Short-Course Raltegravir Intensification Does Not Reduce Persistent Low-Level Viremia in Patients with HIV-1 Suppression during Receipt of Combination Antiretroviral Therapy


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Background. Combination antiretroviral therapy suppresses but does not eradicate human immunodeficiency virus type 1 (HIV-1) in infected persons, and low-level viremia can be detected despite years of suppressive antiretroviral therapy. Short-course (28-day) intensification of standard antiretroviral combination therapy is a useful approach to determine whether complete rounds of HIV-1 replication in rapidly cycling cells contribute to persistent viremia. We investigated whether intensification with the integrase inhibitor raltegravir decreases plasma HIV-1 RNA levels in patients receiving suppressive antiretroviral therapy.

Methods. Subjects (n = 10) with long-term HIV-1 suppression receiving combination antiretroviral regimens had their regimens intensified for 4 weeks with raltegravir. Plasma HIV-1 RNA level was determined before, during, and after the 4-week intensification period, using a sensitive assay (limit of detection, 0.2 copies of HIV-1 RNA/mL of plasma). A 4-week intensification course was chosen to investigate potential HIV-1 replication in cells with relatively short (~1–14-day) half-lives.

Results. There was no evidence in any subject of a decline in HIV-1 RNA level during the period of raltegravir intensification or of rebound after discontinuation. Median levels of HIV-1 RNA before (0.17 log₁₀ copies/mL), during (0.04 log₁₀ copies/mL), and after (0.04 log₁₀ copies/mL) raltegravir intensification were not significantly different (P > .1 for all comparisons in parametric analyses). High-performance liquid chromatography and mass spectroscopy experiments confirmed that therapeutic levels of raltegravir were achieved in plasma during intensification.

Conclusions. Intensification of antiretroviral therapy with a potent HIV-1 integrase inhibitor did not decrease persistent viremia in subjects receiving suppressive regimens, indicating that rapidly cycling cells infected with HIV-1 were not present. Eradication of HIV-1 from infected persons will require new therapeutic approaches.

Trial registration. ClinicalTrials.gov identifier: NCT00618371.
active HIV-1 replication, improving the potency and penetration of drugs that block new cycles of replication is essential. In contrast, if new cycles of viral replication are completely suppressed by current therapy and viremia is derived from reservoirs of long-lived chronically infected cells, new strategies are necessary to cure infection.

Introduction of ART results in rapid and profound decreases in plasma viral RNA levels, followed by continued declines in viral load with slower rates of decline. As previously reported, viral decay rates are the result of death and elimination of infected cells with short (1–1.2-day), intermediate (14-day), and prolonged (39-week) half-lives [25–28]. We previously reported a stable level of persistent viremia (essentially infinite half-life) after the third-phase decline in patients receiving suppressive therapy for >3–4 years [3]. These data suggested that all short- and intermediate-lived HIV-1–infected cells had been eliminated after 3–4 years and that persistent viremia was the product of long-lived cells with integrated proviruses.

To investigate whether ongoing cycles of HIV-1 infection continue during suppressive therapy, we conducted a trial of antiretroviral intensification using inhibitors of HIV-1 reverse transcriptase (efavirenz) or protease (atazanavir–ritonavir or lopinavir–ritonavir) [29]. This study demonstrated that use of efavirenz, lopinavir–ritonavir, or atazanavir–ritonavir to intensify therapy did not lower HIV-1 RNA levels, indicating that additional inhibition of either the reverse-transcription or protease-cleavage steps in viral replication does not further inhibit HIV-1 production. Our findings were consistent with the hypothesis that persistent viremia during therapy is the product of long-lived (>14 days) chronically infected cells.

It is possible that unintegrated HIV-1 DNA generated before ART is started may persist for periods after therapy is initiated and that slow integration of proviruses over time may continue to provide a population of cells that will produce HIV-1 and contribute to persistent viremia. The presence of a substantial number of short-lived cells has been suggested, on the basis of analyses of HIV-1 decay kinetics in patients undergoing therapy with the integrase inhibitor raltegravir [30–32]. To investigate whether inhibition of viral DNA integration decreases persistent viremia, we studied the effects of therapy intensification with raltegravir, a potent HIV-1 integrase inhibitor, in 10 chronically infected patients with persistently detectable viremia who were receiving standard combination ART.

**METHODS**

**Study participants.** HIV-infected individuals >18 years of age who were receiving stable, suppressive ART and had a persistent plasma HIV-1 RNA level of >0.6 copies/mL by single-copy assay at screening were enrolled at the University of Pittsburgh Clinical Trials Unit from December 2007 through January 2009. All participants had screening CD4 T cell counts of >200 cells/μL and were not receiving prophylaxis for opportunistic infections. Study participants had had viral RNA levels of <50 copies/mL of plasma for at least 12 months before screening and had no history of exposure to raltegravir, HIV-1 drug resistance, febrile illness within 3 weeks before enrollment, or vaccination within 6 weeks before enrollment. Participants with a significant comorbid illness, such as chronic hepatitis B virus infection, were excluded. Raltegravir was provided by Merck. Sample size was based on prior experience with single-copy assay results and the number of patients with viral suppression who may undergo further suppression during antiretroviral intensification; we estimated with 90% power that, if 10 participants completing the study had no decline in HIV-1 RNA level, the probability of any individual having persistent viremia that would be suppressible by means of raltegravir is <0.21. On the basis of these estimates, a ceiling of 10 participants completing the study was proposed and approved by the National Institutes of Health (NIH) and the University of Pittsburgh Institutional Review Board.

The study (NCT00618371) was approved by the University of Pittsburgh Institutional Review Board (FWA00006790) and by the NIH Office of Human Subjects Protection and was performed under a Food and Drug Administration investigational new drug application held by the University of Pittsburgh. Participants provided written informed consent.

**Intensification and monitoring.** Study participants had samples collected weekly for the single-copy assay for 3 weeks before intensification. On day 0 of intensification, participants underwent phlebotomy and then initiated raltegravir therapy (400 mg orally twice daily). Plasma samples were obtained on days 7, 14, 21, and 29, the last being the morning after the last dose of raltegravir was administered. After intensification, optional additional sampling was performed on days 29, 30, 35, 42, 49, and 58. Samples for the single-copy assay were pellet immediately after phlebotomy, and plasma was frozen at −70°C. Standard immunophenotyping was performed on samples from study days 0, 29, and 58. Routine safety laboratory studies were performed and graded for severity using the Division of AIDS table (December 2004 version) [33].

**Measurement of HIV-1 RNA level.** HIV-1 RNA level was measured by the Amplicor HIV-1 Monitor assay (version 1.5; Roche) and by single-copy assay, as described elsewhere [2]. Briefly, 10 mL of plasma with RCAS virus added as an internal standard was centrifuged at 100,000 g, and the pellet was extracted and subjected to complementary DNA synthesis followed by real-time polymerase chain reaction amplification of a 79–base pair region of HIV-1 Gag or a portion of the RCAS genome. Sample recovery was measured by Replication Competent ALV LTR with a Splice Acceptor amplification and detection, and HIV-1 RNA levels were determined using a stan-
Table 1. Participant Characteristics

<table>
<thead>
<tr>
<th>Participant</th>
<th>Age at entry, years</th>
<th>Sex</th>
<th>Race</th>
<th>Pretherapy viral RNA level, (\log_{10}) copies/mL of plasma</th>
<th>Duration receiving therapy before intensification, years</th>
<th>CD4 cell count at entry, cells/µL</th>
<th>Antiretroviral regimen*</th>
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<tr>
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<tr>
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<td>...</td>
<td>...</td>
<td>5.0</td>
<td>9.0</td>
<td>473.5</td>
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</tbody>
</table>

**NOTE.** 3TC, lamivudine; ABC, abacavir; EFV, efavirenz; F, female; FTC, emtricitabine; LPV-r, lopinavir-ritonavir; M, male; NA, not available; NVP, nevirapine; TDF, tenofovir; ZDV, zidovudine.

*All antiretrovirals were administered at standard prescribed doses.

Dr. standard curve constructed with HIV-1 of known RNA copy number.

**Measurement of plasma raltegravir concentration.** Samples for determination of raltegravir levels were obtained on days 0, 14, and 29. An assay was developed to quantify raltegravir concentrations in human plasma by means of liquid-liquid extraction paired with high-performance liquid chromatography (HPLC) separation and tandem mass spectrometry–mass spectrometry detection. The dynamic range of the assay was 1–3000 ng/mL, with a coefficient of determination \((r^2, \text{mean} \pm \text{standard deviation})\) of 0.9992 ± 0.0002. The mean precision values for calibration standards ranged from 0.6% to 3.0%, and accuracy values were 96.5%–104.3% [34].

**Statistical analysis.** On the basis of our experience with patients receiving suppressive ART, we anticipated that there would be a nonnegligible number of measurements below the limit of quantitation of 0.2 copies/mL, even using the sensitive single-copy assay. Consequently, we planned for several different analyses to evaluate the effect of therapy intensification. First, we considered plasma HIV-1 RNA level as a binary variable \((\leq 0.2 \text{ or } >0.2 \text{ copies/mL})\) and used repeated-measures regression models with generalized estimating equations to determine whether the proportion of samples with \(<1 \text{ copy/mL}\) increased over time, adjusting for the correlation within individuals. Second, we used parametric mixed-regression models with left-censoring limits that enabled us to more fully model the distribution of the HIV-1 RNA data. We utilized an extension of the model used by Hughes [35] for repeated measures, as implemented by Thiébaut and Jacqmin-Gadda [36]. In this model, we assumed that HIV-1 RNA values \((\log_{10} \text{ copies/mL})\) can be described by a normal distribution but incorporated both censoring below the 0.2 copies/mL limit and correlation from repeated measurements using a random-intercept term.

**RESULTS**

**Study participants.** Thirty-one persons were screened for the study, and 13 participants receiving combination ART who had an HIV-1 RNA level of \(>0.6 \text{ copies/mL}\) were enrolled; the remainder had an HIV-1 RNA level of \(\leq 0.6 \text{ copies/mL}\) or met other exclusion criteria. Ten participants completed the study and were analyzed; 3 participants prematurely discontinued the study for personal reasons.

Baseline demographic characteristics of study participants are presented in Table 1; individuals were predominantly male, had high pretherapy HIV-1 RNA levels (median, 5.0 \(\log_{10}\) copies/mL), and had achieved long-term suppression of viremia during ART (median, 9 years) with stable CD4 T cell counts (median on day 0 before intensification, 473.5 cells/µL). All participants were receiving combination ART; 9 of 10 were receiving efavirenz-based therapy, and 1 was receiving a nucleoside reverse-transcriptase inhibitor, a nonnucleoside reverse-transcriptase inhibitor, and lopinavir-ritonavir.

**Raltegravir intensification.** Before intensification, participants underwent sampling weekly for 4 weeks to establish the baseline HIV-1 RNA level; the median level for all 10 participants was 1.5 copies/mL. All 10 participants had viral RNA levels of \(>1 \text{ copy/mL}\) during the screening visit, but only 9 had viral RNA levels of \(>1 \text{ copy/mL}\) for the majority of time points before intensification. Such variation in HIV-1 RNA level has been noted previously [29]. All participants tolerated the addition of raltegravir without significant adverse events; no laboratory abnormalities exceeding grade 2 and no missed doses
Figure 1. Longitudinal human immunodeficiency virus type 1 (HIV-1) RNA levels in study participants. HIV-1 RNA levels were measured before, during (red), and after raltegravir intensification (400 mg twice daily). Raltegravir intensification did not reduce residual HIV-1 RNA levels in the study participants. White symbols represent measurements below the limit of quantification (0.2 copies/mL).

were reported. Figure 1 shows the longitudinal HIV-1 RNA values for all 10 participants. There was no evidence in any subject of a decline in HIV-1 RNA level during the intensification period or of rebound after discontinuation. There were no significant differences in median HIV-1 RNA levels before (0.17 $\log_{10}$ copies/mL), during (0.04 $\log_{10}$ copies/mL), or after (0.04 $\log_{10}$ copies/mL) intensification ($P > .1$ for all comparisons). As noted above, 1 subject (participant 6) had an HIV-1 RNA level below the limit of quantification for the majority of time points before, during, and after intensification. A secondary analysis excluding data from this subject did not change the results of the comparisons (Figure 2). To summarize, neither the individual participant results nor the aggregate data showed a statistically significant change in median HIV-1 RNA level before, during, or after treatment intensification. These data suggest that, in our study participants, HIV-1 was not derived from the rapidly cycling short-lived cells (half-life, 1–10 days) that are responsible for the majority (>99%) of episodes of viremia observed in untreated individuals.

CD4 T cell counts were determined at entry, on the final day of intensification, and on day 58, 4 weeks after intensification (data not shown). Median CD4 cell counts before, during, and after raltegravir intensification were 580, 623, and 605 cells/µL, respectively; these observed increases were not significantly different from pretherapy counts ($P = .68$ to $P = .86$ for all comparisons).

Plasma raltegravir levels. To investigate whether participants undergoing intensification achieved therapeutic levels of raltegravir, plasma samples obtained before, during, and directly after raltegravir intensification were tested for raltegravir concentrations by HPLC and mass spectroscopy. As shown in Figure 3, preintensification raltegravir levels were below the limit of quantitation. Therapeutic levels of raltegravir were achieved in all subjects by day 14 of intensification (Figure 3).
Figure 2. Human immunodeficiency virus type 1 (HIV-1) RNA levels for all time points for all 9 evaluable participants. Each set of colored symbols represents HIV-1 RNA values obtained for the indicated patient during each phase of the study. White symbols represent measurements below the limit of detection (0.2 copies/mL). Median HIV-1 RNA levels measured before, during, and after intensification are shown by the horizontal bars. The data indicate that raltegravir intensification does not reduce HIV-1 RNA levels. P values for the comparisons between groups are shown above the graph.

The mean raltegravir concentration was 1368.4 nmol/L (range, 55.8–3847 nmol/L), which is 30-fold higher than the reported raltegravir 95% inhibitory concentration ($IC_{95}$) of 33 nmol/L [32]. Raltegravir levels declined after the intensification period but were still present above the $IC_{95}$ at 24 h after discontinuation of raltegravir (Figure 3), consistent with the reported half-life of 7–12 h [37].

DISCUSSION

Currently approved antiretroviral drugs are designed to inhibit new cycles of viral replication but do not block virus production from cells that are already infected. In various combinations, these drugs reduce viral RNA levels >17,000-fold [4]. Despite the potency of these combinations, low-level viremia can be detected in the majority of patients receiving stable suppressive ART for years. Possible sources of this viremia include virus production from long-lived cells containing integrated proviruses [3, 4, 17] or from ongoing cycles of viral replication in sanctuary sites into which some antiretroviral agents penetrate incompletely or not at all [8–13]. To distinguish between these potential sources, we used antiretroviral intensification as a means to determine whether residual viremia is affected. In our first intensification trial, we added potent inhibitors of HIV-1 protease or reverse transcriptase as intensification agents and observed that HIV-1 RNA levels did not decrease during intensification periods of 4–12 weeks [29]. These data strongly suggest that ongoing completes cycles of replication in rapidly cycling cells are not the major source of persistent viremia.

These observations do not exclude the possibility, however unlikely, that unintegrated HIV-1 DNA persists and after integration could serve as a source of virus production despite standard or intensified therapy with HIV-1 reverse transcriptase or protease inhibitors. In this regard, the half-life of unintegrated HIV-1 DNA has been investigated in a number of studies, and the half-life of unintegrated DNA has been estimated to be 4.8 days [38]. Additional support for a similar half-life of unintegrated HIV-1 DNA comes from analysis of viremia decay kinetics after combination therapy with raltegravir [30, 31, 39]. These data suggested that the second phase comprised
cells containing long-lived unintegrated DNA that was blocked from becoming integrated by raltegravir, resulting in the absence of virus production from these cells.

We therefore hypothesized that inhibiting integration by intensification with raltegravir could block the potential contribution of relatively long-lived HIV DNA intermediates to virus production from rapidly cycling cells. The results of the present study, which showed that raltegravir has no effect on residual viremia, do not support this hypothesis. In addition, the results provide additional evidence that new, complete cycles of HIV-1 replication in rapidly cycling cells are not the major mechanism of persistent viremia in patients receiving long-term suppressive ART.

One potential limitation of our study is its small size. We intensified the regimens of 10 participants by adding raltegravir, and no change in HIV-1 RNA level was observed in 9 evaluable participants. It is possible that a larger study would identify a subgroup with residual viremia that is responsive to raltegravir intensification. On the basis of 0 of 9 evaluable participants responding to raltegravir intensification, we estimate that the probability of such a subgroup is <0.21.

The duration of intensification (4 weeks) was chosen to identify changes in viremia arising from virus-producing cells with a short half-life (1–2 days), which comprise >99% of infected cells in untreated HIV-1 infection [25–27]. If all the newly infected cells had a half-life above ~10–14 days, we would be unable to detect a decline in HIV-1 RNA level. This scenario is unlikely, however, because it would require that raltegravir preferentially inhibit new infection of short-lived but not long-lived cells. A larger study with a longer (12-week) intensification period is in progress to investigate the effects of prolonged raltegravir therapy in patients with viral suppression.

The majority of our participants were undergoing reverse transcriptase inhibitor therapy with the combination of tenofovir, emtricitabine, and efavirenz as a single tablet (Atripla), which is the most widely prescribed initial therapy for HIV-1 infection. Our results may not be generalizable to other treatment regimens, although the level of residual viremia observed in the present study is very similar to that observed in patients receiving a variety of suppressive treatment regimens [2–4, 29].

Another potential limitation is that persistent viremia may arise from anatomic sites into which neither the standard regimen nor the intensifying agent penetrates sufficiently to suppress HIV-1 replication completely [18–24]. Ongoing studies, notably the CHARTER study, have investigated drug penetration into sanctuary sites [22, 24]. A gradient of penetration of drug into cerebrospinal fluid has been described for a number of antiretrovirals. Similar studies have not yet been completed with raltegravir, although several ongoing trials will shed new light on this matter. Ongoing HIV-1 replication in such sanctuary sites as the central nervous system, gut-associated lymphoid tissue, and the genitourinary tract are not absolutely excluded by the results of the present study, and differential levels of drug transporter activity, cellular activation, and segregation of immune cells could contribute to differences in antiretroviral drug efficacy in an anatomic-specific fashion. It is notable, however, that participants in the present study achieved suppression for a median of 9 years, or ~1700 HIV generations (generation time, 1.5–2 days). With a mutation rate in the range of $3 \times 10^{-3}$ mutations per site per cycle, even a relatively small population of virus undergoing new cycles of replication would have a reasonable probability of generating drug resistance mutations, resulting in probable drug failure within this period. However, previous genetic analyses of virus in chronically infected individuals who achieve suppressed during ART have not identified the emergence of drug resistance mutations [6, 29].

Although our analyses did not detect changes in HIV-1 RNA levels in this or our previous intensification study, we have detected changes in HIV-1 RNA levels by single-copy assay in individuals undergoing regimen simplification from standard combination therapy to atazanavir-ritonavir monotherapy [40]. Increases in plasma HIV-1 RNA levels occurred 4–12 weeks before viremia became detectable by commercial assays. These data demonstrate that the single-copy assay can detect small changes in residual HIV-1 RNA level in patients who simplify to a less potent regimen.

The absence of a detectable effect of antiretroviral drug intensification on HIV-1 viremia during therapy indicates that neither ongoing, complete cycles of infection nor delayed integration of viral DNA formed before therapy in actively cycling cells is a major source of persistent viremia. Instead, viremia is most likely sustained by long-lived cells that contain integrated proviruses. Antiretroviral drugs that only block new cycles of replication—such as the currently used inhibitors of virion attachment, fusion, reverse transcription, integration, or proteolytic cleavage—are not useful for eliminating long-lived virus-producing cells. Rather, new approaches targeting infected cells directly, such as with immunotoxins [41, 42] or activation- or immune-based therapies, are likely needed to reduce or eliminate important HIV-1 reservoirs.

Acknowledgments

We thank J. Dinoso, R. Siliciano, J. Kovacs, R. Davey, S. Migueles, I. Sereti, R. Leavitt, H. C. Lane, and H. Masur for helpful discussions; Merck for providing raltegravir; and especially the volunteers for participating in this study.

Financial support. National Institute of Allergy and Infectious Diseases, National Institutes of Health (contract HHSN261200800001E to D.M.), and SAIC (contract 20XS190A to J.W.M.). J.W.M. is a research professor of the American Cancer Society with support from the George Kirby Foundation.

Potential conflicts of interest. E.A. is a consultant for Merck, J.W.M. is a consultant for Gilead Sciences, Merck, and Chimerix; has received
grant support from Merck; and owns share options in RFS Pharmaceuticals. All other authors: no conflicts.

References


