavailability, increasing inhibitory quotient and effectiveness of virus replication suppression.

METHODS: Optimization and characterization of NNRTIs were based on structural and biochemical assessments of their binding to HIV-RT, inhibition of HIV-RT activity and inhibition of HIV replication in single- and multiple-cycle replication assays in cell culture. Pharmacokinetics were determined in rats, dogs and cynomolgus monkeys and preclinical safety assessments were conducted in rats and dogs. Plasma exposures of the prodrugs, parents and major metabolites were determined by LC-MS/MS.

RESULTS: RO-5028 represents a new series of NNRTIs with unique HIV-RT binding interactions. RO-5028 is a potent inhibitor of wild-type HIV-1 in cell culture (mean IC_{50}=1.3 nM). The binding affinity to human serum proteins is lower than that of efavirenz. Antiviral potency of RO-5028 in the presence of human serum was reduced 3.9-fold, a smaller effect as compared with efavirenz, etravirine or rilpivirine. RO-5028 potently inhibits replication of HIV-1 variants with common NNRTI resistance mutations (for example, K103N/Y181C, L100I/K103N and G190A/S). In a panel of 50 viruses derived from NNRTI-pretreated patients, RO-5028 inhibited 90% of viruses with human serum binding adjusted IC_{50} values lower than 100 nM. R7180, a prodrug of RO-5028, increased oral bioavailability and dose proportionality of RO-5028, allowing the potential achievement of high inhibitory quotients in humans. In toxicity studies of up to 4 weeks duration in dogs, R7180 was not associated with any significant pathological or clinical findings at dose levels up to 40 mg/kg, while reaching plasma concentrations up to 24 μM (>4,700-fold above human serum adjusted antiviral IC_{50}). No overt signs of toxicity were noted in rats, although plasma concentrations in rats were limited by auto-induction to ~4.4 μM.

CONCLUSIONS: R7180 represents a series of prodrugs for novel NNRTIs with high antiviral potency against wild-type and NNRTI-resistant HIV-1. R7180 is efficiently converted to the active parent RO-5028, achieving high oral bioavailability and well-tolerated in toxicity studies where high plasma exposures were achieved, therefore supporting further development of R7180 for the treatment of HIV infection.
ABSTRACT 28

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HIV-1 susceptibility to the maturation inhibitor bevirimat is modulated by natural polymorphisms at positions 369–371 in Gag spacer peptide 1

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BACKGROUND: The HIV-1 maturation inhibitor bevirimat inhibits cleavage of spacer peptide 1 (SP1) from the C terminus of capsid resulting in defective core condensation. Recent clinical studies found that a subset of patients respond poorly to bevirimat. Genotypic analysis of patient samples suggested that poor response is associated with baseline polymorphisms at positions 369, 370 and 371 in SP1. In this study, we evaluated baseline susceptibility to bevirimat by phenotypic testing of 20 patient-derived HIV-1 isolates.

METHODS: Viral RNA was extracted from virus samples and used to generate Gag-protease (PR) amplicons by one-step reverse transcriptase (RT)-PCR followed by nested PCR. Gag-PR amplicons were sequenced and recombined with an HXB2-based backbone by nucleofection into MT4 cells. Replication-competent recombinant viruses were titrated and subjected to antiviral testing using FDA-approved PR and RT inhibitors and bevirimat. Fold change (FC) values were calculated using IIIB as reference strain. Results were compared with the routine PR-RT genotyping (VircoType) and phenotyping results (Antivirogram) using the same compounds.

RESULTS: Gag-PR and PR-RT were successfully amplified, genotyped and phenotyped from 20 patient isolates. Reference strain IIIB wild-type virus showed an IC50 value for bevirimat of 55 ±16 nM. PR-RT genotyping and phenotyping demonstrated no correlation of bevirimat susceptibility with resistance to PR and RT inhibitors. Gag-PR phenotyping showed three different levels of susceptibility to bevirimat: six highly susceptible viruses had FC values between 1.0 and 4.8, five viruses had intermediate susceptibility (FC values 31.8–71.3) and nine viruses had FC values >140.3. Gag-PR genotyping found wild-type QVT amino acid sequence at positions 369–371 in all six of the highly susceptible viruses, whereas all nine of the least susceptible viruses contained polymorphisms in this region. The five viruses with intermediate susceptibility were either wild-type (three) or polymorphic (two).

CONCLUSIONS: Using a Gag-PR phenotypic and genotypic assay, three levels of susceptibility to bevirimat were observed for a set of 20 patient-derived virus isolates. Reduced susceptibility correlated with polymorphisms at Gag residues 369–371 in SP1. The 11 viruses containing polymorphisms all had the highest FC values. Testing of additional virus isolates should help to further clarify the role of individual polymorphisms in bevirimat susceptibility.
Role of Gag polymorphisms in HIV-1 sensitivity to the maturation inhibitor bevirimat

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BACKGROUND: The HIV-1 maturation inhibitor bevirimat (PA-457) binds to Gag and specifically inhibits CA-SP1 processing. Previous in vitro resistance selection experiments identified four highly conserved residues proximal to the CA-SP1 cleavage site that are crucial for resistance to bevirimat. However, variable clinical responses to bevirimat have not been associated with these resistance mutations, rather the responses appear to correlate with the polymorphisms located in SP1 downstream of the CA-SP1 cleavage site that are present at baseline in a subset of patients. The current study examines how these polymorphisms affect susceptibility to bevirimat in vitro.

METHODS: HIV-1 bearing polymorphisms in SP1 were generated by point mutation or construction of chimeric Gags containing CA-SP1 regions from bevirimat-treated patients. Gag processing in virions released from bevirimat-treated cells was analyzed by SDS-PAGE. Selected clones, as well as whole viruses isolated from bevirimat-treated patients, were also tested for susceptibility to bevirimat in single-cycle infection and virus replication assays using cell lines or primary peripheral blood mononuclear cells.

RESULTS: Viruses containing deletions, substitutions or naturally occurring polymorphisms at Gag positions 369, 370 and 371 in SP1 displayed reduced inhibition of CA-SP1 cleavage by bevirimat in Gag processing assays. These viruses also displayed reduced susceptibility to bevirimat inhibition in single-cycle infection assays and in virus replication assays in which a high level of input virus inoculum was used. For at least a subset of these viruses, this effect appeared to be multiplicity of infection (MOI)-dependent as further titration of the virus inoculum resulted in susceptibility to bevirimat similar to wild type.

CONCLUSIONS: We have identified naturally occurring polymorphisms at Gag positions 369, 370 and 371 in SP1 that reduce the susceptibility of viruses to bevirimat in vitro. These polymorphisms are distinct from previously described resistance mutations in that they have not been observed in resistance selection experiments in vitro and, in contrast to resistance mutations, the effect of the polymorphisms on bevirimat activity appears to be MOI-dependent. The fact that bevirimat is active against the polymorphic viruses under conditions of low viral replication in vitro may help explain why some patients respond to bevirimat despite being infected by virus bearing these polymorphisms.
ABSTRACT 30

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New phenotyping assay demonstrates role of Gag-SP1 polymorphisms in HIV-1 sensitivity to the maturation inhibitor bevirimat

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BACKGROUND: Maturation inhibitors such as bevirimat (PA-457) impair HIV replication by specifically binding to a region of Gag, thereby inhibiting CA-SP1 cleavage by viral protease and subsequent formation of mature virions. Recent analyses of clinical data suggested that patients who responded well to bevirimat generally had wild-type HIV-1 sequences at Gag residues 369, 370 and 371, while patients who responded poorly generally had polymorphisms at one or more of these residues. These polymorphisms in SP1 are distinct from laboratory-generated resistance mutations located at the CA-SP1 cleavage site. The current study examined in vitro sensitivity to bevirimat of HIV strains bearing various SP1 polymorphisms using the replicative in vitro test, dual-enhancement of Cell Infection to Phenotype Resistance (deCIPhR).

METHODS: The HIV-1 genome from the start of capsid through the end of protease was reverse-amplified from 20 patient-derived HIV-1 isolates bearing either wild-type or polymorphic SP1. Recombinant proviruses were engineered to contain this portion of viral genome within an isogenic pNL4-3 background. The deCIPhR assay was used to quantitatively assess inhibition of viral replication by bevirimat (IC₅₀, IC₉₀ and Hill’s coefficient).

RESULTS: When expressed as resistance factor (ratio of recombinant IC₅₀: wild type IC₅₀) it was possible to distinguish three phenotypic groups with different susceptibility to PA-457: most sensitive (resistance factor <2), intermediate sensitivity (resistance factor between 2 and 10) and least sensitive (resistance factor >10). Sequencing of gag from the 20 proviruses demonstrated that all viruses in the most sensitive group had wild-type sequences at Gag 369/370/371 (QVT), while all viruses in the intermediate and least sensitive groups had one or more polymorphisms at these residues.

CONCLUSIONS: The in vitro replicative system deCIPhR reproducibly assessed in vitro susceptibility to the maturation inhibitor bevirimat using clinical virus samples. The level of in vitro bevirimat sensitivity was closely related to the presence or absence of polymorphisms at Gag residues 369/370/371. This supports the clinical correlation observed between the presence of these polymorphisms and patient response to bevirimat. These results suggest that the deCIPhR assay may have utility in further defining the determinants of bevirimat response.
ABSTRACT 31
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Phenotypic and genotypic determinants of resistance to darunavir: analysis of data from treatment-experienced patients in POWER 1, 2, 3 and DUET-1 and 2

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BACKGROUND: Analyses from treatment-experienced patients receiving the protease inhibitor (PI) darunavir plus low-dose ritonavir (DRV/r) in POWER 1–3 (n=458) revealed clinical cut-offs of 10 and 40, and showed a diminished response when ≥3 of 11 DRV resistance-associated mutations (RAMs; V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V and L89V [2006 list]) were present at baseline. New analyses were performed on data from DRV/r-treated patients in POWER 1–3 (n=467) and from DRV/r- and placebo-treated patients in DUET-1 and 2 (n=604).

METHODS: Clinical cut-offs were determined by analyzing viral load change versus baseline DRV fold change (FC) using an ANCOVA model and inverse prediction. DRV RAMs were defined as protease mutations fulfilling at least two of the following criteria: associated with an increased DRV FC; when present at baseline, associated with a diminished virological response at week 24 in patients who did not use/re-used enfuvirtide; developing in ≥10% of rebounders. Genotypes and phenotypes were determined by Virco. Virological response (HIV-1 RNA <50 copies/ml) was determined by time-to-loss of virological response analysis, censoring discontinuations for reasons other than virological failure.

RESULTS: The clinical cut-offs of 10 and 40 were confirmed. Ten of the 11 2006 DRV RAMs (all except G73S) were confirmed and a new mutation T74P was identified, resulting in the 2007 DRV RAMs: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V and L89V. Each of these mutations was present with a median number of 13–15 PI RAMs (2006 IAS-USA list). In patients who did not use/re-used enfuvirtide, a diminished response was found when ≥3 2007 DRV RAMs were present at baseline. ANOVA models on response confirmed the predictive value of the number of 2006 DRV RAMs and showed that the number of 2007 DRV RAMs was slightly more predictive. The prevalence of 2007 DRV RAMs was low in PI-resistant routine clinical samples received at Virco.

CONCLUSIONS: Analyses of a larger clinical dataset confirmed the previously defined phenotypic and genotypic determinants of DRV resistance. An updated DRV RAMs list was defined, containing 10 of the 11 2006 DRV RAMs and one new mutation, T74P.
The non-conventional (folding) protease inhibitor blocks the human immunodeficiency virus type-1 replication without evidence of resistance during *in vitro* passage

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**BACKGROUND:** On the basis of theoretical approaches, we designed a peptide (BRU 83–92) able to interact with a highly conserved folding key sequence of HIV-1 protease. Because of that, the peptide should destabilize the active conformation of the enzyme in a way which is unlikely to bring life to drug resistance. To establish the pattern/rate of emergence of resistance to BRU, we passaged a wild-type clinical isolate in human peripheral blood mononuclear cells (PBMCs) in the presence of drug for up to 9 months.

**METHODS:** *In vitro* long-term susceptibility was performed by passaging in PBMCs of a wild-type HIV-1 isolate in the presence of BRU 83–92 or atazanavir (ATV). Cultures in the absence of drug were maintained as control. The p24 yield was monitored every 3 or 4 days by ELISA assay. In case of viral breakthrough, the concentration of drug was increased and the HIV RNA at that time point was extracted from the supernatant and used to detect appearance of mutations leading to resistance by nested PCR and sequencing of the protease gene.

**RESULTS:** After 9 months of *in vitro* passage, the experiments showed that the peptide was able to steadily inhibit the replication of HIV, whereas the ATV pressure caused increases of p24 production at different time points. Thus, the amount of azapeptide was increased several times in order to lower the replication of the virus. By genotype sequencing, it was seen that BRU 83–92 did not select for any mutation that led to resistance. On the contrary, it was noted appearance of primary and/or secondary mutations (that is, I50L and L10I, respectively) on the protease gene of the isolate under ATV.

**CONCLUSIONS:** The capacity to escape selection of resistance after several *in vitro* passages suggests that primary and/or secondary mutations on the protease gene able to overcome the inhibition of BRU 83–92 are likely to be incompatible with the regular activity of the enzyme. Resistance to protease inhibitors require specific or multiple mutations in discrete regions of the protease and BRU seems to avoid them so far. These data support the investigation of this peptide in the clinical setting.
ABSTRACT 33
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Highly potent HIV protease inhibitors with substituted oxindoles in P2‘
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BACKGROUND: HIV protease inhibitors (PIs) are effective antiretrovirals and one of the major components of highly active antiretroviral therapy regimens. In spite of their success, viral resistance, toxic side effects and suboptimal pharmacokinetic properties of these drugs remain major issues. Therefore, there is a persistent clinical need for discovery and development of potent and broad-spectrum PIs with reduced toxicity for the treatment of multidrug resistant (MDR) HIV strains.

METHODS: We have implemented a structure-based approach for the design of novel PIs with enamino-oxindole substituents in the P2‘ position. The potency of these inhibitors was evaluated against wild type (WT) and MDR mutant forms of HIV protease (PR) in an enzyme-based assay using a fluorogenic substrate and in a cell-based antiviral assay in MT4 cells. The crystallographic structures of representative inhibitors in complex with HIV PR were determined to support the structure activity relationship data.

RESULTS: We designed and synthesized a series of HIV PIs with an enamino-oxindole in P2‘. Several inhibitors of this series were comparable to or, in some instances, superior to darunavir in in vitro enzyme-based and cell-based assays. The best compounds have subnanomolar Ks and antiviral IC50s in the low nanomolar range against WT HIV and retain activity against a panel of eight MDR strains. For example, a tert-butylmethyl substituted analogue exhibited antiviral IC50 of 4.5 nM against WT and average IC50 of 10.1 nM (range 1.5–27 nM) against mutant viruses with average fold change (FC) of 2.3 (range 0.3–6). For comparison, darunavir has antiviral IC50 of 4.7 nM against WT and average IC50 of 28.8 nM (range 1.5–125 nM) against mutant viruses with an average FC of 6.1 (range 0.3–26.6). Unsubstituted indoline and oxindole analogues were less active. The unsubstituted enamine analogue or analogues with small substituents were potent in the enzyme assay but tended to have modest, but similar activity across the MDR panel in the antiviral assays. Analogues with polar substituents (benzimidazole, pyridyl, hydroxy and amino) generally retained good potency against the enzyme but had poor antiviral activity. Analysis of the crystallographic structure revealed a hydrogen bonding pattern that suggests that a tautomer of the enamino-oxindole may explain the high intrinsic binding potency of this class of compounds.

CONCLUSIONS: HIV PIs containing enamino-oxindole substituents in P2‘ are highly active against WT and MDR HIV isolates.
Defining the structural and functional roles of mutations in gp120 associated with the emergence of HIV-1 clinical resistance to the CCR5 antagonist vicriviroc

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BACKGROUND: Previous studies using heterologous chimeric envelopes showed that a C2-V5 domain from a vicriviroc (VCV)-resistant laboratory-adapted HIV-1 strain was sufficient to transfer resistance to a susceptible clone [1]. Here we used the same strategy to analyze the determinants of resistance in HIV env genes isolated from two patients that exhibited viral rebound in a VCV clinical trial.

METHODS: C2-V5 env domains obtained at baseline and at study discontinuation were cloned from two patient isolates into an ADA gp160 expression vector. Point mutations identified in the resistant sequence were reverted to baseline by site-directed mutagenesis. Single-cycle pseudovirus assays were used for VCV susceptibility studies and to measure relative viral infectivity. The baseline and resistant C2-V5 domains were also incorporated into a structural homology model in which gp120 is bound to an N-terminal binding domain of CCR5 [2].

RESULTS: The HIV-1 env gene from one patient (number 91) contained six amino acid changes in the V3 loop and one change in the C4 region. The resistant clone from a second patient (number 8) contained a single change in the V3 loop and two changes in the C4 region. The chimera from patient 91 was completely resistant to VCV, whereas the clone from patient 8 displayed partial susceptibility. Mutation of residues F317L and G321R in the tip and the stem of the V3 loop region from patient 91 VCV-resistant env restored partial and complete susceptibility to VCV, respectively; whereas mutation of V3 loop residues N320D and K328E and the C4 amino acid change R429G significantly reduced pseudovirus infectivity but did not alter the resistant phenotype. Structural modelling results suggest that specific mutations identified in the V3 loop and C4 regions of the resistant clones may influence gp120 binding to the second extracellular loop or N-terminal domains of CCR5.

CONCLUSIONS: While no consistent pattern of resistance mutations to VCV has been identified, results from the two patients’ isolates suggest that mutations in the V3 loop and C4 region can be important determinants of the resistant phenotype. We identified mutations in the stem and tip region of the V3 loop that were associated with resistance level and other likely compensatory changes in the V3 stem, base and C4 region that affected viral infectivity. It will be necessary to analyze a larger number of clinical isolates in order to understand more fully the genotypic and phenotypic characteristics of VCV susceptibility.

REFERENCES

Nucleoside analogues targeted against nucleoside reverse transcriptase inhibitor-resistant HIV-1 reverse transcriptase

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Nucleoside reverse transcriptase inhibitors (NRTIs) are important for the treatment of HIV-1 infections. All of the approved non-NRTIs lack the 3′-OH that is required for extension of viral DNA and act as chain terminators when incorporated by HIV-1 reverse transcriptase (RT). NRTI resistance implies that resistant RTs have an enhanced ability to discriminate between normal dNTPs and the NRTITPs. There are two mechanisms of NRTI resistance. Either the enhanced discrimination takes place at the time the NRTITP is incorporated into DNA (exclusion) or the mutant RT has gained the ability to selectively remove the NRTI after it has been incorporated into DNA (excision). We are trying to develop nucleoside analogues that are effective against the known NRTI-resistant viruses. The excision mechanism used by RT depends on the fact that conventional NRTIs remain at the end of the DNA primer strand. We have been analyzing novel nucleoside analogues that contain a 3′-OH that allows additional normal nucleotides to be incorporated after the analogue has been incorporated, protecting the analogue from excision. We have found that these delayed and kinetic chain terminators are able to inhibit excision-proficient HIV-1 RT mutants in vitro and block the replication of HIV-1-based vectors carrying NRTI-resistant RTs in cultured cells. Recently we have analyzed the efficacy of C4′-methyl-2-deoxyadenosine and C4′-ethyl-2-deoxyadenosine as inhibitors of wild-type and NRTI-resistant HIV-1 vectors. Screening of these compounds in cells infected with HIV-1-based vectors indicates that the compounds have low cytotoxicity and inhibit replication at sub-micromolar concentrations. Both compounds are effective against most NRTI-resistant mutants, with the exception of the exclusion mutant M184V. Analysis of viral DNA isolated from infected cells by real-time PCR indicates the compounds are targeting early steps in viral DNA synthesis. In vitro studies with purified wild-type RT suggest incorporation of C4′-methyl-2-deoxyadenosine triphosphate is efficiently incorporated into viral DNA, but poorly extended. These C4′-modified nucleoside analogues warrant further studies to assess clinical potential.
SESSION 2
Mechanisms of HIV drug resistance
ABSTRACT 36

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Mechanism by which the HIV integrase active-site mutation N155H confers resistance to raltegravir

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BACKGROUND: Raltegravir is the first and currently the only integrase strand transfer inhibitor (InSTI) approved for the treatment of HIV infection. Raltegravir inhibits integrase by binding to and functionally sequestering essential active-site magnesium ions. The binding of the inhibitor to integrase is mediated both by interactions with the metals and via direct contact with the enzyme. The active-site mutation N155H, either alone or with additional mutations, is associated with resistance to raltegravir both in cell-culture studies and in the clinic and has been observed to confer cross-resistance to other InSTIs, including elvitegravir. The mechanism by which this mutation confers resistance has not yet been elucidated. N155 interacts with integrase active-site residues that bind the magnesium ions, and mutation of this residue may confer resistance by perturbing metal binding to the active site.

METHODS: A radioligand binding assay was employed to measure the magnesium-dependent binding of raltegravir to functional complexes of wild-type integrase (Inwt) and mutant integrase (IN_{N155H}) assembled with viral DNA ends.

RESULTS: Raltegravir binding affinities to Inwt and IN_{N155H} were dependent on magnesium ion concentration. The two enzymes displayed similar binding affinities for magnesium and for raltegravir in the absence of metal. However, the binding affinity of raltegravir to integrase at saturating magnesium concentrations was lower with IN_{N155H} than with wild-type enzyme.

CONCLUSIONS: The integrase active-site mutation N155H confers resistance to raltegravir primarily by perturbing the arrangement of the active-site magnesium ions and not by affecting the affinity of the metals or by affecting direct contacts of the inhibitor with the enzyme. These studies lay a foundation for determining the roles of additional mutations in the N155H resistance pathway and for establishing the mechanisms by which other pathways confer resistance to InSTIs.
ABSTRACT 37
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Structural explanations to altered drug resistance pathways in HIV-1 non-clade B proteases
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BACKGROUND: The majority of HIV-1 infections across the world result from non-B clades. CRF01_AE is predominantly seen in Southeast Asia and the protease differs by ~10% when compared with the clade B protease. CRF01_AE polymorphisms are often associated with drug resistance in the clade B protease. CRF01_AE protease has been observed to develop the unique N88S mutation in response to nelfinavir (NFV) therapy, which is not commonly seen in clade B protease. We present here, structural and binding thermodynamic data on CRF01_AE protease in order to explain how sequence polymorphisms within CRF01_AE protease might affect its activity and to explain the altered NFV resistance pathway observed in CRF01_AE.

METHODS: The crystal structure of HIV-1 CRF01_AE N88S protease in complex with darunavir (DRV) was determined to a resolution of 1.76 Å. The structure was compared with the clade B protease–DRV structure to determine effects of sequence polymorphisms on protease structure and substrate binding. Binding constants and thermodynamic parameters for CRF01_AE and clade B protease were determined by isothermal titration calorimetry.

RESULTS: Calorimetric data indicate that wild-type CRF01_AE protease binding is tenfold and twofold weaker to NFV and DRV, respectively. The CRF01_AE structure shows a significant change in the flap hinge region of the protease when compared with the clade B structure. The CRF01_AE flap hinge packs against the core region of the protease via a network of unique hydrogen bonds that are not seen in the clade B structure. The Ser88 side chain in the CRF01_AE structure is involved with a novel network of hydrogen bonds and interacts with the side chain of Asp30. This is likely to disrupt a crucial hydrogen bond required for NFV binding.

CONCLUSIONS: Structural data indicate that sequence polymorphisms in HIV-1 CRF01_AE cause significant structural changes within the protease when compared with clade B protease. Calorimetric data suggest that wild-type CRF01_AE protease might have lower affinity for NFV and DRV. This weakened affinity may permit the alternative pathway for NFV resistance via N88S, which is a mutation outside the active site.
ABSTRACT 38

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How hepatitis C virus NS3-4A protease R155K/T strains can discriminate VX-950 and ITMN-191 but affect differentially SCH-503034

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BACKGROUND: During the selection of hepatitis C virus (HCV) NS3-4A protease mutations under the drug pressure of HCV protease inhibitor (PI) VX-950, mutation R155K/T was selected in patients undergoing treatment, but not in vitro. ITMN-191 shows a reduced susceptibility to HCV NS3-4A R155K strain by 89-fold. Furthermore, it is noteworthy that R155K exhibits similar replication capacity to the wild type and shows selective advantage. Even though mutation R155K is not described in vitro with ITMN-191, mutation R155K has all viral features (replication capacity and high fold resistance) to be selected in vivo. Interestingly, SCH-503034 does not share the same R155K/T resistance profile (FR R155K; 4). The aim of this study is to describe the molecular and structural basis of this different resistance profile between VX-950, ITMN-191 and SCH-503034 against NS3-4A R155K.

METHODS: Structures of drug-resistant HCV NS3-4A protease were obtained from the wild-type structure by mutation of Arg155 to Lys. The resulting ternary complexes formed between HCV NS3-4A protease and each drug were optimized using GenMol software (www.genosciencepharma.com).

RESULTS: The molecular mechanism of HCV NS3-4A R155K/T resistance to VX-950 does not involve only the loss of hydrophobic interactions between its bicyclic P2 and the side chain of residue 155. Indeed, this is not consistent with the SCH-503034 resistance profile. Arg155 participates in the orientation of the VX-950 P4 cyclohexyl towards the Val158 S4 binding pocket. Mutation R155K/T induces the loss of Arg155 orientation function and VX-950 undergoes a destabilization, which occurs during the VX-950 recognition and covalent steps. SCH-503034 undergoes, like VX-950, just minor modifications in the P2–S2 interaction area and SCH-503034 is not strongly affected by the loss of Arg155 P4 orientation function. The molecular mechanism of ITMN-191 resistance against HCV NS3-4A R155K involves a direct resistance mechanism through the modification of the interactions between P2 and S2 binding pocket. Moreover, R155K induces the loss of a key salt bridge between Asp168 and Arg123. Asp168 takes another conformation decreasing indirectly the hydrophobic P4–S4 interactions.

CONCLUSIONS: These data provide new insights into the understanding of the molecular mechanisms of HCV drug escape, potential virological breakthrough and bring predictive potential cross-resistance phenomena with future PIs.
ABSTRACT 39
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Structural basis for K65R function: tenofovir resistance, reduced nucleotide incorporation and excision antagonism

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BACKGROUND: K65R is a primary reverse transcriptase (RT) mutation selected in HIV-infected patients taking antiretroviral regimens containing tenofovir (TFV) or other nucleoside reverse transcriptase inhibitors. The mutant RT has a reduced rate of dNTP incorporation and significantly decreased excision of zidovudine (AZT).

METHODS: Using X-ray crystallography, we determined the crystal structures of K65R mutant RT with double-stranded DNA (27:21-mer) in complexes with tenofovir diphosphate (TFV-DP) at 3.0 Å and dATP at 3.5 Å resolution, respectively.

RESULTS: The crystal structures reveal the molecular mechanism of resistance by the K65R mutation, which is distinct from the steric hindrance mechanism caused by M184V and the ATP-mediated excision mechanism used by thymidine analogue mutations (TAMs). The K65R mutation forms a molecular platform through the interactions of R65 with the conserved amino acid residue R72. The platform can restrict the conformational adaptability of both amino acid residues that is required for DNA polymerization by RT, which can explain the lower rate of nucleotide incorporation by the K65R mutant as well as its decreased excision rate. The amino acid residues R72 and R65 have alternative rotameric conformations in the TFV-DP-bound structure of K65R RT when compared with the dATP-bound structure, which is apparently responsible for discriminating TFV-DP from dATP and allowing for resistance.

DISCUSSION: The R65+R72 platform interfaces with M184 and TAMs at two distinct sites. If the K65R and M184V mutations co-exist, the side chains of R72 and V184 would stack like walls on either side of the ribose ring of a bound dNTP, which further stabilizes the pocket and may explain the lower rate of dNTP incorporation and excision by the double-mutant that results in partial TFV resensitization. The K70R TAM, if it were to co-emerge with K65R, would add additional restrictions to the R65+R72 platform that might be detrimental for dNTP binding, ATP binding (for excision) and/or nucleotide incorporation, which may explain the rare appearance of K65R+K70R mutations. Other TAMs, such as T215Y, which enhance binding of ATP for excision, are antagonistic with K65R because the R65+R72 platform would hinder the proper positioning of the phosphates of ATP, thus decreasing excision and leading to no enhancement of resistance.
ABSTRACT 40

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Zidovudine resistance related connection mutations in HIV-1 reverse transcriptase cause selective dissociation from RNase H competent complexes

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BACKGROUND: Thymidine analogue mutations (TAMs) in HIV-1 reverse transcriptase (RT) cause resistance to zidovudine (AZT) and other nucleoside reverse transcriptase inhibitors by increasing the rate of excision. Recent findings have identified mutations in the connection domain that augment AZT resistance, usually in a background of TAMs. It is widely accepted that these mutations may compromise RNase H cleavage, providing more time for AZT excision to occur. However, the underlying mechanism remains to be elucidated.

METHODS: In this study, we used connection mutations A360V and N348I as a model to address this problem with a combination of complementary biochemical tools. Previous studies revealed that TAMs in combination with A360V and N348I are associated with marked decreases in phenotypic susceptibility to AZT. Here, we employed enzyme kinetics and binding studies to elucidate the molecular mechanism.

RESULTS: Initial experiments confirm that N348I and A360V, in combination with TAMs, decrease the efficiency of RNase H cleavage and increase the amount of rescued DNA product following ATP-dependent excision. The A360T polymorphism is less likely to be associated with treatment and this mutation does not increase rates of excision. Band-shift assays show that the TAM/N348I/A360V mutant accumulates stable, transiently formed 10- to 12-mer hybrids that can rebind to RT. These short hybrids dissociate from the RNase H competent complex and rebind to RT in the polymerase-competent mode that allows excision to occur. The connection mutations selectively reduce substrate binding in the RNase H competent mode. Furthermore, binding in the polymerase-competent mode appears to be facilitated, which is reflected in increases in processive DNA synthesis. As a consequence, mutant enzymes containing N348I, which show the strongest effects in this regard, enhance excision and rescue of DNA synthesis in the presence of pyrophosphate (PPi) or ATP.

CONCLUSION: Selective dissociation of transiently formed hybrids from RNase H competent complexes provides a mechanism for the increase in AZT excision associated with connection mutations N348I and A360V. The combination of diminished RNase H cleavage and increased processivity renders the use of both PPi and ATP advantageous, whereas classic TAMs solely enhance the ATP-dependent reaction.
ABSTRACT 41
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Q509L in HIV-1 reverse transcriptase increases zidovudine resistance by promoting polymerase-competent versus RNase H competent binding on RNA/DNA template/primers with short duplex lengths

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BACKGROUND: A371V and Q509L were selected by zidovudine (AZT) in combination with the thymidine analogue mutations (TAMs) D67N/K70R/T215F and increase AZT resistance 50-fold compared with TAMs alone. Initial biochemical studies show that TAMs/Q509L and TAMs/A371V/Q509L increase AZT monophosphate (AZT-MP) excision from RNA/DNA template/primers (T/P) by decreasing secondary RNase H cleavage events that reduce the RNA/DNA T/P duplex length to <12 nucleotides. However, the precise mechanisms responsible for the decreased RNA cleavage and increased AZT-MP excision have not been defined.

METHODS: Reverse transcriptase (RT) containing D67N, K70R, T215F, A371V and/or Q509L was overexpressed and purified. The rates for RNase H cleavage of AZT-MP terminated RNA/DNA T/P were determined using transient or steady-state kinetic approaches, both in the absence and presence of a nucleic acid trap. The ability of the wild-type or mutant RTs to bind RNA/DNA T/P with duplex lengths <18 nucleotides in a DNA polymerase- or RNase H competent mode was assessed by measuring DNA polymerization or RNase H cleavage at defined times after the addition of a trap to a pre-formed RT–T/P complex.

RESULTS: Initial RNase H cleavages for all enzymes were similar, suggesting that A371V and Q509L do not directly affect the catalytic activity of the RNase H active site. However, the rates for the subsequent RNase H cleavages, which occur after T/P dissociation and rebinding, were reduced 2.2- and 2.3-fold for the TAMs/Q509L and TAMs/A371V/Q509L RTs, respectively. RT-T/P binding assays showed that the Q509L mutation promoted RT binding to short T/P duplexes in a polymerase-competent mode favouring AZT-MP excision, rather than an RNase H competent mode allowing additional cleavages and T/P dissociation.

CONCLUSIONS: The Q509L mutation does not have a direct effect on RT RNase H catalytic activity, but increases AZT resistance by promoting RT binding to RNA/DNA T/P duplexes <18 nucleotides in a polymerase-competent mode that favours excision rather than an RNase H competent mode that favours further cleavage and T/P dissociation. These findings provide new insights into the mechanism by which mutations in the C-terminal domain of RT confer nucleoside reverse transcriptase inhibitor resistance.
ABSTRACT 42

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Evolution of reverse transcriptase connection domain mutations in patients on antiretroviral therapy

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BACKGROUND: Many resistance testing assays do not cover genomic regions beyond currently known International AIDS Society USA (IAS-USA) drug resistance mutations. Yet, there is mounting evidence for mutations in the connection domain (CD) or the RNase H domain of the reverse transcriptase to confer resistance against non-nucleoside reverse transcriptase inhibitors (NNRTI) or nucleoside reverse transcriptase inhibitor (NRTI). We aimed to investigate the evolution of the CD mutations 312Q, 335C, 335D, 348I, 360I, 360V, 365I, 369I, 371V, 376S and 399D in the context of IAS-USA mutations.

METHODS: Reverse transcriptase sequences spanning the first 400 codons were retrieved. Dependencies between IAS-USA and CD mutations were investigated with cluster analysis and Bayesian networks (http://b-course.cs.helsinki.fi).

RESULTS: In total, 1,213 sequences were obtained: 415 (34%) from therapy-naive and 798 (66%) from therapy-experienced patients. Of the latter, 665 had ever failed virologically on thymidine analogues. Frequencies of the following CD mutations were significantly increased among treatment-experienced compared with treatment-naive patients: 348I (11% versus 1%), 360V (4% versus 1%), 365I (5% versus 2%), and 371V (26% versus 19%). Conversely, the frequency of 335D was decreased (11% versus 24%). Dendrograms of mutation patterns from treated patients revealed clustering of mutations from thymidine analogue mutation (TAM)1 or TAM2 pathways, respectively. No CD mutations clustered with TAMs, but the occurrence of N348I was tightly associated with M184V: of 79 tests positive for N348I, M184V was not detected in only seven (9%), although it is likely to be present as a minor species based on treatment history. Bayesian network analyses confirmed a shared pathway for M184V and N348I not directly linked to TAMs, but network structures were inconclusive regarding direction of dependency. Interestingly, patients on first-line therapy containing zidovudine and lamivudine with genotypic tests indicating the presence of both M184V and N348I were found to have been exposed to treatment for longer periods (median 1,000 days; interquartile range [IQR] 334–1426.5; n=12) than those with the M184V mutation alone (median 298.5 days; IQR 181–685; n=18; Wilcoxon P=0.034), suggesting that the emergence of N348I is preceded by M184V.

CONCLUSIONS: The CD mutation N348I does not seem to emerge in the absence of M184V, and contrary to earlier reports was not associated with TAMs. Thus, at this point sequencing of the CD does not seem mandatory.
ABSTRACT 43
Antiviral Therapy 2008; 13 Suppl 3:A48

Delayed chain-termination protects the hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase
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BACKGROUND: Entecavir (ETV) is a potent antiviral drug used to treat infection with the hepatitis B virus (HBV). Recent studies have shown that ETV has anti-HIV activity and can select for the M184V mutation in HIV-1 reverse transcriptase (RT), which limits its clinical use in HIV–HBV-co-infected individuals. However, the mechanism of drug action remains elusive. ETV is a guanosine nucleoside analogue that contains a 3′-hydroxyl group. Thus, the incorporated ETV-5′-monophosphate (ETV-MP) may not act as a classic chain-terminator. Inhibitory effects of ETV-MP can occur at a certain distance from its site of incorporation.

METHODS: We utilized various biochemical tools to elucidate the anti-HIV mechanism of ETV and its implications with respect to major resistance pathways against established nucleoside reverse transcriptase inhibitors (NRTIs).

RESULTS: Incorporation of ETV-MP at position n causes RT pausing at positions n and n+3. Increasing concentrations of natural dNTP pools at positions n+1 and n+4 can eventually overcome enzymatic pausing; however, incorporation of the natural nucleotide at position n+4 is severely compromised. Kinetic measurements revealed a subtle eightfold decrease in efficiency of nucleotide incorporation at position n+1 when ETV-terminated primers are compared with the natural counterpart, whereas nucleotide incorporation at position n+4 is reduced by three orders of magnitude (1,230-fold). High-resolution footprinting experiments show that complexes with HIV-1 RT and a primer/template that mimics the latter situation are as stable as complexes that contain natural primers. Rather, ETV-MP forces the enzyme to slide away from the 3′-end of the primer at position n+3, which provides a plausible mechanism for such ‘delayed chain-termination’. RT enzymes with thymidine analogue mutations (TAMs) can efficiently excise the incorporated ETV-MP at position n, as demonstrated for several established NRTIs. However, ETV is fully sensitive against TAM-containing HIV variants, which shows that the additional nucleotides at positions n+1 to n+3 protect the inhibitor from excision.

CONCLUSION: The results of this study demonstrate that ‘delayed chain-termination’ at position n+3 is the dominant mechanism of action of ETV. The combined data provide a rationale for the development of ETV-like, delayed chain-terminators as anti-HIV compounds that can evade the excision mechanism.
ABSTRACT 44
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Co-evolved protease substrate cleavage site mutations enhance protease inhibitor phenotypic resistance

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BACKGROUND: HIV-1 protease (PR) can acquire a multitude of mutations that confer resistance to protease inhibitors (PIs). These mutations may result in a less efficient PR and impaired viral fitness, leading to selection of compensatory mutations in PR and also in the substrate cleavage sites (CS). However, not much is known about the patterns and impact of Gag mutations on drug susceptibilities in combination with PR mutations.

METHODS: We analysed PI susceptibility of HIV-1 from more than 14,000 clinical samples as determined with the PhenoSense assay (Monogram Biosciences). Results are expressed as fold change (FC) in IC50 (the half maximal inhibitory concentration) of the patient virus relative to that of the drug-sensitive reference, NL4-3. Primary drug resistance mutations at positions 30, 50, 82, 84 and 90 were the main focus of this study, using two levels of stringency for inclusion of secondary mutations. The statistical significance of the difference of median FC between groups with and without the Gag CS mutations was tested using the Mann–Whitney test.

RESULTS: Reduced susceptibility to nelfinavir and saquinavir was observed in viruses with D30N or D30N/N88D in combination with mutations at Gag L449. In addition, with D30N/N88D there were also small changes in susceptibilities in viruses that had mutations at S451, R452 and P453. Reduced susceptibility to lopinavir and indinavir was seen in viruses with V82A in combination with mutations at Gag A431 and L449. Reduced susceptibility to all PIs was observed in viruses with L90M in combination with mutations at Gag A431 (1.2- to 5.3-fold) and K436 (1.3- to 2.3-fold). Finally, changes in susceptibility to saquinavir and ritonavir were observed in viruses with V82A/L90M in combination with a mutation at Gag S451.

CONCLUSIONS: Enhanced PI resistance in HIV-1 is associated with the presence of Gag CS mutations. These studies suggest that substrate co-evolution might contribute to PI susceptibility in highly specific ways based on the particular combinations of mutations and inhibitors.
ABSTRACT 45

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Clonal analysis of protease and gag sequences from HIV-1 subtype C infected patients failing a protease inhibitor

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BACKGROUND: High levels of resistance to protease inhibitors (PIs) have been observed both in vitro and in vivo as a result of mutations in the protease (PR) gene. However, compensatory mutations in the p7/p1 or p1/p6 gag cleavage sites have been shown to restore or improve processing efficiency of the resistant enzyme. In this study we examined minority populations in PR and the two gag cleavage sites in patients who had failed Kaletra therapy.

METHODS: The HIV-1 PR gene and the last 174 amino acids of the gag gene were PCR-amplified from four patients failing Kaletra. Population sequencing demonstrated PR resistance in two of these patients (DR108 and DR112), but wild-type sequences in two other patients (DR106 and DR107). The products were cloned into TOPO TA cloning kit. Clonal sequences were aligned and manually edited using Sequencher 4.5. Phylogenetic analysis of the nucleic acid sequences was performed using Mega 3.1.

RESULTS: Analysis of 100 clonal sequences from DR106 showed that 80% were wild-type in both the PR and gag cleavage sites. Polymorphisms in PR were detected in 17% of clones some of which were thought to be intermediate changes. Three individual clones (DR106.07, DR106.51 and DR106.107) had V82A, N88D and D30N PR mutations, respectively. V82A and D30N are well known PR mutations while N88D facilitates the co-occurrence of D30N and L90M. The PR from DR107 was wild-type by population sequencing and one clone (1%) had the V82A mutation by clonal analysis. This isolate contained the K436R polymorphism at the gag cleavage site by population sequencing and on clonal analysis was also shown to have the I437V mutation in the p7/p1 gag cleavage site in 2% of clones. DR108 showed PI resistance mutations M46I, I54V, V82A, I84V and A431V at the p7/p1 gag cleavage site by population sequencing. All clones showed an identical genotype, although one clone (1%) had an additional mutation (I437T) at the p7/p1 site. No other additional major mutations were found in the PR region by clonal sequencing. DR112 showed mutation M46I and no mutations at the gag cleavage sites by population sequencing. All clones were also wild type at both p7/p1 and p1/p6 cleavage sites. Ninety-five percent of clones (95/100) had the mutation M46I and five (5%) were wild type. One clone (1%) (DR 112.09) had additional mutation I84V in the PR region.

CONCLUSION: Population sequencing underestimates the diversity of PI resistance mutations within minority populations following Kaletra administration. Mutations found in the gag cleavage sites are likely to compensate the mutated PR, but might also contribute to PI resistance in those patients who have wild-type PR.
ABSTRACT 46

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Impact of gag mutations on selection of darunavir resistance mutations in HIV-1 protease

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BACKGROUND: It has been shown in vitro that some protease inhibitors (PI) can select mutations in HIV gag comprising NC-P1/TFP-P6/P6*, without selecting mutations in the protease. Indeed, in vitro selection experiments using darunavir demonstrated phenotypic resistance that could not be explained by resistance mutation in the protease, but could be explained by gag mutations, including a cleavage site mutation at codon 437. Similar results have been found in vivo in isolates with gag mutations (at positions 431, 436 and 437) and which carried or did not carry known resistance mutations in the protease. The aim of this study was to search for genetic factors in the protease and gag regions (NC-P1/TFP-P6/P6*) involved in the selection of darunavir resistance mutations.

METHODS: Forty-eight PI-experienced HIV-infected patients suffering darunavir treatment failure were studied. Viral genotyping at baseline, month 3 and month 6 was used to assess the selection of mutations in the protease and gag regions. A gag–protease region of 323 bp, including the end of NC, P1/TFP and P6/P6* was amplified and sequenced. Darunavir mutations were defined according to International AIDS Society USA (IAS-USA) guidelines.

RESULTS: Patients received four PIs in median before darunavir. The median numbers of antiretroviral drugs, nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) used concurrently with darunavir were four, three and zero, respectively. There were no genotypic differences in the studied gag region between baseline and the latest available rebound isolates. There was an association between the presence of the mutation A431V in the gag sequence and the selection of the L76V mutation in the protease sequence in the latest available rebound. The I437T/V mutation in gag and the L76V mutation in the protease were associated with a lower risk of selecting other darunavir resistance mutations.

CONCLUSION: In these PI-treated patients suffering treatment failure of a darunavir-containing regimen, mutations in the gag region NC-P1/TFP-P6/P6* might influence the selection of darunavir resistance mutations; in particular, the I437T/V gag mutation that confers resistance to PI reduces the selection of darunavir resistance mutations in protease.
ABSTRACT 47
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Gag NC/p1 protease resistance mutations can cause selection of additional NC/p1 changes to optimize cleavage efficiency and replicative capacity

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BACKGROUND: Substrate-based protease inhibitor (PI) resistance due to mutations in NC/p1 is caused by an enhanced processing of the gag protein. We investigated the effect of enhanced gag processing due to NC/p1 resistance mutations on replicative capacity (RC) and the consequences for evolution in the absence of PI pressure.

METHODS: A set of four recombinant viruses containing NC/p1 mutations conferring different levels of PI resistance were generated: HXB2431V, HXB2437V, HXB2437T and HXB2436E+437T. To investigate the effect of enhanced gag processing on RC, viral replication curves were generated. To investigate the potential evolutionary pathways in the absence of PI pressure, multiple individual in vitro evolution experiments were performed, after which complete Gag and protease were sequenced. From the viruses that were selected, RC, gag processing (quantitative immunoblot analysis) and PI susceptibility (MTT assay) were assessed.

RESULTS: Single NC/p1 mutants that displayed only a slight increase in PI resistance did not show an obvious change in RC compared with wild type. This was also reflected in the in vitro evolution experiments where the single NC/p1 mutants showed no signs of evolution, with the exception of the selection of A429K in 1/5 experiments for HXB2431V. In contrast, the double NC/p1 mutant (HXB2436E+437T), which displayed a clear increase in processing efficiency and PI resistance, also demonstrated a clear reduction in RC. Interestingly, in all evolution experiments, amino acid changes in/near the NC/p1 site were observed (2/5, -436E; 2/5, +435R; 1/5, +438R). These selected changes restored the processing efficiency and RC. Furthermore, it was observed that due to the normalization of gag processing efficiency, in parallel, PI susceptibility returned to wild-type level.

CONCLUSIONS: The results from this study clearly demonstrate that there is an optimum rate for HIV-1 gag cleavage. When enhanced gag processing due to PI resistance mutations in NC/p1 reduces RC, HIV-1 can modulate the NC/p1 sequence by selection of additional changes to restore gag cleavage and RC.


ABSTRACT 48
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Relevance of HIV gag cleavage site mutations in failures of protease inhibitor therapies

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BACKGROUND: The evolution of HIV cleavage site (CS) mutations after exposure to protease inhibitors (PI) is generally acknowledged. However, the impact of CS mutations on PI resistance has been controversially discussed. They have been described as either resistance-conferring mutations and/or compensatory mutations. The analysis of HIV CS mutations in PI failures could help to increase knowledge about the relevance of CS mutations.

METHODS: We analysed 188 HIV genotypes of 154 patients in pol gene as well as in gag gene. All genotypes were obtained after at least 90 days (median: 458 days) of antiretroviral therapy with PIs. HIV genotypes were interpreted with HIV genotypic-resistance tools (ANRS, geno2pheno, HIVdb, HIV-Grade and Rega) and classified according the highest agreement. Prediction of lopinavir (LPV) therapy failure was performed with Random Forests using the International AIDS Society-USA list for protease (PR)/reverse transcriptase (RT) as well as CS mutations (431V/437V/449F) and evaluated by 10-fold cross-validation (control group: 102 successful LPV therapies with gag and pol genotypes).

RESULTS: HIV genotypes from PI failures harboured therapy-associated HIV CS mutations and resistance-associated PR mutations in comparable numbers (66% versus 71%, respectively). If genotypic PI resistance was predicted (n=71), therapy-associated CS mutations could be found in 90% (mainly 1 or 2). HIV gag CS mutations 128I, 431V, 437V, 449F, 451T, 452S and 453L accumulated in HIV classified PI-resistant compared to PI-susceptible samples (n=70). HIV interpretation tools gave conflicting results or predicted intermediate resistance in 47 of 188 (25%) HIV genotypes mainly harbouring therapy-associated CS-mutations (42/47). A rules-based system adapted from the HIV Stanford algorithm with weighted PR mutations and CS mutations (431V [15], 437V [10] and 449F [10]) for LPV resistance would have led in 14 of 24 HIV genotypes to a switch in predicted LPV resistance (intermediate resistance to full resistance). Additionally, prediction of therapy outcomes for LPV therapies (n=78) slightly, but significantly, improved with Random Forests analysis (P<0.001) if CS mutations (431V/437V/449F) were considered.

CONCLUSION: Therapy-associated HIV CS mutations were frequently selected under PI therapies and PI-resistant viruses were rarely found without CS mutations. Furthermore, CS mutations were found in PI failures without predicted PI resistance probably pointing to an underestimated genotypic PI resistance. This is especially supported by the fact that outcomes of LPV therapies were predicted more exactly by bioinformatic methods using HIV pol and gag genes.
ABSTRACT 49

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Mutations selected by therapy in HIV-2 reverse transcriptase

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BACKGROUND: Mutations in the reverse transcriptase (RT) gene of HIV-1 are frequently seen under drug-selective pressure, some of which clearly account for therapeutic failure. The mechanisms of resistance development to RT inhibitors in HIV-2 are poorly characterized. Given the growing number of HIV-2 patients under treatment, it is important to elucidate the specific amino acid substitutions conferring resistance to antiretroviral agents in HIV-2 RT.

METHODS: Sequencing of HIV-2 reverse transcriptase was performed with an in-house technique. A total of 120 sequences were obtained for 72 treatment-experienced and 48 drug-naive patients. Amino acid changes were analysed with SeqScape® Version 2.5 software, using ROD HIV-2 strain as reference. Mutations were considered for analysis if they were present in at least 10% of the sequences from the treated group. The sequences from the control group were analysed to uncover the naturally occurring polymorphisms and allow a comparison with the treated population. For statistical analysis, the χ² test was performed (using SPSS® Version 14.0 software).

RESULTS: Several HIV-2 mutations were selected by therapy. Some were present exclusively in treated patients: R22K (10), K40R (9), A62V (7), K65R (19), K70R (7), Q151M (24), M184I/V (59) and Y227F (9). Others were present as polymorphisms in the untreated population, but were significantly more common in patients under therapy: M11T (2 versus 37, P=0.005), K35R (3 versus 19, P=0.005), P51S (2 versus 16, P=0.007), V111I (3 versus 30, P=0.001) and F214L (2 versus 11, P=0.05). Some of these substitutions were not previously reported: M11T, R22K, K35R, K40R, P51S and Y227F. Positions 215 and 219, associated with resistance in HIV-1, were highly polymorphic in the treated population (S215A/C/F/Y, E219D/G/K). S215P and E219D occurred as natural polymorphisms in the untreated group. Even if each amino acid alone did not reach statistical significance, therapy selection cannot be excluded.

CONCLUSIONS: Several mutations selected by antiretroviral therapy were observed in this population of HIV-2-infected patients. Some of them were not previously reported. Further studies are needed to clarify their real effect on nucleoside reverse transcriptase inhibitor (NRTI) resistance.
ABSTRACT 50
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Mutations in the reverse transcriptase connection and RNase H domains exhibit dual resistance to nucleoside and non-nucleoside reverse transcriptase inhibitors

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BACKGROUND: We recently observed that mutations in the HIV-1 reverse transcriptase (RT) connection domains isolated from treatment-experienced patients, as well as mutations of RNase H primer grip residues (which position the primer-template at the RNase H active site), significantly enhance zidovudine resistance. We proposed that a balance between degradation of HIV-1 RNA by RNase H and nucleotide excision is an important determinant of resistance to nucleoside reverse transcriptase inhibitors (NRTI). In this study we sought to determine whether mutations in the connection and RNase H domains can also confer resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI).

METHODS: The level of resistance to the NNRTIs nevirapine (NVP) and efavirenz (EFV) was determined for RTs derived from treatment-experienced patients and RNase H primer grip mutants using a luciferase reporter gene based drug susceptibility assay. RT template switching frequency, which provides a measure of polymerase and RNase H activities, was determined using a green fluorescent protein (GFP) reporter gene based direct repeat deletion assay.

RESULTS: Mutations in the RT connection domain (N348I, G335C, A360I, T369V, A376S and E399G) and RNase H primer grip (Q475A and Y501A), which conferred increased zidovudine resistance, had a similar effect on NNRTI resistance. Template switching frequency of HIV-1 RT was increased in the presence of NVP, but not EFV. The RNase H defective mutant D549N exhibited increased resistance to NVP up to fivefold, but not to EFV. Taken together these data suggest that the observed differences might be dependent on the structure of NNRTIs and/or their influence on RT structure.

CONCLUSION: These experiments demonstrate that specific mutations in the connection and RNase H domains exhibit dual resistance to NRTIs and NNRTIs. These studies indicate that the C-terminal domains of RT significantly contribute to antiviral drug resistance and their inclusion in genotypic and phenotypic analysis of clinical antiviral drug resistance could significantly enhance the accuracy of predicting drug resistance.
ABSTRACT 51
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Prevalence of K65R in different HIV-1 subtypes and its association with thymidine analogue mutations and non-nucleoside reverse transcriptase inhibitor related mutations

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BACKGROUND: K65R causes cross-resistance to didanosine, abacavir, lamivudine, emtricitabine, tenofovir (TDF) and stavudine but hyper-susceptibility to zidovudine. It was found in tissue culture that K65R selection under TDF pressure is more frequent for HIV-1 subtype C. Subtypes B, C and G are the main subtypes in Portugal (B and G) and Israel (B and C). We explored K65R emergence in a large population of treated patients infected with these subtypes.

METHODS: HIV-1 pol sequences from 2,215 drug-experienced patients were collected in Portugal (1,550) and Israel (665). Subtype, drug usage, plasma RNA concentration (viral load) and CD4+ T-cell count were monitored. One sample from each patient was used for the analysis, the first one containing K65R or the last available sample for patients who did not have this mutation. Fisher’s exact test was used for statistical significance.

RESULTS: K65R was found in 3.5% (36/1,030) of subtype B, 3.5% of C (17/487) and 5.6% (31/552) of G patients (P=1.04 and 0.05 for B versus C, G versus C and B versus G, respectively). Regardless of subtype, nearly all samples with K65R carried at least one non-nucleoside reverse transcriptase inhibitor (NNRTI) mutation, with K103N and Y188C the most and least prevalent. Y181C appeared more frequently in subtype B (P=0.05) and G190A/S in C (P<0.02). The prevalence of NNRTI-related mutations was significantly higher compared with that of the general treated population for all subtypes. However, we found differences between B and C in the prevalence of thymidine analogue mutations (TAMs). Although the difference in prevalence of individual TAMs did not reach significance, it was significant for TAM II mutations (11% and 47% in B and C, respectively; P<0.01) and for the total number of TAMs (17% and 53% for B and C, respectively; P<0.02). Interestingly, in subtype C patients, but not in subtype B, K65R also appeared in patients who previously had received monotherapy (mainly zidovudine) as first antiretroviral therapy.

CONCLUSIONS: Differences in prevalence of K65R in B, C and G subtypes can only partly be explained by different treatment history and by differences in pre-existing reverse transcriptase mutations. In particular, the subtype-specific pathways of association with TAM accumulation and with NNRTI-related mutations should be further investigated.
ABSTRACT 52

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Intracellular factors that decrease HIV-1 RNase H activity and increase the rate of zidovudine 5′-monophosphate excision studied for RNA and DNA template dependency

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BACKGROUND: It was recently demonstrated that certain HIV-1 C-terminal mutations, for example, Q509L/A371V or N348I in a thymidine analogue mutation (TAM) background could increase the rate of zidovudine 5′-monophosphate (AZT-MP) excision by reduced RNA template degradation. This increased rate of excision was demonstrated in enzymatic assays using RNA/DNA as template/primer, but not with DNA/DNA. However, as some of the mutations are not in the RNase H domain additional studies are needed to define the exact mechanism. We studied the effect on excision of AZT-MP from terminated primers by changing the assay parameters pH and Mg2+ level. Variations in pH and Mg2+ concentration are known to affect RNase H activity. Such intracellular alterations are considered to occur in lymphocytes used for drug susceptibility assays and/or in human cells.

METHODS: Reverse transcriptase (RT) mutants were constructed containing M41L/D67N/K70R/T215Y/K219Q (TAM), M41L/T69S-SG/L210W/T215Y (69S-SG) or V75I/F77L/F116Y/Q151M (151M). ATP-mediated excision of AZT-MP was studied using non-radioactive multiple incorporation RT assays, with prA300/odT20 or pdA300/odT20 as template/primer, representing RNA and DNA template. Free Mg2+ concentration was reduced from the RT assay optimum of 7 to 1 mM and the pH reduced from 7.5 to 7.0.

RESULTS: In the RT assays, fold resistance to AZT-TP was determined as the ratio of mutant to wild-type IC50. By reducing free Mg2+ or pH, the ATP-dependent fold resistance with RNA template assay for TAM and 69S-SG mutants increased, for example, from approximately 5 to 10 and from 10 to 40, respectively, with lower pH. In the DNA template assay, no changes or only minor increases of resistance were observed when lowering free Mg2+ or pH. With the 151M mutant, representing non-excision mechanism, the same 30-fold resistance was obtained with or without these alterations in either assay. The effect on RNase H activity was also determined and was found to be reduced, in comparison with RT activity, with decreased pH.

CONCLUSIONS: Low intracellular pH or differences between physiological and enzymatic assay Mg2+ levels are, besides other parameters, likely to play a role when measuring AZT resistance. These initial results indicate that these factors are involved in a mechanism promoting enhanced excision with RNA template dependency, and possibly also involve RNase H activity.
ABSTRACT 53

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A new class of tight binding non-nucleoside reverse transcriptase inhibitors inhibit the burst and steady-state phases of wild-type and Y188L HIV-1 reverse transcriptase activity

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BACKGROUND: Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are part of highly active antiretroviral therapy (HAART) regimens to treat HIV-1 infections and inhibit catalysis by binding to a unique allosteric site of the P66 subunit of reverse transcriptase (RT). Previous reports on efavirenz, nevirapine, delavirdine and O-TIBO compounds showed that NNRTIs do not inhibit the steady-state rate of catalysis, but only affect the maximum rate of nucleotide incorporation ($k_{pol}$). In addition, the error-prone nature of RT activity has allowed viruses to evade inhibition by selection of functionally active resistant mutants.

METHOD: A single nucleotide incorporation (SNI) transient kinetic assay was used to determine kinetic parameters of SNI by HIV-RT and to measure the inhibition potency of selected NNRTIs from a new amide series (RO-0335, RO-3048 and RO-3484).

RESULTS: Compounds RO-0335, RO-3048 and RO-3484 inhibited SNI by HIV-RT with IC$_{50}$ values of 11 nM, 60 nM and 533 nM, respectively, and reduced the burst amplitude in a dose-dependent manner. Surprisingly, RO-0335 and RO-3048 were also able to inhibit the steady-state rate ($k_{cat}$), in contrast to the control compound nevirapine and to previous reports with efavirenz, nevirapine and O-TIBO. The weaker binding but structurally similar compound RO-3484 showed a pattern of HIV-RT inhibition similar to nevirapine and different to RO-0335 and RO-3048 (that is, inhibition of burst amplitude without inhibition of the steady-state rate of nucleotide incorporation). Introducing the Y188L mutation in the allosteric site of HIV-RT had no significant effect on inhibition potency of amide RO-0335, reduced the inhibition potency of RO-3048 and abolished inhibition by RO-3484. In agreement with the enzyme inhibition data, mutation Y188L did not affect the HIV-RT binding affinity of RO-0335, but increased the $K_d$ value of RO-3048 and RO-3484 significantly.

CONCLUSION: These data suggest a correlation between the HIV-RT binding affinity of compounds within a new amide series of NNRTIs and their ability to inhibit the burst amplitude and steady-state phases of nucleotide incorporation by HIV-RT. The mechanism of inhibition of tight-binding compounds from a new series of NNRTIs may therefore be different as compared with first-generation NNRTIs.
Identification of lamivudine (3TC) as a novel substrate for human organic cation transporters 1, 2 and 3

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BACKGROUND: Nucleoside reverse transcriptase inhibitors (NRTIs) represent an important component of current antiretroviral combination therapies. Reduced entry of a specific NRTI could compromise optimal intracellular levels of the drug, favouring the emergence of resistant viruses. Human organic cation transporters (hOCT1–3) have been related to the uptake mechanisms of several antiviral drugs in reabsorption and excretion tissues, and are actively expressed in CD4+ T-cells (main targets of HIV-1). This work aims to study the putative involvement of hOCTs in NRTI uptake and the possible cross-inhibition between NRTIs.

METHODS: The interaction of zidovudine (AZT), lamivudine (3TC), abacavir (ABC), tenofovir (TDF) and emtricitabine (FTC) with hOCTs was investigated by [3H]-MPP+ cis-inhibition experiments in Chinese hamster ovary (CHO) stably transfected cells with hOCT1, 2 and 3. Uptake studies were carried out with [3H]-NRTIs in the presence of different inhibitors, either hOCT natural substrates or highly specific inhibitors (D-22). Uptake studies were confirmed with the radiolabelled drugs in Xenopus laevis oocytes.

RESULTS: The NRTIs interacted with high affinity with the three isoforms of the transporter (IC50<0.2 nM for hOCT1, except TDF; IC50<2.0 nM for hOCT2, except FTC; IC50<0.2 nM for hOCT3, except TDF). Studies performed in CHO and Xenopus oocytes, showed that 3TC uptake could be inhibited by all hOCTs substrates (including MPP+), as well as by the inhibitor D-22 (>50% inhibition for all hOCTs). Kinetic studies demonstrated that 3TC is a good substrate for hOCTs with higher affinity for hOCT1 and hOCT2 (Km=505 ±209 µM and Km=137 ±57 µM, respectively) than for hOCT3 (Km=1.38 ±0.25 mM).

Moreover, all NRTIs tested inhibited [3H]-3TC uptake by hOCT1, with ABC and AZT inhibiting up to 50% of 3TC uptake at very low concentrations (K=2.4 nM and 8.5 nM, respectively).

CONCLUSIONS: 3TC is a newly identified substrate for hOCTs, which are expressed both in kidney and immune cells, ABC, AZT, TDF and FTC have a high-affinity interaction with hOCTs, suggesting a putative role of NRTIs as modulators of hOCTs physiological functions. Finally, inhibition of 3TC uptake at low concentrations of ABC and AZT might have implications for 3TC pharmacokinetics.
ABSTRACT 55

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HIV-1 reverse transcriptase connection domain mutations reduce template RNA degradation and enhance nucleoside reverse transcriptase inhibitor excision

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BACKGROUND: We previously identified eight mutations in the HIV-1 reverse transcriptase (RT) connection (cn) domain of treatment-experienced patients that significantly increased 3’-azido-3’-deoxythymidine (AZT) resistance in the presence of thymidine analogue mutations (TAMs). We proposed that these mutations increased AZT resistance by altering the balance between nucleotide excision and template RNA degradation, providing more time for RT to undergo nucleoside reverse transcriptase inhibitor (NRTI) excision. In the current study we have tested the predictions of this model by analysing the effects of the cn domain mutations on in vitro RNase H activity and AZT monophosphate (AZT-MP) excision.

METHODS: Primary and secondary RNase H cleavages were detected by in vitro RNase H assays using 18/18- and 41/77-nucleotide RNA/DNA hybrids. The ability of mutant RTs to excise AZT-MP from blocked primer was tested in an in vitro excision-extension assay using 19/42-nucleotide RNA/DNA and DNA/DNA hybrids and ATP or PPi as a substrate for an excision reaction. All experiments were performed with virion-derived and purified RTs.

RESULTS: In combination with TAMs, the cn domain mutations G335C/D, N348I, A360I/V, V365I and A376S reduced primary and secondary RNase H cleavages on two different RNA/DNA hybrids. When the cn domain mutations were reverted back to wild type, RNase H activity levels were restored and the enhanced AZT resistance was lost. The patient-derived cn domains from all five patients increased ATP-mediated excision of AZT-MP on an RNA template compared with a DNA template. One of the patient-derived cn domains increased ATP-mediated excision on a DNA template. A comparison of the ATP- and PPi-mediated excision on RNA and DNA templates suggested that ATP-mediated excision is more efficient than PPi-mediated excision. These observations, which are currently under further study, suggest that in addition to increasing AZT-MP excision by reducing RNase H activity, the cn domain mutations may directly influence ATP-mediated excision.

CONCLUSION: Overall these studies provide strong support for the model that cn domain mutations increase AZT resistance by reducing the degradation of the RNA template, thus providing additional time for RT to catalyse AZT-MP excision.
The fidelity of HIV-1 reverse transcription: reverse transcriptase variants with altered mutation levels during replication

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BACKGROUND: HIV-1 is an effective pathogen owing in part to its ability to generate sufficient genetic diversity to respond to environmental challenges while maintaining efficient replication. Advantageous mutations introduced during replication make it possible for HIV-1 to evade host defense mechanisms, avoid immune responses and become resistant to antiviral drugs. Mutational diversity can be generated during transcription by cellular RNA polymerase or during first and second strand cDNA synthesis by HIV-1 reverse transcriptase (RT). The portion of diversity due to errors introduced by RNA polymerase is unknown and, given that HIV-1 RT lacks a conventional proof-reading activity and replicates nucleic acids with low fidelity, it has been assumed that most diversity is generated during reverse transcription.

METHODS: To identify error-prone HIV-1 RT variants, we extensively mutated HIV-1 RT and carried out a genetic screen for fidelity variants using a coupled reverse transcription/fidelity assay. In this system, RT activity was monitored with a reverse transcription indicator gene, while fidelity was measured by mutation or reversion of a second selectable gene. Fidelity variants were further characterized in biochemical and viral replication assays.

RESULTS: RT variants with both increased and decreased fidelity were identified. Amino acid substitutions associated with decreased fidelity cluster in three regions: the fingers region, an area previously implicated in nucleotide selectivity; the β7/β8 loop of the p51 subunit; and the connection domain. Several of the RT variants caused decreased fidelity in genetic reversion assays and showed significant levels of misincorporation in biochemical assays. Consistent with the high levels of misincorporation, some of the variants compromise viral replication. Many of the fidelity variants show altered mutational spectra and base substitution specificities after retrotransposition of lacZ-α-containing elements.

CONCLUSION: We identified HIV-1 RT variants that have increased mutation frequencies, altered mutation spectrum and base specificity, and effects on viral replication. These fidelity variants expand the repertoire of enzyme-nucleic acid interactions that influence the quality of replication. Some of these variants may prove useful in distinguishing the contributions of RT and RNA polymerase to HIV-1 diversity. These variants also make it possible to compare the evolution of drug resistance in low fidelity viruses with that of wild type.

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ABSTRACT 57

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Synonymous mutations are involved in the highly ordered regulation of nucleoside reverse transcriptase inhibitor resistance

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BACKGROUND: Synonymous mutations might be involved at the HIV RNA level in HIV-1 drug resistance mechanisms. The goal of this study was to investigate the correlation between synonymous mutations at the HIV RNA level, the known nucleoside reverse transcriptase inhibitor (NRTI) resistance mutations, and viro-immunological parameters.

METHODS: A total of 2,726 HIV-1 subtype B pol sequences from 1,370 drug-naive patients and 1,356 NRTI-treated patients were obtained and analysed. Mutations were defined as differences from the HIV-1 consensus B nucleotide sequence. The association of mutations with NRTI-treatment and viraemia/CD4 was assessed by \( \chi^2 \) tests and median tests, respectively. Covariation analysis was based on the binomial correlation coefficient (phi) and hierarchical clustering. Benjamini–Hochberg methods were used to correct for multiple comparisons (false discovery rate =0.05). RNAfold software was used to study the secondary structure of HIV-1 gag-pol RNA.

RESULTS: Ten synonymous mutations (AUU37AUC, UAC56UAU, AAA65AAG, AAA66AAG, UUU116UUC, AGU162AGC, UAC183UAU, UAU271UAC, AAA287AAG and GAG308GAA) in the viral RNA region encoding for reverse transcriptase were positively associated with NRTI failure and correlated in pairs and in clusters with known NRTI resistance mutations. In particular, very strong correlations were observed for the synonymous mutation AAA65AAG (K65K) with GAC67AAC (D67N) \((P=1.7e^{-15})\), for AAA66AAG (K66K) with GAC67AAC (D67N) \((P=1.0e^{-57})\) and ACT69GAT (T69D) \((P=3.0e^{-13})\) and AAA70AGA (K70R) \((P=3.7e^{-10})\), and for UAC183UAU (Y183Y) with AUG184GUG (M184V) \((P=1.3e^{-3})\). Cluster analysis revealed the existence of a strong cluster involving the thymidine analogue mutations 2 (TAM 2) D67N+K70R+K219Q together with the synonymous mutations AAA65AAG+AAA66AAG (bootstrap =0.91).

At treatment failure, the co-presence of GAC67AAC (D67N) with the synonymous mutations AAA65AAG+AAA66AAG was significantly associated with higher viraemia and lower CD4+ T-cell count compared with GAC67AAC (D67N) alone (4.32 log copies/ml versus 3.41 log copies/ml, \( P=0.004 \); 279 cells/\mu l versus 496 cells/\mu l, \( P=0.003 \)). Consistent with the increase in viraemia, by structural analysis, we observed that the secondary HIV RNA structure is more stable in the presence of GAC67AAC (D67N)+AAA65AAG+AAA66AAG than D67N alone (complete gag-pol \( \Delta G=-1,834.51 \) kcal/mol versus \( \Delta G=-1,827.48 \) kcal/mol; local K64–V75 \( \Delta G=-23.6 \) kcal/mol versus \( \Delta G=-16.0 \) kcal/mol). In addition, the presence of the synonymous mutation AAA65AAG (K65K) at baseline (before antiretroviral treatment) correlates with the appearance of GAC67AAC (D67N) at NRTI failure \((P=0.01)\).

CONCLUSIONS: Besides well-known amino acid mutations, synonymous mutations strongly modulate the evolution of drug resistance. Their knowledge can be crucial for a better understanding of HIV-1 drug resistance mechanisms.
Silent mutations at reverse transcriptase codons 65 and 66 in B and C strains found in Brazil are very strongly associated with treatment and these signatures can impact on zidovudine mutation acquisition in vitro.

**BACKGROUND:** Two ‘silent’ mutations strongly associated with treatment experience in patients carrying thymidine analogue mutations (TAMs), and highly significant after multiple comparison corrections, have been identified. These silent mutations were located at codons K65K and K66K (AAA to AAG, in both cases). Studies using a reverse transcriptase (RT) in vitro assay have shown that the K66K silent mutation introduced into a synthetic RNA template alleviated the pause caused by RT carrying D67N and K70R substitutions (Harrigan et al., 2007). We evaluate the association of these two silent mutations at RT codons 65/66 in sequences from 566 Brazilian patients failing highly active antiretroviral therapy (HAART) and infected with HIV B and C subtypes. We also evaluated the resistance mutation acquisition of HIV-1 clones subtypes B and C submitted to training experiments with zidovudine and lamivudine.

**METHODS:** We analysed 829 RT sequences from Brazilian patients failing on HAART attending for routine genotypic analysis. Among them, we selected 566 patients carrying TAMs and segregated them by subtype (B, n=432; C, n=134). We utilized 225 drug-naive RT sequences from patients from the same geographical region as a control group (B, n=109; C, n=64). The comparisons of silent mutations in TAM and drug-naive groups were performed using the χ² method. The in vitro training with zidovudine and lamivudine was performed in MT4 cells using a stepwise increase of zidovudine and lamivudine concentration (2×) in each passage.

**RESULTS:** As previously demonstrated by Harrigan and colleagues, we found a very strong correlation for the association of TAMs in subtype B isolates with K66K (P<0.000002) and with K65K (0.00037), using the drug-naive data set as control. Contrasting with subtype B data, Brazilian subtype C sequences carry a high frequency of K66K in individuals carrying TAMs (98, 51%) and drug-naive (97, 74%). The same picture was observed in K65K synonymous substitution (TAM group 87, 31% versus drug-naive group 88, 72%; P<0.967). We also evaluated the acquisition of resistance mutations in HIV-1 clones of subtype B and C submitted to training experiments with zidovudine and lamivudine, showing a behaviour of B and C recombinant HIV during the training to look for different differential in vitro kinetics of drug resistance mutation. Subtype B and C isolates followed different mutation pathways when they were under zidovudine selection. Subtype B recombinant isolates accumulate mutation Q151M right after rebounding that was retained all over the culture, and an additional mutation D67N was incorporated when the viral load rebound to original levels before the drug selection. Interestingly, subtype C followed a different route and firstly accumulated D67N before rebounding and adding K70R during the rebound process. The first mutation (D67N) was replaced by T215I after the virus reached a viral load comparable to levels before selection. By contrast, lamivudine selection yielded the same drug resistance mutation acquisition kinetics with the accumulation of the M184I mutation.

**CONCLUSION:** Our findings corroborate and extend Harrigan’s work for subtype B isolates. Additionally, subtype C isolates seem to carry K66K/K65K silent mutations as a natural polymorphism of these variants. These findings can predict a faster selection of TAMs in subtype C, owing to the favourable RNA template for the excision reaction catalysed by TAMs. The in vitro study using a recombinant virus derived from HBX2 shows subtype-specific differences during antiretroviral drug selection in vitro. This could suggest that these silent mutations, as well as specific polymorphisms found in subtype C isolates, can lead to important differences in the biological behaviour of the patients infected with different HIV-1 variants under antiretroviral therapy.
ABSTRACT 59

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In vitro selection and characterization of maraviroc-resistant HIV type 1

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BACKGROUND: Maraviroc, a CCR5 antagonist approved by the Food and Drug Administration (FDA) in August 2007, represents the first of a new class of antiretrovirals targeting HIV-1 coreceptor binding. Resistance selection experiments were undertaken to study maraviroc resistance and to develop reagents for use in cross-resistance studies with other CCR5 antagonists.

METHODS: A dose-escalation method was used to select maraviroc-resistant HIV-1. The HIV-1 JR-CSF molecular clone pYK-JRCSF was used as the starting wild-type virus and was serially passaged in MOLT-4/CCR5 cells using increasing concentrations of maraviroc. Eighteen passages were performed, escalating maraviroc concentrations from 2.0 nM to 40 μM.

RESULTS: Following 15 passages up to 4.0 μM maraviroc, six gp120 mutations were identified by population sequencing (numbering based on HIV-1 JR-CSF gp160; gp120 conserved domains [C] and variable loops [V] indicated): S114N (C1), V253I (C2), T276M/K (C2), N403D (V4), M467I (C5) and I486L (C5). Analysis of specific Env clones identified additional mutations: D77G (C1), Q104K (C1), N195I (C2), T315A (V3), M369T (C3), F378S (C3) and Q434R (C4). The resulting virus was incapable of growing in MOLT-4 cells lacking CCR5, indicating it maintained CCR5 tropism. However, susceptibility testing demonstrated a lack of change in the IC50 value (that is, the half maximal inhibitory concentration) compared with wild-type virus. Passaging was continued, escalating maraviroc concentrations from 2.0 μM to 40 μM.

RESULTS: Following 15 passages up to 4.0 μM maraviroc, six gp120 mutations were identified by population sequencing (numbering based on HIV-1 JR-CSF gp160; gp120 conserved domains [C] and variable loops [V] indicated): S114N (C1), V253I (C2), T276M/K (C2), N403D (V4), M467I (C5) and I486L (C5). Analysis of specific Env clones identified additional mutations: D77G (C1), Q104K (C1), N195I (C2), T315A (V3), M369T (C3), F378S (C3) and Q434R (C4). The resulting virus was incapable of growing in MOLT-4 cells lacking CCR5, indicating it maintained CCR5 tropism. However, susceptibility testing demonstrated a lack of change in the IC50 value (that is, the half maximal inhibitory concentration) compared with wild-type virus. Passaging was continued, escalating maraviroc concentration to 40 μM by passage 18. Population sequencing indicated six additional gp120 mutations emerging by the end of this passage: I192V (V2), S298N (V3), E318D (V3), G375R (C3), E395K (V4) and K432R (C4). Susceptibility testing and sequencing of Env clones from passage 18 are in progress.

CONCLUSIONS: Although a classical shift in IC50 was not observed in susceptibility studies with passage-15 virus, the clear pattern of increasing mutations in gp120 and the ability of passage-18 virus to replicate to significant levels in 40 μM maraviroc (~40,000-fold above the IC50) demonstrate a novel maraviroc resistance genotype and phenotype based on HIV-1 JR-CSF. The results from this study expand existing knowledge of maraviroc resistance.

The reagents generated will be useful for studying the antiviral activity of other CCR5 antagonists.

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ABSTRACT 60
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Escape of HIV-1 from a CCR5 antagonist can reduce envelope-mediated entry

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BACKGROUND: HIV-1 strains resistant to reverse transcriptase, protease and fusion inhibitors often replicate less efficiently than susceptible wild-type viruses. The infectivity of viruses that acquire resistance to CCR5 antagonists has not been well-defined. In this study, we analysed the infectivity and envelope sequences of HIV-1 strains that are resistant to the CCR5 antagonist SCH-C.

METHODS: SCH-C-resistant HIV-1 (JV1083) was selected by sequential passage in peripheral blood mononuclear cells (PBMC) and PM-1 cells. Envelope sequences were amplified from parental and week 35 virus, and transferred to an expression vector. Viral infectivity and entry inhibitor susceptibility of multiple clones from parental and escape virus populations were measured in the PhenoSense™ Entry assay. A series of chimeric envelope clones and site-directed mutants were generated to identify the genetic determinants of drug resistance and altered infectivity, defined as luciferase activity in infected U87-CD4-CCR5 cells in the absence of drug.

RESULTS: Week 35 SCH-C-resistant virus was incompletely suppressed at high SCH-C concentrations (maximum inhibition 60%). Infectivity of the resistant virus was diminished 10-fold compared with the parental virus. Analysis of envelope clones and chimeras revealed genetic determinants of resistance in multiple regions, including surface-exposed protein (gp120) and transmembrane protein (gp41). Two mutations, K305R and G321D, (alone or together) in the V3 region of the resistant virus completely abolished infectivity when introduced into the parental envelope sequence. Additional mutations in the co-receptor-binding region of C4 also conferred reduced SCH-C susceptibility and infectivity. A single substitution (M201I) in the bridging sheet in the C2 domain was sufficient to restore viral infectivity to the resistant virus.

CONCLUSIONS: Variants resistant to the CCR5 antagonist SCH-C can exhibit decreased viral infectivity. Reduced infectivity was associated with mutations in co-receptor-binding regions, including the V3 and C4 domains. Amino acid substitutions in regions associated with co-receptor binding, but distinct from V3, can compensate for the loss of infectivity of resistant virus. These findings may provide important insight into co-receptor binding and resistance to CCR5 antagonists, including vicriviroc and maraviroc.
ABSTRACT 61

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Dual modes of resistance to an HIV-1 fusion inhibitor

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BACKGROUND: HIV-1 fusion inhibitor 5-Helix targets the C-terminal heptad repeat region of the gp41 ectodomain (C-HR). Because this target is exposed transiently during fusion, inhibitory potency depends primarily on the rate of 5-Helix association and duration of C-HR exposure. As a consequence, there is a 20,000-fold disparity between 5-Helix inhibitory potency (IC$_{50}$~12 nM) and its binding affinity (K$_D$~0.0007 nM). Here, we ask how resistance develops to this kinetically restricted inhibitor and if binding affinity influences viral escape mechanism.

METHODS: Env escape mutations were identified in HIV-1NL4–3 serially propagated in 5-Helix and similarly potent 5-Helix variants (IC$_{50}$ range 20–90 nM) with binding affinities that spanned three orders of magnitude (K$_D$ range from 0.05–40 nM). Resistance mechanisms were determined by correlating inhibitory potencies (pseudoviral infectivity assays) with binding affinities and association kinetics (KinExA 3000 binding assays).

RESULTS: Fourfold to 20-fold resistance to the original 5-Helix emerged through mutations at a highly conserved C-HR glycosylation site (Asn637/Thr639; denoted site 1). Site 1 mutations paradoxically enhanced inhibitor binding affinity and increased the dependence of inhibitory potency on association kinetics. These properties are consistent with a kinetic mechanism of resistance, whereby the lifetime of C-HR exposure is shortened to limit the amount of time 5-Helix has to bind gp41. Although the potencies of lower affinity 5-Helix variants were reduced by site 1 mutations, this resistance profile was not selected with these inhibitors. Rather, escape mutations emerged at the highly conserved C-HR residue Asn656 (site 2). Mutations here substantially disrupted binding affinity (>5,000-fold), but did not alter kinetic dependence to inhibition. The correlation between IC$_{50}$ and K$_D$ values pointed to an affinity-based mechanism of resistance. Interestingly, site 1 mutations were much more detrimental to viral fitness than site 2 mutations.

CONCLUSIONS: The data show that similar antiviral agents that inhibit with the same mechanism and with comparable potencies can give rise to vastly different mechanisms of resistance. HIV-1 escape from 5-Helix inhibition involves a complex interplay between binding affinity, Env plasticity and viral fitness that has important implications for the development of inhibitors that target transient intermediate states of HIV-1 entry.
Dissection of the contributions of protease and cleavage site mutations to protease inhibitor resistance: a case of multidrug-resistant virus associated with rapid progression to AIDS

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BACKGROUND: Transmission of dual-tropic multidrug-resistant (MDR) HIV-1 and rapid progression to AIDS has been documented recently [1]. The isolated virus was resistant to all protease inhibitors (PI) and nevirapine. It contained nine reverse transcriptase (RT) mutations, 12 protease (PR) mutations and gag cleavage site mutations (A431V and P453L). It also contained a deletion resulting in the mutation/deletion of five amino acids in both the gag p6 and TF region. Here, we describe the generation and characterization of recombinant clones containing various parts of the viral genome from this patient isolate.

METHODS: Recombinant virus cs-PR-RT M11 was generated by homologous recombination of HXB2 DNA and a 2033 nucleotide (nt) fragment of patient isolate encompassing 259 amino acids of gag protein, protease and 303 amino acids of RT. Molecular clones containing a 270 nt fragment of gag (gag C405–N495, csM11), a 341 nt fragment encompassing protease (PR M11) or a 621 nt fragment containing both gag and PR (csPR M11), were constructed by insertion of corresponding fragments into HXB2. Phenotypic characterization of all mutant strains was performed using a cell-based MTT assay. The replication capacity of viruses was tested in parallel cultures in MT4 cells.

RESULTS: The activities of lopinavir (LPV), atazanavir (ATV), amprenavir (APV), saquinavir (SQV), tipranavir (TPV), darunavir (DRV) and SPI-256 were tested against generated viruses. In general, the presence of gag mutations (csM11) did not significantly affect the potency of tested PIs (a maximum twofold increase in LPV and SQV IC50). PR M11 demonstrated significant resistance to LPV and APV (≥100-fold), a medium (6–15-fold) increase in IC50 for SQV, DRV and SPI-256, and did not affect potency of ATV and TPV. A combination of cleavage site and PR mutations, csPR M11, resulted in a significant further increase in resistance to ATV, SQV and DRV (7–10-fold) with a lesser effect on potency of TPV, LPV and SPI-256 (2–5-fold). The phenotypic profile of cs-PR-RT M11 was similar to that of parental patient viral isolate. The replication capacity of PR M11 and csM11 viruses was lower than csPR M11.

CONCLUSIONS: Generated recombinant viruses allowed us to analyse the relative contributions of cleavage site and protease mutations to phenotypic resistance to PIs and replication capacity. Although no or minimal resistance to all PIs was observed for virus containing only cleavage site mutations, their combination with PR mutations resulted in a synergistic effect. The presence of cleavage site mutations contributed to an improved replication capacity of mutant virus.

REFERENCE:
ABSTRACT 63

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Natural variation of integrase sequences from subtype B and CRF02-AG HIV-1 antiretroviral-naive patients and possible effect on susceptibility to integrase inhibitors

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BACKGROUND: HIV-1 integrase (IN) is an essential enzyme required for viral replication and has great potential as a novel target for anti-HIV drugs. Raltegravir and elvitegravir are integrase inhibitors that are highly active in patients harbouring resistant viruses to other antiretroviral classes. Few data are available concerning the efficacy of these inhibitors on HIV-1 non-B subtype strains such as CRF02-AG, which is mainly prevalent in West Africa and is of increasing prevalence in Europe. The aim of this study was to examine IN sequences from HIV-1 subtype B and CRF02-AG antiretroviral-naive patients for the presence of naturally occurring polymorphisms, and to search for a possible effect on IN structure and sensitivity to IN inhibitors caused by variations between these subtypes.

METHODS: The entire IN gene from 72 HIV-1 subtype B and 66 subtype CRF02-AG antiretroviral-naive patients was amplified and sequenced. Naturally occurring polymorphisms and protein structures from both subtypes were compared. The possible effect on IN structure and sensitivity to IN inhibitors caused by variations between subtypes B and CRF02-AG was addressed within the context of a three-dimensional model of the HIV-1 IN complex.

RESULTS: The analysis of IN amino acid sequences showed that 13 positions (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, L/I234 and S/G283) differed between subtypes B and CRF02-AG. As observed in the three-dimensional model of the pre-integration complex, these differences may affect the functional property of IN. Moreover, most variations are co-localized in three clusters: C1 (Leu101, Thr112, Gly134, Ile135 and Lys136), C2 (Val31, Thr124 and Thr125) and C3 (Val201 and Thr206). Several variations of amino acids in HIV-1 IN subtype CRF02-AG could have a putative effect on IN inhibitor sensitivity. In particular, the cluster formed by Thr125, Thr124 and Val31 contains at least one residue, Thr125, which variation has been involved in resistance to the naphtyridine carboxamide L870,810 IN inhibitor.

CONCLUSION: These results suggest that virological response to IN inhibitors, according to the subtype, needs to be carefully studied in clinical trials. The fact that most variations were found in clusters suggests that some of them could be linked together through compensatory mechanisms.
ABSTRACT 64

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Evaluating the ‘substrate envelope’ hypothesis: structural analysis of novel HIV-1 protease inhibitors designed to be robust against resistance

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BACKGROUND: The major challenge in the treatment of HIV-1 infection is not only to design potent inhibitors against the wild-type and drug-resistant viruses, but also to prevent the virus from evolving further with resistance mutations to that inhibitor. We previously defined the substrate envelope as the overlapping volume occupied by the substrates within the active site of the HIV-1 protease. Most active-site drug-resistant mutations occur where the inhibitors protrude beyond the ‘substrate envelope’ and contact the protease. From this observation, we hypothesized that protease inhibitors that fit within the substrate envelope would be less susceptible to drug-resistant mutations. To test this hypothesis, we designed and synthesized several inhibitors (with and without substrate envelope constraint) and tested them for binding affinity in enzymatic studies against a panel of HIV-1 wild-type and drug-resistant protease variants.

METHODS: Crystal structures of 16 inhibitors with binding affinities ranging from 58 nM to 0.8 pM were determined in complex with wild-type HIV-1 protease. Simplified Lennard–Jones potential was used to estimate total van der Waals contacts between the inhibitors and the active-site residues of the protease.

RESULTS: Analysis of van der Waals contacts in the 16 structures shows that some active-site residues have a differential interaction between the nanomolar and picomolar inhibitors. Hydrogen-bonding analysis revealed that tight binding picomolar inhibitors appear to ‘lock’ into the active site by making hydrogen bonds to particular residues in the active site of the protease. Inhibitors that were designed to fit within the substrate envelope have better resistance profiles than inhibitors that protrude beyond the substrate envelope, validating the substrate envelope hypothesis.

CONCLUSIONS: This is the first systematic analysis performed on a large number of HIV protease–inhibitor complexes. The study shows that, although HIV-1 protease possesses intrinsic structural plasticity, there are some subtle interactions that distinguish nanomolar inhibitors from picomolar inhibitors. Modern drug design ignores the molecular basis for function, resulting in the rapid evolution of drug resistance. With the substrate envelope hypothesis, we have shown that inhibitors can be designed that are less susceptible to resistance.
ABSTRACT 65
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Ninety nine not enough? Characterization of HIV-1 protease mutants with insertions in the flap region
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BACKGROUND: Insertions in HIV-1 reverse transcriptase are well known to cause broad-class nucleoside reverse transcriptase inhibitor (NRTI) resistance. At the same time, little is known about the role of protease insertions. We investigated the prevalence of insertions in the viral protease and the effect on protease inhibitor (PI) susceptibility and function. Furthermore, the effect of these insertions on the structure of the viral protease was investigated.

METHODS: Prevalence of protease insertions was evaluated in a large database consisting of over 208,000 clinical samples. Two representative variants with multiple PI resistance associated mutations and an insertion at protease codon E35EE or L33LL were selected. To dissect the relative contribution of the insertion, corresponding protease mutants with and without the insertion were generated and evaluated with respect to PI susceptibility (MTT assay, inhibition constant \textit{in vitro}) and protease function (viral replication competition experiments, specific proteolytic activity). The protease–inhibitor complexes for crystallization were prepared by mixing recombinant mutant proteases with a fivefold excess of lopinavir. Diffraction data were collected, processed and refined to 1.8 Å resolution.

RESULTS: Prevalence of protease insertions has significantly increased over the past ten years from 0.11% to 0.31% (\(P<0.0001\)). Most protease insertions appeared to be selected at either codon 33 or 35. Investigation of two representative variants with and without insertion revealed that even in a background of known PI mutations both insertions contribute to a further 3–10-fold increase in resistance to most clinically relevant PIs. However, replication capacity (RC) analysis revealed that all variants replicated less efficiently than wild type. Direct comparison of the effect of the insertion on RC revealed that the L33LL decreased RC, whereas the E35EE increased RC. Structural analysis revealed local rearrangements in the protease flap region and in the substrate-binding pockets.

CONCLUSION: Prevalence of protease insertions is very low but increasing over time, which could be a concern because they confer high-level resistance to most clinically relevant PIs. Enlargement of the protease substrate-binding site together with impaired flap dynamics could account for the weaker PI binding by the insertion mutants. These protease insertions could therefore represent an additional mechanism of HIV PI resistance development.
ABSTRACT 66

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HIV p7/p1 cleavage-site mutations are selected by the immune system and/or by antiretroviral therapy

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BACKGROUND: HIV cleavage-site (CS) mutations are associated with exposure to protease inhibitors _in vitro_ and _in vivo_. Cleavage site variability is limited by altered cleavage rates of the viral protease. Nevertheless, natural polymorphisms are found throughout all HIV gag cleavage sites. As the cleavage sites contain cytotoxic T lymphocyte (CTL) epitopes, CTL targeting epitopes within the gag cleavage sites may influence the occurrence of CS mutations. The analysis of driving forces for the selection of CS mutations will enable a better understanding of the mechanisms of sequence adaptation of HIV.

METHODS: We analysed 70 HIV genotypes from therapy-naive patients in the _pol_ gene and in the C-terminal _gag_ gene. Primary resistance mutations were found in 30 patients. All patients were genotyped for HLA class I molecules (HLA-A and HLA-B). In four patients, further HIV genotypes could be analysed after the initiation of antiretroviral therapy and/or therapy failure. Enzyme-linked immunospot technique (ELISPOT) assays and overlapping synthetic peptides were used to analyse the recognition of HLA-B13 epitopes using peptide-stimulated cell lines derived from another cohort of HLA-typed HIV-1-infected patients from Erlangen.

RESULTS: HIV without primary resistance (_n=40_) frequently harboured C-terminal CS mutations, in particular 436R (18%), 437L (8%), 449P (38%), 451N (10%), 453T (10%) and 453L (13%). CS mutations 431V (_n=2_) and 437V (_n=2_) were only observed in HIV with primary resistance (_n=30_). Overall, the presence of CS mutation 436R was significantly correlated with HLA-B13 genotypes (_P<0.05_), although its presence was not limited to HLA-B13-positive patients. 436R came up in one patient (HLA-B13-negative) during suppressive antiretroviral therapy containing lopinavir. 431V located in the same HLA-B13 epitope was detected in two patients with similar PR mutations (53L/82A/90M) of whom only one was positive for HLA-B13. ELISPOT assays showed that the CS mutation 431V could abrogate CTL recognition in some, but not all patients. CS mutation 436R could be recognized but peptide titration experiments showed a moderate decrease of CTL recognition at lower peptide concentrations in some patients, which could explain preferential selection of the 436R mutation in HLA-B13-positive individuals.

CONCLUSION: A variety of CS mutations could be detected in therapy-naive HIV. Different selective pressures contribute to the occurrence of CS mutation 436R. Our data indicate that, in addition to drug therapy, immune selection may influence the development of CS mutations.
Multiply drug-resistant (MDR) variants of HIV-1 can exist within cells as replication-defective quasispecies and be rescued by superinfection

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**BACKGROUND:** RNA viruses generate swarms of similar but genetically distinct variants, many of which can be defective. We have studied whether replication-defective HIV variants, that harbour drug-resistant (DR) mutations, can potentially be rescued by superinfection and recombination.

**METHODS:** We infected cell lines with DR viruses and performed cellular cloning to isolate cells containing defective provirus. The basis for replication incompetence was confirmed by sequencing. We then employed superinfection of cell lines carrying defective viruses to try to rescue DR mutations. The rescued viruses were tested for resistance and sequenced to confirm the existence of DR mutations.

**RESULTS:** Expansion of clonal cell populations shows that defective infections are frequent in cell lines. Furthermore, stable cell lines that harbour defective proviral HIV-1 can be isolated by such cloning. One such cell line, derived from MT2 cells that were infected with a multiply drug-resistant (MDR) variant, was shown to produce non-infectious particles. Superinfection of this cell line by wild-type HIV-1 led to production of replication-competent MDR viruses. In addition, this cell line could also be independently superinfected by a replication-defective, non-revertant virus, mutated in capsid, also leading to production of infectious viral progeny. The successfully superinfected cells should contain two copies of defective proviral genomes and were presumably able to produce functional virions through complementation and assembly of viral proteins produced from the two differently defective proviral genomes. The virus particles were then able to infect new cells, leading to recombination between the complementary HIV genomes during reverse transcription. Sequencing showed that the majority of rescued viruses were wild-type when the infected cells were passaged in culture in the absence of reverse transcriptase (RT) inhibitors, but MDR viruses dominated the cultures if even single RT inhibitors were present.

**CONCLUSIONS:** Cells that harbour defective viruses can produce infectious progeny after superinfection by another defective virus, because of probable recombination during second rounds of infection. These results demonstrate that defective HIV variants possibly represent an important component of the HIV-1 reservoir in terms of both wild-type and drug-resistant viral progeny.
SESSION 3
HIV pathogenesis, fitness and resistance
ABSTRACT 68
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Single cell analysis of HIV DNA from infected patients
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BACKGROUND: The frequency with which host cells are infected in vivo with one, two or multiple HIV-1 proviruses has not been well defined. Furthermore, the genetic relatedness of proviruses within an infected cell and to the extracellular virus population is unknown. To address these issues, we developed a technique to examine viral DNA from a single peripheral blood mononuclear cell (PBMC).

METHODS: PBMCs were diluted in a microtitre plate to much less than one infected cell per well. The cells were lysed and their DNA distributed over 10 wells. A 1.33 kb fragment of DNA encompassing the p6 region of gag, pro and the first 900 nucleotides of reverse transcriptase was amplified and sequenced. The number of viral DNA molecules per infected cell was estimated from the number of positive wells. The relationship of the viral DNA sequences to one another, to DNA in other cells and tissues, and to single genome sequences from plasma virus RNA was determined by phylogenetic analysis.

RESULTS: Analysis of PBMCs from two chronically HIV-infected patients (infected for about 3–5 and 15 years), with viral RNA levels of 12,000 and 1,800,000 copies/ml, revealed an infection rate of one HIV DNA copy per 14,018 and 717 cells, respectively. In both cases, the large majority (80–90%) of wells yielded only a single viral DNA molecule, and the number of wells yielding more than one copy of viral DNA was similar to that predicted by the Poisson distribution. The phylogenetic distribution of these intracellular DNA sequences was indistinguishable from single genome sequences derived from plasma RNA taken at the same time. In addition, the phylogenetic distribution of intracellular DNA sequences and plasma RNA sequences from the second patient remained unchanged 37 weeks after suppressive therapy was initiated (viral load <50 copies/ml).

CONCLUSIONS: Results from two chronically infected patients indicate that most infected mononuclear cells in blood contain only one copy of HIV DNA, implying a limited potential for recombination in this population. The genetic similarity between HIV populations in PBMCs and plasma implies ongoing exchange between these compartments, even following suppressive therapy.
Correlations between transmitted HIV-1 drug resistance mutations and the human leukocyte antigen alleles of therapy-naive HIV patients

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BACKGROUND: Antiretroviral therapy of human HIV-1-infected patients is successful, but limited due to the occurrence of mutations associated with drug resistances. However, in phases of treatment interruption and in therapy-naive patients, the immune system is the main selective force on the viral sequence, selecting mutations that allow the virus to escape recognition by cytotoxic T-cells (CTL). We hypothesize that these CTL-specific HIV-1 mutations can influence transmission and persistence of drug resistance mutations. Preliminary data indicated correlations between transmitted resistance mutations and human leukocyte antigen (HLA) types of RESINA patients.

METHODS: Using programs like SYFPEITHI and ELF, we analysed whether the detected drug resistance mutations are located in putative CTL epitopes. With the help of enzyme-linked immunospot technique (ELISPOT) assays and overlapping synthetic peptides, we investigated the recognition of these assumed epitopes using peptide stimulated cell lines derived from a cohort of HLA-typed HIV-1-infected patients. The stability of investigated resistance mutations is studied by a clinical follow-up of the patients.

RESULTS: Using Fisher’s exact probability test, several correlations between drug resistance mutations and HLA alleles were detected in patients taking part in the RESINA study. Correlations were found between HLA-A*01 and the drug resistance mutation L33F (protease [PR]), between HLA-A*03 and M46I (PR), between HLA-A*11 and V75I (reverse transcriptase [RT]), and between HLA-B*44 and L210F (RT) and K103R (RT). On the basis of these correlations, ELISPOT analyses were performed. Peptides were indeed recognized by the cells of patients carrying the HLA allele, which showed a statistical correlation to the respective drug resistance mutation, indicating that HLA-A*01, HLA-A*03 and HLA-A*11 epitopes are located in the designated regions of PR or RT. More detailed analyses were conducted for the L210F mutation, which is locate in an already described HLA-B*44 epitope. So far the significance of the L210F for the development of drug resistance is not known. However, ELISPOT assays using cells from HLA-B*44 positive RESINA patients revealed the L210F to be an immune escape mutation.

CONCLUSION: Our data suggest that the HLA system seems to play a role in the evolution of drug resistance mutations, even in treatment-naive patients.
ABSTRACT 70

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HIV-1 recombination in patients infected with multiple HIV-1 variants from the same donor

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BACKGROUND: Studies on recombination in vivo are fundamental to understanding the emergence and decay of drug-resistant variants in HIV infection. The contribution of recombination to HIV diversity is currently unknown and difficult to assess in chronic infection. We, therefore, measured the impact of recombination on HIV diversity and evolution in patients recently infected with multiple wild-type or drug-resistant HIV variants.

METHODS: Single genome sequences from gag-pro-pol and env were obtained from longitudinal plasma samples from 15 patients during early HIV infection. Four of these patients had virus populations in acute or early infection resulting from the transmission of multiple HIV variants, two with drug-resistant variants and two co-infected with wild-type variants. The coexistence of genetically distinct variants in acute/early infection provided an ideal opportunity to measure recombination frequency in vivo using SplitTree, Simplot and Highlighter. The contribution of recombination to diversity and evolution was determined by phylogenetic analyses and calculation of average pairwise difference and number of subpopulations.

RESULTS: Recombination between transmitted variants occurred more frequently in gag-pro-pol than in env and more frequently in acute infection than chronic infection. Recombinants in acute infection within the gag-pro-pol fragment were detected at an average frequency of 0.29. Occasionally, crossovers were detected more than once in the same sequence. The majority of these recombinants did not persist over time and the frequency of new recombinants in gag-pro-pol observed after the acute phase of infection decreased approximately twofold (to an average of 0.12). Recombination was found to be infrequent in env in both acute and chronic infection with an overall average frequency of only 0.05. Although most recombinants did not persist in longitudinal samples, one patient infected with two highly distinct variants showed the loss of one gag-pro-pol variant over time but persistence of both env variants, indicating that recombination between these two regions had contributed to viral evolution in this patient.

CONCLUSIONS: These findings show that recombination contributes substantially to HIV diversity in acute infection and facilitates rapid, independent evolution of different regions of the HIV genome.
ABSTRACT 71
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The selection, transmission and persistence of drug-resistant HIV-1 in infants prophylaxed with single-dose nevirapine varies by the timing of infection

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BACKGROUND: Infants infected with HIV-1 despite single-dose nevirapine (sdNVP) often harbour nevirapine (NVP)-resistant HIV (rNVP-HIV-1). We hypothesized that the timing of nevirapine selective pressure relative to the time HIV-1 first infects and expands within infants effects the selection and persistence of rNVP-HIV-1.

METHODS: We are conducted a prospective observational cohort study of 640 Mozambican infants following prophylaxis with sdNVP. The timing of HIV-1 infection was estimated using nested PCR for HIV-1 pol in dried blood spots collected at birth and every 2 weeks during the first 2 months of life, and then every 4–8 weeks until 12 months old. Concentrations of rNVP-HIV-1 were determined using quantitative PCR and quantitative oligonucleotide ligation assays for K103N, V106M, Y181C and G190A.

RESULTS: All 25 infants infected in utero (defined as HIV-1 detected at birth) had wild-type viruses at birth. Nineteen of these had established in utero infection with a high and stable HIV-1 DNA load at birth, and 16 (84.2%) of these had selection of rNVP-HIV-1. rNVP-HIV-1 concentrations peaked between 2–8 weeks of age, and then regressed. In contrast, infants with acute peripartum infection (n=28), with either a small or undetectable viral population at birth that increased before 8 weeks of age, had lower rates of rNVP-HIV-1 (n=11/28; 39.3%). Mutant viruses comprised 100% of the viral populations in seven of these 11 infants (63.6%), which persisted at high concentrations during the first year of life.

CONCLUSIONS: The selection, transmission and persistence of rNVP-HIV-1 in infants following sdNVP varies by the timing of their infection. Resistant viruses are generally selected when infection is well-established prior to birth. In contrast, acute HIV-1 infection during NVP pressure results in virus populations composed of mixed, wild-type, or mutant genotypes, corresponding possibly to virus acquisition slightly prior, immediately prior, or after nevirapine dosing. rNVP-HIV-1 appears to persist longer when transmitted or selected during acute infection, implying a greater risk for failure of NVP-based antiretroviral therapy. Our findings suggest that testing infants exposed to sdNVP for NVP resistance prior to antiretroviral therapy could identify infants that may benefit from NVP- versus non-NVP (that is, protease inhibitor)-containing antiretroviral therapy because of drug resistance.
ABSTRACT 72

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Intensification with efavirenz or lopinavir/ritonavir does not reduce residual HIV-1 viraemia in patients on standard antiretroviral therapy

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BACKGROUND: Currently recommended antiretroviral therapy suppresses plasma HIV-1 RNA to <50 copies/ml in most patients, but persistent, low-level viraemia can be detected with more sensitive assays. This residual viraemia may be produced by long-lived, chronically or latently infected cells, or by ongoing, complete cycles of virus replication. To differentiate between these sources, we conducted a trial of antiretroviral drug intensification in patients with HIV-1 RNA levels suppressed to <50 copies/ml plasma.

METHODS: Patients on combination antiretroviral therapy with non-nucleoside reverse transcriptase inhibitor (NNRTI)- or protease inhibitor (PI)-based regimens including two nucleoside reverse transcriptase inhibitors (NRTIs) and with stable HIV-1 RNA <50 copies/ml plasma for >1 year were screened for residual viraemia using a sensitive HIV-1 RNA assay (single copy assay [SCA]). Six patients with persistent viraemia (>1 copy HIV RNA/ml) were enrolled in a 30 day drug intensification study. Participants on PI-based regimens were intensified with efavirenz (n=4), and those on NNRTI-based regimens were intensified with lopinavir/ritonavir (n=2). Plasma for HIV-1 quantification was obtained weekly before, during and after the 30 day intensification period.

RESULTS: Enrolled patients (five male and one female) had HIV-1 infection for a mean of 9 years (range 4–16) and had received combination antiretroviral therapy for a mean of 4 years (range 1–10 years). The level of residual viraemia before intensification (mean=4.5 copies/ml plasma) was similar to that found in prior studies of patients on standard combination therapy. Drug intensification was well tolerated with no serious adverse events reported. Mean plasma HIV-1 RNA levels during intensification (5.3 copies/ml) and following intensification (5.2 copies/ml) were not significantly different from pre-intensification levels (P=0.76 and 0.79, respectively). Similarly, no significant decreases in HIV-1 RNA levels were observed during or after drug intensification in individual patient analyses. No significant changes in blood CD4+ T-cell counts were detected.

CONCLUSIONS: Antiretroviral intensification with lopinavir/ritonavir or efavirenz did not decrease the level of residual viraemia. This result is inconsistent with the idea that persistent viraemia results from ongoing, complete cycles of viral replication. New therapeutic approaches will be required to eliminate HIV-1 reservoirs.
ABSTRACT 73
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Treatment-emergent gag cleavage site mutations during virological failure of ritonavir-boosted protease inhibitors
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BACKGROUND: Gag cleavage site (CS) mutations can emerge under protease inhibitor (PI) pressure. However, longitudinal data are limited, because published evidence has been derived mostly from cross-sectional analyses.

METHODS: Full-length gag sequences, together with reverse transcriptase (1–335) and protease (1–99) sequences were obtained from: 26 drug-naive individuals newly diagnosed with subtype B HIV-1, including 13 with recent infection and none with transmitted drug resistance; and 28 persons from the MaxCmin1+2 and COLATE trials who were mainly infected with subtype B (26/28), experienced confirmed virological failure (VF) on a PI/r (indinavir, saquinavir or lopinavir), and had matched pre-trial baseline and VF plasma samples. RT and PR resistance mutations were assigned by the International AIDS Society-USA list (Aug–Sep 2007).

RESULTS: Among drug-naive persons, the prevalence of ≥1 CS mutation was 23/26 (88%) for p2/p7 (S373A, A374T, T375A, R380K), 8/26 (31%) for p17/p24 (V128A, Q130H, Q136H), 3/26 (11%) for p1/p6 (449Q, Q450P, R452I/C), 1/26 (4%) for p24/p2 (V362I) and 1/26 (4%) for p7/p1 (K436R). Among PI-experienced individuals, the prevalence was 26/28 (93%) for p2/p7, 6/28 (21%) for p17/p24, 7/28 (25%) for p1/p6, 1/28 (4%) for p24/p2 and 8/28 (29%) for p7/p1. The most prevalent CS mutations in PI-experienced persons were A431V (4/28, 14%) and I437V (2/28, 7%) in p7/p1 and P453L (4/28, 14%) in p1/p6, none of which occurred in treatment-naive persons. Of these, A431V was only found in combination with major PR mutations, whereas I437V occurred both with and without major PR mutations. Although p2/p7 showed similar variability in naive and experienced individuals, S373P and A374S were more common in PI-experienced individuals. Comparison of matched pre-trial baseline and VF plasma samples showed that 23/28 (82%) and 8/28 (28%) of patients had treatment-emergent PR and CS (I437V, T375A, R380K, L449F, N432I, K436R) mutations, respectively, including two (7%) with emerging CS mutations (I437V, L449F) without emerging PR mutations.

CONCLUSIONS: The prevalence and patterns of CS mutations differed in drug-naive and PI-experienced individuals. Mutations in p7/p1 and p1/p6 were more frequent in PI-experienced persons, with A431V, I437V and P453L found exclusively in treatment-experienced patients. Whether CS mutations independently contribute to risk of VF should be further explored.
ABSTRACT 74
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Gag–protease inter-relationships in drug resistance and viral fitness

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BACKGROUND: Resistance to protease inhibitors leads to reduced fitness and gag mutations can reconstitute fitness. However, commonly used replication capacity assay systems incorporate only partial gag sequence (p6, p1 and part of p7); therefore, the full effects of gag with its corresponding protease cannot be explored. The role of full-length gag and non-cleavage site mutations in replication competence remains poorly studied.

METHODS: We developed a single-cycle assay to include full-length sequence of gag–protease from plasma virus, and allowing incorporation of full-length gag, partial gag and protease sequences alone or together. Drug susceptibility and replication capacity have been determined.

RESULTS: We studied a plasma HIV-1 virus derived from a highly drug-experienced patient, incorporating Protease mutations L24I, L33F, 35QNins, M36L, M46I, I54V, K55R, R57K, I64V, L76V and V82A, from Gag an insertion (116TQins) in p17 Matrix, and cleavage site mutations at p2–p7 (five amino acid changes), p7–p1 and p1–p6. Expressing the mutant protease with wild-type gag leads to a 95% reduction in replication capacity. Replication was fully recovered by coexpressing protease with the corresponding patient-derived full-length gag gene. The mutant Gag–Protease virus demonstrated at least 40-fold resistance to a range of PIs (for example, fold-change 50% inhibitory concentration for indinavir: Gag 2.5×, Protease 81× and Gag–Protease 255×, and for amprenavir: Gag 1.9×, Protease 18×, Gag–Protease 40×). We then explored the regions of Gag responsible for the fitness compensation. Incorporation of p6, p1 and part of p7 from patient-derived Gag together with protease recovered replication capacity to around 70%. However, we also identified that, on its own, the 5’ region of gag (incorporating p17 and part of p24) is capable of restoring replication capacity of the mutant protease to >100%, in the absence of cleavage site mutations.

CONCLUSIONS: We demonstrate a complete restoration of replication capacity for a highly mutated Protease by full-length Gag and also a poorly studied region of Gag containing no cleavage site mutations; this suggests a complex inter-relationship between Gag and Protease, and also within Gag, to yield replication competent virus with high-level resistance. We encourage the use of full-length Gag in future studies of protease resistance.
ABSTRACT 75

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Spontaneous mutations in HIV-1 V3 envelope of slow and fast progressors are capable to influence the binding to CCR5 and CXCR4 coreceptors

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BACKGROUND: In this study, we analysed the differences between the V3 region in wild-type virus and isolates from patients at different degrees of HIV disease. Moreover, we aimed at identifying the specific mutations that are able to structurally change V3, and to potentially modify the interaction with the coreceptors and to lead to a different clinical picture.

METHODS: We amplified and sequenced the env gene from HIV RNA of naive patients with viral load >40,000 copies (cp)/ml infected <2 weeks (fast progressors), and from proviral DNA of 14 multifailed patients with viral load ≤800 cp/ml for at least 6 months (slow progressors). By computational models, we analysed the structural, chemophysical and binding features of V3. We performed MD trajectory (Amber 9.0, Pittsburg, PA, USA) in order to characterize the docking. We also investigated V3 from 72 clones derived from fast progressors isolates.

RESULTS: By computational analysis, we detected that V3 from slow progressor showed chemical affinity for CCR5 even if their electrostatic potential seemed to decrease the binding to the coreceptor. However, fast progressors presented both chemical and electrostatic affinities towards CXCR4. Mutated V3 determined a different docking intermediate, but there was no change in the final conformation between the two progressors and the wild-type.

We also identified mutations on GPG V3 crown region on the clonal sequences of fast progressors. Such changes led to a modification in chemophysical properties regarding the interaction between env and coreceptors. Fifty-three percent of clones showed a GRG sequence and a 15% GQG. A clone presented the GPE motif, which was able to completely destabilize the V3 structure. Among these clones, 10% showed an aspartic acid at position 27, which was not evidenced in the bulk sequences and strongly modified the chemophysical properties compared with the others.

CONCLUSIONS: The different mutation pattern between V3 of the two progressors influenced the binding affinity towards the coreceptors. The presence of an aspartic residue instead of a glycine on the tip was able to change the stability and modify the chemophysical properties when present at position 27. Our data will be useful to study the mechanism of interaction between gp120 and coreceptors.
ABSTRACT 76

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Fitness progression and phenotypic susceptibility to raltegravir of HIV-1 integrase are not restricted in long-term HAART-treated patients

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BACKGROUND: Protease (PR), reverse transcriptase (RT) and integrase (IN) share the same precursor polyprotein (Pr160Gag–Pol), and several studies suggest functional interactions between IN and RT. Moreover, natural polymorphisms within IN have been associated with both RT resistance mutations in highly active antiretroviral therapy (HAART)-treated patients and resistance to IN inhibitors. In this study, we aim to elucidate whether long-term HAART targeting PR and RT may preclude IN fitness progression and raltegravir susceptibility.

METHODS: HIV-1 IN population-based sequences, from 45 heavily antiretroviral-treated patients with longitudinal samples separated for a median of 10 years, were obtained to estimate the rate of nucleotide substitutions. IN-recombinant viruses were generated from five patients whose HIV-1 IN accumulated between three and 14 amino acid substitutions over the study period. Changes in viral replication capacity were assayed by competition of intrapatient IN-recombinant virus in the absence of drugs. Phenotypic susceptibility to raltegravir was performed in TZM-bl cells. Additionally, the study included IN-recombinant virus generated from a patient failing a raltegravir-containing regimen and harbouring resistance mutations G140S/Q148H and from the site-directed mutant T66I, resistant to IN inhibitors other than raltegravir.

RESULTS: The rate of nucleotide substitutions within IN was 0.06% per year. Competition experiments showed that IN-recombinant viruses corresponding to IN samples after 10 years of HAART had similar or improved replication capacity than those corresponding to IN-recombinant viruses from baseline samples. Moreover, neither early nor late IN-recombinant viruses showed increase in phenotypic susceptibility to raltegravir. By contrast, recombinant IN from the raltegravir-experienced patient with mutations G140S/Q148H showed a 23-fold increase in drug susceptibility entailing a replication capacity cost. Finally, the site-directed mutant T66I was susceptible to raltegravir, but less replicative in absence of drug than the wild-type virus.

CONCLUSIONS: Long-term drug pressure with PR and RT inhibitors is not enough to restrict fitness progression of IN. Additionally, HIV-1 IN from longitudinal samples obtained from patients treated with IN inhibitor-sparing regimens showed no evidence of genotypic and phenotypic resistance to raltegravir. These data suggest that current antiretroviral regimens do not preclude either IN fitness or efficacy of raltegravir.
ABSTRACT 77

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Relative fitness of raltegravir resistance mutants in HIV-1 integrase
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BACKGROUND: Resistance to raltegravir is associated with two genetic pathways defined by N155H or Q148H and R or K mutations. Additional mutations confer higher levels of resistance (N155 plus L74M, E92Q, G163R or Q148H/R/K plus E138K, G140S/A). Preliminary data suggest that mutations conferring INSTI resistance reduce viral replication capacity, but data on the relative fitness of raltegravir-resistant viruses are limited. To understand the impact of the different raltegravir resistance pathways on viral fitness, we tested recombinant viruses carrying different combinations of raltegravir resistance mutations in growth competition assays.

METHODS: Mutations at IN codons 74, 92, 138, 140, 148, 155 and/or 163 were introduced by site-directed mutagenesis of cloned wild-type (WT) NL4-3 IN. Infectious recombinant viruses carrying the relevant IN mutations and sequence tags in nef were generated by co-transfection in 293T cells. Infectivity titres were measured by the MAGI assay. Replication capacity of the resulting viruses was tested by measuring p24 antigen production in U87-X4 and MT-2 cells in the absence of drug. Relative fitness was assessed by growth competition assay using the recombinant marker virus assay.

RESULTS: HIV-1 recombinants carrying raltegravir resistance mutations replicated slower than WT. For the 148 pathway mutants, the rank order of replication was WT > 140S/148H = 138K/148H = 148R > 148K; the 148H mutant showed minimal replication. For mutants involving the 155 pathway, the rank order was 163R/155H > 92Q/155H = 155H > 74M/155H. Pairwise growth competition assays gave similar results: 140S/148H was more fit (> ) than 148H and 138K/148H > 148H. Similarly, 163R/155H was more fit than 155H and 92Q/155H > 155H. By contrast, 74M/155H was less fit than 155H.

CONCLUSIONS: Mutations Q148H/R/K or N155H impaired viral fitness compared with WT. Although the 148H mutation substantially impaired replication when present by itself, fitness was partially restored by the presence of additional INSTI resistance mutations at positions E138K and G140S. E92Q and G163R, but not L74M, restored fitness of the N155H mutants. It will be interesting to compare the effects of L74M on strand transfer activity and the 3' processing properties of IN with those of other mutations in this region.
Tracking HIV-1 integrase polymorphisms from the pre-antiretroviral therapy era up to the introduction of integrase inhibitors

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BACKGROUND: More than 40 HIV integrase (IN) gene mutations are associated with resistance to integrase inhibitors (INI). Identification of baseline mutations and defining IN polymorphisms in treatment-naive patients will assist in understanding and/or predicting INI drug resistance pathways. Genetic and functional proximity of replication enzymes IN, reverse transcriptase (RT) and protease (PRO) may result in non-INI antiretroviral therapy (ART) leading to changes in the IN gene. To explore these issues we analysed the IN gene of subtype B virus obtained during the pre-ART and pre-INI eras.

METHODS: Baseline IN nucleotide and amino acid polymorphisms were analysed in 71 clinical specimens collected from patients during 1982–1985, before the introduction of ART. IN sequences were compared with those obtained from 127 diagnostic specimens, collected from ART-naive patients, after the introduction of ART but before INI were available (2001–2003). Both nucleotide and amino acid variation was examined to evaluate evolutionary trends using MEGA 4.0.

RESULTS: The nucleotide polymorphism rates in pre-ART and pre-INI groups were 28% and 47% (P<0.05), whereas amino acid polymorphism rates (288aa) were 22% and 47%, respectively (P<0.05). Q148H/K/R and N155H were not detected in either group; however, we identified five other INI-resistance-associated mutations in pre-ART samples (V151I: 5.6%, M154I: 2.8%, K156N: 4.2%, T206S: 1.4% and S230N: 2.8%). In contrast, many more mutations were detected in pre-INI group (L74M/I: 3.2%, T97A: 0.8%, T112I: 6.3%, E138K: 1.6%, V151I: 0.8%, S153A: 0.8%, M154I: 9.4%, K156N: 3.1%, V163I: 0.8%, I203M: 4.7%, S230N: 9.4% and V249I: 0.8%). The V72I and V201I mutations were identified as common polymorphisms in both groups with their frequencies increasing over time (V72I pre-ART versus pre-INI: 52.1% and 78.0%; V201I pre-ART versus pre-INI: 16.9% and 37.8%). Phylogenetic analysis revealed significant genetic distance differences between the two groups, driven predominantly by synonymous mutations.

CONCLUSIONS: The HIV-1 IN gene is intrinsically polymorphic with some INI-resistance-associated mutations present in samples collected in the pre-ART era. Increasing levels of nucleotide and amino acid sequence polymorphisms over time may be due to the influence of ART. Further characterization of these observations is ongoing.
ABSTRACT 79

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Sustaining lamivudine/emtricitabine, abacavir, zidovudine or atazanavir from a previous treatment shows a clinical benefit

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BACKGROUND: Selection of the most appropriate highly active antiretroviral therapy for HIV treatment remains a challenging task. When a therapy switch is forthcoming, there is the question to maintain certain drugs or switch to alternatives. Remaining activity of drugs despite resistance and/or clinical benefits to keep certain drugs has not been well investigated. We addressed the question whether maintaining one of the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs) in a new regimen has a clinical benefit or not.

METHODS: In this study a subset of 20,249 therapy histories from 8,223 patients of the EuResist database were analysed. Therapies were classified as a ‘success’ if the follow-up viral load (VL) measure obtained at 8 (4–12) weeks of treatment was ≤500 copies/ml or alternatively if there was a drop by 2 logs compared with a baseline VL measure obtained at most 90 days before treatment change. If none of these events were observed the therapy was classified as a ‘failure’. The success rate of treatments sustaining a drug from the direct previous regimen was compared with the success rate of treatments that discontinued that drug with the same number of previous treatments. Significance of the difference was determined using the $\chi^2$ test.

RESULTS: The rate of successful current therapies was lower when the following drugs remained in the current regimen: NNRTIs: efavirenz and nevirapine; NRTIs: didanosine and stavudine; and PIs: indinavir and nelfinavir. It was higher when lamivudine/emtricitabine, abacavir, zidovudine or atazanavir were kept in the therapy. For tenofovir, fosamprenavir, lopinavir and saquinavir no statistical significance was reached. In general, the success rate drops with an increasing number of past treatments, more for PIs than for NRTIs. Nevertheless, the difference in success rate between sustaining a certain drug or not remains almost constant with respect to the number of treatments previously received.

DISCUSSION: Significant clinical advantage of drug maintenance was seen for lamivudine/emtricitabine, abacavir, zidovudine or atazanavir. It is unclear whether this is a mechanistic effect or a consequence of biased clinical practice.
ABSTRACT 80
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More frequent detection of lopinavir resistance by single genome sequencing at virological failure of lopinavir/ritonavir maintenance therapy in the OK04 study

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BACKGROUND: Maintenance therapy with lopinavir/ritonavir (LPV/RTV) alone provides a simple twice-daily regimen that preserves nucleoside reverse transcriptase inhibitors (NRTIs) for future use. A major concern with this strategy, however, has been the potential for selection of protease inhibitor (PI) resistance. Standard population genotyping has not frequently detected PI resistance with failure of LPV/RTV maintenance therapy, but more sensitive detection methods have not been used. We, therefore, performed single genome sequencing (SGS) to detect the emergence of low frequency LPV-resistant variants during virological rebound in the OK04 Study (Pulido F., et al. AIDS 2008).

METHODS: OK04 was a randomized, open-label, 144 week trial comparing simplified maintenance therapy with LPV/RTV alone (n=103) versus continued triple therapy with two NRTIs + LPV/RTV (n=102). By study week 48, 15 individuals qualified for standard genotype testing (ViroSeq) by having HIV-1 RNA rebound >500 copies/ml (11 on LPV/RTV alone versus four on triple therapy). SGS of pro was performed on the initial plasma sample with HIV-1 RNA >500 copies/ml from these individuals. We sought to obtain ~29 SGS sequences/sample to have 95% power to detect a resistant variant comprising 10% of the virus population.

RESULTS: HIV-1 RNA at virological rebound ranged from 524–72,300 copies/ml. Standard population genotyping identified major LPV resistance mutations in two individuals from the LPV/RTV-alone arm (M46I and M46I + V82A) and one individual from the triple-therapy arm (V82A). Single genome sequences (321 total) were obtained from 13/15 samples tested (3–31 sequences/sample). Major LPV resistance mutations were found only by SGS in two individuals from the LPV/RTV-alone arm (M46I in 1/30 and V82A in 2/25 sequences) and in 1 individual from the triple-therapy arm (M46I in 1/30 sequences). Minor PI resistance mutations (F53L, V77I and V82I) were found by SGS in three other individuals (two in the LPV/RTV-alone arm).

CONCLUSIONS: Viral variants encoding major LPV resistance mutations were detected more frequently at virological rebound on LPV/RTV therapy by SGS than standard population genotyping. The clinical significance of such low frequency variants warrants further study.
Diversity of the persistent reservoir of HIV-1 associated with low-level viraemia during combined antiretroviral therapy

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BACKGROUND: Most patients receiving a fully suppressive combined antiretroviral therapy (cART) regimen experience transient low-level viraemia (LLV). However, the origin and clinical consequences of LLV remain to be determined. In this study, we examined the complexity of the persistent viral reservoir of HIV-1 and its association with LLV in patients on successful cART.

METHODS: Fifty-seven individuals were randomly chosen from a well-characterized cohort of HIV-1-infected individuals undergoing treatment with cART. All patients were fully suppressed (HIV RNA <20 copies/ml) at baseline and followed for 24 months. Three viraemia groups, based on longitudinal plasma HIV-1 RNA values, were defined as follows: group 1 had viral load persistently <20 copies/ml ($n=18$), group 2 had one or more samples with viral loads ≥20, but <200 copies/ml ($n=29$) and group 3 had one or more samples with viral loads ≥200 ($n=10$). Bulk sequences spanning the C2–V3–C3 region of env were obtained from proviral HIV-1 DNA at baseline and at 6, 12, 18 and 24 months ($n=57$). About 10 additional clonal sequences were obtained per time point in seven patients. Viral diversity was estimated using $p$-distances (clonal dataset) or the number of ambiguity sites per sequence (bulk dataset). Student’s $t$-test was used to compare differences between groups.

RESULTS: In the bulk dataset median baseline diversity was 0.019 (0–0.078), 0.038 (0–0.107) and 0.054 (0.020–0.077) in group 1, 2 and 3, respectively. Viral diversity at baseline was compared among groups and was significantly different between groups 1 and 3 ($P=0.004$) and groups 1+2 and 3 ($P=0.036$). In the clonal data set ($n=7$) median baseline diversity was 0.018 (0.012–0.025) in group 1+2 ($n=5$) and 0.057 (0.053–0.062) in group 3 ($n=2$). Baseline diversity of group 1+2 compared with group 3 was significantly different ($P=0.0003$). Viral diversity in the three groups remained stable with time in both data sets.

CONCLUSIONS: The diversity of the persistent reservoir of HIV-1 was found to be significantly higher in patients experiencing LLV >200 copies/ml than in patients with no LLV or LLV between 20–200 copies/ml. This correlation suggests a link between population diversity and number of potentially virus-producing cells on therapy. Factors driving the differences in diversity will be discussed.

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Recognition of protease by HIV-1-specific cytotoxic T-lymphocyte is influenced by protease inhibitor resistance mutations

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BACKGROUND: Taking the various pathways that have been observed during the development of drug resistance against protease inhibitors into account, it is likely that immunological host factors like the human leukocyte antigen (HLA) system interact with the emergence of resistance mutations.

METHODS: HIV-1 protease sequences and protease-specific cytotoxic T-lymphocyte (CTL) responses were analysed in a cohort of HIV-1-infected, HLA-typed patients in order to determine the influence of HIV-1-specific CTL on the development of drug resistance mutations in HIV-1 protease. Over 80% of the patients who were included in these analyses were protease inhibitor treatment experienced. Univariate statistical analyses were performed to identify correlations between amino acid substitutions and HLA alleles. T-cell activity was measured by ELISPOT and Cr⁵¹ release assays using peptide-stimulated T-cell lines.

RESULTS: Several major (L33F and M46I) and minor (L10I, E35D, I54V, I62V, A71V, and I93L) drug resistance mutations showed associations to HLA class-I alleles. Based on these associations we defined several new CTL epitopes that were restricted by the associated HLA class-I alleles. In most patients, these drug resistance mutations reduced CTL recognition, indicating that resistance-associated mutations often act as CTL escape mutations in patients with the corresponding HLA class-I alleles. Interestingly, some patients were able to generate specific CTL against certain drug resistance mutations, such as the A71V mutation. This suggests that CTL can influence the development of drug resistance mutations.

CONCLUSION: Prospective studies will be needed to explore whether CTL targeting drug resistance mutations in the HIV-1 protease influence the emergence of resistance to protease inhibitors. The interaction between the CTL response and the development of drug resistance mutations could have important clinical implications for the sequencing of protease inhibitors in antiretroviral therapy, the understanding of drug resistance pathways and the design of therapeutic vaccines.
ABSTRACT 83
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Interactions between replication capacity at the end of structured treatment interruption (STI) and pre-STI CD4+ count as predictors of clinical outcome after STI

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BACKGROUND: The CPCRA064 study examined the effects of structured treatment interruption (STI) of up to 4 months followed by salvage treatment in patients failing therapy with multidrug-resistant HIV. Here, we describe interactions between replication capacity (RC) at the end of 4 month of structured treatment interruption (STI; RCend) and the differential effects on on-therapy immunologic parameters based on baseline CD4 stratum.

METHODS: The dataset included 69 patients with RC, CD4+ count, HIV-1 RNA (VL) and protease-RT genotype of plasma samples collected at baseline (BL, month 0) and after 2 and 4 months of STI. Phenotypic susceptibility was tested on 17 drugs (PhenoSense®). Patients were grouped by CD4 BL strata: low CD4 BL (<100, median=25, n=26), med CD4 BL (100–300, median=199, n=23) and high CD4BL (>300, median=384.5, n=20). Association of RCBL, RCend and changes in RC during STI (ΔRC) with CD4 BL, changes in CD4+ after STI (ΔCD4, at 24 months) and time-to-AIDS events or death were determined using Spearman correlation and univariate Cox PH models.

RESULTS: In this population, median CD4 BL was 173 cells/mm3, median RCBL was 38% (interquartile range 19–66) and VL BL was 4.91 (4.32–5.37) log10 copies/ml. After STI, median CD4end was 85 cells/mm3, median RCend was 97% (70–121) and VL end was 5.36 (5.14–5.73) log10 copies/ml. ΔRC and RCend were associated with time-to-AIDS events or death (P=0.008 and P=0.006, respectively), whereas RCBL was not (P=0.83). Other significant predictors included CD4BL, CD4end, interaction CD4BL×RCend, interaction CD4BL×ΔRC. Correlation between RCend and ΔCD4 was negative for med CD4BL (r=-0.70, P=0.0016) and positive for high CD4BL (r=0.35, P=0.19); there was no such correlation in low CD4BL (r=0.24, P=0.45). Higher median ΔCD4 recovery was found for med CD4BL with lower RCend (RCend<100) and for high CD4BL with higher RCend (RCend>100; ΔCD4=269 and 268, respectively). These two groups combined had an average of 5.5 fully susceptible drugs before STI versus 3.5 for other samples. Higher RCend was associated with reversion of 184I/V and 210W to wild-type during STI in both med CD4BL and high CD4BL.

CONCLUSION: These data suggest that multiple subpopulations of HIV-infected patients with different immunologic outcomes after STI can be defined by their baseline CD4 count and the RC of their dominant circulating virus. The impact of viral RC on immunologic recovery appears to be different depending on the immune recovery potential of the host.
ABSTRACT 84
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Modelling the HIV fitness landscape under indinavir treatment pressure using observed evolution in longitudinal sequence data

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BACKGROUND: Our method to model a fitness landscape under protease inhibitor (PI)-selective pressure using cross-sectional data requires many sequences with the PI being used as first in its class. However, for new drugs mainly used in salvage treatment, such data are not available. Therefore, we wanted to provide a proof of principle that fitness landscapes could be modelled using longitudinal data.

METHODS: We learned qualitative epistatic interactions between indinavir (IDV)-selected mutations and background polymorphisms by Bayesian Network learning applied to cross-sectional sequences where IDV was the only PI experienced (PT, n=1,187). A quantitative function F_{cross} to estimate fitness under IDV treatment, was modelled by comparing PT with PI-naive sequences (PN, n=9,116). Similarly three functions were modelled using IDV longitudinal sequences (PL, n=218) while learning epistatic interactions from the same PT (F_{long1}), from cross-sectional PI-experienced sequences (PP, n=3,404; F_{long2}), and from PP but excluding IDV experience (PI, n=1,750; F_{long3}). An independent data set was used for evaluation (PE, n=1,038).

RESULTS: Overall, the sequences belonged to subtypes B (63%), G (12%), C (2.5%) and other (22.5%), but the distribution was significantly different among the populations (subtype B prevalence range: 62–78%). Fitness under the different models was estimated for all PE sequences. There was a good correlation for all pairwise comparisons, which improved significantly when correcting for subtype: F_{cross} versus F_{long1} R^2=0.74, F_{cross} versus F_{long2} R^2=0.75, F_{cross} versus F_{long3} R^2=0.64, F_{long1} versus F_{long3} R^2=0.77 and F_{long1} versus F_{long2} R^2=0.88.

CONCLUSION: This study suggests that drug fitness landscapes can be modelled from longitudinal data, even when epistatic interactions are learned from selective pressure with other drugs acting on the same HIV protein, thus enabling their use for drugs with limited sequence information and mainly used in salvage therapy. Even though the landscapes are subtype-neutral with respect to resistance evolution, they remain fitness landscapes for drug selective pressure and can not capture fitness differences between subtypes. The fact that the correlation was worse when excluding IDV-learned epistatic interactions may indicate that IDV causes protease changes that are not captured by any other PI selective pressure.
ABSTRACT 85

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HIV Nef in long-term non-progressors are defective in the serine/threonine kinase binding site (RR), the glutamic acid cluster and the protease cleavage site (CAW\LEA) as well as in motifs important for MHC class I down-regulation and retention

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BACKGROUND: Infection with HIV-encoding defective nef variants may contribute to a relatively gentle disease progression in long-term non-progressors (LTNP). We have examined the sequence of nef alleles from two independent cohorts: (i) 69 individuals infected with HIV-1 and classified as LTNP (at least 5 years below a viral load of 1,000 copies/ml without any antiretroviral treatment); and (ii) a unique cohort of 24 individuals infected clonally in their childhood (1989) during vaccination due to a lack of sterile working habits.

METHODS: We amplified isolates from 69 LTNP and were able to analyse sequences of 26 patients (set as 100%). We also amplified isolates from 24 individuals and were able to analyse sequences of 15 patients (set as 100%).

RESULTS: We found in 73% of the LTNPs Nef alleles containing a mutation of the RR motif, which is located between amino acid position (aa) 100 and 110. This motif is important not only for binding to a serine/threonine kinase, but also for CD4 down-regulation resulting in an enhanced viral infectivity in vitro and pathogenesis in vivo. In 46% of these patients’ Nef alleles we detected a modified glutamic acid cluster (EEEE aa62–65), which is important for major histocompatibility complex (MHC) I retention within the cytoplasm and PACS-1 binding. In addition, we observed in 35% of the LTNP a change in the protease cleavage site located between aa 54 and 59, which is also important for viral replication and CD4 down-regulation in vitro. In the clonally infected patients, we found that 87% of the Nef alleles contain a mutation of the M20 motif. This motif is important for MHC I down-regulation and the dysfunction could result in an increase in HIV peptide presentation by MHC-I molecules. In 100% of the patients, Nef shows a modification of the glutamic acid cluster (EEEE aa62–65), which is important for MHC-I retention within the cytoplasm and PACS-1 binding.

CONCLUSION: We conclude that both cohorts of LTNP show mutations in motifs that were described as important for viral infectivity. It could be that those mutations contribute to a low viral load and slow disease progression.
Dynamics of emergence of CXCR4-using HIV in clinical samples as detected by Trofile and MT-2 assays

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BACKGROUND: The recent availability of CCR5 antagonists as anti-HIV therapeutics has highlighted the need to identify CXCR4-using (X4) variants in patient samples when considering use of this new drug class. Here, we evaluated the performance of the Trofile and MT-2 assays to detect X4 variants directly in patients’ peripheral blood mononuclear cells (PBMC) and plasma, and compared results with the outcome of Trofile and MT-2 assays on molecular and biological clones isolated from the same samples.

METHODS: Samples were derived from participants of the Amsterdam Cohort Studies on HIV/AIDS who underwent consecutive tropism testing by the Trofile and MT-2 assays at approximately 3 monthly intervals. We evaluated multiple time points per patient that were temporally related to the first detection of X4 variants by the MT-2 assay. Clonal composition of patient PBMC samples was determined using limiting dilution biological cloning. In addition, multiple Env clones were isolated from longitudinal plasma samples to allow tropism testing and sequence analysis.

RESULTS: We previously reported that in HIV-1 isolates the tropism profiles by the MT-2 and Trofile assays demonstrate high concordance (93–95%). In patients with a known moment of first X4 detection by MT-2 assay, a switch from R5 to dual/mixed tropism (DM) was detected at or before this moment in 5/10 patients by standard Trofile and in 9/10 patients by enhanced Trofile. Limiting dilution biological cloning using up to 4 million patient PBMC revealed low-level presence of X4 variants 3 months before their detection in MT-2 assay in 2/13 patients. Clonal analysis of both plasma and PBMC samples showed fluctuating levels of emerging CXCR4-using clones, all of which also used CCR5. The relative ability to use CXCR4 varied between efficient CXCR4 use (designated dual-X clones, detected in all patients) and poor CXCR4 use (designated dual-R clones, detected prior to or concurrent with detection of dual-X clones in 7/9 patients).

CONCLUSIONS: The detection of X4 variants in patients’ plasma (enhanced Trofile) or PBMC (MT-2 assay) is highly concordant. Clonal analysis revealed distinct phenotypes of dual-tropic variants possibly representing Env with different efficiencies of CXCR4 usage during evolution from the R5 to X4 phenotype.
SESSION 4

New resistance technologies and interpretations
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Dynamic HIV-1 escape from vicriviroc therapy in vivo

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BACKGROUND: Novel high-throughput sequencing platforms provide an alternative approach for detecting minor HIV-1 variants. We applied this technology to sequence the gp120 V3 loop-coding region of env in serial plasma samples from four chronically HIV-infected individuals who failed vicriviroc (VCV) therapy.

METHODS: We selected individuals from ACTG 5211, a Phase IIb clinical trial of VCV, who experienced virological failure (VF) with a change in coreceptor usage as determined by a commercial phenotypic assay (Trofile, Monogram Biosciences). Three individuals met the criteria: individuals 18 and 19 had an early change (week 2 after starting VCV) in coreceptor usage, whereas individual 07 developed high-level VCV resistance and late emergence of a minor X4 population. A fourth individual, 47, had VF with no detectable change in coreceptor usage or VCV susceptibility and served as a control. All individuals were receiving VCV at the time of VF. Three time points were analysed for each individual: study entry (week 0), an intermediate time point on study drug and VF. HIV-1 RNA was extracted from plasma and individual-specific primer sets were used to reverse transcribe and amplify plasma V3 loop-coding regions of env. V3 amplicons were then submitted in a blinded fashion for deep sequencing and custom analysis.

RESULTS: Between 25,000–140,000 single genome sequences were obtained per individual per time point.

CONCLUSIONS: V3 loop forms associated with VCV escape, either through CXCR4 use or the emergence of high-level VCV resistance, exist prior to therapy. Minor variants present at <1% of the starting population emerge rapidly in response to VCV treatment and contribute to virological failure.
ABSTRACT 88

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Response to vicriviroc in HIV-infected, treatment-experienced individuals using an enhanced version of the Trofile HIV co-receptor tropism assay [Trofile (ES)]: reanalysis of ACTG 5211 results

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BACKGROUND: Vicriviroc (VCV) demonstrated potent virological suppression in treatment-experienced patients with R5 virus at study screen by the standard Trofile assay (Monogram Biosciences, San Francisco, CA, USA). An enhanced sensitivity Trofile assay (Trofile [ES]), with improved ability to detect CXCR4-using minor variants, could optimize selection of patients who may benefit from CCR5 antagonists.

METHODS: We used Trofile (ES) to determine coreceptor usage at study screen and entry for the 118 individuals on ACTG 5211. We examined virological and immunologic responses by randomized treatment arm (VCV at 5, 10, 15 mg daily or placebo plus optimized background antiretrovirals at day 14) according to tropism results by Trofile (ES). All analyses were intent-to-treat.

RESULTS: Using Trofile (ES), 89 individuals had R5 virus at screening, 25 individuals with R5 virus by the standard assay were found to have dual/mixed (DM) virus and samples from four individuals were not available. Among VCV recipients, respective mean changes in HIV-1 RNA (log10 copies/ml) at 14 days and 24 weeks were improved for individuals with R5 virus by Trofile (ES): -1.10 and -1.85 (5 mg), -1.31 and -2.09 (10 mg), and -0.93 and -1.75 (15 mg), compared with the original results for individuals with R5 virus by standard Trofile: -0.87 and -1.51 (5 mg), -1.15 and -1.86 (10 mg), and -0.92 and -1.68 (15 mg). No difference was found among placebo recipients. Amongst all VCV recipients and according to classification by Trofile (ES), greater reductions in log10 HIV-1 RNA were observed in the 64 individuals with R5 virus at both screening and entry (group 1) compared with the five individuals with R5 virus at screening, but DM virus at study entry (group 2), and the 15 individuals with DM virus at screening (group 3): at day 14, -1.15 versus -0.66 versus -0.09 and at week 24, -1.95 versus -1.20 versus -0.57 (P<0.05 comparing groups 1 and 2, and 2 and 3; P<0.001 comparing groups 1 and 3 for both endpoints).

CONCLUSIONS: Reanalysis of key study endpoints based on Trofile (ES) demonstrates improved antiretroviral activity of VCV and indicates that Trofile (ES) is an improved screening tool for determining patient eligibility for CCR5 antagonist therapy.
ABSTRACT 89

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Improved detection of X4 virus by V3 genotyping: application to plasma RNA and proviral DNA

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BACKGROUND: It would be advantageous to be able to screen patients for HIV tropism by genotypic methods, but previous analyses showed that standard genotype methods detect as few as 30–50% of X4 or dual/mixed virus in clinical samples. In addition, it would be useful to be able to test for tropism when plasma viral load (pVL) is undetectable.

METHODS: Triplicate independent V3 genotype determinations were performed using custom alignment and base-calling software (RE-call) with no manual intervention in 63 samples, in which tropism phenotype was previously determined using the Monogram Trofile assay. HIV tropism was predicted from V3 genotype using protein-specific scoring matrix (PSSM) and/or geno2pheno. In addition, proviral HIV DNA V3 sequence was assessed in 26 R5/X4 and 14 R5 individuals after pVL became undetectable as a result of standard highly active antiretroviral therapy.

RESULTS: This approach led to increased sensitivity and specificity compared with previous population-based V3 approaches. Using a combination of the PSSM and geno2pheno methods, sensitivity and specificity increased to 75.8% and 91.1%, respectively. Furthermore, the Monogram X4 luciferase readout in the phenotypic assay was correlated to the ‘scores’ provided by each genotypic predictor (for example, PSSM, R²=0.54 P<0.001), suggesting that V3 sequence variation alone explains much of the variation in the X4 phenotype parameter. Of samples with undetectable pVL, the sensitivity and specificity using proviral DNA V3 sequence for predicting pre-therapy Trofile results was 76% and 71% for PSSM or 77% and 93% for geno2pheno, respectively. Further assessment of these methodologies using a blinded analysis of an independent HIV RNA dataset (n>300) and also using 454 sequencing is underway.

CONCLUSIONS: Fully automated analyses of multiple V3 sequence assessments detect a much greater proportion of X4 samples than previously possible, regardless of the genotype algorithm used. Using this approach, most patients with X4 virus can be screened out, at a cost of approximately $300–400 per assay. The ability to measure tropism from proviral DNA suggests the possibility of screening for those with suppressed pVL who may wish to switch to CCR5 antagonists for tolerability or other reasons.
ABSTRACT 90
*Antiviral Therapy* 2008; 13 Suppl 3:A100

Improved genotypic prediction of HIV-1 coreceptor usage by incorporating V2 loop sequence variation

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BACKGROUND: The use of the CCR5 or CXCR4 coreceptors is mainly determined by the third hypervariable (V3) loop of gp120. Based on this observation, current methods predict coreceptor usage from the sequence information of V3 alone. However, mutations outside V3, most notably in the bridging sheet, have been repeatedly reported to influence coreceptor usage. The aim of this study was to assess the extent the incorporation of V2 into prediction methods affects their performance, both for clonal isolates and for clinical samples.

METHODS: Nine hundred and sixteen sequences containing V2 and V3 with experimentally determined phenotype were obtained from the Los Alamos Sequence Database and aligned. Statistical significance was determined using Fisher’s exact, Student’s *t*, and Wilcoxon–Mann–Whitney test. Prediction models were trained with support vector machines and evaluated in 10×10-fold cross-validation on clonal data. Epidemiological bias was reduced by allowing only one R5 and one X4 virus per patient in each run. For independent validation, models trained on the whole clonal dataset were validated on 268 therapy-naive, bulk-sequenced isolates and compared with Monogram Trofile results.

RESULTS: Mutations at seven positions within V2 were significantly associated with CXCR4 usage. Cross-validation runs on the clonal dataset revealed that the area under the receiver operating characteristic curve (AUC) was significantly higher (*P*<0.0019) when using V2 and V3 than classifiers trained on V3 alone (V3: 0.914, V2V3: 0.933). Several features, including the charge and the number of positively charged residues of V2 and V3, differed significantly (*P*<0.05) between R5 and X4 viruses. These features, together with sequence information from all clonal samples, were included into prediction engines trained for the validation of clinical samples. Combining V2 with V3, the performance was much higher than using V3 only (V3 AUC: 0.778, V2V3 AUC: 0.841). At a specificity of 90%, the sensitivity increased from 54.2% to 62.8%.

CONCLUSIONS: Mutations in V2 as well as additional features have been shown to be associated with CXCR4 usage and confirm the influence of V2 for coreceptor usage in a large dataset of genotype–phenotype pairs. Incorporation of this information into genotypic predictors leads to modest but significant improvements in prediction quality on an independent clinical dataset.
ABSTRACT 91

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Inferring viral tropism from genotype with massively parallel sequencing: qualitative and quantitative analysis

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BACKGROUND: Genotypic prediction of HIV-1 tropism is an inexpensive and fast alternative to phenotypic approaches. However, using standard sequencing approaches, these advantages are accompanied by lower sensitivities of X4 detection, especially in clinical isolates. As prediction from genotype performs well on clonal data, it is commonly thought that ‘false’ predictions are mainly attributed to the lack of detection of minor variants. In comparison to standard bulk-sequencing, the 454-technology detects single clones, thereby being much more sensitive. The aim of this study was to determine if this method can be successfully combined with bioinformatic approaches to generate a qualitative and quantitative prediction of coreceptor usage from V3 genotype.

METHODS: Plasma samples from 55 antiretroviral-treated patients with tropism documented by the Monogram Trofile assay were sequenced with standard population-based approaches and their tropism predicted with geno2pheno[coreceptor] (default false-positive-rate of 10%). From these, 14 samples (seven R5 and seven X4) were selected for further analysis with massively parallel sequencing (454 Life Sciences/Roche). For quantitative analysis of the R5/X4 distribution, the prediction score of each variant containing the V3 loop was plotted against its frequency within the viral population.

RESULTS: Prediction of coreceptor usage from the 55 bulk sequences showed a sensitivity of 59.1% and a specificity of 90.9%. Among the samples used in the ultradeep analysis, one R5 and four X4 were falsely predicted. Using the 454-technology, 10,000 reads per V3-containing amplicon were generated on average. Minorities of sequences with high confidence in CXCR4 usage were found in all samples, irrespective of phenotypic-determined tropism. For comparison with the Trofile results, a minority cutoff of 5%, reflecting the proposed sensitivity of the standard Trofile assay, was applied. Using the default false-positive rate of geno2pheno[coreceptor], results were concordant except for one sample.

CONCLUSIONS: Combining genotypic prediction with ultradeep sequencing results in a fast and accurate alternative to phenotypic assays. The detection of X4 viruses in all isolates suggests that coreceptor usage as well as fitness of minorities is important for therapy outcome. The high sensitivity of this technology in combination with a quantitative description of the viral population may allow implementing meaningful cutoffs for predicting response to CCR5 antagonists in the presence of X4 minorities.
ABSTRACT 92
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Initiatives for developing genotype interpretation systems: derivation and validation of a didanosine interpretation system using large derivation and large validation datasets

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BACKGROUND: To assess the genotypic determinants of the virological response to didanosine (ddI) in drug-experienced HIV-infected patients using large derivation and validation datasets.

METHODS: The FORUM database on ddI was divided at random into a derivation (n=1,000) and a validation set (n=453). Linear regression models of the week 8 reduction in viral load (VL) from baseline were fitted on baseline VL, exact number of week 8 follow-up and the number of drugs besides ddI to which virus is sensitive according to the ANRS interpretation system. First, a backward selection technique was used to select mutations at any position potentially related to VL response. Second, on 100 bootstrap samples of the derivation dataset, a backward elimination technique was used and mutations that were selected in more than 75% of the models were retained. We then repeated the second step with the mutations selected at step 1 forcing those retained in the second step. Finally a model including mutations retained after the first three steps was fitted on each of the bootstrap samples and the mean of the coefficient estimates calculated to derive a resistance score for ddI. Scores <0.2 were considered as sensitive, scores ≥0.2 and <0.6 as intermediate, and scores ≥0.6 as resistant. The score was then tested on the validation set.

RESULTS: The median baseline VL were 4.31 (interquartile range: 3.74–4.88) and 4.27 (3.68–4.86) in the derivation and validation set, respectively. The weighted score estimated on the derivation set was 0.4*T69D+0.9*D123S+1.0*T139M+1.2*I180V–0.2*M184V+0.9*V189I+0.8*Q207K+0.4*L210W. On the validation set, the proportion of patients for whom this score suggested resistance to ddI was 10.4%, those with intermediate was 26.0% and those with susceptible viruses was 61.6%. The VL reductions according to whether patients had predicted resistant, intermediate or susceptible viruses was 0.76 (0.29–1.22; P=0.002 versus sensitive), 0.95 (0.59–1.32; P=0.047) and 1.45 (1.22–1.67), respectively.

CONCLUSIONS: We derived and validated a genotypic score including mutations that both positively and negatively impact virological response and which contains five mutations never reported in previous studies. Assessment of this new score on external datasets would help to conclude on the role of these new mutations.
ABSTRACT 93
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Assessing the variability in building genotypic score in patients receiving a didanosine-containing regimen: baseline characteristics versus statistical methodology
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BACKGROUND: For a given drug, several distinct rules-based algorithms can be found. We aimed at investigating the variability coming from the statistical methodology used, to that coming from the baseline characteristics of the patients in building a genotypic score for didanosine (ddl).

METHODS: The FORUM database on ddl included >1,400 patients receiving a ddl-containing regimen. Three distinct samples were considered distinctly according to their geographical origin (France n=474, Italy n=440 and USA/Canada n=219). A continuous outcome, viral load (VL) reduction at week 8 (W8), a binary outcome having a viral-load drop of ≥0.6 log10 at week 8 or not were investigated. A statistical procedure based on the Jonckheere's non-parametric test (M1) and regression survival models accounting for censoring (M2) were applied to the continuous outcome. Similarly, a procedure based on the Cochran–Armitage test (M3) and logistic regression models (M4) were applied to the binary response outcome. These four methods were applied to the three distinct datasets based on geographical origin.

RESULTS: The median numbers of previous antiretroviral drugs were five (range 1–12), three, (1–10) and three (1–11) in the France, Italy and USA/Canada dataset, respectively. A higher percent of patients have been previously exposed to ddl in France (48%) than in Italy and USA/Canada (18% and 16%). The median numbers of thymidine-associated mutations was three (0–5), one (0–5) and one (0–5) in the France, Italy and USA/Canada dataset, respectively. Overall, 24, 33, 23 and 18 distinct mutations were selected with the M1, M2, M3 and M4, respectively. The percent of mutations selected in at least two datasets was 8%, 15%, 22% and 6% using the M1, M2, M3 and M4 method, respectively. Similarly, 39%, 32% and 28% of mutations were retained by at least two methods in the France, Italy and USA/Canada dataset, respectively. Of note, the L210W mutation was retained in all genotypic scores using the four methods in each dataset.

CONCLUSION: This study shows that baseline characteristics (geographical origin) contribute to a larger variability in building genotypic score than the statistical method used.
ABSTRACT 94
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Improved interpretation and clinical validation of substitutions in HIV-1 protease predicting the virological response to darunavir/ritonavir

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BACKGROUND: Consensus on the interpretation of genotypic HIV-1 resistance to darunavir/ritonavir (DRV/r) has not yet been achieved. We derived and validated a new DRV/r genotypic score.

METHODS: Protease inhibitor-failing patients starting a DRV/r-based regimen were extracted from the Italian DRV EAP (TMC114-C226) and ARCA. Eligible cases had genotype performed -180/+15 days from DRV/r initiation, baseline and week 4–32 viral load (VL). Virological response (VR) was analysed on the last VL and defined as >2 log drop or VL <50 if at <12 weeks or VL <50 if at >12 weeks. Genotypic sensitivity score (GSS) of the backbone drugs was obtained using ANRS2007. DRV/r resistance was interpreted by updated versions of ANRS, Rega, HIVDB and by scores from Tibotec and ANRS-ATU. A new genotypic DRV/r rule (DRVnew) was derived analysing associations of protease mutations (frequency >4%) with VR and constructing a weighted score based on correlation coefficients on a randomly selected 60% dataset split. The remaining 40% was used for validation.

RESULTS: A total of 192 patients were analysed: 71.4% male, median VL 4.44 log10 copies/ml, CD4+ T-cells 263 cells/μl and time on ART 11 years. Median experienced PIs were 5, 43% experienced enfuvirtide and 4% raltegravir. The median backbone GSS was 1.5, 17.2% used a new class (9.4% enfuvirtide, 8.3% raltegravir). VR was achieved in 99/192 (51.6%). Predictors of VR were baseline VL and new use of enfuvirtide; after adjusting for these, only DRV interpretation by Rega predicted VR (per increase in susceptibility category odds ratio (OR) 1.63; 1.11-2.40, P=0.014). Mutations unfavourably correlated (P<0.20) with VR in the derivation set (n=117) were: W6C/F/G/L, M11I, I13L/K/M/N/V, L33F/I/V, K55R, R41G/K, D60E and N88D/S, whereas L10V, G16E, G48V, F53I/L and V77I were favourably associated with VR. The DRVnew score 11I+33F/I/V+41K+2*55R+2*60E+88D/S-10V-16E-48V-53I/L-77I showed strongest independent correlation with VR in the derivation subset (per score higher, adjusted OR 0.39, P<0.001). In the validation set (n=75, VR=44%) DRVnew score -3–0 yielded VR=53%, 1 VR=43% and ≥2 VR=25%. In a mutually adjusted model this categorization showed stronger association with VR (per resistance category increase OR 0.58, P=0.07), than Rega interpretation (P=0.73).

CONCLUSION: The validated DRVnew score showed improved predictive capacity of VR. The absence of correlation for some ‘classical’ DRV mutation requires more investigation.
ABSTRACT 95
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A rigorous statistical learning method for the estimation and validation of weighted drug susceptibility scores applied to in vivo virological outcome prediction in atazanavir/ritonavir-containing HAART

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BACKGROUND: There is not yet consensus on the interpretation of genotypic resistance to atazanavir (ATV). We assessed both phenotypic and in vivo scores for the prediction of virological outcome to ATV/ritonavir (ATV/r)-containing highly active antiretroviral therapy (HAART).

METHODS: We selected virologically failing patients switching to ATV/r, who had baseline genotype, clinical, demographic and treatment information available. Virological response (VR) was defined as HIV-RNA viral load (VL) \(< 50\) copies/ml or VL \(\geq 2\) log_{10} reduction from baseline VL at the 8th week (range 4–12; VR1) and VL <30 within 24–48 weeks (VR2). Genotypic sensitivity scores were calculated for background treatment and for ATV/r (updated Stanford-HIVDB, ANRS, Rega, Bertoli’s rule HIVDRW2006). A phenotypic sensitivity score (PSS) was independently derived using public genotype–pheno- type data (n=1,266), calculating continuous indicator for a given HAART, by multiple linear regression (MLR), with embedded feature selection (FS). An additional in vivo score (GSSnew) was derived using logistic regression (LR). LR was employed to correlate all scores with VR, adjusting for potential confounders. A basic reference score that counted the number of mutations of the International AIDS Society-USA list (GSSbasic) was used to verify prediction improvements.

RESULTS: We analysed 203 ATV/r-containing regimens. At baseline, median values were VL =4.13 log_{10} copies/ml (interquartile range 3–9), CD4+ T cells =256 cells/µl (interquartile range 150–399). A total of 102/185 patients (55%) had VR1; VR2 was achieved in 114/203 (56%) patients. All ATV/r rules were significantly associated with both VR1 and VR2 in the adjusted models (P-values from 0.0002 of REGA to 0.03 of PSS). GSS, PSS and GSSnew yielded comparable prediction performances (accuracy 0.64–0.73, AUC 0.66–0.76), improving significantly over GSSbasic. MLR-FS for ATV/r phenotype prediction achieved R=0.9 in 10-fold cross-validation (CV). The coefficients were used to create interpretable PSS; new findings and literature confirmations were robustly assessed (for example, hypersusceptibility with 76V, resistance conferred by 10Y, 30N, 58E and 35D). Conversely, GSSnew, though demonstrating good CV performances, reported biased genotypic score (76V associated significantly with failure).

CONCLUSIONS: GSS and PSS scores correlate significantly with VR. CV results in more precise assessment than only multivariable P-values. GSSnew led to potentially misleading mutation interpretation probably due to limited sample size and confounders. Existing and new algorithms outperformed GSSnew. We foresee in PSS an alternative weighted score to catch fine interpretations of resistance/hyper- susceptibility roles of mutations.
ABSTRACT 96

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A combination of bioinformatic tools can be accurately used for the screening of coreceptor usage in clinical samples

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BACKGROUND: Assessment of HIV tropism using bioinformatic tools based on V3 sequences has shown to poorly correlate with the results from phenotypic tropism assays. We have developed a screening strategy combining several bioinformatic tools that shows a good correlation with phenotypic tropism results. Moreover, we have used this strategy for clinical samples and compared it with the results from the current version of the Trofile™ assay.

METHODS: Predictive performance using discriminate analyses and receiver operating characteristic (ROC) were used to compare the percentage of concordance and to select the best predictive combination from PSSM, SVM, C4.5 decision tree generator and C4.5, PART, Charge Rule and Geno2pheno. Using 200 reference sequences, a screening strategy was developed and further evaluated on 54 clinical samples submitted for Trofile™ that were V3 genotyped in parallel and expanded into all possible combinations.

RESULTS: Using the reference sequences, SVM and geno2pheno provided the highest sensitivity for X4 viruses (98.8% and 93.7%, respectively); however, their specificity was relatively low (62.5% and 86.6%, respectively). For R5 tropic viruses, PSSM and C4.5 gave the same results (95.7% sensitivity, 82% specificity). When three out of these four tools reported the same result, levels of sensitivity and specificity were over 90% to predict either R5 or X4 tropic viruses (area under the curve 0.9701; 95% confidence interval 0.9358–0.9889).

Amongst the 54 patients studied, Trofile™ reported 35 (64.8%) as having ‘activity of CCR5’; 10 (18.5%) as ‘no activity of CCR5’ and nine (16.6%) were ‘non-reportable’. Using our screening strategy, 43 (79.6%) were reported as R5 tropic and seven (12.9%) as X4 tropic; in four (7.4%) samples tropism could not be assigned and, therefore, further testing using a phenotypic tropism assay would be required. The number of discordant samples for Trofile reportable sequences was 2/45 (4.4%; one Trofile activity of CCR5/V3 X4 and one Trofile no activity of CCR5/V3 R5).

CONCLUSIONS: An algorithm combining four distinct bioinformatics tools, SVM, geno2pheno, PSSM and C4.5, might improve the prediction of HIV tropism and can be used as a screening tool in clinical practice. In our experience, using this strategy, the number of specimens requiring further phenotypic testing will be substantially reduced.
ABSTRACT 97

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Low rate of tipranavir and darunavir resistance mutations in PI-experienced patients using the new interpretation scores – implications for PI sequencing

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BACKGROUND: New scores for tipranavir/ritonavir (TPV/r) and darunavir/ritonavir (DRV/r) have recently been released. The prevalence of the different mutations recorded in the respective scores and the estimation of susceptible samples among patients who have failed prior protease inhibitor (PI) therapy is unknown.

METHODS: A large clinical database of HIV genotypes derived from patients who have failed antiretroviral drugs in Spain was examined. Only samples collected from patients failing PI were analysed. Resistance to TPV was assessed using the new TPV-weighted score (TWS), which includes 10V, 46L (1 point), 36I, 43T, 84V (2 points), 54A/M/V (3 points), 83D (4 points), 58E, 82L/T (5 points), 47V, 74P (6 points), 24I, 76V (-2 points), 50L/V (-4 points) and 54L (-7 points). The interpretation is as follows: sensitive TWS ≤3; partially resistant TWS =4–10; and resistant TWS ≥11. Resistance to DRV was considered when ≥3 mutations from the following list (11I, 32I, 33F, 47V, 50V, 54L, 54M, 74P, 76V, 84V and 89V) were present.

RESULTS: A total of 1,316 genotypes from PI-experienced patients never exposed to TPV or DRV were identified. The prevalence of TPV and DRV resistance mutations was low. The rate was <10% for all except 33F, 36I, 54V and 84V. The most prevalent mutations were 36I (30.9%), 54V (20.6%) and 84V (13.1%). According to the new resistance interpretation algorithms, 77.4% of patients who had failed PI remained sensitive to TPV, whereas 94.7% retained susceptibility to DRV. DRV resistance (≥3 DRV mutations) was significantly associated to prior failure to fosamprenavir (21.8% versus 4.3%, *P* < 0.001) and LPV (7% versus 4.3%, *P* = 0.042). The selection of TPV hyper-susceptibility mutations was significantly related to prior failure to fosamprenavir (46.2% versus 11.3%, *P* < 0.001) and atazanavir (19.8% versus 12.4%, *P* = 0.0012). In contrast, development of TPV resistance mutations could not be associated significantly to any single PI failure. In a separate dataset, in which 21 patients who failed TPV/r were examined, 76.2% retained complete susceptibility to DRV. On the other hand, from 41 patients who failed DRV, 56.1% remained sensitive to TPV.

CONCLUSIONS: Most PI-experienced patients retain full susceptibility to TPV and DRV, according to the latest genotypic interpretation scores. Moreover, the extent of cross-resistance between DRV and TPV is low, which may permit sequential rescue interventions.
ABSTRACT 98
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Preliminary results of a prospective clinical study of the HIV Resistance Response Database Initiative’s computational models as a treatment decision tool

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BACKGROUND: Virological response to combinations of antiretroviral drugs is highly complex and difficult to predict. We have demonstrated that computational models trained with substantial data from clinical practice can predict response from genotype, viral load, CD4+ T-cell count and treatment history. Here we present interim results from an ongoing prospective pilot study of our system in clinical practice.

METHODS: A random forest model and a committee of 10 artificial neural network models were trained to predict virological response (ΔVL) using data from 3,188 treatment change episodes (TCEs) from multiple clinical sources. These models were tested using an independent set of 100 TCEs and their predictions correlated with the actual ΔVL values with a correlation (r²) value of 0.68 and a mean absolute difference (predicted versus actual ΔVL) of 0.49 log₁₀ copies/ml. An online treatment decision tool was developed through which physicians enter the data for a patient requiring a treatment change and receive a report listing the five combinations of drugs the models predict will result in the largest ΔVL. The physicians were then required to enter their final treatment decision and a follow-up VL at 12 weeks.

RESULTS: Eight patients had completed the study at the time of this analysis. The r² between 12 week ΔVL and the models’ predictions was 0.58 and the absolute difference score was 0.47 log₁₀ copies/ml. In five cases the physician changed their original treatment intention after receiving the RDI report. Physicians indicated that the system was easy to use and would be used ‘quite often’ if generally available.

CONCLUSIONS: These preliminary results indicate that the models are able to predict virological response to highly active antiretroviral therapy with accuracy in clinical practice, that the system affects treatment decision-making and would be widely used if made available. The clinical pilot study continues and an international, multi-centre prospective controlled clinical trial of the RDI system is planned.

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ABSTRACT 99

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Improvement of genotypic algorithms to predict HIV-1 tropism in treatment-experienced patients – correlation with Trofile results

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OBJECTIVES: Viral tropism determination is mandatory before Maraviroc (MVC) prescription. Trofile (Monogram Biosciences, South San Francisco, CA, USA) is considered the gold standard for clinical tropism determination. However, tropism estimation using genotypic tools may be easier, faster and cheaper. We evaluated the accuracy/concordance between Trofile and several genotypic tools in HIV clinical samples collected during the Maraviroc Expanded Access Program in Europe.

METHODS: The env–V3 region was sequenced from plasma HIV RNA. V3 sequences harbouring nucleotide mixtures were translated into all possible amino acid permutations. Coreceptor usage was estimated using several bioinformatics tools: webPSSM (X4R5/SINSI matrixes), wetcat (C4.5, C4.5P8-P12, PART, SVM and Charge Rule) and geno2pheno (1–20% false positive rate [FPR]). Simple rules (11/25, 11/24/25 and Net Charge) were also evaluated. A sample was labelled as X4 if any of all sequence combinations were classified as X4. All predictions were compared with Trofile results derived from the same specimens.

RESULTS: A total of 270 specimens were collected. Paired genotypic/phenotypic results could be obtained for 210 (77%). Genotypic results could not be generated in 6.2%, whereas the phenotypic test failed to give results in 16.8%. The overall concordance between genotypic/phenotypic results was over 75%. The sensitivity for detection of X4 variants using genotypic tools was generally low, ranging from 34% (C4.5 and C4.5P8-P12) to 61% (webPSSMx4r5 and SVM). Conversely, a high specificity was obtained, ranging from 70% (SVM) to 92% (C4.5 and C4.5P8-P12). In order to enhance the sensitivity to detect X4 variants, we introduced a modification for webPSSMx4r5 predictions, changing the cutoff established to label a sample as R5 from -6.96 to -8.00 (named as webPSSMx4r5-8). The sensitivity for X4 variants increased up to 84%, with only slight diminished specificity to 62%. The prevalence of X4-variants using Trofile was 35.4%. An overestimation of X4-strains was seen using webPSSMx4r5-8 (51.6%). In the set of samples which failed to produce results using Trofile, webPSSMx4r5-8 reported half of them as R5.

CONCLUSIONS: The use of webPSSMx4r5-8 showed the best correlation with Trofile to identify X4-variants compared with other genotypic tools, and might assist with therapeutic decisions for using CCR5 antagonists in HIV patients.
Cross-sectional versus cumulative interpretation of HIV-1 drug resistance mutations

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BACKGROUND: Analysis of HIV genotypic drug resistance of consecutive clinical samples is routinely used to select optimized antiretroviral therapy. Nucleotide sequences archived from previous resistance testing provide the opportunity to integrate cumulative mutations in an updated interpretation. The aim of this study is to compare resistance prevalence according to the most recent genotype or to a cumulative analysis approach.

METHODS: From 6,488 archived genotypes from the Geneva AIDS Center (1991–2008), three sets were selected (IDNS™ SmartGene, Switzerland): all patients with at least two sequences of which at least one with nucleoside reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) or major protease inhibitor (PI) mutation (all according to International AIDS Society-USA list). Resistance profiles of the most recent sample of each patient and of cumulated mutations of all of a patient’s samples, including the last one, were generated and compared (validated implementation of ANRS 2007 on IDNS™, χ2 test).

RESULTS: Six hundred and sixteen, 110 and 87 patients were included in the NRTI, NNRTI and PI datasets, respectively. The median number of samples per patient was three (interquartile range [IQR] 2–5) with a median follow-up of 46 months (IQR 20–76). The differences in resistance prevalence between the two analyses were statistically significant for all NRTIs and NNRTIs, and for indinavir and saquinavir. The most significant changes in resistance levels (S to I/R and I to R) were noted for lamivudine (3TC; 31%), saquinavir (SQV) and lopinavir (LPV; 25%), and efavirenz (EFV) and nevirapine (NVP; 20%) followed by abacavir (ABC), zidovudine (ZDV), stavudine (d4T), tenofovir (TDF), entecavir (ETV), amprenavir (APV) and indinavir (IDV; 14%). For patients with differences in resistance levels between cross-sectional and cumulative approach, the median numbers of mutations associated with each drug (cross-sectional/cumulative) were: didanosine, ZDV and d4T: 0/3, 3TC 0/1, TDF 1/3, ABC 2/5, EFV and NVP: 0/2, ETV 2/4, IDV 1/4, SQV 2/4, LPV 5/7 and APV 2/5.

CONCLUSIONS: Cumulative analysis of drug resistance mutations provides an additional tool that improves resistance interpretation as compared with cross-sectional analysis, in particular when multiple drug regimens have been used or antiretroviral drugs have been stopped. This approach takes into account previous failing drug regimens and archived mutations. However, limitations of the cumulative approach may potentially arise from compensatory or antagonistic mutations not necessarily present on a given viral genome.
ABSTRACT 101

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**Automatic identification of resistance-associated mutations using techniques from Human Language Technologies**

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OBJECTIVES: As more antiretroviral drugs and drug resistance data become available, the need for a statistical system to predict HIV phenotype from genotype intensifies. However, most statistical prediction systems use features selected by human experts which lead to the same bottleneck found in maintaining rule-based systems. Here, we developed a method to automatically identify resistance-associated mutations that can perform as well as features selected by human experts in drug resistance prediction.

METHODS: We adapted an approach used to identify keywords in text documents to automatically generate a feature set of resistance-associated mutations for HIV phenotype prediction. Viewing each HIV genotype as a text document in a language without word boundaries, n-grams of varying lengths were extracted at each reading frame as well as position-specific unigrams and their counts were tabulated. For each n-gram count, we derived 20 binary features by considering whether the n-gram occurred at least \( j \) times and computed the \( \chi^2 \) statistic of each binary feature to measure its ability to discriminate between susceptible, low-resistant and high-resistant HIV genotypes. The n-grams were then ranked by the highest \( \chi^2 \) statistic from their derived binary features. The top \( p \) features were used with standard machine learning methods to predict phenotype where \( p \) was optimized for each antiretroviral drug.

RESULTS: Using the same classifier (decision tree) and dataset as a previous comparative study on feature sets for phenotype prediction [1], our position-independent features, position-specific features and their mixture yielded accuracies of 0.787, 0.784 and 0.788, respectively, averaged across all drugs, which were comparable to the reported accuracies of 0.775 from mutations selected by human experts and 0.784 from mutations trained using special treatment history data. Moreover, when used with random forest classifier, our feature sets yielded improved accuracies of 0.809–0.812. Further analysis showed the human expert selected mutations to closely overlap with our \( \chi^2 \) selected features.

CONCLUSIONS: Contrary to a previous study, we developed an automatic method to identify resistance-associated mutations for phenotype prediction that can match the performance of human experts without special treatment history data. This method can remove the human bottleneck in statistical prediction systems relying on human-selected mutations.

REFERENCE

ABSTRACT 102
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Determination of HIV-1 coreceptor usage in German patients – comparison of genotypic methods with the TROFILE phenotypic assay

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BACKGROUND: Maraviroc is a new drug used to treat HIV infection from the new class of drugs called CCR5 entry inhibitors. As the active principle of these drugs is to block the CCR5 receptor on the surface of the target cells, it has to be known if the virus in the patient is using only CCR5 as coreceptor or if there are populations that can also use CXCR4. Therefore an assay to determine the tropism has to be performed before starting a therapy.

METHODS: Besides phenotypic assays like the TROFILE assay by Monogram, used in the approval studies, there exist several genotyping systems like geno2pheno coreceptor, Wetcat (providing five different genotypic tropism schemes) and WebPSSM. We compared the results of 281 patient samples using the geno2pheno coreceptor with the results of the TROFILE assay. Furthermore, 203 sequences without ambiguities were analysed with the other genotyping systems.

RESULTS: The TROFILE assay detected 31% of the samples using as CXCR4- or mixed/dual-coreceptor. The agreement between the TROFILE assay and geno2pheno coreceptor (using a false-positive rate of 20%) was 76% compared with 82% for WebPSSM (Wetcat C4.5: 75%, C4.5 only p8 and p12: 76%, PART: 78%, SVM: 77% and Charge Rule: 82%). Although the performance of geno2pheno coreceptor seems superior compared with the other genotypic systems, this approach only classifies 19% of the 31 CXCR4-tropic samples false as CCR5-tropic, whereas WebPSSM (39%) and Wetcat (C4.5: 83%, C4.5 only p8 and p12: 87%, PART: 80%, SVM: 78% and Charge Rule: 82%) have a higher amount of false R5 classification in this subanalysis (false-negative rates: geno2pheno 27%, WebPSSM 35%, Wetcat C4.5: 77%.

C4.5 only p8 and p12: 78%, PART: 52%, SVM: 47% and Charge Rule: 57%). We also compared in 33 samples the results of proviral DNA, viral RNA and the TROFILE results. The results from proviral DNA and viral RNA showed a divergence of 25%. The comparison with TROFILE showed a slightly higher agreement of proviral sequences (viral: 63% and proviral: 70% in this subset).

CONCLUSIONS: We conclude that geno2pheno coreceptor is a suitable tool to predict HIV-tropism for using CCR5 receptor blocker in clinical practice. Although only a small number of proviral DNA samples were tested, this method seems promising to conduct tropism testing in patients with undetectable plasma viral load.
ABSTRACT 103

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Evaluation of the genotypic prediction of HIV-1 coreceptor tropism in a clinical setting

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BACKGROUND: CCR5 inhibitors have proven efficacy only in R5-tropic HIV-1 isolates. There is a need for evaluating bioinformatic systems of interpretation of genotypic tests for HIV-1 coreceptor usage in a clinical setting.

METHODS: In this multicentre study, plasma samples from antiretroviral-experienced patients were obtained at the screening date of the maraviroc-expanded access programme. The V3 loop sequences were determined and coreceptor usage was predicted using eight genotypic algorithms (PSSMX4R5, PSSMsinsi, Geno2pheno, C4.5, C4.5 p8-p12, PART, SVM, charge rule). All possible amino acid permutations were analysed when mixtures were detected. The genotypic predictions of CXCR4 were compared with the results of a phenotypic assay (Trofile®, Monogram Biosciences) obtained from the same samples. In the analysis, dual/mixed (D/M) variants were considered as X4 variants.

RESULTS: From 111 patients, both genotypic and phenotypic results were obtained from 89 (80%) samples, after exclusion of 11 (10%) patients with non-reportable results with Trofile, seven (6%) patients with failure of sequence determination and four patients (4%) for both reasons. The median viral load was 16,500 copies/ml and the median CD4+ T-cell count 233/μl. Patients had been exposed to a median number of 14 antiretroviral drugs. The majority of viruses (83/89, 93%) clustered with HIV-1 subtype B. According to Trofile, 22 isolates were classified as X4 and 67 were scored as R5. The concordances with Trofile ranged between 75% and 82% for the different algorithms. The specificity and sensitivity of the different algorithms ranged between 88% to 100% and 18% to 50%, respectively. By combining the two most sensitive algorithms (PSSM_sinsi and Geno2pheno) for the detection of X4 viruses, we obtained a concordance of 81%, a specificity of 90% and a sensitivity of 55%. When combining X4 detection with all interpretation systems, the concordance was 71%, the specificity 70% and the sensitivity for X4 detection 73%.

CONCLUSION: Bioinformatic tools can predict coreceptor usage with acceptable concordances with a phenotypic test. The combination of several algorithms can be used for increasing the detection of X4 or D/M viruses. Follow-up data on maraviroc therapy will be of interest to compare the predictivity of genotypic and phenotypic tests on virological response.
ABSTRACT 104
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Direct sequencing of V3 could be a valuable alternative to phenotypic assays for the routine assessment of HIV-1 tropism

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BACKGROUND: Replacing phenotypic assays with simple genotypic predictions of HIV-1 coreceptor usage would make the clinical use of CCR5 antagonists easier. Moreover V3 genotyping allows detecting not only virological failure related to the coreceptor usage switching, but also the occurrence of resistance mutations to CCR5 antagonists.

DESIGN: Paired genotypic and phenotypic determination of HIV-1 coreceptor usage was performed to assess several genotypic approaches for detecting CXCR4-using and CCR5-using viruses in a clinical setting.

METHODS: HIV-1 coreceptor usage was prospectively assessed using plasma samples from 103 patients who were candidates for treatment with a CCR5 antagonist. Direct sequencing of the V3 region and a sensitive recombinant virus phenotypic entry assay were performed in parallel for each patient from the same bulk env PCR product.

RESULTS: Paired genotypic and phenotypic data were obtained for 98 of the 103 patients. For detecting CXCR4-using viruses, the 11/25 genotypic rule was 63% sensitive; this reached 77% using combined criteria from the 11/25 and net charge rules. This combined rule was 96% specific. The Geno2pheno bioinformatic tool was 88% sensitive and 87% specific. The WebPSSM tool was 69% sensitive and 97% specific with the X4/R5 matrix, and 77% sensitive and 94% specific with the SI/NSI matrix. The global concordance between genotypic and phenotypic data was 91% with the combined criteria from the 11/25 and net charge rules.

CONCLUSIONS: The genotypic determination of HIV-1 coreceptor usage based on direct sequencing of V3 could be an efficient, simple alternative to phenotypic assays. This test is indicated before the use of CCR5 antagonists and in the event of therapeutic failure.
ABSTRACT 105
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Detection of low-abundance drug-resistant HIV variants in treatment-experienced subjects
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BACKGROUND: Low-abundance drug-resistant HIV variants can be detected in antiretroviral (ARV)-naive individuals and these resistant variants have been shown to impact clinical responses in individuals initiating antiretroviral therapy (ART). What is less defined is how often these resistant variants go unrecognized by standard sequencing methods in ARV-experienced individuals with detectable viral loads and if these unrecognized variants increase the overall burden of resistance in an infected individual. Our objective was to determine the prevalence and patterns of low-abundance drug-resistant variants in ARV-experienced individuals with viraemia on ART.

METHODS: Samples from ARV-experienced individuals who had a detectable viral load while on ART were evaluated. Standard Sanger sequencing and Ultra Deep sequencing were used to detect drug resistance mutations.

RESULTS: Samples from 24 ARV-experienced individuals with viral loads ranging from 1,380 to >500,000 copies/ml were evaluated. Low-abundance drug-resistant variants at levels ranging from 0.8% to <20% were identified in all 24 samples by Ultra Deep sequencing and were missed in 92% (22/24) of samples by standard sequencing. The number of additional resistance mutations identified at a level <20% ranged from one to 10 per sample (mean of four). The most common additional mutations identified were protease inhibitor (PI) mutations (79%; 19/24 – the majority were ‘minor’ PI mutations), followed by nucleoside reverse transcriptase inhibitor (NRTI; 75%, 18/24) and non-nucleoside reverse transcriptase inhibitor (NNRTI; 50%, 12/24 – eight had either a K103N, Y181C, Y188C or 190A/E/S). Of these additional low-abundance drug-resistant variants, 38% imparted resistance to a single class of ARV, 25% to two classes and 38% to three classes. The proportion of samples that harboured greater overall resistance by increased level of resistance to a drug (or drugs) and by the number of additional resistant drugs were 85% (20/24) and 58% (14/24), respectively.

CONCLUSION: Low-abundance drug-resistant HIV variants in ARV-experienced individuals with viraemia on ART are common. The overall burden of resistance in an ARV-experienced individual is often greater than that reflected in standard genotypes, with NRTI, NNRTI and PI low-abundance resistance variants all being identified in individuals.

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ABSTRACT 106

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Minority quasispecies of drug-resistant HIV-1 leading to early therapy failure in treatment-naive and adherent patients

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BACKGROUND: Early virological failure (eVF) of antiretroviral therapy (ART) associated with the selection of drug-resistant HIV-1 in treatment-naive patients is very critical, because it increases significantly the risk of subsequent failures. Thus, we evaluated the possible role of minority quasispecies of drug-resistant HIV-1, undetectable at baseline by population sequencing, in regard to eVF.

METHODS: We studied four patients with eVF on a first-line regimen of lamivudine, tenofovir and either efavirenz or nevirapine, and 18 patients with similar treatment without virological failure. Adherence was controlled by measuring non-nucleoside reverse transcriptase inhibitor (NNRTI) plasma concentrations. The key mutations K65R, K103N, Y181C, M184V and M184I within the reverse transcriptase were quantified by allele-specific real-time PCR performed blindly and retrospectively on plasma samples prior and during eVF.

RESULTS: None of the viruses prior to treatment showed any evidence of resistance in the genotypic analysis based on population sequencing. Minority quasispecies of either the M184V or the M184I mutation were detected in 3/18 control patients. In contrast, all four failing patients harboured drug-resistant viruses prior to treatment at low frequencies ranging from 0.07 to 2.0%. One to four mutations were detected in each patient. Most of them were rapidly selected and represented the major virus population within weeks after starting ART. All four patients showed good adherence. Two patients had directly observed therapy. NNRTI plasma concentrations were in normal ranges in all four patients on two separate time points.

CONCLUSIONS: Minority quasispecies of drug-resistant viruses detected at baseline can rapidly outgrow and represent the major virus population subsequently leading to early therapy failure. Sensitive assays for key mutations might be a useful tool before starting ART.
ABSTRACT 107
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Development and application of a broadly sensitive genotyping assay for surveillance of HIV-1 drug resistance in PEPFAR countries

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BACKGROUND: As the scaling up of antiretroviral therapy (ART) in resource-limited settings expands, implementation of HIV drug resistance surveillance is important to ensure the continuing effectiveness of first-line ART. US Food Drug Administration-approved genotyping kits are not optimized to genotype multiple HIV-1 subtypes and circulating recombinant forms (CRFs) in the President’s Emergency Plan for AIDS Relief (PEPFAR)-supported countries. Here we report the development and application of a broadly-sensitive genotyping assay that can genotype diverse HIV-1 subtypes and sample types.

METHODS: Between 2005 and 2006, 180 dried blood spots (DBS) were collected under field conditions from newly diagnosed HIV-infected individuals from Malawi (n=59), Tanzania (n=60) and China (n=61). Forty plasma specimens from HIV-1-infected Cameroonians under ART were also collected in 2007. The protease and reverse transcriptase (RT) gene regions gene were amplified with our broadly-sensitive genotyping assay and sequenced. Resistance-associated protease and RT mutations were identified using the Stanford University HIV DR database website (http://hivdb.stanford.edu/).

RESULTS: Of the 180 DBS analysed, 155 (88.3%) were RT-PCR positive. PCR-positive rates were: 92% (54/59) from Malawi, 83% (50/60) from Tanzania and 91% (55/61) from China. Of these amplified samples, 39 from Tanzania and 47 each from Malawi and China were sequenced. Among the 40 Cameroon plasma specimens from individuals taking ART, with known viral load (VL) measurement, 36 were amplified and sequenced including four specimens with VL <400 copies/ml. Four of these sequences had resistance mutations, including a minor PI mutation (E35G), a polymorphic nucleoside reverse transcriptase inhibitor (NRTI) mutation (V118I), and two major non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations (K103N and V106A). Among the newly diagnosed HIV-infected persons from Tanzania, Malawi and China, no specimens demonstrated resistance mutations except one from China with K101E, an NNRTI mutation. Phylogenetic analyses indicate that the predominant strains were subtype C (59, 36%), CRF02 (24, 15%), CRF01 (20, 12%), followed by A1 (8%), B (6%), and CRF07/C and CRF08/C (4%), and many minor subtypes, CRFs and URFs.

CONCLUSIONS: Our results indicate that this broadly sensitive genotyping assay can successfully be used to amplify and genotype both plasma and DBS specimens from individuals infected with diverse HIV-1 subtypes and CRFs. The assay is likely to be useful for HIV drug resistance surveillance in PEPFAR and other developing countries.

ACKNOWLEDGMENTS: The data referred here from Malawi has been accepted for publication in the Antiviral Therapy entitled: Surveillance of Transmitted HIV Drug Resistance with the World Health Organization Method in Lilongwe, Malawi by K Kamoto et al.
ABSTRACT 108
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Presence of minor drug-resistant mutations in HIV-1 subtype C patients failing antiretroviral therapy

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BACKGROUND: Current population-based methodologies for detection of HIV-1 drug resistance do not detect minority variants. The presence of these minority variants may be important in predicting treatment outcome, improve current treatment strategies and further our understanding of pathways leading to drug resistance. This study examined the presence of minority mutations by Ultra Deep sequencing in HIV-1 subtype C-infected patients failing antiretroviral (ARV) therapies.

METHODS: Plasma from 14 previously genotyped HIV-1 subtype C-infected patients failing the first-line (lamivudine, stavudine and efavirenz/nevirapine) or second line (didanosine, zidovudine and Kaletra) regimen on the South African roll-out programme were used in this analysis. Viral loads ranged from 10,000 to 880,000 RNA copies/ml. The protease (PR) and reverse transcriptase (RT) regions were amplified from viral RNA and sequenced using the GS FLX technology. HIV-1 drug resistant mutations occurring in >0.5% of the total population were examined and compared with those detected using standard population-based sequencing.

RESULTS: All mutations detected by population-based sequencing were present at >20% of the total population using the GS FLX technology. Several minor populations where observed that result in resistance to non-nucleoside reverse transcriptase inhibitors, namely, A98G, L100I/E, K103N/R, V106M, V108I, Y179E, Y181C, G190A/E, P225H and T227L. Similarly, minor populations, which result in resistance to nucleoside reverse transcriptase inhibitors, were observed (A62V, K65R, K70R, K219R, T215I). Twelve of the 14 samples contained minor populations of the K65R mutation ranging from 0.5% to 4.4% of the total population. Two samples harboured the V82A mutation in PR region, one of which was below the level of detection of current population-based methods.

Three of the 14 patients, which harboured no drug resistance mutations when sequenced by population-based methods, had mutant populations with the Ultra Deep sequencing technology.

CONCLUSIONS: Ultra Deep sequencing of HIV-1 subtype C samples provided good-quality sequences and detected several minority drug resistance mutations in patients failing ARVs. The presence of the K65R mutation previously not detected using standard population-based sequencing needs to be further investigated to determine the effect it and other minority mutations may have on future ARV treatment strategies.
ABSTRACT 109

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Evaluation of HIV-1 tropism using a new and sensitive system based on recombinant viruses

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BACKGROUND: Assessment of HIV tropism is a mandatory test in HIV-infected patients who are candidates to be treated with CCR5 antagonists. However, minority quasispecies (below 5–10%) in the whole viral population are not detected with available commercial tests. We have developed a system based on replication-competent recombinant viruses that can produce multiple cycles of infection. This strategy increases the sensitivity in the detection of minority viral populations in samples from patients.

METHODS: The sensitivity of the test was assessed using reference R5 and X4 HIV clones mixed in different ratios. HIV tropism was assessed in clinical samples from Spanish patients selected for compassionate maraviroc treatment. Viral RNA was extracted from plasma and amplified with reverse transcriptase-PCR and nested-PCR using primers specific for env. The resulting 2.7 kb amplification products, including the whole open reading frame of the HIV-1 gp160, were ligated into the pNL-lacZ/env-Ren, which carries a luciferase reporter gene in the position of nef. Resulting constructs were transfected into HEK 293T cells to generate chimeric recombinant viruses. In order to assess co-receptor tropism, U87-CD4 cells, expressing either the CXCR4 or CCR5 co-receptor, were infected with these viruses and luciferase activity was measured in cell lysates 48 h post-infection.

RESULTS: Using this protocol, a success rate of 90% for env amplification from plasma was obtained for patients with viral load above 1,000 copies/ml. This system can characterize the tropism of recombinant viruses harbouring different HIV-1 env subtypes. Minority variants of X4 and R5 viruses were detectable when they represented the 1% of the whole viral population. From the first 33 patients analysed from expanded-access program for maraviroc, 18 (53%) presented an R5 phenotype, 14 (44%) carried R5X4 viruses and one patient showed a pure X4-tropic population. Full genotypic and phenotypic tropism data from Spanish patients included in expanded access with maraviroc will be presented at the meeting.

CONCLUSIONS: We describe a new system to assess HIV tropism based on the generation of chimeric replication-competent viruses that increases the sensitivity for detecting minority populations in comparison with available commercial tests.
ABSTRACT 110

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Exploring etravirine resistance among recent routine clinical samples submitted for resistance testing

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BACKGROUND: Phenotypic susceptibility to efavirenz and nevirapine is significantly affected by single mutations in the viral genome resulting in increased fold-change values and loss of clinical efficacy. In the DUET and other trials, etravirine has demonstrated significant clinical efficacy in patients with virus strains resistant to the first generation non-nucleoside reverse transcriptase inhibitors (NNRTIs). In those trials, baseline etravirine phenotypic susceptibility was a strong predictor of virological response. We evaluated etravirine susceptibility among a large dataset of recent routine clinical samples (RCS).

METHODS: Phenotypic susceptibility to NNRTIs (fold change in 50% inhibitory concentration, FC) was calculated from the viral genotype (vircoTYPE v4.2.01) for 93,500 samples submitted to Virco for routine resistance analysis between January 2005 and November 2007. A subset of samples with genotypic evidence of NNRTI resistance was identified. Etravirine FC values were classified as maximal response (MA), reduced response (RR) or minimal response (Min) based on vircoTYPE clinical cut-offs of 1.6 (CCO1) and 27.6 (CCO2) associated with 20% and 80% loss of the predicted wild-type response. Because the RR category spans a broad FC range, a further subdivision based on an etravirine FC of 5.0, associated with a 50% loss of response, was explored.

RESULTS: Among RCS, 73.3%, 24.3% and 2.4% had ETR FC values classified as MA, RR, or Min, respectively. An ETR FC of ≤5.0 was observed in 89.8% of RCS. At least one NNRTI-resistance-associated mutation was detected in 38.4% of RCS. Among these NNRTI-resistant samples, 47.9%, 46.5% and 5.6% had ETR FC values classified as MA, RR, or Min, respectively, and 74.4% had an ETR FC ≤5.0. Among samples in the etravirine RR category, 67.6% of all RCS, and 57% of samples with NNRTI mutations had etravirine FC ≤5.0. In the Duet studies, virological response (undetectable viral load at Week 24) was observed in 69%, 59%, 49%, and 44% of individuals with baseline ETR FC <1.6, 1.6 to ≤5, 5 to ≤27.6, and >27.6, respectively.

CONCLUSIONS: Most RCS, including those with evidence of resistance to first generation NNRTIs, had low ETR FC values, suggesting they might benefit from treatment with etravirine as part of a HAART regimen.
ABSTRACT 111
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No effect of subtype on susceptibility and virological response to TPV/r for treatment-experienced patients

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OBJECTIVES: Differential effects of HIV-1 subtype on tipranavir (TPV) susceptibility for antiretroviral-naive patients have recently been presented. Moreover, the TPV-unweighted score (TUS) predicts reduced response to TPV/ritonavir (TPV/r in some naive, non-B subtype patients. Subtype differences in phenotypic and genotypic susceptibility and virological response to TPV/r in treatment-experienced patients were therefore explored.

METHODS: The RESIST trials entered 120 (55 on TPV/r) non-B-subtype-infected patients enabling a comparison of phenotypic (Antivirogram) and genotypic susceptibility and TPV/r response rates between B and non-B patients. Comparisons of subtype and virtual phenotype were performed on the baseline viruses from the 3,050 treatment-experienced patients screened for participation in RESIST.

RESULTS: Phenotypic susceptibility between subtype B (n=448 with 50% inhibitory concentration [IC50] fold change [FC]) and non-B (n=29 with IC50 FC) patients were similar among RESIST patients, with median (interquartile range) values of 1.0 (0.5–3.5) for non-B and 1.8 (0.8–3.7) for B patients (P=0.3765). Using all screened patients (n=3,050), the virtual phenotypes were also similar between subtype B (n=2,845 with virtual phenotype) and non-B (n=205 with virtual phenotype) patients, with median values of 1.5 (0.9–3.0) and 1.4 (0.9–2.5), respectively (P=0.1413). The TPV-unweighted score (TUS: P<0.0001), TPV-weighted score (TWS: P=0.0018) and ANRS (P<0.0001) had significantly higher scores in non-B patients than B patients. In the RESIST population, there was no association between subtypes and week 48 response rates (VL<50) or effect of treatment regimen (B: difference between TPV/r and CPI/r response rates =13.2%; non-B: difference =12.7%). TWS was the only score (unlike Stanford, Rega and Virtual Phenotype) to be significantly associated with week 8 (VL reduction; P=0.0187) and week 48 response (VL<50; P=0.0212) in non-B patients.

CONCLUSIONS: Phenotypic susceptibility to TPV/r is not reduced among non-B subtypes studied. Although genotypic scores show higher baseline results for non-B than B samples, non-B patients do not have inferior responses, suggesting that this difference is not clinically significant. Further investigation and analysis will be necessary to develop a full understanding of the role of resistance-associated polymorphisms in response to non-B subtype viruses. TPV/r is a viable treatment option for treatment-experienced subtype B and non-B patients.
ABSTRACT 112

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Dynamics of the M184V mutation by allel specific PCR (AS-PCR) in patients receiving lamivudine or emtricitabine monotherapy or undergoing treatment interruption (TI)

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OBJECTIVE: To study the dynamics of M184V mutants by allel specific PCR (AS-PCR) in patients receiving lamivudine (3TC) or emtricitabine (FTC) monotherapy or interrupting combination antiretroviral therapy (highly active antiretroviral therapy [HAART]), and to correlate M184V rates to viro-immunological variables.

METHODS: We examined 67 HAART-failing patients enrolled in the ‘E-184V’ and ‘Break’ studies either receiving once-daily monotherapy with 3TC ($n=28$) or FTC ($n=14$), once-weekly FTC monotherapy ($n=10$), or undergoing complete treatment interruption (TI; $n=15$). At enrollment (BL), direct sequencing showed the presence of M184V mutants in all patients. Plasma M184V-specific PCR was performed at baseline (BL), week 12 and 24, and also at week 4, 8, 16, 20, 36, and 48 in TI patients. The assay accurately measured the rate of M184V mutants within the range of 0.4 to 75%. M184V rates were compared between or within groups by Mann–Whitney or Wilcoxon sign test. Intrapatient changes of M184V rates and viro-immunological variables were calculated by univariate linear regression, as well as changes from BL at each time point.

RESULTS: AS-PCR confirmed the presence of M184V mutants in all patients at BL, with rates <75% in 17/67 patients (range 0.60–72.52). In the monotherapy groups, M184V rates were not different at BL versus week 12 or 24 or between groups at each time point. Patients with < or ≥75% mutant virus at BL or afterwards showed no different changes in plasma HIV-RNA load (VL), CD4+ T-cell or CD8+ T-cell counts, CD4%, CD8% or CD4:CD8 ratios at week 24. In TI patients, M184V% achieved the low detection limit after 4–36 weeks, with a mean slope of -14.83% (SE ±2.43%) over 48 weeks. Following TI, CD4+ T-cell counts decreased less markedly in patients with slower M184V% decays (linear correlation between M184V and CD4+ T-cell slopes, $r=0.582$, $P=0.029$), whereas no relationship was observed between M184V% decay and changes of VL, CD8+ T-cell counts, CD4%, CD8% or CD4:CD8 ratios.

CONCLUSIONS: 3TC or FTC monotherapy, including FTC once weekly, was associated with steady rates of M184V over 24 weeks. The association between slow M184V decay and smaller loss of CD4+ T-cells cells, observed in TI patients, supports the hypothesis that virus harbouring this mutation might be less pathogenic on CD4+ T-cells irrespective of VL.
ABSTRACT 113

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In silico identification of physicochemical properties at mutating positions relevant to reduced susceptibility to amprenavir

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BACKGROUND: We have previously presented high performing artificial neural networks for predicting in vitro 50% inhibitory concentration (IC50) fold-change (FC) values based on genotypic datasets described by the physicochemical properties of the amino acid mutations. Here we, as an example, explore the physicochemical properties that best describe the mutations in the protease enzyme in relation to reduced susceptibility to amprenavir.

METHOD: We used physicochemical properties derived from the amino acid index with additional properties found in literature to describe the mutational change from the HXB2 sequence in a dataset of amino acid sequences. Each sequence is paired with an IC50 FC value for amprenavir. Data was extracted from the Stanford HIV Drug Resistance Database. We performed regression analysis using least angle regression (LARS) with least absolute shrinkage selection operator (LASSO) to derive a model by 10-fold cross-validation in a 1:9 split on our dataset. The model with the lowest mean square error (MSE) of the predicted versus observed IC50 FC values was used to identify the most relevant amino acid positions and their physicochemical properties.

RESULTS: The extracted dataset contained 776 IC50 FC values each paired a protease sequence that was 99 amino acid long. Each amino acid was described with 565 different physicochemical properties. We identified the best model (MSE=0.885, mean correlation coefficient =0.847) to be based on only 22 properties. The 22 properties described 10 of the 99 positions in protease (10, 32, 33, 46, 50, 54, 82, 84, 88 and 90), all within the International AIDS Society-USA drug resistance list of amprenavir except position 88 found to be associated with hypersusceptibility. Categorised into six groups the identified properties are: accessibility (33, 84 and 88), conformational properties (10, 46, 54, 82, 84 and 88), hydrophilicity (10 and 88), hydrophobicity (32 and 50), secondary structure (33, 54 and 90) and polarity (33).

CONCLUSION: We have mathematically identified the important physicochemical properties that drive the change in IC50 FC to amprenavir, although the approach maybe missing properties with high collinearity. Applying this approach to other antiviral inhibitors will provide cost effective in silico knowledge about the physicochemical changes to the affected enzymes and could serve as input for computer-assisted drug designs and improvement of existing drug compounds.
ABSTRACT 114
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Detection of mixtures of hepatitis C virus genotypes

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BACKGROUND: Response to approved treatments for hepatitis C virus (HCV) infection is worse with HCV genotype 1 (the most common genotype) compared with other HCV genotypes. Genotype determination is routine for clinical trials. Detection of mixtures is not currently available; it may be desirable to detect minor populations of HCV genotypes, as this may affect response to therapy.

METHODS: A 15 member virus panel was constructed from patient-derived virus containing genotypes 1b, 2b and 3a, mixed at ratios of 10:90, 30:70, 50:50 with ≤30,000 HCV RNA copies/ml each. Ratio targets were confirmed by clonal analysis (n=50 each). The panel was blinded, randomized, genotyped and reported using the TRUGENE® 5′NC genotyping kit. TRUGENE® FASTA files were downloaded into a novel reporting module, MixuTYPE™ and compared with the original TRUGENE® genotype. A second MixuTYPE™ analysis was performed on 63 patient genotypes previously genotyped by Vanderbilt University Clinic. FASTA sequences obtained from testing with the TRUGENE® 5′NC genotyping assay were downloaded into the MixuTYPE™ reporting module and subsequent results were compared between the two methods. A confirmatory test was also run on these specimens using either HCV Inno-LiPA or HCV Invader assays at Vanderbilt. Specimens containing confirmed mixtures of two different HCV TYPES were cloned (n=50) for complete characterization.

RESULTS: The HCV TRUGENE 5′ NC assay using the integrated OpenGene® reporting system were correctly identified mixtures in 3 of 15 (20%) of the characterized mixture panel, whereas MixuTYPE™ correctly identified 15 of 15 from TRUGENE® FASTA sequences. The correctly identified genotypes were all from the 1b/3a mixture panel set at 30:70, 50:50 and 70:30 ratios. Sixty-three retrospective patient specimen genotypes obtained by HCV TRUGENE® 5′NC assay were sent for re-analysis of the original genotype using MixuTYPE™. No mixtures were identified during the original testing, whereas MixuTYPE™ identified mixtures in five of 63 (7%). Confirmatory testing and subsequent clonal analysis confirmed MixuTYPE™ results in five of five samples.

CONCLUSIONS: HCV-infected patients may have mixed genotypes, reflecting multiple exposures. At present, the ability to detect HCV genotype mixtures may be important for treatment, and if anti-HCV therapies are developed which differ in response rates based on HCV genotype.
ABSTRACT 115

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In vitro analysis of synergism/antagonism effect of different nucleoside/nucleotide analogues combinations on the inhibition of HIV-1 replication

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BACKGROUND: The approval of new nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) has allowed the possibility of using new combinations that have not been evaluated in clinical trials. However, some of these associations have shown unexpectedly high rates of virological failure via mechanisms of action that are not fully understood. To develop an in vitro system to evaluate the synergistic or antagonistic effect of different NRTI combinations might contribute to identify suboptimal NRTI combinations, as well as to determine synergistic combinations to be considered in future therapies, particularly in highly active antiretroviral therapy failing patients.

METHODS: Combination effect was determined in peripheral blood mononuclear cells (PBMCs) to approach a more physiological model than T-cell lines. PBMCs were infected with a full-length luciferase reporter HIV-1 clone in the presence of drugs. The following combinations at an equipotent fixed molar concentration ratio were investigated: zidovudine (ZDV)+(stavudine [d4T], didanosine [ddI] or lamivudine [3TC]), 3TC+(abacavir [ABC], ddI or d4T), tenofovir (TDF)+(d4T, ddI, ABC, 3TC or ZDV), d4T+ddI and ABC+(ddI or d4T). Synergism and antagonism was evaluated with a quantitative method based on the median-effect principle of Chou and Talalay.

RESULTS: A synergistic effect was observed with combinations containing TDF+(ZDV or d4T), d4T+(ddI or ABC) and ZDV+3TC in comparison with all other combinations (P<0.05). In contrast, combinations including ddI+(TDF, 3TC, ABC or ZDV) showed an antagonistic effect in the inhibition of viral replication at all levels of inhibition tested, and was statistically significant (P<0.05) at 50% effective dose (ED50) in comparison with all other combinations. Lower antagonistic effect was found in drug combinations that included 3TC+(ABC, TDF or d4T) and TDF+ABC at ED50. This antagonistic effect gradually turned out to be additive or weakly antagonistic for 90% effective dose levels.

CONCLUSIONS: The results of this new, viable and reproducible method in vitro to evaluate combination of two drugs allow to define synergistic, additive or antagonistic effects among different NRTI combinations. The results suggest that combined therapy, including TDF with thymidine analogues, may be considered for future therapeutics options in failing patients. In contrast to antagonistic combinations such as TDF+ddI or 3TC+ddI that would explain the virological failure happening in clinical studies when these combinations were used.
ABSTRACT 116

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Development of a genotyping assay for HIV-1 integrase compatible with globally available in vitro diagnostic platforms

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BACKGROUND: With recent regulatory approval of the first HIV-1 integrase inhibitor (INI; raltegravir) and continuing development of additional drugs in this class, it is expected that associated drug resistance testing will become important for patient management. We report the development of a genotyping assay (assay not commercially available) for HIV-1 integrase. Primers were designed for coupled amplification and sequencing on the OpenGene® DNA Sequencing System currently used by the TRUGENE® HIV-1 Genotyping Assay for protease and reverse transcriptase (RT) inhibitor resistance testing. Applicability to other platforms, based on dye-terminator cycle sequencing, was also demonstrated.

METHODS: Primers were designed for a 1.2 kbp RT-PCR amplicon and four overlapping regions 350–400 bp in length. Primers were paired to produce two to four overlapping sequencing regions for the entire integrase-coding region or a single region covering the majority of mutations considered clinically significant. Data were generated with OpenGene (Siemens) and ABI Prism (Applied Biosystems) automated DNA sequencers. A software module was developed to report currently known INI–treatment-related mutations. Viral dilutions, subtype panels and HIV-positive patient samples were used to evaluate sensitivity and subtype specificity.

RESULTS: Consistent amplification was demonstrated for subtypes A–H and various circulating recombinant forms. LAV dilutions from 100 to 1,000,000 copies/ml were tested successfully. Plasma samples with viral loads ranging from 68 to 390,000 RNA copies/ml were also genotyped. A set of patient samples from Asia was sequenced in protease (PR), RT and IN regions. In this set, IN polymorphisms that were detected in multiple samples included V31I and T112V for PR or RT mutant samples, and S119R, T125S, and V165I for samples without resistance-associated mutations.

CONCLUSIONS: Although the clinical utility of genotyping from plasma in the range of 50–400 RNA copies/ml has not been established, the sensitivity of this new assay may allow more routine analysis of samples of research interest. Results with samples collected in Beijing in 2005 and 2007 suggest that some HIV-positive populations in China may diverge from previously reported IN polymorphism patterns in addition to grouping with relatively rare subtypes or circulating recombinant forms.
ABSTRACT 117

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Validation of a genotypic and phenotypic recombinant virus assay to determine resistance against HIV-1 integrase inhibitors

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BACKGROUND: With the approval of the first integrase inhibitor (INI) raltegravir (RAL, MK-0518), genotypic and phenotypic resistance assays are required to guide patient therapy and to study treatment failure. In this study, we validated a genotypic and phenotypic recombinant virus assay to determine resistance against HIV-1 INIs RAL and elvitegravir (EVG, GS-9137).

METHODS: Viral RNA extracts were used to amplify the reverse transcriptase (RT)–RNaseH–integrase (IN) region (2,898 bp in HXB2 and referred to as RT–IN). RT–IN was sequenced using an ABI3730xl automated sequencer and cloned into an RT–IN-deleted HXB2-based backbone using In-Fusion technology. After Escherichia coli transformation, DNA was prepared and nucleofected into MT4 cells. The recovered replication-competent recombinant viruses were tested for their susceptibility to INIs RAL and EVG. Viruses carrying reported INI-resistant mutations were generated using site-directed mutagenesis.

RESULTS: The performance of an RT–IN genotypic and phenotypic recombinant virus assay was evaluated at the level of accuracy and reproducibility, and analytical and clinical sensitivity. Overall amplification and sequencing success was 84.4% (433 out of 513) and 94.1% (273 out of 290), respectively. A 90% amplification success rate was observed at a VL of 3.73 log10 copies/ml. Both B and non-B subtypes (C, A1, D, AG and F1) were successfully genotyped and phenotyped (96.5% overall phenotyping success rate [139/144]). Phenotypic accuracy and reproducibility were evaluated by (repeated) antiviral testing of known INI mutations T66I, T66A, E92Q, S147G, E92Q/S147G, E92Q/E157Q/V72I, N155H, N155H/E92Q, Q148R and Q148R/G140S, resulting in fold-change values of 0.6 (T66I) to 387.0 (Q148R/G140S) for RAL and 7.3 (S147G) to >1,909.2 (Q148R/G140S) for EVG.

CONCLUSIONS: A genotypic and phenotypic IN resistance assay was successfully validated, and constitutes a first step in the construction of validated databases in support of the development of an IN resistance predictive algorithm. Since the amplicon contains the RT, RNase H and IN genes, the assay can also be used to study the clinical significance of naturally occurring polymorphisms and nucleoside reverse transcriptase inhibitors- and non-nucleoside reverse transcriptase inhibitors- and INI-selected variants, as well as the interaction between RT and IN.
ABSTRACT 118
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Technical validation of an enhanced sensitivity Trofile HIV coreceptor tropism assay for selecting patients for therapy with entry inhibitors targeting CCR5

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BACKGROUND: The Trofile™ HIV coreceptor tropism assay determines whether a patient’s viral population is CCR5 (R5), CXCR4 (X4) or dual (R5/X4)/mixed (D/M)-tropic, and is useful for selecting patients for appropriate treatment with CCR5 antagonists. The clinical utility of Trofile has been unequivocally demonstrated in clinical trials of CCR5 antagonists, including maraviroc and vicriviroc. Existing studies suggest that detection of patients with lower levels of CXCR4-using variants may further optimize patient selection. We therefore developed an enhanced sensitivity version of Trofile (Trofile [ES]) with an approximate 10–100-fold improved ability to detect low levels of CXCR4-using variants.

METHODS: Experiments were performed to validate the performance of Trofile (ES) for patient management applications in compliance with CAP and CLIA regulations. The coreceptor tropism of a panel of well-characterized viral isolates and patient-derived HIV-1 envelopes (Envs) were evaluated to assess assay accuracy. Precision and reproducibility were assessed by replicate testing. Minor variant sensitivity was determined using mixtures of paired R5 and X4 env clones derived from four patients. R5 and X4 pairs were selected to exhibit similar infectivity of CCR5- and CXCR4-expressing cells, respectively.

RESULTS: Across all assays, X4 clones were detected in 100% of assays when present at 0.3% and in 81% of assays at 0.1%. Notably, detection of X4 variants was env clone pair (patient) dependent and ranged from 0.003–0.3%. Trofile (ES) accurately determined the coreceptor tropism of a panel of 46 well-characterized Envs representing multiple subtypes and clonally analysed patient Env populations, including some with X4 variants below the level of detection of the standard Trofile assay. Intra-assay precision (100%) and interassay reproducibility (99%) were demonstrated from concordant results for 135/135 and 158/160 pair-wise comparisons of R5, X4 and DM Envs clones and from repeat testing of 46 patient Env populations, respectively.

CONCLUSIONS: These validation experiments demonstrate that Trofile (ES) is accurate, precise, reproducible, and has improved sensitivity to detect low levels of CXCR4-using variants in env clone mixtures and patient env populations compared with standard Trofile. These data support adoption of Trofile (ES) as the assay of choice for coreceptor tropism determination of patient virus populations.
SESSION 5
Clinical implications of resistance
Emergence of drug resistance after failure of first-line HAART is associated with intensity of virological monitoring: a systematic analysis of cohort and trial data specifically addressing the WHO public health approach to antiretroviral therapy


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BACKGROUND: Antiretroviral therapy (ART) rollout in the resource poor world is often associated with limited, if any, HIV RNA monitoring. We sought to assess the risk of variable monitoring on resistance after initiating therapy with commonly used nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) combinations.

METHODS: We performed a systematic review of studies and cohort data reporting resistance data to at least 24 weeks in those initiating therapy with two nucleoside analogues (including one thymidine analogue) and an NNRTI, and with a CD4 T-cell count <200 cells/mm³. We used the number of genotypes analyzed (GA) as the denominator following viral rebound to >1,000 copies/ml. Studies were stratified by intensity of virological monitoring (every 3 months or less frequently) and inverse variance-weighting with exact 95% confidence intervals (CI) to compare the percentage of GA with either major NNRTI mutations, M184V, one or more thymidine analogue patterns (TAMs) or K65R.

RESULTS: A total of 4,563 patients from five cohorts and two prospective studies were analyzed from Uganda (n=526), Thailand (n=1,700), Malawi (n=389), Zimbabwe (n=300), Cameroon (n=60), UK (n=1,352) and Switzerland (n=236). The majority of studies reported resistance no longer than 48 weeks after initiation and therefore similar comparative data was used from the UK and Swiss cohorts. Baseline parameters were similar across monitoring groups. The proportion of virological failures successfully genotyped ranged from 91–96% in the infrequently monitored group and from 48–100% in the intensively monitored group. Resistance at virological failure to NNRTI was 91.8% (95% CI 86.3–95.6%) compared with 58.1% (46.9–68.7%), respectively. Lamivudine resistance was 86.3% (79.4–91.5%) and 39.2% (28.8–50.3%), respectively. The prevalence of at least one TAM was 25.2% (18.8–32.5%) and 8.9% (3.9–17.0%), respectively. The results remained unchanged when one cohort with only 80 week data was excluded from the analysis.

CONCLUSIONS: Genotypic resistance to lamivudine, NRTIs (TAMs) and NNRTIs appears substantially higher in infrequently monitored patients as compared with those more intensively monitored. Our data supports the use of sequential drug combinations with non-overlapping resistance profiles in order to maximize the clinical benefit of ART rollout in the context of limited monitoring.
ABSTRACT 120

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Low frequency K103N mutations are strongly associated with inadequate virological responses to non-nucleoside reverse transcriptase inhibitor based therapy

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BACKGROUND: Single-dose nevirapine (sdNVP) used for prevention of mother-to-child HIV transmission is associated with the selection of viral resistance mutations, notably the K103N mutation of reverse transcriptase. In the absence of drug pressure, these variants are shown to fade over time to low levels. Recently developed methods such as the allele-specific PCR (AS-PCR) are capable of detecting lower frequencies of mutant variants than conventional genotyping assays. However, the clinical relevance of these minority variants on treatment outcomes has not been fully established.

METHODS: An observational epidemiological study was conducted in Johannesburg, South Africa, in which initial and sustained virological response to first-line therapy ( stavudine+lamivudine+nevirapine/efavirenz) was compared between 94 HIV-infected women previously exposed to sdNVP 18–36 months earlier and 60 previously pregnant women who were unexposed. Pretreatment samples were tested for K103N mutations using an allele-specific real-time PCR and population sequencing was performed on samples from patients with inadequate virological responses.

RESULTS: K103N mutations were detected by AS-PCR among 10.6% sdNVP-exposed women and 15% unexposed reportedly drug-naive women prior to therapy. Of those with baseline resistance, 57.8% had a subsequent inadequate virological response, defined as either not achieving a viral load <50 copies/ml or not sustaining viral suppression to 78 weeks. However, out of the 30 women in both groups with inadequate virological response, only 36.7% had K103N detected by AS-PCR pretreatment suggesting that other minority mutant populations need to be analyzed. Population sequencing of the earliest failing sample revealed a different profile of mutations between the sdNVP-exposed and -unexposed women: K103N was more common among the exposed (9/18, 50%) than the unexposed group (3/12, 25%) whereas M184V/I was more common among the unexposed (9/12, 75%) than the exposed group (6/18, 33%). However, in both groups the majority had at least one non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutation; 13/18 (72%) among sdNVP-exposed and 10/12 (83%) among unexposed women in their failing sample.

DISCUSSION: Approximately 10% of women participating in this study had K103N mutations detected pretreatment, irrespective of their reported prior exposure to sd-NVP for prevention of mother-to-child transmission. These K103N mutations were shown to be strongly associated with inadequate virological responses to NNRTI-based therapy.
Determination of phenotypic clinical cut-offs for etravirine: pooled week 24 results of the DUET-1 and DUET-2 trials

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BACKGROUND: DUET-1 and DUET-2 are ongoing, randomized, placebo-controlled, double-blind, Phase III trials, demonstrating superior antiretroviral activity at 24 weeks of the non-nucleoside reverse transcriptase inhibitor (NNRTI) etravirine (ETR; TMC125) + background regimen (BR; darunavir/ritonavir + nucleoside reverse transcriptase inhibitors [NRTIs] +/- enfuvirtide) versus placebo + BR in treatment-experienced patients. Phenotypic clinical cut-offs (CCOs) for ETR are presented.

METHODS: In pooled DUET, 599 patients received ETR. Phenotypic CCOs for Antivirogram were determined using ANCOVA models and data-mining techniques in patients not using for the first time (de novo) enfuvirtide and excluding those who discontinued before 24 weeks for reasons other than virological failure (n=403).

RESULTS: Baseline ETR fold change (FC) in EC50 was a significant predictor of response (HIV-1 RNA <50 copies/ml) at 24 weeks. Baseline FC and responses to ETR were characterized by a continuum rather than a bimodal distribution. Inverse prediction of the ANCOVA model, with covariates baseline viral load, baseline CD4+ T-cell count and baseline darunavir FC, NRTI sensitivity and ETR FC, resulted in an initial CCO of 13, based on a 1 log greater response at week 24 versus placebo. As response in patients with baseline FC >13 was still substantial (37%), this value was considered an intermediate CCO. A FC value above which ETR provided no or little additional efficacy benefit (high CCO) could not reliably be established. Data-mining techniques allowed determination of a lower CCO of 3, below which patients exhibited the highest response rate. At baseline, 67%, 18% and 15% of patients had ETR FC ≤3, 3–13 and >13, respectively. At week 24, 71%, 50% and 37% of patients with FC ≤3, 3–13 and >13, respectively, reached <50 copies/ml.

CONCLUSIONS: Response in the ETR arms of the DUET trials decreased with increasing baseline ETR FC. The highest response rate was observed in the group of patients with ETR FC ≤3 (lower CCO). The robust responses observed in a substantial number of patients with baseline ETR FC >13 (intermediate CCO) and the low number of observations in this subgroup did not allow for the determination of a high CCO. These CCOs provide phenotypic guidance for use of ETR in treatment-experienced HIV-1-infected patients.
ABSTRACT 122

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Biological and clinical cut-off analyses for etravirine in the PhenoSense™ HIV assay

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BACKGROUND: The DUET-1 and DUET-2 trials demonstrated the efficacy of etravirine in combination with darunavir/ritonavir in treatment-experienced patients. We explored the lower and upper clinical cut-off (LCCO and UCCO) for etravirine by evaluating week 2, 4, 8 and 24 HIV-1 RNA outcomes from the DUET studies in relation to baseline etravirine fold change (FC).

METHODS: Phenotyping and genotyping was performed on 199 baseline samples (PhenoSense HIV, Monogram Biosciences) from individuals whose optimized background therapy did not include enfuvirtide. The LCCO was defined as the FC above which HIV RNA response was first observed to decline relative to the reference population. The relative contribution of the background therapy was explored by deriving weighted, PhenoSense specific, continuous phenotypic susceptibility scores for new drugs in each regimen. Further exploratory analyses included linear regression and local linear fitting by the function lowess in subsets of the study population. The biological cut-off was defined as the 99th percentile of etravirine FC values from 1,693 viruses lacking known non-nucleoside reverse transcriptase inhibitor (NNRTI)-, nucleoside reverse transcriptase inhibitor- or protease inhibitor-selected mutations.

RESULTS: The etravirine biological cut-off was 2.9. Among the DUET samples the median (range) etravirine FC was 0.75 (0.06–200). Etravirine hyper-susceptibility (HS; FC<0.4) was observed in 67 samples (33.7%). Only 23 (11.5%) samples had both reduced darunavir (FC>10) and etravirine (FC>2.9) susceptibility. At entry only 13% of individuals were on efavirenz, nevirapine or delavirdine but 73% of samples had NNRTI mutations. A modest relationship was observed between etravirine FC and week 4 HIV RNA change in distributions unadjusted for the activity of background therapy (R²=0.05, P=0.02). In models adjusted for the activity of background therapy the activity of etravirine was observed to be reduced at FC>2.9. This finding was supported by regression analyses in samples with reduced susceptibility to darunavir. Etravirine UCCO analyses were limited by the discrete number of samples with higher etravirine FC and these analyses are ongoing.

CONCLUSIONS: In models accounting for the activity of background therapy a LCCO for etravirine was observed at FC 2.9. In this highly treatment experienced population etravirine HS was common, being observed in approximately one third of samples.
ABSTRACT 123
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The CASTLE study 48 week results: the impact of HIV subtypes and baseline resistance on treatment outcomes and the emergence of resistance

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BACKGROUND: AI424138 demonstrated non-inferiority of once-daily atazanavir/ritonavir (ATV/r) (300 mg/100 mg) to twice-daily lopinavir/ritonavir (LPV/r; 400 mg/100 mg), both with once-daily tenofovir/emtricitabine, in 883 randomized antiretroviral-naive HIV-1-infected subjects from 28 countries. The effect of baseline substitutions and HIV subtypes on efficacy and emergent resistance profiles are described.

METHODS: Major/minor resistance-associated substitutions were classified per International AIDS Society USA (IAS-USA) 2007, and polymorphisms per Stanford HIV database. Batched genotypes were performed on baseline samples, and genotype/PhenoSense on paired samples with virological failure (VF) through 48 weeks. VF definition: HIV RNA ≥400 copies/ml through week 48; rebounded without re-suppressing; never confirmed viral load [VL] <400 copies/ml but remained on treatment; discontinued due to lack of efficacy.

RESULTS: Seventeen HIV subtypes were represented: B (66%), C (16%), BF (8%) and AE (7%). Response rates (VL <50 copies/ml) for subtypes B versus non-B were 79% versus 75% for ATV/r and 74% versus 82% for LPV/r. At baseline, 2% versus 3% on ATV/r and LPV/r had ≥1 major protease inhibitor (PI) substitution, 31% versus 39% had ≥1 minor PI substitution, and 67% versus 59% only polymorphisms (non-B [85% ATV/r versus 71% LPV/r], B [58% ATV/r versus 34% LPV/r]); 32% versus 30% had reverse transcriptase (RT) substitutions (17% versus 15% nucleoside reverse transcriptase inhibitors [NRTIs], 1% versus 3% thymidine analogue mutations [TAMs], 19% versus 18% non-nucleoside reverse transcriptase inhibitors [NNRTIs] [2% versus 1% K103N, <1% Y181C]). Frequencies of some baseline PI substitutions varied widely by subtype (for example, M36 >90% in C, BF, AE, <30% in B). The response rates (VL <50 copies/ml) for subjects with 0–2 baseline major or minor PI substitutions were 78% ATV/r and 77% LPV/r; <1% of subjects had ≥3 baseline major or minor PI substitutions. Response rates by number of baseline PI polymorphisms (0–2, 3–4, ≥5) ranged from 76–86%. Two subjects had phenotypic resistance at baseline to study PIs (FC ATV/r 19 and 10; LPV/r 39 and 9). PI substitutions emerged in 10/19 ATV/r and 8/20 LPV/r VF subjects; all were polymorphic except two ATV/r subjects (1 [N88S, M46I]; 2 [L10F, V32I, K43T, M46I, A71I, G73S, L90M]); M184V emerged in 5/19 and 4/20, and TAMs in 1/19 and 1/20, respectively. K65R+K70E substitutions emerged in one ATV/r VF subject (baseline PI: I110V, M36I, I62V, L63P, A71A/T, I93L); (emergent PI: V32I, M46I, L90M, L10F, K43T, A71I, G73S) with on-study phenotypic resistance to ATV (FC:56) and emtricitabine (FC: >56).

CONCLUSION: At baseline, CASTLE had many HIV subtypes and frequent NRTI, NNRTI and PI substitutions, although phenotypic resistance was rare. ATV/r and LPV/r regimens achieved consistent response rates regardless of subtype or number of baseline PI substitutions. The emergence of non-polymorphic PI substitutions was infrequent with both regimens.
ABSTRACT 124

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Mutations selected by other protease inhibitors predict durable response to tipranavir in treatment-experienced patients

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OBJECTIVES: The mutations 24I, 50L, 50V, 54L and 76V have been shown to be associated with increased phenotypic susceptibility and/or better week 8 virological response to tipranavir/ritonavir (TPV/r) and thus receive negative weights in scores used by Monogram Biosciences, Virco and/or the TPV-weighted mutation score. The objective of this work is to explore the long-term effect of these mutations on virological response to TPV/r.

METHODS: The TPV Phase II/III development program was used to assess the prevalence of each of the above mutations and their relationship to TPV phenotype. All TPV/r-treated patients in the RESIST trials were evaluated for short- (week 8) and long-term (week 48) virological response with and without these mutations. The response rates were compared overall and adjusted for optimized background regimen (OBR) activity and TPV-weighted score.

RESULTS: The prevalence of each mutation for all 2,116 patients in Phase II/III trials were 24I (15.5%), 76V (8.4%), 54L (7.1%), 50V (6.9%) and 50L (0.2%). Using all 810 patients with phenotype measured at baseline, the median (interquartile range) TPV IC50 fold changes were 24I (1.3[0.7–2.6]), 76V (1.0[0.5–2.1]), 54L (1.0[0.6–2.5]), 50LV (0.7[0.3–1.1]) compared with (1.8[0.9–3.9]) for patients with none of these mutations. Using RESIST TPV-treated patients, the week 8 response rates (1.5 log drop in viral load [VL]) for patients harbouring 24I, 76V, 54L or 50L/V at baseline were 59.4%, 59.1%, 68.0% and 71.1%, respectively, compared with 44.6% for patients with none of these mutations. The week 48 response rates (VL<400) were 30.3%, 35.8%, 39.3% and 37.8%, respectively, compared with 29.5% for patients with none of the mutations. Presence of at least one of these mutations remained significantly associated with increased week 48 response (P=0.045) even after adjusting for OBR activity and the mutations with positive weights in the TPV-weighted score.

CONCLUSIONS: Patients with at least one of the mutations 24I, 50LV, 54L and 76V demonstrated significantly improved virological response rates to TPV/r-based therapy at week 8 and had strong associations with decreased phenotypic resistance to TPV. The improved response rates decreased over time, but a significant benefit of having these mutations remained throughout the 48 week period.
ABSTRACT 125

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Comparable in vitro susceptibility and virological outcome to darunavir in patients infected with subtype B and non-subtype B HIV isolates participating in the ARTEMIS Phase III trial

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BACKGROUND: Limited data are available concerning the susceptibility and clinical outcome to antiretroviral therapy of HIV-1 patients infected by non-B subtypes. The effect of HIV-1 subtype on the in vitro susceptibility and virological response to darunavir (DRV) was studied against a broad panel of primary isolates and in clinical isolates from treatment-naive HIV-1-infected patients participating in ARTEMIS, an ongoing Phase III trial assessing the efficacy and safety of DRV with low-dose ritonavir (DRV/r) versus lopinavir with low-dose ritonavir (LPV/r).

METHODS: The antiviral activity of DRV was assessed in freshly isolated human peripheral blood mononuclear cells (PBMCs) against a panel of 25 HIV-1 primary isolates, composed of ≥3 viruses from each of the group M subtypes (A to G) and three isolates from HIV-1 group O. In ARTEMIS, patients were randomized to receive DRV/r 800/100 mg once daily (n=343) or LPV/r 800/200 mg total daily dose (n=346), plus a fixed daily-dose of emtricitabine and tenofovir. Subtype determination and phenotyping (Antivirogram) on baseline samples were performed by Virco. Virological response (HIV-1 RNA <50 copies/ml) at week 48 was determined using the time-to-loss of virological response algorithm.

RESULTS: DRV demonstrated high levels of antiviral activity against a broad panel of HIV-1 group M and group O primary isolates in PBMCs with a mean EC_{50} of 1.04 nm (range <0.10–4.28 nM). The majority (61%) of patients in ARTEMIS harboured HIV-1 subtype B; other prevalent subtypes found were subtype C (13%) and subtype CRF01_AE (17%); 9% of patients harboured other subtypes. Median EC_{50} values (range) for DRV were similar for subtype C (1.12 nM [0.23–3.46]) and subtype CRF01_AE (1.27 nM [0.35–4.30]) compared with subtype B (1.79 nM [0.38–5.07]) HIV-1 isolates. Virological response to DRV/r was 81%, 87% and 85% for patients participating in ARTEMIS with subtype B, C and CRF01_AE infection, respectively.

CONCLUSIONS: In vitro susceptibility to DRV was comparable across HIV-1 subtypes in a broad panel of primary isolates in PBMCs and in clinical isolates from the ARTEMIS trial. Accordingly in ARTEMIS, once-daily DRV/r 800/100 mg was highly effective, irrespective of HIV-1 subtype. These results confirm the broad activity of DRV.
ABSTRACT 126

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Re-evaluation of resistance algorithms for lopinavir/ritonavir in the TITAN Trial

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BACKGROUND: Genotypic algorithms used to predict the clinical efficacy of lopinavir/ritonavir (LPV/r) have included a range of mutation lists and efficacy endpoints. HIV clinical trials are normally powered to detect a difference between treatment arms of 10–12% for the end-point of HIV-1 RNA suppression <50 copies/ml. The TITAN trial (TMC114-C214) evaluated LPV/r versus darunavir/ritonavir in treatment-experienced patients with HIV-1 RNA >1,000 copies/ml. Baseline genotypic resistance to darunavir was rare in the TITAN trial.

METHODS: Baseline genotype data were classified using five genotypic resistance algorithms: IAS-USA LPV mutations (cut-off=6), Abbott 2007 (King) mutation list (cut-off=3), ANRS mutations (cut-off=6), FDA mutations (cut-off=3) and IAS-USA major protease inhibitor mutations. Efficacy in the TITAN trial (HIV-1 RNA <50 at week 48) was correlated with the number of mutations from each list to show the ‘efficacy advantage cut-off level’, the number of mutations from each list associated with a difference in efficacy between treatment arms of at least 12%. The linearity of the correlation between mutation count and efficacy of LPV/r was analyzed in TITAN, with sensitivity analysis for the French LPV ATU, BMS-045 and RESIST trials.

RESULTS: In TITAN, the concordance between baseline lopinavir resistance, defined by the mutation scores, ranged from 79–95%. Multivariate analysis identified lower than previously reported genotypic cut-off levels where there was at least 12% lower efficacy for LPV/r versus darunavir/ritonavir. These ‘efficacy advantage cut-off levels’ were IAS-USA LPV mutations cut-off=3, Abbott 2007 cut-off=2, ANRS LPV cut-off=3, FDA LPV mutations cut-off=2 and major IAS-USA PI mutations cut-off=1. There were linear falls in HIV-1 RNA suppression rates with rising mutation counts in the TITAN, French LPV ATU, BMS-045 and RESIST trials.

CONCLUSIONS: The analysis identified more sensitive ‘efficacy advantage cut-off levels’ for four lopinavir genotypic algorithms, below those currently used, at which there is significant efficacy advantage for treatment with darunavir/ritonavir versus LPV/r in the TITAN trial.
ABSTRACT 127
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Week 96 final analysis of resistance mutations in protease gene and in cleavage site of Gag protein for patients failing on a first-line lopinavir/ritonavir single-drug regimen in the Monark Trial

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OBJECTIVE: MONARK is the first 96-week randomized trial comparing lopinavir/ritonavir (LPV/r) monotherapy to triple combination with zidovudine/lamivudine and LPV/r in antiretroviral-naive patients. Our objective was to describe resistance mutations emerging in protease and gag genes in case of virological failure on a first-line LPV/r monotherapy.

METHODS: For the current analysis, patients were eligible for genotypic resistance test if they did not reach an HIV RNA <50 copies/ml at week (W) 24 or if they had a confirmed rebound in HIV RNA >50 copies/ml after reaching an HIV RNA <50 copies/ml at W24. Protease and gag genes were sequenced at baseline and on the same sample obtained at the time of confirmed virological failure. Protease inhibitor (PI) resistance mutations were defined according to 2007 International AIDS Society list. Substitutions in cleavage site of the C-terminal part of Gag protein (p2-NC-p1-p6) at positions V362I, S373P/T/Q, A374T/N/P/S, T375A/N/S, I376V, R380K, G381S, A431V, N436R, I437V, L449P/F, S451N and P453L were analyzed and any changes between baseline and failure noted.

RESULTS: We focus here on the 83 patients randomized to LPV/r monotherapy and followed through to W96. Between baseline and W96, 23/83 patients were eligible for genotypic resistance test: 14 did not reach HIV RNA <50 copies/ml at W24 and nine had confirmed viral rebound >50 copies/ml after reaching HIV RNA <50 copies/ml at W24. PI major resistance mutations were evidenced at the time of confirmed virological failure in five patients (46I+63P at W40, 76V at W44, 13V+46I+76V at W62, 10F+82A at W76 and 76V at W90). Gag sequences are currently available for 15/23 patients with 14/15 having at least one substitution at baseline. Changes in cleavage site of Gag between baseline and failure were noted in 6/15 patients (4/6 with no PI major resistance mutation at failure: R380K, V362I+S373T+T375A, L449F and L449F; and 2/4 patients with PI major resistance mutations at failure: 374T to 374S in one patient and 374P to 374S in one patient).

CONCLUSIONS: Patients (14/15) for whom results are currently available had at least one substitution in cleavage site of Gag at baseline. Changes in cleavage site of Gag between baseline and failure occurred in 6/15 patients. Interestingly, most of these changes were not associated with the emergence of major PI resistance mutations.
ABSTRACT 128

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The rate of accumulation of NNRTI resistance in patients kept on a virologically failing regimen containing NNRTI: a EuroSIDA study

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BACKGROUND: Maintenance of nevirapine or efavirenz therapy in viraemic patients may lead to the accumulation of non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations and constrain the efficacy of second-generation NNRTIs like etravirine. We estimated the rate of accumulation of NNRTI-related (including etravirine-specific) resistance mutations in such patients.

METHODS: Patients in EuroSIDA with pairs of genotypic tests available while being kept on a virologically failing NNRTI-based regimen were included. The first genotype had to contain at least one NNRTI mutation. The overall and etravirine-specific incidence of new International AIDS Society (IAS) NNRTI resistance mutations was calculated as number of new mutations observed at the second genotype divided by the time between genotypes. Multivariable analysis was performed using a Poisson regression model accounting for overdispersion. Etravirine activity was calculated in both genotypes using Rega IS and the average reduction in activity due to accumulation of resistance determined.

RESULTS: A total of 154 patients contributed 326 genotype pairs (174/152 pairs on nevirapine/efavirenz). At the beginning of a pair, 56% received lamivudine, 47% stavudine, 42% didanosine and 61% a protease inhibitor; the median HIV RNA level was 4.1 log₁₀ copies/ml, CD4⁺ T-cell count 224 cells/µl and IAS NNRTI mutations 2 (range 1–9). Median interval between paired genotypes was 5 months and, on average, HIV RNA change over this time was not different from zero (+0.05 log₁₀ copies/ml, P=0.09). Overall, 78 IAS NNRTI resistance mutations accumulated over 180 person-years of follow-up (0.43/year; 95% confidence interval 0.36–0.51). This rate was 0.36/year in patients with a virus predicted to be resistant (R), 1.52/year if intermediate (I) and 2.71/year if susceptible (S) to the NNRTI used. In a multivariable analysis, the rate was threefold/year faster (1.3–7.5) in I and fivefold faster/year (1.6–18.5) in S viruses compared with R viruses. The estimated rate of accumulation of etravirine-specific mutations (41 new etravirine-associated mutations) was 0.23/year (17–30) leading to an absolute mean reduction in estimated etravirine activity of 8%/year (P=0.0001).

DISCUSSION: Whereas the average rate of accumulation of NNRTI mutations was appreciable (1/2.2 years) etravirine-specific mutations accumulated more slowly (1/4 years), leading to an 8% mean reduction in etravirine activity per year. More sensitive viruses accumulated NNRTI resistance mutations faster.
ABSTRACT 129
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Failing therapy with efavirenz results in significantly fewer mutations limiting to etravirine than failing therapy with nevirapine: on-treatment analyses from the CPCRA FIRST Study

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OBJECTIVES: Among HIV-1-infected persons initiating antiretroviral therapy (ART) with efavirenz or nevirapine in the FIRST study, to compare the risk of virological failure (VF; HIV RNA >1,000 copies/ml at or after 4 months) for those on efavirenz compared with nevirapine and to summarize mutations detected at VF by the non-nucleoside reverse transcriptase inhibitor (NNRTI) used at the time of VF.

METHODS: Persons randomized to one of the NNRTI-containing arms of FIRST were seen every 4 months (median follow-up 60 months). Genotypic resistance testing was done at initial VF. The risk of VF was summarized with hazard ratios (HR) from Cox proportional hazards models with time-updated indicators for NNRTI use and adjusted for baseline covariates. Proportions were compared using \( \chi^2 \) tests.

RESULTS: Of the 915 persons randomized to a NNRTI arm of FIRST, the initial ART regimen contained efavirenz or nevirapine for 534 and 381 persons, respectively. The risk of VF and VF with any NNRTI, nucleoside reverse transcriptase inhibitor (NRTI), or any-class resistance was significantly less for those taking efavirenz compared with nevirapine (HR=0.71, 0.53, 0.37, 0.55, respectively; all \( P \)-value \( \leq 0.001 \)). Of those who experienced VF, 149 were on efavirenz at VF and 145 were on nevirapine. Among those failing on efavirenz and nevirapine, respectively, specific NNRTI mutations detected included 103N (47% versus 28%), 106A/M (2% versus 9%), 181C/I (2% versus 30%) and 190A/S (5% versus 14%); all \( P \)-value \( \leq 0.01 \). At least one of the mutations identified as limiting to etravirine (90I, 98G, 100I, 101E/P, 106I, 179D/F, 181C/I and 190A/S) occurred less frequently in persons failing on efavirenz than on nevirapine (58% versus 71%; \( P=0.02 \)).

Despite the differences in rates of VF and VF with resistance favoring efavirenz, immunological and clinical outcomes were similar for those initiating ART with efavirenz or nevirapine.

CONCLUSIONS: The use of efavirenz resulted in less NNRTI, NRTI or any class resistance than the use of nevirapine. Emergent major NNRTI mutations at VF differed by NNRTI used; the mutation patterns suggest that subsequent suppression of HIV RNA with etravirine-containing regimens may be more successful if initial therapy with efavirenz is used rather than nevirapine.
ABSTRACT 130

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HIV-1 genotype algorithms for prediction of etravirine susceptibility: novel mutations and weighting factors identified through correlations to phenotype

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BACKGROUND: Etravirine is a recently approved non-nucleoside reverse transcriptase inhibitor (NNRTI) with activity against many HIV-1 strains resistant to other NNRTIs. The presence of three or more 'etravirine mutations' (V90I, A98G, L100I, K101E/P, V106I, V179D/F, Y181C/I/V and G190A/S) was associated with reduced response in clinical trials. Analysis of larger phenotype/genotype databases can provide additional information, including which mutations have relatively greater effects on resistance than others.

METHODS: The fold change (FC) in etravirine IC50 of 2,588 samples submitted for routine resistance testing was determined (PhenoSense™ HIV, Monogram Biosciences). An extensive list of substitutions, including polymorphisms, potentially associated with NNRTI resistance were considered. Samples containing at least one such mutation, but lacking nucleoside reverse transcriptase inhibitor (NRTI) mutations that are strongly associated with increased susceptibility were studied (n=857). The lower clinical cut-off for etravirine (2.9) was used to define reduced susceptibility (RS). A step-wise, iterative algorithm was used to determine weighting factors (WF). Mutations were entered into the algorithm in order based on relative magnitude of their effect on etravirine susceptibility by univariate analysis. Mutation grouping was determined by minimizing phenotype/genotype discordance rates.

RESULTS: The presence of three or more etravirine mutations was insufficient in predicting RS, with 12% of samples with RS having two or fewer mutations (median FC 6.3, range 3.0 to >200). Four groups of mutations were defined and assigned WF of 1 to 4 with a total score of 4 being required for RS. Mutations with a WF of 4 (sufficient to confer partial reduction in susceptibility on their own) included L100I, K101P, Y181C/I and M230L; Y188L had WF=3; V189I had WF=2; V90I had A98G, K101E/H/Q had V106M; E138A/G/K, V179E and H221Y had WF=1. The revised algorithm reduced the frequency of unexpected RS to 3.5%.

CONCLUSIONS: Existing etravirine genotype interpretation rules underestimate phenotypic RS. The improved scoring system with weighted mutations reduced this type of discordance and should be tested in additional validation studies. The sensitizing effects of NRTI mutations on etravirine resistance or the effects of other potent drugs that are co-administered in clinical studies could be responsible for differences between scoring systems that are based on clinical response or phenotypic susceptibility.
ABSTRACT 131

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HIV drug resistance at baseline of the first highly active antiretroviral therapy trial in Vietnam (ANRS 1210): correlation with poor immunological and virological responses

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BACKGROUND: ANRS 1210 was the first highly active antiretroviral therapy (HAART) trial carried out in Vietnam; its aim was to evaluate efficacy and tolerance of an antiretroviral regimen associating lamivudine/didanosine/efavirenz administered once a day.

METHODS: One hundred HIV-1-infected patients (mostly intravenous drug users [IDU]) with TCD4 counts <200 cells/μl and considered to be antiretroviral drug (ARV)-naive were recruited in Ho Chi Minh City (HCMC). They had a clinical and biological follow-up from initiation of therapy until 18 months (M18) post-initiation when survivors were discharged from ANRS 1210 and taken in charge by ESTHER initiative. The biological follow-up included TCD4 numbering (M3, M6, M9, M12, M15 and M18), viral load quantification (M12, M15 and M18) using the ANRS real-time assay (carried out in HCMC; threshold at 60 copies/ml) plus sequencing of reverse transcriptase (RT) at initiation for characterization of the viruses and potential observation of drug resistance mutations (DRMs) to nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) at baseline.

RESULTS: Eighty-one samples could be sequenced on RT at baseline. All isolates clustered with CRF01_AE. According to IAS-DRM classification, 19.8% and 8.6% of the patients had at least one DRM to NRTIs and NNRTIs, respectively. Globally, the median values of TCD4 were 49.5 and 201 cells/μl at baseline and M18, respectively, while viral loads were <60 copies/ml for 12%, 11% and 26% of the patients, respectively, at M12, M15 and M18. Presence of DRMs at baseline was a highly significant factor in the immunological and virological responses.

CONCLUSIONS: The resistance at baseline has clearly impaired the efficacy of the HAART regimen of this first antiretroviral trial in Vietnam. Some hypotheses can be raised up to tentatively explain the presence of DRMs at baseline: circulation in HCMC of HIV-1 isolates with DRMs, denied ARV treatment before inclusion in ANRS 1210 and close contaminant relationships between patients of IDU group.
ABSTRACT 132

Thymidine analogue mutation patterns from HIV-infected Mexican children after multiple antiretroviral drug failure

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BACKGROUND: Accumulation of thymidine analogue mutations (TAMs) should be extensive in children due to prolonged use of zidovudine/stavudine (AZT/d4T) and non-suppressive antiretroviral drug (ARV) combinations. Data on the frequency of TAMs in children is rare, especially in developing countries. Our objective was to describe the most frequent TAM patterns in Mexican children failing two or more ARV combinations from three tertiary care centres in Mexico City.

METHODS: We included 29 genotypic tests (Viroseq 2.0, Celera Diagnostics) performed in our referral laboratory from three pediatric AIDS clinics in Mexico City. All children had two or more ARV combinations. Patterns of TAMs were correlated to gender, age and time on ARV treatment, AZT/d4T exposure and resistance to tenofovir (TDF) according to the Stanford algorithm.

RESULTS: Resistance tests results from 29 children (16 male and 13 female) with an average age of 10.28 years (2–17) were included. Mean time on ARV treatment was 7 years (1–12.8) while time on AZT/d4T was 6.8 years (1–12.8). Eleven (38%) children had two or more than three ARV combinations and seven (24%) had three. Eight children had TAM pattern 1 (27.5%) and six pattern 2 (20.7%), with an average time of AZT/d4T exposure of 7.78 and 7.38 years, respectively. Of the children with TAM 1, five (62.5%) were male, 87.5% were older than 10 years and only one (12.5%) fulfilled criteria for TDF resistance. Of those with TAM 2, four (66.6%) were female, 66.6% were older than 10 years and all had TDF resistance. No difference in the number of ARV combinations were found between TAM 1 and TAM 2 cases. One child had the Q151M multi-resistance complex. Of the other 14 children, eight had no TAMs and six had 1 to 5 TAMs with no specific pattern, with an AZT/d4T exposure of 5.7 and 7 years, respectively. (P<0.01 between cases with no TAMs and all cases with any TAM).

CONCLUSIONS: TAM development in children is significantly related to the time on AZT/d4T exposure. Pattern 1 is more frequent in males and not frequently related to TDF resistance, while pattern 2 is more frequent in females and always correlate with TDF resistance.
ABSTRACT 133

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Class-wide resistance to antiretroviral drugs is predictive of shorter survival in patients who achieved undetectable HIV RNA after treatment failure

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BACKGROUND: Observational data published by our group demonstrated that class-wide resistance (CWR), particularly if extended to ≥1 drug class, is predictive of faster progression to AIDS and death up to 6 years after treatment failure and genotype resistance test (GRT). Moreover, significant lower risk of progression was observed in patients who achieved viral suppression after GRT. Building on these results, the aim of this analysis is to evaluate which factors may influence disease progression after viral suppression.

METHODS: Patients who underwent GRT between 1999 and 2007 were enrolled at first HIV RNA <50 copies/ml after GRT and followed over time; time to death or new AIDS event were considered study endpoints. Cox model was used for multivariable hazard assessment. Viral rebound as a time-dependent covariate was tested if predictive of progression. Time updated CD4+ T-cell count was added to the final model to stress if the effect over progression was even independent from immunological recovery. CWR was defined as ≥3 main mutations for nucleoside reverse transcriptase inhibitors and protease inhibitors and ≥1 for non-nucleoside reverse transcriptase inhibitors.

RESULTS: Among 1,392 treatment-failing patients, 774 (56%) achieved viral suppression and were included in the analysis: 368 (48%) experienced a subsequent viral rebound. No CWR at previous GRT was found in 52%, 1 CWR in 32% and ≥2 CWR in 16% of patients. After a median of 33 months (interquartile range 19–53), 50 patients experienced a new AIDS event or death. Time-dependent viral rebound was associated with higher risk of clinical progression (hazard ratio [HR] 2.1, 95% confidence interval [CI] 1.1–4.3) suggestive of longer survival related to longer viral suppression. Detection of ≥2 CWR was associated with faster progression (HR 2.5, 95% CI 1.1–5.7). Time updated CD4+ T-cell count, added to the model, was associated with highly significant progressive improvement of survival (up to 12-fold for >50 CD4+ T-cell recovery); ≥2 CWR (HR 2.9, 95% CI 1.3–6.6) was still associated with faster progression together with pre-GRT AIDS diagnosis and older age.

CONCLUSIONS: Our findings suggest that extended resistance to >1 drug class could be overcome by GRT-guided therapy but remains a strong marker of HIV progression, even in patients who achieve viral suppression. Thus, extended class resistance is a marker of advanced disease and should be prevented in order to improve survival.
Response to treatment and resistance patterns in HIV-2-infected patients living in Bissau, West Africa

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BACKGROUND: High rate of virological failures, lower CD4+ T-cell recovery and increased frequency of multi-nucleoside resistance were observed in HIV-2 patients exposed to highly active antiretroviral therapy (HAART) compared with HIV-1. Most of this information derives from small series of HIV-2 patients followed in Western countries and few studies have been conducted in Africa where HIV-2 is endemic. Herein, we describe the outcome in a cohort of HIV-2 patients who initiated HAART in Bissau.

METHODS: Twenty-two HIV-2-positive and seven HIV-1/2-positive patients attending a clinic in Bissau initiated HAART as part of the DREAM program and were followed for 12 months. Plasma HIV-1/2 RNA was retrospectively measured using EasyQ. Protease and reverse transcriptase (RT) were amplified from plasma HIV-2 RNA using specific primers and sequenced for genotypic HIV-2 resistance mutations.

RESULTS: All patients carried HIV-2 subtype A. Most HIV-2-positive patients (15/22, 68%) and almost half (3/7, 43%) of HIV-1/2-positive patients were treated with zidovudine+lamivudine+abacavir as first-line therapy. The rest received zidovudine+lamivudine+nevirapine or abacavir+didanosine+indinavir. Mean baseline plasma HIV RNA was 4.06 log (range 1.9–6.36) and 5.39 log (range 3.9–6.6) in HIV-2-positive and HIV-1/2-positive patients, respectively. Higher mean viral load (VL) in HIV-1/2-positive versus HIV-2-positive patients was most likely due to the HIV-1 RNA contribution. Some HIV-2-positive patients (68%) never reached undetectability and 19/22 (86%) experienced virological failure at 12 months of therapy. On the contrary, 71% of HIV-1/2-positive patients reached and maintained undetectable VL at 12 months; their mean VL drop was 3.39 log (range 2.56–4.51). However, two of these HIV-1/2-positive patients failed therapy thereafter maintaining low-level viraemia (∼2.6 log). As expected, most failures in HIV-2-positive (84%) and HIV-1/2-positive patients (75%) selected M184V in HIV-2-RT. Interestingly, K65R also emerged in three patients (two failing zidovudine+lamivudine+abacavir and one abacavir+didanosine+indinavir). In contrast, none selected Q151M. Protease mutations I82F or L90M were selected in 3/5 HIV-2-positive patients failing indinavir.

CONCLUSIONS: Complete viral suppression of HIV-2 in monoinfected or coinfected patients is not readily achieved and sustained over 1 year with triple antiretroviral regimens and drug resistance develops rapidly. In this study, K65R was selected in the absence of tenofovir in patients failing abacavir+/-didanosine. Selection of potent drug combinations and treatment strategies for HIV-2 merits a differential consideration from HIV-1.
SESSION 6
Epidemiology
Evaluation of transmitted HIV drug resistance using the WHO HIV drug resistance threshold survey model in four Central African countries

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BACKGROUND: The rapid scale-up of antiretroviral therapy (ART) programs in resource-limited settings based on standardized simplified treatment and monitoring protocols is raising concern about the rapid transmission and spread of HIV drug resistance (HIVDR) mutations among newly infected patients. We evaluated the prevalence of transmitted HIVDR in four Central African countries: Chad, Cameroon, Congo and the Central African Republic (CAR).

METHODS: The World Health Organization (WHO) minimum-resource method – termed the HIV drug resistance threshold survey (HIVDR-TS) – was adapted to each country and program reality. Major treatment areas were selected as study sites: Chad (N’Djamena), Cameroon (Yaoundé and Douala), Congo (Brazzaville), and CAR (Bangui). Pregnant women aged <25 years, at first pregnancy, and HIV-positive were enrolled in each site in 2006–2007 and eligible plasma samples sent to the designated regional genotyping laboratory in Cameroon for genotypic testing. The WHO list of mutations for surveillance of transmitted HIVDR was used to identify relevant HIVDR mutations in the protease and reverse transcriptase, and prevalence results for each drug class were categorized as <5%, 5–15% or >15%.

RESULTS: Up to 47 eligible samples were obtained and genotyped from three sites: N’Djamena, Yaoundé and Douala. Samples from Brazzaville and Bangui were excluded because they did not meet eligibility criteria. No drug resistance mutation as per the WHO list was found in N’Djamena and the HIVDR prevalence for all drug classes were categorized as <5%. Two individuals from Yaoundé harboured virus with drug resistance mutations, D67D/N and K103N, respectively, and the HIVDR prevalence was categorized as low <5% for all drug classes. Also, in Douala we found two individuals carrying virus with drug resistance mutations, one with M184V+K101E+G190A and the second with D67D/N.

The frequency of HIVDR transmission in Douala was then categorized as low <5% for protease inhibitors and non-nucleoside reverse transcriptase inhibitors, and moderate 5–15% for nucleoside reverse transcriptase inhibitors. CRF02 (70%) and CRF11_cpx (30%) predominate in Cameroon and Chad, respectively, but co-circulate with many other HIV-1 variants.

CONCLUSION: Although we found a moderate prevalence in Douala, the overall prevalence of transmitted HIVDR among newly infected individuals was low in Chad and Cameroon. This study also highlighted the difficulties of carrying out the WHO generic protocol in field reality.
ABSTRACT 136

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Are patients with acute HIV-1 infection the major source of HIV transmission in France? A 5-year survey in the ANRS PRIMO Network

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BACKGROUND: In France, the stable frequency of HIV-1 transmitted drug resistance in patients diagnosed at the time of primary infection (PHI) over the past decade might be explained by the increasing proportion of chronically treated patients in virological success, who have a low risk of HIV transmission (Chaix et al, HIVWDR, 2007). Here, molecular epidemiological surveillance was used to characterize the HIV transmission chain in France between 2003 and 2007.

METHODS: We included 689 patients diagnosed at the time of PHI (<6 months) from all over France. Phylogenetic analysis of corresponding reverse transcriptase sequences was performed to identify the degree of linkage between patients. Bootstrap values above 950/1,000 were considered significant.

RESULTS: The phylogenetic tree of sequences from 689 viruses revealed 69 clusters including 209 patients (30% of the population). These included 200 men (171 [82%] men who have sex with men [MSM], 23 heterosexual, six with unknown risk factors) and nine heterosexual women. The number of individuals/cluster ranged from 2 to 8: 2 patients (n=35 clusters), 3 (n=13), 4 (n=12), 5 (n=4) 6 (n=4), and one cluster of 8 patients. Eighty-percent of clusters gathered individuals living in the same geographical area. Sixty-two clusters included patients whose PHI occurred within a two-year interval and seven clusters included patients whose PHI date differed from >2 years (one cluster of 2 patients, one of 3 patients, one of 4 patients, three of 5 patients, and one cluster of 8 patients. Sixty-five clusters included B viruses, while three clusters of CRF02_AG viruses were evidenced including 2 patients (2 cases) and 3 patients (1 case). A cluster of 6 MSM infected in Paris in 2006–2007 by a unique recombinant (URF) BC virus suggests a rapid spread of this URF within MSM in the Paris area.

CONCLUSION: At least 30% of PHI in France form phylogenetic clusters, indicating that these individuals may contribute to HIV transmission. A better understanding of HIV transmission dynamics is important to design effective preventive and public health interventions, especially for improving both HIV diagnosis of PHI and chronic infections.
ABSTRACT 137
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Epidemiological trends in genotypic resistance mutations in relation with antiretroviral exposure
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BACKGROUND: Few data on class resistance-associated mutation (RAM) trends in relation to antiretroviral exposure in clinical cohorts are available. Our aim was to describe epidemiological trends of class resistance and RAM patterns as well as their association with drug exposure among HIV-positive patients failing antiretroviral treatment.

METHODS: Catalonia HIV Resistance Network (CHRN) is a centralized database that collects data from all genotypic tests performed since 2002 in HIV-positive patients from Catalonia. We analysed data of adult patients with virological failure (VF > 1,000 copies/ml) that were tested at least once from 2002 to 2005. Mutations were analysed according the International AIDS Society USA (IAS-USA) guidelines (2006). Analysis was performed using Mantel’s χ² test for trend.

RESULTS: Of 2,098 patients (mean age 41.1 years; range 18.2–81.8), 74.5% were male and 37.1% fulfilled AIDS criteria. Median CD4+ T-cell count was 307.5 (interquartile range [IQR] 169.3–483) and median HIV-1 RNA was 13,500 (IQR 3,900–58,925). Prevalence of class resistance dropped from 83.0% and 57.0% in 2002 to 72.8% and 46% in 2005 among those exposed to nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), respectively (P < 0.05 and P = 0.08), but remained stable over time for non-nucleoside reverse transcriptase inhibitors (NNRTIs) (69.4%). Also, triple-class antiretroviral drug resistance decreased over time (30.2% to 15.8%; P<0.001). A significant decrease in the prevalence of some specific thymidine-associated mutations (TAMs) was found: T215YF (49.6% to 37.8%), M41L (48.1% to 30.3%), L210W (30.5% to 18.6%). A rising trend over time in the prevalence of K65R was seen, increasing in 2003 (7.4%) and 2004 (6.8%) with respect to 2002 (3.6%), but decreasing again in 2005 (4.3%; P-value 0.2). This is consistent with an observed decrease in exposure to tenofovir disoproxil fumarate in 2005 (2002 [12.2%],

CONCLUSION: In our cohort, triple-class drug resistance dropped almost by half over time and NRTI class resistance decreased at the expense of TAMs. Reduction in specific mutations are related to changes in antiretroviral use. These results may reflect the efficacy of new drugs, new regimens and/or changes in drug prescription.
ABSTRACT 138

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Virological characterization of HIV strains in recent seroconverters in Spain

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BACKGROUND: Transmission of drug-resistant HIV has reached a plateau and might be declining slightly in developed countries. However, the virological characterization of HIV seroconverters may provide crucial information beyond drug resistance surveillance.

METHODS: All consecutive recent HIV seroconverters (<12 months from exposure) identified from January 1997 to December 2007 at 17 different hospitals distributed across Spain were examined. Major drug resistance mutations associated with transmission [1], HIV subtypes and estimation of co-receptor usage using V3 sequences were analysed.

RESULTS: A total of 504 seroconverters were identified. The median time for HIV exposure was 7 months and 71.5% were men who had sex with men (MSM). At the time of diagnosis, median plasma HIV RNA was 4.6 logs and median CD4+ T-cell count was 564 cells/µl. Drug resistance mutations were recognized in 13.8%, 9.3% being for nucleoside reverse transcriptase inhibitors (NRTI), 5.7% for non-nucleoside reverse transcriptase inhibitors (NNRTI) and 3.3% for protease inhibitors (PI). None carried resistance mutations to enfuvirtide nor integrase inhibitors. Resistance mutations to two and three drug families was seen in 2.4% and 1%, respectively. The prevalence of drug-resistant HIV differed in distinct periods: 29% (1997–1999), 5.3% (2000–2001), 10.7% (2002–2004) and 15.1% (2005–2007) (P=0.02). Whereas 9.3% of seroconverters harboured NRTI-resistant mutations, K65R, L74V and M184V were almost absent, their prevalence being 0, 0 and 0.4%, respectively. In contrast, thymidine analogue mutations (TAMs) were common, T215 revertants and M41L being the most prevalent (5.3% and 3%, respectively). Transmission of NNRTI- and PI-resistant HIV was more frequent in the last period, being seen in 7% and 4.4%, respectively. Furthermore, five patients have acquired HIV with resistance mutations to all three drug families during the past 3 years. Some 47 individuals (9.6%) carried non-B subtypes (11CRF14_BG, 9CRF02_AG, 7CRF12_BF, 6f, 5C, 4G, 3BD, 1CRF01_AE and 1A). Interestingly, they were only recognized since 2001, but represented 15.4% in the last period. No cases of transmission of HIV-2 nor HIV-1 group O were identified, despite circulation of these variants in Spain. X4 or X4R5 viruses were recognized in 16.8% of this population, being more common among intravenous drug users than MSM (36.8% versus 26.5%; P<0.05). No association was noticed with drug resistance mutations.

CONCLUSION: The rate of drug-resistant HIV has slightly increased since 2005 in Spain, being currently 15.1%. PI- and multidrug-resistant viruses have become more frequent in recent years. The presence of X4 viruses does not seem to be associated with transmission of drug resistance mutations.

REFERENCE:
ABSTRACT 139

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The prevalence of multidrug resistance among treated patients in North America and its impact on mortality

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OBJECTIVE: Recent data indicates that the incidence of multidrug failure in clinical practice has declined over the past 10 years. The degree to which the prevalence of multidrug resistance (MDR) has changed over time remains undefined. In a US and Canadian collaboration, we investigated the prevalence of MDR over time in patients who received highly active antiretroviral therapy (HAART).

METHODS: Eight clinical practice-based HIV cohorts contributed data on patients who had initiated HAART. We identified all those who had had two-class or three-class resistance. Resistance was based on the Stanford Genotypic Resistance Interpretation Algorithm and was defined by intermediate level or greater scores.

RESULTS: In 2000, 3,451 patients had a viral load (VL) measured: 1,591 (46%) had VL>1,000/ml and 829 (25% of those with VL>1,000/ml) had genotypic results. In 2005, 2,894 patients had a VL measured: 1,004 (35%) had VL>1,000 copies/ml and 714 (71% of those with >1,000/ml) had genotypic results. The percentage of individuals with resistance to at least one drug in at least two classes declined over time (50% and 33% in 2000 and 2005, respectively; \( P<0.001 \)). The percentage of individuals with resistance to at least one drug in all three classes also declined (24% and 16% in 2000 and 2005, respectively; \( P<0.001 \)). There was, however, no change over time in the frequency of individuals with resistance to all drugs in all three drug classes (9% and 8% in 2000 and 2005; \( P=0.32 \)). Using pan-drug resistance within each class as a predictor, the mortality rate 6 years after the first genotypic test was 12%, 25% and 28% for those with resistance to protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and both PIs and NNRTIs, respectively. Those with wild-type HIV during failure had a 28% mortality at year 6.

CONCLUSION: The prevalence of limited class resistance declined over time while the prevalence of high-level MDR remained stable, at least through 2005. The overall mortality rates in those with resistance was high; this was particularly true for those with NNRTI resistance. Individuals who had no evidence of resistance at the time of genotypic testing also had high mortality rates, presumably as this was a marker of non-adherence.
ABSTRACT 140
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Prevalence of resistance mutations in antiretroviral-naive chronically HIV-infected patients in 2006/2007: a French nationwide study

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BACKGROUND: Our aim was to estimate the prevalence of transmission of drug-resistant HIV-1 variants in antiretroviral-naive chronically HIV-1-infected patients as part of the French ANRS program of HIV-1 primary resistance surveillance.

METHODS: Resistance mutations were sought in samples from 530 newly diagnosed HIV-1-infected patients seen in 31 French AIDS centres from October 2006 to March 2007. The duration between seropositivity and inclusion was <6 months, 6 months to 2 years, 2–5 years and >5 years for 56.2%, 16.1%, 19.7% and 8% of the patients, respectively. According to CDC classification, 12.3% of the patients were at disease stage C. The protease and reverse transcriptase (RT) genes were sequenced and mutations associated with resistance identified from the 2007 Stanford Resistance Surveillance list [1].

RESULTS: At inclusion, median CD4+ T-cell counts and plasma HIV-1 RNA were 352/mm³ (interquartile range [IQR] 242–517) and 4.5 log copies/ml (IQR 4.0–5.0), respectively. Forty-two percent of patients were infected by non-B subtype strains (CRF02 18.3%). The overall weighted prevalence of viruses with RT or protease drug resistance mutations was 9.6% (95% confidence interval [95% CI] 5.9–15.5) and was not statistically different according to the duration of seropositivity (<6 months 9.8%, 6 months to 2 years 11.3%, 2–5 years 10.9% and >5 years 1.8%; P=0.64). The weighted prevalence of protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance-associated mutations was 4.4% (95% CI 1.7–11.1), 5.1% (95% CI 2.4–10.5) and 2.6% (95% CI 1.0–6.5), respectively. Resistance to one and two classes of antiretroviral was observed in 7.1% and 2.5% (PI+NRTI 2.2%). Frequency of resistance was not different in patients infected with B (9.5%) and non-B (CRF02 7.8% and other 11.2%) subtypes, P-value=0.93. Baseline characteristics such as gender, age, transmission group, country of transmission, disease stage, CD4+ T-cell count and viral load were not associated with the prevalence of transmitted drug resistance.

CONCLUSION: In France in 2006/2007, the prevalence of transmitted drug-resistant variants was around 10%. The proportion of non-B subtypes increased from 33% in 2001 to 42% in 2006/2007. Interestingly, prevalence of transmitted drug resistance was comparable in B and non-B subtypes. Prevalence of resistance was similar according to the 2007 International AIDS Society (IAS) list of mutations without taking into account etravirine resistance mutations.

REFERENCE:
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Profiling resistance-related mutations according to the clade assignment in a Brazilian population

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BACKGROUND: A significant portion of the HIV-infected population in Brazil is highly exposed to antiretrovirals, where three distinct HIV-1 clades co-circulate: B, F and C. This study analyzes the resistance-related mutations and pathways to resistance in a well-characterized group of patients failing on antiretrovirals.

METHODS: The pol sequences from 2,474 individuals failing on antiretrovirals from samples collected in Sao Paulo, Brazil from 2002 to 2006 were generated and analysed according to antiretroviral exposure.

RESULTS: Of the patients analysed, 17% were experiencing their first virological failure, 33% their second, and 50% their third or more. Of all patients, 78.3% were clade B, 86% F, 8.2% B/F recombinants, 4.3% C, and 0.5% B/C. Overall, 89.7% of individuals presented resistance to nucleoside reverse transcriptase inhibitors (NRTI), 62.5% to non-nucleoside reverse transcriptase inhibitors (NNRTI), and 67.6% to protease inhibitors (PI), although <1% has three or more darunavir mutations. Wild-type strains were found in 5% of patients, whereas 21% presented resistance to one antiretroviral class, 49% to two classes, and 34% to three classes. Clade B viruses presented less PI mutations than clade C or F, clade C presented less NRTI mutations than clade B or F, whereas clade F presented less NNRTI mutations than clade B. Clade B viruses presented more of a thymidine analogue mutation 1 (TAM1) profile, whereas clade C strains presented more of a TAM2 profile, and clade F viruses presented the same prevalence of both. Two pathways for resistance to NNRTI were identified: patients failing to nevirapine presented more frequently Y181C, K101E, G190A mutations, whereas patients failing to efavirenz presented K103N, L100I, P225H. Viral loads increased proportionally according to the number of resistance mutations.

CONCLUSIONS: Resistance mutations were differently selected in distinct clades in Brazil. According to the TAM profile, cross-resistance to tenofovir will be more prevalent in clade B than in C. It can be predicted that failures to nevirapine will result in more cross-resistance to etravirine than failures to efavirenz, according to the resistance pathway described above. Using viral load as a viral fitness surrogate marker, there is an initial decrease of fitness from selection of resistance mutations, but the viral fitness is restored as the number of mutations increases.
Prevalence and patterns of antiretroviral drug resistance at low plasma HIV RNA load levels

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BACKGROUND: The yield and clinical utility of resistance testing at low viral load (VL) remain uncertain. The study aim was to characterize the population undergoing resistance testing at VL<1,000 copies/ml (‘low-VL group’), to analyse the prevalence and patterns of resistance according to the VL, and to identify risk factors for the detection of resistance.

METHODS: Genotypic resistance results with linked clinical data were obtained from the UK HIV Drug Resistance Database and CHIC Study. Major resistance mutations were scored by the International AIDS Society USA (IAS-USA) list (August to September 2007).

RESULTS: Of the resistance tests recorded in 1999–2005, 781/6766 were performed at low-VL and the relative proportion increased over the years. Test success rate in the centres providing specialized low-VL testing was >87%. Overall, the low-VL group was similar to the whole cohort, but was more likely to have previously achieved a VL<50 copies/ml (75.4% versus 56.5%; P=0.002). Treatment regimens included ≥2 nucleoside reverse transcriptase inhibitors (NRTIs) + non-nucleoside reverse transcriptase inhibitor (NNRTI) (34.1%), ≥2 NRTIs + ritonavir-boosted protease inhibitor (PI/r) (24.6%), 3 NRTIs (16.8%), ≥2 NRTIs + PI (12.4%) or other combinations (5.5%). Years of therapy were 0–3 in 32.8%, 3–6 in 31.6%, 6–9 in 22.3% and >9 in 13.3%; 28.9%, 32.5% and 21.8% of individuals had experienced failure of 1–3, 4–6 and ≥6 drugs, respectively. In the entire cohort, the prevalence of any resistance ranged between 51% and 81%, was highest at VL>1,000–10,000, 77% at VL>300–1,000 and 63% at VL<300. Protease mutations were detected less frequently than reverse transcriptase mutations, and among PI-treated persons their prevalence was 26% at VL<300 and 44% at VL>300–1,000. Protease mutations were detected less frequently than reverse transcriptase mutations, and among PI-treated persons their prevalence was 26% at VL<300 and 44% at VL>300–1,000. In the multivariable analysis, the relative risk (95% confidence interval) for the detection of resistance was 0.91 (0.84–0.98), 0.99 (0.94–1.05), 1.03 (0.99–1.07), 0.93 (0.89–0.97), 0.86 (0.82–0.91), and 0.72 (0.68–0.77) at VL<300, 300–1,000, >3,000–10,000, >10,000–30,000, >30,000–100,000 and >100,000, relative to VL>1,000–3,000, respectively. Independent predictors of

the detection of resistance were earlier calendar year of test, use of NNRTI-containing regimens, increasing number of previously failed drugs, and never reaching a VL<50 copies/ml.

CONCLUSIONS: The likelihood of detection of resistance mutations was highest at VL>300 copies/ml and up to 10,000 copies/ml. Detection remained significant, although reduced, at VL<300 copies/ml and declined progressively as the VL increased >10,000 copies/ml. Resistance testing at low-VL yields useful information.
ABSTRACT 143
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Injection drug users have a higher probability of developing resistance than non-injection drug users regardless of adherence or initial treatment regimen

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BACKGROUND: We previously reported a slightly increased risk of HIV drug resistance to antiretrovirals for drug-naive people with a history of injection drug use (IDU) compared with non-IDUs during a 30 month follow-up period after initiation of highly active antiretroviral therapy (HAART) [1]. Here, we more closely investigate patterns of emergence of resistance after initial HAART between IDUs and non-IDUs in a larger cohort followed for a longer period (median follow-up 4.8 years).

METHODS: Logistic regression was used to model antiretroviral resistance for drug-naive patients initiating HAART between August 1996 and November 2004, followed until November 2005 (n=2,350 individuals; 6,066 genotypes). Baseline factors included viral load, CD4+ T-cell count, age, gender, history of IDU, adherence, year of therapy initiation, and type of initial regimen (non-nucleoside reverse transcriptase inhibitor [NNRTI], protease inhibitor [PI] or boosted-PI). Individual resistance categories were lamivudine, nucleoside reverse transcriptase inhibitor (NRTIs) (excluding lamivudine), NNRTIs and PIs. Adherence was measured by prescription refill percentage in the first year of therapy and was stratified into four groups: 0–<40%, 40–<80%, 80–<95% and ≥95%. To further investigate the relationship between adherence and resistance for IDUs (n=642) and non-IDUs, untimed plasma drug concentrations (UDL) from a subset of individuals who began therapy between August 1996 and September 1999 (n=751) were used as an independent marker of adherence.

RESULTS: Overall, IDUs had a significantly higher probability of developing any resistance than non-IDUs across all adherence strata, even after adjusting for baseline factors (odds ratio [OR] 1.71; 95% confidence interval [CI] 1.39–2.11). Differences in adherence as measured by prescription refill and/or UDL could not account for this difference. The observation of higher risk of developing resistance for IDUs was consistent for both males and females and independent of type of initial regimen. The probability for resistance to each individual drug category was consistently around 1.5–1.6-fold higher in IDUs, except for the NRTIs excluding lamivudine (OR 1.11; 95% CI 0.82–1.50). The probability for resistance to >1 drug category was 1.5-fold higher in IDUs (95% CI 1.15–1.89).

CONCLUSIONS: Even after accounting for baseline parameters and adherence, the estimated probability of developing resistance was higher among IDUs than non-IDUs across all prescription refill and UDL strata (with the exception of resistance to NRTIs other than lamivudine).

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ABSTRACT 144
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Virological outcome and antiretroviral drug resistance in the national antiretroviral therapy program in Cameroon

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BACKGROUND: Antiretroviral therapy (ART) services are growing rapidly in Africa, but virological monitoring is still not feasible for the majority of antiretroviral (ARV)-treated patients. Most people will stay on a virologically failing regimen for longer periods, and rapid or uncontrolled emergence of HIV drug resistance (HIVDR) is feared as a consequence of ART scale-up. Our objective was to evaluate the virological outcome in patients treated according to the national guidelines in a routine HIV/AIDS outpatient clinic in Yaounde, Cameroon.

METHODS: Between November 2006 and October 2007, patients attending the Central Hospital for their medical visit at month 12 and month 24 were consecutively enrolled. A blood sample was taken for serotyping (HIV-1 M, N or O) and viral load testing. Samples with viral load (VL) > 1,000 were tested for genotypic drug resistance by sequence analysis.

RESULTS: At month 12 and month 24, respectively, 249 (176 female, 73 male) and 178 (130 female, 48 male) adults were enrolled. All patients were ARV-naive at ART start. First-line regimens included lamivudine+stavudine/ zidovudine+nevirapine/efavirenz for 418/429 patients. A total of 41 (16.4%) and 40 (22.5%) had VL > 1,000 copies/ml at month 12 and month 24, respectively. Drug resistance mutations were identified in 11 (4.4%) and 28 (16.3%) patients at month 12 and month 24, respectively; 34 were resistant to at least one nucleoside reverse transcriptase inhibitor (NRTI) + non-nucleoside reverse transcriptase inhibitor (NNRTI), one to NRTI only, four to NNRTI only. The 35 NRTI-resistant patients all harboured the M184V mutation conferring resistance to lamivudine, and seven also harboured thymidine analogue mutations (TAMs). An accumulation of NNRTI mutations over time was seen: a single mutation only in all patients at month 12 versus 13/27 (48%) with two or three major mutations at month 24. Virological failure and drug resistance was associated with previous treatment switch or non-adherence: 51/357 (14.3%) versus 29/70 (41%) (P<0.001) for VL > 1,000 and 22/357 (6.2%) versus 19/70 (27.1%) (P<0.001) for drug resistance. Overall, 40/81 (49.3%) patients with VL > 1,000 harboured resistant strains and 36/51 (70.1%) with VL > 10,000.

CONCLUSIONS: Virological failure and drug resistance were relatively low, however, 69% and 18% of the patients with HIVDR mutations were resistant to two or three drugs, respectively, from their regimen. Importantly, only 1/4 and 3/4 of VL failure were associated with drug resistance at month 12 and month 24, respectively. This study underlines the importance of VL but highlights the risk of switching to second-line treatments too early in non-adherent patients in the absence of genotypic drug resistance testing.
ABSTRACT 145
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Predicting the effects on resistance of lack of viral load monitoring in resource-limited settings

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BACKGROUND: The World Health Organization (WHO) recommends a population-based approach to antiretroviral therapy (ART) with switch to second-line regimens based on clinical status and, where available, CD4+ T-cell count rather than viral load. It is important to study the potential consequences of such monitoring strategies in terms of resistance development.

METHODS: A validated computer simulation model of HIV infection and the effect of ART (HIV Medicine, 2007) was used to compare resistance development, use of second-line regimens and survival resulting from different strategies (based on viral load [switch if >500] or clinical observation alone [switch if multiple new WHO stage III or new WHO stage IV clinical symptoms]; CD4+ T-cell count monitoring was also considered, but not included here for space reasons) for determining switch to second-line ART (with lopinavir/ritonavir [LPV/r], didanosine [ddI], zidovudine [ZDV]), for people starting ART with the WHO first-line regimen of stavudine (d4T)/lamivudine (3TC)/nevirapine. Resistance mutations are modelled as the number of thymidine analogue mutations, the presence of M184V, K65R, L74V, non-nucleoside reverse transcriptase inhibitor mutations, and specific protease inhibitor mutations.

RESULTS: The clinical switch criteria was met a median >4 years after the viral load switch criteria. The predicted percent of person-time with presence of resistance over 5 years from start of ART differed little according to viral load/clinical monitoring strategy (20/20, 20/20, 8/9, 1/2 and 0/0, for nevirapine, 3TC, d4T/ZDV, ddi and LPV/r, respectively), the number of active drugs in the second-line regimen was 2.71/2.37. There was a greater difference between monitoring strategies when considering the percent of person-time with the presence of resistance AND viral load >1,000 copies/ml, and hence potentially infectious with resistant virus (10/16, 10/15, 4/8, 1/1 and 0/0). The predicted percent of potential life-years survived was 83% for viral load monitoring compared with 82% for clinical monitoring.

Findings were robust to variations in parameter values in multivariable sensitivity analyses (difference between viral load and clinical strategies in percent person-time with resistance to nevirapine and viral load >1,000: median 4%; 95% range 1–13% over 10,000 runs).

CONCLUSION: For patients on the first-line regimen of stavudine, lamivudine and nevirapine, the benefits of viral load monitoring compared with clinical (or CD4+ T-cell count) monitoring are relatively modest. Development of cheap and robust versions of these assays is important, but widening access to ART (with or without laboratory monitoring) is currently the highest priority.
ABSTRACT 146

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Changes in regional prevalence, clade and epidemiology of HIV-1 drug resistance mutations and clade among antiviral therapy-naive patients in the United States from 2000 to 2007

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BACKGROUND: Pre-existing HIV drug resistance associated mutations (RM) may jeopardize first-line antiretroviral therapy (ART) success. Changes in overall RM, drug class, subtype prevalence and geographical distribution were analysed over time for HIV-infected, ART-naive patients seeking ART treatment from 2000–2007.

METHODS: HIV population-based genotypes and subtypes were analysed from 3,542 ART-naive patients from 36 states (and District of Columbia) enrolling into clinical trials. RM were analysed by 2007 International AIDS Society USA (IAS-USA) and by Stanford ‘surveillance’ definitions. Overall prevalence and prevalence in 2000 and 2007 is presented (range is shown in brackets if different).

RESULTS: Prevalence of major IAS-USA RM was 17% (2000/2007 9/20%). By drug class, nucleoside reverse transcriptase inhibitor (NRTI) was 4% (2000/2007 3/4% [range 3–6%]), non-nucleoside reverse transcriptase inhibitor (NNRTI) 13% (2000/2007 6/15%), and protease inhibitor (PI) 3% (2001/2007 2/3% [range 2–5%]). By Stanford definitions, overall RM was 12% (2000/2007 5/13% [range 5–14%]). Subsetting RM by class, NRTI was 5% (2000/2007 4/5%) [range 3–7%], NNRTI 7% (2000/2007 2/8%), and PI 3% (2001/2007 2/3% [range <1–5%]). By either list, dual-class resistance was 2% (range <1–4%); overall 2000–2007 triple-class resistance was <1% (IAS-USA 16/3, 542; Stanford 15/3, 542). By geographical region, the overall IAS-USA RM for the south was 17% (2000/2007 9/18% [range 9–19%]), northeast 15% (2000/2007 8/24% [range 4–24%]), midwest 15% (2000/2007 14/18% [range 5–20%]), and west 20% (2000/2007 10/32% [range 9–32%]). By Stanford definitions, overall RM 2000–2007 was 12%, 10%, 10% and 13% for the south, northeast, midwest and west, respectively. By race, overall RM prevalence was similar (IAS-USA 16–19%; Stanford 10–14%). The cohort (n=3105, 2001–2007) was 95.3% subtype B, 1.3% subtype C, 1.77% indeterminate, 0.55% subtype G, 0.45% subtype A and <1% chimeric mixes (B with A, D, F or G and A/G). Non-clade B increased from 4.7%–7.9% from 2001 to 2007.

CONCLUSIONS: Although some have predicted that prevalence of HIV RM in ART-naive subjects could be on the decline, in this large US cohort, RM increased from 2000 to 2007, using either IAS-USA or Stanford definitions; the greatest increase was observed for NNRTI RM (15% of subjects in 2007 by IAS-USA). Regional differences in RM prevalence were observed, with the highest in the south and west. Detection of dual-class RM increased slightly over time, although triple-class RM were rare (<1%). Subtype B is still the most common subtype, although non-clade B subtypes increased slightly from 2001 to 2007.
Temporal trend in non-nucleoside reverse transcriptase inhibitor (NNRTI) transmitted drug resistance: relationship with chronically infected potential transmitter population

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BACKGROUND: Transmission of drug-resistant (DR) variants is influenced by the mean levels of viral load (VL) in chronically infected (CI) patients, by sexual behaviour and by intravenous (IV) drug use. Using phylogenetic analysis, we have reported high rates for onward transmission of variants acquired in recent/primary HIV infection (PHI). To further evaluate the relative contribution of the different phases of HIV infection in transmission, this study detailed DR profile changes in the CI population, representing potential transmitters, to newly infected PHI patients.

METHODS: Clinical and virological data were obtained from PHI patients (n=231, <170 days of infection) and CI patients (n=3,402) followed at l’Actuel, a large medical clinic in Montreal. The VL, sociodemographic and risk determinants for HIV acquisition were compared in these two groups. The temporal trends of DR mutational frequency were assessed during three time periods: period I, 1996 to 1999; period II, 2000 to 2003; and period III, 2004 to 2007.

RESULTS: The distribution of men who have sex with men (MSM), heterosexuals and IV drug users was similar over time for the PHI and CI populations (70% versus 71%, 20% versus 17%, 10% versus 12%, respectively). For PHI patients, we observed a marked decrease in the frequencies of transmission of mutations conferring resistance to nucleoside reverse transcriptase inhibitors (NRTIs) (14.0%, 1.8%, 3.2%), protease inhibitors (PIs) (5.1%, 1.8%, 0.0%) and multidrug resistance (MDR) (5.0%, 1.8%, 0.0%), contrasting with an increase in NNRTIs (2.6%, 5.5%, 6.3%) over time. For CI patients, the frequency of NRTIs (67%, 81%, 70%) and PIs (47%, 49%, 41%) were steady, contrasting with an increase in non-nucleoside reverse transcriptase inhibitors (NNRTIs) (18%, 41%, 49%) over time. In 1,279 CI patients harbouring DR, the mean VL decreased (3.6 ± 0.3, 3.0 ± 0.1, 2.5 ± 0.1 log copies/ml) over time.

CONCLUSION: In contrast to NRTIs and PIs, the transmission of variants conferring resistance to NNRTIs has increased over the past decade. This increase of NNRTI transmitted DR cannot be fully explained by the influence of the CI patients harbouring DR, as VL reached very low levels making transmission unlikely. Globally, these results indicate that transmission of DR variants may occur through clustering events observed during early stage infection, fuelling the DR epidemic.

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BACKGROUND: The transmission of drug-resistant HIV has become a critical epidemic in the world today. Surveillance reports from developed countries have shown that 10 to 20% of newly diagnosed HIV/AIDS patients harbour drug-resistant strains. In Japan, where nationwide surveillance has been in effect since 2003, the prevalence of drug-resistant HIV was 4.5% in 2004, one of the lowest rates in developed countries. Here, we summarize the results of a 5-year drug resistance surveillance study showing changes in prevalence and drug-resistance patterns in Japan.

METHODS: Plasma samples were collected from newly diagnosed HIV/AIDS cases from January 2003 to December 2007. HIV-1 protease and reverse transcriptase (RT) sequences were analysed and drug-resistance mutations were defined according to criteria of the International AIDS Society-USA drug-resistance chart and the mutation list proposed by Shafer and colleagues. Viral subtypes for each case were determined from the HIV-1 protease-RT sequence. Time of infection for each case was estimated by the BED assay.

RESULTS: Among 1,945 samples collected during the study period, 1,903 protease-RT sequences (1.3 kbp) were successfully analysed. The population sampled was predominantly male (93.5%), homosexual (71.5%), and carried subtype B (89.2%). Prevalences of drug-resistant cases in 2003, 2004, 2005, 2006 and 2007 were 4.5%, 4.2%, 4.5%, 6.3% and 7.7%, respectively. The mutations observed from 2003 to 2007 predominantly conferred resistance to nucleoside reverse transcriptase inhibitors (3.4–4.7%), for example, M41L, D67N, M184V and T215C/D/E/S. Few protease inhibitor resistant cases (0.3–1.5%) were observed, with M46I being the most commonly detected mutation (0.3–1.3%). The lowest prevalence of mutations during the study period was always among non-nucleoside reverse transcriptase inhibitor resistant cases (0.4–1.0%). Few multiclass resistance cases (0.4%) were observed. Among samples collected in 2007, nearly 30% were classified as recently infected. Drug resistance prevalence did not differ between recently infected (7.2%) and other cases (8.2%), but was slightly higher in heterosexual (10.5%) than in homosexual (7.5%) cases.

CONCLUSION: The prevalence of transmitted drug-resistant HIV cases in Japan increased 1.7-fold from 2003 to 2007. Continuous surveillance is required to understand the epidemiological status of this transmission and to consider strategies to prevent transmission of drug-resistant virus.
ABSTRACT 149

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Impact of clustering on the transmission of HIV-1 variants harbouring drug resistance

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OBJECTIVE: Phylogenetic clustering of half of all sequenced primary/recent infections (PHI) in Quebec is indicative of high rates of forward transmission at early disease stages [1]. We have now evaluated evolving trends in clustering between 2005 and 2007, as well as analysing the cumulative effect on transmission of drug resistance.

METHODS: HIV-1 pol subtype B sequence data from early stage infections (<6 months post-PHI) were obtained from the Quebec PHI cohort and the provincial genotyping programs (n=859, 1998–2007). Phylogenetic analyses determined sequence interrelationships and clustering. Primary infections were stratified as unique, small clustered (2–4 PHI/cluster) or large clustered (≥5 PHI/cluster) transmission events. The distribution of mutations conferring resistance to nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) were ascertained. Phenotypic assays determined drug susceptibilities of transmitted drug-resistant variants.

RESULTS: Clustering has increased from 49% to 56% from December 2005 to June 2007. Whereas the majority (95%, 403/423) of unique and small clusters identified pre-2006 represented dead-end transmissions, PHI in 21 large clusters represented growing transmission cascades that increased from 6.6 ±0.8 to 10.3 ±1.1 PHI/cluster (n=132 and 215, respectively). New unique and small clusters were also observed post-2006 (n=101 and 78, respectively). Viral variants harbouring NRTI mutations and, in particular, 215 revertants were less frequent in clusters (7.9% versus 3.4% versus 1.2% and 5.8% versus 1.7% versus 1.1% for unique, small and large clustered transmissions, respectively). By contrast, higher frequencies of NNRTI mutations (K103N/R and G190A) were observed in large (>5 PHI), as compared with small clusters (1–4 PHI), (12.1% versus 3.3%; P<0.0001). Viruses harbouring NRTI and complex PI mutations showed decreased susceptibilities to relevant antiretroviral drugs.

Of note, a large cluster included 27 infections harbouring the G190A mutation that was associated with >50-fold nevirapine resistance, efavirenz sensitivity, and >10-fold TMC-120/etravirine hypersensitivity.

CONCLUSIONS: The majority of new infections arise from untreated persons at early stages of infection, often unaware of their serostatus. This may result in onward transmission of HIV drug-resistant infections. Improved prevention and diagnostic strategies, as well as routine genotyping, are needed for early stage infection.

REFERENCE:
ABSTRACT 150
Antiretroviral drug resistance patterns from patients failing the national roll-out programme in South Africa

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BACKGROUND: With the South African antiretroviral roll-out program increasing and reaching its fifth year, it is becoming increasingly important to monitor for HIV-1 drug resistance to ensure optimal drug treatment regimens. This is an update of pol mutation patterns arising in patients failing either the first- (stavudine, lamivudine, efavirenz or nevirapine) or second-line (zidovudine, didanosine and Kaletra) antiretroviral (ARV) drug regimens from Johannesburg clinics.

METHODS: Viral RNA was amplified and the protease (PR) and partial reverse transcriptase (RT) regions from 406 patients failing ARV therapy were sequenced and analysed for mutation frequencies. Subtype was designated using the Rega HIV-1 subtyping tool.

RESULTS: All patients analysed appeared to be failing either the first- or second-line regimens as indicated by an increase in viral load. The mutations associated with the majority of patients failing were identified: D67N (17.98%); M184V (64.29%); K70R (10.34%); K103N (42.86%); V106M (26.11%); and G190A (15.76%). These represent signature mutations linked to the drugs used within the national roll-out. Of note, as previously reported, the K65R (5.67%) and Q151M (4.43%) mutations remained at similar frequencies to those reported last year. The occurrence of these mutations with the prescribed ARV drugs is unusual. Comparison of the PR region to subtype B showed the presence of several naturally occurring polymorphisms: namely, K20R (29.06%); M36I (92.197%); D60E (19.70%); L63P (33.00%); H69K (98.03%); and I93L (95.57%). These polymorphisms are linked to decreased susceptibility to PR inhibitors (International AIDS Society USA mutations list) and the presence of them in HIV-1 subtype C is unknown.

CONCLUSION: The HIV-1 subtype C ARV drug resistance pattern remains consistent with that presented previously. The occurrence of both the nucleoside reverse transcriptase inhibitor (NRTI) mutations K65R and Q151M remain at a constant frequency and their presence may impact on future treatment strategies containing tenofovir. Continued monitoring of PR resistance is important as the number of patients accessing Kaletra increases to determine the impact of the subtype C polymorphisms.
Novel amino acids at resistance positions can indicate transmission of drug-resistant variants

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**INTRODUCTION:** Transmitted resistant mutations can evolve to other variants with higher viral replicative capacity. This evolution does not necessarily result in the reappearance of wild-type amino acids. We investigated the appearance of novel amino acids at major resistance positions comparing wild-type HIV-1 with viruses containing drug resistance mutations using a global database of sequences from antiretroviral-naive individuals.

**METHODS:** The WATCH database contains >7,000 sequences from antiretroviral-naive patients from 42 countries across the world. Transmitted drug resistance was defined as at least one major drug resistance mutation including intermediates at position 215 (International AIDS Society list). Amino acids not encoding wild type, well known intermediates (RT 215) or a major mutation were considered novel variants. The prevalence of novel variants at major resistance-related positions was compared between sequences with and without signs of transmitted resistance; \( \chi^2 \) tests were used to compare the prevalence between both groups.

**RESULTS:** Reverse transcriptase (RT) and protease sequences from 6,250 patients were eligible for analysis. Transmitted resistance was more prevalent in subtype B compared with non-B viruses: 7.6% versus 5.7% (nucleoside reverse transcriptase inhibitors [NRTI]; \( P=0.004 \)), 3.5% versus 2.6% (non-nucleoside reverse transcriptase inhibitors [NNRTI]; \( P=0.04 \)) and 3.0% versus 2.1% (protease inhibitors [PI]; \( P=0.03 \)). Novel variants were more frequently observed in subtype B at major NNRTI-related positions (4.8% versus 2.7%; \( P<0.001 \)) but not at major NNRTI positions (3.7% versus 3.6%; \( P=0.81 \)) or major PI positions (1.3% versus 1.1%; \( P=0.39 \)). Patients infected with HIV harbouring ≥1 major mutation (PIDR) more frequently had novel variants at additional major resistance-related positions than patients infected with wild type (PIW): 10.8% versus 3.1% for NRTI positions (\( P<0.001 \)), 5.2% versus 3.8% for NNRTI positions (\( P=0.35 \)) and 4.3% versus 2.4% for PI positions (\( P=0.15 \)). RT genes from PIDR contained more frequently novel variants at thymidine analogue mutation (TAM) positions 67, 210 and 219 than those from PIW (all \( P<0.001 \)). In protease genes from PIDR, positions 46, 47, 48, 50, 54, 88 and 90 contained more novel variants (all \( P<0.001 \)).

**CONCLUSIONS:** Novel amino acids at resistance positions are more frequently found in antiretroviral-naive patients infected with drug-resistant HIV as compared with wild-type virus. The detection of novel amino acids at major resistance positions may serve as an indicator for transmitted drug-resistant viruses.
ABSTRACT 152
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Surveillance of transmitted HIV-1 drug resistance in newly diagnosed HIV-1-infected patients from Shandong Province, China

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BACKGROUND: China is facing an increasing public health threat from the HIV-1 epidemic. To reduce HIV transmission and alleviate suffering of HIV/AIDS-affected populations, the Chinese government launched the ‘Four Frees and One Care’ policy in 2003. Shandong, as a pilot province, has been providing antiretrovirals to eligible patients since early 2003, using first-line regimens recommended by the World Health Organization (WHO). To measure the emergence of transmitted drug resistance (DR) from newly HIV-1 diagnosed patients, a threshold survey was conducted in 2006 following the WHO threshold survey protocol.

METHODS: Blood was collected from 61 newly diagnosed HIV-1 patients between the ages of 18 and 24 in 2006 at voluntary counselling and testing sites. Dried blood spots (DBS) were made and sent to CDC International Laboratory Branch laboratory by express mail. Total nucleic acid was extracted from the DBS. HIV-1 protease and reverse transcriptase (RT) gene regions were amplified by RT-PCR using a broadly sensitive in-house genotyping assay and PCR products were sequenced using Big-Dye terminator cycle sequencing kits. Drug resistance mutations were analysed using calibrated population resistance tool (CPR, Stanford HIV Drug Resistance Database) and phylogenetic analyses were conducted with MEGA software.

RESULTS: Among the 61 DBS, 55 (90.1%) were RT-PCR positive for the HIV-1 protease and RT gene regions; 47 of them were sequenced and analysed. Phylogenetic analyses revealed there were 20 (43%) CRF01-AE, 10 (21%) B, 6 (13%) CRF07-BC, 4 (9%) CRF08-BC and 3 (6%) C. The remaining two each were unique B/C recombinant forms and unclassifiable. Of the 47 DBS specimens, 46 had no DR mutations based on the CPR analysis for transmitted resistance, while one DBS had one non-nucleoside reverse transcriptase inhibitor mutation (K101E). This mutation can cause intermediate resistance to nevirapine and delavirdine and low-level resistance to efavirenz and etravirine.

CONCLUSIONS: Our analysis of 47 drug-naive DBS specimens from newly diagnosed HIV-1 patients collected from Shandong Province, China in 2006 indicates that prevalence of transmitted DR viruses in this population is low, ≤5%. Phylogenetic analysis indicated that the HIV-1 epidemic is characterized by multiple subtypes and CRFs co-circulating. Continued monitoring of the transmitted drug resistance in recently HIV-1-infected populations is warranted to inform the effectiveness of treatment guidelines.
ABSTRACT 153
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Drug resistance in newly HIV diagnosed individuals: transmission rate, clusters and persistence

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BACKGROUND: The aim of this study was to monitor the transmission rate of drug-resistant HIV strains (TDR) in a well-defined urban area between 2000 and 2007. We also aimed to assess whether acquired HIV-resistant strains persist in the absence of drug pressure.

METHODS: All individuals resident in Geneva county (400,000 persons) with a new HIV-1 infection diagnosed between 2000 and 2007 were analysed. Recent infection was defined as an evolving serology or a documented negative screening test ≤1 year before the positive one. Resistance mutations were reported according to the ‘Shafer list’. Phylogenetic analyses were performed on all pol sequences using the maximum likelihood method (PhyML).

RESULTS: Six-hundred and eleven individuals with previously unknown HIV infection, 20% of them with a recent infection, were analysed (51% non-B subtypes). Mutations associated with resistance to at least one drug were detected in 38 (6.2%) individuals (nucleoside reverse transcriptase inhibitor [NRTI] resistance 4.3%, non-nucleoside reverse transcriptase inhibitor [NNRTI] resistance 2.9%, protease inhibitor [PI] resistance 1.0%, >1 drug class 1.8%). The resistance rate was 8% in B and 4.5% in non-B subtypes. An increase of resistance was observed in 2007 (12.2% any drug, 6% NNRTI). The phylogenetic analyses revealed that 30% of individuals (41% with recent infection, 26% with non-B) could be linked to a chain of transmission (2–8 cases) representing 66 different clusters. Five of these transmission chains including 11 individuals were related to TDR. Follow-up samples from 20 people with TDR and without antiretroviral therapy for a median of 24 months (6–49) were obtained and only four (20%) showed reversion of mutations (2×184V, 1×103N, 1×215S). Persistence of 103N was observed in nine (median follow-up 25 months, 6–43) and persistence of thymidine analogue mutations in ten individuals (median follow-up 28 months, 6–49). Two individuals showed persistence of PI mutations (28 and 49 months).

CONCLUSIONS: In a Swiss urban area, resistant strains accounted for 6.2% of newly diagnosed HIV infections. Chains of transmission could be identified in one-third of these cases. In the absence of drug pressure, transmitted drug-resistant strains are persistently detectable for at least two years in the vast majority of individuals. These observations strongly support the need to perform systematic drug resistance testing in any newly HIV diagnosed individual.
SESSION 7
HBV drug resistance
ABSTRACT 154
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Amino acid patterns of hepatitis B virus (HBV) reverse transcriptase from untreated and treated chronically HBV-infected patients in southeastern France

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BACKGROUND: The emergence of hepatitis B virus (HBV) reduced susceptibility to antivirals is a concern for the therapeutic management of individuals chronically infected with HBV. The availability of new nucleos(t)ide inhibitors of the HBV reverse transcriptase (HBVrt) and the widening of sequential and/or combination therapies warrant surveys of HBV genotypic drug resistance. We aimed at analysing amino acid (aa) patterns within HBVrt from patients chronically infected with HBV and followed-up in public hospitals in Marseille.

METHODS: From a 2001–2008 database of 629 HBVrt sequences, those corresponding to the last available sequence from 362 patients were selected for analysis of aa associations (HBV genotype A=43%, D=39%). Moreover, 148 sequences from patients with documented anti-HBV therapy were analysed to identify drug-selected aa (51, 28, 13, and 5 sequences were from patients on lamivudine, adefovir, tenofovir, and entecavir, respectively). Serum HBVrt sequences were obtained using inhouse PCR amplification and direct sequencing protocols. Aa variability and aa patterns were analysed using aa sequence variability analysis program.

RESULTS: Nearly 10% of aa positions displayed aa variability >30% off and on-therapy. Highly variable HBVrt areas, including from untreated patients, were between aa 117–153 (corresponding to the overlapping major hydrophilic region of the hepatitis B surface antigen) and between aa 253–271. The proportion of aa substitutions at positions 181, 215 and 237 was significantly higher for genotype D versus genotype A (P<0.05). rtN236T was observed in 5/6 cases in HBV genotype D. L229V was significantly associated with M204V. Among the 18 aa positions that are most commonly involved in drug resistance, eight (80, 169, 173, 180, 181, 204, 215 and 236) harboured significantly higher aa variability from treated than from untreated patients (P<0.05). Twenty-one other positions (16, 39, 109, 110, 121, 125, 127, 142, 149, 191, 212, 213, 220, 245, 246, 248, 278, 310, 313, 322 and 333) showed higher aa variability from treated patients. Moreover, substitution V142E/A/G/I/D was found in 12 sequences from seven patients on lamivudine, adefovir and tenofovir, whereas K212T/Q was found in six sequences from four patients; these substitutions were frequently associated with drug-resistance mutations to lamivudine or adefovir, and were not observed from untreated patients in the absence of rtM204V.

CONCLUSION: Our data underline the substantial variability of HBVrt and the association of drug-selected aa. They also suggest that drug-selected patterns might differ according to the HBV genotype.
ABSTRACT 155
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HBV genotype constrains the selection of lamivudine resistance profile in monoinfected and HIV-coinfected patients
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BACKGROUND: To investigate the role of hepatitis B virus (HBV) genotypes in development of lamivudine-resistance profiles in HBV- and HBV/HIV-infected patients, and to correlate these mutations with changes in the hepatitis B surface antigen (HBsAg).

METHODS: Full-length sequences of HBV-RT from 89 patients (61 HBV-infected and 28 HBV/HIV-coinfected patients) treated with lamivudine with detectable viraemia were analysed. Genotypes were determined by phylogenetic analysis. The association of mutations with HBV genotypes was assessed by χ² test and multivariate logistic-regression analysis (variables considered: subject demographic, HBV genotype, HIV coinfection, HBeAg status, ADF coadministration and therapy length). Covariation analysis was based on hierarchical clustering.

RESULTS: Patients are failing lamivudine after a median time of 37 months with a median viraemia of 4.1 log₁₀ UI/ml. Genotypes D and A are predominant in HBV-infected patients (90.1%) and in HBV/HIV-infected patients (57.1%), respectively. HBV genotype strongly influences the selection of lamivudine resistance mutations. In particular, A genotype is more prone to develop M204V than M204I (68.2% versus 13.6%, P=0.001). Multivariate analysis supports that A genotype is the only predictor for the emergence of M204V (odds ratio: 14.4 [95%CI:1.3–158], P=0.02). In addition, under lamivudine pressure the selection of L229V is significantly greater in A than D genotype (27.2% versus 7.8%, P=0.003), whereas the selection of L80I and S135Y is significantly lower in A than D genotype (4.5% versus 23.4%, P=0.04; 0% versus 17.2%, P=0.03, respectively). S135Y corresponds to the P127T mutation in the HBsAg ‘a’ determinant, crucial for antibodies binding. Cluster analysis shows that HBV A and D genotypes also differ in the compensatory mutations patterns. Indeed, in D genotype, M204V and L180M strongly cluster with T184A (bootstrap P=0.99; associated also with entecavir resistance), whereas in A genotype M204V and L180M strongly cluster with L229V (bootstrap P=0.95). L229V (corresponding to F220L in the HBsAg) is also associated with the HBsAg mutation S207N (bootstrap P=0.91), both localized near with each other in the fourth transmembrane HBsAg domain.

CONCLUSIONS: The genotype of HBV, which in a fashion is similar to or even more pronounced than HIV, plays a key role in driving both RT and HBsAg evolution under lamivudine treatment. Coinfection with HIV is also relevant in the selection of HBV-mutational pathways. HBV genotype can thus be relevant for therapeutic sequencing, immunological response and disease progression.
ABSTRACT 156

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Use of nucleos(t)ide analogues for treating chronic hepatitis B virus (HBV) infection may result in the selection of HBV vaccine escape mutants

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BACKGROUND: Nucleos(t)ide analogues (NA) are widely used for the management of patients with chronic hepatitis B, but long-term use can result in the selection of drug resistance mutations in the hepatitis B virus (HBV) reverse transcriptase (RT). Given the polymerase and envelope genes overlap in the HBV genome, selection of drug resistance-associated RT mutations can result in HBV surface antigen (HBsAg) changes that could reduce the binding capacity of HBV antibodies elicited by vaccines (putative vaccine escape mutants). The aim of this study was to determine the effects of NA therapy on the selection of putative HBV vaccine escape mutants.

METHODS: One hundred and five sequences (HBsAg 'a' determinant inclusive) were obtained from sera of HIV/HBV-coinfected and HBV-monoinfected patients exposed to NAs (lamivudine, adefovir and/or tenofovir). Relationships between drug resistance, RT mutations and HBsAg mutations, pre- and during treatment cases were evaluated using SeqHepB, Fisher’s exact and the pattern discovery program Magnum Opus.

RESULTS: The majority of sequences studied belonged to HBV genotypes A and D. Of the 228 positions that had base changes, 29% were synonymous. The ratio of synonymous to non-synonymous changes was significantly higher among coinfected (1:5) than among monoinfected patients (1:2) for HBV genotype A (P=0.0086). The reversed was observed for HBV genotype D, with ratios of 1:2 and 1:4 for coinfected and monoinfected patients, respectively (P=0.02951). The majority of samples that harboured drug-resistance-associated RT mutations were HBV genotype A for coinfected patients and HBV genotype D for monoinfected patients. Six patients (12%) coinfected with genotype A had the triple HBV rtV173L+rtL180M+rtM204V (sE164D+sI195M) mutant, that has diminished HBsAg/anti-HBs binding. Of these, two had no prior history of lamivudine treatments. No additional putative vaccine escape mutants were detected. A diagnostic-escape mutation sP120T (rtT128N) was detected in two (10%) patients monoinfected with genotype D.

CONCLUSIONS: Although previously identified vaccine escape mutations were uncommon in this international series of chronic hepatitis B patients failing NA therapy, an association between lamivudine-associated resistance mutations and vaccine escape mutants was observed. Similar associations for adefovir- and tenofovir-associated resistance mutations have not been identified to date. Two patients coinfected with genotype A with the lamivudine-selected triple HBV mutations had no previous treatment history with this drug, suggesting increased likelihood of transmission among vaccinated individuals, particularly in the setting of HIV infection.
SESSION 8
HCV drug resistance
ABSTRACT 157

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Amino acid polymorphism and drug resistance-associated substitutions within hepatitis C virus NS3 protease from protease inhibitor-naive chronically infected patients

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BACKGROUND: Hepatitis C virus NS3 protease inhibitors (HCV-PI) currently represent a great hope for the therapeutic management of patients chronically infected with HCV genotype 1 strains. However, the high level of HCV variability and diversity is an ongoing challenge for these drugs. We aimed to study the natural amino acid (aa) polymorphism within HCV NS3 protease from HCV-PI-naive HCV chronically infected patients and to detect the natural presence of aa substitutions conferring reduced susceptibility to HCV-PI.

METHODS: Serum samples collected from 170 patients chronically infected with HCV were analyzed. PCR amplification then direct sequencing of the HCV NS3 protease gene was performed using in-house protocols. The aa variability and aa patterns were analyzed using aSVARAP.

RESULTS: Eighty-seven and 83 HCV NS3 sequences were obtained from HIV-seropositive and HIV-seronegative patients, respectively. Genotype 1 was in two-thirds of cases. Consensus aa sequences differed at 42 (23%) and 41 (22%) positions between genotype 3 and subtypes 1a and 1b, respectively, and at 16 (9%) positions between subtypes 1a and 1b. The proportion of aa positions that harboured >5% variability was 15%, 15% and 25% for genotypes 1a, 1b and 3a, respectively. The proportion of aa positions harbouring >20% variability was 10% and 26% from HIV-seropositive and HIV-seronegative patients, respectively. This difference might be explained by different distributions of HCV genotypes according to HIV status. Amino acid substitutions were observed at positions 36 (155 and 168) involved in reduced-drug susceptibility. At position 155, a Lys residue (codon AAA), previously found to confer low-level resistance to telaprevir, was harboured by HCV of genotype 1a from one patient on two sequential serum samples. Amino acid substitutions were also observed at sites that have been found critical for the structure or functionality of HCV NS3 protease.

CONCLUSIONS: We identified substantial amino acid polymorphism within the NS3 protease from HCV chronically infected patients with differences according to the HCV genotype and subtype. Moreover, we described the natural presence of aa conferring reduced susceptibility to HCV-PIs. Further studies are needed to confirm and assess the extent of primary resistance to HCV-PI, which would represent a concern for its future use.
ABSTRACT 158
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A novel hepatitis C virus NS3/4A protease assay using a bacteriophage lambda-based genetic screen

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BACKGROUND: Hepatitis C virus (HCV) NS3/4A protease is an ideal target for antiviral therapy because its inhibition is expected to block HCV replication both by direct suppression of viral protein production and by restoration of host responsiveness to interferon (IFN). We have developed a bacteriophage lambda-based genetic screen that can be used to characterize site-specific proteases. This genetic screen system is based on the bacteriophage lambda cI-cro regulatory circuit, in which the encoded repressor cI is specifically cleaved to initiate the lysogenic-to-lytic switch. We have adapted this simple, safe and rapid genetic screen system to predict the activities and phenotypes of HCV NS3/4A protease from both different HCV genotypes and cellular or viral substrates (CARDIF, TRIF, NS4B/5A and NS5A/5B).

METHODS: NS3/4A protease coding regions were amplified by PCR from 10 individuals coinfected with HIV–HCV and two from monoinfected HCV individuals. NS3/4A proteases from different genotypes (1a, 1b, 3a, 4a and 4d) were amplified and analyzed. Resistance mutations to HCV protease inhibitors (A156S, A156T, A156V, D168V and D168A) were introduced in the genotype 1b NS3/4A proteases by site-directed mutagenesis and evaluated with NS5A/5B and NS4B/5A cleavage sites. Finally, NS3/4A proteases from genotypes 1b and 3a were used to screen a protease inhibitor library.

RESULTS: The genotype 1b NS3/4A proteases displayed the highest catalytic efficiencies. However, within this genotype up to threefold difference was observed. As expected, different catalytic efficiencies were observed when different cellular or viral cleavage sites were tested. Resistance mutations A156T and A156V almost abolished catalytic activity of the wild-type genotype 1b enzyme in both the NS5A/5B and NS4B/5A cleavage sites. Four inhibitors, with IC50s within the μM range, were also identified.

CONCLUSIONS: Compared with other protease assay methods, this assay has the following advantages: safe, highly sensitive, highly specific, easy quantification and rapid generation of different protease cleavage substrates using molecular cloning and expression. Characterization of the proteolytic activities of individual NS3/4A proteases should provide clues for understanding HCV-host interactions, as well as assisting in the development of new classes of NS3/4A protease inhibitors.