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Muhammad Esa Seegulam

*Washington University School of Medicine in St. Louis*

Lee Ratner

*Washington University School of Medicine in St. Louis*

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## Integrase Inhibitors Effective against Human T-Cell Leukemia Virus Type 1<sup>▽</sup>

Muhammad Esa Seegulam<sup>1,2</sup> and Lee Ratner<sup>2\*</sup>

Molecular Cell Biology, Washington University School of Medicine, St. Louis, Missouri 63110,<sup>1</sup> and Division of Molecular Oncology, Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110<sup>2</sup>

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**Drugs targeting the viral enzyme integrase have been in use for several years as part of the treatment regimen for patients with human immunodeficiency virus type 1 (HIV-1), and similar classes of compounds have been shown to inhibit human T-cell leukemia virus type 1 (HTLV-1) integration *in vitro*. The current study shows that the clinically approved HIV-1 integrase inhibitor, raltegravir, as well as the more recent diketo acid derivative, MK-2048, are active inhibitors of HTLV-1 infection *in vitro*. These agents were effective in inhibiting cell-free and cell-to-cell transmission of HTLV-1 in lymphoid and nonlymphoid cells. The drugs also inhibited HTLV-1 immortalization of human peripheral blood mononuclear cells. A novel adaptation of the *Alu* assay for viral integration was used to show that the drugs inhibit viral integration without affecting reverse transcription. These data support the administration of raltegravir and other integrase inhibitors as treatments for patients with HTLV-1-associated diseases.**

The retrovirus human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of the malignancy adult T cell leukemia (ATL) and the neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 is endemic in Southern Japan, the Caribbean, and parts of Africa. There are more than 20 million infected individuals worldwide. The rates of seroprevalence range from 37% in some Japanese communities to <0.01% in parts of Europe (reviewed in reference 17). Although relatively rare in North America, HTLV-1 is commonly found among intravenous drug users (9).

Hallmarks of the retroviral life cycle are the reverse transcription of the viral RNA genome to produce a DNA copy that is then inserted into the host cell DNA, a process catalyzed by the virus-encoded enzyme integrase. The integrated viral DNA is referred to as the provirus. HTLV-1 primarily targets CD4<sup>+</sup> lymphocytes and, to a lesser extent, CD8<sup>+</sup> T lymphocytes *in vivo* (15). Upon infection, the virus enters a long clinical latency stage ranging from 10 to >50 years, and infected individuals become asymptomatic, seropositive carriers. After the latent stage, ca. 5% of infected individuals develop ATL or HAM. The majority of ATL patients die within 2 years of presentation with symptoms.

Although host cell factors are believed to contribute to integration site preference, integrase is the principal viral determinant of integration target site specificity (reviewed in reference 3). Chimeric HIV virus carrying murine leukemia virus (MLV) integrase instead of HIV integrase exhibits target site specificity more consistent with that of MLV (11). Integrase catalyzes the insertion of viral cDNA in three stages: complex assembly, 3' processing, and strand transfer (5, 24). In the

complex assembly stage, integrase forms a stable complex with specific viral long terminal repeat (LTR) sequences, after which the 3' ends of the viral DNA are trimmed, removing a dinucleotide and exposing reactive 3'-OH groups. The 3'-OH groups at the viral DNA ends then covalently attack phosphodiester bonds in the host DNA, cleaving the DNA, and covalently linking the 3' viral cDNA ends to the host cell DNA in the final stage known as the strand transfer reaction.

A class of compounds known as diketo acids and which are active inhibitors of the strand transfer reaction of HIV-1 integrase was approved for use in patients in 2007 (7). Recent structural data indicate that strand transfer inhibitors work by creating stable intermolecular interactions with active-site residues of the integrase protein. The binding of the drug results in the displacement of viral DNA from the integrase active site and the prevention of strand transfer (6; reviewed in reference 16). A derivative of these compounds, the pyrimidine carboxamide MK-0518 (raltegravir), has been approved for use in patients with human immunodeficiency virus (HIV) and has demonstrated fewer drug-related clinical adverse events than currently used antiretroviral therapies (13).

Several antiviral agents are currently used in the treatment of ATL, including zidovudine and interferon alpha 2a, but these drugs have proven to be only partially successful. In a clinical trial undertaken to assess the efficacy of chemotherapy, followed by antiretroviral therapy (interferon alpha, zidovudine, and lamivudine) in patients with acute ATL, the majority of patients had high viral RNA levels at presentation or during the course of therapy and, while some patient viral loads remained steady or decreased, several displayed increasing viral RNA levels (19). Along with the changes in viral RNA levels, some patients had proviral sequences harboring mutations in the reverse transcriptase open reading frame indicating that the characteristic error-prone retrovirus replication process may have been reactivated (19). Examination of viral integration sites in blood samples from these patients revealed

\* Corresponding author. Mailing address: Division of Molecular Oncology, Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, Box 8069, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-8836. Fax: (314) 747-2120. E-mail: lratner@dom.wustl.edu.

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the presence of new integration sites with accompanying disease progression.

Given the structural and functional similarities between the HIV-1 and HTLV-1 integrases, some inhibitors that are active against HIV-1 integrase may also be active against HTLV-1 integrase. Rabaaoui et al. (18) were the first to show that two classes of compounds known to act against HIV-1 integrase—diketo acids (DKA) and styrylquinolines—are also active against HTLV-1 integrase *in vitro* and *ex vivo*. We show here that the DKA-based compounds raltegravir and a more recently synthesized inhibitor, MK-2048, display potent antiviral activity against HTLV-1 *in vitro*. We used cell culture models of both cell-free and cell-to-cell transmission of HTLV-1 in a variety of cell types, including the lymphoid cell line Jurkat and isolated human peripheral blood mononuclear cells (PBMC) to demonstrate that raltegravir and MK-2048 actively prevent HTLV-1 viral integration *in vitro*, suggesting that integrase inhibitors may be a valuable addition to treatment regimens for patients with ATL.

## MATERIALS AND METHODS

**Cell lines and media.** Human PBMC were isolated from normal HTLV-1-negative donors, stimulated with 10  $\mu$ g of phytohemagglutinin (PHA)/ml and 50 U of interleukin-2 (IL-2)/ml for 4 days, and maintained in complete RPMI with 50 U of IL-2/ml. Jurkat and MT2 cell lines were maintained in RPMI supplemented with 10% FetalClone III, 2 mM L-glutamine, and 100  $\mu$ g of penicillin and streptomycin/ml. B5Luc cells are B5 cells (derived from the DBS-FRHL rhesus monkey lung fibroblast line) with a stably integrated luciferase reporter gene under the control of a Tax-inducible LTR promoter. B5Luc and 293T cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FetalClone III, 2 mM L-glutamine, 100  $\mu$ g of penicillin and streptomycin/ml, and 1 mM sodium pyruvate.

**Virus stock generation.** 293T cells were seeded at  $4 \times 10^6$  per 10-cm plate and subsequently transfected with 3  $\mu$ g of packaging plasmid, 3  $\mu$ g of GFP-Luc plasmid (both gifts from D. Derse, National Cancer Institute [4]), and 0.5  $\mu$ g of vesicular stomatitis virus G protein (VSV-G) encoding plasmid using TransIT transfection reagent (Mirus) according to established protocols. The transfection mixture was replaced with 8 ml of complete DMEM 4 h after transfection, and virus-containing supernatants were collected 72 h later. Virus stocks were cleared of transfected cells by low-speed centrifugation, followed by filtration through 0.45- $\mu$ m-pore-size filters.

**Cell-free virus infections using recombinant virus vectors.** 293T cells were seeded at  $2.5 \times 10^5$  per well in 12-well plates, followed by incubation in the presence of various concentrations of inhibitors for 4 h. Inhibitors were dissolved in dimethyl sulfoxide. All assays were performed in triplicate. The medium was removed and replaced with 350  $\mu$ l of virus-containing supernatant, and the cells were incubated at 37°C overnight. Complete medium was added to the required final inhibitor concentrations, and the cells were incubated at 37°C for an additional 72 h; they were then lysed, and Promega luciferase assays were performed.

**Cell-to-cell infections.** MT2 cells ( $10^6$  cells lethally irradiated by exposure to 1 Gy) were added to  $10^7$  Jurkat cells that had been incubated for 4 h in medium containing various concentrations of raltegravir or MK-2048. After an initial 48 h of incubation, 75% of the cells from each flask were removed and replaced with fresh medium. The cells were passaged twice more, each time removing 75% of the total cell population. At 10 days postinfection, the remaining cells were counted by trypan blue staining, and DNA was extracted from  $5 \times 10^6$  cells from each flask for analysis by *Alu* assay for viral integration. To determine whether contaminating MT2 cells were contributing to the measured number of integrations, irradiated MT2 cells and Jurkat cells were counted, maintained in separate flasks, passaged separately as described, mixed on day 10, and immediately counted and lysed for DNA extraction. We called this flask the  $t = 0$  time point, representing an infection time of 0 h.

B5 Luc cells were seeded at  $5 \times 10^5$  cells per well in six-well plates and incubated overnight in the presence of various concentrations of inhibitor. Irradiated MT2 cells ( $5 \times 10^5$  per well) were added in medium containing the corresponding drug concentrations and maintained for 6 days by replacement of half the total volume of medium every 48 h. On day 6, the cells were lysed, and Promega luciferase assays were performed.

***Alu* real-time assay for viral integration.** A standard curve was prepared from MT2 cell DNA extracted by using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions and sequentially diluted in water to stocks containing DNA from 30, 300, and 3,000 MT2 cells per 4- $\mu$ l volume. DNA from Jurkat cells infected by cell-to-cell infections, as outlined above, was extracted by the same method, eluted in 100  $\mu$ l of Tris-EDTA (TE) buffer, and diluted in water to the equivalent of DNA from 20,000 cells per 4- $\mu$ l volume. DNA samples were subjected to two rounds of PCR, in which the first round amplified the DNA sequence between the HTLV-1 LTR and the nearest chromosomal *Alu* element and the second nested PCR amplified the first round PCR products. The assay was done with an HTLV-1 LTR sequence-specific probe. First round PCR was performed using the forward primer HTLV-RTF (ATGC CACGTAAGCGAAACTGCGCCGTCTAGGTAAGTTTAAAGCTCAGG; the 5' linker sequence is underlined and followed by sequence complementary to HTLV-1 LTR) and the *Alu* primers (TCCAGCTACTGGGAGGCTGAGG and GCCTCCCAAAGTGCTGGGATTACAG). Primer concentrations for the first reaction were 375 nM for each of the primers in a total reaction volume of 20  $\mu$ l. An initial 95°C denaturation step of 8 min was followed by 12 cycles of amplification (95°C for 30 s, 58°C for 30 s, and 72°C for 2 min). The second-round real-time PCR was performed with the primers L-T (ATGCCACGTAA GCGAACTGC) specific to the linker sequence and RTR2 (GAAACAAAG ACGTAGAGTTGAGCAAGC) complementary to the HTLV-1 LTR and the HTLV-1 LTR-specific probe (56-FAM-AGCTACCTAGACTCAGCCGGCT CTCCACGCTTTG-3IABlkFQ) with iQ Supermix reagent (Bio-Rad) according to the manufacturer's instructions. All samples were performed in triplicate with primer concentrations of 350 nM for the probe and 400 nM for L-T and RTR2 in a final volume of 50  $\mu$ l. This reaction was performed with an initial 95°C denaturation step for 5 min, followed by 40 cycles of amplification (95°C for 30 s, 60°C for 1 min, and 72°C for 30 s). Experimental samples were compared to the MT2 DNA-generated standard curve using a value of three copies of integrated HTLV-1 viral DNA per MT2 cell (1). Reactions were also performed in the absence of *Alu* primers, and these values were subtracted from those obtained in the presence of the primers in order to account for false-positive signals due to linear amplification of products in the first reaction.

**Total DNA assay.** A 2.5- $\mu$ l portion of infected cell DNA prepared as described above was subjected to one round of PCR amplifying a sequence in the *tax* region of the genome. The forward and reverse primers were HTLV-1Fwd (AAGAC TGTTTGCCCAACACC) and HTLV-1Rev (TTGCCAGGCTGTAGCGTG), respectively. Reactions were performed in triplicate with iQ SYBR green Supermix 2 $\times$  mix for real-time PCR applications (Bio-Rad) in 25- $\mu$ l volume reactions with final primer concentrations of 0.25  $\mu$ M each. After an initial 10-min denaturation of 95°C, 40 rounds of amplification were performed (95°C for 15 s, 60°C for 30 s, and 60°C for 12 s). The samples were then held at 72°C for 10 s before melting-curve analysis was performed according to established protocols. For a total DNA assay at 24 h postinfection,  $3 \times 10^6$  293T cells were plated in T25 flasks, followed by incubation in the presence or absence of integrase or reverse transcriptase inhibitor for 4 h. Then,  $10^6$  irradiated MT2 cells were added per flask, and infections were allowed to occur for 24 h at 37°C. DNA was extracted by using a Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's instructions and eluted in 100  $\mu$ l of TE buffer. Total DNA assays were performed as described above with 2.5  $\mu$ l of a 1:10 dilution of infected DNA in water and compared to a standard curve generated by performing the assay on dilutions of DNA extracted from MT2 cells. A value of three copies of viral DNA per MT2 cell was used to compare the total viral DNA across the various samples (1).

**Determining linearity of MT2 standard curve.** Jurkat cells were infected for 10 days by cell-cell infection with irradiated MT2 cells in the absence of inhibitors as described above. DNA from  $2 \times 10^4$ ,  $1 \times 10^4$ ,  $4 \times 10^3$ , and  $2 \times 10^2$  infected cells were diluted, where necessary, in carrier DNA (uninfected Jurkat DNA diluted in water) so that the total DNA contributed from the mixture of infected and uninfected DNA would be equivalent to DNA from  $2 \times 10^4$  cells. *Alu* assays were performed on each sample as described above.

**PBMC immortalization assay.** PBMC ( $5 \times 10^6$ ) were maintained in T25 flasks in 10 ml of complete RPMI containing 50 U of IL-2/ml (no PHA) and raltegravir, in specified concentrations, and then incubated at 37°C overnight. The following day,  $10^6$  irradiated MT2 cells were added to the appropriate flasks. The cells were counted at specified time points by trypan blue staining.

**Cell viability assays.** MTT assays were performed according to established protocols. 293T cells ( $2 \times 10^5$ /well) were plated in a 12-well plate and exposed to various concentrations of inhibitor or staurosporine (positive control for cell death) for 72 h at 37°C. MTT assay reagent was added, and cells were incubated at 37°C overnight. The cells were lysed in isopropanol, and the optical density

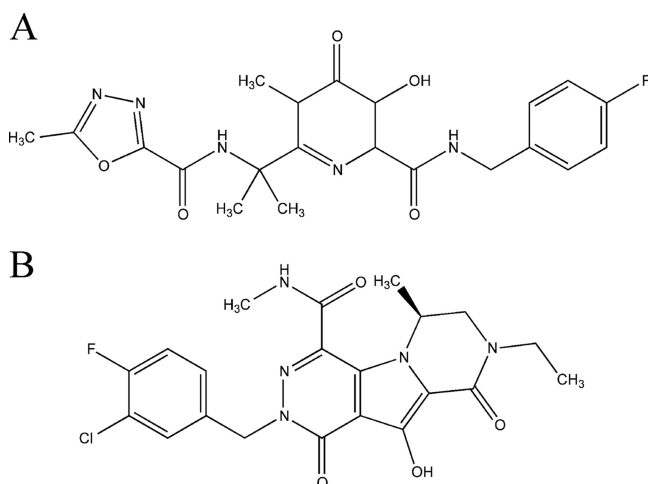


FIG. 1. Chemical structures of the integrase inhibitors MK-0518 (A), also known as raltegravir and Isentress, and MK-2048 (B).

values at 560 nm were measured. CellTiter-Blue (Promega) cell viability assays were performed according to the manufacturer's protocols.

**p19 antigen enzyme-linked immunosorbent assay (ELISA).** Reactions were performed according to standard protocols. A 450- $\mu$ l portion of each supernatant was collected and lysed with 50  $\mu$ l of lysing buffer. Then, 200- $\mu$ l portions of lysed supernatants were placed in duplicate wells of a 96-well plate, and optical densities of each well were read at 450 nm and compared to that of an HTLV-1 antigen standard by using a Tecan Spectrafluor Plus microplate reader.

**Statistical analysis.** The results of all assays were compared by using a two-tailed *t* test. All statistical analyses compared inhibitor-treated cells to cells that were maintained in medium without drug.

## RESULTS

**Determining the efficacy of integrase inhibitors against HTLV-1 infection via cell-free infection with recombinant virus vectors.** We first utilized a cell-free infection assay (4) to gauge the effectiveness of a panel of drugs against HTLV-1 infectivity. Infectious virus was generated from transfected cells as detailed above and used to infect 293T cells exposed to various concentrations of drugs. We verified that the virus produced by this method was inhibited by zidovudine (AZT) with a 50% effective concentration ( $EC_{50}$ ) of 50 nM, in accordance with previously published values (4; data not shown). The experiments were repeated with various concentrations of integrase inhibitors provided by Merck & Co., Inc., including L-870,810, raltegravir (Fig. 1A), and MK-2048 (Fig. 1B). L-870,810 and raltegravir displayed  $EC_{50}$ s near the 50 nM range, and treatment with MK-2048 resulted in an  $EC_{50}$  of 1 nM (Fig. 2 and data not shown). Since raltegravir is already approved for clinical use, we decided to focus the rest of the experiments on this compound, and the more recently developed integrase inhibitor MK-2048. Both compounds showed little or no toxicity against 293T cells, indicating that decreased levels of infection observed in the presence of the inhibitors are not attributable to inhibitor toxicity (Fig. 2C).

**MK-2048 and raltegravir are effective inhibitors of HTLV-1 infection via cell-to-cell transmission.** HTLV-1 infection is primarily achieved through cell-to-cell transmission of virus particles (8). We next tested whether this mode of infection was also subject to inhibition by MK-2048 and raltegravir using

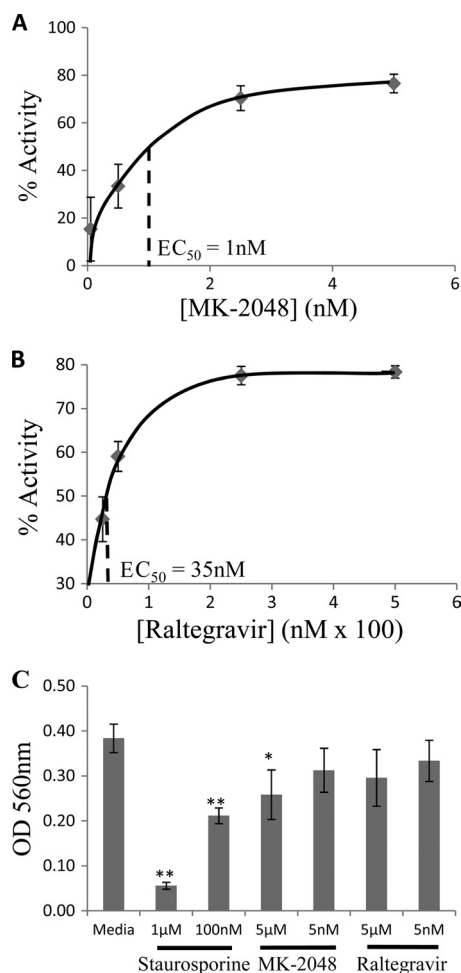


FIG. 2. Raltegravir and MK-2048 significantly inhibit cell-free infection by recombinant HTLV-1 *in vitro* and are not toxic to target cells. The cell-free infection of 293T cells with luciferase-encoding recombinant HTLV-1 in the presence of increasing concentrations of MK-2048 (A) and raltegravir (B) was assessed. (C) MTT assays for cell viability were performed in the presence of each of the two drugs and of the apoptosis-inducing drug staurosporine. Each data point represents the mean of three individual values (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [error bars indicate the standard deviation]).

B5Luc cells, which are B5 cells (primate kidney fibroblasts) containing a stably integrated luciferase gene under the control of an HTLV-1 LTR promoter (20). Infection of this cell line by HTLV-1 activates the Tax-inducible promoter, resulting in luciferase expression. Infection of B5Luc cells by lethally irradiated MT2 cells, an HTLV-1 transformed lymphoid cell line, was significantly impaired in the presence of raltegravir and MK-2048 at concentrations similar to those observed in the cell-free infection assay (Fig. 3).

**Raltegravir and MK-2048 inhibit HTLV-1 integration in target cells.** We examined HTLV-1 integration by adapting the *Alu* assay for viral integration (2) to our HTLV-1 cell-to-cell infection model. Briefly, the assay involves two PCR steps whereby the first step amplifies the DNA sequence between the HTLV-1 LTR and the nearest chromosomal *Alu* element, and the second nested PCR amplifies the first-round PCR products. To our knowledge, this is the first time the *Alu* assay



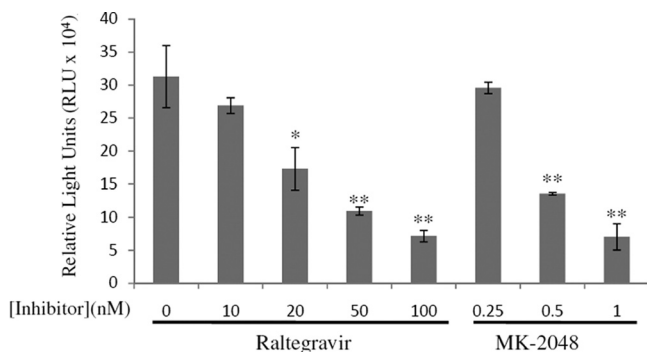
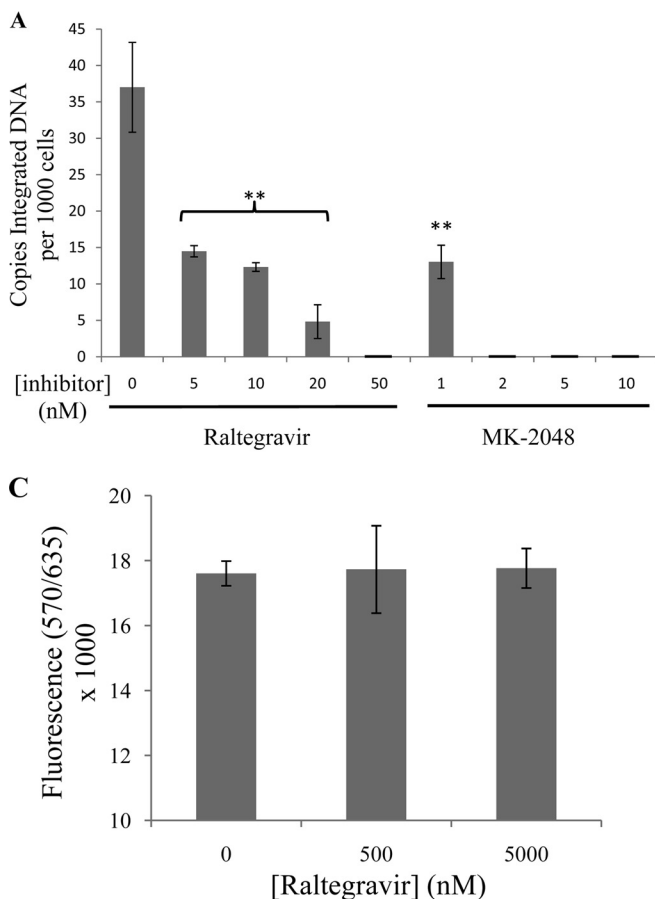


FIG. 3. Raltegravir and MK-2048 significantly inhibit HTLV-1 cell-cell infection of B5 Luc cells. B5Luc cells (fetal rhesus lung cell line clone B5 containing a stably integrated luciferase gene under the control of a Tax-inducible LTR) were coinoculated with lethally irradiated MT2 cells in the presence or absence of the indicated concentrations of MK-2048 and raltegravir. Each bar represents the mean of three values (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [error bars indicate standard deviation]).



for viral integration has been successfully adapted to measure HTLV-1 genomic integration. Total viral DNA was quantified by real-time PCR using primers to the viral tax gene. We tested whether the lymphoid cell line, Jurkat, was affected by the inhibitors in the context of cell-cell viral transmission. As expected, both raltegravir and MK-2048 were effective in reducing the number of integrated viral copies in the target cell line at concentrations within the range identified in the previous cell-free infection, and the B5Luc infection assays (Fig. 4A and B). No integrations were detectable in a sample of MT2 cells after irradiation and cultivation alone for 10 days (data not shown). Raltegravir displayed no toxicity against Jurkat cells, indicating that decreased levels of infection observed in the presence of the inhibitor are not attributable to inhibitor toxicity (Fig. 4C). The standard curve from MT2 cells was reliable in predicting integrations on a linear scale over a 10-fold dilution of infected DNA in carrier DNA (Fig. 4D).

**Raltegravir prevents immortalization of human PBMC in a dose-dependent manner.** MT2 cells produce infectious virus

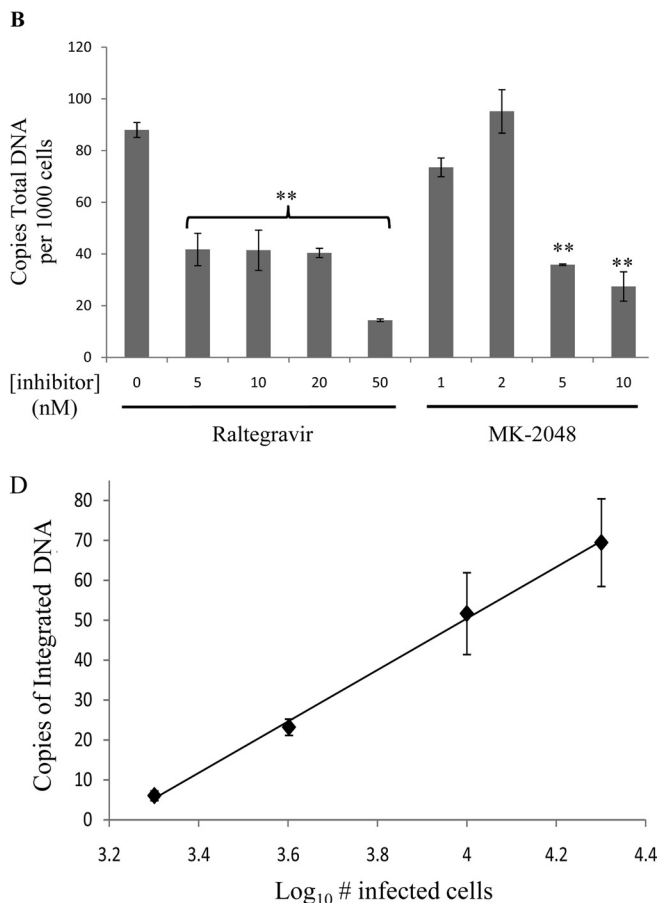


FIG. 4. Raltegravir and MK-2048 reduce the number of viral integration events and total viral DNA levels per cell in cell-cell infections of Jurkat cells and is not toxic to the cells. (A) Jurkat cells were infected by coinocubation with lethally irradiated MT2 cells for 48 h and passed three times over a period of 10 days in the presence or absence of various concentrations of raltegravir and MK-2048. The number of copies of integrated DNA per cell was measured by *Alu* assay (A), while the total DNA per cell was measured by real-time PCR analysis using primers to *tax* (B). Each bar represents the mean of three values. (C) Jurkat cells were either untreated or incubated in medium containing 500 nM or 5  $\mu$ M raltegravir and then passaged for 9 days. A 100- $\mu$ l portion of a 1:5 dilution of each cell category was analyzed by using the CellTiter-Blue cell viability assay (Promega). The data represent the means of triplicate readings with background (medium only) subtracted. (D) Jurkat cells infected by cell-cell contact with irradiated MT2 cells were diluted in carrier DNA and subjected to analysis by *Alu* assay for viral integration. Each data point is the mean of three values (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [error bars indicate the standard deviation]).

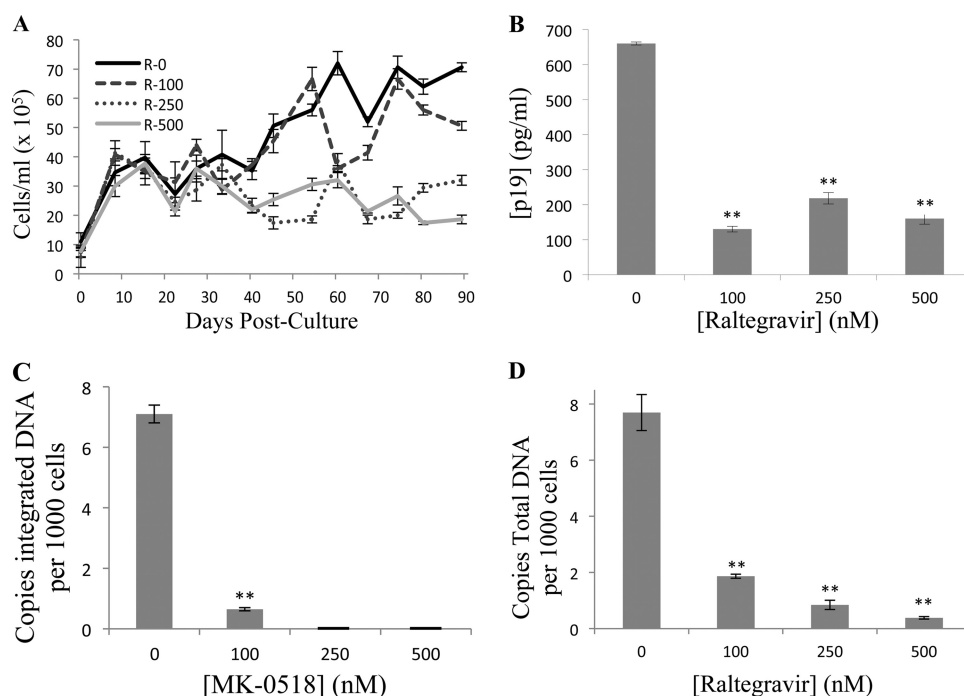


FIG. 5. Raltegravir blocks infection of PBMC by HTLV-1 cell-to-cell transmission from irradiated MT2 cells. PBMC were extracted from whole blood and coincubated with irradiated MT2 cells for 90 days. (A) Cell viability was measured by trypan blue stain resistance at the indicated time points approximately 1 week apart. Each data point is the mean of three cell counts. (B) Viral production was measured from supernatants collected at day 41 after culture and quantified by p19 antigen ELISA (Zeptometrix). Each bar represents the mean of two measurements. The number of integrated proviral copies per cell was measured by *Alu* assay (C), and the total DNA measured by real-time PCR analysis (D) (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [error bars indicate standard deviation]). The reported values are the means of three measurements; R-0, R-100, R-250, and R-500 represent infected PBMC cultured in 0, 100, 250, and 500 nM raltegravir, respectively.

capable of immortalizing uninfected PBMC (21). We tested whether raltegravir would lower the propensity of the PBMC to be immortalized by virus from MT2 cells by coculturing irradiated MT2 cells with PBMC in the presence of various concentrations of raltegravir. Uninfected PBMC proliferated for 4 weeks, after which they steadily decreased in number and were all dead after 9 weeks in culture, whereas the irradiated MT2 cells alone did not proliferate (data not shown). In the absence of inhibitor, PBMC cultured in the presence of MT2 cells proliferated indefinitely ( $>89$  days), and the number of immortalized cells decreased with increasing concentrations of raltegravir (Fig. 5A). HTLV-1 p19 antigen ELISA performed on viral supernatants removed at day 41 after culture indicated that there was significantly less virus production in raltegravir-treated cells compared to the untreated control (Fig. 5B). As expected, cells cultured for 89 days in the presence of inhibitor displayed a significant decrease in the number of copies of integrated DNA and total DNA relative to the untreated control (Fig. 5C and D).

**Antiviral effects of raltegravir and MK-2048 are exhibited at the stage of integration in HTLV-1 cell-cell transmission.** The class of integrase inhibitors described here has been shown to act at the stage of strand transfer in HIV-1 viral integration (7) and in *in vitro* studies of HTLV-1 integration (18). We measured the consequence of this block on the viral replication cycle by measuring the number of integration events in a cell population. However, any antiviral agent that acts at or before the stage of strand transfer would have the same effect on the

number of integrated copies of virus. Treatment of target cells with the nucleoside analog reverse transcriptase inhibitor AZT resulted in a similar decrease in integrated viral DNA in both 293T and Jurkat cells (data not shown). However, because multiple rounds of replication were necessary for these assays, the decrease in integrated DNA was accompanied by a decrease in total viral DNA. If the integrase inhibitors are acting to prevent integration and not at an earlier stage, then we would expect to observe either an increase or no change in total viral DNA at early stages of infection. Therefore, we performed total DNA assays on 293T cells infected with lethally irradiated MT2 cells for 24 h in the presence of MK-2048 and raltegravir, as well as with AZT as a positive control for inhibition of reverse transcription. As expected, total viral DNA levels were significantly diminished by AZT, whereas there was an increase in total viral DNA in raltegravir-treated cells and no significant change in cells treated with MK-2048, indicating that the compounds are effective inhibitors of HTLV-1 viral integration (Fig. 6).

## DISCUSSION

Our data demonstrate that the clinically approved HIV-1 integrase inhibitor raltegravir and the newer DKA-derived drug MK-2048 prevent integration of HTLV-1 *in vitro* for both cell-free and the more physiologically relevant cell-to-cell modes of infection. We utilized a novel application of the *Alu* assay for viral integration to measure HTLV-1 integration

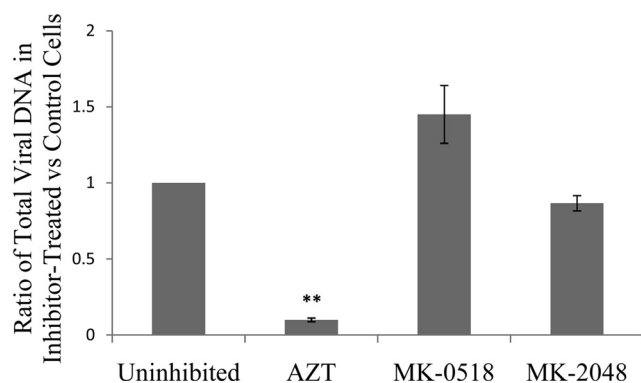


FIG. 6. Raltegravir- and MK-2048-mediated inhibition of HTLV-1 infection *in vitro* occurs at the stage of viral integration. Total DNA assays were performed on MT2 cells infected for 24 h by cell-cell contact with lethally irradiated MT2 cells in the presence of the indicated concentrations of inhibitors (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [error bars indicate the standard deviation, and the values show the means of three measurements]).

events in infected cell populations. The effects of DKA derivatives on HTLV-1 viral integration have not been widely studied. Rabaoui et al. (18) showed that two DKA inhibitors, L-731,988 and L-839,616, prevented HTLV-1 strand transfer with 50% inhibitory concentrations between 50 and 70 nM. These values were obtained from fluorescent PCR quantification of the transfer of synthetic strands of DNA to target sequences in the presence of purified recombinant HTLV-1 integrase in cell-free reactions. In contrast, we used *in vitro* cell culture models to calculate significantly lower  $EC_{50}$ s of 35 and 1 nM for raltegravir and MK-2048, respectively. We also demonstrated that the integrase inhibitor-mediated reduction of the spread of virus by cell-cell transmission, the primary route of HTLV-1 viral propagation, occurred with similar  $EC_{50}$ s for these drugs. As with HIV-1 infection, MK-2048 demonstrated significantly improved potency over the original drug, raltegravir, in the context of HTLV-1 infection (23).

One of the problems associated with studying HTLV-1 infection *in vitro* is the relative difficulty of infecting target cells. Real-time methods to measure total viral DNA are hampered by the presence of contaminating plasmid DNA in infections utilizing cells transfected with the HTLV-1 molecular clone. To circumvent this, we used lethally irradiated cells from the chronically infected HTLV-1 cell line MT2 and, over a period of 10 days, passaged the cells to remove as much background contamination as possible. Over the 10 days, the integrase inhibitors blocked new integrations, reducing the reservoir of integrated virus available for transcription, thus blocking virus spread and decreasing total viral DNA levels. This decrease in total viral DNA in the presence of the integrase inhibitors rendered the antiviral activity of the compounds indistinguishable from the reverse transcriptase inhibitor, AZT, as demonstrated by the *Alu* assay. Since Jurkat cells did not display high levels of infection above background levels up to 36 h postinfection (data not shown), we used 293T cells to show that whereas AZT caused a highly significant decrease in total viral DNA levels 24 h postinfection, the integrase inhibitors either increased or did not change total viral DNA, indicating that

the antiviral effect of the inhibitors is exhibited after reverse transcription.

HIV-1 replicates more efficiently at early stages in infection than HTLV-1 due to its selective targeting to active genes (10, 22). HTLV-1 integration targeted to actively transcribed genomic regions has been associated with more rapid and aggressive disease progression in patients with HAM/TSP (14). ATL patients enrolled in a recent clinical trial displayed high viral RNA levels at presentation or during the course of therapy. Some patients displayed increasing viral RNA levels and proviral sequences harboring mutations in the reverse transcriptase open reading frame, indicating possible viral reactivation (19). A combination of HTLV-1 Tax expression and T cell receptor stimulation have been shown to coactivate viral and cellular gene expression, thereby promoting latent HTLV-1 production (12). The cause-and-effect relationship—whether viral expression is simply a consequence of progression and perhaps a result of lymphocyte activation or whether virus expression and mutation are causing new and more consequential integrations, thus leading to progression—remains unknown.

ATL is a disease with a poor prognosis; the majority of ATL patients die within 2 years of presentation with symptoms and treatment options are currently very limited. The combination of chemotherapy with integrase inhibitors may be a viable treatment option for these patients.

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