

MEETING REPORT

HIV DART 2008: Novel Agents, Strategies, and Assays to Control HIV

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Every 2 years, HIV DART offers basic scientists, drug developers, and clinical researchers a forum for describing ongoing work on therapies to control HIV infection. The 2008 DART edition featured the latest findings on reverse transcriptase, protease, CCR5, and integrase inhibitors that have transformed the management of patients with highly resistant virus, as well as those starting their first regimen. Experimental agents in these classes discussed at HIV DART included the nucleotide reverse transcriptase inhibitor GS-9148, the nucleoside reverse transcriptase inhibitor (NRTI) amdoxovir, and the nonnucleoside reverse transcriptase inhibitors (NNRTIs) IDX899, IQP-0410, and IQP-0528.

Attendees heard reports on several agents in novel classes, including Env-targeted immunotoxins, the postintegration inhibitor BIT225, a fixed-dose dual-agent “virostatic” labeled VS411, and nanoparticle-delivered peptides that target reverse transcriptase dimerization and the reverse transcriptase-integrase interaction. Other investigators detailed new work on explaining how Vpu overcomes host cell restriction, elucidating the impact of arginine methyltransferase on the HIV-1 replication cycle, and detecting mitochondrial toxicity in pancreatic adenocarcinoma cell lines.

Abstracts from HIV DART are published in the *Global Antiviral Journal* and are available online at: http://www.ihlpress.com/pdf%20files/AbstractBook_HIVDART2008_121708.pdf.

Efficacy and Safety of Licensed Antiretrovirals

Integrase inhibitor mechanism may explain unique properties

Raltegravir, a strand-transfer integrase inhibitor, has attracted attention—and wide use—because of its antiviral activity in patients with and without earlier antiretroviral experience [1,2]. Continuing research by Merck investigators who developed raltegravir and by other researchers has uncovered certain unique properties of strand-transfer inhibitors that may be related to their mechanism of action and could explain a number of clinical findings reported to date.

At HIV DART 2008, Merck’s Daria Hazuda proposed that “insights into the mechanism of action and unique intracellular pharmacology of these agents have important implications for understanding the clinical pharmacology of this class and suggest integrase inhibitors could play an important role in eradication strategies as well as post-exposure prophylaxis to prevent the acquisition of HIV-1 infection” [3].

Early clinical trials of raltegravir revealed some intriguing but hard-to-explain effects of this agent in humans. A 10-day placebo-controlled monotherapy trial in antiretroviral-naive individuals showed approximately a 2-log (100-fold) virologic response to raltegravir regardless of whether patients took 100, 200, 400, or 600 mg of raltegravir [4]. A pharmacokinetic/pharmacodynamic study found that 16 of 332 patients had a geometric mean 12-hour raltegravir concentration below 33 nM, but these patients did not have a worse virologic response than patients with higher 12-hour concentrations [5]. Analysis of virologic response in a phase 2 trial that enrolled antiretroviral-naive individuals showed a 70% lower second-phase viral decay with raltegravir than with efavirenz [6].

Trying to explain these findings, Hazuda analyzed details of raltegravir's mechanism in inhibiting HIV-1 replication. Raltegravir and the investigational integrase inhibitor elvitegravir bind to viral integrase after reverse transcription and before formation of the pre-integration complex. Time-of-addition experiments with raltegravir and MK2048, an investigational strand-transfer inhibitor, found that these agents inhibit viral replication in vitro when added up to 10 hours after infection of a CD4-cell line. These studies showed viral replication is inhibited even when raltegravir is removed 8 to 10 hours after infection. In other words, inhibition by raltegravir becomes functionally irreversible approximately 8 hours after infection.

Other work showed that raltegravir has a long residence time (off-rate) in virus-cell complexes assembled in vitro ($T_{1/2}$ 27 h at room temperature, ~7 h at 37°C). In many cases the off-rate exceeded the half-life of the pre-integration complex. This result also indicates that raltegravir-induced inhibition of viral integration in cell cultures is functionally irreversible. Hazuda proposed that these properties probably contribute to the observation that raltegravir efficacy is not linked to trough concentrations in clinical trials.

Results of this work may also have implications for resistance to strand-transfer inhibitors. Mutations selected by raltegravir and elvitegravir in clinical trials are similar to those selected by strand-transfer integrase inhibitors in cell culture. Cross-resistance between first-generation strand-transfer inhibitors such as raltegravir and elvitegravir seems likely. However, second-generation strand-transfer inhibitors being developed by Merck have even longer off-rates than raltegravir in experiments with wild-type HIV-1 or with resistance-conferring N155H mutant virus. As a result, in these washout experiments, N155H mutant virus had no impact on second-generation inhibitors.

Hazuda concluded that the mechanism of action of strand-transfer inhibitors results in a prolonged window for intervention after infection (a crucial consideration in postexposure prophylaxis) and functionally irreversible antiviral activity.

Raltegravir-based salvage therapy: 96-week results

Half of 133 heavily pretreated patients who began a raltegravir-based regimen in a phase 2 placebo-controlled trial had a viral load below 50 copies/mL after 96 weeks of follow-up in a noncompleter-equals-failure analysis [7]. In that time, the mean CD4 count rose more than 100 cells/mm³.

Protocol 005 randomized 133 antiretroviral-experienced patients to take 200, 400, or 600 mg of raltegravir twice daily and 45 to take placebo—all with an optimized background regimen [8]. Everyone had documented genotypic and phenotypic resistance to at least one NRTI, one NNRTI, and one protease inhibitor (PI). Most participants had highly resistant virus: 56% had a genotypic sensitivity score of 0 for their background regimen, meaning that the drugs they took with raltegravir had little or no predicted antiviral activity. A large majority of patients, 89%, had a phenotypic sensitivity score of 0 for PIs.

Median duration of pretrial antiretroviral therapy among all study participants stood at 9.9 years (range 0.4 to 17.3 years). Pre-raltegravir viral load averaged 80,000 copies/mL. By the end of the 24-week double-blind phase, 36 patients in the raltegravir groups (27%) and 38 in the placebo group (84%) discontinued treatment because of lack of efficacy [8]. At that point, 3 people (2%) in the raltegravir arms discontinued treatment because of adverse events, as did 1 (2%) in the placebo arm.

After week 24, participants in any study arm could continue treatment with 400 mg of raltegravir twice daily, the licensed dose of this integrase inhibitor. Ninety-four people originally randomized to raltegravir joined this open-label trial extension, along with 6 from the placebo group. Eight people discontinued treatment during the extension phase (4%), including 3 (2%) for lack of efficacy, 2 (1%) for adverse events, and 3 (2%) for other reasons.

Table 1. HIV RNA and CD4 response to raltegravir salvage at weeks 48 and 96.

	<i>Week 48</i>	<i>Week 96</i>
HIV RNA <400 copies/mL (95% confidence interval [CI])*	68% (59% to 76%)	55% (46% to 64%)
HIV RNA <50 copies/mL (95% CI)*	55% (46% to 64%)	48% (40% to 57%)
Mean gain in CD4 cells/mm ³ (95% CI)†	96 (71 to 121)	104 (76 to 131)

*Noncompleter-equals-failure analysis, n = 133 randomized to raltegravir.

†Baseline values carried forward for virologic failures.

In a noncompleter-equals-failure analysis considering all 133 people randomized to raltegravir, 48% had a viral load below 50 copies/mL at week 96, and 55% had fewer than 400 copies/mL (Table 1). Among 31 people using enfuvirtide for the first time in their background regimen, 26 (84%) had a viral load below 50 copies/mL at week 96. In contrast, 32 of 79 patients (41%) who did not use enfuvirtide and 5 of 14 (36%) who recycled enfuvirtide had a week-96 viral load below 50 copies/mL. Among 68 patients with a baseline genotypic sensitivity score of 0, 31 (46%) had fewer than 50 copies/mL at week 96, compared with 29 of 50 (58%) with a baseline score of 1 to 2.

After week 48, the investigators counted 9 virologic failures (9.6% of 94) with a viral load above 50 copies/mL. Among 6 patients whose virus could be genotyped after failure, 5 had detectable

raltegravir-related resistance mutations at position Q148 or N155, plus secondary mutations (E138A, E138K, G140S, or Y143C).

The investigators reported one serious drug-related laboratory abnormality in the open-label extension phase, decreased platelet count. One malignancy, squamous cell carcinoma in situ, was diagnosed during the extension phase but was not considered related to study drugs. For the entire study period, 4 of 133 patients (3%) taking raltegravir had a grade 3 aspartate aminotransferase (AST) elevation and 2 of 133 (1.5%) had a grade 3 alanine aminotransferase (ALT) elevation. There were no grade 4 AST, ALT, or alkaline phosphatase abnormalities.

Darunavir rescue regimens at 24 weeks in minority US women

Half of the highly treatment-experienced patients in the GRACE trial reached a viral load below 50 copies/mL with a darunavir/ritonavir regimen in the strictest intention-to-treat analysis presented [9]. A relatively high 24-week dropout rate partly explains the 50% intention-to-treat result in this study population consisting largely of African-American and Hispanic women.

The Gender, Race, and Clinical Experience—or GRACE—trial succeeded in its goal of recruiting a high proportion of US minority women by selecting 65 sites with large African-American and Hispanic populations. With 287 women and 142 men enrolled, GRACE is the largest trial of antiretroviral-experienced women in North America. All enrollees had a baseline viral load of at least 1000 copies/mL and a treatment history including a PI- or NNRTI-based regimen for at least 12 weeks. GRACE excluded patients who had already taken an integrase inhibitor, a CCR5 antagonist, darunavir/ritonavir, tipranavir/ritonavir, etravirine (the newest licensed NNRTI), or enfuvirtide. Pregnant or breastfeeding women could not enroll. Study participants began a regimen of standard-dose twice-daily darunavir/ritonavir plus other antiretrovirals selected by study investigators. The rescue regimen could include etravirine.

The 24-week analysis involved 203 patients (47.3% of all enrollees) who completed 24 weeks of treatment or discontinued their regimen before week 24. This group included 180 women (88.7% of 203), 117 of them (65%) African American and 40 of them (22%) Hispanic. When they enrolled in GRACE, these women had a mean age of 42 years (range 19 to 78 years), a mean viral load of 4.67 log₁₀ copies/mL (about 47,000 copies/mL), and a median CD4 count of 206 cells/mm³ (range 1 to 868).

Among women analyzed, 107 (59.4%) had already taken at least two PIs, though 128 of 177 (72.3%) had no primary PI mutations. Forty-five women (25%) had one active drug in the background regimen (excluding etravirine), and 99 (55%) had two or more active background-regimen drugs (excluding etravirine). One third of patients in the 24-week analysis entered GRACE after a treatment interruption lasting 4 weeks or longer.

In the first 24 weeks, 44 women and 2 men (22.7%) discontinued treatment (Table 2). Fourteen women (31.8% of 44) and 1 man stopped treatment because of side effects, while 1 woman (2.3% of 44) and no men stopped because of virologic failure. The remaining discontinuations among women were attributed to loss to follow-up (25% of 44), withdrawn consent (11.4% of 44), noncompliance with protocol (11.4% of 44), or other reasons (18.2% of 44).

Table 2. Discontinuations at 24 weeks in the GRACE trial.

	<i>Women, n = 180 (n, %)</i>	<i>All patients, n = 203 (n, %)</i>
Overall discontinuations	44 (24.4)	46 (22.7)
Adverse events	14 (7.8)	15 (7.4)
Loss to follow-up	11 (6.1)	11 (5.4)
Noncompliant with protocol	5 (2.8)	6 (3.0)
Withdrawn consent	5 (2.8)	5 (2.5)
Virologic failure	1 (0.6)	1 (0.5)
Other	8 (4.4)	8 (3.9)

Exposure to study drugs averaged 33.4 weeks. The most common grade 2 to 4 adverse events at least possibly related to study drugs were nausea (in 5.9%), diarrhea (in 5.4%), rash (in 3.0%, 5 women and 1 man), weight gain (in 3.0%, 5 women and 1 man), dizziness (in 2.0%), and dyspepsia (in 2.0%). Thirty women (16.7%) had a serious adverse event, including pneumonia in 8 women (4.4%).

In a time-to-loss-of-virologic-response (TLOVR) analysis involving only women, 50.6% had a viral load below 50 copies/mL at week 24. This result is comparable to that recorded at 24 weeks in the 318-person phase 2 dose-finding POWER 1 trial of darunavir/ritonavir in treatment-experienced patients [10]. In a TLOVR analysis that excludes patients who discontinued for reasons other than virologic failure, the 24-week response rate in GRACE was 65.5% among women. A noncompleter-equals-failure analysis calculated a 24-week mean CD4-cell gain of 86 cells/mm³.

Carmen Zorrilla (University of Puerto Rico) suggested that the high dropout rate reflects the socioeconomic disadvantages of the study population and the high prevalence of comorbidities such as mental illness. Gender- and race-based comparisons will be made when 48-week results are analyzed.

Response to first-line darunavir versus lopinavir at 96 weeks

Patients starting their first antiretroviral regimen with darunavir/ritonavir—especially those with a higher pretreatment viral load or a lower pretreatment CD4 count—had a significantly better 96-week virologic response than people starting lopinavir/ritonavir [11]. The 96-week findings confirm and extend published 48-week results in the ARTEMIS trial [12].

ARTEMIS is an ongoing 192-week randomized open-label trial comparing once-daily darunavir/ritonavir (800/100 mg) with lopinavir/ritonavir in a total daily dose of 800/200 mg. Of the 346 patients randomized to lopinavir, 77% took 400/100 mg twice daily, 15% took 800/200 mg once daily, and 8% started with twice-daily dosing and moved to once-daily therapy in the first 48 weeks [12]. While 86% of lopinavir-treated patients began with the old capsule

formulation and switched to tablets by week 48, 12% stayed with capsules, and 2% took only tablets. All 689 study participants also took fixed-dose tenofovir/emtricitabine.

Mean pretreatment viral load stood at 70,000 copies/mL and median CD4 count at 225 cells/mm³. One third of study participants entered ARTEMIS with a viral load at or above 100,000 copies/mL, and about 40% entered with fewer than 200 CD4 cells/mm³. A week-48 TLOVR analysis determined that 84% taking darunavir/ritonavir and 78% taking lopinavir/ritonavir had a viral load below 50 copies/mL ($P < 0.001$), a result establishing the noninferiority of darunavir to lopinavir in previously untreated people [12]. Among patients beginning treatment with a viral load above 100,000 copies/mL, the 48-week response rate was significantly higher in the darunavir arm (79%) than in the lopinavir arm (67%) ($P < 0.05$).

After 96 weeks of follow-up, 59 people (17%) randomized to darunavir and 81 (23%) randomized to lopinavir discontinued treatment, including 13 (4%) taking darunavir and 32 (9%) taking lopinavir because of adverse events (Table 3). By week 96, 3 patients (1%) randomized to darunavir and 8 (2%) randomized to lopinavir discontinued because of virologic failure.

Table 3. Discontinuations at 96 weeks in ARTEMIS.

	<i>Darunavir/ritonavir,</i> <i>n = 343 (n, %)</i>	<i>Lopinavir/ritonavir,</i> <i>n = 346 (n, %)</i>
Overall discontinuations	59 (17)	81 (23)
Adverse events	13 (4)	32 (9)
Loss to follow-up	18 (5)	11 (3)
Withdrawn consent	11 (3)	10 (3)
Virologic failure	3 (1)	8 (2)
Pregnancy	6 (2)	3 (1)
Noncompliant with protocol	3 (1)	7 (2)
Other	5 (1)	10 (3)

TLOVR analysis at 96 weeks determined that 79% randomized to darunavir and 71% randomized to lopinavir had a viral load below 50 copies/mL. That result reconfirmed the noninferiority of darunavir to lopinavir (difference 8.4%, 95% CI 1.9% to 14.8%, $P < 0.001$). The 96-week result also established the superiority of darunavir to lopinavir at these doses in antiretroviral-naïve patients ($P = 0.012$). Among patients with paired baseline and failure phenotypes, everyone in both treatment arms remained susceptible to all PIs after failure. Median CD4-cell gain at 96 weeks was equivalent with darunavir (171 cells/mm³) and lopinavir (188 cells/mm³).

Among people beginning treatment with a viral load above 100,000 copies/mL, 76% taking darunavir and 63% taking lopinavir had a week-96 viral load below 50 copies/mL (difference 14%, 95% CI 2% to 25%, $P = 0.023$). Respective response rates for patients starting treatment with a viral load below 100,000 copies/mL were 81% and 75%, a difference that fell short of statistical significance ($P = 0.174$). Among patients with a pretreatment CD4 count below 200 cells/mm³, 79% randomized to darunavir and 65% randomized to lopinavir had a 96-week viral load below 50 copies/mL (difference 14%, 95% CI 4% to 24%, $P = 0.009$). Response rates were statistically equivalent in patients beginning therapy with more than 200 cells/mm³.

Two earlier trials yielded conflicting results on whether patients starting lopinavir/ritonavir with a viral load above 100,000 copies/mL have a diminished response. Study M05-730 compared once- versus twice-daily lopinavir/ritonavir in 664 previously untreated people and discerned no impact of pretreatment plasma viremia on 48-week virologic response [13]. In contrast, AIDS Clinical Trials Group (ACTG) protocol 5073, which compared twice-daily self-administered lopinavir/ritonavir, once-daily self-administered lopinavir/ritonavir, and once-daily directly observed lopinavir/ritonavir in 402 treatment-naïve patients, found that those starting therapy with more than 100,000 copies/mL were less likely to sustain a virologic response through 48 weeks if they self-administered lopinavir/ritonavir once daily rather than twice daily [14].

At ARTEMIS week 96, grade 2 to 4 diarrhea was less common with darunavir than with lopinavir (4% versus 11%). Grade 2 to 4 rash, though rare, was more frequent with darunavir (3% versus 1%). Mean triglyceride levels rose 0.1 mmol/L with darunavir/ritonavir and 0.6 mmol/L with lopinavir/ritonavir. Fifteen patients (4%) randomized to darunavir and 46 (13%) randomized to lopinavir had a grade 2 to 4 triglyceride elevation by week 96. Neutrophil counts were abnormal in 30 patients (9%) randomized to darunavir by week 96, compared with 11 (3%) randomized to lopinavir.

Etravirine DUET results at 48 weeks steady across geographic regions

Salvage regimens combining the NNRTI etravirine with the PIs darunavir/ritonavir yielded similar 48-week virologic responses across diverse geographic regions in the DUET trials [15]. But this regional analysis of 1184 DUET enrollees disclosed a few continental differences.

DUET-1 and DUET-2 randomized 599 treatment-experienced patients to etravirine (200 mg twice daily) and 604 to placebo. Everyone received standard-dose darunavir/ritonavir plus a background regimen that could include the fusion inhibitor enfuvirtide. DUET investigators enrolled 524 patients from North America, 331 from Europe, 329 from Latin America, 15 from Australia, and 4 from Thailand. This analysis did not consider the patients from Australia and Thailand.

All study participants had a baseline viral load above 5000 copies/mL, at least one NNRTI-related mutation, and at least three PI-related mutations. Median pretreatment viral load stood around 65,000 copies/mL in both the etravirine and placebo groups, and median pretreatment CD4 count measured 99 cells/mm³ in the pooled etravirine arms and 109 cells/mm³ in the pooled placebo arms. In a pooled DUET-1 and -2 TLOVR analysis, 61% taking etravirine and 40% taking placebo had a week-48 viral load below 50 copies/mL ($P < 0.0001$) [16,17].

Baseline demographics generally did not vary significantly by region, except that North American clinics recruited a higher proportion of men than clinics in other regions (about 95% versus 85%) and pretreatment median CD4 counts were lower in North America than in Latin America or Europe. The 48-week 50-copy TLOVR response difference between etravirine and placebo was consistent in North America (21%), Latin America (19%), and Europe (25%) (Table 4). But North America had lower overall proportions of responders in the etravirine and placebo arms (55% and 34%) than did Latin America (62% and 43%) or Europe (69% and 44%). Measuring response at a 400-copy cutoff showed a similarly diminished response rate in North American.

The lower overall virologic response rates in North America could reflect the more advanced HIV disease among North American participants as well as a higher proportion of grade 3 or 4 adverse events and discontinuations in North America in both the etravirine and placebo arms than in Latin America or Europe (Table 4).

Table 4. Regional response and safety differences at 48 weeks in the DUET trials.

	<i>Europe</i>		<i>Latin America</i>		<i>North America</i>	
	<i>Etravirine (n = 162)</i>	<i>Placebo (n = 169)</i>	<i>Etravirine (n = 162)</i>	<i>Placebo (n = 167)</i>	<i>Etravirine (n = 266)</i>	<i>Placebo (n = 258)</i>
<50 copies/mL (%)	69	44	62*	43	55†	34
<400 copies/mL (%)	77	49	75†	54	66†	41
Mean CD4-cell gain (cells/mm ³)	105	87	121	91	79	47
Any AE (%)	96	96	95	97	97	95
Any grade 3-4 AE (%)	29	33	25	31	40	40
Any serious AE (%)	20	23	20	22	20	25
Discontinuations due to AE (%)	6	5	6	4	9	8

AE, adverse event.

* $P = 0.0002$

† $P < 0.0001$.

Overall incidence of rash was higher in the etravirine arms than in the placebo arms (19% versus 11%), although rash leading to permanent discontinuation was rare (2.2% with etravirine versus 0% with placebo). Rates of hepatobiliary, hepatic, and neuropsychiatric adverse events did not differ greatly from region to region or when comparing etravirine arms with placebo arms.

In a pooled DUET-1 and -2 noncompleter-equals-failure analysis at 48 weeks, mean CD4 count rose 98 cells/mm³ in the etravirine arms and 73 cells/mm³ in the placebo arms ($P = 0.0006$) [16,17]. The 48-week regional analysis found a similar CD4-cell advantage for etravirine versus placebo regardless of region. Again, however, mean CD4-cell responses were lower in North America (79 cells/mm³ with etravirine and 47 cells/mm³ with placebo) than in Latin America (121 cells/mm³ with etravirine and 91 cells/mm³ with placebo) or Europe (105 cells/mm³ with etravirine and 87 cells/mm³ with placebo) (Table 4).

The DUET investigators suggested that regional response and safety differences may reflect differing treatment practices, host factors, and viral factors.

Etravirine once daily with or without darunavir in treatment naive

Once-daily etravirine yielded adequate concentrations over 28 days in previously untreated people whether given with or without once-daily darunavir/ritonavir [18]. Etravirine is not licensed for once-daily dosing in treatment-experienced patients, in part because an earlier pharmacokinetic (PK) trial in healthy volunteers recorded a 25% lower minimum concentration (C_{min}) with once-daily etravirine than with twice-daily etravirine [19].

Etravirine is licensed at a dose of 200 mg twice daily for treatment-experienced patients, but clinicians are eager to learn more about its activity in patients starting their first regimen. The long half-life of this NNRTI, 30 to 40 hours, suggests that once-daily dosing may be appropriate for treatment-naive individuals. Darunavir is licensed at a dose of 800 mg once daily with 100 mg of ritonavir in previously untreated patients and at a dose of 600/100 mg twice daily for treatment-experienced patients.

This phase 2 trial enrolled 23 antiretroviral-naive people, 20 of them men, without hepatitis B or C virus coinfection or resistance to any study drug. The study group included 9 blacks, 9 whites, and 5 Hispanics with a mean age of 35.7 years, a mean pretreatment viral load of 4.2 log₁₀ copies/mL (about 16,000 copies/mL), and median pretreatment CD4 count of 403 cells/mm³ (range 144 to 895). All study participants took 400 mg of etravirine once daily plus once-daily tenofovir/emtricitabine for 14 days. On days 15 through 28, they added 800/100 mg of darunavir/ritonavir once daily, and from days 29 through 42 they stopped etravirine and continued the other antiretrovirals. All doses were taken with a meal. The investigators collected 24-hour samples on days 14 and 28 to measure drug concentrations.

Mean (and standard deviation [SD]) concentrations of etravirine proved equivalent without and with darunavir/ritonavir: 24-hour area under the curve (AUC) 10,410 (4186) versus 10,720 (5459) ng/h/mL; maximum concentration (C_{max}) 790 (287) versus 801 (327) ng/mL; and C_{min} 233 (130) versus 236 (168) ng/mL. Etravirine C_{min} in this trial was lower than in treatment-experienced patients taking the NNRTI twice daily, but the mean C_{min} was more than 50 times higher than the protein binding-adjusted 50% effective concentration (EC₅₀) of etravirine against wild-type virus, with or without once-daily darunavir/ritonavir.

Mean (SD) values for darunavir were 76,130 (22,080) for 24-hour AUC, 7008 (1514) ng/mL for C_{max}, and 1049 (616) ng/mL for C_{min}. Respective ritonavir concentrations were 4128 (1854) ng/h/mL, 465 (231) ng/mL, and 27 (21) ng/mL. Darunavir levels were slightly lower—and

ritonavir levels slightly higher—than in previously untreated patients at week 4 of the ARTEMIS trial.

Mean viral load dropped 1.7 log₁₀ copies/mL (about 50-fold) with etravirine plus tenofovir/emtricitabine by day 14 and 1.8 log by day 28. At study day 42, mean viral load had fallen 2.0 log₁₀ copies/mL (100-fold). By day 42, the median CD4 count had risen 56 cells/mm³.

Except for one case of grade 3 neutropenia during the first 14 days, clinicians recorded no serious or grade 3 or 4 adverse events, and no adverse events led to treatment discontinuation. The most common side effects at least possibly attributed to etravirine were nausea in 4 patients, headache in 3, rash in 2, and flatulence in 2. Rash can affect patients taking either etravirine or darunavir/ritonavir, and in a few cases these rashes have been serious. Median triglycerides rose by 32.5 mg/dL through day 42. The broad range in triglyceride changes, from -88 to +166 mg/dL, suggests that triglycerides bear watching in patients combining etravirine and darunavir/ritonavir.

Novel Therapeutic Approaches: Antiviral Mechanisms and Predictive Toxicology

New analyses of trial data on CCR5 antagonist vicriviroc

Schering Plough has launched two phase 3 trials of the CCR5 antagonist vicriviroc in treatment-experienced patients, as well as a novel double-therapy trial of vicriviroc plus atazanavir/ritonavir in previously untreated patients [20]. Lisa Dunkle from Schering Plough outlined both trials and offered further analyses of already completed studies.

VICTOR-E1, the largest reported trial of vicriviroc in treatment-experienced patients, randomized study participants to placebo or to 20 or 30 mg of vicriviroc once daily plus a background regimen including a ritonavir-boosted PI [21]. Ritonavir increases vicriviroc exposure 5 to 6 times. All study participants had triple-class experience including at least one reverse transcriptase inhibitor and one PI, and everyone was taking a stable, failing regimen for at least 6 weeks before screening. Coreceptor use screening eliminated people with CXCR4-using virus. After 48 weeks, 59% of study participants randomized to 30 mg of vicriviroc, 50% randomized to 20 mg, and 26% randomized to placebo had an HIV RNA level below 50 copies/mL. As in other salvage trials, virologic response rates were better in patients who could add one or more active drugs to vicriviroc.

Schering statisticians pooled findings from 205 patients who continued to take vicriviroc in extension phases of VICTOR-E1 and ACTG protocol 5211 [22] for up to 216 weeks. Although only about 100 patients had follow-up data after 96 weeks of treatment, the results represent the longest monitoring of people taking a CCR5 antagonist. Mean decrease in plasma viremia continued a downward trend from approximately 1.75 log₁₀ copies/mL at week 48, to approximately 2.0 log at week 96 (n = 108) and about 2.25 log at week 192 (n = 13). Mean CD4-cell count continued a steady upward trajectory over the same period, from a gain of approximately 120 cells/mm³ at week 48 to about 180 cells/mm³ at week 192.

Continuing enrollment and follow-up of treatment-experienced patients in vicriviroc trials has eased concerns about the incidence of neoplasia in people taking this drug. Those concerns arose when ACTG 5211 investigators recorded new malignancies in 6 people randomized to vicriviroc

and 2 randomized to placebo [22]. One of the 2 cancers in the placebo group was diagnosed after the patient switched to vicriviroc. But after those reports, as vicriviroc exposure climbed from under 100 person-years to over 600, only a handful of additional cancers got diagnosed. Except for one 2005 diagnosis of gastric carcinoma, all malignancies have been skin cancers or lymphomas. Malignancies per 100 person-years dropped from a peak of 6 in June 2006 to 3 by September 2008.

VICTOR-E3 and E4 randomized 857 treatment-experienced patients with CCR5-using virus to 30 mg of vicriviroc or to placebo plus an optimized background regimen including a ritonavir-boosted PI. These phase 3 trials have liberal entry requirements that will ensure enrollment of a proportion of patients without highly resistant virus or greatly advanced HIV infection. Participants must have documented resistance to at least two of the first three antiretroviral classes *or* antiretroviral experience for at least 6 months with at least two of the following: one NRTI, one NNRTI, or 2 PIs. Patients coinfecting with hepatitis B or C virus were allowed to enroll.

Undoubtedly as a result of these entry criteria, the mean baseline CD4 count is higher in these vicriviroc trials (246 cells/mm³, range 3 to 1475) than in phase 3 trials of maraviroc (191 cells/mm³, range 2 to 820) or the integrase inhibitor raltegravir (153 cells/mm³, range 1 to 759). In addition, because several antiretrovirals with novel resistance profiles have been licensed in the past few years and are allowed in the vicriviroc trials, patients in VICTOR-E3 and E4 generally have more active drugs in their background regimen than did people in recent salvage trials. For example, more than 90% of enrollees have at least two phenotypically defined active drugs, 19% are taking both darunavir/ritonavir and raltegravir, and 10% are taking raltegravir without darunavir. Fewer than 10% of VICTOR-E3 and E4 participants have a phenotypic sensitivity score of 0 or 1, whereas approximately 40% to 50% had scores that low in phase 3 trials of maraviroc and raltegravir.

Because of these baseline characteristics, it will be more difficult to distinguish the individual contribution of vicriviroc to virologic response in these studies. At the same time, results should offer a more clinically relevant perspective on the potential of vicriviroc in current salvage regimens and in patients with moderately advanced HIV infection.

A trial of vicriviroc in treatment-naïve patients has already enrolled 95 patients in a first stage and aims to enroll 120 more in stage 2. This trial randomized patients to 30 mg of vicriviroc daily plus atazanavir/ritonavir—and no other antiretrovirals—or to atazanavir/ritonavir plus tenofovir/emtricitabine. The first interim analysis will be conducted at week 24. The primary endpoint will be mean change in HIV RNA, and percentage with plasma viremia below 50 copies/mL will be a secondary endpoint. Besides offering patients a simple once-daily regimen, first-line vicriviroc/atazanavir will preserve NNRTIs for future use and delay toxicities seen with NRTIs. The strategy will also test vicriviroc in a population with the highest rate of CCR5-using virus.

Safety profile of new nucleotide in cell and animal studies

GS-9148, an experimental nucleotide reverse transcriptase inhibitor being developed by Gilead Sciences, showed little evidence of mitochondrial or renal toxicity in diverse cell studies or animal toxicology studies [23]. Mitochondrial toxicity resulting from inhibition of mitochondrial

DNA (mtDNA) polymerase-gamma is thought to account for many NRTI-induced side effects, including anemia, neuropathy, pancreatitis, and lactic acidosis. Renal toxicity caused by transporter-mediated renal accumulation of drug is the principal side effect associated with acyclic nucleoside phosphonates, including tenofovir, the only nucleotide reverse transcriptase inhibitor licensed for antiretroviral therapy.

Gilead's Adrian Ray reviewed recent research on the safety profile of on GS-9148 and GS-9131, an ethylalaninyl phosphonoamidate prodrug designed to maximize the delivery of GS-9148 and its active diphosphate metabolite to lymphoid cells and tissues. Ray also reviewed previously reported results on the resistance profile of GS-9148. Susceptibility testing with the Monogram Biosciences phenotypic assay showed that viruses carrying the mutations K65R, K70E, L74V, M184V, or certain combinations of those mutations remained susceptible to GS-9148 [24]. Viruses harboring four or more thymidine analog mutations, including combinations containing M41L and L210W, also remained more susceptible to GS-9148 than to other NRTIs used clinically.

At concentrations 10-fold and 30-fold higher than the EC_{50} against wild-type virus, GS-9148 had no impact on mtDNA accumulation when compared with a no-drug control assay in HepG2 cells [24]. In contrast, didanosine halved mtDNA levels compared with control at a concentration 10-fold greater than the EC_{50} and lowered mtDNA levels even more at a 30-fold greater concentration. Lactate accumulated no more with GS-9148 than in the control assays in experiments lasting 14 or 21 days, while lactate accumulation with didanosine more than doubled control levels. Further biochemistry studies with purified enzyme showed that GS-9148-diphosphate selectively inhibits HIV-1 reverse transcriptase relative to human polymerases, including mitochondrial polymerase-gamma, whereas no inhibition was observed at a concentration greater than 150-fold of the IC_{50} for HIV reverse transcriptase.

Accumulation of antiviral acyclic nucleoside phosphonates in renal proximal tubules depends on their uptake by human renal organic anion transporters type 1 (hOAT1) and 3 (hOAT3). Nucleotide efflux from proximal tubule cells depends on multidrug resistance protein 4 (MRP4), which thus serves a nephroprotective role. Compared with the acyclic nucleotides cidofovir, adefovir, and tenofovir, GS-9148 was transported 60- to 100-fold less efficiently by hOAT1 and was 20- to 300-fold less cytotoxic in cells overexpressing hOAT1 [25]. These studies also documented poor hOAT3-mediated transport of GS-9148 compared with the other nucleotides. Like acyclic nucleoside phosphonates, GS-9148 had reduced intracellular accumulation in cells overexpressing the efflux transporter MRP4.

Consistent with these individual transporter studies, uptake of GS-9148 was inefficient in fresh human renal cortex tissue [25]. A study of ^{14}C -labeled GS-9131 orally administered to dogs showed 6-fold lower dose-normalized accumulation in kidneys relative to tenofovir. These findings probably indicate the mechanism underlying lack of nephrotoxic findings in 28-day studies in rats, dogs, and monkeys at the highest doses tested (300, 20, and 30 mg/kg/day respectively).

Once-daily NNRTI potent in 7-day dose-ranging trial

IDX899, an NNRTI with a novel but still incompletely defined resistance profile, demonstrated good antiretroviral activity with all four doses studied in a 7-day placebo-controlled trial [26]. No discernible patterns of adverse events or laboratory abnormalities emerged in this brief study.

This phase 1b/2a trial randomized 40 antiretroviral-naïve HIV-infected people to 800, 400, 200, or 100 mg of IDX899 once daily or to matching placebo for 7 days. These 37 men and 3 women were infected with HIV-1 subtype B or with B/F recombinants. All but 1 study participant were Hispanic/Latino. The study group had moderately advanced HIV infection. Mean pretreatment viral load measured 4.71 log₁₀ copies/mL (about 50,000 copies/mL), and mean baseline CD4 count was 479 cells/mm³. No one had a CD4 count below 200 cells/mm³ and all had a viral load above 5000 copies/mL.

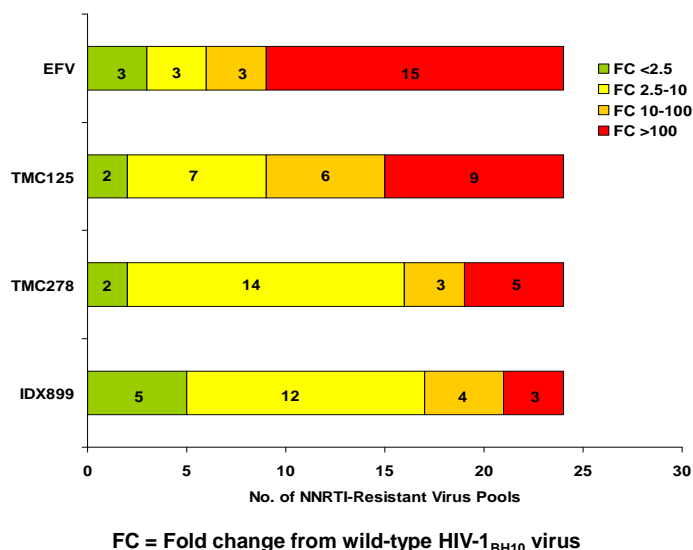
On day 8 mean viral load declined 1.78 log₁₀ copies/mL with 800 mg of IDX899, 1.78 log₁₀ copies/mL with 400 mg, 1.84 log₁₀ copies/mL with 200 mg, and 1.87 log₁₀ copies/mL with 100 mg. Mean viral load rose 0.10 log₁₀ copies/mL in the placebo group. The study revealed no dose-response relationship, probably because steady-state trough concentrations with all four doses of IDX899 exceeded the protein-binding adjusted EC₅₀ and EC₉₀ of IDX899 for wild-type virus. The investigators reported no dose-limiting toxicities, no treatment-emergent serious adverse events, and no grade 3 or 4 laboratory abnormalities; no one discontinued IDX899 during the trial.

Earlier research documented IDX899 activity against mutation-bearing virus selected by efavirenz or etravirine in vitro, including virus with four or five mutations conferring high-level resistance to these two NNRTIs [27]. At least three mutations were required to confer high-level resistance to IDX899. A cross-resistance study testing efavirenz, etravirine, rilpivirine (TMC278), and IDX899 against 24 NNRTI-selected resistant isolates found that 15 had more than 100-fold resistance to efavirenz, 9 had more than 100-fold resistance to etravirine, 5 had more than 100-fold resistance to rilpivirine, and 3 had more than 100-fold resistance to IDX899 (Figure 1).

However, there is some overlap in mutations selected by IDX899 and other NNRTIs. Serial passage studies suggest two primary pathways to resistance to IDX899, involving either E138K or Y181C, which is a primary resistance mutation for efavirenz and nevirapine [28]. No clinically significant drug-drug interactions emerged in studies combining IDX899 with tenofovir/emtricitabine or atazanavir.

PK studies showed that IDX899 has no impact on concentrations of atazanavir, tenofovir, emtricitabine, or coformulated tenofovir/emtricitabine (Truvada). Food enhances absorption of IDX899.

Cross Resistance Profile of 24 NNRTI Selected Virus Pools: Overall Summary



8

Figure 1. IDX899 remained susceptible to more NNRTI-selected mutants than rilpivirine (TMC278), etravirine (TMC125), or efavirenz.

Low-dose zidovudine enhances amdoxovir activity in 7-day study

Amdoxovir (DAPD), a guanosine nucleoside analog, appeared to have synergistic antiviral activity with 200 mg of zidovudine twice daily in a 7-day placebo-controlled trial [29]. Plasma viremia fell nearly 100-fold more with amdoxovir plus 200 mg of zidovudine than with amdoxovir alone.

Amdoxovir is active against virus bearing the M184V mutation or the thymidine analog mutations M41L, D67N, K70R, L210W, T215Y/F, or K219Q/E/N [30]. In vitro studies show that amdoxovir selects the K65R mutation [31], which also confers resistance to tenofovir, abacavir, didanosine, lamivudine, and emtricitabine [28]. Because zidovudine tends to prevent emergence of K65R, these investigators hypothesized that combining zidovudine with amdoxovir will forestall emergence of K65R with amdoxovir. A study of amdoxovir plus zidovudine in human lymphocytes supported this hypothesis and found that the combination also prevents emergence of thymidine analog mutations, which confer resistance to zidovudine and other nucleosides [32].

In silico studies suggest that 200 mg of zidovudine twice daily yields lower AZT-monophosphate levels than 300 mg twice daily and therefore could cause fewer side effects [33]. But the lower zidovudine dose did not result in lower levels of AZT-triphosphate, the active metabolite. Therefore the investigators also theorized that the lower zidovudine dose would result in less hematologic toxicity without affecting antiviral activity when taken with amdoxovir.

The researchers randomized 24 patients not taking or naive to antiretrovirals to 500 mg of amdoxovir twice daily (n = 6), amdoxovir placebo plus 200 mg of zidovudine twice daily (n = 2), placebo or 300 mg of zidovudine twice daily (n = 2), amdoxovir plus 200 mg of zidovudine twice daily (n = 6), amdoxovir plus 300 mg of zidovudine twice daily (n = 6), or placebo (n = 2) for 10 days. Everyone had a pretreatment viral load of at least 5000 copies/mL. Mean viral loads across the six treatment arms ranged from 3.8 to 5.0 log₁₀ copies/mL and mean CD4 counts from 336 to 588 cells/mm³. No one had NRTI-related mutations.

Mean decrease in viral load on day 10 measured 0.65 log₁₀ copies/mL with 200 mg of zidovudine, 0.45 log with 300 mg of zidovudine, 1.07 log with amdoxovir alone, 1.97 log with amdoxovir plus 200 mg of zidovudine, and 1.67 log with amdoxovir plus 300 mg of zidovudine ($P \leq 0.019$ for difference between two amdoxovir-zidovudine arms; $P \leq 0.039$ for 500/200 mg amdoxovir/zidovudine versus 500 mg of amdoxovir without zidovudine). Mean viral load rose 0.10 log in the placebo group.

All 6 people taking amdoxovir plus 200 mg of zidovudine twice daily had more than a 1.5-log decline in viral load, compared with 5 of 6 taking amdoxovir plus 300 mg of zidovudine, and 2 of 6 taking amdoxovir alone. Three of six people in both amdoxovir-plus-zidovudine groups had a viral load below 400 copies/mL after 10 days of therapy. Combining zidovudine with amdoxovir reduced the variability in virologic response seen with amdoxovir alone.

PK data showed that amdoxovir is rapidly cleared from plasma and that its active metabolite, DXG, is adequately delivered to plasma when amdoxovir is taken with zidovudine. The investigators saw no PK interactions between zidovudine and amdoxovir or DXG. Baseline mutations did not change through 10 days of treatment, and no new mutations emerged.

Three of 6 people taking amdoxovir with 200 or 300 mg of zidovudine reported headache, 3 of 6 in each group reported nausea, and 3 of 6 reported muscle contracture. All side effects in this brief study were mild to moderate and transient. No one modified or discontinued study drugs because of adverse events. There were no significant changes in biochemical or hematologic measures in any study arm.

RFS Pharma, the developer of amdoxovir, is considering studies of coformulated amdoxovir and zidovudine.

“Delayed chain termination” proposed as antiviral mechanism of entecavir

Unlike NRTIs that inhibit viral replication by classic chain termination, entecavir appears to work by “delayed chain termination” that occurs when entecavir monophosphate (ETV-MP) forces the enzyme to “slide away” from the 3′-end of the primer at a reverse transcriptase (RT) position designated n + 3 [34]. Because entecavir avoids the excision mechanism that renders virus resistant to classic chain terminators like zidovudine, Matthias Götte and colleagues from McGill University in Montreal proposed that entecavir-like NRTIs be developed as antiretroviral agents that may be less prone to resistance.

Entecavir is licensed for treatment of hepatitis B virus infection, but it has antiretroviral activity and selects the M184V mutation, which confers resistance to the antiretrovirals lamivudine and emtricitabine [35]. M184V diminishes the efficiency of ETV-MP into HIV-1 RT. Götte and

coworkers used an array of biochemical experiments to discern the mechanism of action of entecavir, considering three possibilities (Figure 2):

- De facto chain termination
- Delayed chain termination
- Base-pair confounding

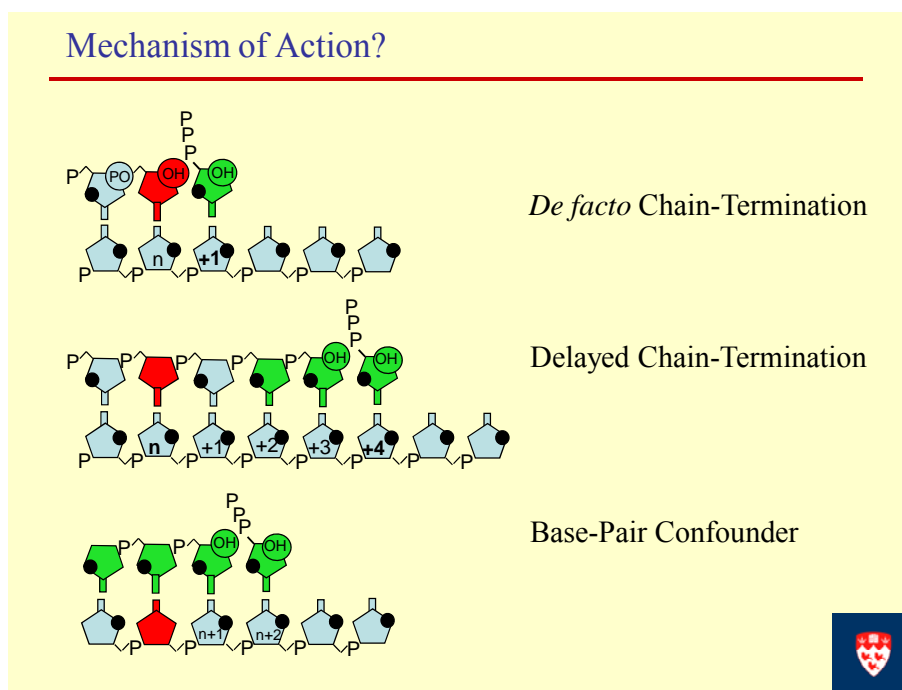


Figure 2. Delayed chain termination proved to be the most likely mechanism of action for entecavir, a guanosine nucleoside analog with activity against HBV and HIV.

These experiments confirmed that ETV-MP competes with deoxyguanosine triphosphate (dGTP) for binding to HIV-1 RT. Increasing natural deoxynucleotide triphosphate (dNTP) concentrations at RT positions $n + 1$ and $n + 4$ overcame RT pausing at the positions designated n and $n + 3$ after ETV-MP incorporation into the template/primer. However, incorporation of the natural nucleotide at position $n + 4$ became severely limited. Nucleotide incorporation at $n + 1$ decreased only 8-fold with entecavir-terminated primers versus natural counterparts, but nucleotide incorporation at $n + 4$ decreased by more than 3 orders of magnitude (1233-fold).

ETV-MP forced RT to “slide away” from the 3′-end of the primer at $n + 3$. The investigators suggested this effect is a plausible explanation of “delayed chain termination,” which provides protection from ETV-MP excision. Thus they proposed “delayed chain termination” as the main mechanism of action of entecavir.

As with classic chain terminators, RT bearing thymidine analog mutations efficiently excised ETV-MP at position n . Despite this excision, entecavir remained fully sensitive to virus bearing thymidine analog mutations. Götte and colleagues published these findings around the time of HIV DART [36].

Differential mitochondrial toxicity across pancreatic cell lines

CAPAN-1 pancreatic adenocarcinoma cells were more reliable and sensitive for in vitro assessment of NRTI-induced mitochondrial toxicity than the PANC-1 pancreatic adenocarcinoma cell line, according to results of studies with five NRTIs [37]. Leda Bassit and colleagues (Emory University and Veterans Affairs Medical Center, Atlanta) believe their findings also help explain cell-specific toxicity with different NRTIs.

Pancreatitis, a dangerous and still-prevalent NRTI-induced mitochondrial toxicity, was responsible for withdrawal of one candidate NRTI. Zalcitabine, stavudine, didanosine, and didanosine plus tenofovir disoproxil fumarate (TDF) have been associated with pancreatitis. Although several available cell-based models can help predict pancreatic mitochondrial toxicity of experimental agents, different variables can affect performance of these models in humans.

Because few studies have assessed mitochondrial cytotoxicity in human pancreatic adenocarcinoma cell lines, the investigators compared PANC-1 cells of pancreatic ductal adenocarcinoma origin with CAPAN-1 cells of both ductal and acinar origin to assess the ability of dextro-thymidine (D-D4FC, Reverset), tenofovir, and TDF to induce mitochondrial toxicity. Cells were exposed to various concentrations of zalcitabine (a positive control) or lamivudine (3TC, a negative control) for 14 days, followed by isolation of total cellular DNA and parallel real-time PCR amplification of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA).

In PANC-1 cells, Bassit and colleagues observed no apparent toxicity for 3TC or tenofovir, while zalcitabine induced mtDNA toxicity at 10 μM . D-D4FC was not toxic at concentrations up to 50 μM , whereas TDF was slightly toxic for both nDNA and mtDNA at a concentration of 50 μM .

Results differed in CAPAN-1 cells. Zalcitabine induced mtDNA toxicity at a concentration of 1 μM . D-D4FC, which was not toxic in PANC-1 cells at 50 μM concentrations, induced selective mtDNA toxicity at a concentration of 10 μM in CAPAN-1 cells. TDF at 10 μM demonstrated slight toxicity for both nDNA and mtDNA. As in PANC-1 cells, tenofovir and 3TC demonstrated no apparent nDNA or mtDNA toxicity in CAPAN-1 cells.

In CAPAN-1 cells, D-D4FC and zalcitabine induced significant mtDNA reduction at an IC_{50} of 0.1 μM for zalcitabine and 4.3 μM for D-D4FC. Compared with results in PANC-1 cells, toxicity in CAPAN-1 cells was 18-fold higher for zalcitabine and 12-fold higher for D-D4FC. TDF displayed similar toxicity in CAPAN-1 cells (IC_{50} 9 μM) and PANC-1 cells (IC_{50} 12 μM).

All drugs were efficiently phosphorylated in PANC-1 and CAPAN-1 cells. After 4-hour exposure of cells to 10 μM of 3TC, TDF, and D-D4FC, nucleoside triphosphate (TP) levels were higher in PANC-1 cells than in CAPAN-1 cells. Intracellular tenofovir-DP concentrations were on average 25 times higher than concentrations of 3TC-TP or D-D4FC-TP in both cell lines. The investigators believe those results suggest there is no direct relationship between NRTI phosphorylation and mitochondrial toxicity.

Bassit and coworkers concluded that the CAPAN-1 cell system offers reliable and sensitive assessment of NRTI-associated mtDNA toxicity and could help predict NRTI-induced pancreatitis.

Pathogenesis and Targeted Design

Electron cryotomography reveals structure of maturing HIV-1 virion

A novel electron tomography analysis of frozen HIV-1 virions—cryo-ET—revealed the size and shape of immature and mature virions and suggested a model of how Gag polyprotein subunits assemble into a lattice framework after viral budding [38]. Although previous work defined the structure of nearly every HIV-1 component, little evidence suggested how individual subunits coalesced into an intact virion until these experiments were performed by Elizabeth Wright and colleagues at the California Institute of Technology in Pasadena. (Wright is now at Emory University in Atlanta.)

Maturation of the HIV-1 virion depends on processing of the Gag and Gag-Pol polyproteins by protease. Unprocessed Gag consists of six radially arranged segments—MA, CA, SP1, NC, SP2, and p6. MA remains bound to membrane during maturation, while processed CA subunits condense to form a conical central capsid encasing NC, the RNA genome, and other viral enzymes. To elucidate the arrangement of Gag subunits during this process, Wright and colleagues halted maturation of HIV-1 virions by inactivating protease, RT, and RNase H. They then flash-froze purified virions onto electron microscopy grids using liquid ethane, imaged the grids, and created three-dimensional reconstructions of the virions.

Cryo-ET has three advantages over other virion-imaging techniques: it samples the virion in its native state, it eliminates specimen preparation artifacts of conventional electron microscopy, and it permits enhanced contrast. Cryo-ET images can be correlated with those of other imaging methods, such as fluorescence microscopy. The technique records two-dimensional projections while samples are tilted incrementally along one or two axes. The three-dimensional structures are then computationally generated with IMOD, which implements a weighted back-projection algorithm.

Visualization of immature virions with cryo-ET showed the concentric shells of the Gag polyprotein. Further analysis revealed that only the CA and SP1 shells contain patches of hexagonal order. The investigators averaged well-ordered units to propose a model for the immature lattice in which a bundle of six SP1 helices stabilizes each CA hexamer.

Wright determined that proteolysis at the CA-SP1 junction during viral maturation removes the six-helix bundle and thereby destabilizes hexamers and the immature lattice. Proteolysis at the MA-CA junction acts as a second structural switch, releasing CA from the membrane and triggering folding of the N-terminal β -hairpin. This restructuring of the CA N-terminus induces formation of the CA-NTD rings seen in the mature lattice. Thus, in contrast with the immature lattice, mature hexamers are held together from "above" by the interactions between helices 1 and 2.

Insights on how Vpu overcomes host cell restriction

Analyses of calcium-modulating cyclophilin ligand (CAML) and tetherin, host-cell restriction factors that inhibit viral replication, disclosed mechanisms by which the HIV-1 Vpu protein overcomes these restriction factors [39]. These experiments also discerned a single residue change in the cytoplasmic domain that may explain why CAML from African green monkeys does not confer restriction, while human CAML does.

Earlier work demonstrated that Vpu, a protein present in HIV-1 but not in HIV-2 or most SIV species, downregulates CD4 and enhances particle release from HIV-infected cells. Recent research revealed two clues to how Vpu enhances release of viral particles. One involves tetherin, the molecule responsible for tethering the virion to the plasma membrane [40]; the second involves CAML. Paul Spearman of Emory University, who showed that Vpu overcomes CAML restriction of HIV-1 release [41], presented results of further studies exploring the mechanisms of these two restriction factors.

Using p24 antigen assays, Western blot, and electron microscopy to analyze the roles of tetherin and CAML, Spearman and coworkers confirmed that both factors restrict particle release and thereby convert permissive cells to a restrictive phenotype. The amount of cDNA required to confer restriction indicated that tetherin is more potent than CAML in restricting particle release. Overexpression of either tetherin or CAML induced particle tethering visible upon electron microscopy.

Coexpression studies demonstrated that tetherin colocalized with Vpu more dramatically than CAML did. This work also showed that Vpu redistributed tetherin from the cell membrane to intracellular sites. African green monkey CAML cloned from Cos-7 cells failed to confer restriction. The investigators mapped this difference from human CAML to a single residue change in the cytoplasmic domain.

Spearman summarized the numerous characteristics of CAML and tetherin as host restriction molecules (Table 5).

Table 5. Characteristics of CAML and tetherin as host restriction molecules

<i>CAML</i>	<i>Tetherin</i>
CAML interacts directly with the Vpu cytoplasmic tail.	Tetherin is inducible by interferon.
siRNA knockdown of CAML in HeLa cells or T cells enhances particle release.	siRNA knockdown of tetherin in HeLa cells or T cells enhances particle release.
Expression of CAML in permissive Cos-7 or HOS cells restricts particle release.	Expression of tetherin in permissive 293T or HT 1080 cells restricts particle release.
Human CAML, but not African green monkey CAML, restricts HIV particle release.	The cytoplasmic tail and GPI anchor are required for restriction of particle release.
CAML colocalizes with Vpu in intracellular sites.	Tetherin colocalizes with Vpu in intracellular sites.
CAML expression in Cos-7 cells recapitulates the electronmicrographic phenotype of tethered Vpu-deficient virions.	Tetherin expression in HT 1080 cells recapitulates the electronmicrographic phenotype of tethered Vpu-deficient virions.
Vpu or HIV-2 Env overcomes a CAML-mediated block to particle release.	Vpu expression leads to downmodulation of cell surface tetherin.

Spearman noted that the mechanism behind the effect of Vpu on tetherin and CAML remains to be determined. Still, he proposed that therapeutic strategies might target Vpu via ion channel inhibitors or small molecules against the cytoplasmic tail. He also suggested it may be possible to mimic or enhance the activity of tetherin.

Nanoparticle delivery of agents targeting RT and integrase

Researchers from the Centre de Recherches de Biochimie Macromoléculaire in Montpellier presented data on development of short peptides that target protein-protein interfaces required for reverse transcription or the RT-integrase interaction [42]. Both peptides can be delivered by a peptide-based nanoparticle system.

Gilles Divita and coworkers designed two families of short peptides that target one of the two steps required for formation of biologically active RT: dimerization (rapid association of the RT palm subunit p66 and the RT finger subunit p51) and maturation (slow conformational changes yielding active RT) [43,44]. The Montpellier group presented results on two peptides: the 9-residue Pep-71B, which targets protein-protein interfaces involved in RT dimerization, and the 10-residue P-16, which blocks the RT-integrase interaction.

To evaluate these novel inhibitors, Divita and colleagues developed a peptide-based nanoparticle system called NANO-VEPEP. The nanoparticles form stable complexes with peptides, enhance their cellular uptake, and improve their *in vivo* stability and distribution. Delivery of these agents by NANO-VEPEP requires intravenous injection.

When delivered by peptide-based nanoparticles, Pep-71B blocked replication of HIV1-LAI in peripheral blood mononuclear cells (PBMCs) at a 50% effective concentration (EC_{50}) of 0.7 nM. Because the Pep-71B sequence is conserved in all HIV-1 subtypes, the peptide exerted potent antiviral activity against isolates representing subtypes A, B, C, D, E, F, and G. Pep-71B also blocked replication of virus bearing multiple mutations induced by RT inhibitors, including 67N, 70R, 215F, and 219Q at an of EC_{50} 0.19 nM; 41L, 74V, 106A, and 215Y at an of EC_{50} 2.9 nM; 181C at an EC_{50} 0.1; T69SSSG, 108I, 210W, and 215Y at an EC_{50} of 0.35 nM; and 62V, 65R, 75L, 100I, 103N, 116Y, 118L, 151M, and 184V at an EC_{50} in a nanomolar range. P-16 also blocked replication of isolates representing subtypes A, B, C, D, E, F, and G as well as viral strains resistant to NRTIs and NNRTIs.

Divita and colleagues then tested NANO-VEPEP-delivered Pep-71B and P-16 in intravenously challenged hCD4/hCCR5 transgenic rats and nude mice. Both peptides blocked viral propagation as well as resistant strains in these models.

Impact of arginine methyltransferase on HIV-1 replication cycle

Protein arginine methyltransferase 6 (PRMT6) disrupts the viral replication cycle of HIV-1 by decreasing the functions of viral proteins Tat and Rev and HIV-1 nucleocapsid (NC), according to results of experiments by Mark Wainberg and colleagues at McGill University in Montreal [45]. They determined that PRMT6 exerts this activity by methylating arginine residues of Tat, Rev, and NC in their basic regions.

Tat activates viral transcription by forming a ternary complex with TAR and cyclin T1. Lysine acetylation, methylation, and ubiquitination all stimulate Tat transcriptional activation. In the

McGill experiments, PRMT6 reduced transcriptional activation of Tat and diminished cyclin T1-dependent Tat transactivation. PRMT6-induced arginine methylation of Tat diminished ternary complex formation with TAR and cyclin T1. Wainberg and coworkers concluded that PRMT6 impairs Tat function.

Rev mediates export of unspliced and partially spliced viral RNA to cytoplasm and thus initiates the late phase of viral replication. Wainberg and colleagues showed that PRMT6 promoted arginine methylation of Rev. However, PRMT6 also induced Rev downregulation independently of its methyltransferase activity. PRMT6 diminished Rev-mediated viral RNA export. The investigators concluded that PRMT6 impairs Rev function.

HIV-1 NC packages viral RNA into virions, mediates strand transfer during reverse transcription, and anneals tRNA^{Lys}₃ to the primer binding site. Again, Wainberg and coworkers demonstrated that PRMT6 fosters arginine methylation of NC. PRMT6 lowered the annealing efficiency of tRNA^{Lys}₃ to the primer binding site and thereby impaired NC function.

Next the McGill team asked whether PRMT6 assists HIV-1 by helping the virus establish latency in host cells. The researchers found that PRMT6 levels were only marginally increased in latently infected U1 cells compared with chronically infected U937 cells. In addition, PRMT6 inhibitors failed to induce viral production in latent U1 cells. Therefore, Wainberg and colleagues concluded that PRMT6 is not a major player in establishing HIV-1 latency.

Finally, the researchers asked whether PRMT6 is part of the host's antiviral defense. Several findings indicated that it is: (1) PRMT6 was strongly upregulated in stimulated PBMCs. (2) HIV-1 counteracted upregulation of PRMT6 levels upon infection of stimulated PBMCs. (3) HIV-1 reduced PRMT6 levels in PBMCs by more than 1 log. (4) HIV-1 abolished PRMT6 levels in chronically infected virus-producing U937 cells. And (5) PRMT6 was downregulated in the presence of Vif, an accessory HIV-1 protein.

Wainberg suggested PRMT6 constitutes an innate anti-HIV-1 immunity mechanism similar to APOBEC3G and that, as with APOBEC3G, the accessory Vif protein counteracts this mechanism.

Glycan deletions in gp120 V1/V2 domain compromise replication capacity

In a study published around the time of HIV DART 2008 [46], researchers from the Rega Institute in Leuven showed that glycan deletions in the V1/V2 loop domain of HIV-1 gp120 compromise replication capacity of the mutant viruses [47]. The investigators believe their findings explain why glycan deletions at these sites rarely occur under pressure of carbohydrate-binding agents (CBAs).

The gp120 glycoprotein of HIV-1 has 24 *N*-linked glycosylation sites. CBAs such as mannose-specific *Hippeastrum* hybrid agglutinin (HHA), GlcNAc-specific *Urtica dioica* agglutinin (UDA), and the nonpeptidic compound PRM-A can select for glycan deletions in all HIV-1 gp120 domains except V1 and V2.

To discern the underlying mechanisms, Joeri Auwerx and coworkers used chimeric virus technology to construct 31 viral strains containing one or several *N*-glycan deletions in the V1/V2 region of gp120 in the CXCR4-tropic strain HIV-1NL4.3. Mutant viral strains analyzed

for coreceptor use consistently used CXCR4, never switching to CCR5 or to dual coreceptor use.

More glycan deletions in the V1/V2 domain resulted in a greater loss of viral replication capacity. Because mutant viruses did not differ in CD4 binding efficiency or sensitivity to soluble CD4 or the CXCR4 antagonist AMD3100, the researchers concluded that loss of replication capacity could not be explained by major structural envelope changes that would compromise binding to CD4 or CXCR4. Auwerx and colleagues found that glycans at positions 156, 160, 186, and 195 are highly conserved.

None of the glycan deletion mutants had markedly decreased sensitivity to the inhibitory activity of HHA, UDA, or PRM-A. In fact, the Rega investigators consistently observed 2- to 10-fold higher sensitivity to these CBAs. They proposed that V1/V2 loop glycans should be considered a “hot-spot” target for specific antiretroviral intervention.

HIV Drug Resistance

Brendan Larder accepted the David Barry DART Achievement Award with an insightful historical review of HIV resistance research and with a touch of irony befitting the occasion [48]. Most people familiar with HIV research know that Larder was the first to isolate zidovudine-resistant virus from people taking the NRTI. He subsequently identified reverse transcriptase mutations in these isolates [49], then proved that these specific mutations make HIV-1 resistant to zidovudine [50] and that resistant virus emerges rapidly in patients taking zidovudine monotherapy [51]. Not as many people realize that, at times, Larder and Barry had anything but a tranquil relationship during their tenures at Wellcome Research Laboratories, as Barry questioned Larder about everything from the way he dressed when presenting his ground-breaking data, to the import of those seminal findings.

Barry, who nursed zidovudine through its development and eventual licensing as the first antiretroviral, was less than jubilant when that his wunderkind scientist discovered its critical flaw—the Achilles heel of resistance that doomed all early monotherapies. Larder noted that the weight of scientific evidence eventually convinced Barry that resistance made monotherapy impossible, and Barry argued for triple therapy at the 1994 International AIDS Conference in Yokohama.

Meanwhile, Larder, collaborating with a Who’s Who in antiretroviral resistance research, including many trained or mentored by him, had gone on to identify a string of RT substitutions, now commonly called thymidine analog mutations or TAMs [52], proving that these mutations alone were sufficient to confer resistance to zidovudine [50]. With Sharon Kemp, he accurately predicted that rapid PCR-based assays could identify resistance-conferring mutations and permit quick clinical detection of failing antiretrovirals [50]. Teaming with Charles Boucher, now at Erasmus Medical Center in Rotterdam, Larder launched the HIV Drug Resistance Workshop, where virtually all key antiretroviral resistance research first faces peer scrutiny.

With the development of HIV-1 resistance genotyping on his résumé, Larder went on to standardize viral phenotyping with Paul Kellam [53], then helped establish Virco, one of the leading antiviral phenotyping concerns. He also devised allele-specific PCR [54] and demonstrated the utility of automated viral sequencing [55].

With Marty St. Clair, Larder showed that the L74V substitution makes HIV-1 resistant to didanosine but re-sensitized to zidovudine [56]. He discovered that the Y181C mutation renders virus resistant to NNRTIs but more susceptible to zidovudine [57]. This dual effect of single RT mutations also held true for the lamivudine-induced M184V [58]. These discoveries, plus clinical trial results, nailed down the rationale for combination therapy, which eventually made HIV infection a manageable disease. At the same time, these studies demonstrated that antiretrovirals with exceedingly low barriers to resistance—like lamivudine and first-generation NNRTIs—could play critical roles in combination regimens.

More recently, Larder founded and became Chair of the HIV Resistance Response Database Initiative (RDI), which is using advanced artificial intelligence computing technology to predict a patient's response to anti-HIV drug combinations relative to viral resistance status [59].

Pyrimidinedione NNRTIs display multiple mechanisms of action

Cell-based studies by investigators at ImQuest Biosciences identified lead compounds from the SAR series of pyrimidinedione NNRTIs that have a high barrier to resistance and activity against virus bearing mutations that confer resistance to multiple current reverse transcriptase inhibitors and PIs [60]. ImQuest's Robert Buckheit Jr. reported that the novel agents appear to inhibit viral replication by multiple mechanisms.

ImQuest investigators identified 68 pyrimidinedione compounds that inhibit virus harboring critical NNRTI resistance mutations, including L100I, K103N, and Y181C, in both cell-based and biochemical RT inhibition assays. The researchers proposed that those findings suggest that pyrimidinediones interact with the RT binding pocket in a way that gives these compounds a high genetic barrier to resistance.

Buckheit and colleagues selected IQP-0410 as the lead therapeutic candidate and IQP-0528 as the lead microbicide candidate. Both candidates inhibited replication of clinical strains representing HIV-1 subtypes A, B, C, D, E, and F. Both compounds also inhibited HIV-1IIIB entry in MAGI cell assays at EC_{50} s below 100 nM. In CEM-SS cells, IQP-0410 and IQP-0528 inhibited fusion and cell-to-cell viral transmission at EC_{50} s of 2nM or less for IQP-0410 and of 7 nM for IQP-0528.

Serial passage studies determined that IQP-0410 initially selects Y181C, a classic NNRTI mutation, followed by mutations in gp120 and gp41. Selection of Y181C and other NNRTI mutations is consistent with the NNRTI class mechanism of action, while selection of gp120 and gp41 mutations is consistent with chemokines receptor engagement and antifusion activity. Pyrimidinediones inhibited entry and fusion of HIV-2 as well as HIV-1 but, like other NNRTIs, these compounds did not inhibit HIV-2 reverse transcription.

Virus resistant to IQP-0410 proved cross-resistant to efavirenz and other pyrimidinedione NNRTIs but susceptible to zidovudine and tenofovir and possibly hypersusceptible to enfuvirtide-resistant virus. Accumulation of mutations in HIV-1 protease yielded increased susceptibility to pyrimidinediones.

ImQuest submitted the IND for IQP-0410 to the FDA on the last day of 2008 and hopes to begin clinical trials in 2009. The company intends to develop IQP-0528 jointly with the International Partnership for Microbicides and CONRAD. Clinical microbicide trials could begin in 2010.

ImQuest aims to develop a next-generation pyrimidinedione with a higher genetic barrier to resistance in 2009.

Analysis of critical NRTI resistance mutations: TAMs and K65R

X-ray crystallography yielded evidence that could add to the understanding of NRTI resistance conferred by thymidine analog mutations (TAMs) and by K65R, which confers resistance to tenofovir and has complex relationships with other NRTI resistance mutations [61].

Eddy Arnold (Rutgers University, Piscataway, New Jersey) explained that resistance to NRTIs implies that mutant HIV-1 RT has an enhanced ability to discriminate between the NRTI and normal nucleosides. That discrimination can occur when the NRTI is incorporated into the template/primer or by enhanced excision of the NRTI after incorporation, in a process called adenosine triphosphate (ATP)-dependent pyrophosphorolysis.

The excision model for resistance to zidovudine (AZT) involves four steps spelled out by Arnold:

- Wild-type HIV-1 RT inefficiently excises AZT-monophosphate (MP) through ATP-dependent pyrophosphorolysis.
- A key AZT resistance mutation, T215Y/F, enhances ATP binding through aromatic interactions of T215Y/F and the adenine ring.
- The other AZT resistance mutations enhance ATP-dependent excision.
- RT finger insertions enhance the ability of AZT-RT to excise other NRTIs.

ATP-mediated excision of AZT-MP forms a dinucleoside tetraphosphate product, AZTppppA. Arnold and colleagues used crystallography to solve and analyze a series of AZT-resistant RT structures, including ternary complexes with a template-primer and AZTppppA. These studies showed that the TAMs K70R and T215Y help bind ATP to mutant RT. Therefore, Arnold and colleagues proposed that K70R and T215Y can be classified as excision-enhancing mutations.

K65R can be selected by tenofovir, abacavir, didanosine, and occasionally stavudine. It confers low-level resistance to all NRTIs, except for zidovudine, which remains susceptible to K65R mutant virus. This mutation has several functions, including (1) decreasing the incorporation rate of dNTPs and NRTIs, (2) decreasing NRTI excision, (3) increasing fidelity, and (4) decreasing viral replication capacity.

Analyzing K65R RT in ternary complexes with tenofovir diphosphate and dATP, Arnold and coworkers found that K65R does not significantly alter the interaction between residue 65 and dNTP and that differential stacking of the guanidinium groups of K65R and R72 creates a “checkpoint” that reduces dNTP incorporation, reduces NRTI excision, and increases fidelity. Because this platform has alternate conformations when tenofovir diphosphate or dATP binds, mutant RT can discriminate between tenofovir diphosphate and dATP, the normal substrate.

Crystallography also yielded evidence suggesting potential mechanisms underlying the complex relationships between K65R and other NRTI mutations, including the excision-enhancing mutations.

HIV-1 can select *gag* mutations that restore optimum cleavage and RC

Experiments with recombinant viruses bearing mutations in the NC/p1 Gag cleavage site showed that HIV-1 can select additional mutations to restore optimum Gag cleavage and replication capacity (RC) [62]. This work by Monique Nijhuis (University Medical Centre Utrecht) and colleagues adds to the growing understanding of how Gag cleavage site mutations contribute to resistance to PIs.

Mutations in viral protease reduce the affinity of protease for the inhibitor. Earlier research by Nijhuis and coworkers showed that resistance to PIs can be conferred by mutations in NC/p1 caused by enhanced processing of the HIV-1 Gag protein [63]. To study the impact of enhanced Gag processing on replicative capacity—and resulting evolution of HIV-1—these investigators generated four recombinant viruses conferring different levels of resistance to PIs: 431V, 437V, 437T, and 436E + 437T (Figure 3). Nijhuis and colleagues used viral replication curves to gauge the impact of enhanced Gag processing on RC, and they performed multiple individual in vitro evolution experiments to investigate potential evolutionary pathways in the absence of PI pressure. They assessed Gag processing and PI susceptibility in mutations revealed by complete sequencing of viral Gag and protease.

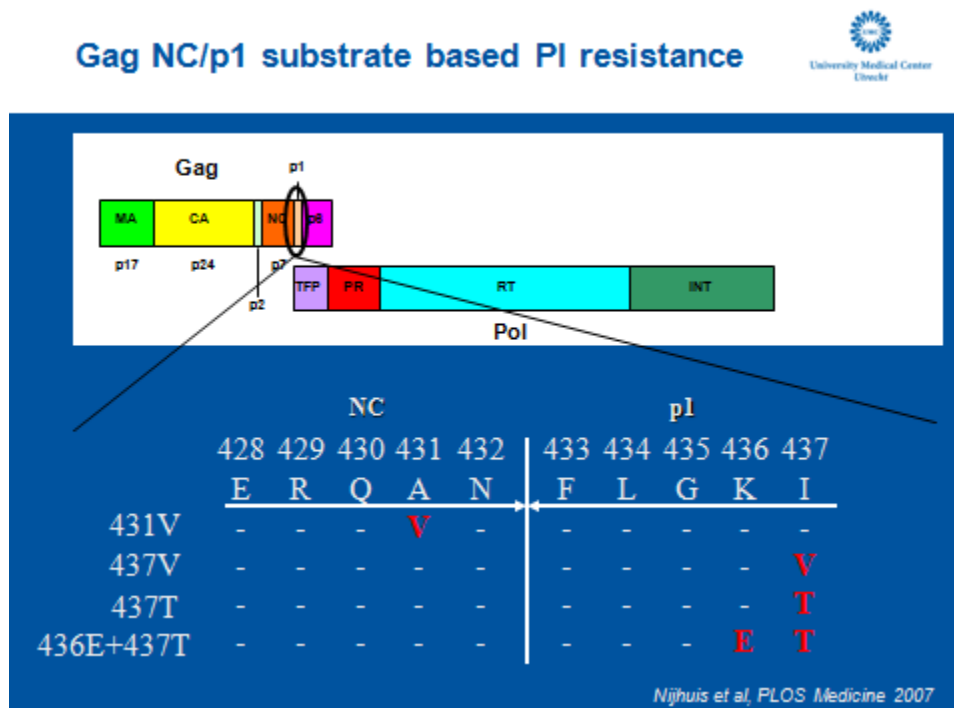


Figure 3. A double Gag cleavage site mutation, 463E plus 437T, increased overall Gag cleavage, conferred resistance to lopinavir and tipranavir, and reduced viral replication capacity.

Single mutations in NC/p1 that conferred only a slight increase in resistance to PIs had no obvious impact on viral RC. The in vitro evolution experiments disclosed no signs of evolution of these single-mutant viruses, except for selection of A429K in one of five 431V experiments. The double NC/p1 mutation 436E + 437T displayed not only a marked increase in overall Gag cleavage and resistance to lopinavir and tipranavir, but also reduced RC.

Nijhuis suggested these findings demonstrate that HIV-1 has an optimum Gag cleavage rate. When NC/p1 mutations enhance Gag processing and reduce RC, HIV-1 can evolve further mutations that restore Gag cleavage and RC.

Toward HIV Eradication

Env-targeted immunotoxins assessed in clearing HIV-1 reservoirs

Engineered toxins that target HIV-1 expressed on the surface of productively infected cells may prove a useful adjunct to antiretroviral therapy that blocks viral replication [64]. Edward Berger (National Institute of Allergy and Infectious Diseases, Bethesda) advanced that proposal on the basis of cell culture and animal studies involving two investigational immunotoxins.

Ample research demonstrates that HIV-1 RNA persists at levels below standard detection limits despite the most potent antiretroviral combinations. This low-level viremia probably does not allow viral evolution, but so far it has proved unresponsive to antiretroviral intensification strategies. As a result, some propose that residual viremia represents release of virus from productively infected cells that may be very long-lived and possibly capable of proliferation. Thus an agent that directly kills productively infected cells would be a valuable ally to current virus replication-blocking strategies that do not affect virus-producing cells.

Berger and colleagues developed two Env-targeted immunotoxins, each of them a recombinant single-chain chimeric protein containing the translocation and cytotoxic domains of *Pseudomonas aeruginosa* exotoxin A linked to a specific protein that binds Env. CD4-PE40 uses soluble CD4 to target Env, while 3B3-PE38, a newer agent, relies on a single-chain antibody fragment (scFv) of the high-affinity 3B3 monoclonal antibody against the CD4 binding site.

CD4-PE40 killed chronically infected cells at an IC_{50} of approximately 2 nM. 3B3-PE38 proved more potent, killing infected cells at an IC_{50} of 0.03 nM. Both agents had negligible activity against parental cells not infected by HIV. 3B3-PE38 also showed greater activity than CD4-PE40 in inhibiting spreading infection from diverse primary HIV-1 isolates tested in PBMCs [65]. In addition, 3B3-PE38 blocked spreading infection in primary macrophages [65].

High-dose CD4-PE40 caused some hepatotoxicity in rhesus macaques, but high-dose 3B3-PE38 did not [65]. This finding is important because CD4-PE40 caused dose-limiting liver toxicity in pre-HAART era trials. Lack of 3B3-PE38-induced liver toxicity in macaques reflects results of recent clinical trials in which a PE-based immunotoxin induced remission of leukemias and lymphomas but caused no liver toxicity [66].

In a thy/liv-SCID-hu mouse model of HIV infection, combination antiretrovirals suppressed viral replication but, as expected, viremia rebounded as soon as the investigators stopped administering antiretrovirals [67]. However, when the investigators administered both antiretrovirals and CD4-PE40 or 3B3-PE38 to these mice, viremia did not rebound for as long as 1 month after drug withdrawal. These findings highlight the value of complementing antiretrovirals with agents that directly kill already-infected cells.

Berger, who discovered that CXCR4 is an HIV-1 coreceptor, expects to begin a clinical trial of immunotoxins plus antiretrovirals soon to see if they help deplete viral reservoirs. But he

cautioned that several questions about this strategy remain, including which cellular reservoirs and anatomic sites can be effectively targeted, whether immunotoxin efficacy will depend on deliberately activating latently infected cells, and whether dose-limiting toxicities will arise.

Postintegration inhibitor slows HIV-1 release from macrophages

Cell studies of a novel antiretroviral candidate, BIT225, show that the compound inhibits HIV-1 release from monocytes/macrophages and viral transfer to CD4 cells, apparently by interfering with a postintegration step involving the HIV-1 Vpu protein [68]. BIT225 had no activity against HIV-2, which differs from HIV-1 in lacking Vpu.

John Wilkinson and Biotron colleagues (Sydney, Australia) isolated CD14 and CD16 monocytes from donors without HIV-1 or HIV-2 and pulsed them for 3 hours with HIV-1Bal on days 0, 2, 5, 7, and 14, then cocultured the pulsed cells with activated CD4 cells from an HIV-1-negative donor for a further 5 days. They used RT-PCR to measure HIV-1 *gag* DNA in nonadherent CD4 cells and adherent monocytes. An RT assay measured viral replication in culture supernatants. The investigators used the TZM-bl indicator cell line to determine infectivity of released virus.

BIT225 reduced HIV-1 spread in CD14 monocytes ($P = 0.016$ versus DMSO control) and CD16 monocytes ($P = 0.076$ versus DMSO control). The compound also inhibited transfer of HIV-1 from monocytes to CD4 cells ($P < 0.05$ versus DMSO control with CD14 monocytes, $P = 0.12$ versus DMSO with CD16 monocytes).

Infecting the TZM-bl cell line with HIV-1 in the presence of BIT225 indicated that the compound acts at a postintegration step. The NNRTI efavirenz, the positive control in this experiment, inhibited viral integration, whereas BIT225 and two negative controls, the PIs lopinavir and saquinavir, did not. BIT225 had no activity against two HIV-2 strains, CBL-20 and CBL-23, which lack Vpu.

Further experiments determined that BIT225 does not affect HIV-1 RT or protease. Electron microscopy of BIT225-treated macrophages revealed an appearance suggesting abnormal packaging. Biotron investigators have begun to characterize these abnormalities.

A phase 1b/2a study of BIT225 for hepatitis C virus infection began in November 2008. A study site and design are being finalized for a phase 1b/2a trial of BIT225 for HIV infection.

Macaque model of HIV infection identifies viral replication and latency sites

A rhesus macaque model of HIV-1 infection being developed to evaluate viral eradication strategies pinpointed sites of ongoing viral replication and latency in animals exposed to a recommended first-line combination—efavirenz, tenofovir, and emtricitabine [69]. University of California, Davis researchers conducted these controlled studies as part of a comprehensive analysis of viral reservoirs and replication dynamics during antiretroviral therapy.

The macaques studied are infected with a chimeric virus of SIVmac239 containing HIV-1 reverse transcriptase instead of SIV reverse transcriptase (RT-SHIV). RT-SHIV is susceptible to NRTIs, NNRTIs, PI, and integrase inhibitors. To assess viral dynamics in these animals, North and colleagues adapted a viral load assay with a detection limit of 1 to 2 viral RNA copies/mL of plasma. They also developed sensitive RT-PCR and PCR assays to measure viral RNA and DNA

in tissues. This study involved 9 antiretroviral-treated macaques, 5 used to measure virus in cells and tissues at necropsy, and 4 used to assess viral rebound dynamics after antiretroviral withdrawal.

Tom North and colleagues suggested several advantages of the RT-SHIV macaque model:

- Animals are infected at the same time with the same virus and can be treated with the same antiretrovirals.
- Questions about the initiation of antiretrovirals can be addressed in controlled experiments.
- Invasive experiments can be performed during therapy to analyze reservoirs, viral latency, and residual viral replication.
- Viral rebound after antiretroviral withdrawal can be studied under controlled conditions to assess clinical benefit of strategies that may contribute to viral eradication.
- Novel drugs or therapeutic strategies can be evaluated.

Analysis of viral RNA and DNA in 28 tissues disclosed distinct differences between the 5 macaques treated with efavirenz, emtricitabine, and tenofovir and 1 untreated control animal. The untreated macaque had detectable RT-SHIV RNA and DNA in nearly all tissues analyzed, including all lymphoid tissues, all regions of the gastrointestinal (GI) tract, and nearly all brain and reproductive tissue sites.

Viral RNA and DNA could be detected in most lymphoid tissue sites of all 5 antiretroviral-treated animals. While viral DNA could also be detected in thymus from all 5 treated animals, only 3 animals had detectable viral RNA in thymus. Most GI tissues of all antiretroviral-treated animals bore viral DNA, but viral RNA could not be detected in some GI samples. Viral RNA loads were lower in GI tissue than in lymph nodes. All other tissues assessed in treated macaques had very low or undetectable levels of viral DNA or RNA. Resting CD4 cells sampled from spleen, lymph nodes, jejunum, and PBMCs had higher levels of viral DNA than did the total cell suspensions from which they were isolated.

North concluded that residual viral replication persists even in animals treated with a highly potent standard antiretroviral combination. Some tissues with detectable SIV DNA but no detectable *gag* RNA are being investigated as potential sites of viral latency.

Fixed-dose agent to inhibit HIV and immune hyperactivation

VS411-2, a coformulation of didanosine and low-dose hydroxyurea, may be the forerunner of a new antiretroviral class called virostatics (*antiviral cytostatics*) that aim both to control HIV-1 replication and to quell hyperactivation of the immune system, which persists even in people with undetectable plasma viremia [70]. Franco Lori and Michael Stevens, who head ViroStatics in Italy and the United States, have begun work on identifying and testing single-molecule virostatic agents that contain neither didanosine nor hydroxyurea.

In the early 1990s, Lori discovered that hydroxyurea inhibits synthesis of deoxynucleotides, which are essential for reverse transcription, and thus promote incorporation of deoxynucleotide analogs such as didanosine into viral DNA [71]. Although hydroxyurea-containing regimens won some adherents among HIV clinicians, the strategy fell from favor as stronger and safer antiretroviral-only combinations became the standard of care.

At HIV DART, Michael Stevens outlined the rationale for a revival of virostatic tactics by explaining the impact of antiretroviral and anti-hyperactivation cytostatic agents on a model of HIV-induced immune deficiency (Figure 4). While antiretrovirals quell immune activation by stopping viral replication, cytostatics such as hydroxyurea limit both the genesis and the impact of HIV-induced and non-HIV-induced immune activation.

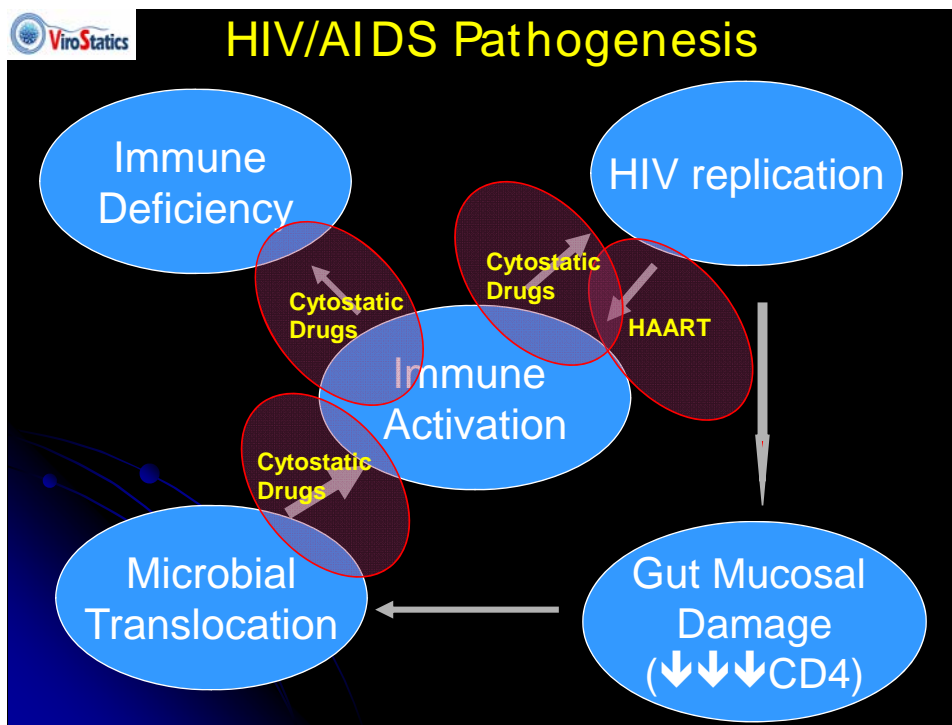


Figure 4. In a five-compartment model of HIV-induced immune deficiency, antiretrovirals disrupt the chain of events at only one point, while a cytostatic drug would act at three additional points, according to investigators from ViroStatics. (Adapted by Michael Stevens from a graphic by Michael Lederman, Case Western Reserve University, Cleveland, Ohio.)

Stevens proposed that an effective virostatic would turn HIV infection in humans into an SIV-like infection in sooty mangabey monkeys. Within the first days of infection, HIV or SIV massively depletes mucosal CD4 memory T cells. The HIV-infected immune system becomes hyperactivated in humans and remains so throughout the course of infection, even when antiretrovirals control viral replication and even in “elite controllers” who maintain relatively low plasma viremia without antiretrovirals. In contrast, SIV-infected mangabeys endure only modest immune activation and maintain higher CD4 counts despite ongoing viral replication. In untreated HIV infection in humans, CD4 counts in blood eventually plummet, whereas peripheral CD4 counts remain high in untreated SIV-infected mangabeys, and they do not acquire the immune-deficiency diseases that define AIDS. Steven and Lori argue that virostatics could effect the same viral-immune equipoise in HIV-infected humans.

Each capsule of VS411-2 combines 300 mg of hydroxyurea with 200 mg of didanosine. Once-daily administration of two capsules would yield a total daily hydroxyurea/didanosine dose of 600/400 mg. In a 12-person four-way crossover trial, VS411-2 yielded a 30% lower didanosine

maximum concentration than standard-dose enteric-coated didanosine, but an equivalent area under the curve for the dosing interval—an intentional pharmacokinetic adaptation designed to limit potential toxicity.

In this study, taking VS411-2 with a high-fat breakfast unexpectedly prolonged absorption and lowered concentrations of hydroxyurea. Hydroxyurea maximum concentration and area under the curve were significantly lower with food than on an empty stomach. Hydroxyurea is typically administered in a fasting state because didanosine must be taken without food.

An ongoing blinded 4-week phase 2 trial in Italy, Russia, Uganda, and Argentina is exploring five doses of hydroxyurea/didanosine in 60 patients: 300/200 mg, 600/200 mg, 900/200 mg, 300/400 mg, and 600/400 mg. Besides suggesting the most appropriate dose, the trial should provide preliminary safety and efficacy data. ViroStatics also has an active discovery and preclinical program investigating agents that combine both antiviral and cytostatic properties in a single molecule.

Novel dose-response curve suggests class-specific limits in inhibitory potential

A novel method to measure HIV-1 inhibition by antiretrovirals, called instantaneous inhibitory potential (IIP), suggests that PIs inhibit virus substantially more than other antiretroviral classes, including integrase inhibitors [72]. Robert Siliciano (Johns Hopkins University, Baltimore) explained how IIP works and outlined results at HIV DART shortly after his group published their findings [73].

Siliciano and colleagues proposed that current dose-response measures such as IC_{50} and inhibitory quotient (drug concentration/ IC_{50}) do not accurately capture the impact of antiretrovirals because they are linear measures trying to gauge the exponential inhibitory action of potent anti-HIV medications. IIP, in contrast, is logarithmic, measuring how much a relevant concentration of an antiretroviral reduces a single round of infectivity in 10-fold units. In the single-round infectivity assay that yields IIP, primary CD4 lymphoblasts are transfected with (1) a plasmid carrying the HIV provirus minus *env* and (2) an X4 *env* expression vector.

Assessing inhibitory potential of all current NRTIs and the nucleotide tenofovir, Siliciano and coworkers found that they yield a dose-response slope approximating 1 log (10-fold) on a logarithmic scale of the fraction of virions inhibited. Raltegravir, elvitegravir, and three experimental integrase inhibitors also have approximately a 1-log slope. Slopes for five NNRTIs (efavirenz, nevirapine, delavirdine, etravirine, and rilpivirine) cluster around 2 log, while slopes for PIs ranged from approximately 2 to nearly 5 log. At maximum concentrations, the most potent PI, darunavir, yields a 10-log (10-billion-fold) reduction in a single round of infection. The Hopkins team had not completed evaluation of CCR5 antagonists at the time of Siliciano's presentation, but he told HIV DART attendees he expects their IIP will be about 1 log.

The investigators showed that antiretrovirals with a substantially higher IIP at 24 hours demonstrated superior virologic efficacy in large, well-controlled clinical trials, although Siliciano noted that an array of other variables clearly contributes to virologic success or failure in the clinic. Still, the Hopkins researchers believe their results explain why most effective antiretroviral combinations include a PI or an NNRTI. But they do not explain why the integrase raltegravir plus lamivudine/tenofovir controlled viral replication faster than efavirenz plus lamivudine/tenofovir in a randomized trial that enrolled previously untreated patients [2].

Siliciano suggested an explanation: Viremia decays rapidly in patients taking raltegravir. That decay reflects turnover of cells still able to produce virus in the presence of drug. One would expect more rapid decay with raltegravir because it blocks HIV replication at a step that leaves a smaller and “older” population of infected cells. But rapid decay may not indicate superior efficacy. Indeed, raltegravir proved no more efficacious than efavirenz in the 48-week trial mentioned [2].

In their *Nature Medicine* report, the Hopkins investigators proposed that the principles of this inhibition model “apply not only to antiviral drugs, but also to vaccine-induced effector mechanisms, including neutralizing antibodies, and any other drugs that target exponential processes, such as the growth of microorganisms or cancer cells.”

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