

MIXED-LINEAGE KINASES: A Target for the Prevention of Neurodegeneration

Leo H. Wang, Cagri G. Besirli, and Eugene M. Johnson, Jr.

*Departments of Neurology and Molecular Biology & Pharmacology,
Washington University School of Medicine, Saint Louis, Missouri 63110-1031;
email: eugene.johnson@wustl.edu*

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■ **Abstract** The activation of the c-Jun N-terminal kinase (JNK) pathway is critical for naturally occurring neuronal cell death during development and may be important for the pathological neuronal cell death of neurodegenerative diseases. The small molecule inhibitor of the mixed-lineage kinase (MLK) family of kinases, CEP-1347, inhibits the activation of the JNK pathway and, consequently, the cell death in many cell culture and animal models of neuronal death. CEP-1347 has the ability not only to inhibit cell death but also to maintain the trophic status of neurons in culture. The possible importance of the JNK pathway in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases provides a rationale for the use of CEP-1347 for the treatment of these diseases. CEP-1347 has the potential of not only retarding disease progression but also reversing the severity of symptoms by improving the function of surviving neurons.

INTRODUCTION

In the United States, an estimated five million Americans are currently afflicted with neurodegenerative diseases. Although the few available therapies afford symptomatic relief, none prevents or delays the pathological neuronal cell death associated with neurodegenerative diseases. The death of these neurons is, in many cases, associated with the activation of the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway. This signaling pathway is important in activating neuronal cell death programs and presents a therapeutic target for drug development. An important component of this pathway is the mixed-lineage kinase (MLK) family of kinases, which activates a neuronal cell death signal transduction pathway. CEP-1347, a semisynthetic MLK inhibitor, is the only experimental tool of well-defined mechanism that has been available to inhibit the JNK pathway to prevent neuronal cell death. CEP-1347 prevents neuronal cell death in cell culture models of trophic factor withdrawal, Alzheimer's disease (AD), and aminoglycoside-antibiotic-induced inner ear hair-cell death. MLK

inhibition is also effective in preventing the neuronal cell death in *in vivo* models of AD, Parkinson's disease (PD), and cochlear hair-cell death. CEP-1347 is currently in clinical trials for PD and may have potential in providing effective therapy for AD, PD, and other neurodegenerative diseases, as well as for hearing loss.

This article will (a) review the biology of the MLK family of kinases and their function in the JNK pathway, (b) review the evidence for the activation of the JNK pathway in neurodegenerative diseases, (c) describe the pharmacological development of CEP-1347, and finally, (d) review the tissue culture and animal studies that examine the neuroprotective actions and mechanisms of this drug.

THE BIOLOGY OF THE MIXED-LINEAGE KINASE FAMILY

MLKs are members of a large family of mitogen-activated-protein kinase (MAPK) kinase kinases (MAPKKKs) that activate MAPKs, including JNK, extracellular signal-regulated kinase (ERK) 1/2, ERK5/BMK1, and p38. Besides the MLK family members, the MAPKKK family includes MAPK/ERK kinase (MEK) kinases (MEKK), apoptosis-inducing kinase 1 (ASK1), and transforming-growth factor β (TGF β)-activated kinase 1 (TAK1) (for more detailed reviews on the structure of the MLK family, see References 1, 2). MLKs can activate the JNK and p38 pathways, although one MLK family member also activates ERK1/2 and ERK5 pathways in overexpression studies (3). Six MLK family members have been identified in mammalian cells, i.e., MLK 1–3, dual-leucine-zipper-bearing kinase (DLK), leucine-zipper kinase (LZK), zipper sterile- α -motif kinase (ZAK); a search of the NCBI protein database identifies the sequence of a seventh member (MLK4). Both MLK4 and ZAK are alternatively spliced to form α and β isoforms, thus increasing the total number of MLKs to nine.

The MLK family can be divided into three subfamilies based on protein-domain structures and amino-acid sequence similarities of the kinase domain: MLK1–4, DLK/LZK, and ZAK. Common to all members are the kinase domain and a leucine-zipper region. The kinase domain shows sequence similarities to both serine/threonine kinases and tyrosine kinases, which led to their name mixed-lineage kinase when the first MLKs, MLK 1 and 2, were identified (4). However, autophosphorylation (5–7) and substrate phosphorylation (i.e., MKK4) (8) on exclusively serine and threonine residues suggest that MLKs are serine/threonine kinases, though tyrosine phosphorylation of some unidentified substrates remains a possibility. Leucine zippers, present in MLK family members, mediate protein dimerization/oligomerization by forming coiled-coil structures. Both MLK3 and DLK form dimers/oligomers mediated by their leucine zippers; this interaction is important for kinase autophosphorylation and JNK activation (9, 10).

The MLK1–4 subfamily has a unique N terminus containing a Src-homology-3 (SH3) domain. SH3 domains are involved in protein-protein interactions by recognizing proline-rich sequences in other proteins and by directing MLK1–4 to

localized signaling environments (4–6, 11–13). In addition, similar to Src tyrosine kinases, MLK3 is inhibited by its own SH3 domain (14), indicating that the SH3 domain may be an important autoregulatory mechanism for other MLKs as well. Besides the SH3 domain, another unique structural feature of the MLK1–4 subfamily is the presence of a Cdc42/Rac-interactive binding (CRIB) motif that follows the leucine zipper domain. Activated Cdc42 increases MLK3 oligomerization, autophosphorylation, and kinase activity in coexpression studies, suggesting that Rac/Cdc42 may be an upstream regulator of the MLK1–4 (15, 16). Members of the DLK subfamily, DLK and LZK, do not have any other recognized structurally distinct motifs in their predicted amino acid sequence besides the kinase domain and the leucine zippers (7, 17, 18). The third subgroup in the mixed-lineage kinase family, ZAK α , contains a sterile- α motif (SAM) N terminal to its leucine zipper, which may be important in regulating homo- or heterodimerization of ZAK α (3, 19, 20).

All MLK family members regulate the JNK signaling pathway by phosphorylation-dependent regulation of the MAPK kinases 4 and 7 (MKK4 and MKK7, see Figure 1). MKK4 and MKK7 are dual-specificity kinases that phosphorylate tyrosine and threonine residues in the catalytic domains of JNKs; these phosphorylation events are required for kinase activity (21, 22). Existence of several family members and splice variants in every step of the JNK pathway (e.g., three JNKs with a total of ten splice variants) suggests a great complexity in MLK-MKK-JNK signaling in mammalian cells. Detailed biochemical characterization of MLK family members provides strong evidence indicating a complex regulation of this signaling pathway. Although some MLKs, such as MLK3 and ZAK, are expressed widely in mammalian cells (3, 11, 19, 20), others such as MLK2 and DLK show a more tissue-specific expression pattern (12, 13, 17, 18, 23). In addition, some MLKs, i.e., ZAK and DLK, show substrate specificity toward MKK4 in *in vitro* and overexpression studies (24, 25). Finally, scaffolding proteins, such as JIP1 and JIP3, interact with specific members of the MLK, MKK, and JNK kinase families, thus providing another level of regulation of JNK pathway activation (26, 27).

Many studies have demonstrated that activation of the JNK signaling pathway is critical for both developmental and pathological neuronal cell death. Genetic deletion of JNK3 attenuates excitotoxic neuronal death in the hippocampus of mutant mice after kainate treatment (28). One of the most critical targets of JNK is the AP-1 transcription factor, c-Jun (29, 30). The N-terminal transactivation domain of c-Jun is phosphorylated by JNK; this phosphorylation increases c-Jun-mediated gene transcription. Activation of the JNK pathway leads to c-Jun induction in many neuronal cell death paradigms. Inhibition of c-Jun activity by dominant-negative c-Jun overexpression, neutralizing antibody injection, or genetic deletion prevents neuronal apoptosis after trophic factor deprivation (31–33). Moreover, hippocampal neurons of mice with a mutant c-Jun gene (*JunAA*) that cannot be phosphorylated by JNK show resistance to kainate injections (34). JNK-mediated activation of c-Jun and perhaps other transcription factors is important for the transcription of proapoptotic genes, such as BH3-only Bcl-2 family members, Bim, and DP5,

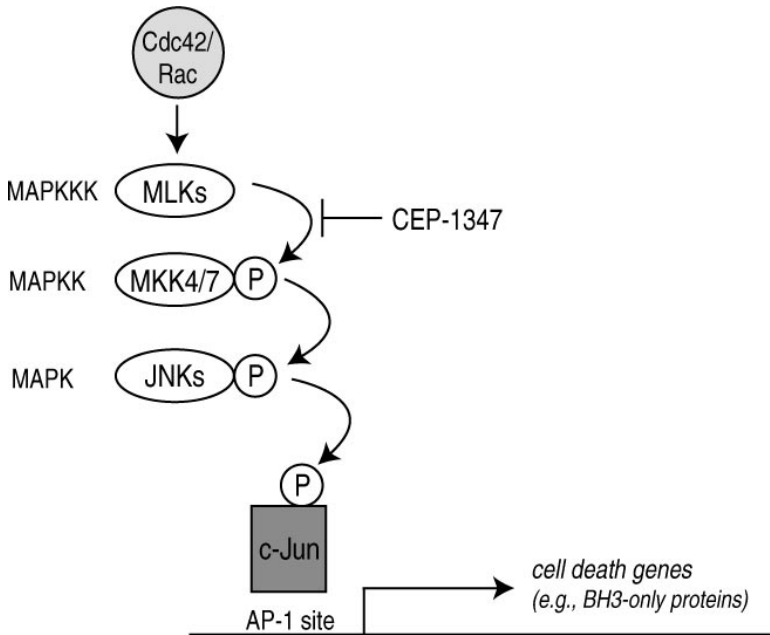


Figure 1 The c-Jun N-terminal kinase (JNK) pathway in neuronal cell death. The JNK pathway is a mitogen-activated-protein kinase (MAPK) pathway that is activated in response to many extracellular stimuli and different forms of environmental stress. The MAP kinase pathway is organized as a cascade of at least three kinases: a MAPK kinase kinase (MAPKKK) first phosphorylates a dual-specificity protein kinase (MAPKK), which in turn phosphorylates the MAPK. The JNK pathway can be activated by small G-proteins, such as Cdc42 and Rac, members of the Rho-family GTPases. In neurons, the MLK family of kinases serves as the major MAPKKKs and phosphorylates MKK4 and MKK7, which in turn phosphorylate the JNKs. Activation of the JNK pathway induces the expression BH3-only members of the Bcl-2 family, critical upstream regulators of neuronal apoptosis. The JNK pathway may also regulate neuronal death through other mechanisms.

during neuronal apoptosis (35, 36). The activation of the JNK pathway may also be important in the cell death seen in neurodegenerative diseases (see below).

NEURODEGENERATION AND THE JNK PATHWAY

The many etiologically diverse neurodegenerative diseases all cause progressive cell death in neuronal populations specific to the disease. The two most common neurodegenerative diseases are Alzheimer's and Parkinson's diseases. Alzheimer's disease (AD) is the most common neurodegenerative disease and the most common

cause of dementia in the elderly, affecting an estimated three to four million Americans. AD is characterized initially by subtle memory loss that slowly leads to progressive loss of cognitive function. Pathologically, the cerebral cortex atrophies and the ventricles enlarge, reflecting a loss of neurons, mostly in the hippocampus, temporal cortex, and nucleus basalis of Meynert in the forebrain. Microscopically, diseased neurons accumulate neurofibrillary tangles and senile plaques. The neurofibrillary tangles are produced from hyperphosphorylation of the tau protein, a microtubule-associated protein, whereas the senile plaques are deposits from β -amyloid protein. Presently, how the tangles and plaques are pathogenic is unclear.

Current treatments for AD are directed at improving cognitive function by increasing the amount of acetylcholine available to targets of basal forebrain cholinergic neurons. The cholinesterase inhibitors are the only currently Federal Drug Administration (FDA)-approved drugs for the treatment of AD. Although studies show retardation in the decline of cognitive function, these drugs likely do not affect disease progression.

Parkinson's disease (PD) is the second most common neurodegenerative disease. Patients suffer from motor dysfunctions characterized by the presence of tremors, rigidity, bradykinesia, and postural and gait disturbances. Pathologically, the disease is characterized by the selective death of dopaminergic, neuromelanin-pigmented neurons in the substantia nigra pars compacta in the midbrain with corresponding loss of coloration. Microscopically, Lewy bodies, with a core of α -synuclein aggregates, accumulate in the diseased neurons. Treatments currently available for PD are directed at increasing the amount of available dopamine neurotransmitters. Levo-dopa, levo-dopa/carbidopa, catechol-O-methyltransferase (COMT) inhibitors, dopamine agonists, monoamine oxidase (MAO) inhibitors, and amantadine are all classes of FDA-approved drugs used to relieve symptoms by increasing the amount of dopamine in the brain. Anticholinergics are also approved for the treatment of Parkinson symptoms. None of these treatments prevents the progression of the disease.

JNK Pathway Activation in AD

Activation of the JNK pathway in AD and PD has been investigated in post-mortem human pathological specimens. Staining with antibodies that recognize phosphorylated JNK proteins reveals JNK activation in brain regions affected by AD pathology. JNK is phosphorylated only in affected regions of patients with AD—in the cerebral cortex (37, 38) and the hippocampus (37, 38)—but not in unaffected regions or control brains.

How and to what extent JNK activation leads to AD pathology remains unclear. Activated JNK phosphorylation of tau and neurofilaments in neurofibrillary aggregates may be important in AD pathophysiology. Although activated JNK staining colocalizes with cytoplasmic phospho-tau immunoreactivity in severe cases of AD (38, 39), most evidence of JNK phosphorylation of tau and neurofilaments are in vitro (40–42). Many kinases appear to be more active than JNKs at

phosphorylating tau, whereas many better substrates exist for JNKs, such as c-Jun. In vivo, the Jnk1-knockout mice (but not the Jnk2-knockout mice) have an age-associated brain neuropathy characterized by increased microtubule disorganization and accumulation of neurofilaments; surprisingly, this neuropathy is associated with increased phosphorylation of tau (and not decreased phosphorylation that one would expect if JNK1 were important in the phosphorylation of tau). This suggests that JNKs are not directly involved in the phosphorylation of tau in vivo (43).

Therefore, a more likely function of activated JNKs in AD pathophysiology is to phosphorylate and activate c-Jun or other transcription factors that are important in neuronal death. Although no studies have specifically examined phospho-c-Jun staining, immunohistochemical analysis of postmortem pathological brain samples finds induction of c-Jun in hippocampal cells (44–46) and the entorhinal cortex (44) of AD patients compared to controls. This suggests that the induction of c-Jun by JNK pathway may be important in the pathophysiology of AD.

Because immunohistochemical studies of postmortem tissues are limited by their ability to analyze only one time point in a chronic disease process, animal and tissue-culture models of the diseases contribute much to our understanding of the pathophysiology of AD. In one tissue-culture model of AD, the extracellular application of aggregated β -amyloid peptide increases MKK4 and JNK phosphorylation in cortical cultures (47) and c-Jun induction in cortical cultures (48) and hippocampal cultures (49). In an animal model of familial AD, JNK phosphorylation colocalizes with a subset of β -amyloid-positive cortical neurons of transgenic mice overexpressing mutant human presenilin-1, a gene that when mutated causes early-onset AD (37).

JNK Pathway Activation in PD

Phospho-JNK staining has been analyzed in brain specimens from patients with PD. Phospho-JNK staining is found in neurons neighboring midbrain neurons with Lewy bodies, but not in neurons with aggregated proteins (50). One animal model of PD is the treatment of rodents with the dopaminergic midbrain neuron-selective neurotoxin, 1-methyl-4-phenyl-tetrahydropyridine (MPTP). By Western blot and immunohistochemical analysis of the midbrain, the treatment of mice with MPTP causes phosphorylation of MKK4 and JNKs (51). However, reports in the literature are conflicting concerning the importance of JNK activity in the treatment of dissociated midbrain neurons with the neurotoxic metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP⁺) (for review, see Reference 52).

JNK Pathway Activation in Hearing Loss

Deafness in the elderly is the result of a chronic progressive disease. In most cases, the auditory impairment is produced by sensory hair-cell death in the organ of Corti of the inner ear. Noise trauma and ototoxic drugs, such as aminoglycoside antibiotics, are two insults that cause hair-cell loss. Gentamicin, kanamycin, neomycin, and streptomycin are examples of aminoglycoside antibiotics that are effective in

treating infections caused by Gram-negative bacteria. Their ototoxic and nephrotoxic side effects limit their usage. However, their low cost leads to common usage, especially in underdeveloped countries. One model of aminoglycoside-induced ototoxicity is the administration of gentamicin to guinea pigs. Gentamicin treatment causes hair-cell death and decreases in auditory function as measured by the auditory brainstem response. Immunohistochemical analyses of the cochlea reveal that JNK is phosphorylated and activated and that the cells are undergoing an apoptotic death (53).

c-Jun Activation is Important in Degeneration of Neurons and Hair Cells

Increased JNK activity and c-Jun induction is observed in stressed cells during various conditions, including degeneration and regeneration. Combined with data from tissue-culture experiments dissecting the JNK pathway, this suggests that activation of the JNK pathway via c-Jun induction or other mechanisms is important in cell-death processes involved in AD, PD, hearing loss, and potentially other neurodegenerative disorders.

PHARMACOLOGICAL DEVELOPMENT OF CEP-1347

The development of the neurotrophic molecule CEP-1347 is based on the observations of the survival-promoting and neurotrophic effects of the naturally occurring small molecule K252a. K252a and its related compounds are metabolites that can be isolated from the culture broth of the actinomycetes group of Gram-positive bacteria. The metabolite was initially isolated from the culture broth of the K252 strain of *Nocardioopsis* bacteria found in a soil sample in Ashahi-cho, Machida-Shi, Tokyo, Japan (54). After structurally similar compounds were isolated from other *Nocardioopsis* strains, the original K252 was renamed K252a. This compound was distinguished in a screen for inhibitors of the Ca^{2+} -messenger system and determined to be a potent inhibitor of protein kinase C (PKC) and calmodulin-activated enzymes (54).

This family of heterocyclic molecules is characterized by an indolocarbazole structure, which is a symmetric fusion of alternating three benzene and two pyrrole rings, with a pyrrolidinone ring fused to the central benzene structure (see Figure 2). A sugar moiety is conjugated to both nitrogens of the indole rings. Each member of the K252a family (a–d and staurosporine) has a different sugar moiety. K252a is a relatively nonselective inhibitor of serine/threonine kinases, inhibiting cyclic AMP- and cyclic GMP-dependent protein kinases (55), phosphorylase kinase (56), myosin light-chain kinase (55), calmodulin-dependent cyclic nucleotide phosphodiesterase (57), and phosphatidylinositol 3-kinase (PI3-kinase) (58).

Because nerve growth factor (NGF) mediates the phosphorylation of certain downstream substrates through the upregulation of PKC activity (59, 60), studies

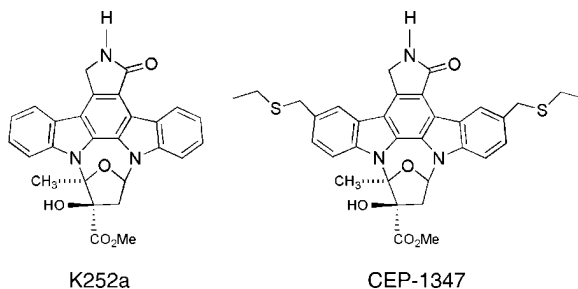


Figure 2 The chemical structures of K252a and its 3,9-bis-[ethylthio(methyl)]-derivative, CEP-1347.

were done using K252a to inhibit the NGF-induced PKC activity. In the pheochromocytoma cell line, PC12, a rat adrenal medulla tumor cell line that differentiates into sympathetic neuron-like cells in response to NGF, K252a inhibits NGF-mediated neurite outgrowth (61, 62).

Because K252a inhibits the short-term actions of NGF in PC12 cells, it was hypothesized that K252a might inhibit a kinase closely associated with the receptor for NGF. The identification of tyrosine receptor kinase, TrkA, as the receptor for NGF prompted the discovery of K252a as a direct inhibitor of the Trk family of neurotrophin receptors. As a competitive antagonist that binds to the ATP-binding site (63), K252a inhibits the autophosphorylation induced by ligands. Specifically, it blocks with an IC_{50} of 3 nM the autophosphorylation of TrkA induced by NGF, TrkB by brain-derived neurotrophic factor and neurotrophin-4/5, and TrkC by neurotrophin-3 (NT-3). Among receptor tyrosine kinases, K252a is relatively specific to the Trk family and does not affect the autophosphorylation of receptors for EGF, PDGF, or the v-src kinase (64–67).

Neurotrophic Activities of K252a

Unexpectedly, K252a not only inhibits the effects of the neurotrophins but also possesses “neurotrophic” activities. In embryonic chicken neurons in vitro, K252a increases the survival of dorsal root ganglion (DRG) and ciliary ganglion neurons in a dose-dependent manner; at the maximal dose tested, K252a maintains similar numbers of DRG and ciliary ganglion neurons as maintained by NGF and ciliary neurotrophic factor, respectively (68). This survival effect persists for at least two weeks in these culture settings. K252a not only promotes survival but also induces morphological changes, such as inducing neurite outgrowth, similar to a growth factor (68).

In rat basal forebrain neuronal cultures, growth factors, such as NGF, promote survival and induce differentiation of these neurons into a cholinergic phenotype as measured by increases in choline acetyltransferase (ChAT) activity. The effects of the growth factors are considered to be neurotrophic. K252a inhibits NGF-stimulated ChAT activity in basal forebrain cultures. However, in lower-density

basal forebrain cultures that are more dependent on exogenous factors for survival, low concentrations of K252a maintain survival (69). With a maximal effect at 50 nM, K252a maintains 60% survival of attached basal forebrain neurons and does so long term (six days). In contrast, untreated neurons are all dead after three days in culture. Most interestingly, low concentrations of K252a also increase ChAT activity threefold compared to cultures not treated with K252a. This suggests that K252a is acting similarly to a growth factor—maintaining survival as well as augmenting the differentiated phenotype.

These effects are seen in other neuronal cell types. In mixed rat spinal cord cultures, which contain primarily motoneurons, K252a prevents the decline in ChAT activity seen in untreated cultures (70). In embryonic rat striatal cultures composed of ~50% GABAergic neurons, K252a (50 nM) maintains long-term survival (~75% survival for seven days) and increases GABA-uptake activity compared to untreated cultures (69).

K252b exerts only indirect neurotrophic effects, requiring the presence of the neurotrophin NT-3. In basal forebrain cultures, K252b inhibits the increase in ChAT activity induced by NGF, BDNF, or NT-3 at concentrations above 200 nM. NT-3 by itself possesses only weak neurotrophic effects. However, in combination with low concentrations of K252b (below 100 nM), NT-3 is able to increase ChAT activity to the same levels maintained by NGF or BDNF (71). K252b was hypothesized to exert its neurotrophic effects through the modification of Trk receptors so that they become more responsive to NT-3. Subsequently, K252b was found to potentiate NT-3 activation of the TrkA receptor (71, 72). Under the conditions studied, neither NT-3 nor K252b alone activates the TrkA receptor, but in combination, the receptor becomes autophosphorylated and downstream effectors of TrkA are activated.

The Synthesis of Semisynthetic K252a-Analogues that Increase ChAT Activity in Basal Forebrain and Spinal Cord Cultures

K252a possesses two activities in neurons. At high concentrations, K252a inhibits the survival-promoting and neurotrophic effects of neurotrophins, whereas at low concentrations, K252a, by itself, promotes survival and differentiation similar to the effects of the neurotrophins. Compounds based on the K252a structure were synthesized to enhance the neurotrophic effects of K252a while decreasing its ability to inhibit Trk phosphorylation. The conjugation of alkyloxy- or alkylthio-side chains to the outer benzene rings of the indolocarbazole structure increases ChAT activity in both rat spinal cord and basal forebrain cultures. The 3,9-bis-alkyloxy-substituted K252a derivatives are 100-fold weaker inhibitors of Trk tyrosine kinase activity than K252a. Even weaker inhibitors are the alkylthio-substituted K252a derivatives, which are at least 500-fold weaker inhibitors of Trk activity than K252a (73).

Therefore, alkylthio-derivatives of K252a are more selective than the alkyloxy-derivatives. Compared to other bulkier alkylthio-derivatives, the 3,9-bis-[ethylthio-(methyl)]-substituted K252a (see Figure 2) has the most potent neurotrophic

TABLE 1 Comparison of the in vitro kinase inhibitory profile of K252a and CEP-1347. PKC, protein kinase C; PKA, cyclic adenosine monophosphate-dependent protein kinase type I; MLCK, myosin light chain kinase; PI3-kinase, phosphatidylinositol 3-kinase; Trk, Trk receptor tyrosine kinase. Reproduced with permission from Reference 58. Copyright 1998 by the Society of Neuroscience.

Kinase	IC ₅₀ (μM)	
	K252a	CEP-1347
PKC	0.028	16.3
PKA	0.072	>10
MLCK	0.19	>10
PI3-kinase	2.0	>10
Trk	0.002	>1

effects. It is the only alkylthio derivative of those studied able to increase ChAT activity in rat spinal cord and basal forebrain cultures at low concentrations. The 3,9-bis-[ethylthio(methyl)]-substituted K252a was named CEP-1347 or KT-7515.

Compared to K252a, CEP-1347 exhibits greater efficacy and potency (EC_{50} = 50 nM) at increasing ChAT activity in rat spinal cord and basal forebrain cultures. Furthermore, CEP-1347 is not cytotoxic above 200 nM, as is K252a, and does not possess the nonselective serine/threonine kinase inhibitor property of K252a, having binding affinities three orders of magnitude lower than K252a to PKA and PKC and myosin light chain kinase (see Table 1). Thus, the semisynthetic derivative of K252a, CEP-1347, has the desired neurotrophic effects while greatly reducing the nonselective inhibitory profile of K252a.

THE DISCOVERY OF MLKS AS A TARGET OF CEP-1347

To determine the molecular target of CEP-1347, Maroney and colleagues examined the effects of the molecule on signal pathways implicated in neuronal death. In trophic factor-deprived motoneurons, they found that CEP-1347 inhibits the increases in JNK pathway activity while having no effect on another MAPK pathway implicated in neuronal death, the ERK pathway (58). To identify the specific molecular targets in the JNK-signaling pathway, Maroney and colleagues utilized coexpression experiments in Cos-7 cells (74). Upstream regulators of the JNK pathway were cotransfected into cells with JNK1 in the presence or absence of CEP-1347, and JNK1 activity was measured by in vitro kinase assay by using c-Jun as a substrate. Rac or Cdc42, potential regulators of MLK1–4, induced 20-fold more JNK1 activity; CEP-1347 significantly blocks this activation. However, no specific binding of CEP-1347 to Rac or Cdc42 was detected, suggesting that potential

targets of CEP-1347 are downstream of these small GTPases. When members of the MKK family were overexpressed to activate JNK1, CEP-1347 showed no significant inhibition of MKK effects, indicating that neither MKKs nor JNKs are targeted directly by CEP-1347. In addition, the possibility of direct JNK inhibition by CEP-1347 was eliminated by showing that MEKK1-mediated endogenous JNK activation in intact Chinese hamster ovary (CHO) cells was unaffected by CEP-1347. In striking contrast, CEP-1347 significantly inhibits the MLK3-induced JNK activity in the same cells. MKKs are activated by MEKKs, ASK1, TPL-2, and MLKs (see Reference 2). However, CEP-1347 significantly inhibits JNK1 activation by only MLK family members (MLK1–3, DLK, and LZK). CEP-1347 is a very potent inhibitor of MLKs (MLK1, $IC_{50} = 38$ nM; MLK2, $IC_{50} = 51$ nM; MLK3, $IC_{50} = 23$ nM) (75). Moreover, CEP-1347 directly inhibits the kinase activities of the MLK family members *in vitro* with IC_{50} values that are in the same range as EC_{50} values for survival promotion in intact cells (58, 76). CEP-1347 competes with ATP to bind MLK1 and inhibit its kinase activity. These results clearly demonstrate that CEP-1347 targets the JNK pathway at the level of MLKs.

Other inhibitors have also been developed to inhibit this pathway by targeting other kinases within the cascade, particularly the JNKs themselves. JNK inhibitors, however, have been little studied in the context of neurodegeneration. This may be partially due to the more general effects that would be produced by inhibiting the terminal kinase in the cascade, as opposed to upstream kinases that have a certain amount of selectivity to neurons. Additionally, inhibitors generally available to the community, in particular SP600125, have a relatively nonselective kinase inhibitory profile that limits the ability to examine the JNK pathway even in model systems (76a, 76b).

TISSUE CULTURE STUDIES OF CEP-1347: PREVENTION OF NEURONAL DEATH

Activation of the JNK pathway has been investigated in several cell culture models of neuronal cell death. The JNK pathway is important in tissue culture models of trophic factor withdrawal from sympathetic neurons, motoneurons, and cerebellar granule cells. CEP-1347 is also protective in tissue culture models of disease, such as β -amyloid-induced or DNA-damaged-induced death of neurons and aminoglycoside-induced death of hair cells. In these models, CEP-1347 inhibits the activation of the JNK pathway and thereby inhibits the cell death.

Trophic Factor Withdrawal

During the development of the nervous system, many more neurons are born than survive to adulthood. The selection of which neurons survive is determined by the limited availability of target-derived growth factors, which act to inhibit programmed cell death. This selection process “sculpts” the nervous system such

that the final number of innervating neurons is matched to the size and presence of the neuronal target. These neurons die in a stereotypic manner; all initiating a cell death program that is apoptotic in character. In tissue culture, this death can be recapitulated by the withdrawal of growth factors.

The model most extensively characterized is that of postnatal rat sympathetic neurons dissociated from the superior cervical ganglion (SCG). SCG neurons are dependent on NGF for survival. NGF-deprived SCG neurons undergo a characteristic protein-synthesis and caspase-dependent programmed cell death. When NGF deprived, SCG neurons decrease TrkA phosphorylation, decrease activation of the ERK and PI3-kinase pathways, and increase activation of the JNK pathway and the induction of the transcription factor c-Jun.

CEP-1347 inhibits the activation of the JNK pathway, as assessed by decreases in phosphorylated MKK4 and c-Jun in NGF-deprived CEP-1347-treated neurons (77). CEP-1347 inhibits SCG neuronal cell death in a dose-dependent manner with complete prevention at 200 nM (76, 77). The latter effect persists for at least ten days. Furthermore, CEP-1347 is also able to inhibit SCG neuronal cell death induced by ultraviolet (UV) irradiation or oxidative stress (76). However, MLK-independent mechanisms of neuronal degeneration exist. In sympathetic neurons, JNK signaling is not increased during cell death induced by some DNA-damaging agents, and DNA-damage-induced neuronal death is not affected by MLK or JNK inhibition (78). Recent studies also show that CEP-1347 activates PI3-kinase activity in SCG neurons, which is important for survival and trophism (79, 80). Roux et al. (80) also find that CEP-1347 inhibits the activity of the src regulator, csk; the importance of this pathway in CEP-1347-mediated effects is unclear.

In the development of the rat spinal cord, ~50% of the motoneurons die between embryonic day 14.5 (E14.5) and 18. This can be recapitulated *in vitro* by plating E14.5 neurons in the absence of trophic factors. These neurons die within 24–48 h. JNK1 activity increases with time in rat embryonic motoneurons deprived of trophic support. CEP-1347 inhibits the increase in JNK1 activity in a dose-dependent manner, with maximal inhibition at 100 nM. The dose-dependent inhibition of JNK1 activity is inversely related to the dose-dependent saving effects of CEP-1347 (58).

In vitro, postnatal cerebellar granule cells can be maintained by potassium-induced depolarization and serum. The deprivation of potassium and serum produces an apoptotic death. Treatment with CEP-1347 inhibits the increases in MKK4, JNK, and c-Jun phosphorylation that result from the deprivation (81). CEP-1347 prevents the death of granule cells in a dose-dependent manner, with a complete effect at 400 nM. However, this survival effect is short lived. In contrast to NGF-deprived sympathetic neurons, most CEP-1347-treated granule cells die after three days. The death in the granule neurons is not because of the activation of the JNK pathway by non-CEP-1347-inhibitable (i.e., non-MLK) pathways, but rather the activation of an alternative death pathway that appears to be unavailable to trophic factor-deprived SCG neurons.

β -Amyloid-Induced Neuronal Death

Whether the neuronal cell death of trophic-deprived neurons recapitulates the death seen in neurodegeneration is unclear, but the neuronal death in neurodegenerative diseases probably shares many of the processes of apoptotic death. One tissue culture model of AD is the treatment of cortical neurons with β -amyloid ($A\beta$), which produces neuronal death associated with the activation of MKK4 and JNK (47). CEP-1347 inhibits the phosphorylation of MKK4 and JNK and, consequently, the cell death in this system.

Hair Cell Models

Neomycin treatment of organotypic cochlear explants of neonatal rats causes JNK and c-Jun phosphorylation and DNA fragmentation (as assessed by TUNEL staining) in hair cells (82). Coincubation with 500 nM CEP-1347 inhibits c-Jun phosphorylation and the cell death induced by the aminoglycosides (82) (see below for *in vivo* studies).

Confirmation with Genetic Inhibitors of MLK in PC12 Cells, SCG Neurons, and Cerebellar Granule Cells

The experiments (74) that defined the site of action of CEP-1347 as the MLK family of kinases were done in a nonneuronal cell line, Cos-7. Recent work confirms these findings in neuronal cell lines and primary neurons. Neuronally differentiated PC12 cells can be induced to die by withdrawal of NGF, exposure to UV irradiation, or subjection to oxidative stress (76). In each case, JNK activity is increased and the presence of CEP-1347 inhibits the death.

Transfection of MLK1–3 or DLK into neuronally differentiated PC12 cells induces an apoptotic cell death (83). This increased death is lessened by the cotransfection of dominant-negative constructs of downstream JNK-pathway targets, such as MKK4, MKK7, or c-Jun. Death induced by overexpression of MLK family members can also be suppressed by cotransfection with dominant-negative constructs of other MLK family members, as well as CEP-1347 treatment.

Similarly, NGF-maintained SCG neurons transfected via gene gun with MLK3 or DLK undergo apoptosis, whereas transfection with dominant-negative constructs of MLK3 or DLK decrease the cell death induced by NGF deprivation for up to 48 h (83). These results support the importance of the MLK family members in initiating the cell death program in neurons and confirm that the inhibition of the MLK-family members obstructs JNK-pathway activation.

In cerebellar granule cells, the transfection of MLK2 or MLK3 into potassium- and serum-maintained neurons overrides the prosurvival signals and induces cell death (84). MLK-induced killing is inhibited by the addition of CEP-1347. The overexpression of downstream members of the JNK pathway, such as MKK4, MKK7, or JNK1, induces cell death that is not inhibited by CEP-1347, confirming the specificity of CEP-1347 in inhibiting the JNK pathway at the level of MLKs.

Similarly, the transfection of dominant-negative constructs of MLK2 or MLK3 inhibits the cell death induced by deprivation of potassium and serum. These studies further confirm the importance of MLKs in mediating the developmental neuronal cell death and the locus of action of CEP-1347 in inhibiting neuronal death in these developmental models.

THE TROPHIC EFFECTS OF CEP-1347 IN SCG NEURONS

In addition to the survival-promoting effects of CEP-1347, our lab has found that CEP-1347 maintains the trophic status of SCG neurons in culture (77). The withdrawal of NGF decreases many neuronal metabolic parameters: the rates of glucose uptake, protein and mRNA syntheses, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (which measures the oxidative-reductive capacity of the neuron). By all these measures, neuronal metabolism is preserved in neurons saved by inhibition of the JNK pathway with CEP-1347. In contrast to the ability of CEP-1347 to maintain metabolism, inhibition of death downstream of JNK activation, by genetic ablation of the proapoptotic molecule *bax* or by inhibition of caspase activity, is not associated with such metabolic maintenance. Therefore, CEP-1347 is acting similarly to the growth factor NGF. These results also demonstrate that JNK-pathway activation in neurons is important in producing the catabolic effects associated with trophic factor withdrawal; a major mechanism by which neurotrophic factors are trophic is their ability to suppress the JNK pathway chronically. In addition to these metabolic measures, CEP-1347 prevents atrophy and even maintains growth of neurons in the absence of growth factor and prevents the loss of cytochrome c from the mitochondria. Thus, the effects of CEP-1347 are in striking contrast to caspase inhibitors, which do not alter the dire metabolic changes nor maintain mitochondrial integrity as evidenced by cytochrome c release. This may be very important in the treatment of neurodegenerative diseases because CEP-1347 may not only save dying neurons but may also maintain their function or even reverse dysfunction.

NEUROPROTECTIVE EFFECTS OF CEP-1347 IN VIVO

To date, MLK inhibitors have been examined in a limited number of animal models addressing neural injury or degeneration.

Motor Neurons

CEP-1347 promotes motor neuron survival in culture (58). This protective effect was further investigated by Glicksman et al. (85) in three in vivo paradigms: developmentally regulated cell death in the chicken embryo and neonatal rat and axotomy-induced cell death in the adult rat.

CEP-1347 blocks naturally occurring, developmental programmed motor-neuron cell death in the chicken embryo and neonatal rat. In chicken embryos, approximately 50% of the spinal cord ventral motor neurons die between E6 and E10. CEP-1347 is administered onto the chorioallantoic membrane daily between E6 and E9. At E10, the embryos are processed and the number of motor neurons is counted in the spinal cord. The two highest CEP-1347 doses tested, 2.3 and 7 $\mu\text{g}/\text{day}$, save approximately 40% of those neurons destined to die. In female neonatal rats, approximately 50% of the motor neurons in the sexually dimorphic spinal nucleus of the bulbocavernosus die perinatally [up until postnatal day 4 (P4)] because of the lack of testosterone. Daily (between P1 and P5) subcutaneous administration of CEP-1347 (at doses of 0.5 mg/kg or 1 mg/kg) over the target muscle reduces the death of the neurons in this population. The reduction in neuronal death is very similar to that produced by the administration of testosterone to female rats. Interestingly, higher doses of CEP-1347 (3 mg/kg) are not protective, indicative of a bell-shaped dose-response curve. This effect on the neuronal population appears direct because CEP-1347 has no effect on the atrophy of the target muscles of this neuronal population (85).

CEP-1347 is also effective in preventing the neuronal cell death in an injury model in adult animals. Transection of the hypoglossal nerve in adult rats results in a dramatic, but reversible, reduction in ChAT levels, as assessed immunohistochemically, in the motor nucleus. Administration of 50 μg CEP-1347 (the maximal effective dose) at the proximal stump of the lesion site maintains detectable ChAT immunoreactivity in 40% of the axotomized neurons. Whether the efficacy is the result of diffusion of the drug back to the soma of the axotomized neurons is unclear. As above, there appears to be a bell-shaped dose-response curve.

Basal Forebrain Neurons

As noted above, CEP-1347 enhances ChAT activity in basal forebrain neuronal cultures. These cholinergic neuronal populations involved in memory functions degenerate in certain neurodegenerative diseases, particularly AD. Although current AD therapy is aimed at increasing cholinergic transmission via these populations of neurons, a more effective AD therapy might involve the inhibition of their degeneration. The neuroprotective effects of CEP-1347 have been examined in two rat models that involve the induction of cell death in basal forebrain neurons: an excitotoxic model with ibotenic acid (86, 87) and the fornix-fimbria transection model (88).

The ibotenic acid model is a model of excitotoxic cell death, and the extent to which apoptotic processes are involved has not been examined. The excitotoxin is injected directly into the rat basal forebrain nucleus basalis of Meynert and CEP-1347 is administered systemically (0.1 mg/kg subcutaneously) (87). CEP-1347 was protective by the criterion of maintenance of ChAT activity in cortex or neuronal number in nucleus basalis of Meynert. Importantly, administration of CEP-1347 could be delayed by at least one day with retention of the effect

(as measured by maintenance of ChAT activity). CEP-1347 did not affect the enzyme activity on the unlesioned side of the animal. Subsequent studies demonstrate that CEP-1347 treatment also produces a long-term prevention of behavior deficits associated with the ibotenic acid-induced lesions (86).

More recently, the ability of CEP-1347 to prevent the death of septal cholinergic neurons in the basal forebrain after axotomy by fornix-fimbria transection was examined (88). The septal cholinergic neurons, a distinct neuronal population from the nucleus basalis of Meynert, degenerate after their afferent projections to the hippocampus are transected in the fimbria-fornix. The transection induces c-Jun expression in the medial septum starting 1 day after the lesion and peaking 5–7 days post-lesion (89, 90). The loss of neurons was partially prevented when the drug was administered subcutaneously by daily injection (1 mg/kg/day for 11 days) or continuous infusion through an osmotic minipump (~0.25 mg/kg/day for 14 days). Regardless of the administrative method, CEP-1347 saves 65%–70% of the cholinergic neurons relative to the number of neurons in the unlesioned control. Untreated animals maintain only ~50% of the cholinergic neurons. At least under the treatment conditions studied, CEP-1347 was not as efficacious as the optimal treatment with intraventricular NGF, which saves 80%–90% of the cholinergic neurons.

Dopaminergic Neurons of the Substantia Nigra

Of particular interest given the current status of the clinical development of CEP-1347 in PD are the effects on dopaminergic neurons in the substantia nigra. The protective effects of CEP-1347 were demonstrated in two widely used animal models of Parkinsonism produced in response to the neurotoxin MPTP. The effects of CEP-1347 on other models that use electrical or mechanical lesions or exposure to 6-hydroxydopamine have not been reported.

When injected into mice, MPTP produces neuronal cell death in the substantia nigra. The dopaminergic neuron-specific neurotoxin inhibits Complex I of the electron transport chain. Cell death is considered to be secondary to Complex I inhibition and displays apoptotic-like morphology in MPTP-treated mice, associated with JNK pathway activation. A single MPTP injection (40 mg/kg) causes the phosphorylation of MKK4 and JNKs, as assessed by immunoblotting of midbrain homogenates (51). The maximal activation of the JNK pathway occurs within hours of MPTP treatment and correlates with the maximal accumulation of the neurotoxic metabolite MPP⁺ in the striatum. The high dose of MPTP causes a marked decrease in the number of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra, with only ~20% TH-positive neuronal survival compared to control (87). CEP-1347 at doses above 0.3 mg/kg decreases this cell loss and maintains 40%–50% of the TH-positive neurons compared to control. Consistent with the importance of the JNK pathway in the death of these neurons, CEP-1347 at 1 mg/kg administered four hours prior to MPTP treatment partially decreases the activation of JNK and MKK4 (51).

The effects of MPTP are dose-dependent. At lower doses of MPTP, dopaminergic terminals are lost without affecting the cell body (91). At the lower dose of MPTP (20 mg/kg), CEP-1347 (0.3 mg/kg), injected four hours prior to and daily afterwards, partially reverses the loss of TH activity in striatum. Lower (0.03 mg/kg) or higher (3 mg/kg) doses of CEP-1347 are not effective. Initiating CEP-1347 treatment seven days after MPTP treatment is not effective in reversing the neuronal cell death. The neuroprotective effects of CEP-1347 result from its interference with the cytotoxic effects of MPTP rather than influencing the pharmacodynamics of the toxin. CEP-1347 does not inhibit MAO-B metabolism of MPTP to MPP⁺ or MPP⁺ uptake into dopaminergic neurons, nor MPP⁺ inhibition of Complex I (91).

MPTP treatment to nonhuman primates produces Parkinsonian syndromes similar to humans and is a more relevant model. Although no full-length article has been published on the effects of CEP-1347 in the primate model, results have been reported in abstract form (92) and in a review by Saporito et al. (93). Cynomolgous monkeys were exposed to low doses of MPTP (a weekly dose of 0.5 mg/kg) for ten weeks or until they reached a predetermined functional deficit as defined by the Laval University Disability Scale (94). Receiving a daily dose of CEP-1347 (1 mg/kg) significantly attenuated the loss of function, which includes bradykinesia, gait disturbances, tremor, and postural instability produced by the toxin. Postmortem examination of these brains showed a decrease in the loss of TH-positive neurons in the substantia nigra. CEP-1347 clearly exerts protective effects on MPTP-exposed neurons, thus providing a rationale for the drug as a therapeutic agent in the treatment of PD.

Auditory Hair Cells

Auditory hair-cell loss is a significant age-associated phenomenon. Recent studies in disparate species demonstrate that hair cell loss in response to noise or exposure to aminoglycoside antibiotics is associated with activation of the JNK pathway and subsequent apoptosis (53). CEP-1347 (1 mg/kg) administered to guinea pigs a few hours before and daily for 2 weeks after 6 h of 120-dB, 4-kHz noise exposure (53, 82) significantly attenuates hair-cell death and hearing loss observed 14 days postnoise exposure.

CONCLUSIONS AND FUTURE DIRECTIONS

A critical role of the JNK pathway in naturally occurring programmed cell death seems firmly established. Apoptosis induced by several insults in cell culture clearly involves the pathway. It is reasonable to propose a similar role for the JNK pathway in human neurodegenerative disease. Evidence is accumulating for this idea in studies both from human pathological material and from animal models of disease. Data reviewed in this article have provided evidence that activation of the JNK pathway in neurons is mediated by the MLK family of MAPKKs in

contrast to many other cell types. Thus, inhibitors of MLKs, such as CEP-1347, have the potential to block neuronal death without altering cell death in most other cell types. CEP-1347 and congeners, as selective inhibitors of MLKs, offer great potential as experimental tools to define the role of the MLKs further (and the consequent activation of the JNK pathway) in a variety of physiological and pathological processes. In addition, CEP-1347 and its analogues offer promise as therapeutic agents in pathological settings, such as neurodegenerative diseases, and, perhaps, in more acute settings of neuronal injury. Their therapeutic potential is enhanced if their ability to maintain and improve neuronal function, seen *in vitro*, similarly occurs *in vivo*.

However, we are in the initial stages of understanding the physiological role of MLKs and the pharmacological and therapeutic potential of inhibitors of this class of enzymes. Several important lines of research need to be expanded:

1. We need to determine which MLK(s) are important in activating the JNK pathway associated with neuronal death. Our understanding of normal MLK-expression patterns is very limited and little data exist on whether the expression may be altered in response to various perturbations. The development of knockout animals for the various MLKs should prove very informative. The demonstration of a cell-death phenotype of cells from such animals would also provide unequivocal support that the mechanism of CEP-1347 and congeners is indeed via MLK inhibition. The production of animals expressing dominant-negative MLKs driven by nervous system-specific promoters will also be valuable tools, especially if some knockouts prove embryonic lethal.
2. The availability of the indolocarbazole inhibitors provides a powerful tool with which to assess the role of JNK activation in the pathology seen in many animal models. As noted above, the compounds have been used in only a few animal models. The degree of activation of the JNK pathway and the potential protective effects of the inhibitors need to be examined in many more settings to gain a better understanding of the role of the pathway in animal models and to assess therapeutic potential.
3. We need information on the function of MLKs in other forms of cell death (nonneuronal) and other physiological processes. Such information may be predictive of side effects associated with chronic MLK inhibition or may provide rationales for the use of the inhibitors in other clinical conditions.
4. Looking ahead optimistically, if found to be clinically effective in neurodegenerative disease, will the inhibitors be effective only in slowing disease progression associated with a decreased rate of neuronal loss, or will the "trophic" effects seen *in vitro* translate into an actual improvement of the function of surviving neurons and reversal of symptom severity?

The experimental tools needed to address these and other questions will become available over the next few years and we can look forward to rapid progress in this area.

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