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Inhibition of Mixed Lineage Kinase 3 Prevents HIV-1 Tat-Mediated Neurotoxicity and Monocyte Activation

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The HIV-1 gene products Tat and gp120 are toxic to neurons and can activate cells of myeloid origin, properties that are thought to contribute to the clinical manifestations of HIV-1-associated dementia (HAD). To investigate the intracellular signaling mechanisms involved in these events, the effect of Tat and gp120 on mixed lineage kinase (MLK) 3 activation was examined. Tat and gp120 were shown to induce autophosphorylation of MLK3 in primary rat neurons; this was abolished by the addition of an inhibitor of MLK3 (CEP1347). CEP1347 also enhanced survival of both rat and human neurons and inhibited the activation of human monocytes after exposure to Tat and gp120. Furthermore, overexpression of wild-type MLK3 led to the induction of neuronal death, whereas expression of a dominant negative MLK3 mutant protected neurons from the toxic effects of Tat. MLK3-dependent downstream signaling events were implicated in the neuroprotective and monocyte-deactivating pathways triggered by CEP1347. Thus, the inhibition of p38 MAPK and JNK protected neurons from Tat-induced apoptosis, whereas the inhibition of p38 MAPK, but not of JNK, was sufficient to prevent Tat- and gp120-mediated activation of monocytes. These results suggest that the normal function of MLK3 is compromised by HIV-1 neurotoxins (Tat, gp120), resulting in the activation of downstream signaling events that result in neuronal death and monocyte activation (with release of inflammatory cytokines). In aggregate, our data define MLK3 as a promising therapeutic target for intervention in HAD.


Mixed lineage kinases (MLKs) are MAPK kinase kinases that contain structural motif suggestive of both serine-threonine and tyrosine kinase activities (hence the nomenclature mixed lineage). In response to diverse stimuli that stress cells, MLKs target JNK and p38 MAPK for activation (1); as a result, these kinases regulate a broad range of cellular processes, including programmed cell death (2–4). Indeed, both of these downstream kinases have been implicated in neurodegenerative disease. For example, activation of p38 has been suggested to play a role in the pathogenesis of HIV-1-associated dementia (HAD) (5), Alzheimer’s disease (6), Parkinson’s disease (PD; Ref. 7), and stroke (8). Similarly, JNK-3 (the JNK isofrom that is primarily expressed in CNS neurons) has been implicated in the pathogenesis of neurodegenerative disorders (9).

MLK3 (also known as MAP3K11) is the most widely expressed MLK family member and is present in neurons (10, 11). It is activated by GTPases of the Ras superfamily, such as Cdc42 and Rac, which trigger protein dimerization via a leucine zipper interface, resulting in autophosphorylation at Thr277 and Ser281 within the protein activation loop and subsequent activation of the enzyme. Endogenous inhibitors of this kinase include the prosurvival protein kinase, Akt (12, 13).

Recent research has focused on inhibition of the MLK/JNK pathway as a therapeutic strategy for PD, with the aim of preventing the apoptotic demise of dopaminergic neurons in the pars compacta of the substantia nigra. Studies using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of parkinsonism have demonstrated the efficacy of a specific MLK3 inhibitor, CEP1347 (14, 15), in treating motor deficits and neuronal degeneration (16–19), and CEP1347-mediated neuroprotection has also been observed in an in vitro model for PD, using methamphetamine-exposed human mesencephalic-derived neurons (16). The preclinical rationale for MLK3 inhibition has been sufficiently compelling for early phase 1 studies to demonstrate the safety and tolerability of CEP1347 in patients suffering from PD (20), as well as to pave the way for larger blinded, placebo-controlled trials of efficacy in patients with early untreated PD (PRECEPT study) (21).

Preclinical studies of CEP1347 have also shown that this agent can protect neurons against a considerable range of insults, including exposure to the Alzheimer’s peptide, Aβ (22), suggesting the possibility that CEP1347 might also be protective in the context of in vitro model systems for HAD. In fact, Bodner et al. (10) have shown that CEP1347 can protect primary rat hippocampal neurons as well as dorsal root ganglion neurons from the otherwise lethal effects of exposure to HIV-1 gp120. However, these studies focused on a single CNS cell type (rat hippocampal neurons) and a single neurotoxin, gp120 (10), prompting the question as to
whether the neuroprotective effects of CEP1347 might extend to other neuronal populations and different candidate HIV-1 neurotoxins. This was an important focus of the present study. In addition, because microglial and macrophage activation has been suggested to play an important role in the pathogenesis of HAD (23–25), it was also of interest to examine whether inhibition of MLK3 might reduce the activation of primary human monocytes after exposure to HIV-1 Tat.

The experiments presented here show that Tat and gp120 activate MLK3 in primary neurons and that this activation contributes to Tat- and gp120-mediated neurotoxicity. Inhibition of MLK3 also attenuates the production of proinflammatory mediators from Tat and gp120-treated monocytes. These cellular events are mediated, in part, via MLK3-dependent activation of partially overlapping signaling mechanisms. Thus, activation of p38 MAPK and JNK mediated Tat-induced neuronal apoptosis, whereas inhibition of p38 MAPK, but not of JNK, was sufficient to prevent Tat and gp120-mediated activation of monocytes. Collectively, these findings further implicate MLK3 as an important molecular target in the pathogenesis and treatment of the neurologic complications associated with HIV-1/AIDS.

Materials and Methods

Reagents

HIV-1 Tat_{1-101}; HIV-1(BaL) gp120; SB203580, SB202190, SP600125, and JNK-inhibitory protein (JNKi); lithium chloride; and staurosporine were obtained from ImmunoDiagnostics, AIDS Research and Reference Reagent Program, Calbiochem, and Sigma-Aldrich, respectively. CEP1347 and CEP11004 were received from Cephalon.

Cell cultures

Cerebellar granule neurons (CGNs). Seven-day-old Sprague-Dawley rats were sacrificed, and cerebellar brain tissue was harvested in accordance with Animal Welfare Act and National Institutes of Health guidelines. The methods used have been described (26). In brief, cerebellum was collected, washed, and separated into a single-cell suspension using gentle trypsinization, trituration with a polished glass pipette, and filtration through a nylon mesh. After Percoll density gradient centrifugation to remove glia, the neurons were collected, washed twice in sterile medium without serum, and then resuspended in DMEM-F-12 medium with 10% horse serum. Cells were then plated on poly-L-lysine (molecular mass, 70–150 kDa; Sigma-Aldrich)-coated 100 mm culture dishes at a density of 3 × 10^6 cells/dish. One day later, 5-fluorodeoxyuridine (20 mg/ml) and uridine (50 mg/ml) were added to eliminate proliferative cells (astrocytes); the purity of the neuronal population was verified by immunocytochemical staining for MAP-2. Under these conditions, the cultures were >95% homogeneous for neurons. Neurons were cultured for ≤7 days at 37°C in a humidified atmosphere containing 5% CO_2 and suspended in serum-free DMEM-F-12 for 24 h before the treatments.

Cortical neurons (CN). Primary neuronal cultures were prepared from embryonic day 18 rats as described (27). In brief, cortices were dissected from a litter of E18 embryonic rats and incubated in 2.0 ml of Ca^2+-Mg^2+-free HBSS (with 10 mM HEPES, pH 7.3) with antibiotics (penicillin, 50 mg/L; streptomycin, 50 mg/L; neomycin, 100 mg/L) plus 0.5 ml of 2.5% trypsin (for 0.25% final) for 15 min at 37°C per brain. After a washing with HBSS and trituration, dissociated cells were counted by trypan blue exclusion for viability and plated in cell culture plates at 0.5–0.6 × 10^5 cells/cm^2, on poly-L-lysine-coated plasticware. The plating and maintenance medium used in these experiments consisted of Neurobasal medium.
with B27 supplement (Invitrogen Life Technologies) as described (28), and as modified for antioxidant-free culture (27). This medium formulation inhibits the outgrowth of glia resulting in a neuronal population that is 98% pure. Cells were cultured for 10–21 days at 37°C in a humidified atmosphere of 5% CO2-95% air, changing medium every 4 days. Cells were used for experiments at days in vitro (DIV) 14–21 unless otherwise indicated. The human cortical neurons used in our experiments were supplied by ScienCell Research Laboratories.

**Human monocytes.** Human peripheral monocytes were isolated from soft-spun buffy coats derived from HIV-1- and hepatitis B virus-seronegative donors by using immunomagnetic isolation methods as described (29); blood was obtained from anonymous donors via the American Red Cross. Briefly, PBMC were isolated fromuffy coats after centrifugation on a Lymphoprep gradient (AXIS-Shield). The cells were further purified by positive selection with anti-CD14 MACS beads (Miltenyi Biotec). These monocytes were then cultured in RPMI 1640 supplemented with 1% autologous plasma for 18–24 h and used for experiments. The monocyte cultures used in our experiments were >95% pure as determined by flow cytometric analyses using FITC-conjugated anti-CD14 Abs.

**Transient transfections.** CN (1 × 105 cells/well) were cultured for 7 DIV and then transiently transfected with either 1.5 μg of plasmid DNA-encoding GFP, MLK3 wild-type (MLK3 wt), or a dominant negative form of MLK3 (MLK3 DN) (gift from Dr. James Woodgett, using Lipofectamine 2000 reagent (Invitrogen Life Technologies); Under these conditions, ~5–10% transfection efficiency was achieved in neurons. Twenty-four hours after transfection, the cells were either left untreated or incubated for 24 h with vehicle alone (control) or with HIV-1 Tat, after which the cells were fixed in 4% formaldehyde and nuclei were stained with Hoechst dye (Molecular Probes) for analysis of neuronal survival.

**Immunoblotting assays**

These assays were performed as described (30). Briefly, after control or experimental treatments, whole cell lysates were prepared using ELB buffer (50 mM HEPES [pH 7], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na3VO4, and 50 μM ZnCl2, supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors), and cellular debris was removed by high speed centrifugation. Cellular lysates were then fractionated by SDS-PAGE and electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were then analyzed for immunoreactivity with primary Abs (as indicated in the figures; all Abs were obtained from Cell Signaling Technology). The bound Abs were detected with HRP-linked sheep secondary Abs (Amersham Biosciences). These assays were performed as described (30). Briefly, after control or experimental treatments, whole cell lysates were prepared using ELB buffer (50 mM HEPES [pH 7], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na3VO4, and 50 μM ZnCl2, supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors), and cellular debris was removed by high speed centrifugation. Cellular lysates were then fractionated by SDS-PAGE and electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were then analyzed for immunoreactivity with primary Abs (as indicated in the figures; all Abs were obtained from Cell Signaling Technology). The bound Abs were detected with HRP-linked sheep secondary Abs (Amersham Biosciences), followed by the addition of ECL reagent (Pierce Biotechnology) and subsequent exposure to x-ray film.

**TNF-α ELISA**

TNF-α levels were measured in culture supernatant (precollected by brief centrifugation) by using a human TNF-α ELISA Kit (Biosource International) according to the manufacturer’s instructions. Briefly, 100 μl of cell culture supernatant was incubated in a 96-well plate precoated with a TNF-α-specific mAb for 1 h. After extensive washing, binding of TNF-α was detected by incubation with biotinylated Abs, followed by streptavidin-peroxidase colorimetric enzyme assays.

**TUNEL assays**

Apoptotic cells were visualized by using an in situ TUNEL assay, according to the manufacturer’s instructions (Intergen) (30). Briefly, the cells were treated with HIV-1-encoded neurotoxins in the presence or absence of
Figure 3. Genetic inhibition of MLK3 confers protection against Tat- and gp120-mediated neurotoxicity. Primary cortical neurons (DIV7) were transiently transfected with either 1.5 μg of plasmid DNA (pcDNA3) encoding GFP, MLK3 wt, or MLK3 DN, using Lipofectamine 2000; all cells also received 1.5 μg of plasmid DNA encoding GFP. Under these conditions, 5–10% efficiency of transfection was achieved. After 24 h, cells were either left untreated or treated with 500 nM Tat or 5 nM gp120 (in the presence or absence of 200 nM CEP11004 or CEP1347). Treatments proceeded for 24 h (A) or 72 h (B), after which the cells were fixed in 4% formaldehyde, and nuclei were stained with Hoechst dye. The percent of apoptotic cells (cells positive for condensed chromatin and/or pyknotic nuclei) was determined by scoring GFP-positive (green) cells for apoptosis based on Hoechst stain. A total of 75–100 GFP-positive neurons were counted in each of these experiments, which were repeated three times. Results for CEP-treated cells were statistically compared with results from untreated cultures transfected with MLK3 wt. Results for Tat (or gp120, B)-treated cells transfected with MLK3 DN were statistically compared with results from Tat (or gp120, B)-treated cells expressing only GFP. Statistical analysis was performed using ANOVA and Bonferroni’s post-test. ***, p < 0.001; **, p < 0.01. Data represent the mean ± SEM of three experiments.

Pharmacologic MLK3 inhibitors protect against Tat-induced apoptosis in rat neurons

In light of a recent report that MLK3 plays a role in gp120-induced death of rodent hippocampal neurons (10), we investigated whether the inhibition of MLK3 activation might be neuroprotective against HIV-1 Tat and gp120 exposure in our culture systems. Rat CGN and primary human CN cultures were exposed to a neurotoxic concentration of HIV-1 Tat or gp120, in the presence or absence of MLK3 inhibitors, CEP1347 or CEP11004, at varying doses. As expected, the incubation of neurons with Tat or gp120 led to the induction of cellular apoptosis (Fig. 2, A, B, and E). Coincubation with CEP1347 or CEP11004 protected these neurons, in a dose-dependent manner, against the otherwise lethal effects of exposure to HIV-1 Tat and gp120 at both 24- and 72-h time points (Fig. 2).

We also conducted experiments to confirm that CEP1347-mediated neuroprotection was not secondary to an effect on microglia present in our neuronal cell culture systems. The rat CGN cultures in our experiments were found to contain only a very low level of microglial contamination (<1%; data not shown), consistent with what one might expect for this highly purified neuronal cell population. To verify that these cells were not in any way responsible for the results observed, we took advantage of a method described by Yabe et al. (33), that was previously used to deplete residual microglia from CGN cultures. Cultures were treated on DIV 4 for 30 min with either vehicle or 20 mM l-leucine methyl ester, a compound that kills microglia. These cultures were then exposed to HIV Tat (500 nM) on DIV 7, in the presence or absence of CEP1347 (320 nM). Despite the removal of microglia (as verified by indirect immunofluorescence staining using anti-OX42), CEP1347 remained able to prevent Tat-induced neuronal apoptosis (data not shown). Thus, the neuroprotective effects of CEP1347 were independent of microglia.

Genetic inhibition of MLK3 also protects against Tat-induced neuronal apoptosis

To understand better the mechanism whereby CEP1347 ameliorates the toxic effects of Tat and gp120, rat CN were transiently transfected with plasmid expression vectors encoding either wild-type MLK3 or a dominant negative mutant isoform of this protein. After 24 h, cells were exposed to a neurotoxic concentration of HIV-1 Tat or gp120 in the presence or absence of the pharmacologic MLK3 inhibitors, CEP1347 or CEP11004 (Fig. 3); neuronal apoptosis was then quantitated with Hoechst staining. These experiments revealed that overexpression of MLK3 WT led to efficient induction of neuronal apoptosis, which was reversed by...
CEP1347 or CEP11004. In contrast, overexpression of the dominant negative MLK3 mutant led to protection of neurons from Tat-induced apoptosis. Primary cultures of rat CGNs were exposed to HIV-1 Tat (Tat, 500 nM), or vehicle alone (NT), in the presence or absence of CEP1347 (320 nM) for 30 min. Cell lysates were then prepared and analyzed using Abs specific either for all p38 MAPK isoforms (A, middle) and JNK (p54) isoforms (B, middle) or for phospho(Thr\(^{180}/\)Tyr\(^{182}\))-p38 MAPK (A, top) and for phospho(Thr\(^{183}/\)Tyr\(^{185}\))-JNK (p54) (B, top). Both sets of lysates were also analyzed using Abs specific for a-tubulin (B, bottom), as a loading control. C. Densitometric analysis of data from three independent experiments that examined p38 MAPK and JNK activation in Tat-exposed CGNs. Fold induction of the various protein species was normalized to levels of a-tubulin and then expressed as a function of the expression level in untreated cells (NT), which was assigned a value of 1. CGNs were also exposed to HIV-1 Tat (Tat, 500 nM) or vehicle alone (NT), in the presence or absence of SB203580 (p38 MAPK inhibitor) (D) or SP600125 (JNK inhibitor) (E) as indicated. Some cells also received Staurosporine (E, which induces neuronal cell death via a JNK-independent pathway). After 24 h, cultures were analyzed for the percentage of apoptotic cells using the TUNEL assay. Data represent the mean \pm SEM for one experiment performed in triplicate; data are representative of two independent experiments. Statistical analysis was performed using ANOVA and Bonferroni’s posttest. D, *, \(p < 0.001\) as compared with NT; #, \(p < 0.001\) as compared with cells exposed to Tat alone; ##, \(p < 0.01\) as compared with Tat (D). E, *, \(p < 0.01\) as compared with NT; **, \(p < 0.05\) as compared with cells exposed to Tat alone (E). Blocking JNK activation by addition of SP600125 prevents neuronal apoptosis by Tat, but not by staurosporine.

Inhibitors of p38 MAPK and JNK protect against Tat-induced neuronal apoptosis

To test whether downstream kinases known to be activated by MLK3 are also involved in Tat-induced neuronal apoptosis, we exposed rat CGNs to a neurotoxic concentration of HIV-1 Tat and then examined the activation of p38 MAPK and JNK. As shown in Fig. 4, A and B, immunoblot analyses revealed that Tat treatment led to increased phosphorylation of p38 MAPK and JNK (including both p54 and p46 isoforms of JNK; only p54 bands are shown in Fig. 4), indicative of kinase activation. Inhibition of MLK3 by coadministration of CEP1347 efficiently blocked activation of p38 MAPK and JNK kinases.

We next examined whether inhibition of p38 MAPK or JNK by pharmacologic agents protected neurons against Tat-induced apoptosis. Rat CGNs were treated with Tat in the presence or absence of p38 MAPK blockers (SB203580 and SB202190), or JNK inhibitors (SP600125 and JNKi), and neuronal apoptosis was measured. Inhibition of p38 MAPK by SB203580 (Fig. 4C), or by SB202190 (data not shown) was found to dose-dependently prevent the lethal effects of Tat. Similarly, inhibition of JNK by SP600125 (Fig. 4D) or by JNKi (data not shown) blocked Tat-induced neuronal apoptosis. To test whether SB203580 and SP600125 might promotes neuronal survival in a nonspecific manner, additional experiments were conducted in which neuronal apoptosis was induced by administration of staurosporine, a toxin that stimulates neuronal apoptosis via p38- or JNK-independent pathways. In contrast to the results noted above (Fig. 4C), SP600125 (Fig. 4D) or SB203580 (data not shown) failed to block staurosporine-induced neuronal apoptosis, suggesting that these reagents did not exert a nonspecific neuroprotective effect in our
culture system. Together, these results support the notion that Tat-mediated activation of MLK3 may promote neuronal cell death, in part, through the activation of p38 MAPK and JNK.

Pharmacologic MLK3 inhibitors reduce Tat-induced activation of human monocytes

The pathogenesis of HAD is believed to involve activation of peripheral monocytes and subsequent recruitment of these cells into the CNS, where they can serve as a sanctuary for HIV-1 replication and production of secretory neurotoxins (35, 36). Therefore, we examined whether pharmacologic inhibitors of MLK3 also blocked Tat- or gp120-induced monocyte activation. In these assays, human monocytes were treated with Tat or gp120 in the presence or absence of CEP1347 and CEP11004, and TNF-α release was measured (as a marker of monocyte activation) in cell culture supernatants using ELISA. The results (Fig. 5) showed that both CEP1347 and CEP11004 abrogated the Tat- and gp120-mediated increase in monocyte-derived TNF-α production. These results show that MLK3 inhibitors can reduce monocyte activation and thereby decrease the production of potentially neurotoxic proinflammatory cytokines.

Inhibitors of p38 MAPK prevent Tat-induced monocyte activation

To test whether downstream kinases that may be activated by MLK3 are also involved in Tat- or gp120-induced monocyte activation, experiments were conducted in which primary human monocytes were exposed to HIV-1 Tat or gp120 in the presence or absence of CEP1347 and CEP11004. Cellular lysates were then prepared and subjected to immunoblot analyses in which phosphospecific Abs against p38 MAPK or JNK molecules were used. As shown in Fig. 6, enhanced phosphorylation of p38 MAPK and JNK (including both p54 and p46 isoforms of JNK; only p54 bands are shown in Fig. 6, C and D), was observed in Tat- and gp120-treated monocytes. Phosphorylation of p38 MAPK and JNK was not detected in cells that were treated with Tat or gp120 in the presence of CEP1347 or CEP11004. These data suggest that Tat and gp120 activate p38 MAPK and JNK in human monocytes, in a MLK3-dependent manner.

To examine the relevance of p38 MAPK and JNK activation to monocyte activation after exposure to HIV-1 Tat or gp120, human monocytes were treated with HIV-1 Tat or gp120 in the presence or absence of p38 MAPK or JNK inhibitors, and TNF-α release was then measured in cell culture supernatants using ELISA. The p38 MAPK inhibitors, SB203580 (Fig. 7, A and C) and SB202190 (data not shown) efficiently abrogated the Tat- and gp120-mediated increase in monocyte TNF-α production. In contrast, the JNK inhibitors (SP600125 and JNKi) had only a very modest effect on Tat- or gp120-induced monocyte-derived TNF-α production (Fig. 7, B and C, and data not shown). These results support the notion that Tat-mediated activation of MLK3 may promote monocyte activation principally via initiation of the p38 MAPK signaling pathway.

Discussion

Current clinical interest in pharmacologic inhibitors of MLK3 is focused on the potential role of MLK3 in the pathogenesis of PD. It is therefore worth noting that several links between parkinsonism and the neurologic aspects of HIV-1 infection have been described, including an initial 1987 case report that described the neurologic symptoms of PD in two patients with AIDS who were treated with the neuroleptic dopamine receptor antagonist prochlorperazine (37). Subsequently, HAD and its milder phenotype, minor cognitive minor motor disease, have been viewed as a subcortical type of neurodegeneration, characterized by defects in dopaminergic pathways that are similar to some aspects of PD (for reviews, see Refs. 38 and 40). Indeed, parkinsonism has been a presenting manifestation of HIV-1 infection, with amelioration of symptoms referable to basal ganglia deficits following HAART (41). Other groups have reviewed neurologic manifestations of HIV-1 infection and concluded that in 42.8% of patients with parkinsonism there were direct effects of HIV-1 on the basal ganglia (42). The interpretation of these clinical findings has been supported by biochemical changes such as a reduction in dopamine

![FIGURE 5. MLK3 inhibitors reduce Tat- and gp120-induced activation of human monocytes. Primary human monocytes were exposed to HIV-1 Tat (Tat, 100 nM; A), or gp120 (Bal, 100 nM; B), or vehicle alone (NT), plus or minus CEP1347 or CEP11004 as indicated. After 4 h, TNF-α levels in culture supernatants were quantitated by ELISA. Data represent the mean ± SEM for one experiment performed in triplicate; data are representative of three experiments. Cotreatment with CEP1347 or CEP11004 led a statistically significant decrease in TNF-α production, relative to cells treated with Tat alone (*, p < 0.01), or gp120 alone (++, p < 0.01; ANOVA and Bonferroni’s posttest).](image-url)
and its major metabolite homovanillic acid in the caudate nucleus of the basal ganglia in persons with HAD (43). This overlap in neuropathologic findings between PD and HAD has been sufficiently compelling to prompt us to consider whether pathologic activation of MLK3 might also occur in preclinical models of HAD (as it does in PD), and if so, whether inhibition of MLK3 might result in neuroprotection.

Bodner et al. (10, 44) previously recognized this possibility and showed that the toxic effects of HIV-1 gp120 on primary rat hippocampal neurons could be reversed by CEP1347. However, their experiments were limited to a single neurotoxin and a single CNS cell population. The data presented here therefore strongly confirm and significantly extend this earlier work, by showing that inhibition of MLK3 also results in the protection of rat CGNs and human CNs from the proapoptotic effects of exposure to HIV-1 Tat and gp120.

The ability of CEP1347 to protect hippocampal neurons (10) as well as CGNs and CNs (Fig. 2) suggests that common signaling mechanisms may be involved in the proapoptotic pathways that are triggered after exposure of neurons to both HIV-1 Tat and gp120. Tat and gp120 are both known to activate ubiquitous neuronal NMDA receptors, leading to calcium flux and neuronal apoptosis (45–49). In the case of Tat, this may occur either directly (45) or indirectly following engagement of widely expressed low density lipoprotein receptor-related protein (50–52). Because activation of NMDA receptors results in p38 MAP and JNK activation in cortical, hippocampal and cerebellar neurons (53–55), this suggests that NMDA receptor activation may trigger common proapoptotic signaling events in different neuronal populations, which CEP1347 is able to block.

HAD is associated not only with the extensive loss of neurons within certain regions of the brain (56–58) and elevated levels of neuronal apoptosis (59, 60) but also with the chronic production of inflammatory mediators that includes a range of cytokines and chemokines and of viral gene products such as Tat and gp120 (61). As noted here, these two viral proteins are toxic to neurons (10, 30, 62) and are also capable of activating uninfected brain-resident macrophages and microglia (63, 64), but the molecular mechanisms that contribute to these processes are incompletely understood.
CEP1347 was therefore used to probe the role of MLK3 activation in Tat-mediated activation of primary human monocytes, because it is known to be a potent inhibitor of this kinase (14, 15). The data presented here demonstrate that CEP1347 reduces monocyte activation (as measured by TNF-α release) after exposure to HIV-1 Tat, in a dose-dependent manner, and our studies further implicate the p38 MAPK pathway (but not the JNK pathway) in this effect. These findings extend previous reports that CEP1347 can reduce the production of proinflammatory cytokines (TNF-α, IL-6) and chemokines (MCP-1) by primary human and rat microglia after exposure to bacterial endotoxins or to the Alzheimer’s peptide, Aβ1–40 (65), as well as similar findings that were obtained using CEP11004 (66). Overall, this suggests that CEP1347 might have the desirable effect of reducing the activation of cells of myeloid lineage within the CNS and periphery of persons with HAD, thereby reducing monocyte recruitment into the CNS and also inhibiting the local generation of neurotoxic effector molecules within the CNS. This is important because of the significant role that has been ascribed to MCP-1, TNF-α, and IL-6 in terms of the pathogenesis and progression of HAD (67–73).

Our analyses of the signaling pathways that contribute to Tat-mediated neuronal cell death differ from a recent report by Singh et al. (62), in which inhibitors of JNK and p38 MAPK failed to prevent Tat-induced apoptosis in cultured rat striatal neurons. The disparities in the two datasets may reflect, at least in part, differences in the composition of striatal vs CGN culture systems. It is also important to note that Singh et al. (62) did find that p38 MAPK inhibition was able to prevent gp120-induced neuronal cell death and neurite loss in the striatal model, although additional studies may be needed to explore more fully the molecular mechanisms that contribute to the neurotoxic actions of HIV-1 Tat.

The mechanisms by which Tat activates monocytes are relatively poorly understood. Tat is known to efficiently induce calcium mobilization, NF-κB activation and cytokine release from monocytes (74–76), but the signaling events and cell surface receptors involved in these processes remain poorly understood. Some groups have reported that Tat triggers release of calcium from IP3-regulated intracellular stores (76, 77), whereas others have implicated extracellular calcium and provided evidence that Tat may interact with L-type calcium channels and/or cell surface chemokine receptors (78, 79). In contrast, gp120 is somewhat better understood and appears to stimulate monocyte activation mainly through the chemokine receptors CCR5 and CXCR4 (80, 81). As in neuronal cells, the downstream signaling events following chemokine receptor engagement by gp120 appear to involve calcium mobilization and activation of p38 MAPK and JNK pathways (80, 81).

P38 MAPK and JNK signaling pathways within the CNS are well recognized to be disrupted in the context of lentivirus infection. In a well-characterized model of SIV-induced neurodegeneration in rhesus macaques, Barber et al. (5) demonstrated that JNK is up-regulated in macrophages and microglia, whereas p38 MAPK is up-regulated in neurons and astrocytes. Additional studies by D’Aversa et al. (82) demonstrated that Tat-induced activation of the p38 MAPK pathway in human microglia can markedly up-regulate production of the chemokines CXCL8 and CCL3, which in turn lead to the recruitment of neutrophils, lymphocytes, and monocytes, respectively. In aggregate, these findings make a


