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Neuroprotective Activities of CEP-1347 in Models of NeuroAIDS

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When the nervous system is infected with HIV-1, it commonly results in neuroinflammation leading to overt neuronal dysfunction and subsequent cognitive and behavioral impairments. The multifaceted disease process, now referred to as HIV-1–associated neurocognitive disorders (HAND), provides a range of molecular targets for adjunctive therapies. One is CEP-1347, an inhibitor of mixed lineage kinases that elicits neuroprotective and anti-inflammatory responses in models of neurodegenerative diseases. Since HAND is associated with inflammatory encephalopathy induced by virus infection and mononuclear phagocytes (perivascular macrophages and microglia) immune activation, we investigated whether CEP-1347 could ameliorate disease in laboratory models of HAND. We now demonstrate that CEP-1347 reduces the levels of secreted proinflammatory cytokines and chemokines in HIV-1–infected human macrophages and attenuates dose-dependent neurotoxicity in rodent cortical neurons. CEP-1347–treated mice readily achieve therapeutic drug levels in peripheral blood. HIV-1 encephalitis (HIVE) mice, where human virus-infected monocyte-derived macrophages are stereotactically injected into the basal ganglia of CB17 severe combined immunodeficient mice, received daily intraperitoneal injections of CEP-1347. Here, CEP-1347 treatment of HIVE mice showed a dose-dependent reduction in microgliosis. Dendritic integrity and neuronal loss were sustained and prevented, respectively. These results demonstrate that CEP-1347 elicits anti-inflammatory and neuroprotective responses in an HIVE model of human disease and as such warrants further study as an adjunctive therapy for human disease.


Commonly, HIV-1 infection leads to immune suppression and CNS disease (1–6). Commonly, cognitive, behavioral, and motor dysfunction falls into a spectrum of neurologic abnormalities now termed HIV-1–associated neurocognitive disorders (HAND) (7, 8). Nonetheless and following the wide spread use of antiretroviral drugs, the severity of disease has diminished and now <10% of infected persons show signs and symptoms of substantive neurologic deficits (9). Although neuropathological correlates of disease have evolved to subtle changes, in its most severe form, HIV-1 encephalitis (HIVE) dominates. This is characterized by the accumulation of virus-infected mononuclear phagocytes (MPs; blood borne macrophages and microglia) in deep gray matter with myelin pallor, astrogliosis and neuronal dropout. Neuronal pathology may be characterized by dendritic pruning and vacuolation (10). Disease ensues as a result of viral infection and MP immune activation with the secretion of viral and cellular neurotoxins (11–15).

In recent years research activities focused on developing adjunctive therapies for disease (16–19). These are important for several reasons. First, the emergence of HIV-1–resistant phenotypes is a common place during chronic antiretroviral therapy (20–24). Second, toxicities of antiretroviral medicines are a frequent cause of noncompliance and treatment failures over time (20, 25). Third, HIV-1 neuropathogenesis, although elicited by virus infection, is fueled by paracrine immune events amplified by disordered innate immunity. This results in a metabolic encephalopathy and subsequent neuronal injury and death (26). Lastly, interests in alternative therapies have increased because similar immune-based pathogenic mechanisms are shared among a number of neurodegenerative disorders, including amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer’s, and Parkinson’s diseases (27–35). Thus, discovery of adjunctive drugs that are effective in improving clinical disease outcomes will have broad applicability. Moreover, it is at least theoretically possible to interrupt disease pathogenesis by therapeutic approaches that do not directly target HIV-1 replication.

Based on these considerations, we pursued the use of CEP-1347 as an adjunctive medicine for the treatment of HAND. CEP-1347 acts as an inhibitor of multilineage kinases (MLKs) and can induce neuroprotective responses through its abilities to downregulate p38 and JNK phosphorylation (32–36). Macrophages are pivotal cells in an immune response through their phagocytic, killing, Ag presentation and secretory functions. The latter occurs through macrophage secretion of immune modulatory factors, including proinflammatory cytokines. In neurons, phosphorylation of p38 and JNK leads to apoptotic changes and apoptosis. In macrophages, phosphorylation and activation of MLKs results in transcription factor activation, leading to the production of proinflammatory cytokines.
cytokines and other immune factors that induce neurotoxic activities (37, 38). CEP-1347 modulates kinase activity after stimulation of MPs and neurons by the viral proteins HIV-1 gp120 and Tat (38, 39). This led to the working hypothesis that CEP-1347 may induce neuroprotection and anti-inflammatory activities for HAND. Our results support this notion and show CEP-1347 can attenuate HIV-1 ADA-mediated neurotoxicity independent of antiretroviral activities. The data, taken from laboratory and animal models of human disease, demonstrate that CEP-1347 treatment can lead to neuroprotective responses for HAND, and as such, may be developed for clinical use.

Materials and Methods

Primary human monocyte isolation and HIV-1 infection

Monocytes were obtained from leukopheresis of HIV-1 and -2 and hepatitis B seronegative donors and purified by countercurrent centrifugal elutriation. Cells were cultured with 10% heat-inactivated pooled human serum, 1% glutamine (Sigma-Aldrich, St. Louis, MO), 10 μg/ml ciprofloxacin (Sigma-Aldrich), and 1000 U/ml highly purified recombinant human macrophage colony stimulating factor (MCSF; a generous gift from Pfizer-Wyeth, Cambridge, MA) in DMEM. After 7 d, the monocyte-derived macrophages (MDM) were infected with HIV-1 ADA (a macrophage tropic viral strain) at a multiplicity of infection (MOI) of 0.01 (40). The ADA strain was used in these experiments as a result of prior and extensive analyses of macrophage function and neurotoxicity based on strain differences (12). We found in these systems used that the levels of viral replication not the strains per se govern the levels of neurotoxicity. Thus, we used the laboratory adapted HIV-1 ADA strain as the levels of viral growth are nearly identical. These experiments as a result of prior and extensive analyses of macrophage function and neurotoxicity based on strain differences (12).

HIV reverse transcription assays

HIV replication was examined by measuring viral reverse transcriptase (RT) activity as previously described (41). In these assays, 90,000 monocytes were cultured in 96-well plates for 7 d, then infected with HIV-1 ADA at a MOI of 0.01 and washed 24 h later to remove the virus. Media was changed every other day. To estimate HIV-1 replication, RT activity was determined by incubating 10 ml of sample with a reaction mixture consisting of 0.05% Nonidet P-40 (Sigma-Aldrich) and 1% SDS (Fisher Scientific, Pittsburgh, PA) containing additional inhibitors of RNA polymerase II (Sigma-Aldrich, St. Louis, MO) in a 96-well plate overnight at 24°C. RT activity was determined by quantifying the incorporation of [α-32P]CTP in the presence of cDNA obtained from an HIV-1 infected cell line. Results are expressed as percent inhibition as compared with untreated controls.

Murine cortical neuron cultures

Cerebral cultures, containing neurons and glia in similar proportions to that found in the brain, were derived from the cerebral hemisphere of embryonic C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) on day 17 of gestation and cultured as described previously (42), after dissociation in 0.02% trypsin. Neuron-enriched cultures were resuspended in neurobasal medium (Invitrogen, Grand Island, NY) with heat-inactivated FCS supplemented with B-27, 500 μM glutamine, and 25 μM glutamate, then seeded at a density of 2.8 x 105 cells/cm2 on poly-D-lysine–coated 96- and 24-well plates.

Cell viability assays

Ninety thousand human MDM were cultured in 96-well plates and treated with 0, 80, 160, or 220 nM CEP-1347 24 h before, at, or 4 h after viral infection. Media was changed every other day and appropriate concentrations of CEP-1347 added. Cultures were monitored for 10 d. Cell viability assays were performed on days 1, 3, 5, 7, and 10 by measuring mitochondrial activity by reduction of tetrazolium salt MTT as described previously (43).

Murine cortical neuron cytotoxicity assays

One million human MDM were cultured in 24-well plates and treated with/without CEP-1347 as outlined previously, cultured for 5 d, and then washed with PBS. Media was replaced with neural basal medium (HBSS with Earle's salts and 1% l-glutamine) every 24 h. At that time, a ratio of 1:5 (supernatant:media) fluids were placed onto murine cortical neurons (MCNs) for 24 h to assess neurotoxicity by neuronal immunohistochemistry. For the neuronal Ag measurements, cells were blocked with 5% normal goat sera for 1 h, then incubated with Abs to microtubule-associated protein 2 (MAP-2) (neural cell bodies, axons, and dendrites, 1:1000) and neuron-specific nuclear protein (NeuN) (1:100) for 1 h (Chemicon International, Temecula, CA). Cells were then washed three times with PBS and then incubated with fluorescent Abs (1:1000 dilutions) to rabbit and mouse Alexa Fluor 488 (green) and 594 (red) as a secondary Ab (Molecular Probes, Eugene, OR) for 1 h. Cells were washed twice with water, and the water was allowed to cover the wells. Plates were read at 617 nM and 519 nM for MAP-2 and NeuN using a fluorescent plate reader, and the data were quantified (four wells/group). Data were expressed as the change in the mean of fluorescence intensity (MFI) in treated versus nontreated MCNs. Lactate dehydrogenase (LDH) levels in neuronal fluids were determined by a cytotoxicity detection kit (Roche, Indianapolis, IN). In this case, MCN culture plates were read at 490 nM. Data were expressed as percent changes in MCN LDH-release among drug and media treatments as compared with 1 nM staurosporin (St)-treated cells (listed as 100%). For characterization of neuronal morphology, double-immunofluorescence staining was carried out using Alexa Fluor 488 (green) and 594 (red) as a secondary Ab (Molecular Probes). Confocal laser scanning imaging was used, which contains Argon and Argon/Krypton lasers allowing for up to four Acousto-Optical Tunable Filter-modulated excitation lines of 488 nm, 514 nm, 568 nm, and 633 nm (Melles Griot, Prairie Technologies, Madison, WI). The lasers are connected to SweptField scanner head (Nikon Instruments, Melville, NY) attached with Cascade 512B back illuminated 12-bit CCD digital camera (Roper Scientific, Duluth, GA), which are connected to the side port of Nikon TE2000U Inverted Microscope with a high-resolution X-Y-Z-motorized stage.

Ab array and flow cytometric analyses of cytokines and chemokines

One million human MDM were cultured in 24-well plates and infected with HIV-1 ADA at an MOI of 0.01 for 1 d, then washed with PBS. Infection was allowed to grow for 5 d. On day 5, cells were treated with 220 nM CEP-1347 for 45 min, washed with PBS, and phenol-red free and serum-free media were added. Supernatants were harvested and assayed for HIV-1 ADA chemokines as previously described by Panomic Ab array 3.0 (Panomics, Fremont, CA) for human cytokines. Panomic Ab array 3.0 measured human cytokines: ApoAI/Fas, Leptin, RANTES, ICAM-1, IL-2, -7, CTLA, MIP1α, TGFβ, VCAM-1, IL-3, -5, -8, Eotaxin, MIP1β, IFNy, vascular endothelial growth factor, IL-4, -5, IL-10, GM-CSF, MIP1, TNFα, IL-1α, -5, -12, IL-10, (p40), epidermal growth factor, MIP-5, TNFRI, IL-1β, -5, IL-15, IL-10, MPM3, TNFRII, IL-1α, IL-6R, and IL-17. For the Cytometric Bead Array flow cytometric of secreted chemokine analysis, one million human MDM were cultured in 24-well plates and infected with HIV-1 ADA at an MOI of 0.01 for 1 d, then washed with PBS. At time of infection, cells were treated with 80, 160, or 220 nM CEP-1347. Half media exchanges were carried out on days 2 and 4 with respective drug concentrations. On days 3 and 5, 50 μl supernatant was harvested for each group by Cytometric Bead Array Human Inflammation Kit and Chemokine Kit (BD Biosciences, San Diego, CA). Human Inflammation and Chemokine Kit measures IL-8, -4, -5, -6, IL-10, TNFα, -12p70, and IL-8, RANTES, MIG, MCP-1, and IP-10, respectively.

Western blot analysis

MDM were infected and cultured with CEP-1347 for 5 d, then lysed using RIPA lysis buffer (Fischer Scientific, Pittsburgh, PA) containing additional protease and phosphatase inhibitors (Calbiochem, San Diego, CA). Protein concentrations of whole cell lysates were estimated using BSA assay and stored at −80°C. Cell lysates were allowed to thaw on ice, then processed for SDS-PAGE by boiling with SDS laemmli buffer (Bio-Rad Laboratories, Hercules, CA), Whole cell lysate (40 μg protein) was loaded into each well of the Bio-Rad SDS gradient PAGE ranging from 4–15% and run at 100 V for 1 h 30 min. Protein was transferred to a polyvinylidine fluoride (PVDF) membrane (Millipore, Billerica, MA) using Bio-Rad semidy transfer apparatus at 25 V for 1 h. The membrane was then blocked with 5% BSA in TBS. Primary Abs used were anti-phosphorylated-JNK (1:1000, Cell Signaling Technology, Danvers, MA), anti-MLK3 (1:1000, Cell Signaling Technology), anti-JNK (1:1000, Cell Signaling Technology), anti-p38 (1:1000, Cell Signaling), anti-phosphorylated-JNK (1:2000, Cell Signaling Technology), anti-p38 (1:1000, Cell Signaling), antiphosphorylated-p38 (1:1000, Cell Signaling Technology), anti-phosphorylated-NF-κB p65 (Ser 468) (1:1000, Cell Signaling Technology) anti-NF-κB p65 (1:1000, Cell Signaling), antiphosphorylated-MAPK/ERK1/2 (Ser935) (1:1000, Cell Signaling), anti-NF-κB p105/50 (1:1000, Cell Signaling), and GAPDH (1:1000, Cell Signaling Technology). Primary Abs were detected with HRP-linked secondary Abs, anti-mouse or anti-rabbit as per Ab used (1:20,000, Chemicon International),
followed by detection by ECL femtodetection reagent (Pierce Biotechnology, Rockford, IL) and subsequent exposure to x-ray films for 5-30 min (for phosphoproteins up to 6 h). Blots were quantified by inverting scanned images of the blots using the program ImageJ (National Institutes of Health) and measuring intensity. Readings were normalized to respective GAPDH expression levels and compared with uninfected, untreated MDM.

Pharmacokinetic study

Five-week-old male CB17/SCID mice and C57BL/6 mice were purchased from Charles River Laboratory, Wilmington, WA. Animals were administered i.p. injections either 1.5 or 15.0 mg/kg CEP-1347. The C57BL/6 mice received both drug dosages, whereas the CB17/SCID received only the lower 1.5 mg/kg dosage. Blood was extracted by cheek puncture at 0, 0.5, 1, 2, 4, 6, or 8 h. Blood samples were centrifuged at 5000 rpm for 10 min at 4°C. Plasma was collected and stored at −20°C pending analysis. The plasma samples were prepared for bioanalysis by adding 10 volumes of acetonitrile containing an internal standard (alpenolol). After the samples were vortexed and centrifuged, the supernatant was transferred to a 96-well plate for analysis by liquid chromatography/mass spectrometry. The amount of CEP-1347 in the sample was quantified using a plasma standard curve made via serial dilution in a concentration range from 10-5000 ng/ml.

SCID mouse model of HIV

Four-week-old male C.B-17/scid/scid (CB17/SCID) mice were purchased from Charles River Laboratory. Animals were maintained in sterile microisolator cages under pathogen-free conditions in the Laboratory of Animal Medicine at the University of Nebraska Medical Center in accordance with ethical guidelines for care of laboratory animals set forth by the National Institutes of Health. HIV-1Ada–infected MDM (1.5 × 10⁶ cells infected at an MOI of 0.1 in 5 μl) were stereotactically injected intracranially after 1 d of viral infection and referred to as HIVE mice (44). The higher multiplicity used in the animal studies as compared with the in vitro experiments reflected the need to infect a larger proportion of cells prior to cell injections. CEP-1347 was then administered i.p. daily for 7 d at doses 0.5, 1.0, 1.5, 5.0, and 15.0 mg/kg/d (n = 4 mice/treatment group). Vehicle only was the control. CB17/SCID mice received intracranial (i.c.) injections of media (sham-operated) and served as additional controls. Seventeen animals were included in each group. Animals were treated with vehicle or CEP-1347 starting 1 d post-i.c. injection and for 7 d after MDM injections and CEP-1347 treatments.

Histopathology and image analysis

Brain tissue was collected at necropsy, fixed in 4% phosphate-buffered paraformaldehyde, and embedded in paraffin. Paraffin blocks were cut until the injection site of the human MDM was identified. HIV-1 p24 Ag (clone Kal-1; Dako, Carpenteria, CA) was used to test for virus-infected human MDM. For each mouse, 30–100 serial (5-μm-thick) sections were cut from the injection site and three to seven sections (10 sections apart) analyzed. Abs to intermediate filaments (clone Vim 3B4; Biocell, Santa Cruz, CA; glial fibrillary acidic protein; GFAP; Dako, Neun, MAP-2 (both from Chemicon International), and H chain (200 kDa) neurofilaments (Dako) were used for detection of neurons. Appropriate secondary Abs and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) were used to complete the immunohistochemical tests. In addition, the polymer-based HRP-conjugated anti-mouse and anti-rabbit Dako EnVision systems were used for secondary detection. For all immunohistochemical assays, 3,3′-diaminobenzidine (Dako) was used as the chromogen with the exception of MAP-2 where the Vector VIP substrate kit (Vector Laboratories, Burlingame, CA) was used. All sections were counterstained with Mayer’s hematoxylin.

Results

CEP-1347 modulates cytokine and chemokine release by MDM

To explore the effects of CEP-1347 on human HIV-1–infected MDM, we performed in vitro studies and evaluated effects of the drug on MDM viability (MTT), viral replication (RT activity), and secretory profile (cytokines). Human MDM were treated from 0 to 220 nM CEP-1347 either 24 h before, at, or 4 h post–HIV-1 infection for up to 10 d. Cell viability was not affected and no morphological changes were seen (data not shown). The levels of viral replication were measured and also showed no significant change (Fig. 1B); only results derived from simultaneous treatment of the cells with CEP-1347 for 24 h are shown, but similar results were obtained in the cells treated with CEP-1347 before or after infection. To explore the immediate effect of CEP-1347 on cytokine production by human MDM, cells were infected with HIV-1Ada for 24 h and then cultured for 5 d. Cells were treated with 220 nM CEP-1347 for 45 min, washed, and incubated for 24 h without CEP-1347, at which time supernatants were removed and analyzed using a commercially available human cytokine/chemokine Ab array. Treatment with CEP-1347 reduced the release of macrophage inflammatory proteins (MIP-1β [CCL4], MIP-4 [CCL18], IL-12 p40, and Stromelysin-1 [MMP-3]) by HIV-1–infected macrophages (Fig. 1A). Upregulation of proteins with anti-inflammatory properties, such as IL-4 and TGFβ, TNFR1, and IL-1R antagonist, was also detected (Fig. 1A). Three experiments using three donors were used, but results were not pooled. Results are from one experiment but are representative of all three experiments. Each Ab on the array was comprised of two dots; statistics were not used. Although there may be differences between groups, it cannot be stated if these differences are significant. The quantitative cytometric bead array performed on supernatants collected during the course of 5 d viral infection with CEP-1347 treatment (Fig. 1C) further revealed that CEP-1347 caused a downregulation of CXCL10/IP-10 and IL-8 secretion by infected MDM. These data demonstrate that CEP-1347 treatment of HIV-1–infected MDM markedly downregulates their proinflammatory phenotype. Three separate experiments using three donors were carried out. Data presented are from one experiment but is representative of all three experiments.

CEP-1347 neuroprotective activities in HIV-1Ada–infected human MDM

The data presented previously suggested that CEP-1347 treatment reduces the neurotoxicity mediated by HIV-1–infected MDM in vitro. To analyze this possibility, we used primary MCN cultures. Supernatants were collected from human MDM that were treated 24 h before, at, or 4 h post–HIV-1Ada infection with CEP-1347 concentrations of 0, 80, and 160 nM and cultured for 5 d in the presence of CEP-1347. On day 4, cells were washed, and media replaced with neural basal media (Invitrogen) for 24 h without CEP-1347. On day 5, supernatants were harvested. These conditioned supernatants were placed on 10-d-old cultures of primary MCN for 24 h, and neurotoxicity was assessed by measuring MAP-2 and Neun staining. The results were then verified by conducting LDH release assays. The morphological changes for
MCN are shown in Figs. 2 and 3 include quantitative MFI measurements for MFI for dendrites and neuronal nuclei (MAP-2 at 617 nm and NeuN at 519 nm wavelength, respectively). Conditioned media-treated MCN was subtracted in all evaluations. In untreated MCN cultures, neurons were evenly distributed and connected with each other with a high density of dendritic branchpoints and long neuritic processes; these cells also contained prominent cell bodies (Fig. 2A). This morphology was unaltered by treatment with CEP-1347 (data not shown). After exposure to HIV-1 conditioned media, neurons (Fig. 2B) displayed a low density of dendritic nodes, shorter neurites, and a loss of connected processes. In contrast, conditioned media from CEP-1347–treated, HIV-1–infected MDM did not elicit these neurotoxic effects (Figs. 2C,2D,3A). In this latter case, neurites were retained and dendritic nodes showed long processes in high density, at similar levels to controls (untreated and uninfected cells); prominent cell bodies were also seen. The greatest increase in MAP-2 immunostaining was seen in cultures that were exposed to supernatant fluids from HIV-1–infected MDM treated with 160 nM CEP-1347 (p < 0.001, compared with cultures exposed to conditioned media...
from untreated HIV-1–infected MDM). MCNs that were exposed to conditioned supernatants from HIV-1–infected MDM treated with 80 nM CEP-1347 also showed increased levels of MAP-2, but this result did not achieve statistical significance ($p < 0.087$). NeuN immunostaining was also significantly increased in MCN cultures that were exposed to conditioned supernatants from HIV-1–infected MDM that had been treated with 160 nM of CEP-1347, versus untreated HIV-1–infected MDM ($p < 0.0005$). MCN exposed to conditioned media from HIV-1–infected MDM treated with 80 nM CEP-1347 also showed elevated numbers of NeuN reactive cells, compared with cultures exposed to supernatants from untreated HIV-1–infected MDM, although this result did not achieve statistical significance. Neuronal protection was also assessed by measuring LDH levels in the extracellular milieu. LDH release from MCN subjected to different MDM conditioned medias were compared with 1 nM St-mediated neuronal destruction (100%, Fig. 3B). LDH release from neurons treated with conditioned media collected from nontreated MDM was subtracted in these evaluations. Conditioned media collected from HIV-1–infected MDM elicited high levels of LDH release in MCN cultures. Levels of LDH release were lower in cultures exposed to conditioned media from HIV-1–infected MDM cultures that were treated with both 80 and 160 nM CEP-1347 ($p = 0.05$ and $p = 0.013$, respectively, Fig. 3B).

**CEP-1347 pharmacokinetic analyses**

To gain insight into the metabolism of CEP-1347 in our SCID mouse model of HIVE, we measured plasma concentrations of CEP-1347 after i.p. administration at dosages of 1.5 mg/kg and 15.0 mg/kg in CB17/SCID mice ($n = 4$ mice/treatment/time point) and collected blood at varying time points as depicted in Fig. 4.

**CEP-1347 affects neuroinflammatory responses in HIVE mice**

Human HIV-1$_{ADA}$–infected MDM were stereotactically injected into the basal ganglia of CB17/SCID mice. Histopathological changes observed in murine brain tissue paralleled those seen for human HIVE. This included HIV-1 infection in perivascular and parenchymal human MDM, the formation of multinucleated giant cells, astrocytosis, and neuronal dropout. Therapeutic efficacy of different dosages of CEP-1347 was evaluated using immunohistochemistry in HIVE SCID mice after administration. Human HIV-1$_{ADA}$–infected MDM were stereotactically injected in the basal ganglia of CB17/SCID mice ($n = 4$ mice/treatment group). CEP-1347 was then administered i.p. daily for 7 d at doses of 0 mg/kg/d (vehicle only), 0.5 mg/kg/d, 1.0 mg/kg/d, 1.5 mg/kg/d, 5.0 mg/kg/d, and 15.0 mg/kg/d. Morphological changes in astrocytes surrounding the lesion site were evaluated using GFAP immunostaining. Quantitation of GFAP expression used three sections from each animal: immediately before, at, and immediately after the lesion site. GFAP expression was quantified by determining GFAP positive area as a percentage of the total image area per microscopy field. Astrogliosis was not unaltered in CEP-1347–treated HIVE mice, when compared with vehicle-only

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Quantitative analyses of CEP-1347 induced neuroprotective activities. Quantitative analyses of MAP-2 and NeuN staining in primary MCN culture treated with conditioned media from CEP-1347–treated, HIV-1–infected MDM. A, Analysis of dendritic density, as assessed by MAP-2 staining and neural density, measured by NeuN staining. Data demonstrate MFI at 617 nm (MAP-2) and 519 nm (NeuN) for culture wells containing treated MCN. Values are mean ± SEM for quadruplicate cultures. Data are representative of three independent experiments using three different donors. B, LDH concentration from replicate primary cortical neural cultures with CM from CEP-1347–treated, HIV-1–infected MDM; values are mean ± SEM of quadruplicate cultures. Data expressed are percentage changes in LDH release compared with MCN treated with 1 nM St, 100%. Data are representative of three independent experiments using three different donor conditioned medias.
CEP-1347 elicits neuroprotective responses in HIVE mice

To determine whether CEP-1347 was neuroprotective in HIVE mice, brain tissue from treated animals was subjected to immunostaining with anti-NeuN and anti-MAP-2 Abs. The area analyzed corresponded to the same area used to assess astrocytosis and microglial activation. Significant neuronal loss was present beyond the lesion site in vehicle-only treated HIVE mice; however, CEP-1347 was neuroprotective in a dose-dependent manner (Fig. 6). Neurons stained with anti-NeuN were counted using several brain sections from each mouse surrounding the lesion area. HIV-1-infected MDM were identified by immunostaining with vimentin (Fig. 5E–H). Serial brain sections were stained with Iba-1 to determine activation of microglia. Microglia stained area was calculated as a percentage of the area of the entire microscopy field. Microglial activation was significantly decreased in all CEP-1347–treated groups when compared with vehicle-only treated HIV-1–infected MDM (2.71 ± 0.61). Results of microglial activation for 0.5 mg/kg/d, 1.0 mg/kg/d, 1.5 mg/kg/d, 5.0 mg/kg/d, and 15.0 mg/kg/d were 0.91 ± 0.16 (p < 0.0001 compared with HIV-1–infected MDM treated with vehicle alone), 1.3 ± 0.26 (p < 0.01), 0.87 ± 0.40 (p < 0.001), 0.75 ± 0.17 (p < 0.001), and 0.41 ± 0.06 (p < 0.0001), respectively (Fig. 5A–C). Microglial activation decreased with increasing concentrations of CEP-1347 (Fig. 5D).

FIGURE 4. Pharmacokinetic analyses of CEP-1347 in plasma of experimentally treated mice. The plasma concentration of CEP-1347 was measured after i.p. dosing of mice with 1.5 or 15.0 mg/kg of CEP-1347. For this experiment, both C57BL/6 and CB17 SCID mice were treated. However, the C57 BL/SCID mice were treated only with the lower 1.5 mg/kg dose of CEP-1347. Blood samples were collected at selected time points (0.5, 1, 2, 4, 6, and 8 h), and plasma was separated for analyses. Samples were analyzed by liquid chromatography/mass spectrometry. Values are mean ± SEM from four animals. Data are represented as a line graph (n = 4 mice/treatment).

CEP-1347 eases pathomorphological changes in HIVE mice

Dendritic loss was significantly decreased in HIVE mice receiving vehicle-only treated HIVE mice (19.5 ± 1.5). Mice receiving 0.5, 1.0, 1.5, 5.0, and 15.0 mg/kg/d had 28.6 ± 1.4 (p < 0.0001 compared with vehicle-treated HIVE mice), 29.3 ± 1.5 (p < 0.0001), 43.2 ± 1.1 (p < 0.00001), 45.5 ± 1.1 (p < 0.00001), and 47.0 ± 1.2 (p < 0.00001), respectively. CEP-1347–treated HIVE mice showed decreased neuronal loss and dendritic processes when compared with vehicle-only treated HIVE mice. A dose-dependent protective effect was observed at CEP-1347 levels up to 1.5 mg/kg/d, but no increased therapeutic advantage was observed for doses above 1.5 mg/kg/d (Fig. 6).

In this study 1.5 mg/kg/d was used for treatments (n = 17 mice/treatment group). SCID mice were stereotactically injected in the basal ganglia with human HIV-1ΔΔΔ-infected MDM and administered CEP-1347 i.p. daily for 7 d. Animals were then sacrificed, and brain tissue removed for immunohistology or RT-PCR. Histopathological changes observed included formation of multinucleated giant cells, astrocytosis, and neuronal dropout (Table 1).

Human MDM were identified by immunostaining with vimentin and were present in the area adjacent to the stereotactic injection site. In HIVE mice treated with 1.5 mg/kg/d CEP-1347, the mean number of MDM was 271.3 ± 121.0 compared with 275.2 ± 123.0 MDMs for HIVE vehicle-only treated animals. HIV-1–infected MDM in brain were immunostained with anti-p24 Ag and quantified. CEP-1347–treated mice had 35.4 ± 19.0 HIV-1–infected MDM, whereas, HIVE vehicle-only treated mice had 61.8 ± 28.4 HIV-1–infected MDM (the difference was not statistically significant). Antiretroviral activity was assessed as a percentage of MDM infected with HIV-1 and compared between CEP-1347–treated HIVE mice (13.0% ± 15.7%) and vehicle-only treated HIVE mice (22.5% ± 23.1%); once again, the difference was not statistically significant.

Using quantitative RT-PCR analysis revealed no difference in HIV-1 RNA expression between groups (data not shown). GAP43 expression was also unaltered, as determined by both histologic and RT-PCR analyses (data not shown). In contrast, microglial activation, as assessed by Iba-1 immunostaining, was significantly decreased in CEP-1347–treated HIVE mice as compared with vehicle-only treated HIV-1–infected MDM mice (3.61 ± 0.17, 8.56 ± 0.24, respectively; p < 0.00001). This result was confirmed by analysis of RNA transcript levels for Mac-1, another marker for activated microglia. HIVE mice treated with CEP-1347 have reduced levels of Mac-1 mRNA, when compared with vehicle-only treated animals (0.09 ± 0.01 and 0.20 ± 0.03, respectively; p < 0.05). We also measured the mRNA expression levels of TNF-α, a proinflammatory cytokine that plays a major role in inflammation, and IL-10, an anti-inflammatory cytokine, in the brains by RT-PCR. TNF-α is upregulated in many neurodegenerative disorders, including HIV and is elevated in HIVE mice (46). Neither TNF-α, nor IL-10 mRNA expression levels were significantly different in CEP-1347–treated HIVE mice compared with vehicle-only treated HIVE mice.

CEP-1347 and MDM signaling by Western blot assays

We next assessed the relationships between HIV-1–infected MDM secreted proteins and cell signaling pathways that are known to be modified by inhibition of MLK activity (47, 48). These experiments were performed based on the fact that perivascular brain macrophages are the principal target cell in disease and responsible for much of the neuronal impairments observed (1). Mechanisms for CEP-1347 actions were sought. To this end, we performed Western blot assays for the kinases ERK1/2, p38, and JNK. These are involved in apoptotic pathways and previously shown to have lower phosphorylation levels in HIV-1gpl20 or Tat-
stimulated MPs after CEP-1347 treatment (38, 39). Levels of phosphorylated to total protein were used to analyze differences between infected and uninfected groups. CEP-1347 (220 nM) treatment groups all showed reduced levels of phosphorylated ERK and p38. However, such reductions were limited. Phosphorylated JNK was increased in HIV and further increased in the HIV/LPS-treated cells. CEP reduced the level of phosphorylated JNK in both conditions but modestly so. The ratio of phosphorylated to total ERK was unchanged in uninfected groups treated with CEP-1347 (Fig. 7). Although HIV infection leads to increased pMLK3, the effect of CEP 1347 is less clear.

In parallel with MLK3 pathway, we also pursued analysis for the NF-κB pathway to determine the potential for the robust anti-inflammatory responses seen by CEP1347. In this context, we analyzed both the cytoplasmic and nuclear fractions of the HIV-1-infected macrophages as well as controls with/without LPS treatment. We observed 1) a modest increase in the p65 and phospho-p105 level in the nuclear fractions with HIV infection as compared with uninfected control, and 2) a significant increase in the case of LPS-treated HIV-infected sample (Fig. 8). We also observed a limited reduction in the level of p65 in CEP1347 treated HIV-infected/LPS stimulated cells (Fig. 8). However, we did not see significant differences in the phospho-p65 and p65 levels in the cytoplasmic fractions. CEP1347 failed to elicit specific response for NF-κB signaling pathway.

Discussion

Progressive HIV-1 infection commonly elicits neurologic impairments despite aggressive antiretroviral regimens, and significant improvements in the quality and duration of life may be balanced with demonstrable cognitive decline (49–54). Such decline is linked, in part, to variable penetration of antiretroviral drugs across the blood–brain barrier, difficulties with drug toxicity and compliance, and viral mutation (50–57).

The complex multifactorial pathogenesis of HAND presents therapeutic opportunities with respect to the development of novel adjunctive therapies. For example, viral infection and immune activation of MPs play an essential role in mediated neuronal...
damage in disease and can be exploited for therapeutic gain (58–60). Medicines that interfere with neuroinflammation or that protect neurons from damage can be expected to have a positive effect on the pathogenesis of HAND. CEP-1347 is a semisynthetic indolocarbazole that inhibits MLKs by acting as a competitive ATP site inhibitor (61). MLKs regulate neuronal-programmed cell death through the MAPK cascade by phosphorylation and activation of the transcription factor c-jun (32, 62). CEP-1347 is also neuroprotective by downregulating p38 and JNK activation in neurons (63) and can prevent the activation of human monocytes after exposure to HIV-1 gp120 and Tat (39). Prevention of phosphorylation of p38 and JNK may lead to decreased secretion of cytokines and inflammatory factors from MPs as well as the overall activation of MPs in response to HIV. Thus, we hypothesized CEP-1347 could attenuate HIV-1-associated neuroinflammation and in so doing protect against neuronal damage and apoptosis seen as a consequence of MPs infection and immune activation.

In this report, we demonstrate that CEP-1347 is a potent inhibitor of the neurotoxic secretome for HIV-1–infected macrophages. First, we found that the drug did not alter the levels of viral replication. Second, we showed that secretion of MPs CCL4/MIP-1β, CCL18/MIP-4, IL-12, and Stromelysin-1 (MMP-3) was reduced by CEP-1347 after 45 min. This may reduce neurotoxicity both by preventing the release of proinflammatory cytokines and chemokines within the brain and by preventing the entry of monocytes into the CNS (64–66). Upregulation of anti-inflammatory cytokines was also observed after exposure of MDM to CEP-1347. Levels of IL-4, TGF-β, IL-1R antagonist, and soluble TNFR1 secretion were all increased in CEP-1347–treated HIV-1–infected MDM, as compared with vehicle-treated HIV-1–infected MDM. These anti-inflammatory molecules may possess neuroprotective activity (67–73). CEP-1347’s upregulation of vascular endothelial growth factor production may also have neuroprotective significance (74).

Productive HIV-1 infection in human macrophages results in increased secretion of proinflammatory cytokines linked to secondary disease processes (11, 12) and suggesting the engagement of multiple kinases (75–77). In prior studies, CEP-1347 was shown to affect the balance between phosphorylation/dephosphorylation

Table 1. **CEP-1347 effects on neuroinflammation and neuroprotection in HIV-1 mice**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CEP-1347*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrogliosis (GFAP immunostaining)</td>
<td>21.4 ± 1.16</td>
<td>27.1 ± 0.66</td>
</tr>
<tr>
<td>Microgliosis (Iba-1 immunostaining)</td>
<td>8.56 ± 0.24</td>
<td>3.61 ± 0.17*</td>
</tr>
<tr>
<td>Dendritic density (MAP-2 immunostaining)</td>
<td>21.1 ± 3.0</td>
<td>29.0 ± 2.3*</td>
</tr>
<tr>
<td>Neuron density (NeuN immunostaining)</td>
<td>32.6 ± 2.2</td>
<td>35.0 ± 2.13</td>
</tr>
<tr>
<td>Degenerating neurons number (Neurofilament immunostaining)</td>
<td>6.5 ± 0.81</td>
<td>2.1 ± 0.65†</td>
</tr>
</tbody>
</table>

Data were assessed by immunohistochemical assays and shown as percent stained brain subregions for GFAP, Iba-1, MAP-2, NeuN (ImagePro program), and for numbers of neuronal bodies as stained by neurofilament Abs.

*CEP-1347 was administered by the i.p. route at a dose of 1.5 mg/kg/d for 7 d after stereotactic i.c. injection of human HIV-1ADA–infected MDM into the basal ganglia CB17/SCID mice (n = 17 mice/treatment group).

* ‡, p < 0.00001, †, p < 0.05, ‡, p < 0.01.
of downstream inflammatory events, including ERK1/2, p38, and JNK (32, 61, 77–79). Thus, we performed cell-based phosphorylation assays for these three kinases. Activation of ERK is known to be associated with cell growth and differentiation (80); whereas, activation of JNK and p38 MAPK are associated with growth arrest, apoptosis, and oncogenic transformation (81). HIV-1 infection was also shown to increase JNK phosphorylation, a proapoptotic pathway (82). In the current report, CEP-1347 showed some effect on JNK activation, consistent with CEP-1347’s activity as a potent inhibitor of JNK activation. HIV-1 infection also increased the phosphorylation (activation) of p38 MAPK, and CEP-1347 reduced this level of phosphorylation although the effects were quite modest. We also examined the effect of HIV-1ADA infection on activation of MLK3. Unexpectedly, CEP-1347 treatment resulted in a decline in the total level of MLK3 within the cells. This suggests the possibility that MLK3 may positively autoregulate its own expression; studies will be required to further examine this hypothesis.

The modulation of anti-inflammatory responses by CEP-1347 also suggested that engagement of MLK was linked to reductions in neurotoxicity mediated by HIV-1–infected MDM. This hypothesis was tested by adding conditioned supernatants from HIV-1–infected MDM that were treated with CEP-1347 or vehicle, to 10-d-old cultures of primary MCN as a commonly used target for HIV-1–mediated toxicity (42, 83). These experiments showed that culture media from CEP-1347–treated HIV-1–infected MDM elicited lower levels of neuronal death and dendritic damage than conditioned supernatants from untreated HIV-1–infected MDM, as assessed by MAP-2 and NeuN immunostaining. Thus, CEP-1347 proved to elicit neuroprotective responses in the HIVE mice model as was previously shown for antiretrovirals and anti-inflammatory drugs (84, 85).

We examined the morphology, activation status, and survival of key CNS cell populations in this model, either in the presence or absence of CEP-1347, including astrocytes, microglia, and neurons. HIVE mice treated with CEP-1347 were found to have lower levels of microglial activation and diminished neuronal loss when compared with untreated HIVE mice in a dose-dependent fashion. The in vivo studies with CEP-1347 involved an initial analysis of the biodistribution and pharmacokinetics of the drug in CB17/SCID mice. This showed relatively low steady-state plasma levels of drug in mice given 1.5 mg/kg/d [a dose similar to that used in human subjects enrolled in the CEP-1347 PRECEPT trial (86)]. The measured plasma levels of 4–32 ng/ml of CEP-1347 in mice over 8 h suggest that the biodistribution and/or pharmacokinetics of CEP-1347 is markedly different in animals treated i.p., as compared with

![FIGURE 7.](http://www.jimmunol.org/) MAPK kinase pathways in HIV-1ADA–infected human MDM treated with CEP-1347. Human monocytes collected after centrifugal elutriation were cultured for 7 d in 1000 U/ml MCSF, then infected with HIV-1ADA Viral stock at an MOI of 0.01 for 6 h with/without 220 nM CEP-1347. CEP-1347 was continued through the experiment. Uninfected MDM with or without CEP-1347 was used as controls. Viral replication continued for 5 d. Viral infection at 5 d showed RT activity 10-fold above background levels. Replicate control and HIV-1–infected MDM were treated with LPS at 100 ng/ml for 24 h when all cells were harvested for Western blot assays. Cell lysates were analyzed using Abs specific MLK3, phospho-MLK3, ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, and phospho-JNK. The data presented represent four independent experiments.

![FIGURE 8.](http://www.jimmunol.org/) Lack of CEP-1347 effect on NF-κB pathways in HIV-1–infected human MDM. Human monocytes collected after centrifugal elutriation were cultured for 7 d in 1000 U/ml MCSF then infected with HIV-1ADA Viral stock at an MOI of 0.01 for 6 h with or without CEP-1347 at 220 nM. CEP-1347 was continued through the experiment. Uninfected MDM with or without CEP-1347 was used as controls. Viral infection at 5 d showed RT activity 10-fold above background levels. Replicate control and HIV-1 infected MDM were treated with LPS at 100 ng/ml for 24 h when all cells were harvested for Western blot assays. Cells were fractionated into cytosolic and nuclear fractions using Nuclear/Cytosol fractionation Kit (BioVision, Cat. No. K266-25). The fractionation was performed according to the manufacturers instructions. Both cytosolic and nuclear fractions were then analyzed by using specific Ab to p65, p105/p50, and its phosphorylated derivatives (Cell Signaling Technology). For all analyses, GAPDH was used as loading control. The data presented represent three independent experiments.
human subjects receiving an oral dose of 10–50 mg CEP-1347, which resulted in plasma levels between 20 and 200 ng/ml. As a result, we performed experiments at a second dose of CEP-1347 (1.5 mg/kg/d), because this was expected to elicit plasma levels of drug equivalent to those in human subjects treated with the CEP-1347. Our experiments also revealed significant differences in the pharmacokinetics of CEP-1347 in C57BL/6 versus CB17/SCID mice. The basis for this is unclear at present.

In light of our analysis of the plasma levels of CEP-1347, we were somewhat surprised to observe that even a low in vivo dose of CEP-1347, capable of eliciting relatively modest steady-state plasma levels of drug (<20 nM), had a striking and statistically significant ability to reduce microglial activation and increase neuronal protection in vivo. This unexpected finding suggests that CEP-1347’s direct neuroprotective activity may work together with its ability to induce anti-inflammatory responses, thereby eliciting a very robust in vivo neuroprotective effect. This in vivo synergy may depend on CEP-1347’s ability to interfere with the treatment of HIV and their topic. Top. HIV Med. 15: 32–39.


