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Combination of inhibitors of lymphocyte activation (hydroxyurea, trimidox, and didox) and reverse transcriptase (didanosine) suppresses development of murine retrovirus-induced lymphoproliferative disease

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Abstract

The ribonucleotide reductase inhibitor hydroxyurea (HU) has demonstrated some benefit as a component of drug cocktails for the treatment of HIV-1 infection. However, HU is notoriously myelosuppressive and often administered only as salvage therapy to patients with late-stage disease, potentially exacerbating the bone marrow toxicity of HU. In this report we have compared the antiviral effects of HU and two novel RR inhibitors trimidox (3,4,5-trihydroxybenzamidoxime) and didox (3,4-dihydroxybenzohydroxamic acid) in combination with didanosine (2,3-didoxyinosine; ddI) in the LPBM5 MuLV retrovirus model (murine AIDS). We also evaluated the effects of these drug combinations on the hematopoietic tissues of LPBM5 MuLV-infected animals. The combination of RR inhibitors and ddI was extremely effective (DX > TX > HU) in inhibiting development of retrovirus-induced disease (splenomegaly, hypergammaglobulinemia, activated B-splenocytes and loss of splenic architecture). In addition, relative levels of proviral DNA were significantly lower in combination drug-treated animals compared to infected controls. Evaluation of femur cellularity, numbers of marrow-derived myeloid progenitor cells (CFU-GM and BFU-E) and peripheral blood indices revealed that TX and DX in combination with ddI were well-tolerated. However, treatment with HU and ddI induced moderate myelosuppression. These data demonstrate that RR inhibitors in combination with ddI provide significant protection against retroviral disease in murine AIDS. Moreover, the novel RR inhibitors TX and DX appear to be more effective and less myelosuppressive than HU when administered with ddI in this model.

Keywords: Lymphocyte activation; Reverse transcriptase; Lymphoproliferative disease

1. Introduction

Inhibition of enzymes involved in DNA precursor biosynthesis has gained recognition as a novel strategy for the treatment of human immunodeficiency virus type-1 (HIV-1) infection. Of particular interest is the ability of antimetabolites to potentiate the antiretroviral activity of 2′,3′-dideoxynucleoside (ddN) reverse transcriptase (RT) inhibitors (Balzarini et al., 1991, reviewed in Balzarini, 2000). This property is attributed to the ability of these compounds to reduce levels of endogenous 2′-deoxynucleotide 5′-triphosphates (dNTPs) (Balzarini, 2000). The active antiviral form of ddNs, 2′,3′-dideoxynucleotide-5′-triphosphates (ddNTPs), compete with the corresponding cellular dNTPs for incorporation by HIV-1 RT into the nascent viral DNA chain. Moreover, the anti-HIV-1 activity of ddNs does not depend solely on the absolute levels of the ddNTP generated, but on the ratio of the intracellular concentrations of ddNTP/dNTP (Johns and Gao, 1998). Therefore, depleting

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cellular dNTP levels with antimetabolites provides conditions more favorable for increased incorporation of ddNTP by RT, resulting in enhanced antiviral efficacy of the ddN (Johns and Gao, 1998).

During investigation of this approach most interest has been focused on hydroxyurea (HU), an inhibitor of ribonucleotide reductase (RR) (Lori et al., 1994). RR catalyses the rate limiting step in the de novo dNTP biosynthetic pathway, and therefore, plays a central role in the formation and control of the optimal dNTP levels required for DNA synthesis and repair (Thelander and Reichard, 1979). Inhibition of RR by HU most effectively depletes the purine dNTP pools, particularly dATP (Bianchi et al., 1986; Slabaugh et al., 1991). Therefore, HU has most often been studied in combination with adenosine ddNs. In vitro studies have demonstrated the ability of HU to enhance the anti-HIV-1 activity of didanosine (Gao et al., 1995a, 1995b), 9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA) and 9-[2-(phosphonylmethoxy)propyl]adenine (PMPA) (Palmer et al., 1999). In addition, results of clinical trials have shown that combinations of HU/ddI (Biron et al., 2000), HU/ddI/d4T (Rutschmann et al., 2000), and HU/ddI/indinavir (Lori et al., 1999a) are associated with suppression of HIV-1 replication in vivo.

In spite of these encouraging data, the clinical appeal of HU for HIV-1 therapy may be limited by its propensity to induce myelosuppression, particularly in heavily pretreated patients or in those with advanced disease (Maserati, 1999). In addition, due to its rapid plasma clearance, frequent administration of HU may be required to achieve optimal effectiveness (Villani et al., 1996).

With the goal of obtaining more effective RR inhibition than that associated with HU, a series of hydroxy-substituted benzohydroxamic acid RR inhibitors have been synthesized (van't Riet et al., 1979). Two of these compounds, didox (DX; 3,4-dihydroxybenzohydroxamic acid) and trimidox (TX; 3,4,5-trihydroxybenzohydroxamic acid) have been shown to inhibit the in vitro activity of partially purified RR enzyme 17- and 100-times more effectively than HU respectively (Elford et al., 1979; Szekeres et al., 1994). In addition, DX and TX have exhibited more effective activity than HU in various murine tumor and retrovirus models (Elford et al., 1979; Szekeres et al., 1994; Ussery et al., 1999; Mayhew et al., 2002a, 2002b). Interestingly, despite more potent RR inhibition compared to HU, our previous studies have shown that therapeutic doses of DX and TX are significantly less myelosuppressive than HU when administered to normal mice (Mayhew et al., 1999). In addition, monotherapy with DX and TX effectively inhibited retroviral disease development without the generation of myelosuppression in the mouse LPBM5 MuLV model of retrovirus-induced immunodeficiency (murine AIDS). This was in marked contrast to HU monotherapy, which effectively inhibited disease progression but also induced significant marrow toxicity (Mayhew et al., 2002b). We have also shown that administration of TX, DX, or HU in late-stage murine AIDS (after development of immunodeficiency and lymphoproliferative disease) partially reverses disease (Mayhew et al., 2002a).

The studies reported here were designed to expand on these previous investigations by evaluating the antiretroviral activity and bone marrow toxicity of DX, TX, and HU in combination with ddI in the murine AIDS model.

MAIDS is induced by inoculation with a complex of retroviruses called LPBM5 murine leukemia virus (MuLV), originally recovered from a radiation-induced lymphoma of C57BL/6 mice (Laterjet and Duplan, 1962). Many of the features of MAIDS are similar to those of HIV. Some of these similarities include development of a profound immunodeficiency characterized by deficits in B- and T-lymphocyte function as well as deficiencies in macrophage function. Early stage disease is characterized by polyclonal activation of lymphocytes and proliferation associated with progressive lymphadenopathy and splenomegaly (Chattopadhyay et al., 1991; Jolicoeur, 1991). Advanced stages of the disease are associated with profound immunodeficiency and enhanced susceptibility to opportunistic infections (Doherty et al., 1995) and development of secondary neoplasms, especially B-cell lymphomas (Buller et al., 1987).

Despite the similarities between HIV and MAIDS, there are several important differences. The major cellular targets for LPBM5 MuLV infection are B-lymphocytes, and not CD4 T-cells as in HIV. LPBM5 MuLV is also much simpler in structure than HIV, lacking the tat, rev, and nef regulatory genes (Magnani et al., 1997). Also, the cause of death in MAIDS is believed to be severe pulmonary compromise secondary to lymphoid infiltration and enlarged thoracic lymph nodes (Jolicoeur, 1991).

MAIDS has been widely used as a model to evaluate experimental anti-HIV compounds (Suruga et al., 1998; Fraternale et al., 2002; Magnani et al., 1997; Sumpter et al., 2004). A particular advantage with the use of MAIDS for evaluation of experimental antiretroviral compounds is that, unlike other murine models of retrovirus infection, the disease progresses over a substantial period of time. This permits administration of experimental compounds for several months, allowing for evaluation of therapeutic benefit versus toxicity. Because B-cell proliferation is a major component of MAIDS infection, it provides a good model for the preclinical study of compounds that have been proposed to be beneficial in HIV infection due in part to their cytostatic properties (Lori and Lisziewicz, 1998).

2. Methods and materials

2.1. Mice

Female C57BL/6 mice aged 8–10 weeks were purchased from Charles River/NCI (Bethesda, MD), and were housed in micro-isolator cages in a temperature- and humidity-controlled environment. Mice were fed Purina Lab Chow and water ad libitum. The University of Kentucky IACUC

committee approved the experimental animal protocol used in these studies.

2.2. Retrovirus infection of mice

The chronically LPBM5 MuLV-infected G6 subclone of the SC-1 cell line was kindly donated by Dr. Donald Cohen, Department of Microbiology and Immunology, University of Kentucky. G6 cells were grown to subconfluence at 37 °C in 5% CO_2 in air. Culture supernatant containing retrovirus particles was then harvested, centrifuged at 300 × *g* for 10 min to remove cell debris and 0.22 μm filtered for inoculation. Animals were inoculated by two 0.5 mL intraperitoneal injections 3 days apart.

2.3. Drug treatment

The RR inhibitors HU (100 mg/kg), TX (175 mg/kg), or DX (350 mg/kg) were administered in combination with ddI (100 mg/kg) to retrovirus-infected mice by daily (7 days per week) i.p. injections for 8 weeks, beginning 1 week postinfection. In order to optimize drug efficacy and due to the apparent lack of toxicity after 4 weeks of combination drug treatment, the daily dose of ddI was increased to 250 mg/kg for weeks 4–8. HU was purchased from Sigma (St Louis, MO). TX and DX were provided Molecules for Health, Inc. (Richmond, VA). ddI was kindly provided by BristolMyersSquibb (Wallingford, CT).

2.4. Sampling and sacrifice

Peripheral blood was sampled at weeks 2, 4, 6, and 8 from the tails of control and drug-treated animals by collection in microhematocrit tubes (Curtin Matheson Scientific, Houston, TX). Peripheral blood indices (total white blood cells and hematocrit) were determined as previously described (Mayhew et al., 2002b). Serum was obtained by centrifugation of blood at 1000 × *g* for 10 min at 4 °C and stored at −20 °C until use. After 4 and 8 weeks of drug treatment, six animals in each experimental group were euthanitized by cervical dislocation after CO_2 anesthesia. Spleens were then removed, weighed, and processed for histological evaluation to assess disease progression. To evaluate drug-induced hematological toxicity one femur was removed from each animal and bone marrow was prepared for evaluation of femoral cellularity. In addition, femur and spleen were assayed for content of committed hematopoietic progenitors, colony forming-unit granulocyte macrophage (CFU-GM) and burst forming-unit-erythroid (BFU-E), as previously described (Mayhew et al., 2002b).

2.5. Detection of activated B-lymphocytes

Single cell suspensions were obtained by crushing spleens in ice-cold PBS and passing splenocytes through a 200 μm mesh screen. Mononuclear cells were then enriched by Ficol-histopaque centrifugation (Sigma; 400 × *g* for 30 min at room temperature). The percentage of activated B cells ($CD43^+$) among total B cells ($CD45^+$) was determined by two-color direct immunofluorescence with monoclonal antibodies directed against murine CD43 and CD45 antigens (Pharmingen, San Diego, CA). Briefly, 1×10^6 splenocytes were washed once by centrifugation at 4 °C with cold FACS buffer (PBS + 0.1% sodium azide) and resuspended in 100 μL fresh FACS buffer. Antimouse CD43 (phycoerythrin (PE)-conjugated) and antimouse CD45R (B220) (fluoroscein isothiocyanate (FITC)-conjugated) antibodies (1 μg each) were then added. Appropriate isotype controls (rat IgG_{2a}, PE or FITC conjugated; Pharmingen) were also included for evaluation. Tubes were gently vortexed and incubated on ice for 30 min. Unbound antibody was then removed by washing three times in cold FACS buffer before stained splenocytes were fixed in 0.5% paraformaldehyde. Splenocyte CD43/CD45 expression was determined using a flow cytometer (FACScalibur, Becton Dickson, Franklin Lakes, NJ).

2.6. Spleen histology

Samples of spleens from three animals per experimental group were fixed in 10% neutral buffered formalin. Tissues were then embedded in paraffin and sectioned at 6–8 μm. Sections were processed through graded alcohols to xylene and stained with hematoxylin and eosin before examination by light microscopy.

2.7. Other determinations

Levels of serum IgG in control and drug-treated animals were determined by an ELISA technique as previously reported (Mayhew et al., 2002b). Genomic DNA was prepared from spleens using the DNeasy tissue kit (Qiagen, Valencia, CA). Semi-quantitative PCR reactions to detect relative levels of integrated provirus in splenic DNA were performed as described previously (Mayhew et al., 2002b), except that PCR product was visualized by ethidium bromide staining.

2.8. Statistical methods

The Student's two-tailed *t*-test was used in this study. A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. The combination of RR inhibitors and ddI inhibits development of retrovirus-induced disease in murine AIDS

Progression of murine AIDS is associated with extensive lymphoproliferation, characterized in part by development of splenomegaly (Morse et al., 1992). All infected control

Table 1
Effect of RR inhibitors in combination with ddI on spleen weight, serum IgG concentration and percent of activated B-splenocytes in LPBM5 MuLV-infected mice

	Spleen weight (mg)		Serum IgG (mg/mL)		Activated splenic B cells (% total splenic B cells)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Normal control	103.2 ± 17.5	87.8 ± 9.45	2.0 ± 0.8	4.8 ± 1.2	8.4 ± 0.5	11.8 ± 0.5
Infected control	316.3 ± 24.8	596.7 ± 120.3	16.4 ± 2.2	92.4 ± 27.2	13.8 ± 0.4	50.7 ± 3.6
Infected + ddI	306.7 ± 61.2	667.7 ± 170.8	12.2 ± 2.4	56.8 ± 9.1	16.3 ± 1.2	51.5 ± 4.3
Infected + TX + ddI	148.6 ± 41.9[a]	111.5 ± 31.7[b]	8.4 ± 3.5[a]	14.5 ± 3.1[b]	11.7 ± 1.3	30.17 ± 2.3[a]
Infected + DX + ddI	119.8 ± 39.8[c]	105.67 ± 20.0[b]	4.6 ± 0.9[b]	10.9 ± 3.5[a]	10.1 ± 0.4[b]	15.73 ± 1.3[a]
Infected + HU + ddI	187.3 ± 40.2[b]	242.5 ± 39.2[b]	8.0 ± 2.3[a]	31.1 ± 4.8[a]	12.8 ± 1.1	39.9 ± 0.7[a]

LPBM5 MuLV-infected animals were treated daily for 8 weeks with RR inhibitors in combination with ddI. After 4 and 8 weeks of treatment six mice in each group were sacrificed. Spleen weight, serum IgG concentration and percent activated splenic B-cells were evaluated as described in Section 2. Values are the mean ± S.D.

[a] $p < 0.05$ vs. infected control.
[b] $p < 0.001$ vs. infected control.
[c] $p < 0.0001$ vs. infected control.

mice in this study developed disease characteristic of murine AIDS. Spleens of infected animals weighed approximately three- and six-fold normal at weeks 4 and 8, respectively. The effect of drug treatment on the spleen weights of infected mice is shown in Table 1. Four weeks of treatment with the combination of TX, DX, or HU + ddI (100 mg/kg/d) effectively inhibited development of splenomegaly (DX > TX > HU). By week 8, after the ddI dose had been increased to 250 mg/kg/d, the spleens of animals treated with DX or TX in combination with ddI weighed less than at week 4, and did not weigh significantly more than normal. The inhibition of splenomegaly by these drug treatments was highly significant ($p < 0.001$ versus infected control). Conversely, the spleen weight of animals treated with HU + ddI increased between weeks 4 and 8 and weighed significantly more (approximately 2.5-fold) than normal at week 8 ($p < 0.001$), despite the higher ddI dose. As monotherapy, 4 weeks of treatment with ddI at 100 mg/kg/d was ineffective and increasing the dose to 250 mg/kg/d did not improve efficacy by week 8.

3.2. Inhibition of development of hypergammaglobulinemia

Similar trends were noted when the serum IgG concentration of each group was evaluated. Infected controls had significantly increased IgG levels at weeks 4 and 8 ($p < 0.0001$). Combination drug treatment and ddI monotherapy significantly inhibited increased serum IgG compared to infected controls at weeks 4 and 8 (Table 1). DX + ddI was the most effective treatment after 4 weeks ($p < 0.001$ versus infected controls). Between weeks 4 and 8 (after the ddI dose was raised) the serum IgG levels increased in animals treated with monotherapy ddI and the combination of HU and ddI. Conversely, by week 8 the serum IgG concentration in DX + ddI treated animals was unchanged and was decreased from four- to three-fold normal in animals receiving TX + ddI.

3.3. Inhibition of B-cell activation by RR inhibitors in combination with ddI

LPBM5 MuLV preferentially infects mature B cells resulting in their activation and proliferation (Huang et al., 1991; Kim et al., 1994). Activated B cells were identified using flow cytometric analysis to investigate the expression of the activation marker CD43 on B cells ($CD45^+$ cells). The percent of activated splenic B cells in infected controls was marginally but significantly increased compared to normal at week 4 ($p < 0.01$; Table 1). Drug treatment had little effect on this small increase, except the combination of DX and ddI, in which the percent of $CD43^+/CD45^+$ cells was significantly lower than infected controls ($p < 0.01$). Between weeks 4 and 8, there was a large increase in the percent of activated B cells in infected controls (five-fold). Activated B cells in all drug treatment groups were also increased compared to normal. However, although ddI monotherapy was ineffective, the percent of activated B cells in combination drug-treated animals was significantly lower than in infected controls. DX + ddI was most effective. The difference in activated B cells in this group, compared to infected controls, was highly significant ($p < 0.0001$) and was increased less than two-fold compared to normal. Although TX + ddI (three-fold increase versus normal) was less effective than DX + ddI, mice receiving this treatment had approximately 10% fewer activated B cells than those treated with HU + ddI (four-fold versus normal).

3.4. Inhibition of defective LPBM5 MULV proviral DNA by RR inhibitors in combination with ddI

After 8 weeks of drug treatment, spleens were excised from animals in each group and genomic DNA was extracted. A semi-quantitative PCR technique was then used to evaluate relative levels of BM5-def proviral DNA in each spleen. RR inhibitor treatment was associated with effective reductions in BM5-def DNA (Fig. 1). The most effective treatment was

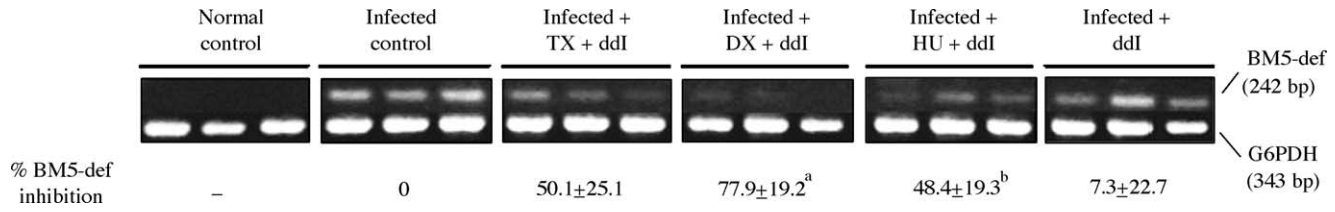

Fig. 1. PCR analysis of BM5def proviral DNA in spleen tissue of C57BL/6 mice infected with LPBM5 MuLV and treated for 8 weeks with RR inhibitors in combination with ddI, or with ddI monotherapy. A 242-bp fragment of the viral p12 *gag* gene was amplified by specific primers from 250 ng of genomic DNA. As an internal control, a 363-bp fragment of the G6PDH gene was amplified. Detection was accomplished by ethidium bromide staining. Percent inhibition of BM5def levels by drug treatment was calculated by dividing the area of BM5def bands by the area of the corresponding G6PDH bands. This figure shows three representative samples from each group of six animals.

the combination of DX and ddI. This treatment reduced relative BM5-def proviral DNA levels by nearly 80% compared to infected controls. TX or HU in combination with ddI inhibited splenic BM5-def levels by approximately 50 and 48%, respectively. Monotherapy ddI was at best, only mildly effective (approximately 7% inhibition). Results from the analysis of proviral DNA after 4 weeks showed similar trends to those observed at 8 weeks, and thus increasing the ddI dose after 4 weeks appeared to have little effect upon proviral DNA levels (data not shown).

3.5. Inhibition of retroviral-induced histological changes by RR inhibitors in combination with ddI

The ability of TX, DX, and HU in combination with ddI to inhibit development of histological changes in the spleens of infected animals was investigated after 8 weeks drug treatment. Compared to normal (Fig. 2A), spleens of infected controls were significantly larger, with complete destruction of follicular architecture, associated with obliteration of the red pulp (Fig. 2B). Approximately 85% of the spleen tissue of in-

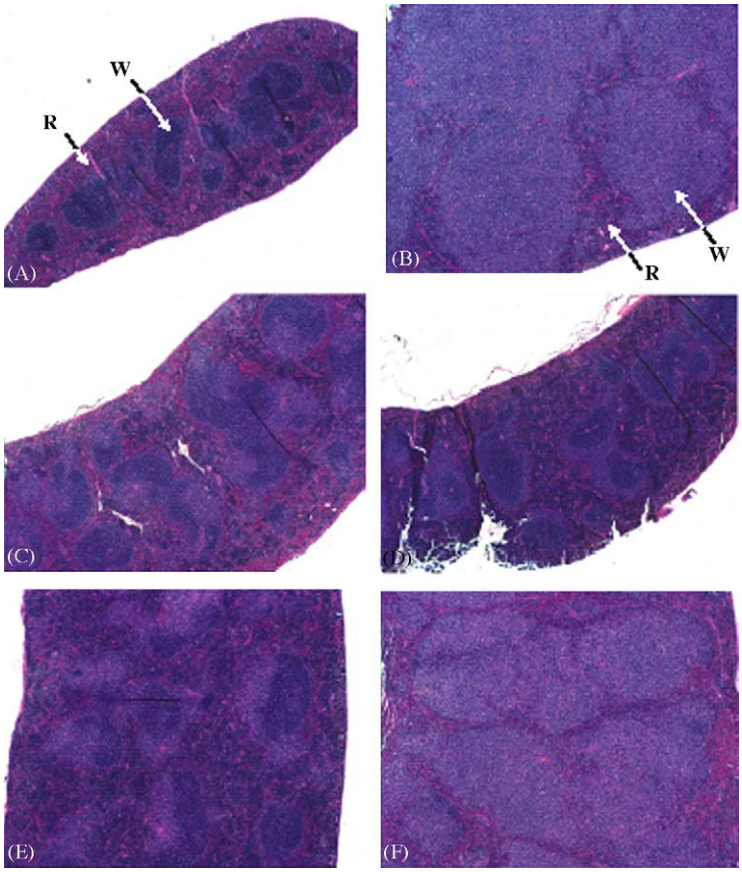

Fig. 2. Gross histological features of spleens from LPBM5-infected mice treated for 8 weeks with RR inhibitors in combination with ddI, or by ddI monotherapy. (A) normal control, (B) infected control, (C) infected + ddI monotherapy, (D) infected + TX + ddI, (E) infected + DX + ddI, and (F) infected + HU + ddI. Spleen sections were stained with hematoxylin–eosin. Note the architecture of the normal spleen with well-defined areas of white (W) and red (R) pulp. In contrast, in addition to the increased size of the spleen, the red pulp is obliterated by massive enlargement of white pulp (lymphoproliferation) in the infected control. Representative images of each group are shown at 4× magnification. This figure shows a representative sample from each group of six animals.

fected controls consisted of a confluent white pulp evidenced by a diffuse proliferation of intermediate to large size lymphoid cells. In marked contrast, drug treatment significantly inhibited destruction of splenic architecture in infected mice (DX + ddI > TX + ddI > HU + ddI). Normal spleens contained approximately 50% white pulp. Spleens of combination TX and ddI treated animals had moderate splenic white pulp expansion (about 60% total spleen area) associated with polymorphous lymphoid cell infiltration with slight compression of the surrounding red pulp (Fig. 2C). Combination DX and ddI almost completely inhibited retrovirus-induced changes in splenic architecture. The follicular architecture and red pulp in the spleens of these animals was completely preserved and apparently normal (Fig. 2D). Conversely, the combination of HU and ddI was less effective. Spleens of these mice contained approximately 70% white pulp marked by follicular enlargement and infiltration of polymorphous lymphoid cells with some confluent areas and significant compression of the red pulp (Fig. 2E). Monotherapy ddI was the least effective treatment. Spleens of ddI treated animals were massively enlarged and associated with marked expansion of white pulp and loss of splenic architecture. Histologically, these spleens appeared similar to infected controls, except that white pulp was not as completely confluent (Fig. 2F).

3.6. Effects of TX, DX, and HU in combination with ddI on peripheral blood indices

The WBC and hematocrit of infected mice treated with TX, DX, or HU in combination with ddI were measured at weeks 2, 4, 6, and 8 and are shown Table 2. Normal control animals had a WBC count ranging between 15,500 and 19,000 cells/μL (Table 2A). The WBC of infected controls was significantly lower than normal at all time points ($p < 0.001$), reaching a nadir at approximately 9700 cells/μL at week 4 and subsequently increasing to approximately 11,400 cells/μL at weeks 6 and 8. Animals receiving ddI monotherapy had WBC counts lower than normal, but not significantly different from infected controls, indicating that ddI treatment had little additional impact on the peripheral WBC count in infected animals. However, addition of TX, DX, or HU to ddI further reduced the WBC slightly (9100–10,600 cells/μL). Although the combination treatments significantly reduced the WBC from normal, TX + ddI was only significantly lower than infected control at week 2. Treatment with DX + ddI and HU + ddI had comparable effects on the WBC count of infected animals throughout the evaluation period and were slightly, but significantly lower than infected controls at week 8 ($p < 0.001$; Table 2A).

The effects of drug treatment on the hematocrit is shown in Table 2B. Four weeks postinfection the hematocrit of infected controls was lower than normal and remained significantly reduced at all time points. Monotherapy with ddI was associated with a reduced hematocrit versus normal controls ($p < 0.05$), but was not significantly lower than infected controls at any time point, indicating that ddI monotherapy had little additional impact on this parameter in retrovirus infected animals. The hematocrit of infected animals treated with TX + ddI and DX + ddI was also slightly lower than normal. Importantly, however, the hematocrit in these animals was not significantly reduced to below that in infected controls. Conversely, treatment of infected animals with the combination of HU and ddI further induced a highly significant

Table 2
Peripheral blood indices of LPBM5 MuLV-infected mice treated with RR inhibitors and ddI

	Weeks of drug treatment			
	2	4	6	8
(A) WBC count (cells/μL)				
Normal control	16247 ± 1099	15460 ± 2010	18987 ± 1304	16058 ± 1864
Infected control	12469 ± 906[b]	9698 ± 1331[b]	11441 ± 1014[b]	11330 ± 665[b]
Infected + TX + ddI	9149 ± 2255[b,c]	10242 ± 1838[b]	10584 ± 1631[b]	9788 ± 1669[b]
Infected + DX + ddI	9325 ± 2068[b,c]	9682 ± 1546[b]	9843 ± 1524[b]	9129 ± 1602[b,c]
Infected + HU + ddI	9218 ± 1369[b,d]	9802 ± 1486[b]	9357 ± 543[b,c]	8938 ± 2271[b,c]
Infected + ddI	11533 ± 1859[b]	10108 ± 1122[b]	10856 ± 1830[b]	10654 ± 1627[b]
(B) Hematocrit (%)				
Normal control	49.00 ± 1.67	48.20 ± 0.45	49.33 ± 1.03	49.83 ± 1.33
Infected control	50.50 ± 1.22	44.50 ± 1.05[a]	46.33 ± 1.03[b]	44.67 ± 1.51[b]
Infected + TX + ddI	49.00 ± 2.45	46.67 ± 1.03[a,c]	45.00 ± 2.97[a]	45.50 ± 2.59[a]
Infected + DX + ddI	47.83 ± 1.94[c]	46.33 ± 1.21[a,c]	43.17 ± 1.17[b]	44.83 ± 2.14[a]
Infected + HU + ddI	45.33 ± 0.82[a,b]	42.83 ± 2.48[a]	38.50 ± 1.97[b,c]	39.67 ± 1.37[b,c]
Infected + ddI	49.33 ± 1.97	46.50 ± 1.38[a,c]	45.50 ± 0.55[b]	44.33 ± 2.94[a]

LPBM5 MuLV-infected mice were treated daily with TX (175 mg/kg/d), DX (350 mg/kg/d), or HU (100 mg/kg/d) in combination with ddI, or with ddI monotherapy. ddI was administered i.p. at 100 mg/kg/d during weeks 0–4, and increased to 250 mg/kg/d during weeks 5–8. At the indicated times peripheral blood was sampled and the (A) WBC and (B) hematocrit were evaluated. Values represent the mean ± S.D. of six animals per group.

[a] $p < 0.05$ vs. normal control.
[b] $p < 0.001$ vs. normal control.
[c] $p < 0.05$ vs. infected control.
[d] $p < 0.001$ vs. infected control.

Table 3
Bone marrow parameters of LPBM5 MuLV-infected mice treated with RR inhibitors and ddI

	Femur cellularity ($\times 10^6$)		CFU-GM per femur		BFU-E per femur	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Normal control	24.1 ± 0.33	24.2 ± 0.18	47718 ± 6494	49852 ± 3646	5543 ± 754	4840 ± 354
Infected control	23.5 ± 0.44	22.2 ± 0.14	43240 ± 8114	32856 ± 2042*	4230 ± 793*	2664 ± 165*
Infected + ddI	22.7 ± 0.31	24.5 ± 0.17	54480 ± 7392	52675 ± 3698*	6129 ± 831*	3675 ± 258*
Infected + TX + ddI	22.4 ± 0.32	19.8 ± 0.36*	54656 ± 7808*	48510 ± 8942*	6048 ± 864*	3690 ± 730*
Infected + DX + ddI	21.5 ± 0.22	21.8 ± 0.56	69660 ± 6966*	63656 ± 16325*	6235 ± 623*	4142 ± 1064*
Infected + HU + ddI	19.1 ± 0.18*	23.0 ± 0.16	47559 ± 4606	38870 ± 2687*	3247 ± 314*	1840 ± 127*

LPBM5 MuLV-infected animals were treated daily for 8 weeks with RR inhibitors in combination with ddI. After 4 and 8 weeks of treatment six mice in each group were sacrificed. Numbers of nucleated cells per femur and numbers of colony-forming units granulocyte-macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E) per femur were determined. Values are the mean ± S.D.

* $p < 0.05$ vs. normal control.

reduction in the hematocrit ($p < 0.001$ versus infected control weeks 6 and 8).

3.7. Effect of TX, DX, and HU in combination with ddI on the bone marrow in murine AIDS

The effects of RR inhibitors in combination with ddI on the bone marrow parameters in LPBM5 MuLV-infected mice is shown in Table 3. Normal femur cellularity was approximately 24×10^6 cells per femur and retroviral infection alone failed to significantly reduce the number of cells per femur (Table 3A). Additionally, none of the drug treatments had a major impact on the femur cellularity. There were, however, slight but significant reductions in HU + ddI and TX + ddI treated animals at weeks 4 and 8, respectively ($p < 0.05$ versus normal). None of the drug treatments induced significant changes in femur cellularity compared to infected controls.

In contrast to the femur cellularity, retroviral infection and drug treatment induced significant changes in the numbers of CFU-GM and BFU-E per femur (Table 3). Compared to normal, infected controls had significantly reduced numbers of CFU-GM per femur at week 8 ($p < 0.0001$). Animals treated with DX in combination with ddI had increased numbers of CFU-GM compared to both normal and infected controls at weeks 4 and 8. Animals treated with TX in combination with ddI had femoral CFU-GM numbers that were increased compared to normal and infected controls at week 4, and between normal and infected control levels at week 8. However, the number of CFU-GM per femur in animals treated with HU + ddI was significantly lower than normal at week 8 ($p < 0.01$). Monotherapy with ddI slightly increased femoral CFU-GM numbers compared to normal controls.

Retroviral infection also significantly depleted femoral BFU-E (Table 3). BFU-E per femur in infected controls were significantly lower than normal at weeks 4 ($p < 0.05$) and 8 ($p < 0.0001$). Compared to normal controls, animals treated with TX or DX in combination with ddI had slightly higher numbers of BFU-E per femur at week 4 and slightly lower numbers at week 8. However, the numbers of BFU-E per femur in these animals were significantly higher than infected controls at both time points ($p < 0.05$). Conversely, HU + ddI reduced femoral BFU-E to levels significantly lower than normal and infected controls at weeks 4 ($p < 0.01$) and 8 ($p < 0.0001$).

4. Discussion

Based upon the results of this study, three important observations can be made. First, RRIs administered in combination with ddI effectively suppress development of murine retrovirus-induced immunodeficiency. Second, there is a good correlation between drug efficacy (as measured by inhibition of splenomegaly/proviral DNA levels) and ability of drugs to inhibit lymphocyte activation (as measured by serum IgG levels and $CD43^+/CD45^+$ Splenocytes). Third, only minimal bone marrow toxicity was observed with TX/ddI, and DX/ddI. This is in contrast to HU, which induced low-grade anemia associated with depletion of committed erythroid progenitor cells from the bone marrow.

Inclusion of HU in HIV-1 drug combination therapy has been demonstrated to have benefit in patients, particularly those in early stage infection (Rutschmann et al., 1998; Lori, 1999; Rodriguez et al., 2000). However, HU use is complicated by its propensity to induce hematopoietic toxicity. This is particularly evident when HU is used as a component in salvage therapy (Maserati, 1999). The recent demonstration that addition of HU to an effective regimen results in treatment failure because of increased toxicity, not virus rebound (Havlir et al., 2001) enhances concern about the use of HU in HIV-patients. Since the use of HU in HIV-1 therapy has a strong theoretical rationale, particularly the ability of HU to potentiate the activity of ddNs (Gao et al., 1994), the search for other compounds with similar properties but reduced toxicity compared to HU is of critical importance.

We have been evaluating the novel RR inhibitors TX and DX as potential antiretroviral agents in the murine AIDS and HIV-SCID HU mouse retrovirus models and have shown in addition to potent antiretroviral activity TX and DX have significantly less bone marrow toxicity than HU (Broud et al., 1998; Ussery et al., 1999; Mayhew et al., 1997, 1999, 2002a, 2002b; Sumpter et al., 2004). Importantly, in the HIV SCID-HU model system we demonstrated that RRI treatment was able to decrease HIV-1 viral titer in vivo (Ussery et al.,

1997), and potentiated the anti-HIV activity of ddI (Ussery et al., 1996). The data presented in this study supports and extends these previous observations by demonstrating the potent antiviral efficacy and lack of bone marrow toxicity of TX and DX in combination with ddI in the murine AIDS model.

Activation and proliferation of CD4 and CD8 cells is an absolute requirement for HIV replication and pathogenesis. Experimental and mathematical models have proposed blocking activation of T-cells to limit target cell availability as a means of controlling HIV replication (Corey, 1995; De Boer and Boucher, 1996; Biron et al., 2000). Combination of a cytostatic agent like HU with an agent that suppresses HIV replication such as ddI has been proposed as a means of inducing maximal antiviral effects and improved immune functions (Lori et al., 1999b). Extending our previous demonstration of anit-retroviral activity of TX and DX in the murine AIDS model (Mayhew et al., 2002b) we demonstrate here that DX and TX have the ability to inhibit B-cell activation and reduce lymphoproliferative disease in retrovirus-infected mice. These data support the concept that inhibition of cellular proliferation by RR inhibitors may be an important contributor to antiretroviral activity and provides reason for optimism that TX and DX may be of benefit to HIV infected patients when combined with ddNs.

Recent demonstration that addition of HU to an effective regimen results in treatment failure because of increased toxicity, not virus rebound (Havlir et al., 2001) enhances the fears about the use of HU in HIV-1 patients. Since the approach of using HU has strong theoretical benefit (increased activity of ddNs; free radical scavenging; blocking NF kappa B upregulation of HIV-1 LTR activity; iron chelation; cytostatic properties) the search for other compounds with similar properties but reduced toxicity compared to HU is important. Our results have clearly shown that TX and DX do possess potent antiviral effects (better than those of HU) with significantly less toxicity (Mayhew et al., 1997, 1999, 2002a, 2002b; Sumpter et al., 2004).

In addition to inhibition of RR and cytostatic activity, TX and DX have several other properties that may be beneficial in HIV-1 infection. For example, TX and DX have been demonstrated to be more potent free radical scavengers than HU (Elford et al., 1980; Rauko et al., 1997; Ussery et al., 1999). This property of TX and DX may be of benefit because it has been reported that HIV-1 patients are under chronic oxidative stress that can contribute to HIV pathogenesis (Pace and Leaf, 1995). Moreover, both TX and DX are effective chelators of iron (Fritzer-Szekeres et al., 1998; Szekeres et al., 1995). The use of iron chelating agents for the treatment of HIV-1 infection has been suggested because excess iron levels may enhance oxidative stress, impair already compromised immune systems and directly promote the growth of microbial agents (Boelaert et al., 1996). Additionally, the increased body iron stores associated with progression towards advanced HIV-1 infection may contribute to shorter survival times (de Monye et al., 1999).

An additional property of DX and TX with perhaps the most potential clinical benefit to HIV patients is the difference observed in the effects upon dNTP pools between these two compounds and HU (Elford and van't Riet, 1985). HU has consistently been shown to only reduce the purine dNTP pools (dATP, and dGTP) in various cell lines (Snyder et al., 1984; Bianchi et al., 1986; Gao et al., 1995a, 1995b). In contrast, TX and DX have been shown to reduce pools of both purine and pyrimidine dNTPs in vitro (Elford et al., 1980; Szekeres et al., 1994), and in vivo (Elford and van't Riet, 1985). The potential ability to influence both pyrimidine and purine dNTP pools may result in a greater array of combination treatment options for treatment of human AIDS. The recent demonstration that TX and DX enhance the activity of the guanine-analog abacavir in the MAIDS model (Sumpter et al., 2004) supports this hypothesis. Further studies are currently under way in our laboratory to investigate the potentiation of other ddNs in vivo.

In conclusion, our group has demonstrated that TX, DX, and HU provide effective treatments in murine AIDS, with TX and DX producing several benefits compared to HU (i.e. greater efficacy with less toxicity alone and in combination with NRTIs). Given the wide range of properties of DX and TX that make them potentially useful in HIV-1 treatment (differential effects upon dNTP pools, antioxidant/free radical scavenging properties, iron chelation, etc.) further studies are needed to determine the mechanisms of action of these drugs in retroviral infection.

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