Motexafin gadolinium (Gd-Tex) selectively induces apoptosis in HIV-1 infected CD4+ T helper cells

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Here, we show that motexafin gadolinium (Gd-Tex), a compound that promotes intracellular oxidative stress, selectively induces apoptosis in HIV-1-infected CD4$^+$ T cells in IL-2-stimulated cultures of peripheral blood mononuclear cells infected *in vitro* with HIV-1. This selective induction of apoptosis, which we detect by FACS analysis of intracellular HIV/p24 and concomitant surface and apoptosis marker expression, is abrogated by the glutathione precursor, *N*-acetyl-L-cysteine. Importantly, it occurs at Gd-Tex concentrations that are not cytotoxic to uninfected cells in the culture. These findings suggest that Gd-Tex may have therapeutic utility as an anti-HIV agent capable of selectively targeting and removing HIV-infected cells in an infected host.

glutathione | redox modulation

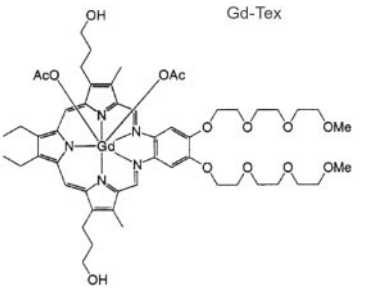

Fig. 1. Structure of motexafin gadolinium (Gd-Tex). Chemical structure of Gd-Tex. OAc, axially coordinated acetate ligands.

Texaphyrins are synthetic porphyrin-like ligands that complex large metal cations, including the trivalent cations of the lanthanide series (Fig. 1 and ref. 1). One derivative, motexafin gadolinium (Xcytrin, or Gd-Tex), has been reported to localize to tumors and to enhance radiation response in animal tumor models. This drug is currently in late stage clinical development as an adjuvant for radiation therapy of brain metastases (2, 3).

The enhancement of the tumor radiation response mediated by Gd-Tex seems to proceed by means of an oxidative stress-increasing mechanism in which redox cycling by the electron affinic Gd-Tex complex results in the catalytic oxidation of intracellular reducing metabolites such as glutathione (GSH) and ascorbate. This increased consumption of reducing metabolites decreases protection against reactive oxygen species such as superoxide and hydrogen peroxide. Thus, it renders cells more sensitive to the lethal effects of ionizing radiation (4, 5) and, more generally, to the damaging effects of oxidative stress. This finding suggests that Gd-Tex might be specifically toxic to cells that are already under oxidative stress.

Studies here were motivated by the demonstrated linkage between oxidative stress and HIV-1 infection (6–8). Previous studies have shown that low intracellular GSH promotes HIV-1 expression, whereas maintenance of normal GSH levels inhibits HIV-1 transcription and replication in *in vitro* systems (9–11). In addition, the production and release of a key HIV protein (HIV-TAT) has been shown to decrease the activity of manganese superoxide dismutase and to lower intracellular GSH levels in uninfected T cells (6, 12–15). These findings, coupled with evidence discussed above indicating that Gd-Tex may be toxic for GSH-depleted cells, suggest a potential role for Gd-Tex in HIV infection.

Here, we report that Gd-Tex selectively induces apoptosis in primary human CD4 T cells infected *in vitro* with HIV-1 and thereby inhibits both HIV-1 production and replication in these cells. In contrast, Gd-Tex at the same dose does not induce detectable apoptosis in uninfected CD4 T cells or in CD8 T cells, even when present in cultures containing HIV-infected CD4 T cells that are undergoing apoptosis.

We discuss the mechanism responsible for the Gd-Tex induction of apoptosis in the HIV-infected CD4 T cells in terms of evidence, also presented here, showing that both HIV infection and high doses of Gd-Tex deplete GSH from peripheral blood mononuclear cells (PBMC). These findings, we suggest, indicate that the combination of HIV infection and low-dose Gd-Tex specifically induces lethal oxidative stress in the HIV-infected cells. We discuss this possibility and its implications for using Gd-Tex, which has already been shown to be safe for patients with brain metastases, as a therapeutic agent in HIV disease.

Materials and Methods

Biological and Chemical Reagents. Monochlorobimane, annexin-V conjugates, Cascade Blue, Cascade Yellow, Alexa Fluor 430, and Alexa Fluor 594 were purchased from Molecular Probes. R-phycoerythrin (PE) and allophycocyanin (APC) were purchased from ProZyme (San Leandro, CA). FITC was purchased from Pierce. Cy5, Cy5.5, and Cy7 were obtained from Amersham Pharmacia. Tandem conjugate protocols for Cy5PE, Cy5.5PE, Cy7PE, Cy5.5APC, and Cy7APC can be found at www.drmr.com/abcon. All antibodies in this study were obtained through PharMingen and conjugated to indicated fluorophore as needed. *N*-acetyl-L-cysteine, L-buthionine–(S,R)-sulfoximine (B50), ethidium bromide, acridine orange, and propidium iodide were purchased through Sigma. Human recombinant IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infections Diseases, National Institutes of Health. Gd-Tex was synthesized and provided by Pharmacyclics (Sunnyvale, CA).

Cell Culture and Primary Cell Isolation. For preparation of primary cells, mononuclear cells were isolated from blood of healthy donors (Stanford Blood Bank, Stanford, CA). Human peripheral

Abbreviations: Gd-Tex, motexafin gadolinium; GSH, glutathione; PBMC, peripheral blood mononuclear cells; moi, multiplicity of infection; FACS, fluorescence activated cell sorter; NAC, *N*-acetylcysteine; RT, reverse transcriptase.

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blood monocytes were obtained by Ficoll-plaque density centrifugation (Amersham Pharmacia) of whole blood and depletion of adherent cells by adherence to plastic culture dishes. Isolated cells were maintained in complete media [RPMI medium 1640, 10% (vol/vol) FCS, 1% (vol/vol) penicillin-streptomycin glutamate (PSQ)] at 37°C and 5% CO_2. Cells were activated with human recombinant IL-2 for 24 h before HIV-1 infection. BSO treatments were preformed at 5 mM for 72 h and NAC treatments were performed at 5 mM for 24 h, as described (9, 19). Treatment samples were synchronized for time and processed simultaneously. All culture reagents were replenished every 3 days. Quantitative cell counts were obtained by using TruCount beads (Becton Dickinson).

HIV-1 Strains and Infection. HIV-1 strains are referred to as R5, X4, or R5X4 depending on the coreceptor used for viral entry. The M-tropic R5 prototype strain (BaL) was used in these studies; primary isolates were obtained from the National Institutes of Health AIDS reagent program. Virus-containing supernatants were harvested at 3, 6, 9, and 12 days and stored at −80°C. Tissue culture 50% infective dose ($TCID_{50}$) was determined in IL-2-stimulated PBMC. Cells were cultured for 24 h in IL-2 and then infected by a 2-h incubation with HIV-1_{BaL} at two doses (1,500 $TCID_{50}/1 \times 10^6$ cells, and 300 $TCID_{50}/1 \times 10^6$ cells). Every 3 days, cells were split and replenished with all stimuli. Cell-free supernatants were saved for p24 and RT activity assays, and cells were processed for flow cytometry. All HIV-1 based work was conducted at the Center for AIDS Research facility at Stanford University under BSL-3 guidelines.

Flow Cytometry and FACS Analysis. Intracellular and extracellular staining was performed as described (http://herzenberg.stanford.edu/protocols/ICCStaining.htm) but modified for monochlorobimane stain as described (20) and p24 stain. Intracellular p24 staining was achieved by directly conjugating a human anti-p24 mAb antibody to Alexa Fluor 488 (Molecular Probes). Ethidium monazide was used to discriminate between live and dead cells for intracellular staining. PBMCs ($\approx 1–10 \times 10^7$) were treated with IL-2 (100 units/ml) for 24 h and were treated subsequently with Gd-Tex, NAC, or BSO as indicated and prepared for flow cytometry. Cells were washed with buffer A (PBS, pH 7.4/0.5 mM EDTA/2.5 mM Na_2PO_4), surface-stained in buffer B (deficient RPMI/4% FCS) and stained for 20 min by using pretitred antibodies (0.1–0.8 μg of ab/1×10^6 cells). Cells were fixed and resuspended in buffer C [1% (vol/vol) paraformaldehyde in PBS]. In-between washes were performed in PBS (0.5 mM EDTA, pH 7.4). Isotype control match antibodies were used for all antibodies. Eleven-color data acquisition was collected on a modified FACStarPlus (Becton Dickinson) connected to MoFlo electronics (Cytomation, Fort Collins, CO). Data were collected by FACS-DESK software and compensated, analyzed, and presented by using FLOWJO software.

p24 Elisa and RT Assay. Supernatants from HIV-1-infected and treated cultured cells were subjected to a p24 ELISA as described by the manufacturer of the p24 ELISA kit (NEN). p24 levels were monitored at 3-day intervals and quantified by using a p24 standard curve prepared with recombinant p24. RT assays were performed as described (21) or by an RT activity assay kit (Molecular Probes), according to the manufacturer's instructions.

Results

***In Vitro* HIV Infection Depletes GSH in PBMC Cultures.** PBMC isolated from healthy donors and activated in culture with recombinant human IL-2 are readily infected *in vitro* by HIV at multiplicities of infection (mois) of 30 to 150. Single-cell fluorescence activated cell sorter (FACS) analysis of intracellular viral production (p24) indicates that >98% of $CD4^+$ cells harvested 6 days after infection are producing virus, and that virus production does not (under these conditions) in and of itself induce apoptosis (Fig. 2).

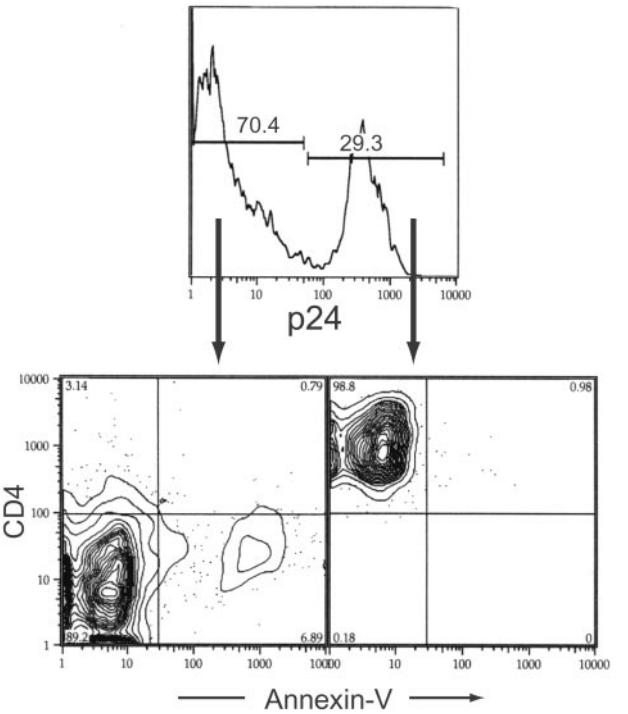

Fig. 2. Single-cell HIV-1 infection detection by intracellular p24 staining. IL-2-activated PBMC were HIV infected ($TCID_{50} = 300/1 \times 10^6$ cells), cultured for 6 days, and stained for surface-marker CD4, annexin-V, intracellular p24, and ethidium monoazide (EMA). Live gated (EMA negative) cells were gated for p24 levels and correlated with CD4 and annexin-V markers.

Concomitant analysis of GSH with the monochlorobimane assay (16) demonstrates that even at the lowest HIV dose tested, GSH levels in the infected cells are decreased roughly 8-fold for CD4 T cells and 2-fold for coresident CD8 T cells (Fig. 3A, elaborated below). This HIV-infection-mediated GSH depletion does not seem to be highly detrimental, because it does not result in apoptosis induction and does not decrease the cell yield relative to uninfected cultures (see Fig. 2).

Because GSH levels do not drop more than 10% in uninfected cultures (data not shown), the GSH decrease in CD8 T cells in the infected cultures must be a consequence of the infection. Most likely, it represents the GSH depleting activity of HIV-TAT (17), which is known to be released in HIV-infected cultures.

High-Dose Gd-Tex Depletes GSH and Is Toxic to T Cells in PBMC. At high Gd-Tex doses (>400 μM), nearly all IL-2 activated PBMC T cells die within 24 h when cultured in the presence of 1 mM Gd-Tex (Fig. 3C). However, toxicity is substantially decreased at lower Gd-Tex doses (<400 μM) and is essentially undetectable at Gd-Tex concentrations below 250 μM (Fig 3C).

The Gd-Tex toxicity that occurs at high Gd-Tex concentrations affects both CD4 and CD8 T cells and is accompanied by a decrease in GSH in both T cell subsets (Fig. 3B). This toxicity is directly related to the GSH decrease because it is significantly alleviated when *N*-acetylcysteine (NAC) is added to the culture. Indeed, NAC addition raises the maximum tolerated Gd-Tex concentration to almost 1 mM (Fig. 3C). Furthermore,

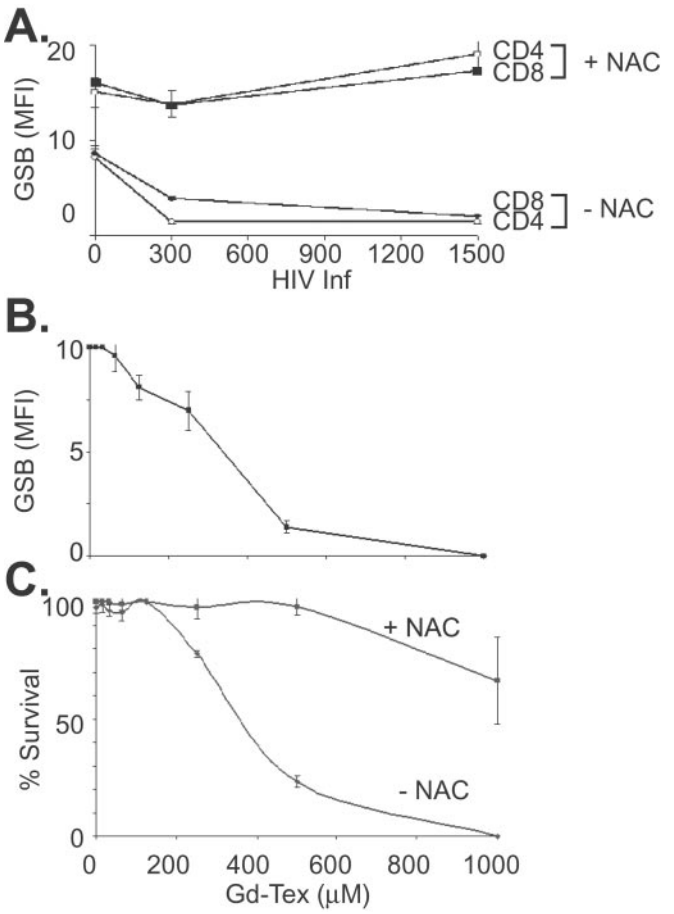

Fig. 3. HIV-1 infection and high concentrations of Gd-Tex lowers redox levels *in vitro*. (*A*) GSH levels in uninfected, HIV-1 low moi ($TCID_{50} = 300/1 \times 10^6$ cells) and HIV-1 high moi ($TCID_{50} = 1500/1 \times 10^6$ cells). PBMC were isolated and HIV-1 infected as described in *Materials and Methods*. Intracellular GSH levels were assessed by using monochlorobimane fluorescence, and samples were analyzed by flow cytometry. Median fluorescence intensity (MFI) values for monochlorobimane fluorescence (GSH-s-bimane, GSB) of uninfected, HIV-1-infected T cells ($TCID_{50}$ displayed on *x* axis). Cells were treated with (+NAC) and without NAC (−NAC) (5 mM, 24 h). (*B*) GSB levels of IL-2 activated PBMC as a function of Gd-Tex concentration. (*C*) Gd-Tex dose-response curve on whole PBMC treated in the presence (+NAC) or absence (−NAC) of NAC (5 mM, 24 h). Survival was determined by using PI-exclusion flow cytometry assay.

BSO treatment, which depletes GSH, renders PBMC more sensitive to killing with Gd-Tex (data not shown). Thus, consistent with previous indications (4), Gd-Tex toxicity in PBMC is caused by GSH depletion and the consequent induction of oxidative stress.

Low-Dose Gd-Tex Selectively Kills HIV-Infected CD4 T Cells. At lower Gd-Tex doses (<250 μM), GSH depletion and Gd-Tex toxicity are minimal (see Fig. 3*C*). However, for HIV-infected cells, even 3 μM Gd-Tex is toxic. In essence, although CD8 T cells and uninfected CD4 T cells are not affected by the presence of Gd-Tex in this dose range, HIV-infected CD4 T cells are selectively killed (elaborated below).

These findings are shown in Fig. 4 and Fig. 5, in which data are shown for subset-defining cell-surface marker expression, HIV infection, intracellular GSH, and Gd-Tex toxicity (induction of apoptosis and the breaking of the cell permeability barrier), all measured simultaneously for individual cells by 11-color, 13-parameter Hi-D FACS.

Fig. 4 demonstrates HIV-infected (moi 150; referred to as high) CD4 T cells are depleted in GSH at low Gd-Tex concentrations, compared with CD8 T cells in the same culture (Fig. 4*A*). GSH levels of two HIV-infection doses (moi 30, low; moi 150, high) of CD4 T cells decreases as a function of Gd-Tex concentration, contrasting that observed for uninfected CD4 T cells at concentrations <400 μM in IL-2 stimulated PBMC cultures (Fig. 4*B*).

Fig. 4*C* shows the Gd-Tex-mediated selective induction of apoptosis in CD4 T cells in IL-2 stimulated PBMC cultures infected with HIV. CD8 T cells in these cultures show no evidence of apoptosis induction whether measured as a percentage of cells in control culture (no Gd-Tex) harvested on the same day (Fig. 4*C*) or a percentage of initially cultured cells (data not shown).

Fig. 4*D* shows that Gd-Tex induces apoptosis in more than half the CD4 T cells in IL-2-stimulated PBMC cultures infected with HIV at two dose levels, moi 30 and moi 150. Cultures infected at these dose levels in the absence of Gd-Tex, in contrast, show <10% (Fig 4*D*). Data were similar whether computed as a percentage of apoptotic cells (Fig. 4*D*) or as cell yields (data not shown). In both cases, CD4 T cells were selectively killed/depleted by Gd-Tex. Low GSH levels correlated with induction of apoptosis as a function of Gd-Tex concentration (Fig. 4*E*). These findings are reflected in data from HIV reverse transcriptase (RT) and p24 ELISA analyses (Fig. 5 *A–B*), which show clearly that viral production ceases in Gd-Tex cultures as a function of time and concentration.

Finally, Fig. 5*C* shows that in HIV-infected cultures grown in the presence of Gd-Tex, only the CD4 T cells expressing intracellular p24 stain with annexin-V, which is roughly 50% of the p24+CD4 T cell population in the presence of 50 μM Gd-Tex. Titrations with Gd-Tex indicate that 3 μM is sufficient to induce apoptosis, as determined by annexin-V stain in the p24 positive CD4 T cells (Fig. 5*D*). Thus, Gd-Tex selectively induces apoptosis in CD4 T cells that are successfully infected with the HIV virus.

Discussion

In this report, we demonstrate that high-dose Gd-Tex depletes GSH in IL-2 activated human PBMC in short-term culture. This Gd-Tex-mediated GSH depletion occurs equally in CD4 and CD8 T cells and is equally toxic for both types of T cells. Adding NAC to the cultures prevents the Gd-Tex toxicity and thus implicates the induction of oxidative stress in the mechanism through which high-dose Gd-Tex kills cells.

Low-dose Gd-Tex, in contrast, does not deplete GSH and is not toxic to uninfected PBMC. However, in HIV-infected PBMC cultures, Gd-Tex doses as low as 3 μM selectively kill HIV-infected (p24+) CD4 T cells.

The mechanism responsible for this selective killing does not seem to depend solely on induction of oxidative stress. Although we have shown that HIV infection depletes GSH, this depletion is not as marked as the GSH depletion caused by high-dose Gd-Tex. It does not, by itself, kill a significant number of cells. Furthermore, it occurs equally in CD4 and CD8 T cells, whereas the low-dose Gd-Tex selectively kills CD4 T cells. In fact, low-dose Gd-Tex only kills HIV-infected CD4 T cells that are propagating the virus (as determined by the intracellular p24 stain), suggesting that HIV replication is itself in some way required to enable low-dose Gd-Tex toxicity.

Could Gd-Tex be useful for treating HIV infection? Perhaps it could be useful. Phase II trials of Gd-Tex in patients with brain metastasis show that it can be safely used at doses up to 6.3 mg/kg, which was used to reveal metastatic sites in MRI scans (2, 18). Pharmacokinetic studies on the steady-state concentrations of Gd-Tex from Phase II clinical trials indicate that a single i.v. dose of ≈5 mg/kg yields a maximum plasma

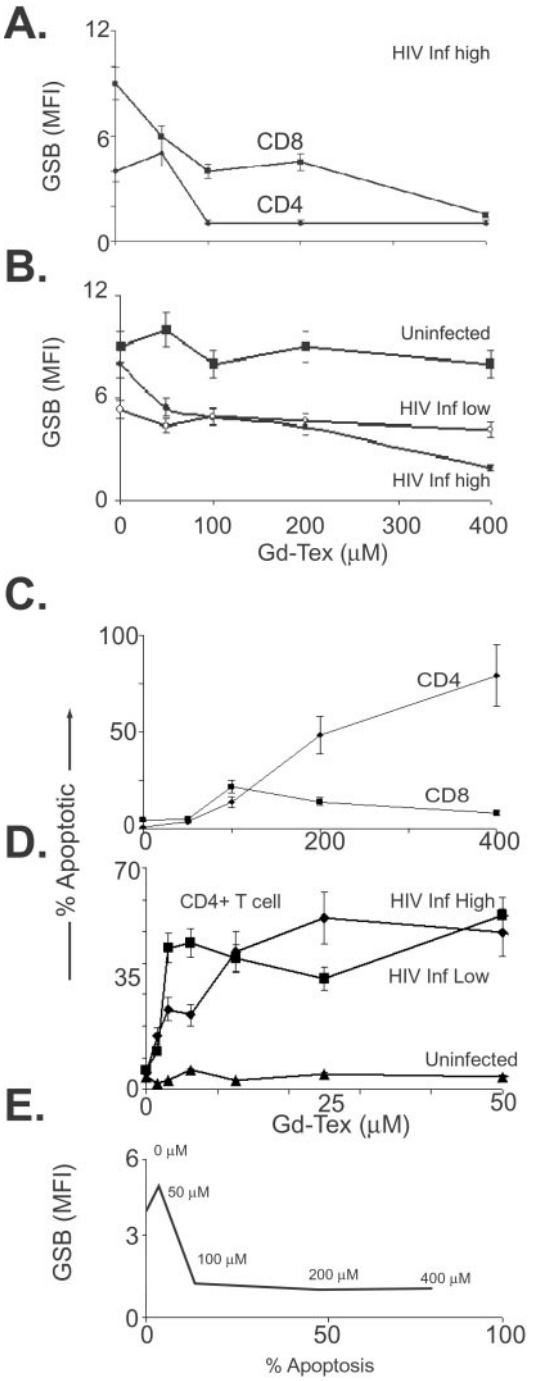

Fig. 4. Gd-Tex cytotoxicity is enhanced in CD4 T cells infected with HIV-1. (*A*) Median GSH levels of HIV-1-infected ($TCID_{50} = 1500/1 \times 10^6$ cells) CD4 and CD8 T cells as a function of Gd-Tex concentration (post 6 days). (*B*) Median GSH levels of uninfected, high HIV-1-infected and low HIV-1-infected CD4 T cells as a function of Gd-Tex treatment (post 3 days). Error bars denote SD of nine independent experiments from 12 healthy donors. (*C*) Gd-Tex induces apoptosis selectively in HIV-1-infected CD4 T cells. PBMC were infected with a high HIV-1 dose, incubated for 3 days with indicated concentrations of Gd-Tex, and assessed for annexin-V by flow cytometry. Live CD3 cells were gated, and apoptotic percentages were quantified for CD4 and CD8 cells. (*D*) Gd-Tex-induced apoptosis in live CD4 T cells infected with HIV-1. PBMC were infected with high and low HIV-1 doses and treated for 6 days with the indicated concentrations and processed as indicated above. (*E*) Low median GSB is proportional to the induction of apoptosis as a function of Gd-Tex concentration. Median GSB values and percent apoptotic cells for CD4 T cells infected with HIV-1 (low) were plotted as a function of Gd-Tex concentration after a 6-day treatment. Error bars denote SDs of at least three independent experiments.

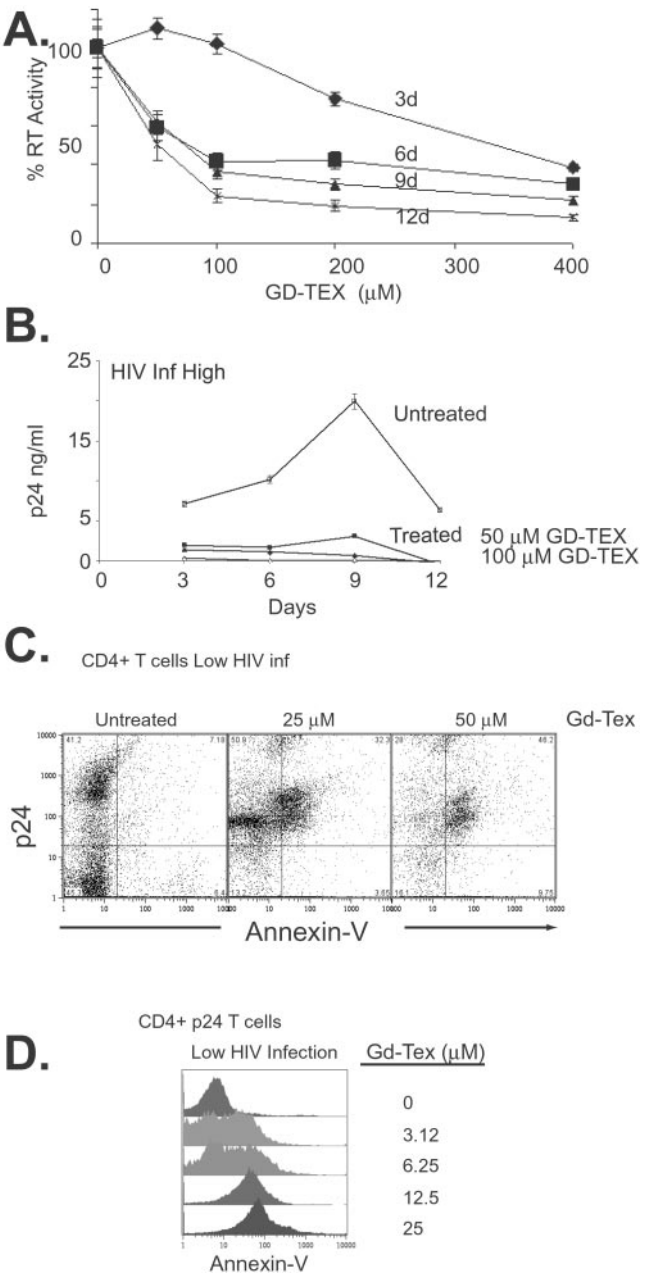

Fig. 5. Inhibition of HIV-1 production by Gd-Tex. (*A*) RT activity assay of HIV-1-infected PBMC ($TCID_{50} = 300/1 \times 10^6$ cells) as a function of Gd-Tex concentration and time. HIV-1-infected PBMC were incubated with Gd-Tex at the indicated concentration, and cell-free supernatants were collected after 0, 3, 6, 9, and 12 days. Diluted supernatants were spotted in 96-well plates, and RT activity was determined by an RT activity assay kit (Molecular Probes) and previously used conventional radioactive RT activity measurements (data not shown). Values are normalized to HIV-1-infected Gd-Tex untreated cells. (*B*) p24 levels over time as a function of Gd-Tex treatment for HIV-1 infection ($TCID_{50} = 300/1 \times 10^6$ cells). p24 levels were determined by p24 ELISA and normalized to a p24 standard curve. Error bars denote SD of at least three independent experiments from nine healthy donors. (*C*) Intracellular p24 stain of CD4 T cells infected with HIV-1 and treated with Gd-Tex at indicated concentrations for 6 days. Live CD4 T cells were gated and analyzed for annexin-V and p24 stain. Note decrease of p24 cells in the 50-μM treated culture, likely because of their depletion under Gd-Tex culture conditions. (*D*) Titration of p24+ CD4 T cells with Gd-Tex. IL-2-activated, HIV-infected cultures were treated with Gd-Tex at the indicated concentrations for 6 days and processed for flow cytometry. Cells were gated for live p24 positive CD4 T cell and displayed for annexin-V stain. Histograms are representative of four independent experiments.

concentration of 62.5 μg/ml (18). *In vitro* conditions reported here demonstrate effective killing of HIV-infected cells at levels ranging from 3 to 100 μM (3.4 to 114.8 μg/ml). If HIV-infected cells take up Gd-Tex as well as tumor cells, and if this uptake is sufficient to kill the infected cells *in vivo*, then Gd-Tex could prove quite useful for removing HIV-producing cells. Future studies will help to evaluate these possibilities. Identification of the mechanism responsible for the selective killing of HIV-infected cells by Gd-Tex will clearly contribute to this effort.

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1. Sessler, J. L. & Miller, R. A. (2000) *Biochem. Pharmacol.* **59,** 733–739.
2. Radford, I. R. (2000) *Curr. Opin. Investig. Drugs* **1,** 524–528.
3. Rosenthal, D. I., Nurenberg, P., Becerra, C. R., Frenkel, E. P., Carbone, D. P., Lum, B. L., Miller, R., Engel, J., Young, S., Miles, D. & Renschler, M. F. (1999) *Clin. Cancer Res.* **5,** 739–745.
4. Magda, D., Lepp, C., Gerasimchuk, N., Lee, I., Sessler, J. L., Lin, A., Biaglow, J. E. & Miller, R. A. (2001) *Int. J. Radiat. Oncol. Biol. Phys.* **51,** 1025–1036.
5. Bump, E. A. & Brown, J. M. (1990) *Pharmacol. Ther.* **47,** 117–136.
6. Israel, N. & Gougerot-Pocidalo, M. A. (1997) *Cell. Mol. Life Sci.* **53,** 864–870.
7. Herzenberg, L. A., De Rosa, S. C., Dubs, J. G., Roederer, M., Anderson, M. T., Ela, S. W. & Deresinski, S. C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1967–1972.
8. James, J. S. (1997) *AIDS Treat. News***,** 1–5.
9. Staal, F. J., Roederer, M., Raju, P. A., Anderson, M. T., Ela, S. W. & Herzenberg, L. A. (1993) *AIDS Res. Hum. Retroviruses* **9,** 299–306.
10. Raju, P. A., Herzenberg, L. A. & Roederer, M. (1994) *AIDS Res. Hum. Retroviruses* **10,** 961–967.
11. Roederer, M., Staal, F. J., Raju, P. A., Ela, S. W. & Herzenberg, L. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4884–4888.
12. Lachgar, A., Sojic, N., Arbault, S., Bruce, D., Sarasin, A., Amatore, C., Bizzini, B., Zagury, D. & Vuillaume, M. (1999) *J. Virol.* **73,** 1447–1452.
13. Opalenik, S. R., Ding, Q., Mallery, S. R. & Thompson, J. A. (1998) *Arch. Biochem. Biophys.* **351,** 17–26.
14. Baier-Bitterlich, G., Wachter, H. & Fuchs, D. (1996) *Science* **271,** 582–583.
15. Lee, R., Beauparlant, P., Elford, H., Ponka, P. & Hiscott, J. (1997) *Virology* **234,** 277–290.
16. Anderson, M. T., Trudell, J. R., Voehringer, D. W., Tjioe, I. M. & Herzenberg, L. A. (1999) *Anal. Biochem.* **272,** 107–109.
17. Sawaya, B. E., Khalili, K., Gordon, J., Taube, R. & Amini, S. (2000) *J. Biol. Chem.* **275,** 35209–35214.
18. Carde, P., Timmerman, R., Mehta, M. P., Koprowski, C. D., Ford, J., Tishler, R. B., Miles, D., Miller, R. A. & Renschler, M. F. (2001) *J. Clin. Oncol.* **19,** 2074–2083.
19. Staal, F. J., Anderson, M. T., Staal, G. E., Herzenberg, L. A. & Gitler, C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3619–3622.
20. De Rosa, S. C., Zaretsky, M. D., Dubs, J. G., Roederer, M., Anderson, M., Green, A., Mitra, D., Watanabe, N., Nakamura, H., Tjioe, I., *et al*. (2000) *Eur. J. Clin. Invest.* **30,** 915–929.
21. Yahi, N., Baghdiguian, S., Moreau, H. & Fantini, J. (1992) *J. Virol.* **66,** 4848–4854.