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Biochemical Pharmacology 61 (2001) 1145–1151

Biochemical Pharmacology

κ-Opioid receptor agonist suppression of HIV-1 expression in CD4$^+$ lymphocytes

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Received 3 July 2000; accepted 8 September 2000

Abstract

Synthetic κ-opioid receptor (KOR) agonists have been shown to suppress HIV-1 expression in acutely infected macrophages. In the present study, we examined the effects of the KOR ligand *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488) on HIV-1 expression in CD4$^+$ lymphocytes, the main target cell of this virus. When U50,488 was added to activated CD4$^+$ lymphocytes, HIV-1 expression was inhibited in a concentration- and time-dependent manner with maximal suppression (≈60%) at 10^{-7} M U50,488. The KOR selective antagonist nor-binaltorphimine (nor-BNI) had no effect by itself on viral expression but blocked the antiviral property of U50,488, suggesting that U50,488 was acting via a KOR-related mechanism. Support for the involvement of KOR was provided by the findings that 34% of activated CD4$^+$ lymphocytes were positive for KOR, using an immunofluorescence technique, and that seven additional synthetic KOR ligands also inhibited HIV-1 expression. The results of this study broaden understanding of the antiviral properties of KOR ligands to include cells outside of the nervous system and suggest a potential role for these agents in the treatment of HIV-1 infection.

Keywords: AIDS; HIV; κ-Opioid receptor ligands; CD4$^+$ lymphocytes; Opioids; U50,488

1. Introduction

The introduction in 1996 of highly active antiretroviral therapy (HAART) has resulted in a striking reduction in HIV-1-associated mortality [1,2]. Despite the enormously beneficial effects of HAART, however, many HIV-infected patients have not had adequate responses to the combination drug regimens, or they cannot tolerate their toxic side-effects. Also, the emergence of strains of HIV that are resistant to currently available drugs is a widespread and growing problem [2]. Thus, finding new therapies for HIV infection remains a high priority.

Along with greatly expanded knowledge of the immunopathogenesis of HIV has been research demonstrating that the major cell types infected by this virus, i.e. CD4$^+$ lymphocytes and mononuclear phagocytes, possess receptors for, and are impacted functionally by, opiates and opioid peptides [3,4]. Work in our laboratory has focused on the search for opioid compounds that might alter the expression of HIV in these cells. In the course of these studies, we have shown that the synthetic KOR ligand *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (U50,488) inhibits replication of the monocytotropic HIV-1_{SF162} strain in human microglial cell [5] and monocyte-derived macrophage [6] cultures. In the present study, we examined the effect of KOR ligands on the expression of HIV-1 in acutely infected CD4$^+$ lymphocyte cultures.

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Abbreviations: Ag, antigen; AZT, zidovudine; BSS, balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC-AA, 2-(3,4-dichlorophenyl)-*N*-[1-(3-aminophenyl)2-(1-pyrrolidinyl)ethyl] acetamide; GPI, guinea pig ileum; IL, interleukin; KOR, κ-opioid receptor; nor-BNI, nor-binaltorphimine; PBMC, peripheral blood mononuclear cells; and PHA, phytohemagglutinin.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00574-3

2. Materials and methods

2.1. Reagents

2.1.1. KOR ligands

The KOR selective ligand U50,488 was provided by the Pharmacia Upjohn Co. The fluroescein-isothiocynate-coupled arylacetamide κ-opioid selective agonist FITC-AA was obtained from J. Bidlack (University of Rochester). Anti-fluorescein, biotin-conjugated, was obtained from Molecular Probes, and extravidin R-phycoerythrin was purchased from the Sigma Chemical Co. The κ-selective antagonist nor-BNI was provided by P.S. Portoghese (University of Minnesota). Eight additional KOR ligands were synthesized by A.-C. Chang [7], X. Fang, and P.S. Portoghese. All of these compounds were evaluated on the electrically stimulated GPI preparation, as described previously [8].

2.1.2. Other reagents

All other reagents were purchased from the indicated sources: FBS (HyClone Laboratories); Lymphocyte Separation Medium (ICN Biomedical Inc.); human recombinant IL-2 (Boehringer-Mannheim); AZT, DMEM, RPMI 1640, phytohemagglutinin (PHA-P), l-glutamine, penicillin, streptomycin, and all other culture reagents (Sigma).

2.2. Lymphocyte activation and purification

Six healthy, HIV-1-seronegative laboratory personnel served as donors of venous blood for this study. From heparinized venous blood, PBMC were obtained by Ficoll-Hypaque gradient centrifugation using Lymphocyte Separation Medium. For most experiments, PBMC were then activated for 3 days with 4 μg/mL of PHA in RPMI 1640 supplemented with 10% heat-inactivated FBS, 5 U/mL of IL-2, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. In one experiment, PBMC were incubated in culture medium for 3 days in the absence of the activation signals (i.e. PHA and IL-2). $CD4^+$ lymphocytes were then isolated from the activated or non-activated PBMC using Dynabeads® (Dynal) according to directions supplied by the manufacturer. Briefly, magnetic polystyrene beads coated with primary monoclonal antibody to CD4 were incubated with PBMC for 45 min at 4° on an orbital rotator at a 1:4 ratio (cell:bead). The lymphocytes that bound to the beads were separated using a magnet (Dynal MPC) and washed four times with PBS containing 2% FBS. After isolation, DETACHaBEAD® was used to remove the isolated $CD4^+$ cells from the Dynabeads® (1 U/100 μL of cell suspension was used to detach positively selected lymphocytes from the magnetic beads using a Dynal-MPC magnet). Isolated $CD4^+$ lymphocytes were ≥ 98% pure by FACScan analysis and were ≥ 98% viable by trypan blue dye exclusion criteria.

2.3. Indirect immunofluorescence and phenotypic labeling

FITC-AA labeling and amplification procedures were performed using previously described techniques [5,9]. Briefly, $CD4^+$ lymphocytes were washed twice by centrifugation at 200 *g* for 10 min at 4°, followed by resuspension in HEPES-BSS (pH 7.4). Lymphocytes at 1×10^5/mL were incubated with 30 μM FITC-AA for 30 min at 25° for optimal staining. A high-affinity, κ selective antagonist, nor-BNI, at a final concentration of 500 μM was included to measure nonspecific fluorescence. Samples were chilled on ice, diluted with 1 mL of HEPES-BSS, and centrifuged at 400 *g* for 3 min at 4°. After aspirating the supernatants, cells were washed twice and resuspended in a final volume of 100 μL of HEPES-BSS, containing 10 μL biotin-conjugated anti-fluorescein IgG. For the FITC-AA and phycoerythrin only control groups, antibody was omitted. After a 30-min incubation at 4° in the dark, cells were washed twice, and then were resuspended in 40 μL of medium and 10 μL of extravidin-conjugated R-phycoerythrin. This incubation took place for 15 min on ice, and the cells were then washed twice before resuspension in a final volume of 1 mL for flow cytometric analysis. At least 10,000 events were analyzed in each sample using a Coulter XL100. The data were evaluated for the percentages of $CD4^+$ cells bearing KOR, using the software (EPICSr XL) provided by the manufacturer (Coulter Corp.).

2.4. HIV-1_{AT}

The HIV-1_{AT} isolate used in this study was originally recovered from the peripheral blood of an asymptomatic HIV-1-infected patient and prepared as previously described [10]. This viral isolate has characteristics most suggestive of a T-tropic strain, i.e. it replicates readily in the T cell line IIIB and in primary activated $CD4^+$ lymphocytes but is not expressed in cultures of human microglial cells, which are primary brain macrophages that are productively infected by M-tropic but not by T-tropic HIV-1 strains.

2.5. Drug treatment and HIV-1 infection of $CD4^+$ lymphocytes

Purified activated $CD4^+$ lymphocytes were incubated with KOR ligands or AZT at various concentrations for the indicated time periods prior to or post-infection with HIV-1_{AT} at a multiplicity of infection of 0.02. After 2 hr of incubation with HIV-1 at 37°, $CD4^+$ lymphocytes were washed three times with PBS and resuspended in culture medium (RPMI 1640, 10% FBS, penicillin/streptomycin, 2 μg/mL of PHA) containing indicated KOR agonists. In one experiment, $CD4^+$ lymphocytes that were isolated from non-activated PBMC were infected with HIV-1. Three days post-infection,

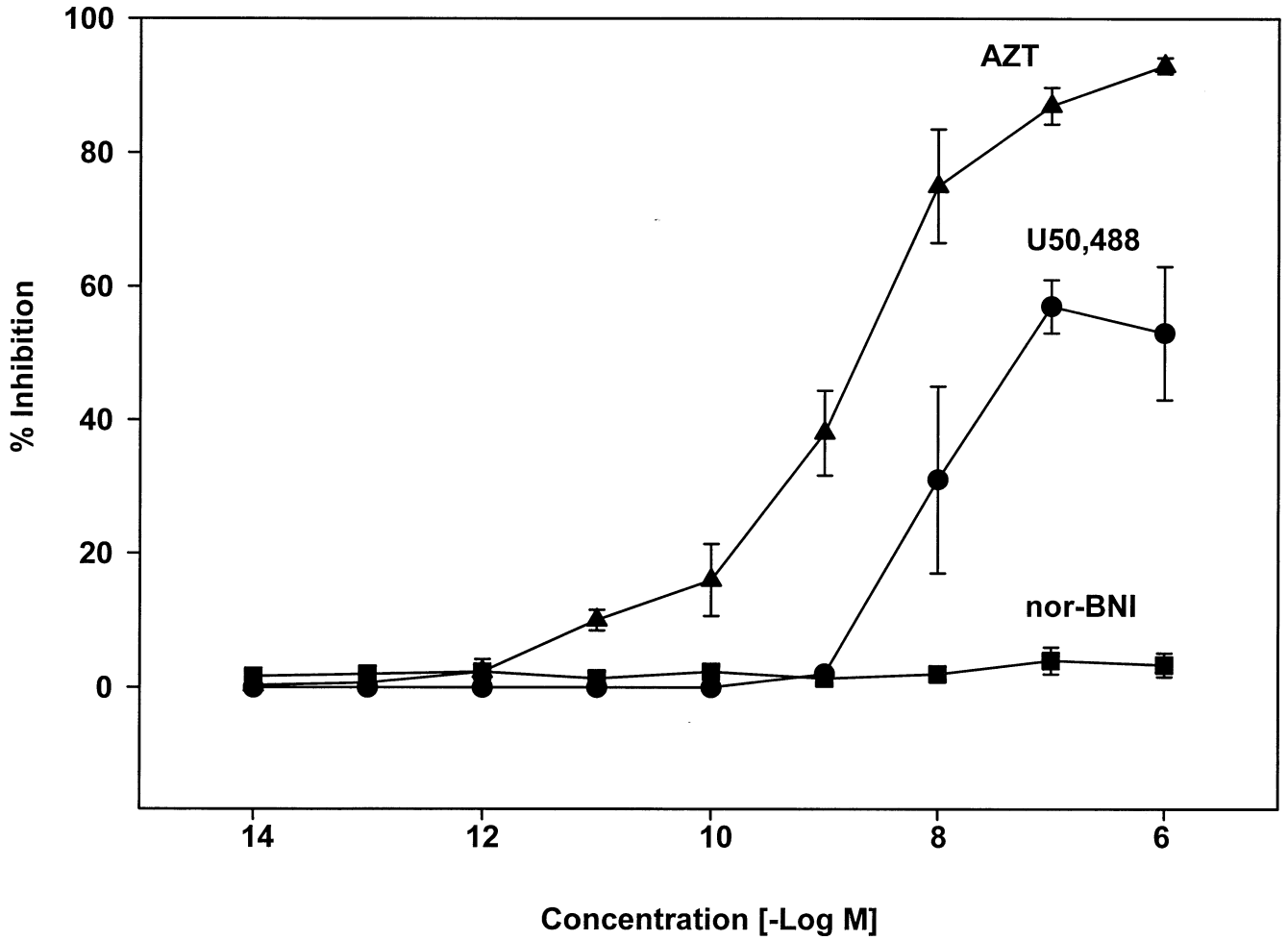


Fig. 1. Concentration–response effect of U50,488 on HIV-1 expression in $CD4^+$ lymphocytes. Cultures of activated $CD4^+$ lymphocytes were incubated in the absence (control) or presence of U50,488, nor-BNI, or AZT, at the indicated concentrations, for 24 hr prior to infection with HIV-1. Following 3 days of incubation, culture supernatants were collected for the measurement of p24 Ag levels. Data (means ± SEM of three separate experiments using $CD4^+$ lymphocytes from three donors) are expressed as a percentage of the control value (779 ± 142 pg/mL of p24 Ag).

culture supernatants were collected in duplicate for HIV-1 p24 Ag assay.

2.6. HIV-1 p24 Ag assay

HIV-1 p24 Ag levels were measured using an enzyme-linked immunoassay (Abbott Laboratories), as previously described [5]. A standard dilution curve derived from known amounts of p24 Ag was used to quantify the Ag levels in culture supernatants. The sensitivity of this assay is 30 pg/mL.

2.7. Statistical analysis

The effects of various test compounds on HIV-1 replication are expressed as a percentage of p24 Ag levels in control (untreated) cultures. Where appropriate, data are expressed as means ± SEM of the indicated number of separate experiments. To compare the means of two groups, Student's *t*-test was used.

3. Results

3.1. Effect of U50,488 on HIV-1 expression in $CD4^+$ lymphocytes

To determine whether KOR ligands would alter the expression of $HIV\text{-}1_{AT}$ in acutely infected $CD4^+$ lymphocytes, U50,488 was added to activated $CD4^+$ lymphocytes at concentrations ranging between 10^{-14} and 10^{-6} M for 24 hr prior to viral infection. For comparison, the KOR antagonist, nor-BNI, and the reverse transcriptase inhibitor, AZT, were incorporated at the same concentration as U50,488 in $CD4^+$ lymphocyte cultures. While nor-BNI had no effect on viral expression, treatment of $CD4^+$ lymphocytes with U50,488 or AZT inhibited HIV-1 expression in a concentration-dependent manner (Fig. 1). Maximal suppression of HIV-1 expression by U50,488 was observed at 10^{-7} M (57 ± 4%) inhibition to 10^{-6} M (53 ± 10%) inhibition. By comparison, at 10^{-6} M, AZT inhibited the expression of $HIV\text{-}1_{AT}$ by 93 ± 1%.

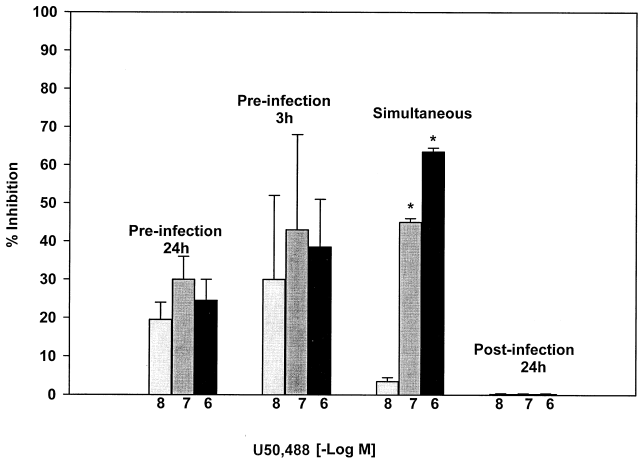


Fig. 2. Effect of U50,488 exposure time on antiviral activity. Cultures of activated $CD4^+$ lymphocytes were incubated in the absence (control) or presence of U50,488, at the indicated concentrations, for 3 or 24 hr prior to, simultaneous with, or 24 hr post-infection with HIV-1. Following 3 days of incubation, culture supernatants were collected for measurement of p24 Ag levels. Data (means ± SEM of three separate experiments using $CD4^+$ lymphocytes from three donors) are expressed as a percentage of the control value (735 ± 107 pg/mL of p24 Ag).

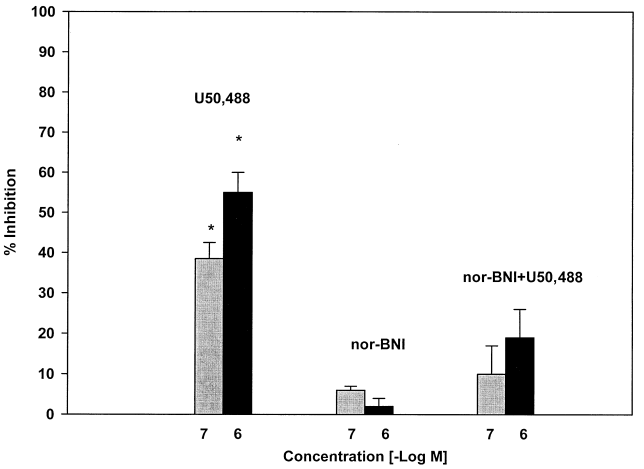


Fig. 3. Effect of nor-BNI on antiviral activity of U50,488. Prior to infection with HIV-1, cultures of activated $CD4^+$ lymphocytes were incubated for 24 hr in the absence (control) or presence of 10^{-7} or 10^{-6} M U50,488, or nor-BNI, or were pretreated with nor-BNI for 30 min prior to adding U50,488. After 3 days of incubation, culture supernatants were collected for the measurement of p24 Ag levels. Data (means ± SEM of three separate experiments using $CD4^+$ lymphocytes from three donors) are expressed as a percentage of the control value (817 ± 121 pg/mL of p24 Ag). Key: (*) $P < 0.05$ vs control value.

Next, we investigated the influence of varying the time of exposure of $CD4^+$ lymphocytes to U50,488 on its inhibitory effect on HIV-1 expression. For this experiment, U50,488 was added at concentrations of 10^{-8}, 10^{-7}, or 10^{-6} M to activated $CD4^+$ lymphocyte cultures for 24 or 3 hr prior to infection, simultaneously with HIV-1, or 24 hr post-infection. As shown in Fig. 2, the inhibitory activity of U50,488 was found to be both concentration- and time-dependent. When added simultaneously with HIV-1, 10^{-6} M U50,488 potently inhibited viral expression, whereas 10^{-8} M U50,488 was inactive at this concentration. However, exposure of $CD4^+$ lymphocytes to 10^{-8} M U50,488 for either 3 or 24 hr prior to infection resulted in more pronounced inhibitory activity but in a diminished antiviral effect at 10^{-6} M U50,488. At all concentrations tested, U50,488 had no inhibitory activity if it was added to $CD4^+$ lymphocyte cultures 24 hr after infection (Fig. 2). These findings with U50,488 contrasted sharply with those with AZT, which inhibited viral expression by over 85% at all three of these drug concentrations whether it was added simultaneously, for 3 or 24 hr prior to infection, or at 24 hr post-infection (data not shown).

To evaluate whether the inhibitory activity of U50,488 was operating through a KOR-related mechanism, activated $CD4^+$ lymphocytes were treated with nor-BNI for 30 min prior to exposure to equimolar concentrations of U50,488. At the two concentrations tested, nor-BNI blocked the viral inhibitory property of U50,488 (Fig. 3).

3.2. Flow cytometric analysis of KOR on $CD4^+$ lymphocytes

Blockade of the viral inhibitory activity of U50,488 by nor-BNI suggested that U50,488 was acting via a KOR-related mechanism. To confirm the presence of KOR in the $CD4^+$ lymphocyte preparations used in these experiments, we used FITC-AA, a KOR selective ligand, in a fluorescence-activated cell sorter assay. This analysis revealed that activated $CD4^+$ lymphocytes that were prepared as for the HIV-1 infection experiments (i.e. PBMC were first activated with PHA and IL-2 for 3 days prior to isolation of activated $CD4^+$ lymphocytes) bound FITC-AA, and that this binding could be partially blocked by treatment of $CD4^+$ lymphocytes with nor-BNI (Fig. 4). Calculation of the number of activated $CD4^+$ lymphocytes that were positive by FITC-AA binding showed that 34 ± 1% of cells bound this KOR ligand, while only 19 ± 2% of $CD4^+$ lymphocytes treated with nor-BNI were found to be positive, i.e. nor-BNI blocked the binding of FITC-AA by 46 ± 4% ($P < 0.01$, N = 3 donors). To determine if cell activation was required for expression of KOR, $CD4^+$ lymphocytes were isolated from PBMC cultures that had been incubated for 3 days in the absence of activation signals (i.e. PHA and IL-2). These non-activated $CD4^+$ lymphocytes neither expressed KOR, as determined by FITC-AA analysis, nor expressed virus after 3 days of culture (38 ± 9 pg/mL of p24 Ag, N = 3 donors).

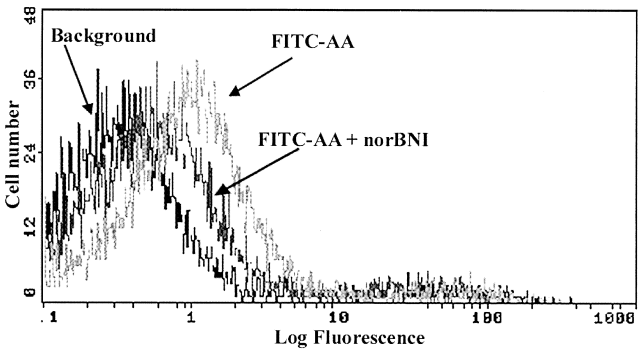


Fig. 4. Flow cytometric analysis of phycoerythrin amplification of FITC-AA labeling of KOR. Activated $CD4^+$ lymphocytes were incubated with or without 500 μM nor-BNI for 30 min prior to adding FITC-AA. FITC alone (only biotinylated anti-fluorescein IgG and extravidin-coupled R-phycoerythrin) was included as a background control. Data are representative of three separate experiments using $CD4^+$ lymphocytes from three donors.

3.3. Effect of KOR ligands structurally related to U50,488

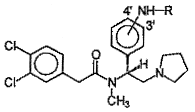
To determine whether synthetic KOR agonists related to U50,488 were also capable of inhibiting HIV-1 expression in $CD4^+$ lymphocytes, activated $CD4^+$ lymphocytes were treated with various concentrations of eight such compounds (Table 1) for 24 hr prior to infection with HIV-1_{AT}. Conjugates V-56, V-50, III-223, and III-117 exhibited potent agonist activity in the GPI smooth muscle assay, whereas intermediate potency was observed for V-60 and XF-115. The guanidine analogues (XF-229 and XF-225) functioned as partial agonists in the GPI smooth muscle assay, with maximal responses of 52 and 24%, respectively, at a concentration of 1 μM (data not shown). At 10^{-6} M, seven of eight of these compounds suppressed viral expression by between 16 and 41% (Table 1). At lower concentrations, two of these compounds (V-56 and V-60) lost their antiviral activity. Similar to the findings with U50,488, a bell-shaped concentration–response effect was observed with several of these KOR agonists (III-223, III-117, V-60, and XF-225) with maximal suppression occurring in the nanomolar range. Of note, there was no apparent correlation between the potency of the eight KOR ligands in the GPI smooth muscle assay and their antiviral activity. For example, V-56 and V-50, the most potent compounds in the GPI assay, were among the least effective in suppressing HIV-1 expression in $CD4^+$ lymphocytes, and XF-225, which had relatively poor activity in the GPI assay system, was one of the most active compounds in terms of viral suppression.

4. Discussion

Previously, we have shown that the KOR ligand U50,488 is capable of suppressing the expression of a monocytotropic HIV-1 strain in human microglial cell [5] and blood monocyte-derived macrophage [6] cultures. The results of the present study indicate that U50,488 can also inhibit viral expression in human $CD4^+$ lymphocytes, the primary target cell of HIV. The finding that this antiviral property could be blocked by nor-BNI, a KOR selective antagonist, suggests that U50,488 acts via a KOR-related mechanism.

Suppression of HIV-1 expression by seven additional synthetic KOR ligands supports a linkage between KOR activation and the down-regulation of viral expression. Although the rank order potencies of the arylacetamides in the GPI preparation did not parallel the suppression of HIV-1 expression in $CD4^+$ lymphocytes, it is highly likely that this effect is mediated via KOR for two reasons. First, ligands in

Table 1
Comparison of effects of KOR ligands on HIV-1 expression versus their activity in a GPI smooth muscle assay



Compound[a]	R	GPI IC_{50} (nM)	% Inhibition HIV-1[b]				
			Log [M]				
			−10	−9	−8	−7	−6
V-56	3′-L-Glutamyl	0.34	6	9	3	4	31
V-50	3′-γ-D-Glutamyl	0.39	1	2	9	3	36
III-223	3′-β-L-Aspartyl	0.49	9	58	44	30	27
III-117	3′-L-Aspartyl	0.62	15	34	36	42	21
V-60	3′-D-Glutamyl	4.2	20	36	54	58	16
XF-115	3′-SO_3Na	11	2	4	13	42	41
XF-229	4′-(C═NH)NH_2	>100	0	0	0	7	0
XF-225	3′-(C═NH)NH_2	>100	21	45	51	41	29

[a] Compounds are listed by ranking of their potency in the GPI assay.

[b] Data are mean values of three separate experiments using $CD4^+$ lymphocytes from three donors and are expressed as percentage inhibition of viral expression relative to control (untreated) cultures (967 ± 61 pg/mL of p24 Ag).

this series have structural features in common with a well-established class of κ agonists. Second, the suppression was antagonized by the selective κ opioid antagonist nor-BNI. While we do not have an explanation for the discrepancy between the opioid activity and suppression of HIV-1 expression, two possibilities that come to mind are (a) the presence of KOR subtypes, and (b) the association of KOR as dimers or oligomers with other receptors [11].

The signaling pathway between KOR activation of $CD4^+$ lymphocytes and the suppression of viral replication has not been elucidated. The observation that U50,488 had no effect on HIV-1 expression if it was added post-infection, which contrasted sharply with the antiviral effect of AZT, suggests that U50,488 may inhibit viral expression at an early step in infection, e.g. by blocking viral entry into $CD4^+$ lymphocytes. HIV-1 gains entry into $CD4^+$ lymphocytes and mononuclear phagocytes via interacting with CD4 and chemokine coreceptors [12]. Although there is no known homology between KOR [13] and chemokine receptors, chemokine receptors may be down-regulated via downstream events as a consequence of KOR activation. Other investigators have demonstrated that inhibition of chemokine-induced monocyte chemotaxis by δ- and μ-opioid receptor ligands is due to heterologous densitization through phosphorylation of chemokine receptors [14]. A similar process could be involved in the inhibitory effect of KOR agonists upon HIV-1 entry in $CD4^+$ lymphocytes.

Support for the hypothesis that a KOR-related mechanism is involved in U50,488-mediated suppression of HIV-1 expression was also provided by the finding that 34% of activated $CD4^+$ lymphocytes bound the highly KOR selective ligand FITC-AA. Non-activated $CD4^+$ lymphocytes, on the other hand, were found to neither bind FITC-AA nor support viral infection. These findings are consistent with earlier reports suggesting that cells of the immune system possess KOR [15]. Other groups of investigators, using molecular or immunofluorescent labeling techniques, have demonstrated that murine T lymphocyte lines, thymoma cells, and splenic $CD4^+$ lymphocytes express KOR [9,16–19]. Of more direct bearing on the present study, human $CD4^+$ lymphocytes also have been reported to express mRNA transcripts for KOR [20]. With the same FITC-AA probe that was used in the present study, Ignatowski and Bidlack found that approximately 60% of immature ($CD4^+/CD8^+$) murine thymoma cells bound FITC-AA [19], whereas less than 25% of mature murine splenic $CD4^+$ lymphocytes ($CD4^+/CD8^-$) express KOR [9]. The results of the present study suggest that a similar percentage of mature human $CD4^+$ lymphocytes express KOR following immunologic activation. The finding that U50,488 is capable of suppressing viral replication by about 60% may mean that additional $CD4^+$ lymphocytes express KOR during the course of a 3-day incubation with HIV-1.

In previous studies, U50,488 and other KOR ligands have been shown to alter various functional characteristics of T lymphocytes [21,22], mononuclear phagocytes [23–26], and polymorphonuclear leukocytes [27]. Our earlier observations that U50,488 can suppress the expression of HIV-1 in primary human microglial cell cultures [5] and that this KOR ligand also potentiated the antiviral activity of tumor necrosis factor-α in mixed glial/neuronal cell cultures [28] suggested to us that KOR agonists could have a therapeutic role in HIV-1-related brain disease. Because the major target cell of HIV-1 is the $CD4^+$ lymphocyte, the results of the present study have substantially broader therapeutic implications. However, most of the work on the functional effects of KOR ligands on cells of the immune system, including our studies, has been carried out with *in vitro* systems. Whether these *in vitro* findings have any biological relevance in intact animals is largely unknown, and *in vivo* studies are sorely needed to elucidate the impact of opioid agonists on cells of the immune system within the context of their role in infectious diseases [29]. Given the profound need for additional therapeutic modalities for HIV-1 infection, consideration should be given to the development of KOR ligands with a satisfactory pharmacologic profile for trials in animal models of retroviral infection, as well as in clinical trials of HIV-1-infected patients.

Acknowledgments

This work was supported, in part, by U.S. Public Health Service Grants DA09924, DA01533, K05-DA 00360, and DA04355.

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