Workshop report

Clinical implications of resistance to antiretrovirals: new resistance technologies and interpretations

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Understanding resistance to antiretroviral therapy plays an ever more crucial role in managing HIV infection as new agents – including several in new antiretroviral classes – promise better control of multidrug-resistant virus in the developed world. Yet these new drugs have different, and often complex, resistance profiles. At the same time, resistance has assumed a key role in developing countries as access to additional antiretrovirals expands in the face of first-line regimen failures. Every year the International HIV Drug Resistance Workshop gathers leading investigators and resistance-savvy clinicians to share unpublished, peer-reviewed research on the mechanisms, pathogenesis, epidemiology, and clinical implications of resistance to licensed and experimental antivirals. The 2007 workshop, held on 12–16 June, proved particularly notable for its exploration of resistance to two new antiretroviral classes, integrase inhibitors and CCR5 antagonists, as well as to agents that control hepatitis C virus (HCV) infection. This report summarizes most oral presentations from the workshop and many posters.

Introduction

The XVI International HIV Drug Resistance Workshop afforded leading antiviral researchers a forum to present new data on resistance to new antiretroviral agents; HCV/HBV drug resistance; the epidemiology of antiviral resistance; clinical implications of antiretroviral resistance; HIV pathogenesis, fitness, and resistance; mechanisms of antiretroviral resistance; and new resistance technologies and interpretations. This article by Workshop organizers analyses studies selected by the programme committee for oral presentation and other key research.

Resistance to new antiretroviral agents

Resistance and cross-resistance to integrase inhibitors

Developers of two HIV integrase inhibitors, raltegravir (MK-0518) and elvitegravir (GS-9137), presented the first in vivo resistance selection data on these agents [1,2]. Resistant virus emerged in 35/38 patients in whom raltegravir failed virologically [1] and in 28/30 in whom elvitegravir failed [2]. Findings from both groups suggest that cross-resistance between these potent antiretrovirals is likely.

At week 24 of a Phase II, placebo-controlled trial of raltegravir at 200, 400 or 600 mg daily, 70% of antiretroviral-experienced patients randomized to raltegravir plus an optimized background regimen (OBR) had a plasma HIV RNA <400 copies/ml compared with 16% randomized to placebo plus an OBR in a non-completer-equals-failure analysis [1]. Testing site-directed mutants of treatment-induced integrase substitutions for susceptibility to raltegravir revealed two genetic pathways to resistance, one stemming from N155H (n=14) and the other from Q148H/R/K (n=20). High-level resistance to raltegravir depended on evolution of further mutations (L74M, E92Q and G163R with N155H, or E138K and G140S/A with Q148H/R/K).

Hazuda et al. proposed that the Q148 path to resistance may be preferred for three reasons: resistance to raltegravir was greater with Q148 mutations (especially with an additional mutation) than with N155H, plasma HIV RNA returned to baseline when raltegravir failed with a Q148 mutation, and some patients in whom N155H evolved appeared to retain
average susceptibility to etravirine in clinical samples of HIV-infected patients and a lower virological set point failure, perhaps because of partial raltegravir activity against N155H mutants or reduced fitness of the mutant virus [1].

HIV with mutations at positions N155 or Q148 also conferred resistance to diverse strand-transfer integrase inhibitors including elvitegravir, a finding consistent with the shared mechanism of these agents. Two resistance patterns – G140S plus Q148H and G140S plus Q148R – rendered virus highly resistant to both raltegravir and elvitegravir. Other mutation combinations decreased susceptibility to both drugs to a lesser degree.

N155H and Q148H/R/K also emerged upon failure of elvitegravir in a Phase II trial, as did other mutations that reduced susceptibility to this integrase inhibitor [2]. Experimental assays determined baseline and post-failure genotype and phenotype in 28/30 patients with virological failure by week 24 of elvitegravir therapy. E92Q, E138K, Q148R/K/H and N155H were the most common integrase mutations at failure, emerging in 11/28 patients (39%). S147G evolved in 9/28 (32%) patients and T66I/A/K in 5/28 (18%) patients.

Baseline susceptibility to elvitegravir ranged from a 0.91- to 2.53-fold change in median inhibitory concentration (IC50). Fold-change in susceptibility to elvitegravir at failure averaged at >151-fold (range 1.02–301-fold). Numerous single and double mutations conferred cross-resistance to elvitegravir and raltegravir (Table 1).

Genotypic predictors of response to investigational NNRTIs

The Resistance Workshop offered a 24-week analysis of the Phase III DUET-1 and -2 trials showing that three or more newly identified etravirine (TMC125)-induced mutations compromised response to this investigational non-nucleoside reverse transcriptase inhibitor (NNRTI) [3]. A separate study by Barcelona clinicians found low rates of mutations that would confer complete resistance to etravirine in clinical samples of HIV-infected patients resistant to nevirapine or efavirenz [4].

Tibotec researchers analysed 44 baseline NNRTI resistance mutations in HIV isolated from 406 patients with decreased virological response to etravirine, defined as a ‘worse’ response than in patients with no detectable NNRTI mutations at baseline [3]. All trial participants, however, had a history of resistance to efavirenz or nevirapine. The resistance analysis excluded patients who discontinued etravirine for any reason other than virological failure and patients taking enfuvirtide for the first time. The DUET trials randomized antiretroviral-experienced patients to 200 mg of etravirine twice daily or placebo plus a background regimen including ritonavir-boosted darunavir.

Of the 44 mutations analysed, 26 (59%) occurred in virus of five or more patients at study entry. Univariate analysis identified 13 baseline mutations correlating with decreased response to etravirine: V90I, A98G, L100I, K101E, K101P, V106I, V179D, V179F, Y181C, Y181I, Y181V, G190A and G190S. Notably, this list excludes the K103N mutation, which confers resistance to both efavirenz and nevirapine.

V179F, G190S and Y181V had the greatest effect on virological response, but occurred in only seven, 14 and six patients, respectively. Virus including one of five mutations – A98G, K101E, Y181C, V179D or G190A – had a higher NNRTI mutation total than virus without these mutations. V179F never occurred without Y181C. That these findings rest on a univariate analysis may limit clinical use of the proposed score without further validation. Earlier work found no single NNRTI mutation associated with a <10-fold change in susceptibility to etravirine [5]. When K101P, V179E, V179F, Y181I, Y181V, G190S or M230L appeared with up to four other mutations, they did correlate with a <10-fold change in susceptibility to this drug.

The number of baseline International AIDS Society (IAS)-USA NNRTI-related mutations or the number of total baseline NNRTI resistance mutations did not predict virological response to etravirine. Three or more of the identified etravirine-related mutations did diminish virological response in an analysis corrected for baseline HIV RNA, CD4+ T-cell count, number of active antiretrovirals in the regimen and fold change in susceptibility to darunavir; however, only 15% of patients had three or more of these mutations at baseline. In contrast, 70% of patients had none of the etravirine-linked mutations or only one before starting this NNRTI.

Development of novel NNRTIs continues with a new class of diphenylether agents active against NNRTI-resistant virus. R1206, the prodrug of a diphenylether NNRTI labelled RO-0335, yielded high oral bioavailability (33–100%) and low clearance (3–6 ml/min/kg) of RO-0335 in rats, dogs and

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**Table 1. Mutations conferring cross-resistance to elvitegravir and raltegravir**

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Elvitegravir</th>
<th>Raltegravir</th>
</tr>
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<tbody>
<tr>
<td>G148K</td>
<td>67</td>
<td>34</td>
</tr>
<tr>
<td>Q146R</td>
<td>118</td>
<td>30</td>
</tr>
<tr>
<td>N155H</td>
<td>38</td>
<td>23</td>
</tr>
<tr>
<td>T66I+G140S</td>
<td>143</td>
<td>33</td>
</tr>
<tr>
<td>G140S+Q148H</td>
<td>166</td>
<td>135</td>
</tr>
<tr>
<td>E92Q+N155H</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>E138K+S147G+Q148R</td>
<td>175</td>
<td>34</td>
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Viral replication capacity fell from a median 108% in 25 baseline samples to 54% in 27 virological failure samples, most of them harbouring resistance mutations (P<0.005). Of the 44 mutations analysed, 26 (59%) occurred in virus of five or more patients at study entry. Univariate analysis identified 13 baseline mutations correlating with decreased response to etravirine: V90I, A98G, L100I, K101E, K101P, V106I, V179D, V179F, Y181C, Y181I, Y181V, G190A and G190S. Notably, this list excludes the K103N mutation, which confers resistance to both efavirenz and nevirapine.

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monkeys [6]. Safety studies in rats and dogs did not find significant pathological or clinical events with R1206, which reached plasma exposures of ≤12 µM in rats and ≤78 µM in dogs.

Roche researchers reported that RO-0335 has a mean IC₅₀ of 1.2 nM against wild-type virus in cell culture and retains activity against virus bearing common NNRTI mutations. Virus harbouring the G190S, K103N plus Y181C or L100I/K plus K103N mutation had high-level resistance to efavirenz, whereas V179F plus Y181C had high-level resistance to etravirine; however, these mutants remained susceptible to RO-0335. Whereas efavirenz and etravirine inhibited 0% and 62% of a 50-virus panel derived from NNRTI-treated patients, respectively, RO-0335 inhibited 92% of those viruses.

The requirement for compensatory Gag cleavage site mutations contributes to the high genetic barrier to protease inhibitor (PI) resistance. Tibotec researchers detected three baseline Gag cleavage site mutations in ≥10 patients who had reduced virological responses to darunavir in the POWER 1, 2 and 3 trials [7]. Only one of these mutations, the novel substitution V128I, emerged in >10% of POWER patients who had a virological rebound while taking 600/100 mg of darunavir/ritonavir.

Resistance profile of a novel hepatitis C virus inhibitor

Most new anti-hepatitis C virus (HCV) agents now in clinical trials fall into three classes – NS3 PIs, NS5B nucleoside inhibitors and NS5B non-nucleoside inhibitors. Resistance to all these agents has been documented [8], and certain mutations (such as A156T in HCV protease) confer cross-resistance to investigational agents. However, ACH-806 (GS 9132), a novel acylthiourea NS5A antagonist, retains activity against variants resistant to PIs in development, because it acts by a mechanism different from HCV PIs.

Wyles et al. combined ACH-806 with small-molecule compounds and with interferon-α to determine which combinations are synergistic (combination index [CI]<0.9), additive (CI 0.9–<1.1) or antagonistic (CI >1.1) [9]. They combined agents for 48 h in 96-well plates using cells stably expressing an HCV genotype 1b luciferase reporter replicon at fixed-ratio combinations of two twofold serial dilutions above and below the IC₉₀ of the individual compounds.

IC₉₀ (±SEM) measured 116.8 nM (±5.4) for ACH-806, 4.5 IU/ml (±0.6) for interferon-α, 310.3 nM (±48.4) for the Vertex PI, 9.3 nM (±0.7) for the peptidomimetic PI BILN-2061, 301 nM (±23.9) for the GlaxoSmithKline PI, 446.8 nM (±46.2) for the nucleoside 2′-C-methyladenosine and 3.5 (±0.4) µM for the GlaxoSmithKline non-nucleoside. CIs (±SEM) at the 70% inhibitory concentration level proved mildly to moderately synergistic for all small-molecule compounds and additive for interferon: interferon-α 0.93 ±0.12; Vertex PI 0.79 ±0.03; BILN-2061 0.81 ±0.08; GlaxoSmithKline PI 0.68 ±0.06; 2′-C-methyladenosine 0.72 ±0.07; and GlaxoSmithKline non-nucleoside 0.73 ±0.10.

This analysis uncovered no antagonism between ACH-806 and NS3 PIs.

| Table 2. Gag cleavage site mutations correlating with reduced response to darunavir/ritonavir |
|---------------------------------|------------------|------------------|
| Gag CS mutation at baseline*  | Isolates, n (%) | % of overall response |
| All                            | 326              | 44               |
| E428G (CS4)                    | 16 (5%)          | 19               |
| S451T (CS5)                    | 18 (6%)          | 22               |
| R452S (CSS)                    | 16 (5%)          | 19               |

*Detected in ≥10 patients before treatment with darunavir/ritonavir (600/100 mg twice daily) and associated with a response rate <75% of the overall response rate. CS, cleavage site.
Entry inhibition

Resistance to maraviroc in MOTIVATE 1 and 2

Pfizer and Monogram Biosciences researchers identified diverse V3 loop mutations that evolved when regimens including maraviroc, a CCR5 antagonist, failed in the Phase III MOTIVATE 1 and 2 trials [10]. However, resistance-conferring mutations differed from patient to patient and the researchers could not discern a phenotypic marker of resistance in two-thirds of patients in whom maraviroc failed.

Mori et al. analysed virus from 38 patients in whom maraviroc failed with CCR5-tropic virus and further evaluated resistance in 12 viral samples that met one of three failure criteria: plateau maximal percentage inhibition (MPI) < 95%, IC50 fold change in susceptibility to maraviroc ≥ 1.95 than a twofold change in IC50 between baseline and on-treatment viral isolates. In earlier in vitro studies, maraviroc dose–response curves with reduced MPI correlated with the detection of maraviroc-resistant viruses that used either unbound CCR5 or maraviroc-bound CCR5 as a co-receptor. Earlier work by these investigators showed that reduced maximal inhibition with maraviroc – that is, an MPI plateau – reflects viral binding to an already maraviroc-bound CCR5 [11].

No MPI plateau lay below 95% in 38 baseline isolates or in 25 isolates from patients in whom a placebo regimen failed. Susceptibility to maraviroc measured as fold change in IC50 versus the reference virus JRCSF did not correlate with maraviroc failure. However, MPI plateaus were < 95% in 4/12 patients in whom a maraviroc regimen failed during the blinded phase of the trials. Although V3 loop mutations differed among the four individuals with an MPI plateau below 95%, all four with mutations had changes in V3 position 13 or 26.

Site-directed mutants created with mutations detected in one person determined that the mutations 13S plus a 16A insertion mutation were sufficient to render virus resistant to maraviroc in pretreatment virus and necessary for resistance in failure isolates. Site-directed mutants created to reflect V3 substitutions in a second person determined that the mutations 20F plus 25D plus 26V were sufficient to cause resistance in the pretreatment virus and necessary to cause resistance in the failure virus. The mutations 11S plus 26V, found in a third patient, were not sufficient to cause resistance in the pretreatment virus, but necessary for resistance in the failure virus. A site-directed mutant containing the 13S mutation, found in the fourth person, showed that this mutation alone was sufficient to render pretreatment virus resistant to maraviroc and necessary to make failure virus resistant.

The Pfizer and Monogram researchers proposed that reduced MPI can be used as a phenotypic marker of resistance to maraviroc in some patients taking this drug. However, 8/12 patients in whom maraviroc failed in this analysis had no phenotypic marker of resistance to this CCR5 antagonist.

CXCR4-tropic HIV that emerged during treatment with maraviroc or placebo regimens in MOTIVATE 1 and 2 trials usually came from minority pretreatment X4-tropic populations, according to results of clonal sequencing analysis by Pfizer and Monogram Biosciences [12]. The findings indicate that emerging X4-tropic virus does not arise as a result of a tropism switch caused by mutations in CCR5-tropic HIV during treatment. The investigators performed clonal analyses of virus isolated from 16 patients taking maraviroc and four taking placebo plus an OBR in the Phase III MOTIVATE trials of treatment-experienced people. The Trofile assay detected X4-tropic virus during treatment in all 20 of these patients, Env sequences from 192 baseline clones and 48 on-treatment clones were aligned, and phylogenetic trees were constructed to determine the origins of the X4-tropic HIV.

On-treatment sequences of X4-using virus from 14 of the 20 patients proved similar or identical to baseline sequences. Baseline X4-tropic clones represented 1–6% of the viral population in 10 patients and > 10% in the remaining four. On-treatment X4-tropic clones in the other six patients proved phylogenetically distinct from baseline clones and also distinct from on-treatment R5-tropic clones, results indicating that the X4 virus did not evolve from R5-tropic HIV during therapy. The on-treatment X4 clones and on-treatment R5 virus differed from baseline sequences at 7–17 amino acid sites in the 35 amino acid V3 loop.

Clonal screening also verified almost complete loss of R5-tropic virus in on-treatment samples from the maraviroc group. Four of 16 patients taking maraviroc and 2/4 patients taking placebo continued to respond to their salvage regimen at week 24.

No consistent IC50 increase with vicriviroc failure

Amino acid substitutions in the V3 loop emerged in 5/9 patients in whom vicriviroc or placebo plus an OBR failed in the Phase IIb ACTG A5211 trial [13]. However, only 1/5 patients with V3 loop mutations had HIV with decreased susceptibility to this CCR5 antagonist, and the study revealed no consistent increase in IC50 with virological failure.

ACTG investigators determined co-receptor use, env sequences and susceptibility to vicriviroc in eight randomly selected patients with virological failure at week 16: two patients from the placebo group and two patients each from the 5, 10 and 15 mg vicriviroc arms. They also performed clonal sequencing of virus from one
patient in the 10 mg vicriviroc arm with evidence of progressive loss of susceptibility to vicriviroc. Virus from the eight randomly selected patients with virological failure had no consistent increase in vicriviroc IC50 (maximum increase measured only 2.83-fold). Amino acid substitutions arose in four of these eight patients: two in the 5 mg group and two in the 10 mg group. These changes involved different substitutions and evolved at different amino acid positions in all four patients, and this diversity mirrored that observed among patients taking maraviroc in the MOTIVATE trials [10]. No V3 mutations arose among the four people taking 15 mg of vicriviroc or placebo in ACTG A5211.

Multiple V3 loop sequence changes emerged in the one patient with phenotypic evidence of resistance to vicriviroc. Five months after this patient stopped taking vicriviroc, viral samples were R5-tropic and showed a return to baseline V3 loop sequences and susceptibility to vicriviroc. These findings suggested a fitness advantage for wild-type virus over vicriviroc-resistant virus. Further analysing env clones from patients in whom vicriviroc failed virologically, the ACTG researchers found a plateau in vicriviroc inhibition. This finding suggested improved viral entry via CCR5 coreceptors already bound by vicriviroc. Enhanced viral growth during vicriviroc failure implied that HIV adapted to enter CD4+ T-cells more efficiently through vicriviroc-bound CCR5 than through unbound CCR5.

Separate work by Schering Plough investigators yielded further evidence indicating that vicriviroc-resistant HIV has increased affinity for CCR5 that is already bound by the drug [14]. Buontempo et al. generated two vicriviroc-resistant viruses by serial passage in the presence of escalating vicriviroc doses, and they generated control viruses by serial passage in the absence of drug. They infected U87.CD4.CCR5 cells with increasing doses of resistant or control virus in the presence of increasing CCR5 antagonist concentrations. They then determined dissociation constants for drug and virus in independent experiments for each drug–virus combination.

These experiments disclosed no shift in viral tropism to CXCR4 coreceptors. Non-resistant control viruses had decreased affinity for drug-bound CCR5, whereas resistant viruses had increased affinity for drug-bound receptor. These findings confirmed that one mechanism of HIV resistance to CCR5 antagonists involves selection of virus with a high affinity for antagonist-bound receptors.

Mutations outside V3 drive resistance to Schering C

Work by Huang et al. uncovered further complexities in resistance to CCR5 antagonists by exploring mutations that arise outside the V3 loop region of viral env [15]. The study involved resistant virus generated by serial passage in the presence of Schering C (SCH-C), a CCR5 antagonist no longer in development. The researchers amplified full-length gp160 from parental and week 35 passaged virus, then cloned and sequenced the amplified gp160. They generated chimeric envelopes to analyse the effect of V3 and non-V3 mutations on resistance to SCH-C. Virus with high-level resistance to the CCR5 antagonist had 15 or 16 amino acid changes in gp120, two or three substitutions in gp41, and only minor or no changes in V3. Analysis of the chimeric envelopes showed that these minor changes in V3 and in V3-flanking regions did not play a major role in resistance to SCH-C. In contrast, mutations in the C4 coreceptor-binding region of gp120 had a major impact on susceptibility to the compound, especially when these changes were accompanied by other substitutions in gp41 or gp120. Mutations in both V3 and C4 decreased viral infectivity, and further changes in V1 or V2 restored infectivity. The results suggested that HIV can evolve multiple mutations outside V3 that in combination contribute to escape of control by CCR5 antagonists.

CCR5 binding dynamics, inhibition and resistance

Individual CCR5 antagonists differ in coreceptor binding dynamics, and those differences suggest that antagonists without cross-resistance can be developed, according to results of competitive binding experiments [16]. This work by Roche investigators also confirmed that resistant HIV can ‘learn’ to bind to CCR5 already bound by an inhibitor. Jekle et al. generated maraviroc-resistant virus by serial passage of CCR5-tropic HIV in peripheral blood mononuclear cells (PBMCs). The researchers analysed susceptibility of passaged virus to various inhibitors in a peripheral blood mononuclear cell infection assay and used competitive binding methods to determine binding interactions and kinetics with wild-type and mutant CCR5 receptors.

Passaged virus had 8,000-fold resistance to maraviroc and 12,000-fold resistance to vicriviroc. Yet it remained susceptible to fusion inhibitors, CCR5 antibodies and RO-1752, a small-molecule CCR5 antagonist. Modelling of receptor-ligand interactions suggested that maraviroc and RO-1752 share the same binding site, but differ in specific interactions with this site. These interactions stabilize receptor conformations that wild-type HIV-1 cannot recognize.

The conformational changes also slowed binding kinetics and binding dynamics differed for maraviroc and RO-1752. The Y108A mutation on helix III eliminated time-dependent binding for maraviroc, but a Y251A mutation on helix VI did not eliminate time-dependent binding for RO-1752.

The Roche investigators proposed that differences in binding dynamics between CCR5 antagonists yield differences in CCR5 receptor conformation and that...
active antagonists cause conformational changes that wild-type HIV-1 cannot recognize; however, resistant mutants can still bind to inhibitor-bound receptors.

Tropism algorithms fall short in predicting phenotype. Accurate genotype-based algorithms to predict HIV coreceptor tropism would be welcome because they would shorten turnaround time and lower costs when compared with Trofile-based phenotyping. In theory, genotyping may be a useful approach to determining tropism because viruses using CCR5 differ from those using CXCR4 in their envelope sequence. However, a comparison of algorithm-based tropism calls and actual phenotypes suggested that these tropism predictors must be improved before they see use in the clinic [17].

Researchers from Canada, Germany and the USA determined the sensitivity and specificity of six tropism predictors against a panel of 977 viral isolates from chronically infected antiretroviral-naive individuals. They stratified coreceptor phenotype according to Trofile-generated relative light units (RLUs). Compared with Trofile-generated phenotypes, tropism-calling specificity of most algorithms proved high, but sensitivity was low: 11/25 charge rule (30.5% sensitivity, 93.4% specificity), position-specific scoring matrices (PSSM) SI/NSI (33.8% sensitivity, 95.3% specificity), PSSM X4/R5 (24.5% sensitivity, 96.9% specificity), neural network (44.4% sensitivity, 87.5% specificity), support vector machine (SVM) geno2pheno (44.7% sensitivity, 90.6% specificity).

Adjusting the analyses by more aggressively categorizing samples that are CXCR4-capable improved sensitivities only into a range of 24–50%.

The sensitivity of genotyping methods was proportional to the CXCR4 RLU readout of the Trofile assay ($P < 0.05$); in other words, algorithm sensitivity increased as Trofile detected CXCR4 tropism more readily. Clonal analysis of 40 clones from each of eight viral samples suggested that undetected X4-tropic minority species could explain some of the discordance between the prediction algorithms and Trofile results. Although bulk genotyping results strongly agreed with clonal analysis for most samples, when the proportion of CXCR4-using virus in a viral population fell <23%, bulk genotyping became less reliable in detecting minority viral variants. The researchers suggested these algorithms can be improved by ‘training’ them on clinical samples, enhancing prediction with clinical data such as CD4%, optimizing cutoffs and quantitatively assessing genotype signal strength to enhance detection of minority species. It is important to note that these studies and the data available to conduct them apply only to subtype B HIV-1.

Clinical implications of resistance

Resistance disadvantage with PI/NNRTI regimen
Lopinavir/efavirenz without nucleoside reverse transcriptase inhibitors (NRTIs) proved as potent as efavirenz plus two NRTIs, but failure of the NRTI-sparing regimen resulted in significantly more NNRTI resistance than failure of efavirenz plus NRTIs [18]. Resistance to two classes emerged more upon failure of efavirenz/NRTIs than upon failure of lopinavir/NRTIs in this 112-week analysis of ACTG 5142 by Haubrich et al. [14]. ACTG investigators randomized 753 treatment-naive individuals with a median CD4+ T-cell count of 191 cells/mm3 and a median plasma HIV RNA of 4.8 log10 copies/ml to efavirenz (600 mg daily) plus lopinavir/ritonavir (533/133 mg twice daily) or to standard doses of either of those drugs plus lamivudine and one of three other NRTIs – zidovudine, stavudine or tenofovir. Defining virological failure as a confirmed early rebound or a plasma load >200 copies/ml after week 32, Haubrich et al. counted 94 failures among 253 people (37%) randomized to lopinavir/NRTIs, 73/230 (29%) randomized to lopinavir/efavirenz, and 60/250 (24%) randomized to efavirenz/NRTIs after a median 112 weeks of follow-up ($P = 0.006$ for efavirenz/NRTIs versus lopinavir/NRTIs).

Among all randomized patients, 39/250 (16%) taking lopinavir/efavirenz, 22/230 (9%) taking efavirenz/NRTIs and 16/253 (6%) taking lopinavir/NRTIs had one or more major mutations upon failure ($P < 0.05$ for lopinavir/efavirenz versus the other arms). Comparing only patients with a genotype available at failure, the ACTG investigators found significantly higher rates of one or more major mutations with lopinavir/efavirenz (39/56, 70%) than with efavirenz/NRTIs (22/46, 48%; $P = 0.03$) or with lopinavir/NRTIs (16/78, 21%; $P < 0.001$). PI mutations arose only in patients taking lopinavir/efavirenz (L33F in one patient and L90M in another).

Among patients genotyped at failure, NNRTI mutations (71% of them K103N) proved more frequent with lopinavir/efavirenz (66%) than with efavirenz/NRTIs (44%; $P = 0.03$). Mutations to two drug classes were more frequent with efavirenz/NRTIs (26%) than with lopinavir/efavirenz (7%; $P = 0.01$) or lopinavir/NRTIs (1%, $P < 0.001$). Fewer patients randomized to lopinavir/NRTIs than to efavirenz/NRTIs had the K65R mutation (0% versus 7%, $P = 0.05$), but the frequency of thymidine analogue mutations (TAMs), M184V or any NRTI mutation did not differ significantly between these two arms.

Lower resistance barrier with lopinavir monotherapy
Sequencing viral isolates collected at virological rebound from patients taking lopinavir/ritonavir...
monotherapy or lopinavir/ritonavir plus zidovudine/lamivudine, MONARK trial investigators concluded that the monotherapy regimen has a lower genetic barrier to resistance than lopinavir/ritonavir as part of a standard triple regimen [19].

MONARK researchers randomized 83 treatment-naive people to lopinavir/ritonavir alone and 53 to lopinavir/ritonavir plus the two NRTIs. Everyone had a pretreatment HIV RNA load ≤100,000 copies/ml and a CD4+ T-cell count ≥100 cells/mm³. Of the 133 baseline genotypes successfully amplified, 36% were not subtype B and 40% of the non-B viruses were circulating recombinant form (CRF)_02. Resistance testing was performed whenever HIV RNA rebounded >500 copies/ml or at an investigator’s request. Through 96 weeks of follow-up, 32 patients taking lopinavir/ritonavir monotherapy (38.5%) and seven taking a triple regimen (13.2%) had a resistance test. Four of seven patients tested in the triple-therapy arm had a change from their baseline protease sequence, but none of those changes involved a major PI mutation.

Among the 32 patients genotyped during monotherapy, 17 (53%) had changes from their baseline protease sequence and five of those 17 (15.6% of those genotyped) had major PI mutations, including: M46I plus L63P at week 40, L76V at week 44, I13V plus M46I plus L76V at week 62, L10F plus V82A at week 76 or L76V at week 90. All three patients in whom L76V evolved were infected with CRF_02. L76V is a darunavir-associated mutation also sometimes seen with (fos)amprenavir or lopinavir. Median (range) plasma HIV RNA within 4 weeks of genotyping was 2.9 log₁₀ (about 8,000) copies/ml (range 2.8–3.1 log₁₀ copies/ml). Neither baseline plasma HIV RNA nor week 4 load correlated with emergence of any mutation. Among the five patients with emergence of one or more major PI mutations, four had phenotyping at baseline and rebound. The increase in lopinavir IC₅₀ at rebound averaged only 1.64-fold (range 1.13–2.69-fold). All patients in whom a major PI mutation evolved continued either monotherapy or added zidovudine/lamivudine and suppressed viraemia <400 copies/ml.

Insights on resistance to darunavir, Trizivir/tenofovir and NNRTIs

Several other studies presented at the Resistance Workshop offered results with a potentially immediate effect on clinical practice, including studies of resistance to the PI darunavir, the fixed-dose combination of zidovudine, lamivudine and abacavir (Trizivir) plus tenofovir, and the NNRTIs efavirenz and nevirapine.

De Meyer et al. offered an analysis of POWER 1, 2 and 3 comparing 48-week responses in patients starting 600/100 mg of darunavir/ritonavir twice daily with high baseline resistance to amprenavir, fosamprenavir, atazanavir, indinavir, lopinavir, nelfinavir, saquinavir or tipranavir [20]. The investigators defined high baseline resistance as a predicted fold change in 50% effective concentration (EC₅₀) to the PI above the VircoTYPE upper clinical cutoff, which predicts minimal response to that PI.

A non-completer-equals-failure analysis determined that 31% of patients with high baseline resistance to amprenavir or fosamprenavir reached a plasma HIV RNA <50 copies/ml with darunavir/ritonavir (Table 3) and that the 48-week decrease in plasma HIV RNA averaged 1.30 log₁₀ copies/ml in these individuals. When predicted (fos)amprenavir fold change in susceptibility lay below the upper clinical cutoff, week 48 mean HIV RNA with darunavir/ritonavir dropped 2.24 log₁₀ copies/ml. In patients with high baseline resistance to other PIs, 37–45% reached a plasma HIV RNA <50 copies/ml during 48 weeks of darunavir/ritonavir therapy (Table 4), and HIV RNA declines averaged 1.50–1.64 log₁₀ copies/ml.

Salvage therapy research over the past few years makes it abundantly clear that an effective rescue regimen must include at least two, preferably three, antiretrovirals to which HIV remains susceptible. While waiting for enough active agents to become available, many clinicians try to construct a 'holding

<table>
<thead>
<tr>
<th>Baseline PI</th>
<th>BL-predicted FC &gt;CCO, n</th>
<th>Week-48 HIV RNA &lt;50 copies/ml, %</th>
<th>BL-predicted FC &gt;CCO and using PI at BL, n</th>
<th>Week-48 HIV RNA &lt;50 copies/ml, %</th>
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<tbody>
<tr>
<td>(Fos)amprenavir</td>
<td>276</td>
<td>31</td>
<td>62</td>
<td>34</td>
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<tr>
<td>Atazanavir</td>
<td>261</td>
<td>40</td>
<td>44</td>
<td>39</td>
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<td>Indinavir</td>
<td>193</td>
<td>37</td>
<td>5</td>
<td>50</td>
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<td>Lopinavir</td>
<td>295</td>
<td>40</td>
<td>158</td>
<td>42</td>
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<tr>
<td>Nelfinavir</td>
<td>422</td>
<td>45</td>
<td>7</td>
<td>57</td>
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<tr>
<td>Saquinavir</td>
<td>206</td>
<td>39</td>
<td>55</td>
<td>44</td>
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<tr>
<td>Tipranavir</td>
<td>126</td>
<td>40</td>
<td>39</td>
<td>49</td>
</tr>
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</table>

BL, baseline; CCO, upper clinical cutoff (predicting minimal response to that protease inhibitor [PI]); FC, fold change in susceptibility compared with wild-type virus.

Table 3. Week-48 response to darunavir with high baseline resistance to other PIs
regimen’ that prevents immunological or clinical progression without giving rise to further resistance mutations. Llibre et al. reported that a holding regimen combining Trizivir (fixed-dose zidovudine, lamivudine and abacavir) with tenofovir meets those requirements for many patients [21].

This retrospective multicentre study involved 28 patients with a record of multiple failures, including failure of a boosted PI. All had virus with the M184V mutation when they began Trizivir/tenofovir; one patient had one TAM, three had two TAMs and the rest had three or more TAMs. After 24 weeks of follow-up, no patient had clinical progression and only one had a possible abacavir hypersensitivity reaction. Median plasma viraemia declined from 4.08 to 3.12 log10 copies/ml during follow-up and median CD4+ T-cell count fell 43 cells/mm3.

Among 28 patients with persistent viral replication while taking Trizivir/tenofovir, 11 had a follow-up genotype. In these patients the median number of reverse transcriptase mutations did not change between baseline and week 24 when counted as nucleoside analogue mutations, TAMs or accessory mutations at reverse transcriptase positions 44 and 118.

Numerous studies document the emergence of resistant virus during or after treatment interruptions (TI). A retrospective analysis of 50 virological responders who took a CD4-guided TI yielded evidence suggesting that the pattern of NNRTI-related mutations that emerged in seven of these patients (14%) during the TI depended on NNRTI plasma levels just before the interruption [22]. Plasma concentrations close to the lower limit of proposed therapeutic ranges for efavirenz (<1,500 μg/ml) or nevirapine (<3,400 μg/ml) correlated with the evolution of the primary NNRTI resistance mutations K103N, Y181C or G190S. In contrast, the mutations V106I/A, K103R/E or Y188C/D tended to emerge in patients with consistently subtherapeutic NNRTI concentrations. This latter pattern also appeared at the end of TI periods, when NNRTI selective pressure had disappeared.

The meaning of subtherapeutic levels in patients not interrupting treatment is unclear, because clinical and research experience indicates that therapeutic levels of nevirapine and efavirenz rarely fall so low in patients who do not skip doses or take another drug that interacts with NNRTIs. The correlation of mutations at positions 103 and 106 with subtherapeutic levels is especially surprising because K103R and V106I are common polymorphisms. In addition, K103E and Y188D are highly unusual mutations that have not been described earlier.

Compared with stable or rising NNRTI concentrations, decreasing concentrations from one assay to the next quadrupled the risk that a major NNRTI mutation would emerge (odds ratio 4.22, 95% confidence interval [CI] 1.15–15.6, P=0.03). When the lower second concentration lay below the therapeutic range for efavirenz or nevirapine, the risk of a major mutation evolving rose 4.4 times (95% CI 1.18–16.32, P=0.027). Darwich et al. suggested that NNRTI concentrations close to the low end of the therapeutic range may pose a threat of resistance even in virological responders.

New resistance technologies and interpretations

Hidden pretreatment NNRTI mutations threaten response

Antiretroviral-naive patients starting an NNRTI-based regimen in the FIRST trial [23] had more than a three times higher risk of virological failure if they had pretreatment NNRTI mutations detected by Ultra Deep sequencing, which can uncover minority viral variants representing only 1% of a patient’s viral population [24]. Without considering cost, Kozal et al. believe their findings justify validation of an ultrasensitive genotypic assay for clinical practice.

Nearly 1,400 FIRST participants began antiretroviral therapy with a PI plus NRTIs, an NNRTI plus NRTIs, or with a PI, an NNRTI and one or more NRTIs [23]. In a subset of 258 FIRST participants, these investigators compared baseline IAS-USA mutations or Stanford
All 11 study participants who began an NNRTI/NRTI regimen with an Ultra-Deep-detected NNRTI mutation experienced virological failure during the trial. Conventional genotyping missed these NNRTI mutations in 9/11 patients. The risk of virological failure was 3.38 times higher when Ultra Deep sequencing detected an IAS-USA NNRTI mutation than when it did not (95% CI 1.65–7.36, \( P < 0.0001 \)). Respective rates of baseline Stanford database NNRTI, NRTI and PI mutations were 14.3%, 14.3%, and 4.7% with Ultra Deep sequencing versus 6.6% (\( P = 0.03 \)), 5.8% (\( P < 0.0001 \)), and 2.3% (\( P < 0.0001 \)) with standard genotyping.

Researchers from the CDC and World Health Organization successfully amplified 104 of 119 dried blood spot specimens (87.4%) from newly infected individuals in Malawi (92% sequenced) and Tanzania (83% sequenced) [28]. The 86 sequenced specimens contained no detectable resistance mutations. Phylogenetic analysis determined that 65% of the samples were HIV-1 subtype C, 15% subtype A1, 7% subtype C/CRF_08, 4% subtype D, and the remainder C/A1, D/CRF_10, A1/D, A1/CRF_15 and C/D. Buckton et al. found that the collection paper matrix and extraction procedure used had a significant effect on overall sensitivity of dried blood spots [29]. Preliminary findings indicate that Whatman 903 paper coupled with Boom extraction yields the best extraction results. These investigators called for a consensus on optimal collection matrices, nucleic acid extraction techniques and amplification strategies to make dried blood spot technologies feasible for low-income countries.

**Implications of resistance to antiretrovirals**

**Effect of resistance on mortality**

Studies correlating resistance with clinical outcome are difficult to plan and interpret because of the diverse variables that affect a patient’s response to therapy. Consequently, studies with different designs may yield
differing, but perhaps not conflicting, results. Even the most carefully planned and judiciously analysed study cannot untangle the effect of resistance from the effect of access to treatment and resistance testing, the year therapy commenced, adherence and potentially other relevant covariates.

Recent analysis of a cohort in British Columbia, where antiretroviral therapy is free, determined that emergence of resistance to any antiretroviral class in people beginning therapy between 1 August 1996 and 30 September 1999 raised the risk of death by 75% [30]. Furthermore, the emergence of resistance to NNRTIs tripled the risk of death in this population. However, researchers from San Francisco reported at the Workshop that resistance did not predict increased mortality in that city [31].

The San Francisco study was a case-control comparison of AIDS patients who died or survived from 2001 to 2006, matching year of AIDS diagnosis (1989–2006) and year of resistance testing (2001–2006). Merging two patient databases yielded 943 unduplicated records including 127 people (13%) who died and 816 (87%) who did not. Resistance prevalence in the combined group stood at 45.4%, and among those with resistance 46.5% had dual-class resistance and 14.7% multiclass resistance.

The case-control comparison involved 40 patients who died and 239 controls who did not. Bivariate analysis determined that injecting drug use raised the risk of death 2.26 times (P=0.011) and having public health insurance raised the risk 2.56 times (P=0.035). A multivariate model adjusting for resistance, age at AIDS diagnosis and CD4+ T-count within 6 months of beginning treatment, found injecting drug use the only independent predictor of death at an adjusted odds ratio of 2.75 (P=0.022). Resistance had a non-significant protective effect, lowering the risk of death. Truong et al. hypothesized that resistance – and resistance testing – are surrogates for access to care, which allows for changes in antiretroviral regimens to cope with resistant virus.

Trends in transmission of drug-resistant HIV

Analysis of virus sampled from 126 people recently infected in the state of North Carolina showed that resistance patterns in this largely rural population reflected patterns in US metropolitan areas [32]. The study group was mostly male (81.0%), 46.0% black, 43.7% white and 5.6% Hispanic, and 38.1% identified themselves as men who have sex with men. Only three individuals (0.2%) identified as women. The most frequently detected mutations were those conferring resistance to NNRTIs, found in 9.5% according to the IAS-USA list and 7.9% according to the surveillance list. Only two individuals (1.7%) had mutations that make HIV resistant to drugs in two antiretroviral classes.

People diagnosed with HIV during the acute retroviral syndrome had nearly a four times higher risk of infection with mutations from either list. This univariate analysis also determined that women had a 3.46 times higher risk of infection with a surveillance-list mutation (95% CI 1.15–10.2) and a 4.26 times higher risk of an IAS-USA mutation (95% CI 1.48–12.24); however, women made up only 19% of this study group. As HIV continues to spread in non-urban areas of the USA, the researchers believe these findings suggest clinicians in rural areas should take the same precautions taken in cities when considering first-line antiretroviral therapy.

Subtype effect on resistance in the US

A survey of 186,381 viral samples collected for genotypic resistance testing in a US diagnostic laboratory showed that the proportion with HIV-1 subtypes other than B increased linearly from 0.18% in 1998 to 2.75% in 2006 (r²=0.96), a 0.34% annual rise [34]. The non-B subtypes found most often were C (38.7% of non-Bs), AG (28.4%), and A1 (7.7%). The investigators had treatment histories for none of the patients whose samples they analysed.

Whereas 2.2% of B subtypes analysed had a tipranavir resistance score ≥5, 5.1% of non-B subtypes had a tipranavir score that high (odds ratio 2.4, P<0.0001). The highest rates of tipranavir scores ≥5 occurred in subtypes A1 (7.9%), AG (6.8%) and G (4.5%). On the other hand, an atazanavir resistance score ≥3 was 53% less likely in non-B subtypes (P=0.0001), a darunavir score ≥3 was 83% less likely in non-B subtypes (P<0.0001) and a lopinavir score ≥6 was 78% less likely in non-B subtypes (P<0.0001).

Resistance to NRTIs, NNRTIs and PIs, and predicted multidrug resistance, proved consistently lower for non-B subtypes than for subtype B (Table 4). Furthermore, resistance rates for both B and non-B subtypes fell consistently over the past few years.

Ross et al. reported a rise in non-B subtype prevalence from 4.7% in 2001 to 6% in 2006 among 2,984 previously untreated people enrolling in clinical trials in 35 US states and the District of Columbia [35]. During this period the prevalence of clade C virus rose from 1.7 to 2.5%. Prevalence of resistance mutations in non-B virus remained low – 1.4% for subtype C, 1% for indeterminate subtypes, 0.6% for G, 0.4% for A and <0.1% for other subtypes and circulating recombinant forms.
HIV pathogenesis, fitness and resistance

Viral evolution during repeated STIs

Repeated structured treatment interruptions (STIs) appeared to create a sustained evolutionary bottleneck in env sequence diversity, according to the results of a post hoc analysis of the Swiss–Spanish Intermittent Therapy Trial (SSITT) [36]. Expansion of distinct viral lineages during repeated STIs suggested chance reactivation of different long-lived latently infected cells rather than expansion of a low-level replication population.

Joos et al. analysed env diversity in the C2-V3-C3 region in nine patients who took four 2-week STIs, each followed by 8 weeks of therapy. After the last STI, all patients suspended antiretroviral therapy indefinitely. Pretreatment env diversity ranged from 0.7 to 4.9% and averaged 2.5%. After 2.7 ±0.4 years of suppressive therapy followed by four STIs, diversity declined to 1.2% ±1.0%. However, rebounding virus often proved phylogenetically distinct from one rebound to the next in individual patients.

During the final prolonged STI, env diversity rose steadily but slowly; it took 1–1.5 years before diversity in six patients reached pretreatment diversity. Two to four years after the first STI, diversity stabilized in most patients. By that time four years after the first STI, diversity ranged from 0.7 to 4.9% and averaged 2.5%. After 2.7 ±0.4 years of suppressive therapy followed by four STIs, diversity declined to 1.2% ±1.0%. However, rebounding virus often proved phylogenetically distinct from one rebound to the next in individual patients. During the final prolonged STI, env diversity rose steadily but slowly; it took 1–1.5 years before diversity in six patients reached pretreatment diversity. Two to four years after the first STI, diversity stabilized in most patients. By that time env sequences diverged markedly from baseline quasispecies.

Effect of enfuvirtide mutations on neutralizing antibodies

Enfuvirtide-associated resistance mutations in the HR1 region of gp41 increased viral sensitivity to HR2-directed, broadly neutralizing antibodies 2F5 and 4E10 [37], a result consistent with a previously proposed hypothesis of enfuvirtide activity. Enfuvirtide is derived from the HR2 region of gp41 and blocks HIV fusion to CD4+ T-cells by binding to HR1.

Barry et al. undertook this study because laboratory strains of virus bearing the G36D and V38M enfuvirtide resistance mutations are more sensitive to the neutralizing antibodies 2F5 and 4E10 than wild-type virus [38]. The mutations also make one HIV laboratory strain more sensitive to neutralization by sera of HIV-infected patients. These findings led to the hypothesis that these HR1 mutations (without compensatory HR2 mutations) increase exposure of these two key epitopes to neutralizing antibodies. Other work correlates mutations at V38 with continued immunological benefit in patients taking an enfuvirtide-containing regimen after virological failure [39].

To test the hypothesis, Barry et al. assessed the sensitivity of enfuvirtide-resistant virus clones to 2FS and 4E10 in U87 cells expressing CD4 and CXCR4 receptors. In most cases, the HR1 mutations led to an increase in sensitivity to HR2-directed neutralizing antibodies. HR2 mutations mitigated this effect. Barry et al. proposed that their findings support the hypothesis that certain mutations conferring resistance to enfuvirtide alter the interaction kinetics between HR1 and HR2 and the lifetimes of fusion intermediates.

HIV likely to escape DIS inhibitors

HIV-1 can probably mutate to escape small-molecule inhibitors that interfere with dimer initiation signalling (DIS), according to the results of an in vitro inhibition study by Moore et al. [40]. In these experiments, blocking the base-pairing of DIS did not prevent dimer formation and had little impact on viral replication in a single-round assay.

Because HIV-1 puts two copies of RNA in each virion, Moore et al. hypothesized that base-pairing at the DIS largely determines which RNA partners are selected for copackaging in the virion. The investigators studied the effect of DIS mutations on viral replication by engineering six mutants in such a way that perfect DIS base-pairing could involve only RNAs from partner viruses, not RNAs from the same virus. They then tested the capacity of mutant constructs to replicate on their own or when paired with partner virus. It was found that all mutants, when analysed singly, produced new virus and encapsidated their RNA with few defects, and newly produced virus had infectivity close to that of wild-type virus and recombined with the same frequency as wild-type virus. These results indicated that HIV-1 RNA forms dimers even in the absence of complementary DIS.

Moore et al. also determined that virus pairs with compensatory DIS mutations attained almost twice the recombination rate seen with wild-type virus. That finding suggested heterozygous virion formation is enhanced in viruses with compensatory DIS mutations. The investigators believe their results demonstrate that base-pairing of the DIS plays a key role in selecting the copackaged RNA and that altering base-pairing of the DIS can change the proportion of heterozygous viruses in a population.

Mechanisms of HIV drug resistance

Pathway to first-line lopinavir resistance

A resistance pathway involving the L76V and M46I mutations may promote failure of lopinavir/ritonavir regimens in previously untreated individuals, which have been rare until now [41]. The possible contribution of mutations at the Gag cleavage site remains to be explored in detail.

Wensing et al. described three previously untreated patients in whom first-line lopinavir/ritonavir failed with the L76V and M46I mutations. The V82A mutation in protease and the A431V mutation at the Gag NC/p1
cleavage site later emerged in two patients. One of the three patients took lopinavir/ritonavir with no other antiretrovirals, whereas the other two took it as part of a standard combination.

Constructing site-directed mutants to study the effect of these mutations, the investigators determined that L76V alone significantly reduced viral replication capacity. When added to L76V, M46I enhanced viral replication to yield a virus with 12-fold resistance to lopinavir. This mutant proved cross-resistant to amprenavir (5.3-fold increase in IC_{50}) but not to darunavir. The L76V/M46I mutant was hypersusceptible to atazanavir (0.4-fold change), saquinavir (0.3-fold change) and tipranavir (0.5-fold change).

Analyzing a US database, which included more than 180,000 mostly subtype B isolates, these investigators found a significant correlation between the number of lopinavir prescriptions and the increase in samples bearing L76V with predicted resistance to any PI (from 0.4% in 2000 to 3.3% in 2006, p=0.89). Of the L76V isolates, 80% also carried M46I.

‘Silent’ mutations are associated with NRTI resistance. ‘Silent’ AAA-to-AAG substitutions at reverse transcriptase positions K65 and K66 are associated with TAM-related resistance to NRTIs, according to results of two database analyses and kinetic assays by Harrigan et al. [42]. The findings apparently represent the first evidence of an RNA-level mechanism directly relevant to antiretroviral resistance. Because there is a strong correlation between treatment experience and the silent mutations, the investigators searched for these substitutions in 9,593 isolates from 2,990 individuals with known treatment histories. Harrigan et al. found the AAA-to-AAG substitution at codon 65 in 812 isolates from 351 patients and at codon 66 in 2,219 samples from 829 patients. In subtype B isolates they also discerned a strong association between these silent mutations and TAMs. Whereas 7% of samples wild-type at codon D67 had silent mutations, more than 80% of D67N isolates had the silent mutations – a result strongly suggesting coselection of the silent mutations and D67N. The researchers confirmed these findings in an independent dataset from Virco.

The silent mutations could have particular importance in certain non-B subtypes in which AAG is the wild-type amino acid at codons 65 and 66. These silent substitutions may also directly influence viral replication of TAM-bearing virus. For example, Harrigan et al. observed that reverse transcriptase has a strong tendency to pause or dissociate at codon 66 in virus bearing D67N and K70R. Pausing and dissociation proved much less common when the AAG substitution at K66 preceded D67N and K70R. The pol gene-coding sequence explains why the K65R mutation evolves more rapidly in subtype C HIV-1 than in subtype B, according to results of DNA synthesis studies by Coutsinos et al. [43]. This faster emergence of K65R with subtype C has global implications for antiretroviral planning, because subtype C is the most widespread HIV-1 clade and tenofovir is becoming more available for second-line therapy in low- and middle-income countries. K65R is a primary resistance mutation for tenofovir.

Earlier work suggested more rapid evolution of K65R in subtype C both in cell culture [44] and clinical studies [45], but biochemical analyses of C and B virus did not explain the mechanism. Coutsinos et al. expressed recombinant subtype C and B reverse transcriptase in purified Escherichia coli and used gel-based nucleotide extension assays to analyse DNA synthesis for different natural and synthetic DNA and RNA templates spanning the K65-to-M184 region of pol.

Using subtype C reverse transcriptase to synthesize DNA from subtype C templates, the investigators found preferential pausing at the nucleotides responsible for the AAG-to-AGG mutation that yields K65R. The AAG-to-AGG substitution appeared to be more frequent than an AAA-to-AGA substitution. Using subtype B reverse transcriptase to synthesize DNA from a subtype B substrate, Coutsinos et al. documented pausing at the nucleotides spanning positions 65–67, but the strongest pausing occurred at position 67, a position important in development of the TAMs pathway for excision-based resistance.

To determine whether this difference between subtype C and subtype B involves biochemical or enzymatic properties of the reverse transcriptase enzyme, the investigators analysed subtype C reverse transcriptase with a subtype B template and subtype B reverse transcriptase with a subtype C template. Results suggested that the differences lie solely in the pol coding sequence and that the RTs themselves have little or no effect on the more rapid development of K65R in subtype C than B. Similar analyses of M184V evolution did not find that the pol gene-coding sequence favours emergence of this mutation in either subtype.

The researchers suggested that long-term follow-up of patients taking nucleosides in subtype C-endemic areas may elucidate the potential clinical relevance of these findings.

Ribonuclease H (RNase H) cleaves the RNA portion of viral RNA/DNA hybrids and is essential for viral replication. Several workshop studies evaluated the impact of RNase H on resistance to antiretrovirals and obstacles in developing RNase H cleavage inhibitors. The Q509L mutation in the RNase H domain of reverse transcriptase and A371V in the connection domain enhance resistance to zidovudine by decreasing DNA-dependent RNase H cleavage and increasing the efficiency of zidovudine-monophosphate (AZT-MP)
excision from the template primer, according to experiments by Brehm et al. [46]. The findings build on earlier work by the same investigators showing that zidovudine selects Q509L and A371V in vitro and that those mutations increase resistance to zidovudine approximately 10–50-fold when combined with the TAMs (M41L/L210W/T215Y or D67N/K70R/L215F), but had a minimal (1.7-fold) effect without TAMs [47,48].

To define the mechanism of this enhanced resistance, Brehm et al. measured adenosine triphosphate-mediated excision of AZT-MP using a radiolabelled DNA/DNA template primer and a radiolabelled RNA/DNA template primer. They measured multiple-round excision and incorporation with a 178-nucleotide RNA or DNA template annealed to a radiolabelled DNA primer. Furthermore, they assessed the relative rate of DNA-dependent RNase H cleavage by measuring the formation of secondary cleavage products from the RNA/DNA template primer in single-round AZT-MP excision assays.

Compared with control virus carrying only TAMs, viruses carrying A371V/Q509L plus TAMs increased AZT-MP excision in a multiple-round excision assay on RNA/DNA template primers, but not on DNA/DNA template primers. The investigators interpret these results as meaning that the effect on excision is specific for RNA/DNA duplexes. A371V/Q509L decreased formation of the -10 RNase H cleavage product (that is, the 10-nucleotide RNA/DNA duplex). Thus, Brehm et al. proposed that AZT-MP excision is inefficient on the short 10-nucleotide RNA/DNA duplex, while preserving longer RNA/DNA duplexes increases AZT-MP excision. A371V/Q509L also increased AZT-MP excision on the short RNA/DNA duplex approximately twofold. However, when Brehm et al. used a DNA template in multiple-round assays or either a DNA or RNA template in single-round assays, A371V/Q509L did not increase the efficiency of AZT-MP excision.

Taking a different approach to these issues, Delviks-Frankenberry et al. demonstrated that mutations in the RNase H primer grip region increase resistance to zidovudine in TAM-containing virus [49,50]. This finding adds to earlier work by the same group showing that mutations in the connection domain of reverse transcriptase increase resistance to zidovudine by >500-fold, while decreasing reverse transcriptase template switching [51]. A decreased template switching rate suggested that these mutations increase resistance to zidovudine by altering the balance between nucleotide excision and template RNA degradation. However, mutations in the RNase H primer grip region alone had only a modest impact on resistance to zidovudine.

Several residues in the HIV-1 connection domain form an RNase H primer grip structure that helps situate the template primer at the active sites of RNase H and polymerase. To test the hypothesis that connection domain mutations further resistance to zidovudine by acting on the RNase H primer grip, Delviks-Frankenberry et al. determined how alanine substitutions in primer grip residues affect resistance in wild-type virus or virus containing TAMs or K65R. When combined with TAMs, 10/11 primer grip mutations (G359A, A360K, K390A, K395A, E396A, T473M, Q475A, K476A, Y501A, Y501C and I505A) increased resistance to zidovudine from 20-fold to 243-fold compared with wild-type virus. All 11 mutations significantly decreased template switching, a finding suggesting that they reduce RNase H activity. These mutations also increased RNase H cleavage in vitro.

Eight connection domain mutations (E312Q, G335C, G335D, N348I, A360I, A360V, V365I and A376S) and two RNase H active site mutations (D549N and H539N) also increase resistance to zidovudine in the presence of TAMs, while decreasing template switching and RNase H cleavage.

Delviks-Frankenberry et al. concluded that most mutations in the HIV-1 RNase H primer grip that enhance resistance to zidovudine also decrease RNase H activity. Most patient-derived C-terminal domain mutations that further resistance to zidovudine also decrease RNase H activity. The investigators believe these findings support the model that altering the balance between nucleotide excision and RNase H activity increases resistance to zidovudine.

RNase H research has prompted speculation that RNase H inhibitors may be useful adjuncts to antiretroviral therapy. However, work by Götte et al. uncovered possible difficulties in developing such agents [52]. These investigators analysed the mechanisms of two potential RNase H cleavage inhibitors, dihydroxy tropolone β-thujaplicinol (which shows properties of an active site inhibitor) and a vinylogous urea called VU447 (which lacks such properties). This work confirmed the differing mechanisms of these agents and yielded evidence suggesting that small molecules that bind close to the metal binding sites of the active site, like β-thujaplicinol, have difficulties in overcoming competition with the nucleic acid substrate. The nucleic acid substrate has free access to its binding channel in HIV-1 reverse transcriptase and may simply displace small molecules that bind to the RNase H active site. Götte et al. also found that allosteric inhibitors such as VU447 may be able to bypass this obstacle.

New method to design resistance-resistant PIs
Synthesizing candidate PIs that avoid contacting viral protease at key residues may point the way to developing
compounds less prone to resistance than current agents, work by Schiffer and colleagues suggests [53–55]. The researchers pursued a hypothesis based on their crystallographic studies visualizing how HIV-1 PIs fit into viral protease [56,57]. This work showed that viral protease recognizes various substrate sequences through a conserved shape that the researchers call the ‘substrate envelope’ (not to be confused with the envelope gene or protein of HIV-1).

Schiffer and colleagues found that most crucial viral mutations conferring resistance to PIs occur when the inhibitors protrude beyond the substrate envelope and contact the protease. These residues are prime drug resistance sites because they are more important than other sites for PI binding than for substrate binding. Thus, the investigators hypothesized that PIs fitting within the substrate envelope would be less susceptible to resistance mutations.

To test this hypothesis, Schiffer and colleagues used computer-based design to predict inhibitors that fit inside the substrate envelope, then made these inhibitors via synthetic chemistry. They used enzymatic and calorimetric studies to assess inhibitory activity of these candidates against a panel of wild-type and resistant proteases. Initial candidate libraries used a structure similar to that of amprenavir and darunavir. The most successful compound had a subnanomolar binding profile to viral variants including the signature protease inhibitor mutations D30N, G48V, I50V, V82A and I84V. Crystallography showed that, as hypothesized, this inhibitor fit within the substrate envelope.

Summary

Since the conclusion of the XVI International HIV Drug Resistance Workshop, two of the novel agents explored at the workshop – maraviroc and raltegravir – have been licensed or recommended for licensing in the United States and the European Union. With Phase III trials of other new antiretrovirals well under way, their resistance profiles will continue to merit scrutiny from experts in this field. Yet this workshop draws the interest of everyone from basic scientists to HIV clinicians because of its broad focus on all issues relevant to resistance, not just on new agents. Continued expansion of antiretroviral access in low- and middle-income countries will raise new resistance questions that will be addressed in studies presented at future workshops.

Disclosure statement

MM declares no competing interests. DR has served as a consultant to Anadys Pharmaceuticals, Inc, Biota, Bristol-Myers Squibb, Boehringer Ingelheim Pharmaceuticals, Inc., Gilead Sciences, Inc, Idinex, Koronis Pharmaceuticals, Merck & Co, Inc, Monogram Biosciences, Inc, Pfizer, Inc, Roche Pharmaceuticals and Tobira Therapeutics, Inc. BL is a consultant to RDI. JM reports that he is a scientific advisor to Gilead Sciences, Merck, Idinex and Panacos, and owns stock in Pharmasset. CB has served as a consultant to Bristol-Myers Squibb, Boehringer Ingelheim Pharmaceuticals, Inc., Abbott Inc, Merck & Co, Inc, Pfizer, Inc, Roche Pharmaceuticals and Glaxo.

References

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Accepted for publication 06 February 2008