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# Suppression of retrovirus-induced immunodeficiency disease (murine AIDS) by trimidox and didox Novel ribonucleotide reductase inhibitors with less bone marrow toxicity than hydroxyurea

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**Abstract**

Recently, the use of the ribonucleotide reductase (RR) inhibitor hydroxyurea (HU) in combination with nucleoside analogs has gained attention as a potential strategy for anti-HIV-1 therapy. However, appeal for the long-term use of HU in HIV-1 infection may be limited by its propensity to induce hematopoietic toxicity. We report a comparison of the efficacy and bone marrow toxicity of HU (400 and 200 mg/kg/day) with the novel RR inhibitors and free radical-scavenging compounds didox (DX; 3,4-dihydroxybenzohydroxamic acid; 350 mg/kg/day) and trimidox (TX; 3,4,5-trihydroxybenzamidoxime; 175 mg/kg/day) in the murine AIDS (LPBM5 MuLV) model of retrovirus infection. Infected mice received daily drug treatment for 8 weeks. Efficacy was determined by measuring drug effects on retroviral-induced disease progression (i.e. development of splenomegaly and hypergammaglobulinemia) and by evaluating splenic levels of proviral DNA. Bone marrow toxicity was evaluated by measuring peripheral blood indices (WBC, hematocrit and reticulocyte counts), femoral cellularity and by determining the numbers of hematopoietic progenitor cells (CFU-GM, BFU-E) per femur and spleen. Compared to infected controls receiving no drug treatment, disease progression was significantly suppressed by TX, DX and HU. However, HU was associated with mortality and

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induced significant hematopoietic toxicity in a time- and dose-dependent manner. Conversely, TX and DX effectively inhibited retrovirus-induced disease but did not induce hematopoietic toxicity. These results suggest that due to their reduced hematopoietic toxicity and ability to inhibit disease progression in murine AIDS, TX and DX may offer effective alternatives to HU therapy in HIV-1 infection. 

*Keywords:* Ribonucleotide reductase; Murine AIDS; Bone marrow toxicity; Hydroxyurea; Didox; Trimidox

## 1. Introduction

The importance of developing alternative therapeutic strategies for the treatment of human immunodeficiency virus type-1 (HIV-1) infection has been highlighted by the demonstration of low level viral replication (Dornadula et al., 1999) and generation of multi-drug resistant HIV-1 strains (Shafer et al., 1998) in patients receiving highly active anti-retroviral therapy (HAART). In addition, HAART may be associated with induction of significant metabolic abnormalities (reviewed by Vigouroux et al., 1999). These problems are compounded by complicated drug administration schedules, often resulting in poor compliance, and the high cost of therapy, precluding the use of HAART in the countries hardest-hit by the AIDS crisis. One alternative therapeutic strategy that has received attention is the use of hydroxyurea (HU), an inhibitor of the cellular enzyme ribonucleotide reductase (RR). Initial studies demonstrated the ability of HU to inhibit in vitro HIV-1 proviral DNA synthesis (Lori et al., 1994), and its capacity to potentiate the anti-retroviral activity of several dideoxynucleoside (ddN) analogs (Lori et al., 1994; Meyerhans et al., 1994). Clinical studies have subsequently demonstrated the long-term anti-HIV-1 efficacy of HU in combination with ddI (Biron et al., 2000) and ddI+d4T (Rutschmann et al., 2000) in some patients infected with HIV-1. However, the hematopoietic toxicity commonly associated with HU therapy may limit its overall effectiveness as an anti-HIV-1 agent. Indeed, when HU was evaluated as monotherapy for HIV-1 treatment, there was no change in viral load and patients developed significant hematopoietic cytopenias (Giacca et al., 1996). In patients with advanced disease, in those receiving other anti-retroviral drugs that suppress hematopoiesis (e.g. AZT), or in heavily pre-treated patients, bone marrow suppression associated with HU may be exacerbated (Maserati, 1999).

In an effort to achieve greater inhibition of RR than HU, several more potent RR inhibitors have been developed (Elford and van't Riet, 1985). Two of these compounds, trimidox (TX) and didox (DX), are particularly effective in vitro RR inhibitors, inhibiting RR activity 100- and 17-fold more potently than HU, respectively (Elford and van't Riet, 1985; Szekeres et al., 1994b). TX and DX also have more potent in vivo anti-tumor activity than HU in several mouse tumor models (Elford et al., 1979; Szekeres et al., 1994b). In addition, results from our laboratory and others have also shown that TX and DX have anti-retroviral activity, both alone and in combination with ddI, in the murine AIDS (Mayhew et al., 1997) and HIV-infected HuPBMC SCID (Broud et al., 1998; Ussery et al., 1999) mouse retrovirus models. We have also demonstrated that at equimolar concentrations to HU, TX and DX are less inhibitory to the in vitro growth of normal human and murine hematopoietic progenitor cells and also induce fewer perturbations to in vivo murine hematopoiesis than HU (Mayhew et al., 1999). The structures of HU, DX and TX are shown in Fig. 1.

Murine AIDS has been widely used as a model to evaluate experimental anti-HIV-1 drug strategies (Fraternale et al., 2000; Palamara et al., 1996). Disease is induced by infection with a complex of retroviruses, termed LPBM5 murine leukemia virus (MuLV). Many features of LPBM5 MuLV-induced disease in murine AIDS are similar to those in human AIDS, including development of profound immunodeficiency, abnormal T- and B-lymphocyte function, polyclonal B-cell proliferation, lymphadenopathy, splenomegaly and hypergammaglobulinemia. Advanced disease is associated with enhanced susceptibility to oppor-

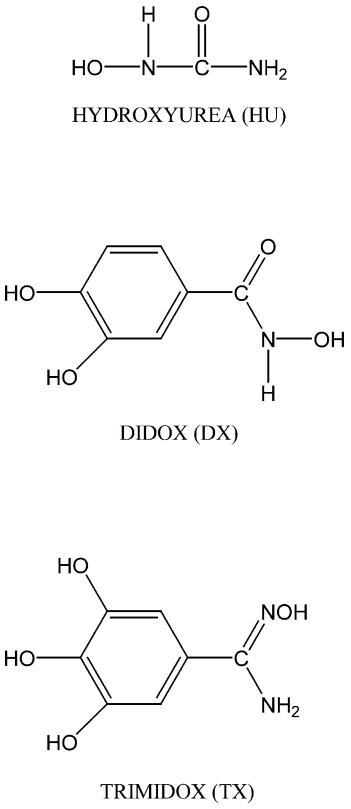


Fig. 1. Structures of the RR inhibitors HU, TX and DX.

tunistic infections and development of secondary neoplasms (Morse et al., 1992; Mosier, 1996). Despite these similarities, there are important differences between LPBM5 MuLV and HIV-1 infection. For example, LPBM5 MuLV is not a lentivirus and is simpler in genetic structure than HIV, lacking the *tat*, *rev* and *nef* regulatory genes (Mosier, 1996). Additionally, the major cellular targets for LPBM5 MuLV infection are B-lymphocytes, not $CD4^+$ cells. However, as infected B-lymphocyte proliferation is a major component of LPBM5 MuLV retrovirus-induced disease, murine AIDS provides a good model for pre-clinical evaluation of compounds like HU that have been proposed to be beneficial in HIV-1 infection due in part to their cytostatic properties (Lori and Lisziewicz, 2000).

Another advantage with the use of murine AIDS for evaluation of experimental anti-retroviral agents, unlike other mouse models of retrovirus infection (e.g. HIV-infected HuPBMC SCID), is that the disease progresses over a substantial period of time (Morse et al., 1992). This permits administration of experimental drugs for several months and evaluations of therapeutic benefit versus toxicity in the context of retroviral infection, more closely resembling the human situation, can be performed.

In view of the limitations of HU, and the potential efficacy of TX and DX in the treatment of human AIDS, we conducted a comparative evaluation of HU, TX and DX as anti-retroviral therapy in murine AIDS. In addition to evaluating anti-retroviral activity, a particular focus of this investigation was to evaluate the propensity of these drugs to induce bone marrow suppression in retrovirus infected mice.

## 2. Materials and methods

### 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 experimental animal protocol used in these studies was approved by the University of Kentucky IACUC committee.

### 2.2. Infection of mice with LPBM5 MuLV

The G6 subclone of chronically LPBM5 MuLV infected SC-1 cells was kindly provided by Dr Donald Cohen, Department of Microbiology and Immunology, University of Kentucky. G6 cells were grown in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MI) and 1% penicillin–streptomycin (Gibco, Grand Island, NY). Virus was prepared for inoculation from sub-confluent G6 cells by removal of the cell supernatant. The cell monolayer was subsequently lysed (by repeated freeze-thaw cycles) to yield intra-cellular virus. This was then combined with the cell supernatant, centrifuged at $300 \times g$ for 10 min and 0.45 μm filtered.

Inoculation was performed by two separate 0.5 ml intraperitoneal (i.p.) injections per mouse, 3 days apart.

### 2.3. Treatment of LPBM5 MuLV infected mice with RR inhibitors

LPBM5 MuLV infected animals were randomly assigned in groups of 12 to receive either no drug treatment (infected controls), HU 400 or 200 mg/kg/day, DX 350 mg/kg/day or TX 175 mg/kg/day. Twelve non-virus infected mice that did not receive drug treatment served as normal controls. DX and TX doses used approximated to those previously demonstrated to have in vivo anti-tumor and -retroviral activity in mice (Broud et al., 1998; Elford and van't Riet, 1985; Elford et al., 1979; Mayhew et al., 1997; Ussery et al., 1999). Using inter-species dose scaling (Freireich et al., 1966), the HU doses used in mice for this study were calculated to correspond to approximately 1000 and 2000 mg/day in a 60 kg human. These doses are similar to the highest dose of HU evaluated as monotherapy (1500 mg/day *q.d.*) for anti-HIV efficacy in humans (Giacca et al., 1996). Drug treatment commenced 7 days after the first virus inoculation and continued daily for 8 weeks. HU, TX and DX were injected i.p. at a volume of 0.5 ml. Drugs were prepared weekly, sterile filtered 0.45 μm, stored at 4 °C and warmed to body temperature before injection. HU was purchased from Sigma. TX and DX were provided by Dr Bart van't Riet, Molecules for Health, Richmond, VA.

### 2.4. Procurement of tissues for analysis for antiviral drug efficacy and hematopoietic toxicity

Every 2 weeks during the evaluation period, mice in each experimental group were bled to evaluate peripheral blood indices (white blood cells, hematocrit and reticulocyte count) and serum IgG levels. Blood was obtained from the tail of each animal by collection in a heparinized microhematocrit tube (Curtin Matheson Scientific, Houston, TX). The white blood cell count (WBC) was performed using a Coulter Counter (model ZM, Coulter Electronics, Hileah, FL). The hematocrit was evaluated after micro-capillary centrifugation. Blood was also stained with new-methylene blue (Sigma) and a smear prepared for quantitation of peripheral reticulocytes. One thousand red cells on each smear were counted and positive reticulocytes scored. Serum was obtained by centrifugation of blood at $1000 \times g$ for 10 min. After 4 and 8 weeks of treatment, several mice in each group were sacrificed by cervical dislocation for further evaluation of anti-viral efficacy and bone marrow toxicity (except HU 400 mg/kg/day; due to five early deaths from toxicity, the seven remaining mice were sacrificed at week 4). The spleen and one femur from each animal were rapidly removed and immediately placed on ice. The contents of each femur were flushed in ice-cold PBS using a 22-guage needle. A single cell suspension was then obtained by repeated gentle flushing through a 19-guage needle, an aliquot of cells taken, and after lysis of red cells with Zapoglobin (Coulter), nucleated cells were counted using the Coulter counter. Individual animal femoral cellularity was then calculated and bone marrow cells were pooled for evaluation of the femoral content of hematopoietic progenitor cells in a colony-forming assay (Mayhew et al., 1999). Spleens were processed as follows. Spleens were weighed and cut approximately in half. One-half was immediately frozen in a dry-ice/ethanol bath and stored at −80 °C until used for DNA extraction. Splenocytes were obtained from the other half by disruption in 5 ml ice-cold PBS followed by passage through a sterile nylon screen. An aliquot was taken and after lysis of red cells, nucleated splenocytes were counted using the Coulter counter. Pooled splenocytes were then used for assay of splenic content of hematopoietic progenitors.

### 2.5. Enzyme-linked immunosorbant assay for murine IgG

Serum was obtained every 2 weeks as described above and frozen at −20 °C until use. A standard enzyme-linked immunosorbant assay technique (ELISA) was used to quantify levels of murine IgG. Ninety-six-well microtitre plates were coated overnight at 4 °C with 1.5 μg/ml goat *anti*-mouse

IgG (Fc-chain specific; Jackson ImmunoResearch, West Grove, PA) in 0.1 M $NaHCO_3$. After washing and blocking with 0.1% bovine serum albumin in PBS, diluted serum samples and serially diluted murine IgG standards of known concentration (Sigma) were added to plates and incubated for 2 h at room temperature. After washing, 0.3 μg/ml alkaline phosphatase-labelled *anti*-mouse IgG (Jackson ImmunoResearch) in PBS was added and incubated at room temperature for 1 h. Finally, after washing, *p*-nitrophenyl phosphate substrate (Sigma) was added and incubated for 15 min at 37 °C. Reactions were stopped by addition of 3 M NaOH and the absorbance in each well was read at 405 nm. Serum IgG concentrations were determined by comparison with the absorbance of the murine IgG standards.

### 2.6. Extraction of genomic DNA

Genomic DNA was extracted from three randomly selected spleens from each experimental group using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturers recommendations. Spleen size was not taken into account when selecting tissue for DNA extraction. RNA was removed from tissue extracts by incubation with 2 mg/ml RNase A (Qiagen). DNA was eluted from spin columns and stored at 4 °C until use in PCR reactions. The yield and purity of extracted DNA was quantified spectrophotometrically.

### 2.7. Semi-quantitative polymerase chain reaction for integrated proviral DNA

The following primers, specific for the $p12^{gag}$ region of the LPBM5 defective virus (BM5-def) genome, were used for PCR amplification: 5′-CCT TTT CCT TTA TCG ACA CT-3′ (sense), and 5′-ACC AGG GGG GGA ATA CCT CG-3′ (antisense). The expected size of the amplified product was 246 base pairs. A second pair of oligonucleotide primers, designed using the Oligo (version 6.15) computer software (Molecular Biology Insights Inc., Cascade, CO), was used to amplify a region of the murine *G6PDH* gene which served as an endogenous control. The G6PDH primer sequences were: 5′-TGA TTG GGG GCT CCA AGC A-3′ (sense) and 5′-AAG GGT TCA TGA ATG GAT GCT-3′ (antisense) and amplified a product of 363 base pairs. Primers were end-labeled with $^{\gamma 32}P$ using T4 polynucleotide kinase (Gibco), according to the manufacturers recommendations. PCR amplification in the linear range was performed in a final volume of 25 μl, containing 0.25 μg genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM each labeled primer, 200 μM each dNTP and 1 unit of AmpliTaq Gold (Perkin–Elmer, Foster City, CA). BM5def was amplified by initial denaturation at 95 °C for 10 min followed by 22 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1.5 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 8 min. G6PDH was amplified by initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. $^{32}P$ labeled PCR products were separated by 10% polyacrylamide gel electrophoresis and post-stained with 2.5 μg/ml ethidium bromide to ensure amplification of product of the correct size. Gels were then dried and bands visualized by autoradiography. The areas of autoradiographic bands were quantified by laser scan densitometry (Kodak Digital Science 1D, version 2.0.2). Each BM5def band was normalized to its corresponding G6PDH band before comparison between experimental groups.

### 2.8. Assay of colony-forming units-granulocyte-macrophage (CFU-GM) and burst forming units-erythroid (BFU-E)

The mean content of committed hematopoietic progenitors (CFU-GM and BFU-E) per femur and spleen of normal and infected controls and infected mice treated with RR inhibitors was determined as follows. A single cell suspension of pooled bone marrow or splenocytes from each experimental group was prepared as described above. Then $5 \times 10^4$/ml bone marrow cells or $2.5 \times 10^5$/ml splenocytes were added to methylcel-

lulose media (Stem Cell Technologies; Vancouver, BC, Canada) containing 1% methylcellulose in Iscove's minimal Dulbecco's media, 15% FBS, 1% bovine serum albumin, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin (iron saturated), $10^{-4}$ M β-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant murine (rm) stem cell factor, 10 ng/ml rm-interleukin-3, 10 ng/ml recombinant human (rh) interleukin-6 and 3 units/ml rh-erythropoietin. After vortexing, 1 ml of media was plated in duplicate in a six-well tissue culture plate and incubated at 37 °C in air containing 5% $CO_2$ for 10 days. Using an inverted microscope, CFU-GM and BFU-E were identified on the basis of their morphology and colonies containing greater than 50 cells were scored. The numbers of CFU-GM and BFU-E per femur was determined using the following calculation: mean number of hematopoietic colonies scored multiplied by the mean number of cells per femur, divided by 50 000. Numbers of CFU-GM and BFU-E per spleen were determined relative to normal controls by dividing the mean number of colonies scored by the mean spleen weight of each experimental group.

### 2.9. Statistical analysis

To determine whether the differences observed between experimental groups were statistically significant, a two-tailed *t*-test was performed. A *P*-value of $\leq 0.05$ was considered to be statistically significant. *P*-values are given in tables and figures where appropriate.

## 3. Results

The novel RR inhibitors DX and TX were comparatively evaluated with HU for anti-retroviral efficacy and the induction of hematopoietic toxicity in murine AIDS.

### 3.1. Gross toxicity

Daily treatment with HU at both doses resulted in development of gross toxicity (e.g. failure to groom and lack of response to handling), which was particularly evident at the higher dose. After 4-week treatment with HU 400 mg/kg, the mean body weight was reduced to approximately 83% control (Table 1), and all mice were either moribund or had died. HU 200 mg/kg was better tolerated, and although body weights were not significantly different from normal controls, several mice were moribund after 8-week drug treatment and one animal had died. Conversely, TX and DX were well tolerated. All TX and DX treated animals survived 8 weeks of drug administration with no gross toxicity. However, body weights of mice treated with TX and DX were slightly lower than normal controls at weeks 4 and 8. Body weights of DX treated mice were not statistically different from normal at either timepoint, although at week 8 the body weight of TX treated animals was significantly lower than normal.

### 3.2. Inhibition of splenomegaly by RR inhibitors

Mice infected with LPBM5 MuLV develop profound immunodeficiency disease which has many similarities to human AIDS, including lymphoproliferation characterized in part by development of splenomegaly (Morse et al., 1992). In the present study, all infected control mice developed the characteristics typical of murine AIDS, including extensive peripheral lymphadenopathy and splenomegaly. Spleen weights of mice from each experimental group are shown in Table 1. Spleens of infected control animals weighed approximately fourfold greater than normal at week 4 and nearly eightfold normal at week 8. Four weeks of HU 400 mg/kg administration was extremely effective in inhibiting splenomegaly in infected animals with spleens weighing only slightly more than normal. Treatment with HU 200 mg/kg was less effective, but although splenomegaly was not significantly inhibited at week 4, there was a negligible increase in spleen weight between weeks 4 and 8 ($P < 0.05$ vs. infected control; week 8). TX and DX also significantly inhibited development of splenomegaly in infected animals, with spleen weights only slightly greater than normal at weeks 4 and 8 ($P < 0.02$ vs. infected control). Although effectively inhibiting

Table 1
Effect of HU, TX and DX on the body weight and spleen weight in murine AIDS

| | Body weight (g) | | Spleen weight (mg) | |
|---|---|---|---|---|
| | Week 4 | Week 8 | Week 4 | Week 8 |
| Normal control | 22.87±1.36 | 23.82±1.81 | 83.0±10.7 | 104.6±5.6 |
| Infected control | 23.17±0.49 | 25.80±2.4 | 332.4±60.2[b] | 780.2±337.5[a] |
| Infected+HU 400 mg/kg | 18.97±3.76[a,c] | Dead | 90.8±58.0[c] | Dead |
| Infected+HU 200 mg/kg | 22.43±1.06 | 20.20±4.82 | 251.1±115.6[b] | 260.3±200.7[c] |
| Infected+TX 175 mg/kg | 21.93±0.55[d] | 21.36±0.76[a,c] | 105.0±15.8[a,c] | 125.8±25.0[c] |
| Infected+DX 350 mg/kg | 21.93±0.9[c] | 22.42±0.83[c] | 134.0±20.3[b,c] | 133.6±22.4[a,c] |

LPBM5 MuLV infected mice were treated daily with RR inhibitors at the indicated doses. After 4 and 8 weeks of drug treatment, body and spleen weights of animals in each experimental group were evaluated. Values represent the mean±standard deviation of five to seven animals per group. The mean body weight of animals at the start of the experiment was 19.83±1.22 g.

[a] $P < 0.05$;
[b] $P < 0.01$ vs. normal control;
[c] $P < 0.05$;
[d] $P < 0.01$ vs. infected control.

splenomegaly compared to infected controls, HU treatment resulted in a greater variability in spleen weight than TX and DX.

### 3.3. Inhibition of hypergammaglobulinemia by RR inhibitors

Early progression of murine AIDS is associated with polyclonal activation and proliferation of B-lymphocytes resulting in increased serum immunoglobulin concentrations (hypergammaglobulinemia) (Klinman and Morse, 1989). Development of hypergammaglobulinemia in LPBM5 MuLV infected mice was evaluated by measuring serum IgG concentrations at weeks 2, 4, 6 and 8 (Fig. 2). Increased serum IgG in infected controls was evident by week 2 and compared to normal controls was significantly increased at all time points ($P < 0.05$). Treatment with RR inhibitors was associated with decreased serum IgG compared to infected controls. HU 400 mg/kg was extremely effective ($P < 0.02$ vs. infected control), with minimally increased serum IgG concentrations at week 4. HU 200 mg/kg, TX and DX were slightly less effective than the higher dose of HU, with serum IgG levels increasing gradually with time. However, serum IgG in all surviving drug treated groups was only one-third the level of infected controls at week 8 ($P < 0.01$).

### 3.4. Inhibition of defective LPBM5 MuLV proviral DNA by RR inhibitors

Relative levels of proviral DNA (BM5def) in spleens were evaluated by semi-quantitative PCR (Fig. 3). After 4 weeks of drug treatment, levels of BM5def proviral DNA in spleens of infected animals treated with RR inhibitors were significantly reduced compared to infected controls. HU 400 mg/kg was most effective, resulting in BM5def levels 78.4±14.4% lower than infected control ($P < 0.01$). At week 4, DX, TX and HU 200 mg/kg inhibited levels of proviral DNA by 60.1±5.2, 58.3±18.4 and 51.8±18.4%, respectively ($P < 0.05$). After 8 weeks of treatment, HU 200 mg/kg resulted in the greatest reduction in splenic proviral DNA (66.7±7.5%; $P < 0.01$). TX and DX inhibited BM5def proviral DNA levels by 56.6±18.5 and 45.0±21.6%, respectively at week 8 ($P < 0.05$).

### 3.5. Effect of RR inhibitors on peripheral blood indices

The WBC and hematocrit of controls and infected mice receiving RR inhibitors were evaluated at weeks 2, 4, 6 and 8 and are shown in Table 2A. The WBC of normal controls ranged between approximately 7000 and 12 500 cells/μl

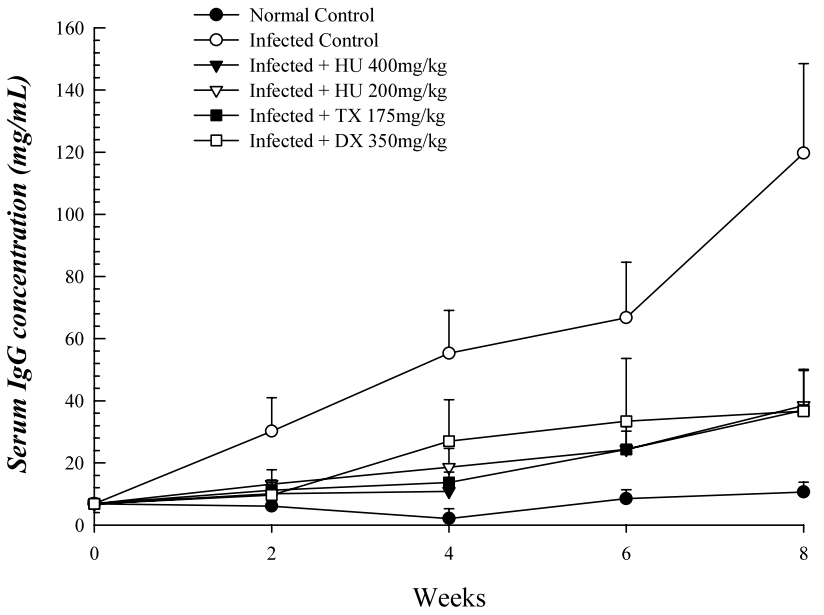


Fig. 2. Inhibition of LPBM5 MuLV induced B-lymphocyte activation and proliferation by RR inhibitors. Infection with LPBM5 MuLV induces activation and proliferation of B-lymphocytes, resulting in hypergammaglobulinemia. Infected animals were treated daily with HU 400 or 200 mg/kg, TX 175 mg/kg or DX 350 mg/kg, commencing 1-week post-infection. At the indicated times after initiation of treatment, blood was sampled from mice in each treatment group and the extent of hypergammaglobulinemia evaluated by measuring serum IgG using a standard ELISA technique. Values are expressed as mean ± standard deviation of five to seven mice per group. Serum IgG of infected animals receiving drug treatment was significantly different ($P < 0.05$) compared to infected controls at each time point except week 6.

during the period of evaluation and reflects the dynamic process of leukocyte production and release from the bone marrow. The WBC in LPBM5 MuLV infected controls was depressed compared to normal at all time points, reaching a nadir of approximately 5000 cells/μl at week 4, subsequently increasing slightly to approximately 6000 cells/μl at week 8. RR inhibitor treatment of infected mice was associated with further suppression of the WBC compared to infected controls (HU 400 mg/kg > HU 200 mg/kg > DX > TX) and was significantly decreased at all time points compared to normal ($P < 0.02$). HU 400 and 200 mg/kg suppressed the WBC to approximately 3700 cells/μl at week 2. Subsequently at week 4, the WBC had declined to less than 1700 cells/μl in moribund animals receiving HU 400 mg/kg. The WBC in mice receiving HU 200 mg/kg declined more slowly but was also reduced to less than 1700 cells/μl by week 8, a decrease of greater than 65% compared to the infected control group. At weeks 2 and 4, the WBC of infected mice receiving TX and DX was not significantly different from infected controls. However, in contrast to HU, between weeks 4 and 8 TX and DX treatment did not further reduce the WBC, indeed in mice receiving DX the WBC increased slightly.

Effects of RR inhibitor therapy on the hematocrit of LPBM5 MuLV infected mice are shown in Table 2B. By week 8, the hematocrit of infected controls was suppressed by approximately 10% ($P < 0.01$ vs. normal). Treatment of infected mice with HU resulted in rapid induction of anemia. This was evident by week 2, when HU 400 mg/kg and HU 200 mg/kg had reduced the hematocrit by approximately 50 and 35%, respectively ($P < 0.01$ vs. normal). By week 4, compared to normal HU 400 mg/kg had suppressed the hematocrit by over than 60% ($P < 0.01$). Treatment with HU 200 mg/kg reduced the hematocrit at each subsequent time point, and by week 8 had reduced the hematocrit by greater than 70% ($P < 0.01$). Conversely, TX

and DX had minimal effects on the hematocrit during 8 weeks of daily administration to infected animals. The greatest reduction was observed at week 6, when TX and DX reduced to the hematocrit by approximately 10% ($P < 0.01$). However, the hematocrits in these groups did not decline further and at week 8 were above levels seen in infected controls.

The reticulocyte count of controls and drug treated mice was evaluated at weeks 4 and 8 (Table 2C). HU 200 mg/kg induced reticulocytosis, with reticulocyte counts nearly threefold normal at week 8 ($P < 0.05$). In contrast, TX and DX did not significantly elevate the reticulocyte count at either week 4 or 8. These results indicate that active erythropoiesis as a result of HU treatment was observed in animals in response to HU. However, no reticulocytosis, i.e. rebound erythropoiesis, was observed in animals treated with either TX or DX demonstrating that these compounds induced minimal adverse effects on the production of erythrocytes in infected mice.

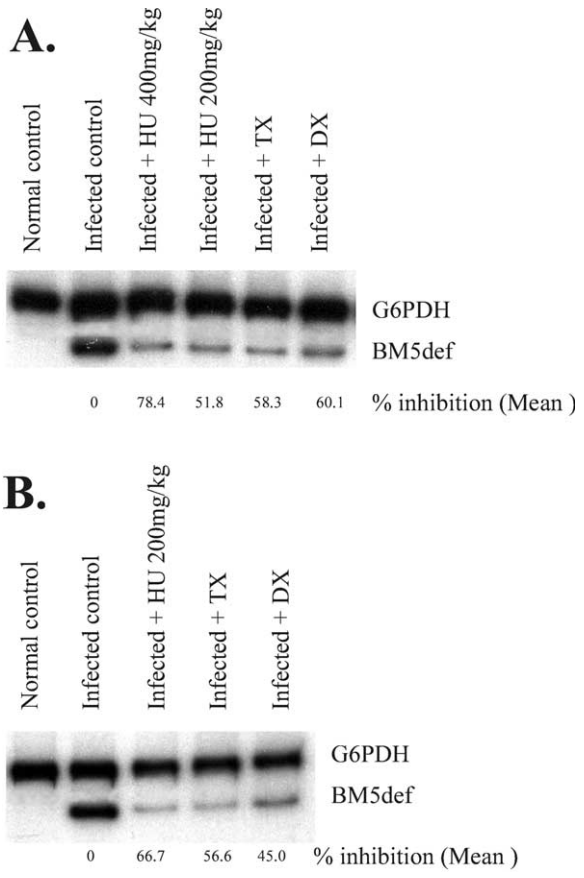


Fig. 3

### 3.6. Effects of RR inhibitors on femur cellularity and hematopoietic progenitor cells

The effects of RR inhibitor treatment on femur cellularity is shown in Fig. 4A. Infection with LPBM5 MuLV had no significant effect on the numbers of nucleated cells per femur. However, numbers of femoral cells were dramatically decreased in infected animals after 4 weeks treatment with HU 400 mg/kg ($P < 0.0001$ vs. normal). There was also a time-dependent decrease of greater than 60% in femur cellularity in the HU 200 mg/kg group by week 8 ($P < 0.01$ vs. normal). TX and DX, on the other hand, had no significant effect on the numbers of nucleated cells per femur. There was a slight decrease compared to normal controls associated with TX and DX treatment at week 4, but levels rebounded to, or slightly above control, by week 8.

Fig. 3. Polymerase chain reaction analysis of relative BM5def proviral DNA levels in spleens of LPBM5 MuLV infected mice treated with RR inhibitors. Infected mice were treated daily with HU 400 or 200 mg/kg, TX 175 mg/kg or DX 350 mg/kg, commencing 1-week post-infection. Genomic DNA was extracted from the spleens of three randomly selected animals from each experimental group after (A) 4 and (B) 8 weeks of drug treatment*. A 252-bp sequence of the defective retroviral $p12^{gag}$ gene was amplified by PCR using specific $^{32}$P-labelled primers from 0.25 μg genomic DNA, as described in Section 2. As an endogenous control, a 363-bp sequence of the murine *G6PDH* gene was amplified. Detection was accomplished by autoradiography and bands were quantified using laser-scan densitometry. Percent of inhibition of BM5def proviral DNA in spleens of drug-treated animals compared to infected controls was calculated as follows: BM5def band intensity was divided by its corresponding G6PDH band. The mean BM5def/G6PDH ratio for infected controls was then normalized to 100% and the same multiplication factor applied to each of the infected/drug treated BM5def/G6PDH ratios to yield percent inhibition. Representative BM5def and G6PDH PCR product from a single animal from each group at weeks 4 and 8 is shown. *HU 400 mg/kg was not evaluated at week 8 because all mice in this group were moribund by week 4 and were sacrificed.

Table 2
Effect of HU, TX and DX on the peripheral blood indices in murine AIDS

| Weeks | 2 | 4 | 6 | 8 |
|---|---|---|---|---|
| (A) WBC (cells/μl) | | | | |
| Normal control | 12 386 ± 733 | 7222 ± 1902 | 10 388 ± 1707 | 12 397 ± 1928 |
| Infected control | 9577 ± 3239 | 4891 ± 1363[a] | 5484 ± 720[b] | 6246 ± 926[b] |
| Infected + HU 400 mg/kg | 3773 ± 1321[b,c] | 1647 ± 1243[b,d] | Dead | Dead |
| Infected + HU 200 mg/kg | 3716 ± 842[b,c] | 3450 ± 1749[b] | 2399 ± 1065[b,d] | 1641 ± 728[b,d] |
| Infected + TX 175 mg/kg | 7400 ± 499[b] | 4689 ± 1227[a] | 4518 ± 312[b,c] | 4905 ± 701[b,c] |
| Infected + DX 350 mg/kg | 5731 ± 1038[b] | 3432 ± 1560[b] | 3988 ± 729[b,c] | 4263 ± 771[b,d] |
| (B) Hematocrit (%) | | | | |
| Normal control | 46.40 ± 0.55 | 46.00 ± 3.21 | 48.20 ± 0.84 | 47.20 ± 1.48 |
| Infected control | 47.80 ± 2.68 | 45.21 ± 2.16 | 43.60 ± 1.52[b] | 41.40 ± 2.41[b] |
| Infected + HU 400 mg/kg | 24.40 ± 4.34[b,d] | 17.86 ± 15.91[b,d] | Dead | Dead |
| Infected + HU 200 mg/kg | 31.00 ± 6.24[b,d] | 23.33 ± 6.19[b,d] | 18.60 ± 7.99[b,d] | 12.75 ± 7.76[b,d] |
| Infected + TX 175 mg/kg | 47.60 ± 2.19 | 45.29 ± 2.06 | 43.80 ± 1.48[b] | 43.80 ± 1.30[b] |
| Infected + DX 350 mg/kg | 46.40 ± 2.30 | 43.00 ± 3.79 | 42.80 ± 3.27[a] | 44.60 ± 1.34[a,c] |
| (C) Reticulocytes[e] (%) | | | | |
| Normal control | | 5.70 ± 1.30 | | 4.10 ± 1.70 |
| Infected control | | 8.70 ± 0.60[a] | | 5.90 ± 2.20 |
| Infected + HU 200 mg/kg | | 10.50 ± 5.50 | | 11.10 ± 3.00[a] |
| Infected + TX 175 mg/kg | | 5.10 ± 0.60 | | 4.40 ± 0.50 |
| Infected + DX 350 mg/kg | | 5.70 ± 0.40 | | 5.90 ± 2.70 |

LPBM5 MuLV infected mice were treated daily with RR inhibitors and at the indicated times peripheral blood was sampled and the (A) WBC, (B) hematocrit and (C) reticulocyte count were evaluated. Values represent the mean ± standard deviation of five to seven animals per group except reticulocytes ($n = 3$).

[a] $P < 0.05$;
[b] $P < 0.01$ vs. normal control;
[c] $P < 0.05$;
[d] $P < 0.01$ vs. infected control.
[e] Reticulocyte count of animals receiving HU 400 mg/kg/day was not evaluated.

The effect of RR inhibitor treatment on the numbers of granulocyte-macrophage progenitors (CFU-GM) per femur is shown in Fig. 4B. Infected controls had significantly decreased femoral CFU-GM at weeks 4 and 8 ($P < 0.05$, vs. normal). Treatment with both doses of HU was also associated with significant depletion of the femoral CFU-GM pool. HU 400 mg/kg reduced femoral CFU-GM by 95% at week 4 ($P < 0.0001$ vs. normal). In animals receiving HU 200 mg/kg, the number of CFU-GM per femur was reduced to 20% of control at week 4 ($P < 0.001$). There was no further decline by week 8, although levels remained significantly lower than normal ($P < 0.0001$). Conversely, TX and DX had no significant effect on numbers of femoral CFU-GM. Indeed, after 8 weeks of daily treatment with DX, levels of CFU-GM were above normal. Similar trends were observed when the numbers of erythroid progenitors (BFU-E) per femur were evaluated (Fig. 4C). HU 400 mg/kg significantly depleted femoral BFU-E by week 4 ($P < 0.0001$). HU 200 mg/kg also significantly reduced femoral BFU-E by approximately 80% by week 4 ($P < 0.001$), with a slight increase observed at week 8. Treatment of infected mice with TX and DX did not significantly alter the numbers of BFU-E per femur.

Evaluation of splenic hematopoiesis revealed large increases (30-fold) in the numbers of both CFU-GM and BFU-E in infected controls, compared to normal at week 8 (data not shown). This likely represents a splenic contribution to new blood cell formation due to reduced marrow progenitor numbers as a consequence of viral disease progression (Fig. 4B and C). These changes

were not observed to the same extent in drug treated animals. However, at week 4 HU 400 mg/kg had increased splenic CFU-GM threefold versus normal (data not shown). At week 8, TX increased both CFU-GM and BFU-E approximately threefold. There was also a modest increase in splenic CFU-GM and BFU-E in HU 200 mg/kg and DX treated animals (seven- and sixfold, respectively) at week 8. These results do not suggest the lack of an anti-viral effectiveness but indicate that splenic hematopoiesis was present, potentially to contribute to the restoration of normal hematopoiesis in drug treated animals.

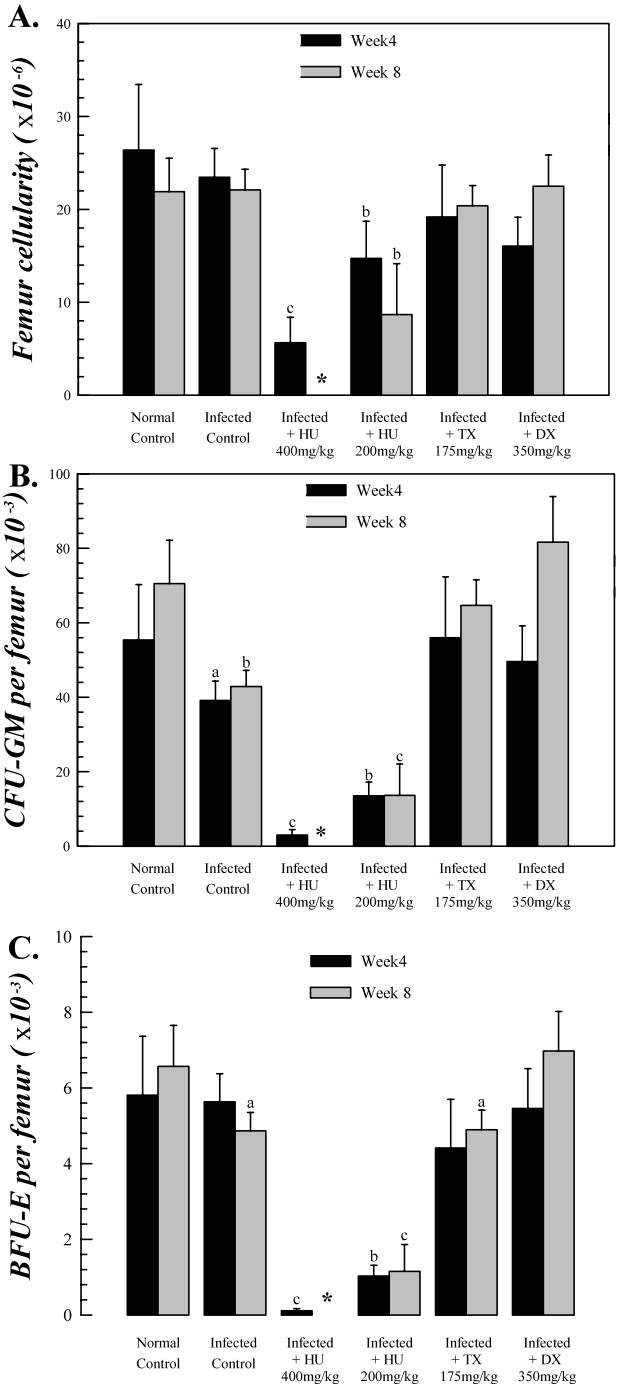


Fig. 4

## 4. Discussion

The cellular enzyme RR controls the rate-limiting step in de novo DNA biosynthesis (Thelander and Reichard, 1979). Inhibition of RR activity results in depletion of the cellular dNTP pools, and cessation of DNA synthesis. Due to the extremely high rate of HIV-1 replication in vivo and its lack of the enzymes required for dNTP synthesis, HIV-1 proviral DNA synthesis has an absolute requirement for large quantities of host cell derived dNTPs. It was therefore the ability of inhibitors of RR to deplete dNTP pools that first prompted the investigation of RR as a target for anti-HIV-1 drug therapy (Gao et al., 1993). The data presented here demonstrates that HU and the novel RR inhibitors TX and DX are effective drug treatments with the ability to influence development of several parameters associated with disease progression in the murine AIDS model of retrovirus-induced immunodeficiency.

Fig. 4. Effects of RR inhibitor therapy on the bone marrow of LPBM5 MuLV infected mice. Infected animals were treated daily with HU 400 and 200 mg/kg, TX 175 mg/kg or DX 350 mg/kg, commencing 1-week post-infection. After 4 and 8 weeks of drug treatment, animals in each experimental group were sacrificed to evaluate the effects of drug treatment on the bone marrow. (A) Nucleated cells and the numbers of: (B) CFU-GM; and (C) BFU-E per femur were evaluated as described in Section 2. Values are expressed as mean ± standard deviation of five to seven animals per group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.0001$ vs. normal control at each time-point. *HU 400 mg/kg was not evaluated at week 8 because all mice in this group were moribund by week 4 and were sacrificed.

TX and DX doses evaluated in this study were based upon those previously demonstrated to have optimal anti-retroviral efficacy in mouse models (Broud et al., 1998; Mayhew et al., 1997; Ussery et al., 1999). Therefore, the foundation for toxicity comparisons in this study was an evaluation of drug induced myelosuppression in the context of effective inhibition of disease progression. Of particular note therefore is the demonstration that although HU was associated with effective anti-viral activity it was toxic and induced, in a dose- and time-dependent manner, significant perturbations in all aspects of hematopoiesis examined. Conversely, the novel RR inhibitors TX and DX were associated with significant inhibition of viral-induced pathophysiology, but did not induce demonstrable hematopoietic toxicity in retrovirus infected mice after 8 weeks of drug treatment. Importantly, inhibition of disease progression by TX and DX was similar to that associated with HU 200 mg/kg, particularly those parameters associated with lymphoproliferation, demonstrating a greater benefit to toxicity ratio for TX and DX compared to HU at the doses examined.

The WBC of TX- and DX-treated animals was significantly lower than normal. However, these changes were not reflected in the bone marrow compartment. This observation separates TX and DX induced effects from those of HU, which not only reduced the numbers of peripheral white cells but also depleted the marrow of committed hematopoietic progenitors. This observation in retrovirus-infected mice supports our previous demonstration that administration of HU to normal mice induced more significant perturbations of in vivo hematopoiesis than either TX or DX, and that HU was more growth inhibitory to normal murine and human hematopoietic progenitor cells than TX or DX (Mayhew et al., 1999). Taken together, these data suggest the novel RR inhibitors TX and DX have effective in vivo anti-retroviral and -proliferative activity at doses that do not influence hematopoiesis to the degree seen with HU.

Since HIV-1 infection is characterized by T-cell activation with the most efficient virus replication only occurring in activated T cells (Stevenson et al., 1990), the cytostatic property of HU has been proposed as the mechanism for its anti-HIV-1 action (Lori, 1999). In support of this hypothesis are mathematical models which propose that inhibition of $CD4^+$ cell proliferation and therefore HIV-1 target cell availability, accounts for the anti-HIV-1 effect of the combination of HU and ddI (De Boer et al., 1998). These models also propose that limiting target cell availability reduces the growth rates of drug resistant mutants (De Boer and Boucher, 1996), and may explain the continued efficacy of HU and ddI, even in the presence of ddI-resistant mutant HIV-1 strains (De Antoni et al., 1997; Lori et al., 1997). Additionally, the numbers of activated $CD8^+$ cells increases dramatically in HIV-1 infection (Giorgi et al., 1993), and $CD8^+$ cells have been implicated to be responsible for the immunopathology in HIV infection (Zinkernagel and Hengartner, 1994). As RR inhibitors it is possible that TX and DX may have in common with HU the potentially beneficial effects seen on $CD4^+$ and $CD8^+$ cell activation in human AIDS (Lori, 1999). In support of this notion is the observation that TX, DX and HU effectively blocked virus-induced B-lymphocyte activation and proliferation (characterized by hypergammaglobulinemia and splenomegaly) in this study.

Although LPBM5 MuLV viremia was not directly evaluated in this study, effects of drug treatment on viral replication were indirectly measured by evaluation of levels of proviral DNA in spleens of infected animals treated with RR inhibitors. HU inhibited BM5def levels most effectively. However, this suppression was only moderately better than that achieved with DX and TX, and was associated with significant toxicity. In addition, previous experiments by our group in the mouse HuPBMC SCID model of HIV-1 infection have demonstrated the ability of TX and DX to reduce HIV-1 RNA titers in vivo (Broud et al., 1998; Ussery et al., 1999). Therefore, in HIV-1 infected individuals, these compounds may be effective not only by virtue of their cytostatic properties, but by a direct inhibitory effect on HIV-1 replication.

A property of TX and DX that may have additional therapeutic benefit in HIV-1 infection

compared to HU is their differential effect on the physiological dNTP pools. The anti-HIV activity of ddN analogs does not depend solely on absolute levels of ddNTPs, but instead on the ratio of ddNTP to its corresponding physiological dNTP (i.e. ddATP/dATP, ddCTP/dCTP etc.) (Perno et al., 1992). Therefore, reducing the levels of physiological dNTPs may prove as effective as increasing levels of ddNTPs. Thus far, HU has been demonstrated to consistently deplete only the cellular dATP pool. Therefore, HU most effectively potentiates the activity of adenosine analogs (particularly ddI), with much lesser potentiation of the activity of thymidine and cytosine analogs (e.g. AZT, ddC) (Gao et al., 1994; Lori et al., 1994). In contrast to HU, TX and DX have been demonstrated to reduce not only the dATP pool, but also the dCTP and dGTP pools in various cell lines (Elford and van't Riet, 1985; Szekeres et al., 1994a; Tihan et al., 1991). Based on this information, we speculate that TX and DX may have the ability to increase the anti-HIV-1 activity of a larger number of ddNs than HU, potentially providing more therapeutic options in the treatment of human AIDS. However, it has been suggested that HU not only increases the activity of nucleoside RT inhibitors like ddI, but can also potentiate the toxicity of these compounds (Moore et al., 2001). For RR inhibitors like TX and DX, which may perturb a broader array of dNTP pools than HU, there may be potential for increased toxicity from a larger range of nucleoside RT inhibitors. Therefore, careful monitoring of patients treated with combinations of RR inhibitors and nucleoside RT inhibitors is warranted. Experiments to evaluate the ability of TX, DX and HU to potentiate the activity and toxicity of different nucleoside analogs in murine AIDS are currently underway in our laboratory.

In addition to their RR inhibitory activity with subsequent beneficial effects on the dNTP pools, TX and DX are significantly more potent free-radical scavengers than HU (Elford and van't Riet, 1985; Rauko et al., 1997). Administration of antioxidants has been demonstrated to inhibit retroviral disease progression in murine AIDS (Palamara et al., 1996). Therefore, this property of TX and DX may partly explain their activity in this model. The free-radical scavenging ability of TX and DX may also provide additional therapeutic advantage compared to HU in HIV-1 infection, because oxidative stress has been shown to contribute to several aspects of HIV disease pathogenesis (reviewed by Pace and Leaf, 1995). In support of the potential for TX and DX to influence redox regulated reactions which modulate HIV-1 replication, was a study by Lee et al., which demonstrated that TX potently inhibited NF-κB activity and suppressed HIV LTR expression in vitro (Lee et al., 1997).

With respect to their enhanced in vitro RR inhibitory activity compared to HU, TX and DX might therefore be expected to have greater in vivo hematopoietic toxicity. The data presented here clearly demonstrates that at the doses we evaluated this is not the case. TX and DX exhibited inhibition of viral-induced pathophysiology to a similar degree to that seen with HU 200 mg/kg. However, TX and DX had minimal effects on hematopoiesis whilst HU ablated the bone marrow, suggesting that additional properties of TX and DX in addition to RR inhibition may be important in their mechanism of action in vivo. The mechanism for the activity of TX and DX in the murine AIDS model is under investigation in our laboratory.

In summary, the anti-retroviral activity of HU and the novel and more potent RR inhibitors TX and DX was comparatively evaluated in the murine AIDS model. Results demonstrated that all three drugs have effective in vivo anti-viral activity and the ability to inhibit retrovirus induced lymphoproliferation in this model. However, HU was associated with induction of significant hematopoietic toxicity. In contrast, TX and DX demonstrated effective anti-retroviral activity at doses that did not induce hematopoietic toxicity. Myelosuppression is a common side effect associated with HU therapy of HIV-1 (Maserati, 1999), therefore these studies clearly highlight the potential of TX and DX for HIV-1 therapy. In light of these data and their other potential beneficial pharmacological properties (i.e. free-radical scavenging and inhibition of NF-κB activation), it is possible that TX and DX may prove

to be useful as components of drug combinations in the treatment of human AIDS.

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