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Meeting Report from AIDS 2006

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Prevention and Therapeutics

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This year’s 16th International AIDS conference in Toronto, ON, Canada, and ICAAC in San Francisco, CA, USA, brought two themes to the fore: prevention and new treatments. The rising burden of HIV infection is disproportionately impacting lives, life expectancy, and economies in many of the world’s poorest nations, creating a vicious cycle. The limited availability and relatively high cost (relative to gross domestic product or health spend) of antiretroviral drugs has not been sufficient to act as a public health measure, although they do provide benefits to many individuals. Similarly, the disappointingly slow progress on vaccine development means that this option remains distant, at best. As the majority of HIV transmissions occur through sexual intercourse, easy access to an inexpensive topical product, such as a microbicide preparation that is protective against HIV transmission and not an irritant to the vaginal or rectal mucosa, would be highly attractive. Furthermore, microbicides offer the advantage of a means of protection that the passive party in a relationship can control and potentially use without their sexual partner’s knowledge. Such an agent would complement other preventative measures including condoms, circumcision, and one day, hopefully, vaccinations. Importantly, as described in the Commentary by Per Johan Klasse (Cornell University, Weill Medical College, New York, NY, USA), HIV-specific microbicides such as those based around HIV-entry inhibitors do not necessarily need to act as spermicides and thus may allow safer conception [1]. Despite the number of obstacles that Dr Klasse describes, the use of small-molecule entry inhibitors in topical microbicides represents an important potential avenue of research, as several of these agents, such as HIV attachment inhibitors and CCR5 and CXCR4 antagonists, possess attractive properties suitable for further evaluation.

In addition to effective HIV prevention strategies, there is a pressing need for new agents in the management of patients with multi-class resistant HIV. Studies of the mechanism of HIV entry into CD4+ cells has yielded multiple targets for the development of new entry inhibitors, including HIV attachment inhibitors, CCR5 antagonists, and fusion inhibitors. As reviewed by Jacob Lalezari (Mount Zion Hospital of University of California and Quest Clinical Research, San Francisco, CA, USA) and colleagues, a number of these compounds are parenteral formulations that offer the potential of improved pharmacokinetic properties and/or sustained release profiles, which might allow once-weekly, or even once-monthly, dosing. Although parenterally administered agents are unlikely to compete as first- or second-line agents in most settings, their emergence could result in greater treatment choices for patients in need of salvage therapy.

As a journal of viral entry, the scope of this issue expands to include a comprehensive discussion by Zhu-Nan Li and David A Steinhauer (Emory University School of Medicine, Rollins Research Center, Atlanta, GA, USA) of the mechanism of entry of the influenza virus. The interaction between influenza viral hemagglutinin (HA) and host cell surface glycoproteins containing sialic acid results in conformational changes within HA that produce a fusion peptide – a helical coiled coil structure – that, like gp41 of HIV, is propelled to the target cell surface to mediate entry into the cell. Interestingly, as noted by Li and Steinhauer, this paradigm for viral entry is not only seen with influenza and HIV, but is shared with other viruses, including simian immunodeficiency virus and ebola virus. Given the ongoing threat of an avian flu pandemic, it is hoped that a more complete understanding of the mechanism of influenza entry will yield additional antiviral agents to protect humanity from this scourge.

References

Recent Results with CCR5 Antagonists

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Progress in the development of small-molecule, oral CCR5 antagonists as therapeutic agents was reported during the 16th International AIDS Conference. This new class of drugs includes maraviroc, currently in Phase III clinical trials and vicriviroc, in Phase IIB trials. A third member of the class, aplaviroc, was discontinued due to hepatic toxicity; however, this problem has not been observed with the other two agents in ongoing development.

Although the expectation is that CCR5 antagonists will be of most use in individuals carrying only R5 HIV, the activity of maraviroc in individuals with mixed- or dual-tropic (X4/R5) virus was assessed in a Phase IIB study reported at the conference (Mayer H, et al. Abstract THLB0215). Patients were randomized to receive placebo, maraviroc 150 mg once-daily, or maraviroc 150 mg twice-daily, with ritonavir assumed as a component of the optimized background regimen (OBR). The OBR consisted of three to six approved drugs, and was selected on the basis of resistance testing. Patients entering the study were triple-class experienced, had a viral load of >1000 HIV RNA copies/mL to facilitate tropism evaluation, and any CD4+ cell count. Baseline characteristics indicated that the patients had very advanced infection with a mean CD4+ cell count of <100/mm³ and a mean viral load of >5 log₁₀ copies/mL. The primary analysis presented was 24-week data, which showed a change in viral load in the placebo, maraviroc 150 mg once-daily, and maraviroc 150 mg twice-daily groups of −0.97, −0.91, and −1.2 log₁₀ copies/mL, respectively (differences not statistically significant). In individuals who also received the fusion inhibitor enfuvirtide, the changes were −0.89, −1.26, and −1.44 log₁₀ copies/mL, respectively. These data suggest antiviral synergy between maraviroc and enfuvirtide, as has been inferred from in vitro studies of combinations of entry inhibitors.

With regards to secondary endpoints, the percentage of patients achieving a viral load of <400 copies/mL in the placebo, maraviroc 150 mg once-daily, and maraviroc 150 mg twice-daily cohorts were 24.1%, 24.6%, and 30.8%, respectively. For the ‘<50-copies’ assay, the results were 15.5%, 21.1%, and 26.9%, respectively. Although the reductions were relatively similar, changes in CD4+ cell counts favored the maraviroc treatment groups, with mean increases of 35.7, 59.6, and 62.4 cells/mm³ in the respective cohorts. Emergence of X4 virus was observed in two of 58 placebo patients, 12 of 57 maraviroc once-daily patients, and 12 of 52 maraviroc twice-daily recipients. Interestingly, CD4+ increases of 48 and 33 cells/mm³ were noted with maraviroc once-daily and twice-daily dosing, respectively, suggesting that the X4 virus emergence did not unfavorably affect CD4+ cell numbers.

Results from the ACTG 5211 study, assessing the activity of vicriviroc in combination with an OBR in individuals exclusively harboring R5 virus were also discussed at the conference (Gulick R, et al. Abstract THLB0217). Patients were triple-class experienced, had a median viral load of 4.56 log₁₀ copies/mL, and a median CD4+ cell count of 146 cells/mm³. The vicriviroc arms each included 30 individuals; 28 individuals were randomized to the placebo group. During the first 14 days, patients received either vicriviroc 5, 10, or 15 mg once-daily, or placebo, after which background therapy was optimized based on resistance testing. The mean change in viral load was −0.87, −1.15, −0.92, and +0.06 log₁₀ copies/mL in the vicriviroc 5, 10, 15 mg-once-daily, and placebo arms, respectively. At week 24, the changes were −1.51, −1.86 and −1.68 log₁₀ copies/mL in the vicriviroc treatment arms, respectively, and −0.29 log₁₀ copies/mL in the placebo group. An impressive CD4+ recovery of +84, +142, and +142 cells/mm³ was also seen in the vicriviroc arms, respectively, compared with a decline of 9 cells/mm³ in the placebo group. Emergence of X4 virus, either as a mixture or as the dominant quasi-species, was detected in 27%, 10%, and 7% of the vicriviroc recipients, respectively, and 4% (one individual) in the placebo group. The 5 mg-vicriviroc arm was discontinued and further development of vicriviroc will use the higher doses examined in this study.

Taken together, the data from these two small studies indicate that CCR5 antagonists demonstrate good activity in individuals who screen positive for R5 virus only, and may contribute to both antiviral efficacy and CD4+ recovery in persons with X4/R5-tropic virus. Data from large scale studies with CCR5 antagonists are expected shortly.
Parenteral Inhibitors of HIV Entry in Current Development

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The availability of highly active antiretroviral therapy (HAART) has, since its introduction in the mid-1990s resulted in a marked improvement in outcomes for patients with HIV type 1 (HIV-1) infection. However, the success of currently available antiretroviral therapy is limited by the emergence of drug-resistant viruses, the necessity of sustained adherence to complex drug regimens, and the potential for toxicity.

Multi-class resistance has rapidly become a problem in the treatment of HIV; 1–2% of newly infected patients in the US and EU harbor virus that is resistant to all three major therapeutic classes: the protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and nucleoside reverse transcriptase inhibitors (NRTIs) [1]. Resistance among the treatment-experienced population is also widespread, with an estimated 20% of these patients having virus that has lost susceptibility to two of these three drug classes [2]. This, in combination with the need for simple regimens and drugs with fewer adverse effects, clearly illustrates the urgency for novel classes of safe and effective antiretroviral agents.

Studies of the mechanisms of HIV-1 entry into CD4⁺ cells have yielded multiple targets for new drug development. Processes of specific interest include virus attachment to CD4, binding of the virus to cellular coreceptors (CCR5 and CXCR4), and viral–cell fusion. Drugs that block these stages of the viral life cycle, the entry inhibitors, have demonstrated potent antiretroviral activity in preclinical and early stage clinical trials. Owing to their novel mechanisms of action, these new agents also remain active against viruses resistant to both reverse transcriptase and protease inhibitors. Following the example of enfuvirtide, the first entry inhibitor to gain approval for clinical use, a rich pipeline of parenterally delivered entry inhibitors is now in development and offer the potential of further diversifying treatment options as they initiate an era of once-weekly to once-monthly dosing regimens. These investigational agents include TNX-355, a monoclonal antibody that binds to CD4 to prevent post-attachment conformational changes required for entry; PRO-140 and HGS004, two monoclonal antibodies that block HIV binding to the CCR5 coreceptor; and the second-generation fusion inhibitors, TRI-999 and TRI-1144, the latter of which was recently selected for further development. J Viral Entry 2006;2(2):48–56.

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Studies of the mechanisms of HIV-1 entry into CD4⁺ cells have advanced the understanding of the cellular and molecular basis for HIV-1 infection, and identified multiple new targets within the extracellular stage of the viral life cycle that precedes penetration of the target cell membrane (reviewed in [3]). Viral entry involves a series of interactions between viral envelope molecules and receptors on the cell surface, including:

- Virus attachment to CD4.
- Virus binding to a cellular coreceptor (CCR5 or CXCR4).
- Virus–cell fusion.

Each of these processes has become a target for the development of drugs belonging to a novel class of antiretrovirals, collectively referred to as entry inhibitors. Owing to their novel mechanisms of action, entry inhibitors remain active against viruses resistant to both RTIs and PIs. Furthermore, for the same reason, cross-resistance to other classes of antiretrovirals is likely to be limited (reviewed in [4], and there is also evidence of significant synergistic interactions between different entry inhibitors [5].

Enfuvirtide (also known as T-20), a synthetic peptide that targets gp41-mediated fusion of the viral envelope with the host cell membrane [6], was the first entry inhibitor to gain
approval by the US Food and Drug Administration (FDA) for the treatment of HIV infection [7]. The success of this agent has recently prompted a more aggressive goal of achieving undetectable viral load in triple-class experienced patients [8], and underscores the potential benefits of drugs that act on different steps in the viral life cycle.

Following the example set by enfuvirtide, a number of entry inhibitors are now in the pipeline, offering the potential of further diversifying treatment options. In addition, several of these drugs have improved pharmacokinetic properties and/or have been designed as sustained-release formulations that potentially allow once-weekly, or even once-monthly, dosing. Current investigational agents in development that require parenteral administration include TNX-355, which is a monoclonal antibody (MAb) that binds to CD4 to prevent post-attachment conformational changes required for entry; PRO-140 and HGS004, both MAbs that inhibit HIV binding to the CCR5 coreceptor; and TRI-999 and TRI-1144, two second-generation fusion inhibitors that, like enfuvirtide, interfere with virus–cell fusion. This article discusses the present status of these novel compounds as they move from proof of concept to pivotal Phase III clinical trials.

TNX-355

TNX-355 is a humanized MAb in Phase II clinical trials. It was reconstructed from a murine progenitor antibody, 5A8, into a humanized immunoglobulin G subtype 4 (IgG4) molecule (Hu5A8 or TNX-355) that retains identical functional specificity and activity of the predecessor antibody [9,10]. TNX-355 blocks entry of HIV into target cells by binding to a conformational epitope on domain 2 of the extracellular portion of the CD4 receptor (Fig. 1). The binding site is distinct from the site required for interaction of the receptor with major histocompatibility complex proteins (domain 1) [10]. Unlike anti-CD4 receptor antibodies that target domain 1, TNX-355 does not interfere with immunological functions involving antigen presentation [9,10]. Binding of the MAb prevents entry of the virus by blocking post-attachment conformational changes between gp120 and CD4 prior to chemokine coreceptor interactions [9,10].

Preclinical data demonstrate potent antiviral activity against CCR5- and CXCR4-tropic viruses

TNX-355 has been shown to inhibit the infectivity of a diverse panel of HIV-1 variants in vitro, including CCR5- and CXCR4-tropic (R5 and X4, respectively) primary isolates [11]. In preclinical studies, TNX-355 reduced viral loads in rhesus monkeys infected with the simian immunodeficiency virus of macaques (SIVmac) [12,13]. In a further study, virus load was decreased by up to 1.7 log10 RNA copies/mL in SIVmac-infected rhesus and cynomolgus monkeys after approximately 10 days of treatment with 3 mg/kg TNX-355 twice weekly [14]. Sustained binding of TNX-355 to CD4+ cells was observed without absolute decreases in the CD4+ cell counts, and without immunosuppression, in uninfected rhesus monkeys and in monkeys infected with SIVmac [9,14].

Phase I clinical trials show good tolerability and significant viral load reductions

In the Phase IA protocol, Hu5A8.01, five sequential cohorts of six treatment-experienced HIV-positive patients received single-dose intravenous infusions of 0.3, 1, 3, 10, and 25 mg/kg TNX-355 [15]. Safety, pharmacokinetics, and antiviral activity were assessed. When administered as a single dose, TNX-355 was well tolerated. No serious adverse events were reported in the study. In this monotherapy setting, TNX-355 demonstrated antiviral activity as measured by reductions in viral load: median nadir reductions of ≥1 log10 RNA copies/mL were seen following treatment with the 10 mg/kg and 25 mg/kg doses. Clinically significant viral load reductions (>0.5 log10 RNA copies/mL) were maintained for 2–3 weeks following single infusions of the 10 mg/kg and 25 mg/kg doses. Mean CD4+ cell counts initially increased, then returned towards baseline over the course of the follow-up.

In protocol TNX-355.02, a Phase IB trial, multiple doses of TNX-355 were studied [16]. Nineteen patients were randomized to receive a 10 mg/kg initial dose, followed by nine doses of 10 mg/kg weekly or five doses of 6 mg/kg twice weekly. Additionally, three non-randomized patients
were enrolled and received five doses of 25 mg/kg twice weekly. In this study, TNX-355 was administered to patients who were on a stable, failing regimen of HAART, or was given as a single agent. Again, TNX-355 was well tolerated. Four serious adverse events were reported in the study; however, none were related to the study drug. As essential monotherapy, TNX-355 demonstrated clinically significant median viral load reductions (approximately 1.0 log_{10} ) at nadir measurements.

Phase II clinical trials confirm significant viral load reductions and show increased CD4+ cell counts
A Phase II protocol, TNX-355.03 was consequently conducted, to assess the safety and antiviral activity of TNX-355 in the setting of combination therapy [17]. This 48-week randomized, double-blind, placebo-controlled, three-arm study enrolled 82 treatment-experienced HIV-positive patients with viral loads \( \geq 10\,000 \) RNA copies/mL and \( \geq 50 \) CD4+ cells/mm\(^3\) at baseline. The investigators selected a resistance test-guided, optimized background regimen (OBR) for each patient. Patients were subsequently randomized to receive 15 mg/kg TNX-355 twice weekly, 10 mg/kg TNX-355 weekly for 9 weeks followed by 10 mg/kg twice weekly, or placebo. The primary endpoint of the study was the mean change in viral load at week 24 between either of the TNX-355 treatment arms and placebo.

At 24 weeks, the mean changes in viral load for the intent-to-treat population using last observation carried forward analysis for non-completers were (Fig. 2):

- TNX-355 15 mg/kg: \(-0.95 \log_{10}\) (p=0.003 compared with placebo).
- TNX-355 10 mg/kg: \(-1.16 \log_{10}\) (p<0.001 compared with placebo).
- Placebo: \(-0.20 \log_{10}\).

Treatment with the 10 mg/kg and the 15 mg/kg regimens in combination with OBR continued to produce statistically significantly greater mean viral load reductions than the placebo-plus-OBR treatment at 48 weeks (Fig. 3). CD4+ cell-count increases were significantly greater in the TNX-355-treated patients than in the placebo group (Fig. 4) [18]. Further studies of TNX-355 in treatment-experienced patients are planned.

**CCR5 antagonists**
Rationale for development of anti-CCR5 antibodies for the treatment of HIV-1 infection
CCR5 is the chemokine receptor for the ligands macrophage inflammatory protein-1 (MIP-1) \( \alpha \) and \( \beta \), RANTES (regulated on activation normal T cell expressed and secreted), and monocyte chemotactic protein-2. CCR5 is also the primary coreceptor for HIV-1 transmission and replication, from the early stages of disease through progression to AIDS. In all stages of HIV-1 infection, R5 and dual-tropic (R5/X4) viruses comprise the majority of viral strains detected, while X4-exclusive viruses constitute a minority of cases. Naturally occurring host defects in CCR5 expression have demonstrated the importance of this receptor in HIV-1
infection, as it has been observed that individuals with a homozygous deletion (CCR5Δ32) show resistance to the virus [19]. In addition, CCR5 is a potentially safe target in HIV-1 treatment, as people lacking CCR5 expression appear healthy. Because MIP-1β and RANTES are proinflammatory, another potentially interesting role for this class of agents is their use in altering the immune activation that has been shown to promote disease progression in HIV-1 [20].

**HGS004**

The development of HGS004 was initiated with the generation of fully human MAbs from Xenomice (animals genetically engineered to produce completely human MAbs; Abgenix, Thousand Oaks, CA, USA) immunized with mouse cells that expressed CCR5. Binding specificity, affinity, antagonism of ligand binding, signaling, and antibody effector functions were determined using human peripheral blood mononuclear cells (PBMCs) or CCR5-expressing cell lines.

Preclinical data show potent inhibition of R5 viruses and synergy with other antiretrovirals

The antiretroviral activity of HGS004 was evaluated in HIV-1 envelope-dependent cell–cell fusion assays and with the PhenoSense HIV entry assay (Monogram, Inc., South San Francisco, CA, USA). Novel CCR5 MAbs were identified that specifically bind to CCR5, fail to induce intracellular signaling, block HIV entry, and inhibit HIV envelope dependent cell-cell fusion. HGS004 was selected as the lead candidate based on potency in HIV-1 infectivity assays, and was converted to an IgG4 backbone.

The ability of HGS004 to block infection with R5 HIV-1 was evaluated in a series of *in vitro* assays. HGS004 was observed to potently inhibit:

- CCR5-dependent entry of 30 HIV-1 viruses representative of clades A–G (median IC₅₀ [the concentration required for 50% inhibition of viral replication] 6 nM).
- Enfuvirtide-resistant HIV-1.
- Ligand binding (IC₅₀ 0.41 nM).
- Cell–cell fusion (Fig. 5).
- Viral replication.

In addition, HGS004 did not induce signaling or mediate any cellular toxicity upon binding to CCR5, nor did it induce antibody-dependent, cell-mediated cytotoxicity, or complement-dependent cytotoxicity in human cells [21].

To assess the risk of resistance development, two primary R5 virus isolates (RM and JC) were passaged *in vitro* for >24 weeks in the presence of HGS004. When compared with control cultures (grown without the antibody) that had been through a similar number of passages, there was no significant difference in IC₅₀ values between the viruses, suggesting a low likelihood of causing development of resistance [22].

Furthermore, *in vitro* combination studies with current antiretroviral agents representing each of the drug classes
NRTIs (zidovudine and lamivudine), NNRTIs (efavirenz), PIs (indinavir), and fusion inhibitors (enfuvirtide), were performed with the RM and JC isolates [22]. The results demonstrated that HGS004 acts synergistically with all currently approved classes of antiretroviral agents.

Phase 1 clinical trial demonstrates safety and efficacy of HGS004 single-dose intravenous injections

HGS004 is currently being evaluated in a Phase 1 clinical trial. The study is a randomized, placebo-controlled, dose-escalation, multi-center investigation in patients who are infected with HIV-1 and not receiving concurrent antiretroviral therapy. The primary objective is to evaluate the safety and tolerability of escalating doses of a single intravenous infusion of HGS004. The secondary objectives are to determine the pharmacokinetics of HGS004, and to assess its effect on plasma viral load, CD4⁺, and CD8⁺ T cell counts over time.

A total of 63 HIV-1-positive patients (R5 virus, HIV-1 RNA >5000 copies/mL, CD4⁺ cell counts >250/mm³) were randomized to one of five dose cohorts receiving 0.4, 2, 8, 20, or 40 mg/kg HGS004 [23]. A single dose of MAb or placebo was administered as an intravenous infusion. Blood samples were obtained pretreatment, post-infusion, and on days 1, 2, 4, 7, 14, 21, 28, 42, and 56. Adverse events, laboratory assessments (clinical chemistry and hematology), drug levels, CD4⁺ cell counts, viral load, receptor occupancy, and change in tropism or isolate sensitivity (IC₅₀) were assessed. Overall, HGS004 was well tolerated and no dose-limiting toxicities were observed. No grade 3–4 adverse events were noted, although two treatment-related events of moderate severity, involving infusion-related allergic reactions at the 2 mg/kg dose, necessitated a protocol change to include premedication with oral diphenhydramine. The study further demonstrated:

- Short-term increases in CD4⁺ and CD8⁺ cell counts.
- Non-linear pharmacokinetics at doses of 0.4–40 mg/kg (Cₘₐₓ increased proportionally with dose while area under the curve increased more than proportionally with the dose).
- High levels of receptor occupancy of >80% through 28 days in the 8, 20, and 40 mg/kg-dose cohorts.
- Day 14 HIV-1 RNA reductions of >1 log₁₀ in 14 of 26 (54%) subjects in the 8, 20, and 40 mg/kg-dose cohorts (Fig 6).
- Day 28 HIV-1 RNA reductions of >1 log₁₀ in four of 10 subjects in the 40 mg/kg cohort (Fig 6).
Preliminary data on post-treatment susceptibility of viral isolates showed a shift in tropism to dual-mixed populations in five subjects.

HGS101
Further research has subsequently identified HGS101, an alternative anti-CCR5 MAb candidate. *In vitro* data suggest that HGS101 is 5.5-fold more potent against the clinical isolates from the Phase I trials. Other attributes of HGS101 are similar to those of HGS004, including favorable pharmacokinetics, strong *in vitro* evidence of anti-viral activity that is additive or synergistic in combination with approved therapeutic agents, and a low likelihood of causing development of resistance based on long-term *in vitro* culture.

PRO 140
PRO 140 is a humanized IgG4κ MAb against CCR5, developed by Progenics Pharmaceuticals, Inc (Tarrytown, NY, USA) [24,25]. It recognises a multidomain epitope on CCR5 and binds human PBMCs *in vitro*. The antiviral activities of humanized and murine PRO 140 have been shown to be essentially indistinguishable, and the two mAbs are equally potent in blocking HIV-1 envelope-mediated cell–cell fusion [24].

Preclinical studies show subtype-independent inhibition of R5 viruses, without CCR5 antagonism PRO 140 has demonstrated broad-spectrum activity against primary R5 strains of HIV-1 in a variety of preclinical settings. In an evaluation using a panel of six primary HIV-1 isolates selected for their genotypic and geographic diversity, the antibody was compared with RANTES [24]. Like RANTES, murine PRO 140 demonstrated potent and subtype-independent inhibition of each of the viruses tested, with IC₉₀ values of 2–3 µg/mL. Murine PRO 140 inhibited HIV-1 replication in both isolated PBMCs and macrophage cell cultures, whereas RANTES was a poor inhibitor of replication in macrophages. Thus, unlike the natural ligand for CCR5, murine PRO 140 protects both T cells and macrophages from HIV-1 infection *in vitro*. Tests to assess the CCR5 antagonistic effect of PRO 140 *in vitro* demonstrated effective suppression of viral replication at concentrations that have little or no effect on the natural activity of CCR5.

The antiviral activity of both murine and humanized PRO 140 were subsequently examined *in vivo*, using hu-PBL-SCID mice (a human lymphocyte-engrafted, severe combined immunodeficient model) of chronic HIV-1 infection and therapy [26]. Viral loads were reduced to undetectable levels (<400 copies/mL) in all animals (n=5) treated with 1 mg-daily doses of humanized PRO 140 for 8 days. The mean viral load reduction exceeded 1.5 log₁₀ and was statistically significant (p<0.01), while the virus levels remained high in control animals. Murine PRO 140 was also effective in controlling viral replication in this model. PRO 140-treated animals had, on average, higher levels of human CD4+ cells than untreated animals, possibly reflecting diminished levels of virus replication and cell killing.
in the presence of PRO 140. These findings indicate that the antiviral effects of PRO 140 were related to CCR5 blockade rather than to depletion of target cells.

To investigate a potential synergistic activity against HIV, the effect of combinations of PRO 140 and the small-molecule CCR5 antagonists, SHC-C, vicriviroc, and TAK-779, were assessed in a fluorescence resonance energy transfer assay (a well-established model of HIV entry) [27]. The antiviral activity consistently increased by at least four-fold, as measured by the reduced drug concentration necessary to block 50% of HIV membrane fusion. Synergy was observed only with the addition of PRO 140 and not with combinations of two small-molecule CCR5 antagonists alone.

In additional laboratory studies, humanized or murine forms of PRO 140 were tested for activity against viruses selected for resistance to small-molecule CCR5 inhibitors in vitro. Viruses were cultured in the presence of small-molecule CCR5 inhibitors until drug-resistant isolates emerged. The viruses acquired mutations that enabled them to use CCR5 in the presence of the small-molecule inhibitors; however, these mutations did not affect viral susceptibility to PRO 140.

**Phase I clinical trial suggests infrequent dosing requirements**

To assess PRO 140 in humans, a single-dose, double-blind, placebo-controlled dose-escalation study was conducted in healthy volunteers to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics following intravenous injection [28]. Four dose cohorts of five subjects each (four receiving drug and one placebo) were administered a single dose of 0.1, 0.5, 2, or 5 mg/kg PRO 140. No dose-limiting toxicities or patterns of drug-related toxicities were observed. Preliminary pharmacokinetic analyses suggest a serum half-life of 2–3 weeks, which could support dosing as infrequently as monthly or bimonthly. Preliminary pharmacodynamic analyses demonstrate that a single 2.0 or 5.0 mg/kg dose of PRO 140 significantly coated CCR5-positive blood lymphocytes for 2 and 8 weeks, respectively. This extended period of coating was observed without depletion of CCR5-positive lymphocytes from the circulation. Since coating of CCR5 is the mechanism by which PRO 140 inhibits HIV replication in vitro, these data support the view that PRO 140 has the potential to inhibit viral replication in HIV-infected patients.

PRO 140 has been given a “fast track” designation by the FDA for the treatment of HIV infection. The Fast Track Development Program facilitates development and expedites regulatory review of drugs intended to address an unmet medical need for serious or life-threatening conditions. An ongoing Phase IB study is the first to explore the safety and activity of PRO 140 in patients with HIV-1 disease.

### Next-generation fusion inhibitors: TRI-999 and TRI-1144

Clinical trials have verified the effectiveness and safety of the fusion inhibitor enfuvirtide. It is administered by twice-daily subcutaneous injection, with injection-site reactions being a common, but seldom treatment-limiting, adverse effect [29]. With the aim to improve the convenience of peptide-based antiretroviral therapies, two next-generation fusion inhibitors (NGFIs), TRI-999 and TRI-1144, are therefore currently being developed, in another collaboration between the manufacturers of enfuvirtide, Trimeris-Roche (Trimeris, Inc., Morrisville, NC, and Roche, Nutley, NJ, USA).

To improve the potency and durability of the new agents, two peptides were derived from an optimized gp41 heptad repeat (HR) 2 region, partially overlapping the enfuvirtide amino acid sequence (Fig. 7) [30]. The natural sequences were modified using independent strategies, resulting in two compounds from distinct chemical series with promising potency, genetic barrier to resistance, and pharmacokinetic properties.

### Structural modifications to improve pharmacokinetics

**TRI-999** is a 36-amino acid peptide containing a polyethylene glycol linker-fatty acid moiety attached to an internal lysine side chain. The peptide sequence overlaps the sequence of enfuvirtide by 24 residues and contains 12 additional HR2 residues N-terminal to the sequence from which enfuvirtide is derived. These N-terminal residues make contact with the deep pocket on the HR1 viral target [30–33]. The C-terminal fatty acid increases non-covalent binding to serum proteins; this protects the peptide from renal elimination and thus confers improved pharmacokinetic properties. The precise fatty acid position on the peptide and the length and type of linker between the peptide and fatty acid were chosen to minimize loss of potency when serum proteins are present in the HIV assay.

**TRI-1144** is a 38-amino acid peptide engineered to adopt a helical conformation in solution. The starting peptide sequence is similar to that of TRI-999, and the wild-type residues that contact the viral target have been either retained or conservatively changed. Residues that are not involved in HR1 binding have been replaced with helix-promoting motifs.

### Preclinical data demonstrate superiority over enfuvirtide

To assess antiviral activity, the peptides were tested against a panel of 12 fusion inhibitor-sensitive clinical isolates.
TRI-999 had a geometric mean IC$_{50}$ of 1 nM – seven times better than enfuvirtide, while TRI-1144 was similar to enfuvirtide at 7 nM [30]. Furthermore, the modified peptides were found to have a markedly narrower range of IC$_{50}$ values than enfuvirtide. TRI-999 and TRI-1144 were subsequently tested against a panel of eight virus isolates that were resistant to enfuvirtide and other peptide fusion inhibitors. The geometric means of the IC$_{50}$ values demonstrated >250-fold and >150-fold greater effectiveness of TRI-999 and TRI-1144, respectively, relative to enfuvirtide [34].

These improvements in potency suggest higher genetic barriers to generation of resistance than that for enfuvirtide. To verify this, passaging experiments were performed [30]. In _in vitro_ selections with three different viruses in the presence of increasing concentrations of the peptides for >70 days showed only one- to eight-fold reductions in drug susceptibility. Similar experiments with enfuvirtide generated viruses with >10-fold losses in susceptibility, revealing a comparatively superior durability of TRI-999 and TRI-1144 _in vitro_.

Furthermore, subsequent pharmacokinetic experiments in cynomolgus monkeys demonstrated four- to six-fold slower clearance rates (11 and 7 mL/kg/h) for TRI-1144 and TRI-999, respectively, compared with enfuvirtide (40 mL/kg/h), following intravenous administration [30]. These improvements in pharmacokinetic parameters allow the possibility of once-weekly dosing when coupled with a sustained-release formulation. Early experiments in rodents with TRI-1144 demonstrated steady sustained release of peptide over 1 week following a single subcutaneous injection [35].

Recently, the TRI-1144 peptide was advanced as the lead preclinical candidate for continued development due to its overall suitability as a candidate for HIV-1 therapy.

**Conclusion**

As we enter the third decade of HIV therapy, the success of antiretroviral drug development has been remarkable. Refinements in formulations and co-formulations along with ritonavir boosting of PIs have provided many patients with the opportunity to treat HIV as a chronic manageable illness with the convenience of once-daily oral dosing. Much-anticipated data on the small molecule CCR5 antagonists and integrase inhibitors will likely translate into further options to control viral replication with convenient oral regimens.

Against this backdrop, it is fair to ask where the parenteral agents currently under investigation for treatment...
of HIV might fit. For some (TNX-355), the advent of an agent with a completely novel mechanism of action could have an important role in salvage therapy irrespective of the need for intravenous infusion. For others (Tri-1144, the CCR5 MAbs), the trade-off might be to eventually forego some, or even all, daily therapy in favor of weekly subcutaneous dosing and/or monthly intravenous infusions. One thing is for certain: the rapidly expanding antiretroviral arsenal, including the parenterally administered entry inhibitors in development, will offer clinicians and patients further options in constructing successful regimens to control HIV.

Disclosures
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References
Influenza A viruses are enveloped viruses with segmented single-stranded RNA genomes of negative polarity. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins, along with the M2 protein, are incorporated into the viral membrane, and each plays a significant role in the virus life cycle (Fig. 1). Influenza A viruses attach to host cells via interactions between HA and cell surface glycoproteins and glycolipids that contain terminal sialic acid. Following receptor-mediated endocytosis, the acidification of endosomes by cellular proton pumps leads to two events that are critical for virus entry. One is the triggering of irreversible conformational changes in HA that are required to mediate fusion of the viral and endosomal membranes. The other involves the proton channel activity of the viral M2 protein, which, in response to the proton influx into endosomes, induces a concurrent acidification of the interior of virus particles. This results in the dissociation of the viral matrix (M1) protein from the nucleocapsids, a process that frees them to enter the nucleus, where viral transcription and replication take place. At the end of the replication cycle, the sialidase activity provided by NA cleaves host cell and viral surface sialic acid to remove functional receptors. In this way, NA plays a critical role in the release and dissemination of newly assembled virus particles. The structural and functional information available on HA, NA, and M2 make them ideal targets for antiviral intervention, and these topics form the focus of this review.

Structure of HA
HA is a type I glycoprotein that is synthesized as a precursor polypeptide of approximately 550 amino acid residues, referred to as HA0. In the endoplasmic reticulum, HA0 forms homotrimers that are stabilized by non-covalent interactions. Each monomer of the trimer is subsequently cleaved into the disulfide-linked subunits HA1 and HA2, a process required for virus infectivity. The receptor-binding activity of HA is provided by the HA1 subunits in the membrane-distal head domains of the molecule, while the HA2 subunit is required for fusion (Fig. 2). High-resolution X-ray crystal structures are now available for many of the 16 antigenic subtypes of HA, and several structures also exist for HA complexes with receptor analogs [1–6]. In addition, for the H3 subtype, the structure has been determined for the HA0 precursor, the native cleaved protein, and the low-pH conformation that the molecule assumes during the process of membrane fusion [6–8]. Thus, this article will concentrate on the H3 subtype, but the concepts involved are equally applicable to the other subtypes.

Influenza A viruses continue to cause serious medical, commercial, and social problems. Vaccines provide a degree of protection when the strains used for their production are good matches with the circulating viruses; however, they are least effective in the populations that need them the most – the very young and the elderly. In addition, there is the ominous threat posed by the emergence of antigenically novel viruses and the possibility that they may spread in the human population before effective vaccines can be developed, manufactured, and distributed. In such situations, antiviral drugs may be useful. For these reasons, antivirals against influenza have a significant role to play in modulating the severity of disease during the normal flu seasons, as well as in the strategic planning for future pandemics. Two classes of anti-influenza drugs are currently employed on a relatively large scale, M2 inhibitors and neuraminidase inhibitors. While both clearly are beneficial when administered in a timely fashion, each also suffers from drawbacks. Hence, there is an ongoing effort to improve existing and design new antiviral compounds as an alternative means for controlling influenza – an omnipresent moving target. J Viral Entry 2006;2(2):57–66.
Figure 1. The influenza virus life cycle, illustrating virus binding of sialic acid-containing receptors on the host cell, internalization, and the processes that occur inside the endosome. For simplicity, the details regarding the synthesis of the viral glycoproteins in the endoplasmic reticulum, trafficking through the Golgi, and transport to the cell surface are not represented. As endosomes are acidified, the virion interior is also acidified, owing to the proton channel function of the M2 protein. This releases the nucleocapsids, which transfer to the nucleus following fusion of the viral and endosomal membranes. The drug amantadine blocks M2 channel function, which inhibits the dissociation of ribonucleoproteins from the matrix (M1) protein and prevent nucleocapsids from entering the nucleus. The inset image portrays a model of the transmembrane portion of the M2 tetramer and how amantadine might block the influx of protons into the virus particle. It also shows the position of one of the four mutations (Serine 31) on the inside of the channel that are commonly associated with drug resistance. The figure also depicts assembly and budding of progeny virions. Inhibition of viral neuraminidase at this stage results in aggregation of virus particles at the infected cell surface and inhibition of virus dissemination.

Reproduced with permission from [92].
**Receptor binding**

The HA trimer extends approximately 135 Å from the membrane to the tip of the trimer, and the receptor-binding site is a shallow pocket located at the distal end of each monomer. The site is composed of a small helix (the 190 helix) at the membrane-distal edge of the pocket, an extended chain (the 130 loop) at the front of the site, and the 220 loop, which constitutes the left side of the binding domain. Residues Y98, W153, H183, and Y195 form the base of the pocket. Several of the residues in the binding site are highly or relatively well conserved, suggesting their involvement in stabilizing the structural integrity of the region, receptor interaction, or both (Fig. 3).

HA binds to sialic acid components at the ends of carbohydrate chains on glycoproteins or glycolipids of host cells [9,10]. Avian influenza A viruses mainly bind to receptors with terminal sialic acids attached to galactose via α2,3 linkages, whereas human influenza A viruses generally prefer receptors with α2,6-linked sialic acid [11,12]. The specificity of binding reflects, to a great degree, the preponderance of receptors at the site of replication in the host. The digestive tract of bird species are rich in α2,3-linked sialic acid receptors, while the human respiratory tract contains receptors with α2,6-linked sialic acid [13]. Interestingly, most of the α2,6-linked sialic acid receptors in human airways reside on non-ciliated cells, while avian receptors are predominantly expressed on the ciliated cells [14]. In addition, mucins in the human respiratory tract primarily contain sialic acids with α2,3 linkages, thus providing a natural barrier to avian viruses [15].

Studies on viral and bacterial ‘receptor-destroying enzymes’ in the 1940s led Burnet and colleagues to postulate that such enzymes might be used therapeutically to remove influenza receptors from the respiratory tract, or that competitive inhibitors of these receptors might provide a barrier to infection [16–18]. These are still good ideas. Several lines of reasoning suggest that inhibition of receptor binding offers an attractive option for the design of antiviral compounds. For example, an abundance of data exists on how HAs bind to various receptor analogs. Furthermore, the binding site is accessible on the viral surface, so drugs would not have permeate cellular or viral membranes. Ideally, such inhibitors should have the following properties:

- Bind more tightly to HA than sialic acid.
- Be capable of forming multivalent HA–receptor contacts.
- Be resistant to the action of viral NAs.

However, the binding of HA to monovalent sialosides is not exceptionally strong, exhibiting dissociation constants ($K_d$) of around 2–4 mM [19]. Interestingly, the $K_d$ values for monovalent analogs such as sialyllactose with an α2,3 or α2,6 linkage indicate only a very slight difference in binding, despite their specific HA preferences. By comparison, several viral attachment proteins have a much greater affinity for their specific host cell receptors. For example, the HIV type 1 (HIV-1) envelope protein binds to CD4 with dissociation constants in the micromolar range [20].

As monovalent receptor–HA interactions are inherently weak, approaches involving monovalent inhibitors include the synthesis of sialic acid compounds with substituents designed to interact more strongly than natural sialosides [21]. For example, derivatives with substituents that interact with hydrophobic regions and polar residues adjacent to the binding site result in a 10- to 100-fold increase in binding affinity relative to naturally occurring monovalent sialosides. A second sialic acid binding site at an HA1–HA2 interface toward the bottom of the head domains on the HA structure has also been defined with crystallography using α2,3-linked sialyllactose and other sialic acid derivatives. Although these structures suggest that the site is not biologically relevant for influenza binding to receptors, sites such as this could be exploited to enhance the affinity of polyvalent compounds designed for antiviral activity [22].

Approaches with multivalent inhibitors have been explored extensively [23]. Numerous naturally occurring proteins that are heavily glycosylated are known to inhibit
influenza viruses by interfering with receptor binding [21]. For example, horse serum is rich in α2-macroglobulin, which functions as a potent inhibitor of many influenza strains that preferentially recognize α2,6-linked receptors. This is due to its high content of α2,6-linked sialosides, a significant percentage of which contain the 4-O-acetyl derivative of sialic acid that is resistant to neuraminidase [24]. If grown in the presence of horse serum, changes in the HA receptor binding region that alter receptor preference to those containing an α2,3 linkage are selected [25]. Given that natural molecules such as α2-macroglobulin have antiviral properties, it stands to reason that it might be possible to synthesize low-molecular-weight compounds that appropriately present multiple α-sialosides for the purpose of binding inhibition. Bivalent and tetravalent compounds increase the affinity of binding and probably interact with more than one trimer on the viral surface [26,27]. A number of sialyl glycopolymer compounds have also been generated and shown to inhibit binding [21], but these have not been advanced to the stage of clinical availability.

**HA-mediated membrane fusion**

The cleavage of HA0 into HA1 and HA2 is a critical prerequisite for HA fusion activity and influenza virus infectivity (Fig. 4) [28,29]. Highly pathogenic avian strains often have HA cleavage sites that contain the furin recognition motif (R-X-K/R-R), whereas the non-pathogenic avian strains and human strains usually have only a single arginine residue at the cleavage site [30]. Because the polybasic furin sites are recognized and cleaved
intracellularly by ubiquitously expressed proteases, viruses with these motifs can cause systemic infections in the host, whereas replication of viruses that have HAs with single arginine residues is generally restricted to locations at which appropriate extracellular proteases are present (e.g. the lumen of the respiratory tract in humans).

A conformational change involving HA1 residues 323–328 and HA2 residues 1–12 accompanies proteolytic cleavage of HA0 [7]. The liberated HA2 N-terminal domain is a highly conserved hydrophobic region, and due to its role in membrane fusion, it has been designated as the fusion peptide. After cleavage, the fusion peptide relocates into an adjacent cavity and contacts several ionizable residues. The positioning of residues 1–3 of the relocated fusion peptide also blocks access to another internal cavity in the trimer interior that contains additional ionizable residues [1,3]. It is thought that the relocation of the fusion peptide and the changes in the environment of ionizable residues in this region after cleavage may set the trigger for subsequent conformational changes required for fusion.

Acidification of native cleaved HA in the endosome leads to three prominent structural refolding events (Fig. 5). The globular membrane-distal heads of HA, de-trimerize, but remain tethered to the trimeric HA stalk through residues 28–43 of HA1 [8,31]. In the HA2 subunit, the fusion peptide is extruded from its buried position in the interior of native HA trimers, and two structural rearrangements take place in the HA stalk [8]. One is the refolding of the extended chain that links the long alpha helix with the shorter one in the native HA hairpin loop structure. These residues become helical and lead to an N-terminal extension of the coiled coil. As a result, the small helix is also recruited into the newly formed coiled coil to insert into the target membrane. The other conformational change involves residues 106–112 in the membrane-proximal segment of the native HA coiled coil. These unfold to form a loop structure that causes residues at the C-terminal end of each α-helix to reorient by 180°. The short helical domains pack antiparallel to the coiled coil, and residues C-terminal to this extend towards the same end of the structure as the fusion peptide. The resulting structure is a rod-shaped trimeric molecule with the N- and C-termini at the same end [32]. Similar structures have been observed for many other viral fusion proteins, such as those of Moloney murine leukemia virus, HIV-1, simian immunodeficiency virus-1, Ebola virus, the paramyxovirus SV5, parainfluenza virus 5, and for the vesicle (v) - and target (t)-SNARE complexes involved in

**Figure 4.** A: Ribbon diagram of the conformational changes that accompany precursor cleavage. The HA1 residues shown in grey and the HA2 residues shown in blue do not change. The residues of the HA0 loop structure (light blue, left panel) relocate following cleavage at residue 329 (arrow) such that the N-terminus of HA2 is inserted into the trimer interior of the native cleaved HA (right panel). B: Space-filling model of the region of the cleavage loop showing HA0 (left) and native HA (right). The cleavage loop structure is oriented toward the reader in the left panel and the residues that become the C-terminus of HA1 (white circle) and N-terminus of HA2 (black circle) are shown. The cavity that becomes occupied by the fusion peptide is located to the left of the loop in HA0. The right panel shows the locations of the HA1 C-terminus (white circle) and HA2 N-terminus (black circle) after cleavage.

Reproduced with permission from [7]. HA: hemagglutinin.
synaptic fusion [33,34]. The formation of the rod-like structures is suggested to provide both a mechanism for the close apposition of the prefusion membranes and the driving force for the fusion of the bilayers.

**Potential targets for HA fusion inhibition**

The steps involving HA cleavage, formation of the native HA structure, and the subsequent conformational changes that lead to membrane fusion are all vital for viral replication, and provide attractive phases of the entry process for potential intervention by antiviral drugs. For example, HA cleavage is required for membrane fusion activity and, thus, virus infectivity [28,29], and there are examples in which inhibitors such as peptidyl chloromethylketones that mimic the cleavage site have been shown to block cleavage activation of HA in cell culture [35]. The calcium-specific ionophore A23187 has also been found to inhibit cleavage of HAs processed intracellularly by the calcium-dependent protease furin, presumably by interfering with the calcium concentration in organelles where HA cleavage takes place [36]. However, most strategies involving protease inhibition are likely to encounter problems with toxicity.

On the other hand, approaches designed to prevent post-cleavage folding or acid-induced conformational changes, using low-molecular-weight compounds with limited toxicity, might be feasible. The HA cleavage loop resides adjacent to a cavity that is lined by ionizable residues, as shown in Fig. 4 [7]. Following HA cleavage, the residues that make up the lower (membrane-proximal) half of the loop are liberated and insert into the cavity, burying the conserved residues D109 and D112 in HA2 and the partially conserved H17 in HA1. In addition, this relocation of the HA2 N-terminal domains occludes another cavity in the interior of the trimer from access to bulk solvent. The second cavity also contains ionizable residues that may play a role in fusion. The relocation of the HA2 N-terminal fusion peptide and the change in the ionization state of nearby residues is thought to set the trigger for the subsequent acid-induced conformational changes required for fusion, as low pH fails to induce the equivalent conformational changes in uncleaved HA0 [7].

It is possible that compounds designed to bind in these cavities could either prevent the cleaved fusion peptide from inserting appropriately to prime HA for fusion, or might influence the structure or ionization state of nearby residues such that they are not induced to initiate conformational changes upon acidification. Compounds have been identified using structure-based drug-design strategies such as these, and some demonstrate antiviral activity against certain HA subtype viruses. Inhibition of H3 subtype viruses by benzo- and hydroquinones was found to be due to the inhibition of acid-mediated HA conformational changes [37]. Similar results were seen with quinolizidine-linked benzamide compounds [38,39], which were shown to prevent HA conformational changes of H1 and H2, but not H3, HA. Inhibition of H1 and H2 conformational changes was also reported using an N-substituted piperidine compound [40]. Photo-affinity labeling experiments [41] and mutant selection studies [40] suggest that, as predicted, these compounds probably act by binding to the HA stalk region in proximity to...
the native pH fusion peptide. In another report, it was shown that diiodofluorescein can inhibit virus infectivity by facilitating HA conformational changes, presumably by triggering the irreversible HA structural rearrangements before fusion function is called for [42].

As stated above, a number of viral fusion proteins adopt stable rod-shaped structures during the fusion process. Like HA, these contain a trimeric central coiled coil and an antiparallel polypeptide chain that functions to draw the viral and cellular membranes together. For many of these fusion proteins, the antiparallel chain is in the form of a second group of three α-helices that pack against the outside of the coiled coil to form what is referred to as a six-helix bundle. For these proteins, which include the fusion proteins of paramyxoviruses and retroviruses, strategies have been undertaken to utilize helical peptide inhibitors designed to competitively prevent six-helix bundle formation and membrane fusion [43,44]. Such approaches are less straightforward for influenza HA, as the antiparallel polypeptide chain in the low pH structure does not form a true six-helix bundle. In HA, the hairpin loop is followed by only approximately four turns of helix in the antiparallel direction, the protein then adopts an extended chain structure that traces along a groove in the central coiled coil. While it remains possible that peptide-based compounds might be designed to prevent proper formation of HA helical rods, the approaches could be more problematic than for the six-helix bundle proteins. However, benzotriazole-based compounds, which appear to block formation of the trimer of hairpins in a manner similar to peptide inhibitors, show promise in respiratory syncytial virus [45,46], and it may be possible to develop similar compounds for other viruses that form trimeric rod structures during fusion, such as influenza.

**The proton channel function of M2 and action of amantadine and rimantadine**

The viral M2 protein is synthesized as a 97-amino acid polypeptide chain with a 19-residue transmembrane sequence, an N-terminal extracellular domain of 24 residues, and a relatively long cytoplasmic domain of 54 amino acids. It associates as homotetramers and higher order oligomers, which are expressed abundantly at the infected cell surface but are sparingly incorporated into budding virus particles [47,48]. M2 is a proton channel that functions during virus entry to acidify the interior of virus particles when they are in the endosome, a critical step that frees ribonucleoproteins to enter the nucleus following membrane fusion (Fig. 1). It also functions at late stages of infection to help regulate the pH of trans-Golgi transport vesicles.

It is the proton channel activity of M2 that is the target for inhibition by α-adamantane compounds. Interestingly, these compounds were recognized for their capacity to inhibit influenza A viruses, and amantadine (1-amino-1-adamantane hydrochloride) and its counterpart, rimantadine (1-methyl-1-adamantane methylamine hydrochloride), were being utilized clinically long before their mode of action was understood. In fact, the recognition of M2 as a proton channel derived in large part from studies on influenza mutants selected for resistance to these drugs.

Amantadine has two antiviral effects on influenza A virus [49]. At high concentrations (>0.1 mmol/L), the drug increases the pH of endosomes such that HA conformational changes are not triggered during virus entry. This is analogous to that of other weak bases, such as ammonium chloride and chloroquine, and is non-specific, as the antiviral activity of high concentrations of amantadine and other lysosomotropic compounds is observed with influenza B as well as several other enveloped viruses. This results in the selection of HA mutants with an elevated pH during membrane fusion [50].

Lower concentrations (0.1–5 μmol/L) of either amantadine or rimantadine exhibits strain-specific antiviral effects on influenza A viruses. For most susceptible virus strains, the effect is seen at early stages of infection, where the drugs block the proton-dependent proton channel activity of M2 in endosomes (Fig. 1). This abrogates the acidification of the virus interior, which is necessary for the dissociation of nucleocapsids from the M1 matrix protein and the subsequent uptake of nucleocapsids into the nucleus [51–54]. An effect late in infection can be seen with viruses that contain HAs that are cleaved intracellularly, such as pathogenic H5 and H7 avian strains. In these, M2 ‘chaperones’ the cleaved HAs to the cell surface by preventing the acidification of trans-Golgi vesicles. This stops the cleaved HAs undergoing a conformational change prior to reaching the plasma membrane. In the presence of amantadine, intracellularly cleaved HAs are expressed on the cell surface in the low pH conformation and virus release is impaired [55,56].

Analysis of amantadine-resistant mutants revealed mutations in the transmembrane domain of the M2 protein [49], and along with observations that the proton ionophore, monensin, antagonizes the effects of M2 by equilibrating proton gradients within the cell, suggested that M2 might act to modulate the pH of intracellular compartments [57]. The finding that M2 forms tetramers allowed for modeling of the transmembrane domains in the form of a helical tetrameric channel in which the positions implicated in drug resistance orient towards the interior, as shown in Fig. 1 [58,59]. Direct evidence for the ion channel activity of M2 was obtained by electrophysiology studies using M2-expressing *Xenopus* oocytes, which have no
channel activity of their own [60]. Importantly, subsequent studies showed that the channel activity was selective for H+ ions [61].

Amanadine has been in clinical use since the 1960s and rimantadine was widely used in Russia long before it was approved in the US, in 1993 [62]. These two drugs have been shown to be effective against many influenza A viruses, both prophylactically and therapeutically. They are reasonably well tolerated at low doses, but at higher concentrations, amantadine exhibits a greater frequency of neurological and gastrointestinal side effects compared with rimantadine [63].

Amantadine and rimantadine could prove useful during the initial stage of a pandemic provided that the virus strains involved are susceptible to the drugs. This would be especially important before vaccines could be developed and manufactured, particularly for segments of the population such as healthcare providers. These agents are also less expensive than the neuraminidase inhibitors discussed below, and have a long shelf-life so are suitable for stockpiling. However, both drugs suffer from the drawback that they are strain-specific and that resistant mutants arise at a relatively high frequency in virus-infected patients receiving treatment [64,65]. A worrying feature of H3N2 viruses is the recent dramatic increase in resistance to M2 inhibitors among strains circulating in the US, from <2% of isolates examined to >90% over the past 2 years [66]. Among the avian H5N1 strains that have spread from Southeast Asia in recent years, a lineage that is currently prevalent in Indonesia and through China, Mongolia, Russia, and Turkey seems to be sensitive, while others are resistant [67]. Thus, it will be necessary to continue monitoring emerging strains to develop plans to control them.

**NA and its inhibitors**

Although this review focuses on virus entry, it is appropriate to briefly discuss the critical balance between the HA receptor binding and NA receptor-destroying functions, together with currently available NA inhibitors. NA may have some functions in virus entry, e.g. to counteract the inhibitory effects of mucus that block access to epithelial cells of the respiratory tract [68]. However, the primary function of the viral NA is to cleave sialic acid from host cell and viral surfaces at the end of the virus life cycle (Fig. 1). Studies on temperature-sensitive mutants [69], and viruses with truncated NA proteins [70] show that infection, RNA replication, and protein synthesis are not affected in the absence of NA function, but that virus particles accumulate at the surface of infected cells. This is due to the presence of an abundance of functional receptors at these sites, and their interactions with sialic acid binding sites of the viral HA glycoproteins.

The first crystal structures of NA were reported in 1983 [71], and the active site interactions with sialic acid were shown by the structure of complexes in 1992 [72]. This led to the development of the NA inhibitor zanamivir, which was designed to bind to this active site of the molecule [73], and several such inhibitors have now been characterized. Of these, zanamivir – which is administered as an inhalant – and its orally administered counterpart, oseltamivir, have been in clinical use since 1999.

The NA inhibitors lead to reduced virus dissemination in a fashion that is reminiscent of NA-defective viruses, and can be effective against viruses of all subtypes. They do not block virus infection, but are effective at reducing the severity of disease if administered within the first few days after contracting the infection. Several studies have described NA inhibitor-resistant viruses, and the mutations leading to resistance map not only to the vicinity of the NA active site, as would be expected, but also to HA regions predicted to affect virus attachment [74]. Thus, the virus can circumvent the inhibitor-mediated block to virus shedding by mutating HA to bind less tightly, or possibly by mutating to alter glycosylation. This provides further evidence for the existence of a functional balance between HA and NA proteins in influenza virus infections [75]. Unlike the situation with amantadine and rimantadine, NA inhibitor-resistant mutants are not readily isolated from patients receiving these drugs [76,77]. To date, zanamivir-resistant viruses have not been isolated from immunocompetent patients, and oseltamivir-resistant mutants arise at levels of approximately 1% for adults and 5% for children. In addition, the resistant mutants that have been analyzed are often found to be debilitated [78–81]. However, the situation could change and vigilance is crucial in continuing to monitor clinical isolates and emerging strains for susceptibility to NA inhibitors. In the event of a pandemic, these inhibitors may provide a critical line of defense, particularly if the strains are resistant to M2 inhibitors.

**Conclusion**

The processes involved in the entry of influenza viruses into host cells and NA function at the end of the virus life cycle provide many steps that might be exploited for the design of antiviral drugs. Among existing agents, the M2 inhibitors, amantadine and rimantadine, and the NA inhibitors, zanamivir and oseltamivir, have been most widely used clinically; several review articles detail their modes of action, effectiveness when administered therapeutically or prophylactically, and their advantages and disadvantages [62,77,82–84]. However, it is vital to persevere with research into novel inhibitors of influenza, such as those discussed here, and others that modulate pathways such as...
cell signaling [85], endosomal trafficking [86], and those involving various stages of the RNA "life cycle" [87–90]. The continued expansion of knowledge of the structure, function, and biology of influenza viruses and their components should lead to the further development of improved methods for the control of these viruses.

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The Mechanism and Inhibition of Influenza Virus Entry


What is the Role for Entry Inhibitors in Microbicides for Blocking Transmission of HIV-1 via the Cervico–Vaginal or Rectal Mucosa?

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Microbicides are designed for topical application on the vaginal or rectal mucosa in order to block the transmission of HIV type 1 (HIV-1) virus. Diverse substances are currently being considered for such use, based on their in vitro activity against HIV-1 infection; some have been tested in animal models and a few are already in clinical trials. Entry inhibitors interfere with the earliest events in the viral replication cycle: the viral attachment to susceptible cells, docking onto receptors, subsequent interactions with coreceptors, and fusion of the viral envelope with the cytoplasmic membrane of the target cell, all of which are mediated by the viral envelope glycoprotein. Entry inhibitors act either on the viral or the cellular side, binding to different subunits of the envelope glycoprotein or to receptors and coreceptors. These agents are very diverse and belong to a range of different classes of molecules: antibodies, recombinant receptor fragments, peptides, chemically modified chemokines, carbohydrate-binding proteins, polyanions, and small organic molecules. This article considers some important questions in developing effective microbicides that include entry inhibitors. Is inhibition of infectious entry into target cells in the mucosa sufficient to prevent entry into the organism? Can HIV-1 bypass a mucosal microbicidal barrier by getting captured by trafficking dendritic cells? These issues are discussed against the background of in vitro experimentation, macaque transmission models, and the biology of human infection with HIV-1. J Viral Entry 2006:2(2):67–72.

With an incidence of approximately 4 million HIV-1 infections per year [1], the world urgently needs effective preventive measures to curb the spread of this virus. The majority of transmissions occur through sexual intercourse. As a satisfactory vaccine is unlikely to become available within the next few years, one preventive strategy now focuses on microbicides, which are agents designed to prevent infection when applied topically to the cervico-vaginal or rectal mucosa.

Transmission to women is currently more common than to men; among 15- to 24-year-olds in sub-Saharan Africa, females are three times more likely to be HIV-1-infected than males [2]. Microbicides offer the advantage of a means of protection that women can control; they may be used without their sexual partners’ knowledge and do not exclude the possibility that intercourse may lead to conception. Hence, there are many reasons to regard microbicides as a valuable complement to condoms.

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In the future, it may also be possible to combine these agents with vaccines. So far, the most intractable difficulty in HIV-1 vaccine development has been to elicit high enough levels of neutralizing antibodies. HIV-1-specific cytotoxic lymphocytes can readily be induced, but this is not considered sufficient for sterile immunity [3]. However, cellular immunity in combination with a microbicidal barrier that substitutes for mucosal antibodies might provide adequate protection [4].

Different classes of active agents in microbicides have been tested in cell culture and animal models of viral transmission, and some have progressed to clinical studies. One candidate, Nonoxinol-9 (nonylpenoxypolyethoxyethanol [N-9]), a non-ionic surfactant that disrupts the viral lipid envelope, has already been assessed and rejected in a clinical trial, as it was found to damage the vaginal mucosa and thus enhance the risk of infection [5]. This failure notwithstanding, other surfactants are currently being evaluated in human trials, as are various pH modifiers, polyanionic gels, and inhibitors of HIV reverse transcriptase [6–9]. This review, however, will focus on another diverse
group of substances, namely molecules that specifically interfere with viral entry into susceptible cells.

Molecular interactions of viral entry into the host cell

HIV-1 particles, or virions, are studded with spikes of the envelope glycoprotein, Env, which mediate virus entry [10]. Each protomer of Env consists of a surface moiety (SU) and a transmembrane protein (TM) that anchors the complex in the phospholipid bilayer of the viral membrane. The SU and TM are linked to each other through non-covalent bonds. Trimers of these SU–TM heterodimers stud the surface of the virion.

An infectious cycle begins when a virion approaches the target-cell surface and a trimer binds to the main cellular receptor for the virus, CD4. The binding induces conformational changes in the Env oligomer that promote interactions with a cellular coreceptor – CCR5 for R5 viral strains, or CXCR4 for X4 strains (for reviews, see [10–12]). The coreceptor interactions in turn trigger a refolding of TM into a coiled coil, which promotes the fusion of the viral and cellular membranes [13,14]. Each individual step in the entry process, from attachment to fusion, can be blocked by specific inhibitors that bind to Env, the receptors, or the coreceptors (Table 1).

Viral ligands

Sulfated polyanions interact with positively charged side chains in Env, particularly the hypervariable V3 region in X4 viral strains [15]. Other compounds, such as Cyanovirin-N (CV-N), coat virus particles through interactions with the abundant and mannose-rich carbohydrate moieties on Env [16]. This type of binding may inhibit infection by increasing the repulsion of virions from target cells and by interfering with viral binding to ancillary cell-surface attachment molecules. It may also block binding to specific receptors through steric hindrance [17]. The use of these molecules in microbicides has been reviewed elsewhere [18–20].

Several specific ligands for Env are also being considered for microbicide use. However, there are only a few potent neutralizing antibodies (NAbs) that are active against multiple strains of HIV-1, partly because of the extensive sequence variation of the env gene [21]. Among these broadly neutralizing antibodies, some appear quite promising. The NAb b12, directed against the CD4-binding site (CD4bs) on gp120, has been shown to provide protection against vaginal challenge with a simian–human immunodeficiency virus (SIV/HIV) strain. Other promising NAbs include 2G12, 447-52D, 2F5, 2G12, and 4E10. These antibodies are being developed for use as microbicides and may be used in combination with other inhibitors to block different steps in the viral entry process.
Interestingly, the innate immune peptides, challenge with SHIV in the macaque model [25]. An anti-gp120 peptide, C52L, has shown partial protection from vaginal challenge with SHIV in the macaque model [25]. Interestingly, the innate immune peptides, α- and θ-defensins, bind to gp120 with dissociation constants in the nanomolar range and also block HIV-1 infection [26]. In addition, θ-defensins have been shown to bind to gp41 and to block the formation of the fusogenic six-helix bundle [27].

It remains to be seen whether the production of NAbs or other proteins for use in microbicides can be made economically feasible [9]. One strategy may be to colonize the vagina or rectum with recombinant bacteria in order to produce the inhibitors in situ [28–31]. However, whether this approach can yield sufficient local concentrations for production of inhibition is debatable [9]. Aside from their high cost, proteins also have other disadvantages: they may degrade, denature, and elicit immune responses. Inflammation in the mucosa would be likely to augment the risk of infection through recruitment of susceptible cells (Fig. 1). Therefore, small-molecule ligands of Env are attractive microbicide candidates. One such molecule, BMS-378806, which binds to the CD4bs on gp120 and blocks viral docking onto the receptor [32], has shown protection in the macaque model [25]. Small-molecule gp41 ligands that mimic the fusion-blocking peptides are now also being identified [33], and would provide valuable additions to the microbicide arsenal.

Viral ligands can only bind once the infectious inoculum is deposited on the mucosa. They must then attach rapidly, when the semen and microbicide solution mix by diffusion, in order to prevent infectious encounters with the first potential target cells. Those ligands that depend on receptor interactions with Env for the induction of their binding sites must be present at sufficiently high concentrations in the relevant tissue compartment at that late step in entry. The situation is principally different for the counterpart inhibitors on the cellular side.

Cell surface-receptor ligands

HIV-1 infection depends on the binding of gp120 to CD4. This interaction tethers the viral envelope to the cell membrane and triggers the subsequent steps in the entry process. The blocking of CD4 with monoclonal antibodies (MAbs) such as TNX-355 – a humanized MAb currently in Phase II clinical trials for systemic use – has therefore been considered for microbicide application [7]. The defensins, mentioned above, bind to CD4 in addition to binding to Env, and this may contribute to their inhibition of infection [26]. A small-molecule ligand for CD4, NSC 13778, that prevents gp120 binding has also been described [34]. Inhibitory occupancy on cell surface receptors can be achieved before exposure to the virus, and, if dissociation is slow, protection will be maintained for hours after application [9].

The next step in host cell entry is the interaction with the coreceptor. PSC-RANTES, a modified version of the chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted), and the small molecule CMPD167, both of which bind to CCR5, have shown good protection in the macaque model of vaginal R5 SHIV transmission [25,35]. This is encouraging, as almost exclusively CCR5-using strains are transmitted [11,12]. However, X4 virus can be present in the semen of an infected individual. It would be of grave concern if the blocking of R5 transmission gave a relative preference to the transmission of X4 variants, as presence of X4 virus is associated with a more rapid progression to AIDS. Hence, there is a rationale for also trying to block X4 infection with microbicides. Some inhibitors, such as the sulfated polyanions that interact more strongly with the X4 gp120 than the R5 gp120, would inhibit X4 at least as efficiently as R5 virus. Others, including the cross-neutralizing antibodies, small-molecule gp120 ligands, soluble CD4-constructs, fusion-blocking peptides, and CD4 ligands would block most strains of either tropism. And, in analogy with blocking CCR5, the coreceptor for X4 virus, CXCR4, could also be blocked. This can be achieved with the physiological ligand for CXCR4, stromal cell-derived factor 1, or possibly by certain defensins [36]. The small-molecule CXCR4 ligands, AMD3100 and AMD3465, inhibit X4 infection in vitro, and could be combined with CCR5 ligands and general blocking agents in a microbicide [7,9].

Combinations of inhibitors

In addition to the aforementioned worry that blockade of the CCR5 receptor might somehow promote transmission of X4 virus, there is also a potential risk that resistant strains could be preferentially transmitted, or amplified in tissues with suboptimal concentration of inhibitor. This is a further reason to include several inhibitors in a formulation, a strategy that would also generally enhance the effectiveness at obtainable concentrations. Indeed, combining the CCR5 ligand,
Figure 1. The female genital mucosa: the locale where microbicides may prevent transmission. The squamous stratified epithelium of the vagina forms an efficient barrier to viral penetration when intact. The columnar epithelium of the cervix uteri provides a weaker barrier against infection, but cervical mucus may trap virions [45]. However the plug of mucus would not protect ectopic cervical epithelium. Ex-plant experiments suggest that HIV cannot cross the epithelial barrier in the absence of a rift. The figure depicts virus in infectious semen that has been deposited in the vagina. Both CCR5- and CXCR4-using virions (blue and grey, respectively) are present. With few exceptions, R5 virus comes to dominate in a newly infected host; it may be preferentially transmitted or disproportionately amplified early in infection [11,12]. The dendritic cell (DC) to the left has captured a virion from the lumen. DCs express CD4 and CCR5 and can thus be infected by HIV; deeper in the tissue, an infected DC has progeny virions budding from the cell surface (sections studded with black Env spikes). DCs also express DC-SIGN (DC-specific ICAM-3 grabbing non-integrin) and other mannose C-type lectins (MCLR) on their surface, which are used for binding to the virus and subsequent internalization. Whether DCs contribute to natural infection by conveying virus bound to MCLR across the epithelial barrier is not known [37]. To establish an infection, the virus must reach a number of CD4+CCR5+ lymphocytes as shown in the propagation step in the middle of the figure. Infected macrophages (shown on the right) are also considered to be an important source of progeny virus [46]. The arrows show different routes of traversing the mucosa, infection of the first target cells, and migration of progeny virus or DCs with captured virus to the lymph nodes, via the afferent lymphatics. The lymph nodes are rich in CD4+ lymphocytes. From these target cells and infected macrophages, new generations of progeny virus cascade to the next level of lymphatic tissue. Viremia peaks when the virus reaches the gut-associated lymphoid tissue (GALT); its subsequent decline may be due to a depletion of the reservoir of susceptible cells in the GALT. Eventually, the virus disseminates via efferent lymphatics and blood to the spleen, brain, liver, and lungs.

Reproduced with permission from [4].
CMPD167, or the gp120 ligand, BMS-378806, with the fusion-blocking peptide, C52L, has shown improved protection in the macaque model compared with using single drugs. This might be explained by the synergy observed in vitro between C52L and either CMPD167 or BMS-378806 [25].

Is entry block enough?
Studies have shown that HIV-1 can bind to and infect dendritic cells (DCs) [37]. If dendrites of these cells become exposed in the lumen of the vagina, for example through coitally-induced microabrasions, virus particles may be picked up, transported to regional lymph nodes with the trafficking DCs, and there infect lymphocytes (Fig. 1). The half-life of DC-trapped virus is prolonged and DCs may also enhance the infectivity of the virus it presents to lymphocytes compared with the corresponding amounts of virions in cell-free suspension.

Furthermore, it has been observed that HIV-1 can cross some epithelia through transcytosis and infect underlying lymphocytes in the lamina propria. This would be a more plausible mechanism for transmission across the rectal or colonic epithelium, than via the female genital tract [7,38]. If this route of infection is relevant, it would be insufficient to maintain sterilizing concentrations of inhibitors in the lumina of the vagina or the rectum. The odds for protection by cell-surface-receptor ligands would seem even worse than for viral ligands in such a scenario. The latter might stay bound to the DC-captured virus, or even prevent the capture. However, if infection of DCs – as opposed to their mere capture of virus – is an obligatory step in crossing the mucosal barrier, at least agents preventing R5 infection would be effective. In any case, viral ligands that bind only after receptor docking, such as the fusion-blocking peptides, do protect in the vaginal macaque model. This would suggest that cells accessible by viral diffusion from the lumen are important targets in infection. Whether protection could be further enhanced by also blocking the interactions with Env-binding mannose C-type lectin receptors (MCLRs) on DCs is another question. The interaction of virus with MCLRs, such as DC-SIGN (dendritic-cell-specific intercellular adhesion molecule 3-grabbing non-integrin), can be blocked by mannann and CV-N [16] and perhaps by defensins, which have lectin activity [26] (Table 1). Such inhibitors should therefore be tested in combination with others.

The high ratio of inhibitory concentrations in vivo/in vitro
It has been observed that inhibitor concentrations several orders of magnitude higher are required to protect a proportion of macaques from vaginal or rectal challenge, than to block a similar fraction of infectivity in vitro. Tentatively, this difference is particularly pronounced for inhibitors that bind to the cell as opposed to the virus [8,9]. How can this be explained? The viral doses used in the two situations are usually comparable; furthermore, if some 90% of the infectivity of the inoculum were blocked in the lumen or on the mucosa, this should be reflected in a substantial degree of protection, since when 10-fold lower challenge doses than the usual are used in the macaque model, significantly lower frequencies of infection ensue. Hence, the explanation does not seem to be that a greater extent of inhibition is required in vivo than in vitro [9,18,22,35,39-44]. Nor does capture by DCs seem to be a major explanatory factor, since an agent such as CV-N, which blocks both infection and interactions with MCLRs, also evinces high in vivo/in vitro ratios [9,16,39,40].

What does appear relevant is the concentration gradient of inhibitor that is created by diffusion from the lumen of the vagina or rectum to interstitial spaces deep in the mucosal tissue. This explanation is compatible with a greater in vivo/in vitro ratio for cell surface receptors than for viral ligands. The receptor ligands would have to reach sufficiently high concentrations in the deeper tissues to yield blocking occupancies there; the viral ligands, on the other hand, would achieve their blocking occupancies in the lumen, then slowly dissociate as the coated virions diffuse or traffic with DCs into deeper tissues. Other considerations that apply equally to cell surface-receptor and viral ligands involve the diffusion of inhibitor into the luminal fluids, both before and after deposition of the inoculum. Substantial losses and dilution would be expected, as would variations in local distribution due to incomplete mixing [9].

Conclusion
It may seem that the earlier in the viral replication cycle an inhibitor interferes, the better. But interference with any step obligatory to infection is sufficient. Potency, extent, and breadth of inhibition, rather than the mechanism, are of the essence. Hence, many entry blockers are attractive microbicide candidates but not prima facie more promising than, for example, reverse transcriptase inhibitors. Due to the extreme genetic variation of HIV-1, and the palpable risk of selecting for viral escape, combinations of inhibitors may be the wisest strategy. Regardless, it must be shown that the drug formulations do not induce inflammation or in other ways compromise mucosal integrity, which would increase the risk of infection. Candidates that out-compete others based on breadth of inhibition in vitro and degree of protection against prototype strains in the macaque model may qualify for human clinical trials. But they must also be easy to use and relatively cheap [9]. The potency and molecular disparity of the entry inhibitors mean that some may well fit the bill for inclusion in future microbicides.
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Coreceptors

Long-lasting CCR5 internalization by antibodies in a subset of long-term nonprogressors: a possible protective effect against disease progression
Pastori C, Weiser B, Barassi C et al.

The authors of this report discuss the possibility that antibodies against CCR5 may be preventing HIV infection, or slowing down or abrogating disease progression. In a cohort of patients with and without highly active antiretroviral therapy, anti-CCR5 antibodies were only seen in long-term non-progressors, in whom they occurred as a fifth of the selected sample. Loss of these antibodies was associated with progression to disease.

Blockade of the coreceptor CCR5 is now possible with several antiretroviral agents and appears to reduce the viral burden. However, naturally occurring antibodies that recognize an epitope in the first extracellular loop of the receptor have been identified, and found to down-regulate CCR5 on the target cell surface as well as render CD4+ cells resistant to infection by CCR5-tropic (R5) virus strains.

A selection of 290 subjects from cohorts in Italy and the US were assessed for the presence of anti-CCR5 antibodies and their benefit in preventing progression of HIV disease. The only subjects in whom anti-CCR5 antibodies were found were long-term non-progressors (23.5% of this group), while they were not seen in age-, sex-, and risk factor-matched controls with AIDS or equally matched controls with chronic HIV-infection who were receiving highly active antiretroviral therapy (p<0.001).

Since almost all viruses transmitted are R5 variants, these strains may have a role in preventing infection. In this study, the presence of antibodies targeted to CCR5 was associated with a lack of progression, and in nine of 20 long-term non-progressors who lost these antibodies over the follow-up period, viral burden increased (>1 log10 rise in HIV RNA) and disease progression occurred. This was in the absence of any change in viral phenotype, suggesting that the protection afforded by these anti-CCR5 antibodies is independent of viral strain and may be an attractive target for potential vaccine development.

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Drug Interactions

An anti-CCR5 monoclonal antibody and small molecule CCR5 antagonists synergize by inhibiting different stages of human immunodeficiency virus type 1 entry
Safarian D, Carnec X, Tsamis F et al.
Virology 2006;352:477–84.

In this study, Safarian and colleagues document that sensitivity to two small-molecule inhibitors (SMIs) of CCR5-mediated viral entry, SCH-C and TAK-779, is regulated by the hypervariable V3 crown of gp120, differ in the timing of inhibition from the monoclonal antibody PA14, and that both SMIs synergize with PA14 to inhibit CCR5-tropic strains of HIV type 1.

Following binding of HIV gp120 to the cell surface CD4 receptor, the former undergoes a conformational change to expose the chemokine receptor binding site. At least two contact points occur between gp120 and the CCR5 coreceptor:

- The second extracellular loop of CCR5 (ECL2) abuts the crown of the gp120 hypervariable V3 loop.
- The CCR5 N-terminal domain binds the V3 stem and bridging sheet of gp120.

In previous work, this group has shown that both SCH-C and TAK-779 bind the transmembrane domain of CCR5, in a
pocket distinct from the gp120 binding site, and non-
competitively inhibit gp120 binding to CCR5 [1,2]. Monoclonal
antibodies (MAbs) to ECL2, such as PA14, are potent inhibitors
of viral entry without blocking gp120 binding.

In this study, the investigators documented small-
molecule inhibitor (SMI) and MAb IC_{50} values to HIV type 1
(HIV-1) reporter viruses pseudotyped with envelope
glycoprotein from 13 different primary CCR5-tropic (R5)
viruses. In contrast to a narrow six-fold range in sensitivity to
PA14, sensitivity to SCH-C and TAK-779 spanned four orders
of magnitude. Safarian and colleagues then showed that
modifications in the V3 crown determine sensitivity to SMIs,
that the greater sensitivity to both SMIs is due to reduced
binding of the gp120 V3 crown with the coreceptor, and that
this is linked to features of the V3 crown.

Furthermore, synergy studies demonstrated that combinations of either SMI with PA14 synergize to inhibit envelope-mediated entry. Finally, an assay was established to determine the time course of viral inhibition of HIV-1
entry. The time required to achieve half-maximal inhibition
with SCH-C was 9 min and with PA14 32 min, compared
with 46 min for the gp41-membrane fusion-inhibitor
enfuvirtide. Together, these data indicate significant
differences in the mechanism of action for SMIs and PA14,
and thus, excellent potential as combination HIV
chemotherapy warranting further study.

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molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human

Synergistic in vitro antiretroviral activity of a
humanized monoclonal anti-CD4 antibody
(TNX-355) and enfuvirtide (T-20)
Zhang XQ, Sorensen M, Fung M et al.

A number of new agents are currently being investigated
for blocking entry of HIV type 1 into the host cell. In this
study, synergy was demonstrated in vitro between a
humanized monoclonal antibody against an epitope of
CD4 (TNX-355) and the fusion inhibitor enfuvirtide.
The fact that this dual attack on the entry cascade
provides increased benefit may help to design successful
combinations of agents that act on the entry process.

Drugs with activity against the HIV-receptor, CD4, and
coreceptors, CCR5 and CXCR4, as well as fusion inhibitors,
are now beginning to reach the clinic. It has previously been
speculated that they might act in tandem to reduce the
ability of HIV to enter target cells effectively. One of
the agents currently in development is TNX-355, a humanized
monoclonal antibody, which targets a unique epitope in CD4
that is involved in a conformational change allowing virus
entry after CD4 binding.

In this report, a series of experiments were conducted using
activated peripheral blood mononuclear cells from HIV-
negative individuals, exposed to six strains of HIV and one of
human T-cell leukemia virus-III, to examine the effect of single
or continuous exposure to TNX-355 at varying concentrations
of enfuvirtide. Synergistic antiretroviral activity was seen across
all experiments with TNX-355 and enfuvirtide, and appeared
greatest when both agents were present throughout the
culture period. The fact that there are now three tightly linked
targets around viral entry, all with candidate drugs, might allow
a three-way antiviral synergy against this staged process by
which the virus gains a foothold in the cell.

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Drug Resistance and Susceptibility

Characterization of envelope glycoprotein gp41
genotype and phenotypic susceptibility to
enfuvirtide at baseline and on treatment in the
Phase III clinical trials TORO-1 and TORO-2
Melby T, Sista P, DeMasi R et al.

This article presents further data on the genotype and
phenotype of viruses isolated at baseline and after 48
weeks of enfuvirtide therapy within the TORO (T-20
versus Optimized Regimen Only) studies. Since
enfuvirtide resistance has been seen both in vitro and
in vivo, where it develops rapidly in the face of ongoing
viral replication, the identification of amino acid changes
resulting in loss of susceptibility is clinically important.

Enfuvirtide works by competitively inhibiting interactions
between the heptad repeat (HR) 1 and HR-2 domains
of gp41; thus preventing the reconfiguration that allows the
virus and cell surfaces to meet and fusion to occur. Changes
in the conserved sequence of gp41 at positions 36–45 may
result in loss of enfuvirtide activity.

The TORO trials demonstrated a wide range of baseline
susceptibilities with a 3 log_{10} difference across the patients
included. Few polymorphisms in this region were identified,
the most common, seen in 16% of subjects, was at
position 42. However, changes in the 36–45 stretch of amino acid residues were observed in 92.7% of the patients who lost virological control during the studies, and in 98.8% of those with a greater than four-fold reduction in susceptibility to enfuvirtide.

While coreceptor tropism and viral subtype had no effect on viral response – although there were limited numbers of non-B subtypes in these studies – the 3.5% of viruses using the CXCR4 receptor showed higher IC_{50} values (the concentration required for 50% inhibition of viral replication) than CCR5-using or dual-tropic viruses. The presence of the N42S polymorphism had an independent association with lower baseline IC_{50}, however, it was not possible to follow changes in susceptibility over time in this cross-sectional study.

Taken together with the results of previous studies, those shown here attest to the relative fragility of enfuvirtide when given alone and argue for its use with an effective backbone regimen of drugs to which the patient remains sensitive. With the impending availability of CCR5 receptor blockers and integrase inhibitors, the future seems rosy indeed.

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Viral Variants

**Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4- using virus reservoir**

Westby M, Lewis M, Whitcomb J et al.


The current study describes the coreceptor tropisms observed before, during, and after 10 days’ treatment with the CCR5 antagonist maraviroc. The emergence of CXCR4-using virus was rare and was the result of pre-existing variants that reverted to predominantly CCR5-using virus following discontinuation of maraviroc therapy.

The development of CCR5 receptor antagonists has been complicated by concern over the emergence of CXCR4-using (X4) viral variants with the potential for more accelerated disease progression, which could arise from selective pressure during treatment with these agents.

The current publication describes 10-day monotherapy, dose-escalation studies with the CCR5 antagonist maraviroc, and the viral tropism changes before, during, and after therapy. Following treatment, the virus remained CCR5-tropic (R5 virus) in 97% (62 of 64) of the assessed patients.

At day 11, two subjects receiving maraviroc 100 mg once daily had CXCR4-using (X4) virus. The baseline CD4+ cell counts and viral load reductions in these subjects were indistinguishable from the rest of the study group. Clonal analysis by sequencing of gp160 revealed that a minority population of dual-tropic virus existed at baseline in one subject. Phylogenetic analysis of each subject showed that the X4 virus was genetically distinct from the R5 clones, and thus emerged from pre-existing viral reservoirs rather than resulted from mutations in the R5 isolates. A third subject was incorrectly enrolled into the trial with a mixed-tropic virus on day 1 and experienced no drop in viral load following 10 days’ treatment with maraviroc 100 mg twice daily. This subject had persistent X4 viral clones throughout the study period that were genetically distinct from the CCR5-using isolates. In all three subjects, circulating virus reverted to predominantly CCR5-using variants following discontinuation of maraviroc.

These results suggest that the development of CXCR4- using virus is uncommon following short-term maraviroc monotherapy, and occurs as a result of pre-existing viral reservoirs. Whether or not pre-existing CX4-using variants will evolve under pressure from highly active antiretroviral therapy containing multiple agents in combination with a CCR5 antagonist has yet to be determined. However, based on the findings of this study, the screening for viral tropism prior to use of CCR5 inhibitors is warranted.

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Antiretroviral Therapy

**A pharmacokinetic–pharmacodynamic model to optimize the Phase IIA development program of maraviroc**

Rosario MC, Poland B, Sullivan J et al.


The results of this modeling study indicate that it is possible to accelerate drug development by replacing some arms or trials using simulations based on data from existing studies. In this report, the development plan for the CCR5 inhibitor maraviroc was assisted by results from the first monotherapy study in HIV patients to plan the doses to be studied thereafter.

The authors of this article examine the use of a modeling approach to the pharmacokinetics and pharmacodynamics of the CCR5 receptor blocker maraviroc (formerly UK-427,857). Clinical data were used from a monotherapy study (A4001007), in which 44 HIV type 1 (HIV-1)-infected
subjects received maraviroc, given without food, at a dose of 25 mg/day, or 50, 100, or 300 mg twice daily, or placebo, for 10 days.

Using the day-10 data, HIV-1 RNA changes and viral susceptibilities of the individuals over the study period were incorporated in a mixed effects-modeling approach to develop a pharmacokinetic–pharmacodynamic model. The parameters from this model were then used to calculate a variety of outcomes, including average viral inhibition fraction and decay rate of actively infected cells. These data were entered into a Monte Carlo simulation, which predicted the viral load changes over a number of doses to be examined in a future study, A4001015.

The advantage of a model is that it can be updated using new clinical data when available as well as data from the literature. The model predicted that inhibition of the virus would range from 0.15–0.38 for the 25-mg once-daily dose, to 0.88–0.96 for the 300-mg twice-daily dose. The mean decline rate of HIV-1 RNA was similar or greater than published data for monotherapies of other antiretrovirals, but occurred more slowly than in patients receiving combination therapy. This has been reported as a good predictor of subsequent clinical outcomes.

There was good agreement between the predicted and observed data, although the effect of food on maraviroc levels (approximately 50% reduction) was overestimated; the model predicted a 14% reduction in viral load benefit, whereas only an 8% reduction was recorded in the subsequent study. It may be that this discrepancy is due to a lack of pharmacokinetic data on this fed dose for use in the model. Overall, however, this simulation approach helped to reduce unnecessary evaluation of particular doses of maraviroc.

Characterization of the molecular pharmacology of AMD3100: a specific antagonist of the G-protein coupled chemokine receptor, CXCR4
Fricker SP, Anastassov V, Cox J et al.


This article confirms that AMD3100 binds to CXCR4, inhibiting binding of the receptor’s natural ligand, stromal cell-derived factor-1 (SDF-1; CXCL12), chemotaxis, and SDF-1-mediated calcium flux. No inhibition of calcium flux was observed in cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5, or CCR7.

The chemokine receptor CXCR4 is present on many different cell types and plays an important role in leukocyte chemotaxis. The sole ligand for this receptor is stromal cell-derived factor-1 (SDF-1, also called SDF-1α, or CXCL12), a chemotactic cytokine that is constitutively produced in numerous tissues. This receptor–cytokine axis has important roles in diverse aspects of the body including T lymphocyte functions (lymphopoiesis, maturation, trafficking to bone marrow throughout the body), B cell growth and development, vascularization of the gastrointestinal tract, and normal cerebellar development. AMD3100 is a specific inhibitor of CXCR4 that has been widely studied, not only to validate CXCR4 as a target for HIV therapy, but also for mobilization of hematopoietic stem cells in patients with multiple myeloma and non-Hodgkin’s lymphoma. Animal studies indicate that CXCR4 blockade with AMD3100 may have beneficial effects in inflammatory diseases, including rheumatoid arthritis and asthma.

Fricker and colleagues provide detailed data characterizing the specificity of action of AMD3100 receptor binding, and the lack of agonist effects following receptor binding, and of cross-reactive binding with cells expressing CXCR3, CCR1, CCR2b, CCR4, CCR5, and CCR7. The article demonstrates that AMD3100 binding to CXCR4 is tight and slowly reversible, without allowing:

- SDF-1 binding.
- SDF-1-mediated calcium flux.
- SDF-1-stimulated chemotaxis.

The use of AMD3100 for inhibition of HIV has been limited by the requirement for continuous intravenous infusion, dose-related side effects including gastrointestinal symptoms, paresthesias, and premature ventricular contractions. The authors note that AMD3100 does not result in significant declines in HIV-viral load, despite specific activity against CXCR4-tropic virus.

The report cites recent clinical trials in multiple myeloma and non-Hodgkin’s lymphoma, which indicate that AMD3100 expands myeloid cells in the bone marrow, resulting in release of stem cells into the circulation. These studies suggest that AMD3100 may be safe and effective in improving stem cell mobilization when combined with granulocyte colony-stimulating factor (G-CSF), as compared with the current use of G-CSF alone. It remains unclear whether the clinical issues raised with AMD3100 in HIV patients were unique to the agent used, or a consequence of successful CXCR4 blockade. The presence of the CXCR4 receptor system in diverse tissues warrants particularly careful assessment of short- and long-term safety of AMD3100, and any other agent that interferes with CXCR4.

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Earlier this summer I gave a series of lectures in Canada, one of which was in the same hotel in Vancouver where almost exactly 10 years earlier we heard the first seriously good results from protease inhibitor (PI)-based highly active antiretroviral therapy (HAART). The treatment of HIV/AIDS has changed dramatically since then and now includes better-tolerated drugs and formulations, fewer pills per regimen, and more once-daily dosing. In my cohort, the last death from AIDS in a patient with no therapy options left happened in May 2000; the major cause of HIV disease and death in the UK is late presentation. This, of course, is not the case for the majority of people in the world, and the 16th International AIDS Conference Conference reflected this in the prominence rightly given to access and empowerment issues, such as the plight of the South African population, where, as a result of the government’s AIDS policy, more infections occur daily than patients enter into HAART programs.

Although this meeting was not primarily scientific, there was a selection of data for many of the new compounds in development (Table 1).

### Entry inhibitors

**TNX-355**
Thomas Duensing (Tanox, Inc., Houston, TX, USA) presented new 48-week data from a randomized, double-blind, placebo-controlled study of triple-class-experienced patients treated with the novel entry inhibitor TNX-355 [1]. TNX-355, a monoclonal antibody that prevents the conformational change in gp120 needed to expose the coreceptor binding site, is given as a weekly intravenous infusion. Eighty-two

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**Table 1. Antiretroviral agents discussed at AIDS 2006 (some of these drugs are still under development).**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Class</th>
<th>Abstract number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNX-355</td>
<td>Tanox, Inc.</td>
<td>Entry I</td>
<td>TUPE0058, THLB0218</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>Pfizer</td>
<td>CCR5 I</td>
<td>THLB0215</td>
</tr>
<tr>
<td>Vicriviroc</td>
<td>Schering-Plough</td>
<td>CCR5 I</td>
<td>TUPE0074, THLB0217</td>
</tr>
<tr>
<td>TRI-999, TRI-1144</td>
<td>Trimeris, Inc./Roche</td>
<td>Fusion I</td>
<td>THPE0021, THAA0303</td>
</tr>
<tr>
<td>Fosavudine</td>
<td>Heidelberg Pharma</td>
<td>NRTI</td>
<td>THPE0025, THLB0216</td>
</tr>
<tr>
<td>Etravirine (formerly TMC125)</td>
<td>Tibotec, Inc.</td>
<td>NNRTI</td>
<td>TUPE0061, TUPE0082, TUPE0084, TUPE0086, THPE0136</td>
</tr>
<tr>
<td>Rilpivirine (formerly TMC278)</td>
<td>Tibotec, Inc.</td>
<td>NNRTI</td>
<td>TUPE0087</td>
</tr>
<tr>
<td>GS9137</td>
<td>Gilead Sciences</td>
<td>Integrase I</td>
<td>TUPE0080, TUPE0088</td>
</tr>
<tr>
<td>MK-0518</td>
<td>Merck &amp; Co.</td>
<td>Integrase I</td>
<td>THPE0020, THPE0027, THLB0214, THAA0302</td>
</tr>
<tr>
<td>PL-100</td>
<td>Merck Pharmaceuticals</td>
<td>PI</td>
<td>THAA0304</td>
</tr>
<tr>
<td>Brecanavir</td>
<td>GlaxoSmithKline</td>
<td>PI</td>
<td>TUAB0105, THPE0023</td>
</tr>
<tr>
<td>Darunavir (formerly TMC114)</td>
<td>Tibotec</td>
<td>PI</td>
<td>TUAB0104, TUPE0060, TUPE0062, TUPE0063, TUPE0069, TUPE0078, TUPE0083, TUPE0086, THPE0136</td>
</tr>
</tbody>
</table>

I: inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; NRTI: nucleoside reverse transcriptase inhibitor; PI: protease inhibitor
patients with CD4+ T cell counts >50 cells/mm³ and viral loads >10 000 copies/mL were randomized to one of the following three groups:

- TNX-355 10 mg weekly for nine doses, given every 2 weeks thereafter.
- TNX-355 15 mg twice every 2 weeks.
- An optimized background regimen (OBR).

Virological failures were rolled over onto TNX-355 plus a new OBR. The viral load declines were approximately 0.2 log₁₀ less than the equivalent 24-week data shown previously and data on a withdrawn poster (Fig. 1) [2] (D Norris, Comprehensive Research Center, Tampa, FL, USA). The mean increase in CD4+ T cell count was 51 cells/mm³ for the 15-mg group and 48 cells/mm³ for the 10-mg group, compared with only 1 cell/mm³ for the placebo arm (p<0.05).

The time to loss of virological response was similar in the two treatment arms at 8 months (Fig. 2), but no data were presented on the outcomes of patients who subsequently received open-label drug. No serious adverse events (SAEs) were attributed to TNX-355, and the mild side effects reported were similar to those in the OBR group.

A poster by S Weinheimer (Tanox, Inc.) provided further data on the activity of this agent, in this case against virus with enfuvirtide-resistance substitutions (G36D, V38A, and N43D) generated through site-directed mutagenesis [3]. Compared with wild-type virus, HIV envelopes with these mutations showed an 11–32-fold reduced sensitivity to enfuvirtide but less than a two-fold reduced susceptibility to TNX-355. This suggests that cross-resistance between these two drugs may not represent a major problem.

Vicriviroc

On a poster by Angela Sanone (Schering-Plough Research Organization, Kenilworth, NJ, USA) the effect of the CCR5 receptor antagonist vicriviroc in combination with a range of ritonavir-boosted PIs (atazanavir, saquinavir, indinavir, fosamprenavir, and tipranavir) and unboosted nelfinavir was evaluated in a pharmacokinetic study in healthy volunteers [4]. It appears that the addition of any of these agents has a negligible effect on the key vicriviroc pharmacokinetic parameters, and no significant adverse events were reported. Clearly, additional data with boosted lopinavir and darunavir (formerly TMC114) are required to complete the evaluation.

After the disappointment of the earlier vicriviroc studies, it was reassuring to hear the positive news given by Roy Gulick (Cornell University, New York, NY, USA) in a late-breaker presentation on the ACTG 5211 study [5]. Three doses of vicriviroc were assessed against placebo in a highly treatment-experienced patient population with CCR5-tropic (R5) virus. One hundred and eighteen subjects (median viral load 36 380 HIV RNA copies/mL and CD4+ T cell count 146 cells/mm³) were randomized to receive vicriviroc (5, 10, or 15 mg/day) or placebo (Fig. 3). Viral load declines were significantly greater in all three vicriviroc arms compared with placebo at day 14 and week 24 (p<0.01, intention-to-treat analysis) (Table 2; Fig. 4). Although not statistically significant, the lower dose of 5 mg/day resulted in a greater
number of patients with virological failure and emergence of CXCR4-using (X4) virus. Thirty-three percent of participants had previous enfuvirtide experience, 92% were male, 66% Caucasian, 20% black, and 12% Hispanic. At study entry, 86% of patients harbored R5 virus exclusively and 10% had dual- or mixed-tropic variants. Considering that only a few weeks earlier, tropism screening had shown all patients as having R5 virus, this reveals some of the limitations of the current technology to exactly determine the tropism of a patient’s virus. However, emerging data that mixed- or dual-tropic virus responds to CCR5 receptor blockers may make the use of screening assays less vital.

When the response was analyzed according to baseline tropism, good suppression was seen even in the X4/R5-tropic populations.

**Maraviroc**

Howard Mayer (Pfizer Global Research and Development, New London, CT, USA) discussed the 24-week results from the A4001029 study, in the only talk to present new data on the CCR5 inhibitor maraviroc [6]. A4001029 is an ongoing, double-blind, placebo-controlled, exploratory Phase IIB trial in subjects with triple drug-class failure, >5000 HIV RNA copies/mL, and non-R5 virus – a patient population in which...
one may expect the drug to have only limited benefit. A total of 190 subjects were randomized to maraviroc (150 mg once or twice daily) or placebo, all with OBRs. Patient characteristics at baseline are shown in Table 3; tropism testing revealed that most subjects had dual- or mixed-tropic virus. Approximately 50% of the patients received concurrent enfuvirtide therapy, 60% in each group had between two and four active drugs in their OBR, baseline CD4+ T cell count was approximately 95 cells/mm^3, and viral load was 5.0 log_{10} copies/mL. As would be expected, mean viral load changes did not differ greatly between the groups (Table 4). However, what was surprising was that CD4+ T cell increases were greater in the maraviroc-treated patients than in those who received placebo; this reached the lowest level of statistical significance when all randomized subjects were included; Table 5. In addition, the use of enfuvirtide in the OBR pointed towards a trend of a greater than additive benefit. This raises the question of whether maraviroc may actually be useful in a broader range of patients than was first thought. No drug-specific toxicity was noted; in fact, a greater number of grade 3 and 4 abnormalities in liver function were recorded in the placebo group.

**New-generation fusion inhibitors**

Two new fusion inhibitors, TRI-999 and TRI-1144, are being developed through a joint venture between Trimeris, Inc. (Morrisville, NC, USA) and Roche (Nutley, NJ, USA), a collaboration that has already produced enfuvirtide. In a poster presentation by DK Davidson (Trimeris, Inc.), the ability of these new compounds to inhibit virus isolates resistant to enfuvirtide and the previous lead compound, T-1249, was examined [7]. Through *in vitro* selection, a series of mutations developed in the heptad repeat 1 (HR1) domain, and, less frequently, in HR2 of gp41. While all enfuvirtide-resistant isolates demonstrated a <10-fold loss of susceptibility to T-1249, mutations arising through selection with T-1249 were highly resistant to enfuvirtide. When these isolates were tested against either TRI-999 or TRI-1144, they retained sensitivity to these new agents. Both required a greater number of mutations to acquire resistance. Furthermore, virus breakthrough was seen at concentrations 30-fold lower than those required of enfuvirtide or T-1249, suggesting a much-enhanced genetic barrier against resistance development.

Mary Delmedico (Trimeris, Inc.) then showed exciting data on advances towards a once-weekly formulation of the two lead compounds, administered by subcutaneous injection [8]. The researchers’ aim was to develop formulations that require a minimum dose with maximum drug exposure. Using a rat model, they first tried a peptide–organic salt complex. For TRI-1144, this resulted in 13% bioavailability, but a long half-life and an improved area under the curve (AUC) when the release rate of the compound was increased.
However, when a modified gel formulation of the other lead compound, TRI-999, was assessed in a rabbit model, bioavailability was increased to near 100% and the half-life greatly extended (Fig. 6). These data are exciting, as they demonstrate that a once-weekly injectable fusion inhibitor is achievable. This is a big leap towards improved clinical use of parenteral compounds that appear to overcome many of the current problems with enfuvirtide. When asked if enfuvirtide was a candidate for an extended-release formulation, Dr Delmedico said that its pharmacokinetic profile did not lend itself to this and no work in this direction is planned. This makes the rapid development of these two new compounds all the more vital.

**Nucleoside reverse transcriptase inhibitors**

*Fosalvudine tidoxil*

Pedro Cahn (Fundacion Huesped, Buenos Aires, Argentina) showed data from a Phase I study conducted in Argentina.

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### Table 3. Baseline patient characteristics of the study population in the maraviroc Phase IIB trial, A4001029.

<table>
<thead>
<tr>
<th></th>
<th>Placebo+OBR (n=62)</th>
<th>Maraviroc once daily+OBR (n=63)</th>
<th>Maraviroc twice daily+OBR (n=61)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in HIV-1 RNA (log_{10} copies/mL)</td>
<td>0.87</td>
<td>1.15</td>
<td>0.92+0.06</td>
</tr>
<tr>
<td>Mean age (years), (range)</td>
<td>44.6 (23–65)</td>
<td>42.7 (16–59)</td>
<td>42.5 (16–62)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>9 (14.5)</td>
<td>10 (15.9)</td>
<td>6 (9.8)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– White</td>
<td>40 (64.5)</td>
<td>46 (73.0)</td>
<td>44 (72.1)</td>
</tr>
<tr>
<td>– Black</td>
<td>18 (29.0)</td>
<td>17 (27.0)</td>
<td>13 (21.3)</td>
</tr>
<tr>
<td>– Other</td>
<td>4 (6.5)</td>
<td>0 (0)</td>
<td>4 (6.6)</td>
</tr>
<tr>
<td>Tropism, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– X4</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>– R5</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>– Not phenotyped, or not reported</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>– Dual/mixed-tropic</td>
<td>58</td>
<td>57</td>
<td>52</td>
</tr>
</tbody>
</table>

OBR: optimized background regimen (three to six antiretroviral drugs).
on the safety, tolerability, and pharmacokinetics of fosalvudine tidoxil in HIV patients [9]. This agent is a prodrug of the nucleoside reverse transcriptase inhibitor (NRTI) alovudine, and has a narrow therapeutic window. In a previous study, 4 weeks’ treatment with alovudine (7.5 mg once daily) resulted in a median reduction of 1.88 log_{10} in HIV RNA levels in patients with multiple thymidine-associated mutations [10]. Another early trial of alovudine was stopped after a case of liver failure. At the end of the session, the investigator of that particular study, Charles Flexner (John Hopkins University School of Medicine, Baltimore, MD, USA), commented that this was an idiosyncratic hepatic necrosis, and went on to advise investigators of this compound to be very careful. After the experience with lodenosine, which caused high rates of liver changes, the present author agrees. Nevertheless, the present study in 24 healthy volunteers showed that fosalvudine tidoxil has a half life of 5–7 h at single doses of 5, 10, 20 (molar equivalent of 7.5 mg alovudine), and 40 mg. The prodrug is thought to be safer than the parent compound, as animal and in vitro studies have shown that it has minimal bone marrow distribution and high protein-binding properties [11] (Frank Reuss, Heidelberg Pharma GmbH, Ladenburg, Germany), and, apart from headaches, no adverse events were reported in the present study.

Non-NRTIs

Etravirine

Data on non-NRTIs (NNRTIs) at the conference exclusively concerned etravirine (formerly TMC125) and rilpivirine (formerly TMC278) from Tibotec, Inc. (Yardley, PA, USA). The only new information on the latter agent was a healthy volunteer study (n=16) that examined the interaction with ketoconazole, an inhibitor of the liver enzyme cytochrome P3A4 (CYP3A4) [12] (Rolf van Heeswijk, Tibotec BVBA). Rilpivirine is a substrate for CYP3A4, and concomitant administration with ketoconazole increased exposure to rilpivirine by 49% (AUC_{24h}) , probably by inhibiting its metabolism in the liver. The results suggest that the combination of rilpivirine and inhibitors of cellular cytochromes may require dose modifications.

Two pharmacokinetic studies of etravirine, which is further along in development than rilpivirine, were presented by M Schöller-Gyüre, (Tibotec BVBA). In the first of these, there seemed to be no significant interaction between this drug and methadone in healthy, HIV-negative volunteers who were stabilized on methadone therapy [13]. In a 14-day study of 16 individuals, no clinically relevant changes in drug handling of either agent were seen and no significant symptoms of methadone withdrawal (which can be a problem with the currently licensed NNRTIs) were observed.

Table 4. Efficacy results in the maraviroc Phase IIB trial, A4001029.

<table>
<thead>
<tr>
<th></th>
<th>Placebo+OBR (n=58)</th>
<th>Maraviroc once daily +OBR (n=57)</th>
<th>Maraviroc twice daily +OBR (n=52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean decrease in HIV-1 RNA (log_{10} copies/mL)*</td>
<td>-0.97</td>
<td>-0.91</td>
<td>-1.20</td>
</tr>
<tr>
<td>Treatment difference (maraviroc–OBR) in HIV-1 RNA decrease (log. copies/mL) (97.5% CI)</td>
<td>+0.06 (-0.53,0.64)</td>
<td>-0.23 (-0.83,0.36)</td>
<td></td>
</tr>
<tr>
<td>HIV RNA &lt;400 copies/mL (%)</td>
<td>24.1</td>
<td>24.6</td>
<td>30.8</td>
</tr>
<tr>
<td>HIV RNA &lt;50 copies/mL (%)</td>
<td>15.5</td>
<td>21.1</td>
<td>26.9</td>
</tr>
<tr>
<td>Mean decrease in HIV-1 RNA in patients using enfuvirtide** (log. copies/mL)</td>
<td>-0.89</td>
<td>-1.26</td>
<td>-1.44</td>
</tr>
</tbody>
</table>

*Primary end point. **Last observation carried forward analysis. OBR: optimized background regimen.

Table 5. Changes in CD4+ cell count in patients with dual/mixed-tropic HIV type 1 at screening in the maraviroc Phase IIB trial, A4001029.

<table>
<thead>
<tr>
<th>CD4+ cell change from baseline (cells/mm³, mean)</th>
<th>Placebo+OBR (n=58)</th>
<th>Maraviroc once daily +OBR (n=57)</th>
<th>Maraviroc twice daily +OBR (n=52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All treated patients with dual/mixed-tropic HIV-1 (at 24 weeks, last observation carried forward)</td>
<td>+36*</td>
<td>+60</td>
<td>+62</td>
</tr>
<tr>
<td>Patients with only X4-tropic HIV-1 detectable at time of virologic failure</td>
<td>-104 (n=2)</td>
<td>+48 (n=12)</td>
<td>+33</td>
</tr>
</tbody>
</table>

* Data for patient 4 is missing.
A second bioavailability study of etravirine in 18 healthy, HIV-negative volunteers assessed co-administration of the agent with the H₂ antagonist ranitidine and the proton-pump inhibitor omeprazole [14]. Drug–drug interactions with medications used for ulcer pain and gastritis have been problematic for some other HIV drugs, notably, unboosted atazanavir. However, neither ranitidine nor omeprazole had any clinically relevant effect on etravirine in this study, although etravirine exposure was increased by 41% by concomitant omeprazole, possibly as a result of the inhibition of CYP2C19.

Clinical data on the TMC125-C223 trial were discussed by Cal Cohen (Community Research Initiative of New England, Boston, MA, USA). A total of 199 subjects with documented NNRTI resistance were randomized to etravirine (400 mg or 800 mg, twice daily), or a standard-of-care control regimen [15]. Baseline resistance analysis showed a mean of two NNRTI mutations and a phenotypic mean fold change of only 1.7 for etravirine, compared with 41 for efavirenz and 61 for nevirapine. At week 48, the viral load change was –0.88 log₁₀ and –1.04 log₁₀ for the two etravirine arms, compared with –0.14 log₁₀ for the control arm, in which 78% of patients had withdrawn due to virological failure (Fig. 7). When the response was assessed against baseline resistance to NNRTIs, the number of mutations predicted the viral response (Fig. 8). However, even with three mutations, the response to etravirine remained greater than the active control.

Following pharmacokinetic data presented by Marta Boffito (Chelsea and Westminster Hospital, London, UK) [16] in London last year, which defined the optimal doses of the combination of darunavir/ritonavir and etravirine as 600 mg/100 mg + 200 mg, respectively, twice daily, this combination has now entered into two large Phase III studies (DUET 1 and 2) for safety and efficacy assessment.

Thomas Kakuda (Tibotec, Inc.) and co-workers reported a formal two-way crossover trial to further assess pharmacokinetic interactions in 32 HIV-negative volunteers, randomized to two groups [17]. All volunteers received etravirine 100 mg twice daily for 8 days. After a washout period of 14 days, they were given darunavir/ritonavir 600 mg/100 mg twice daily for 16 days. From day 9 to 16, group A received etravirine 100 mg twice daily, while group B received 200 mg twice daily. Concomitant administration of etravirine and darunavir/ritonavir was found to have no clinically significant effect on the pharmacokinetics of darunavir or ritonavir. However, the etravirine 100 mg twice-daily exposure was decreased by 37%, with similar decreases in Cₘₐₓ and Cₘᵢₙ (32% and 49%, respectively). Pharmacokinetic parameters for etravirine observed after co-administration of etravirine 200 mg twice daily with darunavir/ritonavir was slightly lower than the historical control for healthy volunteers given the same dose and formulation (AUC₁₂₉ₐ, 7638±2254; Cₘₐₓ, 876±2336). Overall, the safety profiles for etravirine, darunavir/ritonavir, and their co-administration

![Figure 7. Change in viral load at 48 weeks in the TMC125-C223 trial assessing therapy with etravirine.](image-url)
were similar. Apart from a rash, which was seen more frequently during co-administration, use of etravirine with darunavir/ritonavir in healthy volunteers was generally safe and well tolerated.

A number of groups around the world have early data on compassionate-use groups receiving the two drugs simultaneously. One of the more clinically relevant studies for salvage therapy was a poster by Julio Montaner (British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada) on the outcomes of combining darunavir/ritonavir with etravirine [18]. In four heavily pre-treated subjects administered this combination, three of whom also received enfuvirtide as a new drug, a drop of 2 log_{10} in HIV RNA was recorded. No adverse events were reported. These results are similar to data obtained by the present author from patients in London, UK (unpublished data). This triple regimen offers a reasonable new HAART for patients who cannot wait for novel classes such as integrase inhibitors to become available.

**Integrase inhibitors**

**MK-0518**

MK-0518, the first integrase inhibitor developed by Merck & Co., Inc. (Whitehouse Station, NJ, USA), is an agent that prevents HIV from incorporating its genetic information into that of the host cell, thereby preventing the establishment of infection in T cells. It has shown good efficacy in vitro and acts synergistically with many available antiretroviral drugs.
patients with multi-drug resistance [19] (Vincenzo Summa, IRBM-Merck Research Laboratories, Pomezia, Rome, Italy). In addition, favorable pharmacokinetic and metabolic properties suggest that MK-0518 may have good safety and efficacy profiles in HIV-positive patients [20] (Ralph Laufer, MSD/IRBM, Merck, Pomezia, Rome, Italy). Furthermore, Michael Miller (Merck Research Laboratories, West Point, PA, USA) provided an update of the biochemical and antiviral activity of MK-0518, which charted the history of the development of this drug over the past 12 years, thanks to the tenacity of Daria Hazuda (Merck Research Laboratories) and her team [21]. MK-0518 has been shown to:

- Be highly specific for HIV integrase.
- Be active against HIV-1 and HIV-2.
- Have either additive or synergistic effects with all tested antiretroviral agents.

No data are available on the use of either tipranavir or darunavir in combination with MK-0518; however, no negative effects/interactions are expected. A member of the audience asked whether the drug crosses the blood–brain barrier, but this has not yet been assessed.

Exciting new data on MK-0518 were presented by Martin Markowitz (The Rockefeller University, Aaron Diamond AIDS Research Center, New York, NY, USA) from a pivotal study in antiretroviral-naive subjects [22]. The study design is shown in Figure 9. Considering that efavirenz and the combination tenofovir/emtricitabine (Truvada, Gilead Sciences, Inc., Foster City, CA, USA) have proved to be both safe and highly effective in treatment-naive patients, it is quite a gamble to compare a new drug with these. Thus, it was reassuring to see that MK-0518 performed well, especially since the 10-day monotherapy lead-in could jeopardize the outcome if resistance were to emerge (Fig. 10). The enrolled subjects had a mean age of 36 years, 80% were male, 69% were non-white, and 34% had AIDS. Not only did MK-0518 outperform efavirenz at each dose tested, but it also appeared to result in a greater proportion of patients with undetectable virus at an earlier time point (Fig. 11).

The tolerability profile of MK-0518 was at least as good as that for efavirenz (Table 6), if not better, and so the longer-term follow-up of this study will be very interesting and could potentially lead to a new option for patients who are naïve to antiviral therapy.
GS-9137
GS-9137 is the potent HIV integrase inhibitor from Gilead, which, at a dose of GS-9137/ritonavir 50 mg/100 mg, resulted in a >2.0 log_{10} decrease in HIV RNA in a 10-day monotherapy study recently published by Edwin De Jesús (Orlando Immunology Center, Orlando, FL, USA) and co-workers [23]. At this conference, two pharmacokinetic interaction studies conducted by Brian Kearney (Gilead Sciences Inc.) and his group assessed whether zidovudine or tenofovir/emtricitabine demonstrated meaningful interactions with this drug [24,25]. Although GS-9137 is primarily cleared by the cytochromes in the liver, it is also, like zidovudine, excreted renally through glucuronidation, indicating a potential for drug–drug interactions. In a standard crossover design, 28 subjects were given the highest planned dose of GS-9137/ritonavir (200 mg/100 mg once daily) concomitantly with zidovudine or tenofovir/emtricitabine (Fig. 12). No clinically significant alteration of either drug level was apparent, co-administration was safe and well tolerated, and the half-life of this dose of GS-9137 was 9.1 h, supporting once-daily dosing. In a similarly designed study, a further 26 subjects were enrolled to establish whether GS-9137 at the same dose can be administered concurrently with tenofovir/emtricitabine (Fig. 13). No grade 3 or 4 events, and no meaningful interactions were noted during the 27-day dosing period, suggesting that these agents can be given together with impunity.

Protease inhibitors
PL-100
Those who went to the late-breaker presentations on new drug development were rewarded with news of an exciting new PI – PL-100 [26] (Serge Dandache, Ambrilia Biopharma,
Inc., Verdun, QC, Canada). Developed by Ambrilia Biopharma Inc., in conjunction with Mark Wainberg (McGill University, Montreal, QC, Canada), this agent was recently acquired by Merck Pharmaceuticals. PL-100 is a potent orally bioavailable PI with a simple synthesis process of only five stages. The precursor, PPL-100, is converted into PL-100, which has a half-life of approximately 35 h and does not require ritonavir boosting. In vitro selection studies produced viruses with reduced sensitivity to PL-100 (mutations selected were novel and included K45R, M46I, T80I, and P81S), and

Figure 12. Pharmacokinetic interaction studies assessing concomitant administration of the highest planned dose of GS-9137/ritonavir (200 mg/100 mg once daily) and zidovudine. Plasma concentration–time profiles are shown.

- Data are mean ± standard deviation (n=24)
- No clinically relevant drug–drug interaction
at least four were required to generate a 10.8-fold reduction in drug sensitivity. There appears to be no cross-resistance with currently licensed PIs, and the T80I mutation results in hypersensitivity to saquinavir and nelfinavir. A Phase I study was undertaken in healthy volunteers, using doses of 300, 600, 1200, and 2400 mg once daily, either fasted or with a light meal. Although food reduced the $C_{\text{max}}$, it had no effect on the AUC. No significant toxicity was seen (no grade 3 or 4 events) and the compound is now moving forward to clinical development.

**Figure 13.** Pharmacokinetic interaction studies assessing concomitant administration of the highest planned dose of GS-9137/ritonavir (200 mg/100 mg once daily) and tenofovir/emtricitabine. Plasma concentration–time profiles are shown.
The second PI heading towards clinical use is brecanavir. Data presented by Charles Craig (GlaxoSmithKline, Stevenage, UK) discussed the susceptibility in vitro of a panel of 105 “worst case scenario” viruses from Monogram Biosciences, Inc. (South San Francisco, CA, USA), along with other viruses with single, double, or triple PI resistance-associated mutations at positions 32, 33, 46, 47, 50, 54, 82, 84, and 90 [27]. Throughout this panel, brecanavir retained activity at sub-nanomolar levels (median IC₅₀ 300 pM), suggesting that increased numbers of resistance mutations will be required for the drug to fail. Amino acid substitutions at residues 32, 47, and 50 were associated with the greatest reduction in sensitivity (six-fold).

Data from three multiple-dose studies on the interactions of brecanavir (doses of 300 mg or 600 mg with 100 mg ritonavir boosting) with a variety of PIs were shown by Mark Shelton (GlaxoSmithKline) [28]. In the first study with brecanavir 300 mg and lopinavir/ritonavir, no significant changes in the levels of either agent were seen, and no unexpected adverse events were reported. In the second, with brecanavir 300 mg and atazanavir/ritonavir 300 mg/100 mg, both agents showed increases in Cₘₘₙ (38% for brecanavir and 48% for atazanavir) and AUC (41% for brecanavir and 21% for atazanavir). Some bilirubin changes were noted with atazanavir that probably resulted from the increased drug levels.

The final study, with tipranavir/ritonavir 500 mg/200 mg and brecanavir 600 mg, was discontinued prematurely as seven out of 12 healthy volunteer subjects developed elevated alanine aminotransferase (ALT) levels (grade 1 [n=3], grade 2 [n=2], grade 3 [n=2]); this was believed to indicate a high risk of poor drug–drug interactions. All changes in the volunteers returned to normal following drug cessation. In conclusion, from a pharmacokinetic perspective, brecanavir seems best partnered with lopinavir/ritonavir (Kaletra, Abbott Laboratories, Abbott Park, IL, USA) and should not be used with tipranavir.

**Darunavir**

Among the PIs, the largest body of new data was for darunavir, the next anti-HIV drug to be licensed in many countries. Fitting that most of the results came from the pivotal POWER 1 and 2 trials, augmented by POWER 3; all but one of the presentations were posters and several were combined analyses.

Starting with clinical pharmacology, Vanitha Sekar (Tibotec, Inc.) and co-workers presented an overview of all studies that have led to the recommended dose of darunavir/ritonavir of 600 mg/100 mg twice daily [29]. In healthy volunteers, ritonavir boosting increased the absolute oral bioavailability of darunavir from 37% to 82%, and systemic darunavir exposure by approximately 14-fold. Administering a higher dose of 200 mg of ritonavir did not increase darunavir levels to any greater extent. Darunavir is 95% plasma protein-bound, mostly with alpha-1-acid glycoprotein, and is metabolized almost exclusively by CYP3A4. A mass balance study showed that darunavir was excreted mainly in feces (80%) and to a lesser extent in urine (12%), with a terminal half-life of 15 h. Although darunavir exposure was approximately 30% lower under fasted than fed conditions, it was unaffected by meal type (Fig. 14).

Tolerability data for the three studies combined were compared with data of those who received the comparator PIs (CPIs) [30] (José Valdez Madruga, Centro de Referência e Treinamento DST/AIDS, Mariana-São Paulo, Brazil). The number of patient-years of exposure was greater in the darunavir/ritonavir group than the CPI group (310 vs. 75 person-years), due to the longer mean duration of treatment and the larger number of patients treated in this group. Eleven percent of the darunavir/ritonavir group and 81% of the CPI group discontinued the drug because of adverse events, and 3% and 67%, respectively, discontinued the drug following virological failure. Darunavir/ritonavir was generally well tolerated and no specific toxicity was associated with the treatment. The incidence of diarrhea was
lower in the darunavir/ritonavir arm than in the CPI arm (16% vs. 28%), as was headache (11% vs. 20%). Approximately 29% of the patients in each treatment group reported grade 3/4 adverse events. Fifteen percent of darunavir/ritonavir-treated patients and 14% of CPI-treated patients reported at least one SAE, the most common being pneumonia and metabolic acidosis; individual SAEs occurred in <1% of patients. Eleven patients died in the darunavir/ritonavir arms. Causes of death were illicit drug overdose, acute and chronic pulmonary emboli, anal cancer, multiple organ failure due to sepsis, bacterial endocarditis, acute respiratory failure, meningoencephalitis, septic shock, chronic cryptosporidial diarrhea, brain edema, and unknown cause; all deaths were considered by the investigator to be unrelated to the study medication. One patient died in the CPI group 2 weeks after follow-up.

An integrated analysis of laboratory abnormalities in the same population was performed by Tony Vangeneugden (Tibotec BVBA) [31]. The incidences of graded and non-graded laboratory abnormalities were generally low and similar in darunavir/ritonavir- and CPI-treated patients. Most were grade 1–2, and discontinuations because of these changes were uncommon (1% of patients in both groups). The commonest grade 3/4 changes in the darunavir/ritonavir group were:

- Decreased white blood cell count (6% vs. 7% in the CPI group).
- Increased triglycerides (9% vs. 7%).
- Increased amylase (7% vs. 5%).
- Increased total cholesterol (5% vs. 2%).

Changes in lipids over the first 12 weeks are shown in Fig. 15 and illustrate low rates of meaningful abnormalities over this short follow-up. Longer periods of therapy will be needed to clarify the comparative status of darunavir in this area.

Finally, a poster abstract by D Dubois (Johnson & Johnson Pharmaceutical Services, Beerse, Belgium) evaluated quality-of-life changes from POWER 1 and 2 with the validated Functional Assessment of HIV Infection (FAHI) score, and demonstrated that clinically significant improvements were overall more frequent in the darunavir arms (20–40% for various scores) compared with the CPI group (15–25%) [32].

Two posters showed data for POWER 3 alone, a follow-up of POWER 1 and 2, in which all subjects received darunavir/ritonavir 600 mg/100 mg from the outset. POWER 3 was a non-randomized open-label trial that enrolled 327 patients; of these 75% were Caucasian, 87% male, the mean entry viral load was 4.62 log₁₀ HIV RNA copies/mL, and the mean CD4⁺ T cell count was 115 cells/mm³. To assess whether there was a relationship between drug exposure and efficacy or safety, trough and peak pharmacokinetic samples were taken at week 4. In addition, the baseline resistance measurement was used to calculate the inhibitory quotient (IQ) of the drug, i.e. how much drug is required to overcome resistance to it [33]. Not surprisingly, it appears from these data that the darunavir IQ is a strong predictor of
virological outcome, and that this is mainly driven by the baseline resistance fold change to the drug and not darunavir exposure levels. With regard to safety, there seemed to be no association of darunavir drug levels with any adverse events. It is comforting that signature toxicity does not seem to be a hallmark of this compound.

The second POWER 3 analysis [34], presented by Jean-Michel Molina (Hospital Saint-Louis, Paris, France), showed results at week 24 to be broadly similar to the first two studies:

- A >1 log₁₀ reduction in HIV RNA in 65% of patients.
- Less than 50 HIV RNA copies/mL reached by 40% of patients.
- A mean reduction in HIV RNA of 1.65 log₁₀.

The most common adverse events were diarrhea (14%), nasopharyngitis (11%), and nausea (10%). Grade 3 or 4 triglyceride, cholesterol, and ALT and aspartate aminotransferase elevations occurred in 6%, 4%, 2%, and 2% of patients, respectively; these rates are similar to those seen in previous studies with darunavir/ritonavir.

Sharon Walmsley (Toronto General Hospital, Toronto, ON, Canada) gave an oral presentation on the week-48 combined analysis of POWER 1 and 2, and compared the results from 241 subjects treated with darunavir with data from 244 patients who received CPI regimens [35]. In the darunavir group, 91 patients had <50 HIV RNA copies/mL, compared with 22 in the CPI group (p<0.001). The breakdown by baseline subgroups clearly demonstrated that use of enfuvirtide in the back group is important for patients with virus that is still sensitive to the drug (Fig. 16).

CD4⁺ T cell responses were good in the darunavir group compared with the CPI group in both POWER 1 (mean increase of 92 vs. 17 cells/mm³; p<0.001) and POWER 2 (mean increase of 102 vs. 19 cells/mm³; p<0.05). Adverse events were similar to those seen earlier, with approximately 20% having some diarrhea and 15% headaches; the majority of adverse events were grades 1 and 2. It should be noted, however, that it is always difficult to assess side effects in this group of late-stage patients since immune reconstitution can produce many constitutional symptoms.

**Conclusion**

Overall, a hopeful set of results were presented at AIDS 2006, putting into perspective the widening gap between those who have access to efficacious drugs and those that do not. However, despite the great advances that have produced new therapies, it remains vital that doctors and healthcare professionals know how to use these treatments wisely to avoid the emergence of resistance, which has been the hallmark of poor clinical practice in the past.
Acknowledgement

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