Neuroprotective Activities of CEP-1347 in Models of NeuroAIDS

Dawn Eggert, Prasanta K. Dash, Santhi Gorantla, Huanyu Dou, Giovanni Schifitto, Sanjay B. Maggirwar, Stephen Dewhurst, Larisa Poluektova, Harris A. Gelbard and Howard E. Gendelman

*J Immunol* 2010; 184:746-756; Prepublished online 4 December 2009;
doi: 10.4049/jimmunol.0902962
http://www.jimmunol.org/content/184/2/746

References  This article cites 83 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/184/2/746.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Neuroprotective Activities of CEP-1347 in Models of NeuroAIDS

Dawn Eggert,* Prasanta K. Dash,* Santhi Gorantla,* Huanyu Dou,* Giovanni Schifitto,† Sanjay B. Maggirwar,‡ Stephen Dewhurst,‡ Larisa Poluektova,* Harris A. Gelbard,† and Howard E. Gendelman*

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. The Journal of Immunology, 2010, 184: 746–756.
cytokines and other immune factors that induce neurotoxic activity (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<sub>ADA</sub>-mediated neurotoxicity independent of retroviral 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<sub>ADA</sub> (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<sub>ADA</sub> strain as the levels of viral growth are nearly uniform and not dependent on host cell differences. This ensured that the data acquired was reproducible from one experiment to another regardless of macrophage donor.

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<sub>ADA</sub> 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 [3H]dTTP (2 Ci/mmol; Amersham, Arlington Heights, IL) in Tris-HCl buffer (pH 7.9) for 24 h at 24-well plates.

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 0.05% trypsin. Neuron-enriched cells were resuspended in neurobasal medium (Invitrogen, Grand Island, NY) with heat-inactivated FCS supplemented with 0.027% trypsin. Media was replaced with neural basal media (Invitrogen, Grand Island, NY) every 24 h. On days 3 and 5, cells were treated with 80, 160, or 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 cytokines and chemokines per ml by Panomic Ab array 3.0 (Panomics, Fremont, CA) for human cytokines. Panomic Ab array 3.0 measured human cytokines: ApoE/Fas, Leptin, RANTES, ICAM-1, IL-2, IL-7, CTLA, MIP1α, TGFβ, VCAM-1, IL-3, IL-8, Eotaxin, MIPβ, IFNY, vascular endothelial growth factor, IL-4, IL-10, GM-CSF, MIP4, TNFα, IL-1α, IL-5, IL-12, TNFβ, epidermal growth factor, MIP-5, TNFRI, IL-1β, IL-6, IL-15, IFNγ, MP3M, TNFRII, IL-1α, IL6R, 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<sub>ADA</sub> 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 removed 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, IL-1β, IL-6, IL-10, TNFα, IL-12p70, and IL-8, RANTES, MIG, MCP-1, and IFN-γ, 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 laemmlti 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 polyvinylidene 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 and washed in TBS. Primary Abs used were anti-NF-kB p65 (1:1000, Cell Signaling Technology, Danvers, MA), anti-MKL3 (1:1000, Cell Signaling Technology), anti-JNK (1:1000, Cell Signaling Technology), anti- phospho-JNK (1:2000, Cell Signaling Technology), anti-p38 (1:1000, Cell Signaling), anti-phosphorylated-p38 (1:1000, Cell Signaling Technology), anti-phosphorylated-NF-κB p65 (1:1000, Cell Signaling Technology), anti-p65 (1:1000, Cell Signaling Technology), antiphosphorylated-NF-κB p65 (Ser 468) (1:1000, Cell Signaling Technology), anti-p65 (1:1000, Cell Signaling Technology), antiphosphorylated-NF-κB p65 (Ser 536) (1:1000, Cell Signaling Technology), anti-phosphorylated-p65 (1:1000, Cell Signaling Technology), anti-phosphorylated-p65 (1:1000, Cell Signaling Technology), anti-phosphorylated-p65 (1:1000, Cell Signaling Technology), 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 Laboratories, 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/ScCr-ScID/Dk (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 stereotypically 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, Carpinteria, 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 used to delineate intermediate filaments (cleaved VIM 3B1; Chemicon, Indianapolis, IN) were used to detect and localize human cells in mouse brains. Mouse microglia were detected by Abs to Iba-1 (WAKO, Osaka, Japan), and astrocytes were detected by Abs for 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. The numbers of human MDM and HIV-1 p24 Ag-positive cells were counted with a Nikon Microphot-FXA microscope. All obtained images were imported into Image-Pro Plus, v. 4.0 (Media Cybernetics, Silver Spring, MD) for quantifying area (%) of GFAP, Iba-1, MAP-2, and NeuN positive staining.

**Real time PCR**

The levels of HIV RNA in the injected hemisphere were determined by real time PCR using ABI 7000 prism (Perkin-Elmer, Applied Biosystems, Foster City, CA). Briefly, total RNA was extracted from the brain tissues. The HIV-1 gag RNA specific primers and probe were used as previously described (45) and are: forward, 5′-ACA TCA AGC CAT GCA AAT-3′; reverse, 5′-ATC TGG CCT GGT GCA ATA GG-3′; and probe, 5′-CAT CAA TGA GGA AGC TGC AGA ATG GGA TAG A-3′. The reverse primer was used to make cDNA from RNA, which was further amplified using primers and probe at 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Separate GAPDH amplifications were used as an endogenous control to ensure that equal amounts of RNA were used. For GAPDH, Mac-1, TNF, GFAP, and IL-10 Taqman gene expression assays were used (Applied Biosystems). Results were expressed as mean copy number ± SEM. Statistical analysis Data were analyzed using Excel (Macintosh, 1994) with Student t test for comparisons. p < 0.05 was designated statistically significant.

**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 the 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). Uprogation 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-1 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 ($p < 0.0001$, compared with cultures exposed to conditioned media

**FIGURE 1.** CEP-1347 and MDM secretory and viral replication activities. Effect of CEP-1347 on HIV-1 replication, growth factor, chemokine, proinflammatory cytokine and enzyme secretion by human MDM. A, TransSignal human cytokine Ab array 3.0 by Panomics and results table. At 5-d post-HIV-1_ADA infection, human MDM were treated with 220 nM CEP-1347 (upper blot) or vehicle (lower blot) for 45 min. Cells were washed to remove CEP-1347, and supernatants were harvested 24 h later. Each cytokine on array is composed of two horizontal dots. Data are representative of three independent experiments (mean ± SEM). B, CEP-1347 was administered to cultures at a concentration of 0–220 nM 24 h before, at the time of, and 4 h after HIV-1_ADA infection. Monocytes were cultivated for 7 d, then infected at a MOI of 0.01. Supernatant fluids were collected and assayed for RT activities from zero to 10 d after viral infection. Values represent counts per minute (mean ± SEM) of samples from six independent cultures and are representative of three independent separate experiments when CEP-1347 was added simultaneously with infection. The same results were obtained with drug administration before and after infection. C, In addition, Cytometric Bead Array showed downregulation of IL-8 and CXCL10/10. Values represent concentrations of chemokines in supernatants collected from three independent experiments, when CEP-1347 was added simultaneously with infection (mean ± SEM). # $p < 0.05$ difference between uninfected and HIV-1–infected MDM. * $p < 0.05$, differences between untreated and treated infected cells. ** $p < 0.01$, differences between untreated and treated infected cells.
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 media was 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<sub>ADA</sub>–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<sub>ADA</sub>–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
Microglial activation decreased with increasing concentrations of CEP-1347 (Fig. 5D). Microglial activation was significantly decreased in all CEP-1347–treated groups when compared with vehicle-only treated HIV-1-infected MDMs (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 ± 0.26, 0.87 ± 0.40 (p < 0.0001), 0.75 ± 0.17 (p < 0.0001), and 0.41 ± 0.06 (p < 0.00001), respectively (Fig. 5A–C). Microglial activation decreased with increasing concentrations of CEP-1347 (Fig. 5D).

CEP-1347 elicits neuroprotective responses in HIV-1 infected MDMs

To determine whether CEP-1347 was neuroprotective in HIV-1 infected MDMs, 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 astrogliosis and microglial activation. Significant neuronal loss was present beyond the lesion site in vehicle-only treated HIV-1-infected MDMs; 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 MDMs treated with CEP-1347 had significantly increased numbers of neurons surrounding the lesion when compared with vehicle-only treated HIV-1-infected MDMs (13.9 ± 3.3; HIVE mice receiving 0.5, 1.0, 1.5, 5.0, and 15.0 mg/kg/d of CEP1347 had neuron counts of 29.3 ± 3.6 (p < 0.05 compared with vehicle-treated HIV-1-infected MDMs), 37.0 ± 4.0 (p < 0.001), 52.7 ± 3.7 (p < 0.00001), 51.8 ± 4.6 (p < 0.0001), and 51.1 ± 5.2 (p < 0.0002), respectively. Dendritic processes, stained with anti-MAP-2 from the same field as neurons were counted and were quantified as a percentage of the area of the entire microscopy field. Several brain sections were used to determine dendritic process loss surrounding the lesion area. Dendritic loss was significantly decreased in HIV-1 infected MDMs receiving CEP-1347 compared with HIV-1 infected MDMs receiving vehicle-only treatment (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 HIV-1-infected MDMs), 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 HIV-1-infected MDMs showed decreased neuronal loss and dendritic processes when compared with vehicle-only treated HIV-1-infected MDMs. 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).

CEP-1347 was further assessed in the same HIV-1 infected MDM model for tests that included both histology and quantitative real time RT-PCR. 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-1ADA–infected MDM and administered CEP-1347 i.p. daily for 7 d. Animals were then sacrificed, and brain tissue was removed for immunohistology or RT-PCR. Histopathological changes observed included formation of multinucleated giant cells, astrocytosis, and neuronal dropout (Table 1).

Human MDMs were identified by immunostaining with vimentin and were present in the area adjacent to the stereotactic injection site. In HIV-1-infected MDMs treated with 1.5 mg/kg/d CEP-1347, the mean number of MDMs was 271.3 ± 121.0 compared with 275.2 ± 123.0 MDMs for HIV-1–infected vehicle-treated MDMs. HIV-1–infected MDMs in brain were immunostained with anti-p24 Ag and quantified. CEP-1347–treated mice had 35.4 ± 19.0 HIV-1–infected MDMs, whereas, HIV-1–infected vehicle-treated mice had 61.8 ± 28.4 HIV-1–infected MDMs (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 HIV-1-infected MDMs (13.0% ± 15.7%) and vehicle-only treated HIV-1-infected MDMs (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). GFAP 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 HIV-1-infected MDMs compared with vehicle-only treated HIV-1-infected MDMs (3.61 ± 0.12, 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. HIV-1–infected MDMs 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 HIV-1–infected MDMs (46). Neither TNF-α, nor IL-10 mRNA expression levels were significantly different in CEP-1347–treated HIV-1–infected MDMs compared with vehicle-only treated HIV-1–infected MDMs.

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-1 gp120 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 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...
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 HIVE mice**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment Groups</th>
<th>CEP-1347*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrogliosis (GFAP immunostaining)</td>
<td>Control</td>
<td>21.4 ± 1.16</td>
</tr>
<tr>
<td>Microgliosis (Iba-1 immunostaining)</td>
<td></td>
<td>8.56 ± 0.24</td>
</tr>
<tr>
<td>Dendritic density (MAP-2 immunostaining)</td>
<td></td>
<td>21.1 ± 3.0</td>
</tr>
<tr>
<td>Neuron density (NeuN immunostaining)</td>
<td></td>
<td>32.6 ± 2.2</td>
</tr>
<tr>
<td>Degenerating neurons number (Neurofilament immunostaining)</td>
<td></td>
<td>6.5 ± 0.81</td>
</tr>
</tbody>
</table>

Data were assessed by immunohistochemical assays and shown as percent stained brain subregions for GFAP, Iba, 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-1ΔΔα 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 HIV mouse 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. HIV mice treated with CEP-1347 were found to have lower levels of microglial activation and diminished neuronal loss when compared with untreated HIV 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.](image1) MAP kinase pathways in HIV-1ΔΔα–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-1ΔΔα Viral stock at an MOI of 0.01 for 6 h with or 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.](image2) 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-1ΔΔα Viral stock at an MOI of 0.01 for 6 h with or without 220 nM CEP-1347. 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 its ability to induce anti-inflammatory responses, thereby reducing microglial activation and increasing immune competence in macrophages.

Regardless of these in vitro considerations, the major take-home message of this study is that CEP-1347 elicits significant neuroprotective effects in vivo, proving support for the use of mixed lineage kinase inhibitors in HAND treatment. In conclusion, we demonstrate that the mixed lineage kinase inhibitor, CEP-1347, elicits an anti-inflammatory phenotype in HIV-1–infected human MDM and that this results in a reduction in neurotoxic activities. Our data also show that, in a murine model for HIV-1 infection, CEP-1347 reduces microglial activation and dendritic damage and speeds neuronal survival. Collectively, these findings support the idea that CEP-1347 can be of therapeutic potential for HAND.

Acknowledgments

We thank Robin Taylor for outstanding administrative and computer support. We thank Drs. Deborah Galinas, Lisa Aimone, and Rebecca Morey for plasma sample and pharmacokinetic analyses and Dr. Donna Bozyczko-Coyne of Cephalon (Brandywine, PA) for thoughtful discussions and research design. We thank Nan Gong for cortical neuron culture and Michael T. Jacobson for confocal microscopy.

Disclosures

The authors have no financial conflicts of interest.

References
