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U50,488 inhibits HIV-1 Tat-induced monocyte chemoattractant protein-1 (CCL2) production by human astrocytes

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Abstract

κ-Opioid receptor (KOR) ligands have been reported to alter many cell functions and to exert an immunomodulatory role in the CNS. Astrocytes, the predominant brain cell type, have been implicated in the neuropathogenesis of human immunodeficiency virus type 1 (HIV-1). HIV-1 nuclear protein Tat has been reported to induce production of the chemokine monocyte chemoattractant protein-1 (MCP-1 or CCL2) and to activate nuclear factor κB (NF-κB) in human astrocytes. In the present study, we investigated whether the synthetic KOR ligand *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate (U50,488) would down-regulate MCP-1 production in primary human astrocytes stimulated by Tat. Treatment of astrocytes with U50,488 inhibited Tat-induced MCP-1 production in a concentration-dependent manner. The KOR-selective antagonist nor-binaltrophimine (nor-BNI) completely blocked the inhibitory effect of U50,488, indicating involvement of KOR. While U50,488 alone had a partial inhibitory effect on constituent NF-κB activation, it potently suppressed Tat-induced NF-κB activation. These findings suggest that KOR ligands could have an anti-inflammatory effect in the CNS and thereby be beneficial in the treatment of HIV-1-associated brain disease.

Keywords: Astrocytes; κ-Opioid receptor; MCP-1; Tat; U50,488

1. Introduction

The effects of opioids are mediated mainly through opioid receptors classified as mu (μ), kappa (κ), and delta (δ) opioid receptors. A number of opioid agonists and antagonists have been evaluated in *in vitro* studies and for therapeutic potential in HIV-1 infection. In our laboratory, we have found that treatment of microglia [1] or activated $CD4^+$ lymphocytes [2] with the synthetic KOR agonist *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate (U50,488) down-regulated HIV-1 replication through a KOR-mediated mechanism. U50,488 also suppressed the neurotoxicity mediated by supernatants derived from HIV-1-infected microglia [3].

HIV-1 Tat, a transactivating and non-structural viral protein, has been implicated in the neuropathogenesis of HIV infection [4]. Tat can be detected in the serum and brains of HIV-1-infected patients [4–6]. It also has been reported to be neurotoxic [7,8] and is capable of stimulating cytokine and chemokine production by glial cells [9–11]. These Tat-induced cytokines and chemokines could further exert autocrine or paracrine effects on surrounding cells. Tat also has been shown to evoke intracellular Ca^{2+} flux in microglia [12,13], which could potentially result in a cascade of events to disrupt cellular functions and network communication in the brain.

One of the chemokines induced by Tat is MCP-1 (also known as CCL2), which is important in recruiting monocytes and activated lymphocytes to sites of inflammation [14–16]. Infiltration of these cells into the CNS may contribute to the development of HIV-1-related dementia. Up-regulation of MCP-1 production in human MDM infected with HIV has been reported [17]. MCP-1 has been found to be expressed in the brains and detected in the cerebrospinal fluid of patients with HIV-1-associated dementia [18,19]. In the CNS, both microglia, the resident

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Abbreviations: FBS, fetal bovine serum; GFAP, glial fibrillary acid protein; HIV-1, human immunodeficiency virus type 1; KOR, κ-opioid receptor; MCP-1 (or CCL2), monocyte chemoattractant protein-1; MDM, monocyte-derived macrophages; NF-κB, nuclear factor κB; nor-BNI, nor-binaltrophimine.

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macrophages of the brain, and astrocytes are sources of MCP-1 production upon activation, and Conant *et al*. [18] have detected MCP-1 positive cells as astrocytes and neurons in brain sections of patients with HIV-1-related dementia.

In the CNS, astrocytes are the predominant cell type, accounting for approximately 70% of the cells within the brain. Astrocytes play important supportive functions, including serving as a glutamate sink to protect neurons against excitotoxic injury. Even though astrocytes are relatively inefficient in supporting HIV-1 replication, they may contribute substantially to the pathogenesis of HIV-1 infection [18]. In this study, we investigated whether U50,488 exerted any effect on Tat-induced MCP-1 production in human astrocytes.

2. Materials and methods

2.1. Reagents

The following reagents were obtained from the sources indicated: recombinant human MCP-1 and anti-MCP-1 antibodies (R&D Systems); anti-GFAP (an astrocyte marker) antibody (Dako); donkey anti-goat IgG horseradish-peroxidase conjugate (Jackson Immunoresearch); HIV-1 Tat (NIH AIDS Research and Reference Reagent Program); U50,488 (a gift from The Upjohn Co., now the Pharmacia Corp.); nor-BNI (provided by P.-S. Portoghese, University of Minnesota); Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salts (HBSS), trypsin, BSA, polyoxyethylenesorbitan monolaurate (Tween 20), PBS, pyrrolidinedithiocarbamate (PDTC), Trypan Blue, methylthiazoletetrazolium (MTT), penicillin and streptomycin (Sigma); ApopTag *in situ* apoptosis detection kit (Intergen); FBS (HyClone); and K-Blue substrate (Neogen).

2.2. Astrocyte cultures

Human fetal brain tissues were obtained from 16- to 22-week-old aborted fetuses under a protocol approved by the Human Subjects Research Committee at our institution. Brain tissues were cleared of meninges, triturated, and incubated with 0.25% trypsin for 30 min at 37°. A final concentration of 10% FBS was added, and the tissues were centrifuged at 600 *g* for 10 min at room temperature and washed twice with HBSS. After 5–10 passages of tissue fragments in DMEM (containing 10% FBS, penicillin [100 U/mL], streptomycin [100 μg/mL]) through a 5-mL pipette, the cell suspensions were seeded into 75 cm^2 flasks at a density of $(80-100) \times 10^6$ cells/flask and incubated at 37° in a 10% CO_2 incubator. Culture medium was changed at a weekly interval. On day 21, flasks were shaken at 180–200 rpm for 16 hr followed by washing and trypsinization with 0.25% trypsin in HBSS for 30 min at 37°. After adding FBS (final concentration 10%), centrifugation, and washing, cells were seeded into new flasks with DMEM followed by medium change after 24 hr. The subculture procedure was repeated four times at a weekly interval to achieve highly purified astrocyte cultures (>99% of cells stained with anti-GFAP antibody).

2.3. ELISA

To assay MCP-1 in astrocyte culture supernatants, ELISA plates (96-well) were coated with mouse anti-human MCP-1 (1–2 μg/mL) antibodies overnight at 4°. The plates were blocked with 1% BSA in PBS for 1 hr at 37°. After washing with PBS supplemented with Tween 20 (0.05%) (PBST), astrocyte culture supernatants or serial dilutions of MCP-1 (as standard) were added to the wells for 2 hr at 37°. Following washing, detection antibody (goat anti-human MCP-1 antibody, 1:1000) was added for 90 min at 37° followed by donkey anti-goat IgG horseradish-peroxidase conjugate (1:10,000) for 45 min. A chromogen substrate, K-Blue, was then added at room temperature for color development, which was stopped with 1 M H_2SO_4. The plate was read at 450 nm to generate a standard curve for MCP-1 concentration extrapolation.

2.4. NF-κB assay

Following treatment of astrocytes with medium or Tat with or without U50,488 for designated periods of time, cell extracts were used for Trans-AM^{TM} NF-κB assay according to the protocol of the manufacturer (Active Motif).

2.5. Statistical analysis

Data are expressed as means ± SEM. For comparison of multiple means, ANOVA was used, followed by Fisher's protected least significant difference (PLSD) test.

3. Results and discussion

MCP-1 production by Tat-stimulated astrocytes (10–300 ng/mL) was found to be concentration-dependent, and maximal levels (8.93 ± 0.58 ng/mL, mean ± SEM, $N = 4$) were achieved with 100 ng/mL of Tat (data not shown). Thus, throughout the study 100 ng/mL of Tat was used, unless otherwise noted. A low level of constitutive MCP-1 production (1.38 ± 0.02 ng/mL, mean ± SEM, $N = 6$) by unstimulated astrocytes was detected.

3.1. Effect of U50,488 on Tat-induced MCP-1 production

Astrocytes were incubated with U50,488 at concentrations ranging from 10^{-16} to 10^{-6} M for either 24 or 6 hr prior to or simultaneous with the addition of Tat for 24 hr. As shown in Fig. 1, astrocytes that were pretreated with

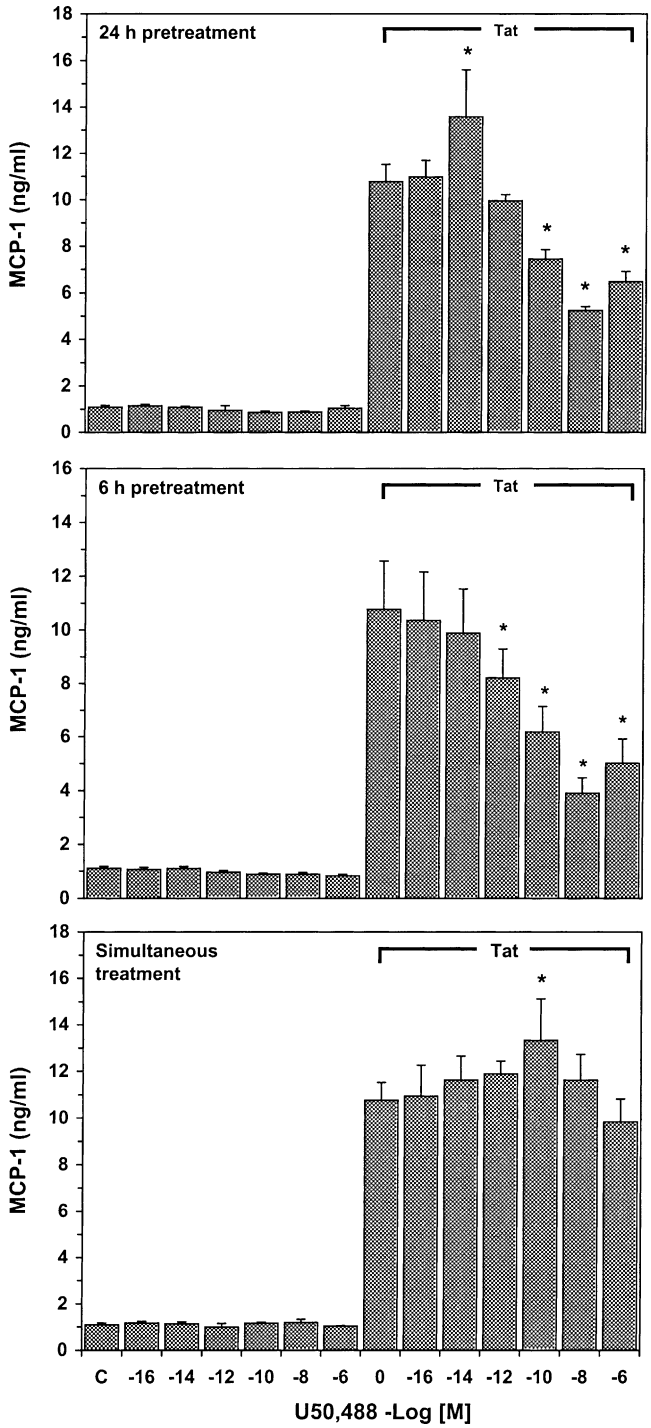

Fig. 1. Inhibition of Tat-induced MCP-1 production by U50,488. Human fetal astrocytes (10^5 cells per well) were treated with the indicated concentrations of U50,488 for 24 or 6 hr prior to or simultaneous with the addition of medium alone (C) or medium containing Tat (100 ng/mL) for 24 hr before harvesting supernatants for MCP-1 assay. Data are means ± SEM of triplicates of two separate experiments. Key: (*) $P < 0.05$ vs. Tat alone (ANOVA with Fisher's PLSD test).

U50,488 for 24 or 6 hr prior to Tat exposure displayed a concentration-dependent inhibition of MCP-1. However, U50,488 had no effect on MCP-1 production when it was added at the same time as Tat (Fig. 1). Thus, pretreatment of astrocytes with U50,488 before Tat stimulation was necessary to achieve the inhibitory effect. U50,488 treatment alone had no effect on constitutive MCP-1 levels (Fig. 1). There was no evidence of cytotoxicity by U50,488 as determined by Trypan Blue dye exclusion assay, MTT assay, and oligonucleosomal ELISA for histone-associated DNA fragments at the concentrations of U50,488 used in this study.

In the macrophage cell line p388 D_1 and in murine peritoneal macrophages, other investigators have demonstrated that treatment with U50,488 inhibited lipopolysaccharide (LPS)-induced interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) production [20,21]. We also found that U50,488 inhibited TNF-α-induced MCP-1 production in human astrocytes (unpublished observation). Thus, it appears that U50,488 has a non-discriminatory inhibitory effect on many stimuli-induced responses of glial cells.

3.2. Effect of nor-BNI on the inhibitory effect of U50,488

We have reported previously that human astrocytes express KOR [22]. To ascertain whether U50,488 was acting through a KOR-related mechanism, we used nor-BNI, a KOR selective antagonist. Astrocytes were pretreated with nor-BNI (10^{-6} M) for 1 hr prior to the addition of U50,488 (10^{-6} M) for 6 hr and then were stimulated with Tat for 24 hr. As shown in Fig. 2, nor-BNI completely reversed the inhibitory effects of U50,488 on Tat-induced MCP-1 production. These results suggest that the inhibitory effect of U50,488 on MCP-1 production is mediated through KOR.

3.3. U50,488 inhibition of Tat-induced NF-κB activity

Tat has been shown previously to activate NF-κB [23–26]. Also, MCP-1 production is known to be mediated through NF-κB activation since the MCP-1 promoter contains an NF-κB binding site [27,28]. In our astrocyte cultures, treatment with Tat (30 ng/mL) induced an approximately 2-fold increase of NF-κB activation (Fig. 3). When astrocytes were pretreated with U50,488 for 6 hr prior to Tat stimulation, U50,488 was found to inhibit Tat-induced NF-κB activation completely (Fig. 3). Interestingly, U50,488 treatment alone down-regulated constitutive NF-κB activation (Fig. 3). Adding PDTC (1–30 μM), an inhibitor of NF-κB, to astrocyte cultures abrogated Tat-induced MCP-1 production (data not shown). These results suggested that inhibition of NF-κB activation could be one of the mechanisms responsible for the inhibitory effect of U50,488 on MCP-1 production.

NF-κB plays important roles in immunity, the inflammatory process, and apoptosis. This regulatory factor can be activated by many stimuli including bacteria, viruses, viral proteins, cytokines, and chemokines. Activation of NF-κB, which occurs rapidly within minutes after exposure to such stimuli, could also up-regulate many genes

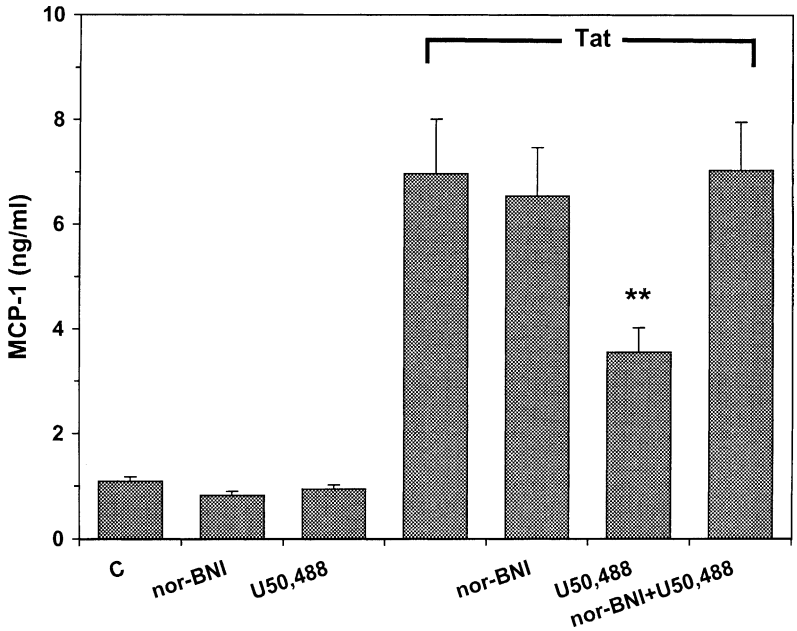

Fig. 2. Nor-BNI blockade of the inhibitory effect of U50,488 on Tat-induced MCP-1 production. Human astrocytes (10^5 cells per well) were treated with medium (C), nor-BNI (10^{-6} M), or U50,488 (10^{-6} M) alone, or were pretreated with nor-BNI (10^{-6} M) for 1 hr prior to adding U50,488 (10^{-6} M) for 6 hr, prior to stimulation with Tat (100 ng/mL) for 24 hr and harvesting supernatants for MCP-1 assay. Data are means ± SEM of triplicates of three separate experiments. Key: (**) $P < 0.01$ vs. Tat alone and nor-BNI + U50,488 + Tat (ANOVA with Fisher's PLSD test).

including those for cytokines, chemokines, and apoptotic factors [29]. As in the case for the MCP-1 gene promoter, NF-κB binding sites are also found in the promoter regions of the proenkephalin and prodynorphin genes, which encode opioid peptide precursors [30,31]. The murine δ-opioid receptor gene sequence also has a potential binding site for NF-κB [32]. These findings suggest that activation of NF-κB plays a pivotal role in the expression of many genes involved in inflammation and opioid expression.

The interaction/regulation between opioids and NF-κB activity has not been fully characterized. Inhibition of LPS-induced NF-κB activation by morphine, a μ-opioid receptor (MOR) agonist, at micromolar concentration was observed in murine macrophages showing significant accumulation of the inhibitory protein of NF-κB, i.e. IκB, which could lead to an increased sequestration of NF-κB in the cytoplasm [33]. In human neutrophils and monocytes, morphine was found to attenuate LPS-induced NF-κB activation, and

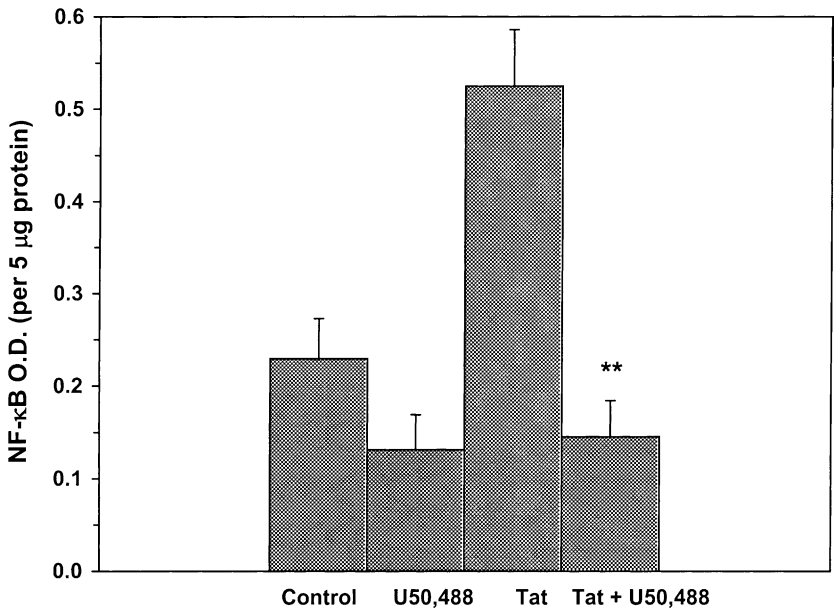

Fig. 3. Inhibition of NF-κB activation by U50,488. Human astrocytes (5×10^5 cells per well) were pretreated with medium (Control) or U50,488 (10^{-6} M) alone for 6 hr prior to an additional 2 hr of incubation in the absence or presence of Tat (30 ng/mL). Whole cell extracts (5 μg protein) were used in NF-κB ELISA. Data are means ± SEM of triplicates of three separate experiments. Key: (**) $P < 0.01$ vs. Tat (ANOVA with Fisher's PLSD test).

the inhibitory action was mediated by nitric oxide release, although morphine alone had no effect on NF-κB [34]. However, D-Ala^2, Gly, *N*-Me-Phe^4, Gly-ol^5 (DAMGO), a MOR agonist, was found to increase NF-κB activity in primary rat cortical neurons [35]. Similarly, endomorphin-1 and -2, the natural ligands for MOR, were reported recently to potentiate LPS-induced NF-κB binding in the human cell line THP-1 [36]. It appears that cell type, characteristics of the MOR agonist, and treatment regimen contribute to the differential response of NF-κB. To our knowledge, no data have been reported regarding the interaction of KOR agonists and NF-κB activity. Thus, the detailed mechanism of inhibition of NF-κB activation by U50,488 found in our study awaits further investigation.

Although astrocytes are not productively infected by HIV-1, astrocytes are known to produce an abundant amount of the β-chemokine MCP-1 upon stimulation with Tat [18]. MCP-1 has been shown to be the most potent chemokine for directing migration of monocytes and lymphocytes to sites of inflammation [14,37,38]. During HIV-1 infection, infiltrating monocytes or lymphocytes into the brain in response to MCP-1 released from astrocytes could play a role in HIV-1-related neuropathogenesis. Since MCP-1 is expressed in the brain and can be detected in cerebrospinal fluid of patients with HIV-1-associated dementia [18], developing a strategy to control astrocyte MCP-1 production may be fruitful in delaying progression of HIV-1 encephalopathy. The results of this study suggest that KOR ligands could be candidate compounds for this therapeutic purpose.

Acknowledgments

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