Activity of a Novel Combined Antiretroviral Therapy of Gemcitabine and Decitabine in a Mouse Model for HIV-1

Christine L. Clouser, Colleen M. Holtz, Mary Mullett, Daune L. Crankshaw, Jacquie E. Briggs, M. Gerard O’Sullivan, Steven E. Patterson, and Louis M. Mansky

Institute for Molecular Virology, Department of Diagnostic & Biological Sciences, MInCReST Program, School of Dentistry, Center for Drug Design, Department of Microbiology, Medical School, and Masonic Cancer Center Comparative Pathology Shared Resource, University of Minnesota, Minneapolis, Minnesota, USA; Department of Food Science and Nutrition, VA Medical Center, Minneapolis, Minnesota, USA; and Department of Veterinary Population Medicine, College of Veterinary Medicine, St. Paul, Minnesota, USA

The emergence of drug resistance threatens to limit the use of current anti-HIV-1 drugs and highlights the need to expand the number of treatment options available for HIV-1-infected individuals. Our previous studies demonstrated that two clinically approved drugs, decitabine and gemcitabine, potently inhibited HIV-1 replication in cell culture through a mechanism that is distinct from the mechanisms for the drugs currently used to treat HIV-1 infection. We further demonstrated that decitabine inhibited replication of a related retrovirus, murine leukemia virus (MuLV), in vivo using the MuLV-based LP-BM5/murine AIDS (MAIDS) mouse model at doses that were not toxic. Since decitabine and gemcitabine inhibited MuLV and HIV-1 replication with similar potency in cell culture, the current study examined the efficacy and toxicity of the drug combination using the MAIDS model. The data demonstrate that the drug combination inhibited disease progression, as detected by histopathology, viral loads, and spleen weights, at doses lower than those that would be required if the drugs were used individually. The combination of decitabine and gemcitabine exerted antiviral activity at doses that were not toxic. These findings indicate that the combination of decitabine and gemcitabine shows potent antiretroviral activity at nontoxic doses and should be further investigated for clinical relevance.

The efficacies of current anti-HIV-1 therapies are limited by the emergence of drug-resistant virus, which necessitates the development of drugs that exploit novel drug targets. The development of anti-HIV-1 drugs has been relatively rapid and efficient, which has expanded the repertoire of drugs available to HIV-1-infected individuals. The HIV-1 mutation rate represents a potential drug target that has yet to be successfully exploited. Compounds that intentionally increase the mutation rate have been shown to render the virus unable to replicate with enough fidelity to remain infectious, a process termed lethal mutagenesis (4, 9, 12, 18, 27). Although a number of compounds have been shown to lethally mutate HIV-1 and one has even made it to clinical trials, none have yet been efficacious and safe enough to be approved for clinical use. The current model is not a perfect model for HIV/AIDS, we show here that the similarities between the potency of...
decitabine and gemcitabine against HIV and murine leukemia virus (MuLV) in cell culture validate the use of this model for examining the efficacy and toxicity of decitabine and gemcitabine. Here we demonstrate the in vitro and in vivo activities of decitabine and gemcitabine using the MAIDS model. In cell culture, both gemcitabine and decitabine potently inhibit MuLV replication. In vivo, the combination of decitabine and gemcitabine inhibited progression of MAIDS at doses that did not demonstrate toxicity, as determined by changes in body weight and liver histopathology. Additionally, certain indicators of disease progression indicate that the drug combination was more effective than the individual drugs, as would be predicted on the basis of the cell culture data.

MATERIALS AND METHODS

Materials. U373-MAGI-CXCR4<sub>CEM</sub> cells (14, 29) and SC-1/MuLV LP-BM5 cells (6, 15, 20) were obtained through the NIH AIDS Research and Reference Reagent Program. 293T cells were obtained from the American Type Culture Collection (ATCC). C57BL/6 mice were purchased from Jackson Laboratories. Dulbecco modified Eagle medium (DMEM) was purchased from MediaGlow. Gemcitabine and decitabine were obtained from Carbosynth (Berkshire, United Kingdom). The IgM enzyme-linked immunosorbent assay kit was from Assay Designs (Ann Arbor, MI). The plasmids pCR-DEF and pCR-185 were a kind gift from Mauro Magnani (University of Urbino, Urbino, Italy) and have previously been described (5). The plasmid pRES2-EGFP was obtained from Clontech (Mountain View, CA). The plasmids pMIGR1, pJK3, pL-IVS-G, and CMV-Tat were kind gifts from Vineet Kewal Ramani (NCI-Frederick). The plasmid pMIGR1 is an MuLV vector containing an internal ribosome entry site (IRES)-green fluorescent protein (GFP) element (22); pJK3 contains Gag Pol driven off the HIV-1 long terminal repeat (LTR) (1), pL-IVS-G is also driven off the HIV-1 LTR (1), and CMV-Tat allows transcription off the HIV-1 LTR (1).

Cell culture. SC-1/MuLV LP-BM5 cells and 293T cells were maintained in DMEM containing 10% fetal clone 3 (FC3) serum (HyClone, Logan, UT) and penicillin-streptomycin at 37°C in 5% CO<sub>2</sub>. U373-MAGI-CXCR4<sub>CEM</sub> cells expressing the CD4 receptor and the CXCR4 coreceptor were maintained at 37°C in 5% CO<sub>2</sub> in selection medium composed of DMEM with 10% FC3, 1 μg/ml puromycin, 0.1 mg/ml hygromycin, and 0.2 mg/ml neomycin.

Transfection of 293 T cells. For production of MuLV, pMIGR1 (13.9 μg), pJK3 (6.9 μg), pL-IVS-G (5.54 μg), and CMV-Tat (1 μg) were transfected into 293T cells using the calcium phosphate method. Forty-eight hours after transfection, cell culture supernatant was collected and frozen at −80°C. Similar conditions were used to produce envelope-deficient HIV-1, except that 293T cells were transfected with the envelope-deficient HIV containing GFP, as previously described (8), and the CXCR4–HIV-1 envelope.

Drug treatment/flow cytometry. U373-MAGI-CXCR4<sub>CEM</sub> cells (62,000) were plated in 12-well dishes 24 h prior to drug treatment. Twenty-four hours later, gemcitabine, decitabine, or the combination of the two was added to the cultures to achieve the concentrations indicated in Fig. 1, when taking the total volume (500 μl of medium and 500 μl of virus) into account. Two hours after drug treatment, 500 μl of virus (either MuLV or HIV) was added to each culture such that 20 to 30% of cells were infected in the absence of drug. Twenty-four hours later, the medium containing drug (or dimethyl sulfoxide) was removed and replaced with fresh medium that did not contain drug. Twenty-four hours after the medium change, U373-MAGI-CXCR4<sub>CEM</sub> cells were collected and analyzed by flow cytometry to determine the percentage of cells expressing GFP. Infectivity was normalized for each individual experiment by setting the infectivity of the untreated cells to 100 for each experiment and then multiplying the data from the other individual treatments by the number used to convert the no-drug-treated cells to 100. The 50% effective concentrations (EC<sub>50</sub>s) were determined in GraphPad Prism software by plotting the log of drug concentration versus the percent infectivity. The data were fit by nonlinear curve fitting using GraphPad Prism. The EC<sub>50</sub>s and 95% confidence intervals were determined in GraphPad using the log inhibitor-versus-response analysis.

Mice. Female C57BL/6 mice aged 8 to 10 weeks were purchased from Jackson Laboratories (Sacramento, CA) and were housed in standard rodent shoebox cages without a filter top at 22 ± 1°C with a 12-h-light/12-h dark cycle, 60% ± 5% humidity, and 12 air changes/h. Mice were fed lab chow and water ad libitum. The experimental protocol was approved by the U.S. Department of Veterans Affairs Medical Center (Minneapolis, MN) IACUC committee (IACUC protocol number 0803A28341).

Infection of mice with LP-BM5 MuLV. LP-BM5 was produced from confluent SC-1 cells by filtering the cell supernatant through a 0.25-μm pore-size syringe. The filtered viral supernatant was maintained at −80°C until inoculation was performed. C57BL/6 mice were inoculated with two intraperitoneal injections of 0.25 ml of viral supernatant or DMEM spaced 3 days apart.

Drug treatment of LP-BM5 MuLV-infected mice. C57BL/6 mice were randomly divided into the groups shown in Table 2. All mice were treated daily with either gemcitabine, decitabine, both decitabine and gemcitabine, or phosphate-buffered saline (PBS) for 8 weeks beginning at 1 week postinfection. Animals were weighed daily to achieve proper dosing and to detect changes in body mass due to toxicity or infection. Animals did not receive drug treatment 24 h prior to sacrifice.

Sacrifice of animals. Animals were euthanized 8 weeks after drug treatment. Animals were weighed and given 100 mg/kg ketamine plus 10 mg/kg xylazine prior to blood collection from the submandibular vein. After euthanasia, necropsy was performed to assess gross abnormalities in any organs. Spleens were removed, weighed, and sectioned for histopathological analysis as well as for the quantification of proviral levels. Samples of lymph nodes (when detected) and liver were collected. Tissues for histopathological analysis were fixed in 10% neutral buffered formalin, routinely processed into paraffin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin.

Spleen and lymph node histology. Histological examination and scoring of spleen and lymph nodes were performed by an ACVP board-certified veterinary pathologist (M.G.O.) from the University of Minnesota Masonic Cancer Center Comparative Pathology Shared Resource. The severity of lymphoproliferation was scored using a previously published scoring system (7).

Determination of provirus levels from spleen. At 8 weeks posttreatment, defective BM5 (BM5def) DNA content was assayed from genomic DNA isolated from spleen. Total cellular DNA was isolated using a Roche kit as per the manufacturer’s instructions. BM5def DNA was quantified by real-time PCR assay as previously described (5). The amount of BM5def DNA in spleen was calculated by interpolation of the experimentally determined provirus standard plasmid and was normalized to the amount of 18S rRNA. SYBR green master mix (Applied Biosystems) containing genomic DNA (1 μl) and 25 pM primers was used. The primers used to detect BM5def were 5′-CCTTTATTCGACCACCTCCTTTTTTTTTT-3′ and 5′-TG GCGAGGAGGGAAGTT-3′. The primers used to detect 18S rRNA were 5′-GTAACCGGTTGAACCCCATTT- (forward) and 5′-CCATCACAACG GTAGAAG (reverse). Conditions for amplification of BM5def and 18S RNA included an initial heat activation of the polymerase at 95°C for 13 min, followed by 40 cycles of 95°C for 15 s and 62°C for 1 min. Samples were then heated to 72°C for 5 min and 95°C for 1 min, prior to performing a melt curve analysis from 55°C to 95°C. Data were used for analysis only when the standard curve for each primer set yielded an R<sup>2</sup> value of >0.99 with an efficiency of 90 to 110%, single peaks were observed in the melt curves, and template controls gave no detectable amplification. Provirus levels normalized to 18S rRNA levels were determined in triplicate for each set of reactions, and 2 to 3 independent assays were performed with each sample, depending on the amount of tissue available for analysis.
**TABLE 1** Anti-MuLV and anti-HIV-1 activities of decitabine and gemcitabine in cell culture

<table>
<thead>
<tr>
<th>Virus</th>
<th>Drug</th>
<th>EC_{50} (nM)</th>
<th>95% confidence interval</th>
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<tbody>
<tr>
<td>HIV-1</td>
<td>Decitabine</td>
<td>354</td>
<td>208–601</td>
</tr>
<tr>
<td>MuLV</td>
<td>Decitabine</td>
<td>361</td>
<td>127–1,029</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Gemcitabine</td>
<td>16.3</td>
<td>8.7–31</td>
</tr>
<tr>
<td>MuLV</td>
<td>Gemcitabine</td>
<td>1.6</td>
<td>1.1–2.5</td>
</tr>
</tbody>
</table>

a The data shown are the average from three independent experiments.

**RESULTS**

The combination of decitabine and gemcitabine inhibits MuLV infectivity in cell culture. Our previous studies showed that both decitabine and gemcitabine have potent anti-HIV-1 activity in cell culture (8) and that gemcitabine alone has anti-MuLV activity using the MAIDS model (7). To determine if the LP-BM5/MAIDS model was an acceptable model with which to examine the efficacy and toxicity of decitabine and gemcitabine, we first examined the anti-MuLV activities of the drugs alone and in combination in cell culture. To do this, MuLV expressing GFP was pseudotyped with vesicular stomatitis virus glycoprotein G (VSV-G) and then used to infect target cells that had been pretreated with decitabine, gemcitabine, or the combination of the two. Flow cytometry was then used to determine the percentage of infected cells. Figure 1A and B show that both decitabine and gemcitabine potently inhibit MuLV infectivity, with EC_{50} values in the nanomolar range. Figure 1C further demonstrates that the antiviral activity of decitabine and gemcitabine was potentiated when they were used in combination. Although the potentiation was not as great as what is seen with HIV-1 (8), similarities in the potencies of these drugs (Table 1) suggested that the MAIDS model would be a valid model for examining the efficacy and toxicity of these drugs.

The combination of decitabine and gemcitabine inhibits progression of MAIDS as detected by spleen weight and histopathology. The data shown in Fig. 1 demonstrate that decitabine and gemcitabine inhibit MuLV infectivity in cell culture. To determine if decitabine and gemcitabine inhibit MuLV infectivity in vivo, mice were infected with LP-BM5 and treated daily with saline (control), a low dose of decitabine, a low dose of gemcitabine, or low doses of both decitabine and gemcitabine (Table 2). The dose of gemcitabine was chosen on the basis of our previously published dose-response analysis performed in the MAIDS model (7). The dose of decitabine was chosen on the basis of our preliminary studies showing that 0.15 mg/kg decitabine decreased the ratio of spleen weight to body weight, while 0.1 mg/kg had minimal effect on the spleen weight to body weight in mice with MAIDS (data not shown). These low doses that demonstrated minimal anti-MuLV activity were chosen so that any potentiation of the drugs when used in combination would be easily observed, as we have previously described (8).

Infection of mice with LP-BM5 is characterized by lymphopenia, with EC_{50} values in the nanomolar range. Figure 1C further demonstrates that the antiviral activity of decitabine and gemcitabine was potentiated when they were used in combination. Although the potentiation was not as great as what is seen with HIV-1 (8), similarities in the potencies of these drugs (Table 1) suggested that the MAIDS model would be a valid model for examining the efficacy and toxicity of these drugs.

**FIG 1** Decitabine and gemcitabine have anti-MuLV activity in cell culture. MuLV containing GFP was produced from 293T cells and used to infect U373-MAGI-CXCR4_{CEM} cells that were treated with decitabine (A) or gemcitabine (B) at the indicated concentrations. The data represent the average ± SD of three independent experiments. (C) Potentiation of anti-MuLV activity when decitabine (Dec) and gemcitabine (Gem) are used in combination. U373-MAGI-CXCR4_{CEM} cells were treated with the indicated concentrations of decitabine, gemcitabine, or the combination of the two for 2 h prior to infection. The data represent the average ± SD of three independent experiments. Statistical significance was assessed using one-way ANOVA with the Tukey-Kramer posttest. Data with the same letters indicate no statistical difference, while data with different letters indicate a statistical difference (P < 0.05). For example, the results for the decitabine and gemcitabine treatment groups are not statistically different, while the result for the gemcitabine treatment group is significantly different from that for cells treated with both decitabine and gemcitabine. ND, no drug.
liferation, which is detected histologically as a marked expansion of white pulp in the spleen. This expansion also leads to splenomegaly, which is another indicator of disease progression (30). Thus, to examine the extent of disease progression, spleens were removed and weighed immediately after sacrifice. The effect of drug treatment on disease progression was examined by comparing the ratio of spleen weight to body weight. As expected, infected but untreated animals demonstrated splenomegaly, as indicated by the high ratio of spleen weight to body weight compared to the uninfected animals (Fig. 2). Treatment with the individual drugs had no effect on the ratio of spleen weight to body weight, whereas the combination of decitabine and gemcitabine significantly decreased spleen weight compared to the infected but untreated group. Spleens from these animals were further examined for histopathological changes associated with LP-BM5 infection. Lymphoproliferation was scored using a previously described system (7, 15). Briefly, LP-BM5 causes proliferation of lymphoid cells, leading to marked expansion of the spleen’s white pulp (Fig. 3; compare Fig. 3A and B). Therefore, the histopathological changes in the spleen are based on the ratio of white pulp to red pulp, which increases with disease progression. Animals with normal spleen histopathology were rated with an N, while animals with increasing lymphoproliferation were given a score of 1, 2, or 3, indicating mild, moderate, and severe lymphoproliferation, respectively. Table 3 demonstrates the number and percentage of animals identified as having significant lymphoproliferation (as indicated by a score of 2 or 3). None of the uninfected animals had significant lymphoproliferation (Table 3 and Fig. 3A), whereas all spleens from the infected but untreated animals had significant lymphoproliferation (Table 3 and Fig. 3B). Treatment with a low dose of decitabine or gemcitabine alone had little effect on spleen histopathology compared to that for the untreated but infected animals. In contrast, the combination of decitabine and gemcitabine reduced the number of animals identified as having significant lymphoproliferation within the spleen (Table 3 and Fig. 3C).

FIG 2 Ratio of spleen weights to body weights from mice infected with LPBM5 MuLV. Each symbol (circles, squares, and triangles) represents one mouse. The average ± SD within each treatment group is shown. Treatment groups that were statistically significantly different from one another, as determined by one-way ANOVA with the Tukey-Kramer posttest (P < 0.05), are indicated by different letters. For example, the uninfected, untreated group and the group uninfected but treated with gemcitabine are labeled with “a,” indicating that there is no statistically significant difference. Dec, mice treated with 0.1 mg/kg decitabine; Gem, mice treated with 1 mg/kg gemcitabine; Dec + Gem, mice treated with 0.1 mg/kg of decitabine and 1.0 mg/kg gemcitabine. Each data point represents a single measurement of spleen weight and body weight from an individual animal.

FIG 3 Effect of drug treatment on spleen histopathology. Spleen tissue was fixed in 10% neutral buffered formalin, routinely processed into paraffin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin. Each panel shows spleen at the same magnification, and arrows indicate the white pulp. (A) Spleen from an uninfected animal; (B) spleen from an infected but untreated animal showing marked expansion of the white pulp (arrow); (C) spleen from an infected animal treated with 0.1 mg/kg decitabine and 1.0 mg/kg gemcitabine showing marked attenuation of MuLV-induced lymphoproliferation. Bars = 500 μm.

Treatment with the combination of decitabine and gemcitabine decreases MAIDS-associated lesions in the lymph nodes. Infection with LP-BM5 markedly alters lymph node architecture, making lymph node histopathology a useful indicator of disease progression (30). When detected, the lymph nodes were also evaluated for lymphoproliferation, which was scored on the basis of the extent to which the lymph node architecture and content differed from that expected of a normal, uninfected animal (7). Similar to the spleen scoring system, lymph nodes from animals with...
increasing lymphoproliferation were given scores of 1, 2, or 3, indicating mild, moderate, and severe lymphoproliferation, respectively. As expected, lymph nodes were difficult to detect in the uninfected animals, whereas lymph nodes were enlarged and easily detected in infected but untreated animals. In fact, all of the infected but untreated animals demonstrated moderate to severe lymphoproliferation (Table 3). Treatment of animals with low doses of either decitabine or gemcitabine alone had little effect on the percentage of animals with scores of 2 or 3 (Table 3). In contrast, the combination of decitabine and gemcitabine reduced the percentage of animals with lymphoproliferation scores of 2 or 3 compared to animals treated with the drugs individually (Table 3).

**Effects of decitabine and gemcitabine on provirus levels.** The decrease in LP-BM5-mediated spleen lymphoproliferation and splenomegaly suggests that treatment with decitabine and gemcitabine reduced disease progression, which is expected to correlate with a reduction in provirus levels in animals treated with the combination of decitabine and gemcitabine. To examine provirus levels in the infected mice, genomic DNA was isolated from spleen sections and proviral DNA was assessed by real-time quantitative PCR using the 18S rRNA gene for normalization. Consistent with the histopathology data, neither treatment with decitabine alone nor treatment with gemcitabine alone reduced provirus levels compared to those in the infected but untreated animals (Fig. 4). In contrast, the combination of decitabine and gemcitabine significantly reduced provirus levels compared to those in infected but untreated animals (Fig. 4). Although the average provirus level from animals treated with both decitabine and gemcitabine was lower than the average provirus level from gemcitabine-treated mice, the averages were not statistically significantly different between these two treatment groups.

**Toxicity of decitabine and gemcitabine determined by changes in body weight and liver histopathology.** Loss of body weight is often used as an indicator of drug-associated toxicity in mice. Therefore, all mice were weighed daily, and the change in body weight during the course of the study was compared among the treatment groups. None of the treatment groups showed a significant loss of body weight during the study, indicating that the drugs were well tolerated (data not shown). Similarly, no significant changes in liver histopathology were seen in animals treated with the combination of decitabine and gemcitabine (data not shown), further supporting the suggestion that these drugs were well tolerated in these animals.

**DISCUSSION**

The high mutation rate combined with the rapid replication of HIV-1 drives the emergence of drug resistance under suboptimal treatment conditions. Resistance to one drug often confers drug resistance to other drugs in the same drug class, emphasizing the need for new drugs that exploit novel drug targets. Our previous studies demonstrated the anti-HIV-1 activity of two FDA-approved drugs, decitabine and gemcitabine (8), that appear to inhibit HIV-1 replication through lethal mutagenesis, a process where the mutation rate prevents the virus from replicating with enough fidelity to remain viable. In this study, we examined the antiretroviral activity of decitabine and gemcitabine in vivo using the LP-BM5 MuLV model (a murine AIDS model). This is an efficient model with which to examine the in vivo efficacy and toxicity of potential anti-HIV-1 compounds and has been validated with approved anti-HIV-1 drugs (10, 21, 24, 25). The disease induced by LP-BM5, the infectious agent of MAIDS, has striking similarities to that induced by HIV-1, including (i) a dependence on CD4+ T cells for disease initiation, (ii) early onset of hypergammaglobulinemia, (iii) loss of B and T cell responses with disease progression, (iv) splenomegaly, and (v) increased susceptibility to opportunistic infections with disease progression. However, there are notable differences in the diseases induced by MuLV and HIV-1, which emphasizes the need to confirm the anti-MuLV activity in cell culture before moving into the animal model.

The results presented here show that decitabine and gemcitabine inhibited MuLV in cell culture at nanomolar concentrations (Fig. 1 and Table 1), indicating that the LP-BM5/MAIDS model would be a suitable model for examining the efficacies and toxicities of these drugs in vivo. While the potency of the individual drugs was similar when comparing MuLV and HIV-1, the combination of decitabine and gemcitabine did not synergistically inhibit MuLV replication, as determined by the FIC method of eval-

<table>
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<th>Treatment group</th>
<th>spleen</th>
<th>lymph node</th>
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<tbody>
<tr>
<td>Not infected, no drug</td>
<td>0/4 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Not infected, decitabine + gemcitabine</td>
<td>0/4 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>Infected, no drug</td>
<td>4/4 (100)</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>Infected, gemcitabine</td>
<td>4/5 (80)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>Infected, decitabine</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>Infected, decitabine + gemcitabine</td>
<td>2/8 (25)</td>
<td>2/8 (25)</td>
</tr>
</tbody>
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*See Materials and Methods for scoring system. NA, not enough tissue from these animals was obtained for histopathological analysis.

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**TABLE 3 Effect of treatment on spleen histopathology**

**FIG 4 Provirus levels in mice infected with LP-BM5 MuLV.** Genomic DNA was extracted from mouse spleen and quantitative real-time PCR was performed to detect the defective MAIDS provirus. Provirus levels were normalized to the level of the 18S rRNA gene. Each symbol (squares, circles, and triangles) represents one animal. The average ± SD for each treatment group is shown. The ∆∆CT (threshold cycle) method was used to assess gene expression. Statistical significance was assessed by one-way ANOVA with the Tukey-Kramer posttest, with a P value of <0.05 designated significant. Treatment groups labeled with different letters are statistically different from one another, while treatment groups labeled with the same letter are not statistically different from one another.
ating synergy (11, 13). This is in contrast to HIV-1, where the combination of decitabine and gemcitabine synergistically inhibits HIV-1 replication (8). This difference may indicate that one or both drugs use a different mechanism of action to inhibit MuLV compared to the mechanism used to inhibit HIV-1. Similar to the cell culture data, the antiviral activities of decitabine and gemcitabine were potentiated in vivo according to certain indices of disease progression, including the histopathology of spleen and lymph nodes. While the average spleen weight (Fig. 2) was reduced in animals treated with the combination of decitabine and gemcitabine, the provirus levels were not statistically significantly different from those in animals treated with gemcitabine alone. Similarly, the provirus levels were not statistically significantly different when comparing the animals treated with gemcitabine alone to the animals treated with both decitabine and gemcitabine. This lack of potentiation in vivo may be due to the variation within each group as well as the small number of animals per treatment group, or it may indicate a different mechanism of action when comparing the mechanism for these drugs in MuLV infection to those in HIV infection.

Although MuLV and HIV-1 have similar life cycles and mechanisms of replication, they also demonstrate some notable differences that may explain why the combination of decitabine and gemcitabine does not act synergistically to inhibit MuLV replication in vivo. Since each drug exhibited similar (decitabine) or lower (gemcitabine) potencies when comparing activities against MuLV and HIV-1 (Table 1), the difference in the ability of these drugs to work in combination suggests a difference in their antiviral mechanism of action when comparing the mechanism against HIV-1 to that against MuLV.

As previously described (8), decitabine and gemcitabine are proposed to inhibit HIV-1 replication by lethal mutagenesis, a process where the mutation rate is increased to a level that prevents viral replication with enough fidelity for the virus to remain viable. In the proposed model, decitabine is incorporated into HIV-1 DNA during reverse transcription, where it forms noncanonical base pairs, thereby increasing the mutation frequency, which can be detected experimentally as an increase in G-to-C mutations in the provirus. Gemcitabine's anti-HIV-1 activity is attributed to its inhibition of ribonucleotide reductase, which alters deoxyribonucleoside triphosphate (dNTP) pools, where it potentiates decitabine in one of two ways: (i) gemcitabine reduces dCTP levels, thereby increasing incorporation of decitabine, a cytidine analog, or (ii) gemcitabine increases mutation frequency simply by altering dNTP pools, as would be suggested by previous studies that showed that alterations in dNTP pools alone increased HIV-1 mutation frequency (2, 19).

While gemcitabine’s reduction or alteration of dNTP pools is likely to increase the HIV-1 mutation frequency and/or increase incorporation of decitabine, gemcitabine may have a different effect on MuLV replication. For example, the $K_m$ of MuLV reverse transcriptase (RT) for dNTPs is higher than that for HIV RT, indicating that MuLV is less efficient at reverse transcribing viral DNA under conditions of low dNTP levels. Thus, gemcitabine’s reduction in dNTP pools may inhibit MuLV reverse transcription since MuLV is unable to efficiently reverse transcribe under lower dNTP concentrations. In contrast, HIV-1 is able to efficiently reverse transcribe viral DNA in the presence of low dNTP levels, supporting the suggestion that gemcitabine’s anti-HIV activity is likely due to an increase in mutation frequency either through alterations in dNTP pools or by increases in the incorporation of decitabine. Further biochemical studies will be performed to determine if gemcitabine’s anti-MuLV activity is due to inhibition of the enzymatic activity of reverse transcriptase.

While the antiviral mechanism of gemcitabine is likely to differ when comparing MuLV to HIV-1, we do not believe that the antiviral activity of decitabine would differ between the two viruses. Decitabine is a cytidine analog that is incorporated into viral DNA during reverse transcription. Since the EC$_{50}$ of decitabine for MuLV and HIV-1 are similar and gemcitabine does not have an altered sugar moiety (a common feature of nucleoside RT inhibitors that are less potent in MuLV) (3, 28), there is no reason to believe that decitabine would have a different affinity for MuLV RT than HIV-1 RT.

Although both decitabine and gemcitabine are used as anticancer drugs, the concentrations needed to inhibit viral replication are significantly lower (100 to 1,000 times lower) than the concentrations needed to inhibit cell growth in vitro. Our results show that the doses needed to inhibit viral replication in vivo were well tolerated, with no signs of toxicity. The dose of gemcitabine used in cancer chemotherapy in humans is 1,000 mg/m$^2$ once per week for 7 weeks with a 1-week break (26). In contrast, the dose of gemcitabine used in this study, 1 mg/kg, is equivalent to 3 mg/m$^2$ in humans when using the body surface area method to convert mouse dosing to human dosing (23). The standard decitabine dosing is 15 mg/m$^2$ every 8 h for 3 days, with this cycle repeated every 6 weeks for a minimum of 4 cycles. In contrast, the dose of decitabine used in this study, 0.1 mg/kg, is equivalent to 0.3 mg/m$^2$, which, like the dose of gemcitabine, is well below the therapeutic dose used to inhibit cell growth in humans.

One limitation for the use of decitabine and gemcitabine as antiretrovirals is that both are currently administered intravenously. However, Eli Lilly has developed a gemcitabine prodrug that is currently in clinical trials. Although no decitabine prodrugs are currently in clinical trials, it has been demonstrated that with appropriate oral dosing, it is possible to achieve pharmacologically relevant plasma concentrations for cancer treatment (17). Since the dose of decitabine needed to inhibit HIV replication is expected to be lower, it is possible that a prodrug would not be needed, although without a prodrug, it is likely that decitabine would have to be taken multiple times per day, which is not reasonable for anti-HIV drugs.

In summary, the findings presented here indicate that the combination of decitabine and gemcitabine has potent antiretroviral activity in vivo and ex vivo using the LP-BM5/MAIDS model. These findings, along with previous HIV-1 studies in cell culture with gemcitabine and decitabine, suggest that this drug combination has broad antiretroviral activity that is well tolerated at doses that exhibit antiviral activity.

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