Diazepam-Mediated Inhibition of Human Immunodeficiency Virus Type 1 Expression in Human Brain Cells

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Diazepam (Valium) is a benzodiazepine (BDZ) analog that readily crosses the blood-brain barrier (BBB) and is widely prescribed for anxiety disorders (2, 13). Most of diazepam’s anxiolytic and sedative attributes are manifested through a central BDZ receptor, an allosteric site on the gamma-aminobutyric acid A receptor located in the central nervous system (CNS) (22, 32). However, diazepam also binds to a pharmacologically distinct peripheral BDZ binding site that has been identified in various tissue and cell types (5, 7, 19). BDZ receptor agonists are classified according to their affinity for one or both of these receptors, either pure central (e.g., clonazepam), pure peripheral (e.g., RO5-4864), or mixed (e.g., diazepam) agonists. In addition to its anxiolytic attributes, diazepam possesses immunomodulatory and anti-inflammatory properties (4, 21, 36).

The exact function of the peripheral BDZ receptors is unclear, but it is believed that they mediate the immunomodulatory properties of BDZs in mononuclear phagocytes (11). Peripheral as well as mixed BDZ ligands show dose-dependent suppressive effects on lipopolysaccharide-induced tumor necrosis factor alpha production by murine peritoneal macrophages (21, 35). In vivo treatment of mice with peripheral (RO5-4864) and mixed (diazepam), but not central, BDZs significantly impairs the ability of peritoneal and splenic macrophages to produce several key mediators of inflammation (36). In addition to central BDZ receptors, peripheral BDZ receptors have recently been found on rodent brain cells including astrocytes (3, 16, 23) and microglia (24). Microglia, the resident macrophages of the brain, are a primary cell type permissive for human immunodeficiency virus type 1 (HIV-1) infection within the human CNS (18, 28, 34). Because of the immunomodulatory properties of BDZs and the presence of peripheral BDZ receptors on glial cells, we tested the hypothesis that diazepam treatment would alter HIV-1 expression in human brain cells.

In mixed glial-neuronal cell cultures (27), a culture system that mirrors the brain microenvironment in that it contains primarily astrocytes and neurons with interspersed microglia and oligodendrocytes, diazepam was found to be a potent inhibitor of HIV-1 strain SF162 (9) p24 antigen (Ag) production with a 50% inhibitory concentration (IC50) of approximately 8 μM (Fig. 1A). Representatives of the other two classes of BDZ receptor ligands, included here for comparison, also inhibited HIV-1 expression in these cultures, but not as potently as diazepam. The pure central ligand (clonazepam) and the pure peripheral ligand (RO5-4864) each inhibited viral p24 Ag production with IC50s of approximately 10 and 27 μM, respectively (Fig. 1A). Similar results were obtained in three separate experiments using glial-neuronal cell cultures derived from different brain specimens. None of these BDZs, at the concentrations used in these experiments, appeared to be cytotoxic, as assessed by trypan blue dye exclusion or by lactate dehydrogenase release assay (4, 8, 43, and 4.5 U/ml for 100 μM diazepam, RO5-4864, and clonazepam, respectively, versus 4.2 U/ml for untreated control).

Because microglial cells are a primary site for HIV-1 replication within the CNS (14, 18, 28, 34), and we have previously demonstrated that this is also the only cell type in our mixed glial-neuronal cell cultures that is permissive for viral replication (20), we next evaluated the effect of diazepam on HIV-1 expression in highly enriched human microglial cell cultures (8). Diazepam treatment was again found to potently inhibit HIV-1 p24 Ag production in a dose-dependent manner with an IC50 of approximately 3 μM (Fig. 1B). The pure central ligand (clonazepam) and the pure peripheral ligand (RO5-4864) each inhibited viral p24 Ag production with IC50s of approximately 65 and 10 μM, respectively (Fig. 1B). Similar results were obtained in three separate experiments using microglia isolated from different brain specimens. No evidence of BDZ-induced toxicity, as assessed by trypan blue dye exclusion, was observed.

Because chronically infected monocytes are thought to be a vehicle for viral entry into the CNS (the so-called “Trojan Horse” hypothesis) and to be a reservoir of HIV-1 within the brain, experiments were also performed with chronically infected promonocytes. U1 cells, a promonocytic line that contains integrated copies of HIV-1 proviral DNA, produce very little HIV-1 p24 Ag under basal conditions; however, HIV-1 expression can be induced by plating these cells at a high density. We found that diazepam treatment also inhibited HIV-1 expression in these chronically infected cells (Fig. 1C). This suggests that the diazepam-induced suppression of HIV-1 occurs after infection. Although diazepam inhibited HIV-1 expression in U1 cells, the pure peripheral agonist RO5-4864 was the most potent among the three ligands tested, with an approximate IC50 of 3 μM versus 12 μM for diazepam (Fig. 1C). Also, in contrast to the findings in mixed glial-neuronal
and enriched microglial cell cultures, the pure central ligand (clonazepam) had a nominal antiviral effect in U1 cells (Fig. 1C). Similar results were obtained when HIV-1 expression was induced by treating the U1 cells with 20 pg of tumor necrosis factor alpha per ml (i.e., diazepam inhibited p24 expression with an IC50 of approximately 12 μM, and clonazepam treatment had little effect).

Taken together, the data from mixed glial-neuronal and enriched microglial cell cultures suggest that diazepam inhibits HIV-1 expression during acute infection of human brain cells. The presence of multinucleated giant cells (syncyia), which result from fusion of microglia, is the histopathological hallmark of HIV-1 encephalitis (6, 10). Hence, we next investigated the effect of diazepam on multinucleated giant-cell formation in HIV-1-infected microglial cell cultures. In contrast to uninfected control cells (Fig. 2A), microglial cells formed syncyia in response to HIV-1 infection (Fig. 2B), and immunofluorescence staining with an anti-p24 monoclonal antibody (Dako, Carpinteria, Calif.) demonstrated that these syncyia were active sites of viral replication (Fig. 2B). Infected microglial cell cultures treated with diazepam (20 μM) displayed marked reduction in syncytium formation as well as p24 Ag staining (Fig. 2C).

The mechanism underlying BDZ-induced inhibition of HIV-1 expression is unclear. It is well known that cellular activation is a key requirement for HIV-1 replication in infected cells, and transcription factors that participate in HIV-1 expression could be key therapeutic targets for antiviral agents. Nuclear factor kappa B (NF-κB) is an important transcriptional activator of inflammatory mediators, including certain cytokines, chemokines, and adhesion molecules (30), and it is well established that activation of this transcription factor is necessary for self-perpetuated HIV-1 replication in mononuclear phagocytes (1, 17, 26, 29, 33). Thus, we hypothesized that the mechanism of diazepam’s anti-HIV-1 effect involved an inhibition of NF-κB activation. Nuclear extracts (31) were obtained from 5 × 10^6 chronically infected U1 cells which were induced by high-density plating and treated with diazepam, as well as the other prototypical classes of BDZs, to determine the effect on NF-κB activation. Although equal amounts of nuclear extract (2 μg) were added for different binding reactions, a decrease in NF-κB binding was observed in the diazepam- and RO5-4864-treated cultures, whereas clonazepam treatment had little effect (Fig. 3A and B). This differential effect of the three BDZs was consistent with their effects on p24 Ag production in U1 cells, presented above.

Because acutely infected monocytes may also serve as a vehicle for HIV-1 entry into the brain, we next examined the effect of diazepam treatment on HIV-1-induced NF-κB activation in acutely infected monocyte-derived macrophages (MDM). Using nuclear extracts from these cells (2 × 10^6), we again found evidence of decreased activation of NF-κB after diazepam treatment (Fig. 3C). The reduced activation of NF-κB in MDM was associated with an 84% suppression of p24 Ag production at 14 days after infection, following treat-
ment with 20 μM diazepam, compared to untreated HIV-1 infected MDM. These data suggest that the anti-HIV-1 effects observed with diazepam are mediated, at least in part, through inhibition of activation of the NF-κB transcription factor.

The prototypical BDZ drugs used in this study (clonazepam, diazepam, and RO5-4864) differ from the BDZ-derived TIBO [tetrahydroimidazo(4,5,1-jk)](1,4)-benzodiazepin-2(1H)-one and -thione] compounds (e.g., R14458) and the BDZ-derived Tat inhibitors (i.e., RO5-3335 and RO5-24-7420), whose anti-HIV-1 activity has been well studied in cell lines (reviewed in references 15 and 25). TIBO compounds inhibit HIV-1 reverse transcriptase yet are devoid of any BDZ-like pharmacological effects (25). Previous experiments have shown that TIBOs and other reverse transcriptase inhibitors (e.g., zidovudine) do not suppress HIV-1 expression in chronically infected promono-

FIG. 2. Effect of diazepam treatment on the formation of multinucleated giant cells (syncytia). (A) Uninfected human microglia display no positive staining for viral p24 Ag and no syncytium formation after 14 days in culture. (B) Immunofluorescence staining with a monoclonal antibody to HIV-1 p24 capsid protein shows that the syncytia formed by fusion of human microglia, 14 days after infection with HIV-1pGluc, are active sites of viral expression (intense green fluorescence). (C) Absence of syncytia and decreased p24 Ag levels in the presence of 20 μM diazepam.

cytes (U1 cells) which already contain two copies of integrated provirus. Regarding Tat inhibitors, the affinity of RO5-3335 for the BDZ receptor is less than 1% of that of diazepam (15), and HIV-1 replication during chronic infection of macrophages is not inhibited by BDZ-derived Tat inhibitors (12). It is currently unknown if diazepam possesses any anti-RT or anti-Tat activity.

Because many of the presently used antiretroviral drugs do not readily cross the BBB, an urgent need exists to identify novel approaches for stopping HIV-1 replication within this viral sanctuary. These in vitro studies show that diazepam, which is currently one of the most widely prescribed medications in the world (13), markedly inhibits HIV-1 expression in human brain cells, as well as in cells that may carry HIV-1 into the brain. This inhibition occurs at clinically achievable concentrations (2) and appears to be mediated through a cellular mechanism involving decreased activation of NF-κB. Interruption of HIV-1 expression by psychotropic agents such as diazepam, which readily cross the BBB and interfere with activation of cellular transcription factors, could offer novel therapeutic approaches to the management of HIV-associated neurological disease.

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