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*Short Communication*

# Diazepam inhibits HIV-1 Tat-induced migration of human microglia

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**During HIV-1 encephalitis, the chemotaxis-inducing activity of Tat may enhance the viral life cycle through recruitment of additional susceptible microglial cells to foci of infection. Benzodiazepines (BDZs) readily penetrate the blood–brain barrier and are known to possess anti-inflammatory properties. Pretreatment of human microglial cells with peripheral (Ro5-4864) and mixed (diazepam), but not central (clonazepam), benzodiazepine receptor ligands was found to potently suppress HIV-1 Tat-induced chemotaxis. Application of Tat to microglial cells evokes an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) that rapidly desensitizes the cells. Diazepam's inhibitory effect was associated with its ability to block Tat-induced $[Ca^{2+}]_i$ mobilization. These data support the notion that through their effects on microglia, peripheral BDZ receptor ligands could alter the neuropathogenesis of HIV-1.** *Journal of NeuroVirology* (2001) **7,** 481–486.

**Keywords:** diazepam; HIV-1; microglia; Tat

## Introduction

HIV-1 Tat is known to induce chemotaxis of monocytes and macrophages and may facilitate their infiltration into the CNS paremchyma during AIDS dementia (Lafrenie *et al*, 1996; Albini *et al*, 1998a). Tat has been shown to be secreted extracellularly by infected cells (Ensoli *et al*, 1993; Westendorp *et al*, 1995), and when injected intraventricularly into rats it causes an influx of inflammatory cells including neutrophils, macrophages, and lymphocytes (Jones *et al*, 1998). Tat may stimulate chemotaxis of monocytes in the CNS directly through Cys-Cys-Phe domains, a motif characteristic of numerous $\beta$-chemokines (Albini *et al*, 1998b), or indirectly through the induction of monocyte-chemoattractant protein (MCP)-1 production by neighboring astrocytes (Weiss *et al*, 1999). Immunohistochemical studies of brain tissue from patients with HIV-1 encephalitis have demonstrated that Tat is primarily localized within and adjacent to microglial cell nodules (Bonwetsch *et al*, 1999). Thus, Tat secretion, and its corresponding chemotaxis stimulating activity, may enhance the HIV-1 life cycle through the recruitment of additional susceptible microglial cells, the principal cell type supporting HIV-1 replication in the brain, to foci of viral infection.

Benzodiazepine (BDZ) receptor ligands, such as diazepam (Valium), are extensively prescribed drugs for anxiety disorders. A number of investigators over the past several decades have demonstrated that BDZs also possess immunomodulatory properties. 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 tissues (Braestrup and Squires, 1977; Le Fur *et al*, 1983; Canat *et al*, 1993). Prototypical BDZ receptor ligands are grouped based on their selective affinity for one or the other of these receptors: classified as either central, peripheral, or mixed agonists. Most of the sedative properties of these drugs are

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manifested through the central receptor, an allosteric site on the gamma-aminobutyric acid ($GABA_A$) receptor. The exact function of the peripheral receptors is unclear, but it is believed that they mediate the immunomodulatory properties of BDZs in mononuclear phagocytes (Bessler *et al*, 1992; Drugan, 1994; Matsumoto *et al*, 1994). In the present study, we tested the hypothesis that ligands of the peripheral BDZ receptor type would inhibit the migration of human microglia towards HIV-1 Tat.

*Human microglial cells migrate towards HIV-1 Tat*

Before investigating whether BDZ receptor ligands affect Tat-induced migration, we first determined the chemotactic effect of Tat on human microglial cells at various concentrations (ranging between 1–100 ng/ml). For these studies, a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD) was used to measure the migration of microglia towards medium (random migration) or HIV-1 Tat protein. The human microglial cell cultures were prepared using a previously described technique (Chao *et al*, 1994). Human fetal brain tissues were obtained from aborted fetuses under a protocol approved by the Human Subjects Committee at our institution. Chemotaxis was measured as previously described (Yao *et al*, 1990; Chao *et al*, 1997) with minor modifications. Briefly, a 5-$\mu$m polyvinalpyrrolidone-free filter separated the upper and lower compartments of the chamber. Microglial cells were added to the upper chamber and, after a 3-h incubation period, the nonmigrating cells were gently scraped from the upper surface of the filter. Cells on the lower surface were fixed in methanol and stained with Diff-Quik (Baxter, McGraw Park, IL). An investigator blind to the experimental conditions counted the number of cells migrating to the underside of the filter microscopically. Five high-power fields (HPF)/well of triplicate wells were examined at 400×, and cell numbers were averaged. Data are expressed as mean ± SEM.

In these experiments, microglial cells were found to migrate towards HIV-1 Tat in a concentration dependent manner with both 30 and 100 ng/ml resulting in robust stimulation (Figure 1). Based on these data a Tat concentration of 30 ng/ml was chosen for use in further experiments to assess the effects of BDZs on microglial cell chemotaxis. In addition, the Tat-stimulated directional movement of microglial cells was found to be pertussis toxin-sensitive and cholera toxin-insensitive, indicating that this chemotactic response was mediated largely by $G_i$ proteins (Figure 1). Studies from other laboratories on the role of G proteins in Tat-mediated chemotaxis of monocytes have yielded similar results (Albini *et al*, 1998b).

*Peripheral, but not central, benzodiazepine receptor ligands inhibit Tat-induced chemotaxis*

Pretreatment of microglial cells with mixed (diazepam) and peripheral (Ro5-4864), but not central (clonazepam), BDZ receptor agonists, 30 min prior to Tat exposure (30 ng/ml), was found to potently suppress Tat-induced chemotaxis (Figure 2). This BDZ-mediated effect was concentration-dependent with highly significant ($P < 0.01$) inhibition at a dose of 4 $\mu$M; 74.9% and 75.3% inhibition for diazepam and RO5-4864, respectively; and maximal inhibition at a concentration of 20 $\mu$M (86% and 83.2% inhibition for diazepam and RO5-4864, respectively; Figure 2). None of the BDZ receptor ligands alone had a significant effect on random microglial cell migration (Figure 2).

Because the suppressive effects of BDZ treatment on chemotaxis towards Tat were mediated by peripheral, but not central, BDZ receptor ligands, we performed displacement studies on human microglial cells with $^3$H-diazepam. In these experiments, a 30-min pretreatment of microglial cells with the peripheral agonist Ro5-4864 blocked the subsequent

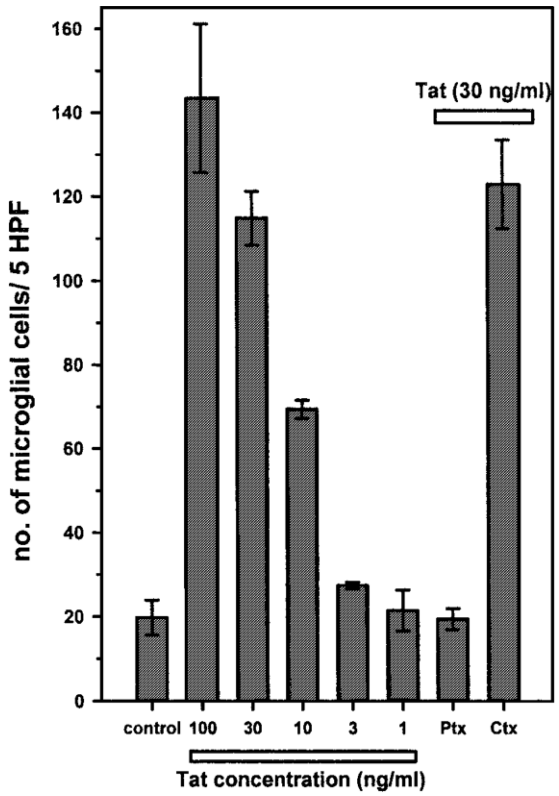


**Figure 1** Concentration-dependent induction of microglial cell migration towards increasing amounts of HIV-1 Tat. Human microglial cells were loaded into the upper wells of chemotaxis chambers that contained HIV-1 Tat (at the indicated concentrations) or medium alone (control) in the lower wells. After a 3-h incubation period, cells that migrated from the upper to lower compartments were collected, fixed, and stained. Five high-power fields (HPF) of triplicate wells were examined at 400×, and cell numbers were averaged. A 30-min pretreatment of the microglial cells with pertussis toxin (Ptx, 10 ng/ml) caused a marked decrease in chemotaxis to HIV-1 Tat (30 ng/ml), cholera toxin (Ctx, 10 ng/ml) did not significantly affect microglial cell migration. Data are mean ± SEM of triple values and are representative of at least three independent experiments using microglia from different brain specimens.

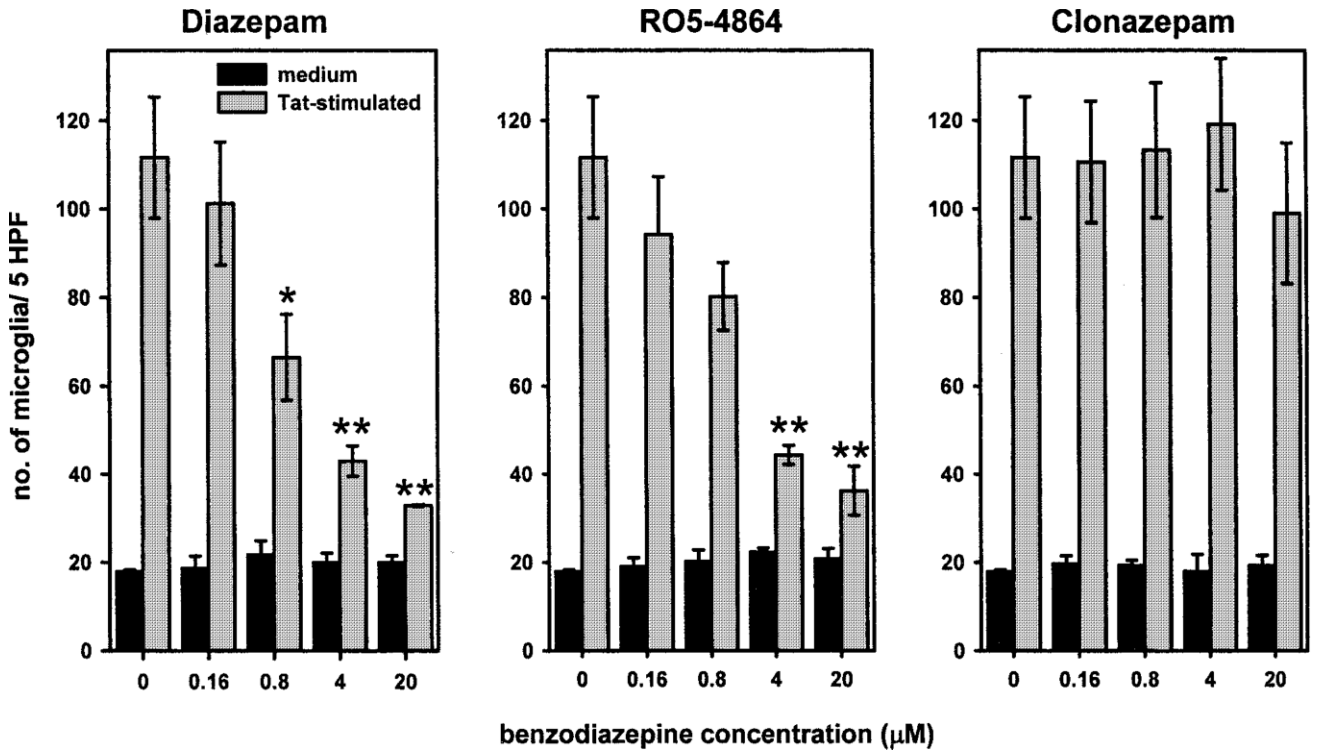


**Figure 2** Effect of BDZ receptor ligands on Tat-induced migration. Microglia were incubated in medium alone or medium containing either mixed (diazepam), peripheral (Ro5-4864), or central (clonazepam) BDZ receptor ligands, at the indicated concentrations, for 30 min prior to being loaded into the upper wells of chemotaxis chambers which contained HIV-1 Tat (30 ng/ml) or medium in the lower wells. Results are mean ± SEM number of cells that migrated through filters after 3-h incubation, assessed by examining 5 HPF, and are pooled data derived from three separate experiments using microglia from different brain specimens. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, Student's *t*-test.

binding of $^{3}$H-diazepam (88 ± 3.8% displacement, n = 3). In contrast, a 30-min pretreatment with the central agonist clonazepam had no significant effect on subsequent $^{3}$H-diazepam binding (5.7 ± 3.6% displacement, n = 3). Thus, microglial cells appear to possess peripheral, but not central, BDZ binding sites.

*Diazepam treatment blocks Tat-induced increases in intracellular calcium $[Ca^{2+}]_i$*

Changes in $[Ca^{2+}]_i$ serve, in general, as a good indication of the activation of G protein-coupled receptors. Because the chemotactic response of phagocytic cells is known to be associated with changes in intracellular calcium concentration ($[Ca^{2+}]_i$), we next tested the hypothesis that diazepam would block Tat-induced $[Ca^{2+}]_i$ changes as a potential mechanism underlying its inhibitory effects on microglial cell migration. In these experiments, $[Ca^{2+}]_i$ was measured using a previously described dual emission microfluorimeter (Werth and Thayer, 1994) to monitor the fluorescent chelator, indo-1 (Grynkiewicz *et al*, 1985). Briefly, microglial cells were loaded with indo-1 by incubation with 2 $\mu$M indo-1/acetoxymethyl ester for 45 min at 37°C, in HHSS, containing 0.5% BSA. Loaded cells were then mounted in a flow through chamber for viewing (Thayer *et al*, 1988). The superfusion chamber was mounted on an inverted microscope and cells were superfused with HHSS at a rate of 1.0–1.5 ml/min for 15 min prior to starting an experiment.

We have previously shown that application of HIV-1 Tat to microglial cells evokes an increase in ($[Ca^{2+}]_i$) that rapidly desensitizes (Hegg *et al*, 2000). In the present study, Tat (50 ng/ml) again triggered a robust change in $[Ca^{2+}]_i$ in microglial cells (Figure 3). Diazepam treatment (4 $\mu$M) for 10 min prior to the addition of Tat (2 min) blocked the ability of these cells to mobilize $Ca^{2+}$ (Figure 3). Thus, diazepam's inhibitory effect on microglial cell chemotaxis was found to be associated with its ability to block Tat-induced $[Ca^{2+}]_i$ mobilization.

*Diazepam treatment inhibits Tat-induced upregulation of the CCR5 chemokine receptor on human microglia*

Flow cytometry was used to determine the effect of diazepam treatment on the regulation of chemokine receptors on microglial cells. Microglial cells were cultured in Teflon vials in the absence (control) or presence of diazepam (20 $\mu$M) for 30 min prior to exposure to HIV-1 Tat (30 ng/ml). The microglial cells were stained using a FITC-labeled MAb to human CCR5 (Pharmingen, San Diego, CA). As shown in Figure 4A, there was a prominent shift in the

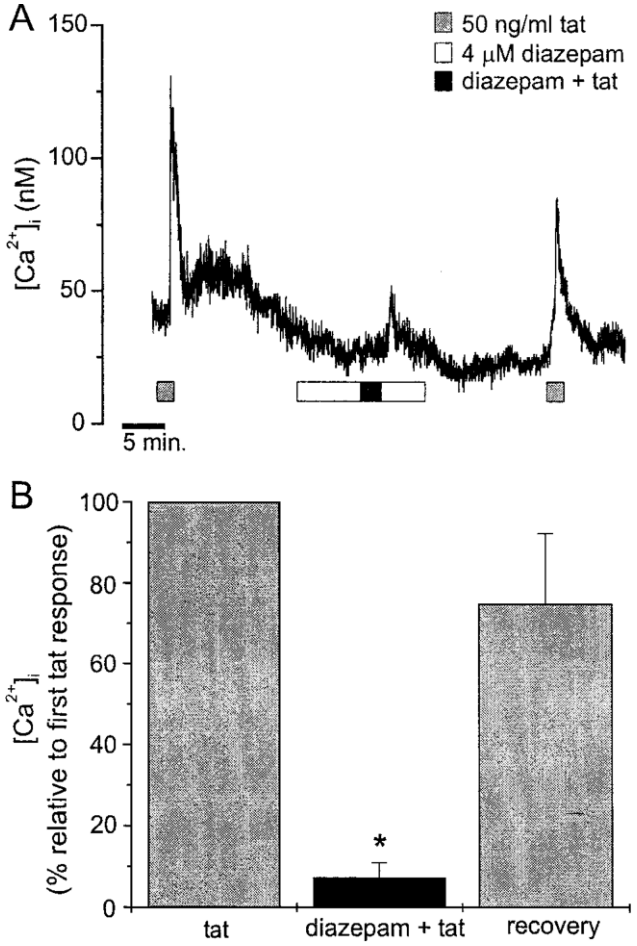


**Figure 3** Diazepam treatment blocks Tat-induced increases in intracellular calcium. Changes in intracellular calcium ($[Ca^{2+}]_i$) were measured as follows: after acquiring a 5-min $[Ca^{2+}]_i$ baseline, Tat (50 ng/ml) was superfused for 2 min. Following return to basal $[Ca^{2+}]_i$ the solution was changed to 4 $\mu$M diazepam in HEPES-buffered Hank's Salt Solution (HHSS) and superfused for 10 min. Tat was then applied in the presence of diazepam for 2 min. Diazepam was superfused for an additional 6 min before washing with HHSS. A third Tat response was elicited 20 min later. (A) Tat application evoked an increase in $[Ca^{2+}]_i$ that rapidly desensitized. (B) Data were normalized to the first Tat-evoked $Ca^{2+}$ transient, averaged and expressed as mean ± SEM ($n = 4$ experiments with microglia from different brain specimens). $^*P < 0.05$, Student's *t*-test.

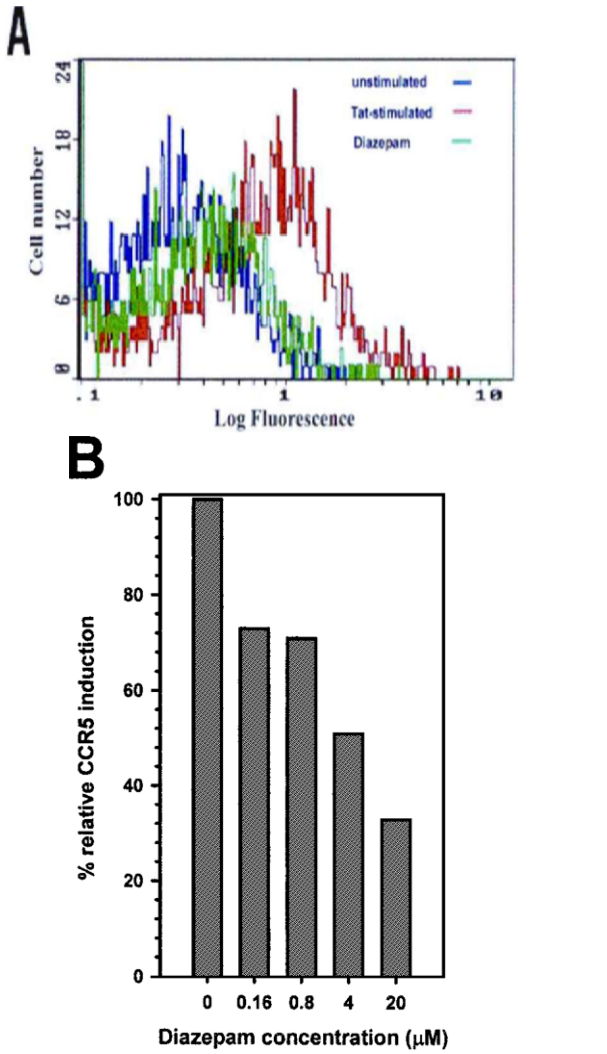


**Figure 4** Diazepam inhibits Tat-induced upregulation of the CCR5 chemokine receptor on human microglia. (A) Surface expression of the CCR5 chemokine receptor on total ungated microglia analyzed by flow cytometry. Microglial cells were cultured in Teflon vials without Tat stimulation, or stimulated with HIV-1 Tat (30 ng/ml), or were pretreated for 30 min with 20 $\mu$M diazepam prior to exposure to HIV-1 Tat, for 48 h. The cells were then stained using a FITC-labeled MAb to human CCR5 (Pharmingen, San Diego, CA). 10,000 events were recorded. (B) % of cells positive for Tat-induced CCR5 expression following 30-min diazepam pretreatment at the indicated concentrations (0.16–20 $\mu$M). Results are representative of two experiments using microglia from different brain specimens.

fluorescence profile following Tat-treatment, indicating that Tat induces upregulation of the $\beta$-chemokine receptor CCR5. CCR5 surface expression was detected on 14% of the untreated microglia (total ungated cells, n = 2 experiments), which increased to 24.3% following Tat induction. However, histograms obtained from microglial cells subjected to diazepam treatment prior to Tat induction do not show the shifted fluorescence profile, with 12.9% positive following 20 $\mu$M diazepam (Figure 4A). Interestingly, Tat treatment had little effect on the CCR3 chemokine receptor, which was detected on 6.9% of the untreated microglia. Additional experiments showed that the diazepam-mediated suppression of CCR5 expression was concentration-dependent, with 67% suppression of CCR5 at 20 $\mu$M diazepam (Figure 4B).

Results generated during this study further support the idea that peripheral BDZs possess immunomodulatory properties. Peripheral as well as mixed BDZ ligands have been shown to have concentration-dependent suppressive effects on lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-$\alpha$ production by murine peritoneal macrophages (Matsumoto *et al*, 1994; Zavala *et al*, 1984). Furthermore, *in vivo* treatment of mice with peripheral and mixed, but

not central, BDZs significantly impairs the capacity of peritoneal and splenic macrophages to produce several key mediators of inflammation including reactive oxygen intermediates, interleukin (IL)-1, IL-6, and TNF-$\alpha$ (Zavala *et al*, 1990).

PK 11195 is described as a selective antagonist that blocks ligand binding to the peripheral benzodiazepine receptor (Le Fur *et al*, 1983). This reagent has been reported to antagonize the suppressive effect of 4′-chlorodiazepam on LPS-induced TNF-$\alpha$ production from murine macrophages (Matsumoto *et al*, 1994). However, PK 11195 has also been reported to behave itself as a potent agonist inhibiting concanavalin A-induced interleukin (IL)-3 production by human PBMCs (Bessler *et al*, 1992). In our hands, in various assays PK 11195 gives variable results, sometimes acting as an antagonist and sometimes acting as an agonist. At high concentrations, it has agonist activity and at low concentrations it is often not sufficient to block the effects of peripheral benzodiazepines. Taken together, these results demonstrate that the antagonistic effects of PK 11195 do not always follow the same trend.

In addition to their immunomodulatory properties, we have previously shown that BDZs possess direct antiviral effects on HIV-1 p24 production in human microglial cells (Lokensgard *et al*, 1997). Furthermore, this inhibition of viral protein production is associated with decreased activation of NF-$\kappa$B (Lokensgard *et al*, 1997). Thus, therapeutic targeting of microglia with peripheral BDZs may induce a state of general cellular "deactivation" resulting in decreased activation of transcription factors, reduced chemotactic ability, inhibition of $Ca^{2+}$-mediated signaling, and diminished production of cytokines, as well as decreased HIV-1 protein production.

Ideal therapeutic agents for the treatment of AIDS dementia would attenuate HIV-1 neuropathogenesis through both direct inhibition of viral expression and suppression of neuroinflammation. Both of these properties have been demonstrated with BDZs. The results of this study suggest that it may be possible to exploit peripheral BDZs and capitalize on their demonstrated ability to penetrate the blood-brain barrier for the purpose of treating brain damage associated with AIDS dementia, as well as other neuroinflammatory diseases.

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