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Benzodiazepines, Glia, and HIV-1 Neuropathogenesis

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Abstract

Although the precise mechanisms whereby HIV-1 infection induces neurodegeneration have yet to be determined, a great deal of evidence has incriminated glial cells and the production of proinflammatory mediators in this pathologic process. For this reason, ideal therapeutic agents for the treatment of AIDS dementia would attenuate HIV-1 neuropathogenesis through both direct inhibition of viral expression and suppression of brain cell-produced immune mediators. Benzodiazepines (BDZs), such as Valium, are extensively prescribed drugs for anxiety disorders, which readily cross the blood–brain barrier and have demonstrated immunomodulatory properties. BDZs bind to primary human microglial cells, the principal site of HIV-1 replication in the brain, and inhibit lipopolysaccharide (LPS) induced tumour necrosis factor (TNF-α) production by these cells in a concentration-dependent manner. Treatment of HIV-1-infected primary human microglial, as well as mixed glial/neuronal, cell cultures with BDZs inhibits the expression of HIV-1 p24 antigen. BDZ-induced inhibition of HIV-1 expression in chronically infected promonocytic (U1) cells has been found to be associated with decreased activation of the nuclear transcription factor kappa B (NF-κB). Because HIV-1 expression is critically dependent on the cellular transcription machinery, inhibition of the activation of transcription factors, which participate in both HIV-1 expression and the production of neurotoxic immune mediators, by BDZ analog may provide new therapeutic options for AIDS dementia.

Index Entries: Benzodiazepines; diazepam; HIV-1; cytokines, NF-κB.

Introduction

BDZs are a class of widely prescribed anxiolytic drugs, which also possess immunomodulatory and anti-inflammatory properties. Patients who are infected with HIV-1 or have developed AIDS have a high incidence of anxiety or adjustment disorders with anxious mood, and BDZs (e.g., Valium) are commonly used to treat such anxiety. Here, evidence is reviewed that suggests that BDZs, which are already used in many HIV-1 patients, may have additional beneficial effects for treatment or prevention of AIDS-associated neurological disease.

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Benzodiazepines (BDZs)

Classification of BDZs

Two pharmacologically distinct types of BDZ receptors have been described: a central receptor, found in the CNS (Mohler and Okada 1977; Squires and Braestrup, 1977) and a peripheral receptor, found primarily on the outer mitochondrial membrane of cells from various somatic tissues (Braestrup and Squires, 1977; Le Fur et al., 1983; Canat et al., 1993). Prototypical BDZ agonists are grouped based on their selective affinity for one or both of these receptors: classified as either central (e.g., clonazepam); mixed (e.g., diazepam); or peripheral (e.g., RO5-4864) agonists. Most of the sedative properties of these drugs are manifested through the central BDZ receptor, an allosteric site on the gamma-aminobutyric acid ($GABA_A$) receptor located in the CNS (Mohler and Okada, 1977; Squires and Braestrup, 1977). 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 (Bessler et al., 1992; Matsumoto et al., 1994; Drughan, 1994).

In addition to central BDZ receptors (Braestrup and Squires, 1977; Squires et al., 1979; Schoemaker et al., 1981; McCauley et al., 1995), the presence of peripheral BDZ receptors has also been reported on cells of the CNS. This receptor has been found on a murine microglial cell line (Park et al., 1996) as well as on cultured mouse and rat astrocytes (Bender and Hertz, 1987; Oh et al., 1992; Itzhak et al., 1993). We have recently initiated studies of the interaction of BDZs with primary human microglial cell cultures. As seen in Fig. 1, [H^3] diazepam binds specifically to human fetal microglial cells, and this binding is incubation time-dependent.

The fact that BDZs readily cross the blood–brain barrier potentially gives them a major therapeutic advantage in the treatment of diseases characterized by production of neurotoxic immune mediators. Ideal therapeutic drugs for the treatment of AIDS dementia should not only readily cross the blood–brain barrier, but should also possess both direct antiviral as well as anti-inflammatory activities. Both of these properties have been demonstrated with BDZs.

It should be pointed out that the prototypical BDZ drugs discussed in this article (i.e., clonazepam, diazepam, and RO5–4864) differ from the BDZ derivative tetrahydroimidazo [4, 5, 1-jk][1,4]-benzodiazepin-2(1H)-one and thione (TIBO) compounds (e.g., R14458) or the BDZ-derived Tat inhibitors (i.e., Ro 5-3335, and Ro 5–24–7420), whose anti-HIV-1 activity has been well studied (reviewed in Pauwels, 1993; Hsu and Tam, 1993). TIBO compounds inhibit HIV-1 reverse transcriptase yet are devoid of any BDZ-like pharmacological effects (Pauwels, 1993). Previous experiments have shown that TIBOs and other RT inhibitors (e.g., zidovudine) do not suppress HIV-1 expression in the chronically infected promonocyte U1 cell line, which contains two copies of integrated provirus. Regarding Tat inhibitors, Ro 5–3335's affinity for the BDZ receptor is < 1% of that of diazepam, and Ro 5–24–7420 has < 0.1% of the affinity of diazepam for CNS BDZ receptors (Hsu and Tam, 1993). The ability of Tat inhibitors to suppress HIV-1 expression during chronic infection is controversial, and probably depends on the type of infected cell and differences in cellular cofactors required for optimum Tat function (Dunne et al., 1994). At the present time, it is unknown if the prototypical BDZ analogs discussed in the present article possess any anti-RT or anti-Tat activity.

Immunomodulatory Properties of BDZs

The concept that BDZs possess immunomodulatory activities is consistent with the known effects of psychological stress on immune function. Experimentally, BDZs have been shown to have a number of immunoregulatory and anti-inflammatory properties both in vitro and in vivo. Peripheral as well as mixed BDZ ligands show dose-dependent suppressive effects on lipopolysaccharide (LPS) induced tumor necrosis factor-α (TNF-α) production by murine peritoneal macrophages

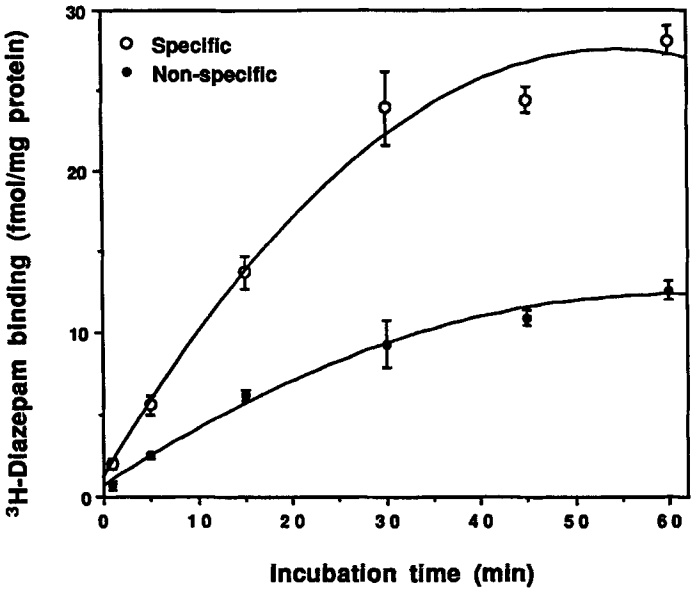

Fig. 1. ^{3}H-diazepam binding to primary microglial cells. Human fetal microglial cells (10^5 cells) were incubated with^{3}H-diazepam (5 n*M*) for the indicated time periods prior to harvesting. Nonspecific binding was determined through the simultaneous addition of 3 μ*M* unlabeled diazepam to the microglial cultures. Data (mean ± SEM) shown are representative of two independent experiments using different microglial cell specimens.

(Zavala et al., 1985; Matsumoto et al., 1994). In vivo treatment of mice with peripheral (RO5–4864) and mixed (diazepam), but not central (clonazepam) BDZs significantly impairs the capacity of peritoneal and splenic macrophages to produce several key mediators of inflammation (i.e., reactive oxygen intermediates [ROI], interleukin [IL]-1, TNF, and IL-6) (Zavala et al., 1990). In human peripheral blood mononuclear cell (PBMC) cultures, peripheral and mixed BDZ suppress both phytohemagglutinin (PHA) and concanavalin A- (Con A) induced lymphocyte proliferation as well as IL-3 secretion (Bessler et al., 1992). Taken together, these findings support the notion that BDZs have anti-inflammatory properties.

Glia

BDZ Inhibition of Glial Cell Cytokine Production

Proinflammatory cytokines produced by glial cells (i.e., microglia and astrocytes) appear to be an integral part of the host immune response to CNS insults. However, excessive production of these cytokines may also lead to some aspects of the pathophysiology associated with neuroAIDS. Microglia, when activated, are a rich source of cytokines (Benveniste, 1994; Chao and Hu, 1994), several of which (e.g., TNF-α IL-1β, and IL-6) have been implicated in neurological disease (Dickson et al., 1993). Astrocytes comprise about 75% of all the cells within the cerebral cortex, and although monocytotropic strains of HIV-1 do not appear to replicate within astrocytes, limited expression of lymphocytic viral strains does occur in these cells (Nath et al., 1995). Whether they support HIV-1 replication or not, astrocytes have clearly been implicated in viral pathogenesis (Nottet et al., 1995; Ranki et al., 1995). Like microglia, activated astrocytes release cytokines that appear to be involved in the neurodegeneration, which is characteristic of AIDS dementia complex (Benveniste, 1994; Chao et al., 1995).

Because excessive production of proinflammatory cytokines may lead to neuronal cell

injury, treatments that suppress the production of these cytokines in microglial cells may also attenuate HIV-1 neuropathogenesis. We have recently found that peripheral and mixed, but not central BDZ receptor ligands inhibit LPS-induced TNF-α production by primary human microglial cells (Fig. 2). This finding suggests that BDZs will affect the production of TNF-α, as well as other proinflammatory cytokines, in brain cells stimulated with HIV-1.

Nitric oxide (NO) is another neurotoxic immune mediator, which is produced during inflammatory responses. NO production by cytokine-activated monocytes has been implicated in recent studies of HIV-1-related neuropathogenesis (Bukrinsky et al., 1995). Research on human microglial cells suggests that activated microglia release NO (Ding et al., 1997), but it may not be in sufficient quantities to kill neurons, which contrasts sharply with the neurotoxic capability of murine microglia in this regard (Peterson et al., 1994). However, cytokine-activated human astrocytes produce high levels of NO, and substantial amounts of nitrite, a metabolite of NO, can be detected in supernatants from activated astrocyte cultures (Lee et al., 1993; Hu et al., 1995). IL-1 is the critical, if not the only, cytokine that is capable of activating astrocyte-inducible nitric oxide synthase (iNOS) and NO production (Lee et al., 1993). Astrocyte-produced NO is neurotoxic when evaluated in an in vitro human fetal neuronal culture system (Chao et al., 1996). ND has been implicated in several neurodegenerative diseases. However, the precise mechanism underlying the NO-induced neuropathogenesis of AIDS dementia is still largely unknown (Adamson et al., 1996). We have examined the presence of nitrites (NO_2^-) in astrocyte cultures that have been treated with interferon-γ and IL-1β in the presence of diazepam. Significantly less nitrite (approx 40%) was detected in BDZ-treated astrocyte cultures (unpublished observation). It has been found that IL-1-induced astrocyte NO production is mediated through a nuclear transcription factor (NF-κB) mechanism (Chao et al., 1997), a finding that suggests that diazepam's mechanism of action may be mediated through the inhibition of transcription factor activation.

HIV-1 Neuropathogenesis

Neuro AIDS

Even though the precise mechanisms whereby HIV-1 induces neurodegeneration have yet to be determined, a large body of evidence has incriminated glial cells and proinflammatory cytokines in this phenomenon. The complex interplay of these and other factors has been the subject of intensive investigation in several laboratories (Gendelman et al., 1989; Atwood et al., 1993; Chao et al., 1993; Lipton and Gendelman, 1995; Masliah et al., 1996). Proinflammatory cytokines, as well as other mediators, released by activated glial cells during HIV-1 infection within the CNS modulate viral expression and induce neuronal injury (reviewed in Gendelman et al., 1997).

Studies of the brains of patients with HIV-1-related dementia typically reveal:

1. Microglial nodules associated with multinucleated glial cells (the histopathologic hallmark of AIDS dementia),
2. Astrogliosis
3. Extensive changes in subcortical gray and white matter, as well as substantial loss of neurons in the frontal, parietal, and temporal cortex (Kaufman 1992; Masliah et al., 1994). Recent autopsy findings suggest that neuronal cell death in AIDS dementia occurs via an apoptotic pathway associated with elevated expression of TNF-α, IL-1, IL-4, and IL-6 (Petito and Roberts, 1995; An et al., 1995). Additionally, TNF-α has been shown to potentiate glutamate neurotoxicity (Chao and Hu, 1994), and IL-1 plus TNF-α synergistically mediate neurotoxicity via a mechanism involving astrocyte production of the neurotoxic free radical NO and NMDA receptors (Chao et al., 1995).

Viral Replication in the CNS

The findings that HIV-1 is not tropic for neurons and that a paucity of intact virus is found within the brains of patients with AIDS

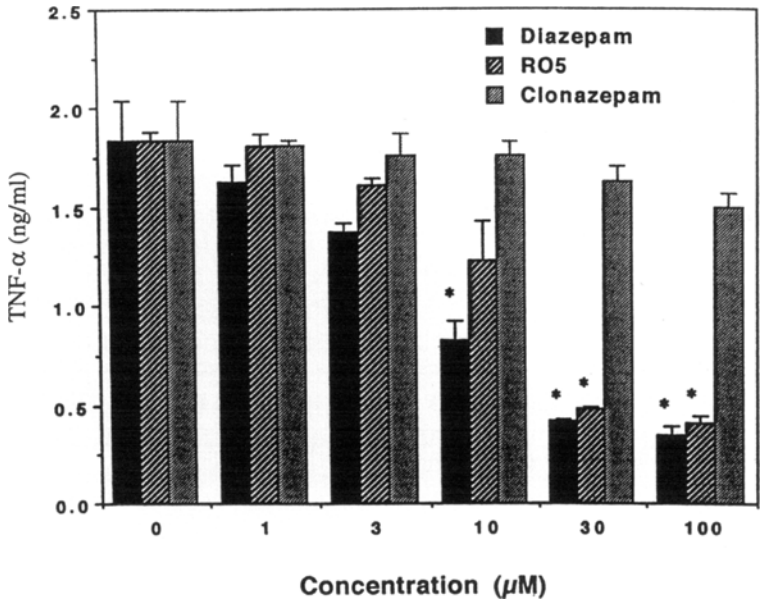

Fig. 2. Concentration-dependent BDZ-mediated inhibition of LPS-induced TNF-α production by human microglial cells. Highly enriched (>99%) human microglial cells (5×10^4 cells/well) were pretreated for 30 min in the presence or absence of the indicated concentrations of each class of BDZ agonist. Following pretreatment, the cells were stimulated with 1 μg/mL LPS and the supernatants were assayed 8 h poststimulation for TNF-α production (ng/mL). Results shown are representative of two independent experiments using different brain cell specimens.

dementia have supported the concept of an indirect mechanism of HIV-1-induced neurodegeneration involving the cells and mediators discussed above. However, recent evidence, primarily obtained by *in situ* PCR, has indicated that AIDS dementia is associated with massive, activated HIV-1 infection of the brain including microglial cells and astrocytes, as well as neurons themselves (Nuovo and Alfieri 1996). It has also been reported that levels of HIV-1 RNA (i.e., viral load) in the cerebrospinal fluid correlate directly with the stage of AIDS dementia (Brew et al., 1997). In addition, several research groups have provided evidence (using neuronal cells from nonhuman sources or tumor cell lines and transgenic mouse models) that the HIV-1 proteins gp120 (Brenneman et al., 1988; Dryer et al., 1990; Lipton, 1992), gp41 Adamson et al., 1996; and Tat (Sabater et al. 1991) can induce neuronal cell injury. Also, Tat has been shown to be toxic to cultures of human fetal neurons via an NMDA receptor mechanism (Magnuson et al., 1995), and both gp120 and Tat have been demonstrated to induce cytokine production by mononuclear phagocytes (Clouse et al., 1991; Scala et al., 1994; Koka et al., 1995) Although it has not been proven that viral load in the CNS plays a critical role in neurodegeneration, there is mounting evidence that AIDS dementia is associated quantitatively with the amount of HIV-1 expression in the brain. However, it is doubtful that HIV-1 infection is the only factor responsible for AIDS dementia. Therefore, successful therapeutic strategies for AIDS dementia must be designed not only to arrest viral replication within the brain, but also to reduce viral-induced cellular activation, along with the associated production of inflammatory mediators that precipitate the process of neurodegeneration.

BDZ Effects on HIV-1 Expression in Brain Cells

Because of the demonstrated immunomodulatory properties of BDZs and the presence of peripheral BDZ receptors on glial cells, we investigated whether BDZ treatment would alter HIV-1 expression in human brain cells. All classes of BDZ ligands were found to be potent inhibitors of HIV-1 p24 Ag expression in mixed human glial/neuronal cultures, as well as purified human microglial cells, and in both systems, the suppressive effects of these drugs were dose-dependent (Fig. 3) (Lokensgard et al., 1997a), Further experiments have determined that the approximate IC_{50}s for the BDZs in mixed human glial/neuronal cultures are 8, 10, and 27 μ*M* for diazepam, RO 5–4864, and clonazepam, respectively. None of these BDZs appeared to be cytotoxic to the brain cell cultures, as assessed by trypan blue dye exclusion or by lactate dehydogenase release assay (4.8, 4.3, and 4.5 U/mL for 100 μ*M* diazepam, RO5-4864, and clonazepam, respectively, vs 4.2 U/mL for untreated control).

Because microglial cells are a primary site for HIV-1 replication within the CNS (Koenig et al., 1986; Price et al., 1988; Watkins et al., 1990; He et al., 1997), 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 (Lokensgard et al., 1997b), we next evaluated the effect of diazepam on HIV-1_{SF162} expression in highly enriched human microglial cell cultures. BDZs were also found to inhibit HIV-1 expression in purified microglial cells with IC_{50}s of 3, 10, and 65 μ*M* for diazepam, Ro 5–4864, and clonazepam, respectively (Fig. 3) (Lokensgard et al., 1997)

Involvement of NF-κB in BDZ-Induced HIV-1 Suppression

The mechanism underlying BDZ-induced inhibition of HIV-1 expression is unclear. Following HIV-1 infection, the virus integrates into the cellular genome, and expression of this integrated provirus is regulated by cellular transcription factors through the same mechanisms that control activity of endogenous cellular genes. Gene expression is regulated at the transcriptional level by binding of nuclear factors to *cis*-acting DNA motifs in the promoters of both cellular and viral genes. Subsequently, these transcription factors activate gene expression, depending on the particular complement of nuclear factors with which they interact.

Cellular activation is a key requirement for productive HIV-1 replication in microglial cells, (reviewed in Gendelman et al., 1997). NF-κB is an important transcriptional activator of inflammatory mediators, including certain cytokines, chemokines, and adhesion molecules in mononuclear phagocytes, (reviewed in Roulston et al., 1995). Activated NF-κB consists of a heterodimer of two proteins: p65 and p50. An inactive form, which consists of the p65/p50 heterodimer complexed to an inhibitory protein (I-κB), occurs in the cytoplasm. Extracellular signals (e.g., cytokines) activate various kinases, that phosphorylate I-κB and induce its dissociation from the heterodimer. Free NF-κB heterodimer is then translocated to the nucleus, where it binds to specific DNA motifs in the promoter region of its target genes. Because anxiolytic drugs downregulate TNF-α production and the synthesis of this cytokine is initiated through a mechanism involving NF-κB (Collart et al., 1990), it has been proposed that the immunomodulatory action of these drugs is mediated through inhibition of transcription factor activation. In addition, it is well established that activation of the NF-κB transcription factor is necessary for self-perpetuated HIV-1 replication in cells of the mononuclear phagocyte lineage (Suzan et al., 1991; Bachelerie et al., 1991, Paya et al., 1992; Roulston et al., 1993; Jacque et al., 1996; Folgueira et al., 1996). Thus, the intimate involvement of this transcription factor with HIV-1 replication makes it a potentially important target for therapeutic manipulations.

We examined the hypothesis that the mechanism of BDZ-induced anti-HIV-1 activity involved an inhibition of NF-κB activation (Lokensgard et al., 1997a) Nuclear extracts (Schreiber et al., 1989) were obtained from 5 ×

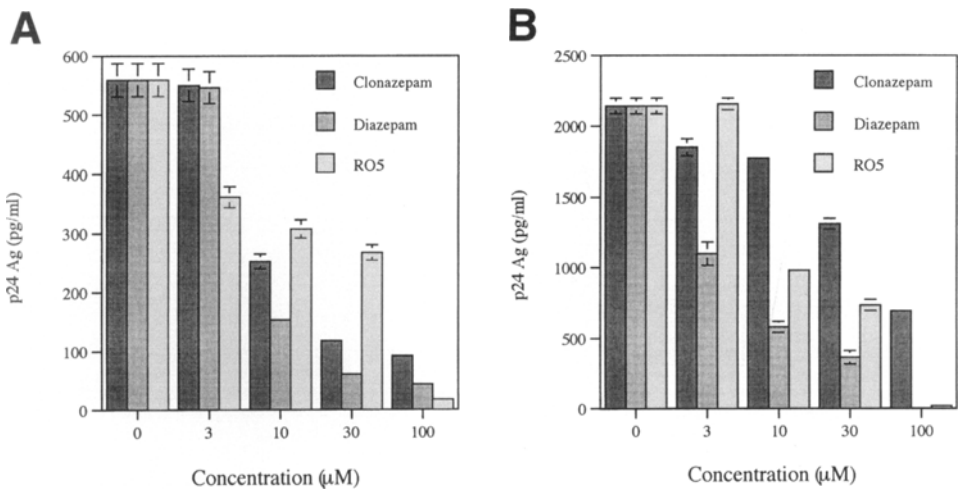

Fig. 3. Concentration-dependent BDZ-mediated inhibition of HIV-1 p24 Ag expression in human brain cell cultures. (**A**) Mixed glial/neuronal cell cultures (1×10^5 total cells/well) were infected with HIV-1_{SF162} in the presence or absence of each class of BDZ receptor ligand at the indicated concentrations for 14 days. Supernatants were then assayed for p24 Ag levels. Results shown are representative of three independent experiments using different brain cell specimens. (**B**) Highly enriched (>99%) human microglial cells (1×10^5 cells/well) were infected with HIV-1_{SF162} in the presence or absence of BDZ receptor ligands. Following infection, cultures were incubated at the indicated concentrations for 14 d; the supernatants were then assayed for p24 Ag levels. Results shown are representative of three independent experiments using microglial cells derived from different specimens (redrawn from Lokensgard et al. [1997a]).

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 to each binding reaction, a decrease in NF-κB binding was observed in the diazepam- and RO5-4864-treated cultures, whereas clonazepam treatment had little effect (Fig. 4A,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. 4C) (Lokensgard et al., 1997a). The reduced activation of NF-κB in MDM was associated with an 84% suppression of p24 Ag production at 14 d after infection, following treatment with 20 μ*M* diazepam, when 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 current data show that BDZs suppress HIV-1 expression by a mechanism involving inhibition of cellular transcription factor activation, including NF-κB. Through this mechanism, TNF-α and other neurotoxic, proinflammatory molecules are likely to be concurrently inhibited.

Summary

The anti-HIV-1 agents presently being used (i.e., nucleoside analog and protease inhibitors) directly target enzymes involved in viral replication. However, with the exception of Tat (the viral *trans*-activator), HIV-1 expression is criti-

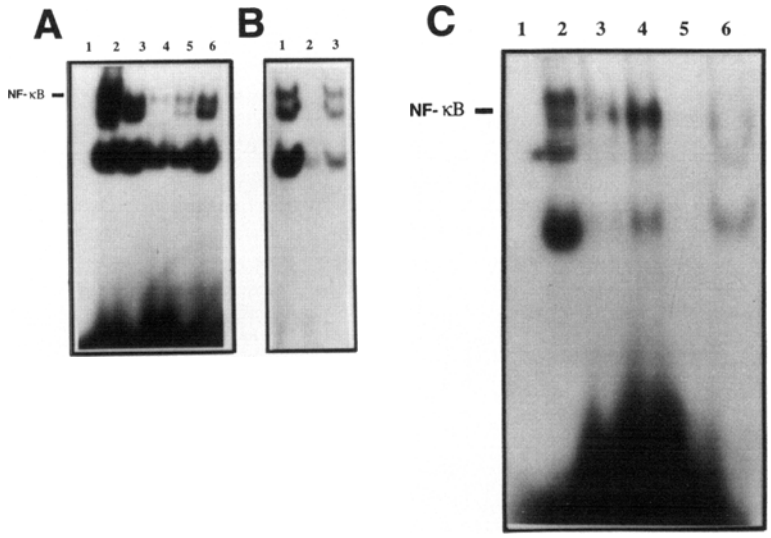

Fig. 4. Effect of diazepam treatment on activation of NF-κB. (**A**) Electrophoretic mobility shift assay using U1 cells. Nuclear extracts (2 μg) were probed for transcription factor binding to a [^{32}P] NF-κB-specific oligonucleotide; lane 1, probe alone; lane 2, HeLa cells (control for NF-κB binding); lane 3, untreated; lane 4, RO5-4864-treated (10 μ*M*); lane 5, diazepam-treated (10 μ*M*); lane 6, clonazepam-treated (10 μ*M*). (**B**) Specificity controls; lane 1, untreated; lane 2, untreated with 50-fold excess unlabeled NF-κB probe; lane 3, untreated with 50-fold excess unlabeled mutant NF-κB binding site. (**C**) Band-shift assay using extracts from MDM. Nuclear extracts (1 μg) were probed for NF-κB binding activity: lane 1, probe alone; lane 2, HeLa cell extract; lane 3, uninfected MDM cell extract; lane 4, HIV-1-infected, 3 d after infection; lane 5, HIV-1-infected, 3 d after infection in the presence of 20 μ*M* diazepam; lane 6, HIV-1-infected, 3 d after infection (same extract as lane 4), with anti-p65 antibody (reprinted from Lokensgard et al. [1997a] with permission from the publisher).

cally dependent on host cell transcription machinery. Thus, therapeutic targeting of cellular factors that participate in HIV-1 transcription may provide additional ways to inhibit viral neuropathogenesis. In future studies, the anti-HIV-1 activity of related, and possibly more potent, BDZs that act within the CNS could be examined. Interruption of HIV-1 expression, as well as the production of neurotoxic proinflammatory mediators, through BDZs, which readily cross the blood brain barrier and interfere with activation of cellular transcription factors, could offer novel therapeutic approaches to the management of HIV-associated brain disease.

Acknowledgments

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