Motoneuron Apoptosis Is Blocked by CEP-1347 (KT 7515), a Novel Inhibitor of the JNK Signaling Pathway

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Neurons undergoing apoptosis can be rescued by trophic factors that simultaneously increase the activity of extracellular signal-regulated kinase (ERK) and decrease c-Jun N-terminal kinase (JNK) and p38. We identified a molecule, CEP-1347 (KT7515), that rescues motoneurons undergoing apoptosis and investigated its effect on ERK1 and JNK1 activity. Cultured rat embryonic motoneurons, in the absence of trophic factor, began to die 24–48 hr after plating. During the first 24 hr ERK1 activity was unchanged, whereas JNK1 activity increased fourfold. CEP-1347 completely rescued motoneurons for at least 72 hr with an EC_{50} of 20 ± 2 nM. CEP-1347 did not alter ERK1 activity but rapidly inhibited JNK1 activation. The IC_{50} of CEP-1347 for JNK1 activation was the same as the EC_{50} for motoneuron survival. Inhibition of JNK1 activation by CEP-1347 was not selective to motoneurons. CEP-1347 also inhibited JNK1 activity in Cos7 cells under conditions of ultraviolet irradiation, osmotic shock, and inhibition of glycosylation. Inhibition by CEP-1347 of the JNK1 signaling pathway appeared to be selective, because CEP-1347 did not inhibit p38-regulated mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP2) activity in Cos7 cells subjected to osmotic shock. The direct molecular target of CEP-1347 was not JNK1, because CEP-1347 did not inhibit JNK1 activity in Cos7 cells cotransfected with MEKK1 and JNK1 cDNA constructs. This is the first demonstration of a small organic molecule that promotes motoneuron survival and that simultaneously inhibits the JNK1 signaling cascade.

Key words: motoneurons; indolocarbazole; CEP-1347; survival; apoptosis; c-Jun N-terminal kinase; mitogen-activated protein kinase; p38

During development, populations of neuronal embryonic cells *in vivo* undergo a predetermined process of programmed cell death (PCD) (for review, see Oppenheim, 1991). In particular, between embryonic days 14.5 and 18 ~50% of rat spinal cord motoneurons undergo a form of PCD morphologically identified as apoptosis. Cultures of motoneurons isolated from E14.5 rat spinal cord also apoptose *in vitro* (Comella et al., 1994; Milligan et al., 1994). Cell death in this relatively pure population of neurons can be partially prevented by the addition of growth factors such as brain-derived neurotrophic factor and insulin-like growth factor-1 (Henderson et al., 1993; Hughes et al., 1993). Because the environmental cues leading to *in vivo* apoptosis of motoneurons are not well understood, cultures enriched for motoneurons provide a useful, relatively homogeneous model for identifying neuronal survival agents and examining the biochemical events that govern survival.

Growth factors may mediate neuronal survival by regulating signaling cascades downstream of the small GTP binding proteins ras, rac, and cdc42 (for review, see Denhardt, 1996). Activation of the small GTP binding proteins leads to modulation of serine/threonine kinases in the mitogen-activated protein kinase (MAPK) family. Specifically, activation of ras leads to phosphorylation and activation of extracellular receptor-activated kinase (ERK), which has been linked biologically to growth and differentiation processes, whereas stimulation of rac/cdc42 leads to an increase in the activity of JNK and p38, a response that is associated with stress and apoptosis. In neuronally differentiated PC12 cells, withdrawal of NGF causes apoptosis that is preceded by a decrease in ERK activity and an increase in JNK/p38 activity (Xia et al., 1995). These results suggest that ERK and JNK/p38 are tightly coupled in an opposing relationship to each other. However, more recent studies indicate that inhibition of ERK activation failed to block NGF-dependent survival of superior cervical ganglion (SCG) neurons (Creedon et al., 1996; Virdee et al., 1996). Furthermore, insulin promotes survival of fetal chick forebrain neurons concomitant with inhibition of p38 in the absence of an effect on ERK and JNK activity (Heidenreich and Kummer, 1996). Clearly, the activity of several MAPK members is altered during apoptosis, but at present it is uncertain which, if any, of these activities is necessary and/or sufficient for neuronal apoptosis.

In an effort to identify small molecules that promote neuronal survival, we selected CEP-1347, also known as KT7515, for its ability to induce choline acetyl transferase (ChAT) activity in cultures prepared from embryonic spinal cord and basal forebrain tissue (Kaneko et al., 1997). CEP-1347 is a semisynthetic derivative of the fermentation product K-252a, an indolocarbazole that promotes neuronal survival in chick dorsal root ganglion cultures (Borasio, 1990) and ChAT activity in cultures of rat embryonic spinal cord (Glicksman et al., 1993), basal forebrain, and striatal

Received June 11, 1997; revised Oct. 6, 1997; accepted Oct. 15, 1997.

We thank J. Silvio Gutkind for providing the MEKK and JNK vectors, Anne Camoratto for helpful review of this manuscript, and Judy Richardson for manuscript preparation.

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neurons (Glicksman et al., 1995); it also protects hippocampal, septal, and cortical cultures against glucose deprivation-induced death (Cheng et al., 1994). CEP-1347 effectively protects motoneurons in several *in vivo* models of PCD such as the postnatal rat motoneurons of the spinal nucleus of the bulbocavernosus, adult rat hypoglossal axotomy, and chick lumbar motoneurons *in ovo* (M. Glicksman, unpublished observation). To elucidate the mechanism by which CEP-1347 promotes survival of motoneurons *in vivo,* we examined the survival activity and determined the effect of CEP-1347 on members of the MAPK family in cultures enriched for embryonic motoneurons.

MATERIALS AND METHODS

Materials. CEP-1347, also known as KT7515, is a semisynthetic derivative of K-252a provided by Kyowa-Hakko Kogyo (Tokyo, Japan) (Kaneko et al., 1997). CEP-1347 was dissolved in cell culture grade dimethylsulfoxide (DMSO) and stored in the dark at 4°C. All dilutions of CEP-1347 were made in DMEM containing 1% bovine serum albumin. c-Jun N-terminal kinase 1 (JNK1) antibody (catalog #sc-474-G) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK1 antibody (catalog #06-182), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP2) antibody (catalog #06-534), and MAPKAP2 peptide substrate (catalog #12-240) were purchased from Upstate Biotechnology (Lake Placid, NY). HA antibody was purchased from Babco (Richmond, CA). AP-1 (*c-jun*) substrate was purchased from Promega (Madison, WI). Myelin basic protein substrate, Hoechst dye, and tunicamycin were purchased from Sigma (St. Louis, MO). SB203580 was custom-synthesized by RIT International Technology (Snellville, GA). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Rat spinal cord cultures enriched for motoneurons. Spinal cords were dissected from Sprague Dawley rat fetuses (Charles River Laboratories, Wilmington, MA) of embryonic age (E) 14.5–15. Cells from only the ventral portion of the spinal cord were dissociated and further enriched for motoneurons by centrifugation on a 6.5% step metrizamide gradient, as described previously (Henderson et al., 1993), and were analyzed for purity by staining with low-affinity neurotrophin receptor antibody (IgG-192, Boehringer Mannheim, Indianapolis, IN) (data not shown). Cells were seeded onto 96-well plates previously coated with poly-L-ornithine and laminin (5 μg/ml each) at a density of 6×10^4 cells/cm^2 in chemically defined serum-free N2 medium (Bottenstein and Sato, 1979). To separate attachment from survival effects, we added CEP-1347 to the cultures after an initial attachment period of 1–3 hr. Neuronal survival was assessed after 4 d by using calcein AM (Molecular Probes, Eugene, OR) in a fluorimetric viability assay (Bozyczko-Coyne et al., 1993). Microscopic counts of neurons correlated directly with relative fluorescence values. In brief, culture medium was diluted serially in DPBS (Dulbecco's PBS), and a final concentration of 6 μM calcein AM stock was added to each 96-well plate. The plates were incubated for 30 min at 37°C, followed by serial dilution washes in DPBS. The fluorescent signal was read with a plate-reading fluorimeter from Millipore (Cytofluor 2350; Bedford, MA) at an excitation equal to 485 nm and an emission equal to 538 nm. For each plate, mean background derived from wells receiving calcein AM, but containing no cells, was subtracted from all values. Linearity of the fluorescence signal was verified for the concentration and incubation time for the range of cell densities in these experiments.

For assessing apoptotic nuclei, we plated enriched motoneuron cultures onto eight-chamber slides (Lab-Tek, Nunc, Naperville, IL); they were fixed with 4% paraformaldehyde for 20 min, rinsed with DPBS, and then stained with 1 μg/ml Hoechst dye for 15 min. After staining, cells were rinsed again and coverslipped, using the aqueous mountant Fluoromount (Vector Laboratories, Burlingame, CA), and then they were examined and photographed with a Nikon Diaphot microscope (Garden City, NY).

Cos7 cell culture. Green monkey kidney Cos7 cells were obtained from American Type Culture Collection (CRL 1651; Rockville, MD) and maintained in DMEM containing 10% bovine serum, 2 mM glutamine, 1 mM pyruvate, and 50 U/ml penicillin/streptomycin at 37°C in 10% CO_2/90% air atmosphere. Cos7 cells were detached for passaging by adding 0.25% trypsin.

In vitro *kinase activity.* The inhibitory activities of various concentrations of K-252a and CEP-1347 were measured in kinase assays. Partially purified protein kinase C (PKC) was prepared from rat brain, and the holoenzyme of cyclic adenosine monophosphate-dependent protein kinase type I (PKA) was partially purified from rabbit skeletal muscle as described (Kase et al., 1987). Phosphoinositol serine- and Ca^{2+}-dependent PKC activities were assayed under the conditions described, using 200 μg/ml histone H-I and 5 μM[γ-^{32}P]ATP (Kase et al., 1987). The activity of PKA was assayed in the presence of 100 μg/ml histone II-AS and 10 μM [γ-^{32}P]ATP by the methods in Kase et al. (1987). Myosin light chain kinase (MLCK) was purified from chicken gizzard and assayed as previously described by HPLC analysis (Nakanishi et al., 1991). Phosphatidylinositol-3 kinase (PI3K) was partially purified from calf thymus and assayed as described in Yano et al. (1993). The tyrosine kinase activity of the cytoplasmic domain of human recombinant trkA was assessed in an ELISA assay (Angeles et al., 1996). The IC_{50} values reported in Table 1 were calculated from plots of the percentage of inhibition versus $\log_{10}$ concentration of the compound.

Immunoprecipitation and kinase assay from whole cells. Purified motoneurons were plated at a density of 6×10^4 cells/cm^2 in 16-mm-diameter wells. Cells were allowed to attach for 2 hr before treatment. Cells were treated with either 0.0125% DMSO or 500 nM CEP-1347 for the indicated time points in defined N2 medium. Then cells were rinsed with ice-cold PBS, followed by lysis in 0.4 ml of Triton buffer (1% Triton X-100, 50 mM sodium chloride, 10 mM Tris, pH 7.6, 0.1% bovine serum albumin, 30 μM sodium pyrophosphate, 50 mM sodium fluoride, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml leupeptin, and 1 mM sodium vanadate). Immunoprecipitation and kinase assay were performed as previously described (Maroney et al., 1995). Lysate from motoneuron cultures was normalized to cell number, and lysate from Cos7 cells was normalized to protein concentration. For JNK1 and ERK1 immunoprecipitations, antibodies were used as recommended by the providers. Immunoprecipitates were rinsed three times with Triton buffer, followed by a final wash in kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM sodium vanadate). Reactions were incubated in kinase buffer containing 1 μM ATP and 5 μCi [γ-^{32}P]ATP and substrate (20 μg/sample of myelin basic protein for ERK1 or 1 μg/sample of AP-1 for JNK1) for 15 min at 30°C. The kinase reaction was stopped by the addition of sample buffer. Samples were heated to 80°C for 5 min and loaded onto polyacrylamide gels. Quantitation of results was performed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

For the MAPKAP2 assay, Cos7 cells were grown to confluency in a 60 mm dish. Cells were preincubated in serum-free medium containing either 0.0125% DMSO or 500 nM CEP-1347 for 1 hr, followed by treatment with 500 mM sorbitol for 1 hr. Then cells were rinsed with ice-cold PBS and lysed in Triton buffer; lysate was normalized to protein concentration. Lysate was immunoprecipitated with the MAPKAP2 antibody and assayed for kinase activity with 0.125 mM MAPKAP2 peptide substrate, as described by the provider. The kinase reaction was stopped with 75 mM phosphoric acid, loaded onto phosphocellulose filters (Pierce, Rockford, IL), and washed with 75 mM phosphoric acid, followed by elution with 1N NaOH. Radioactivity from the eluate was counted in a Beckman LS3801 (Fullerton, CA).

Stress-induced JNK1 activity in Cos7 cells. Cos7 cells were grown to confluency in 60 mm plates. The cells were preincubated with 0.01% DMSO or 500 nM CEP-1347 for 1 hr, followed by the following stresses: 500 mM sorbitol for 1 hr; exposure to ultraviolet irradiation from the UV2400 Stratolinker (Stratagene, La Jolla, CA) for 5 min, followed by 1 hr incubation at 37°C; and 50 μg/ml tunicamycin for 5 hr. The cells were rinsed with cold PBS, lysed with Triton buffer, immunoprecipitated with the JNK1 antibody, and assayed for kinase activity as described above.

Overexpression of mitogen-activated kinase kinase kinase 1 (MEKK1) and JNK1 in Cos7 cells. Cos7 cells were plated to 80% confluency and transfected with 2 μg each of cDNA constructs, using Lipofectamine as recommended by the provider (Life Technologies, Gaithersburg, MD). A truncated cDNA of mouse MEKK1 (corresponding to amino acids 817–1493 of full-length rat MEKK1) and full-length human JNK1, kindly provided by J. Silvio Gutkind (National Institutes of Health, Bethesda, MD), were subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA). The JNK construct contained a hemagglutinin tag. After a 48 hr transfection, the cells were treated with DMSO or 500 nM CEP-1347 for 2 hr, followed by immunoprecipitation with the HA antibody. Immunoprecipitates were normalized for protein and assayed for kinase activity in the presence of *c-jun* substrate as described above.

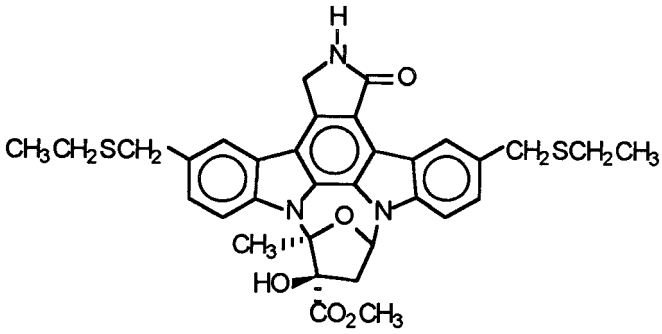

Figure 1. Structure of CEP-1347. CEP-1347 was synthesized by derivitization of K-252a, an indolocarbazole isolated from culture broths of *Nocardiosis*.

RESULTS

Low nanomolar concentrations of K-252a, a natural product indolocarbazole of the bacterium *Nocardiosis* species, induced ChAT activity in spinal cord cultures (Glicksman et al., 1993). The ChAT enzyme catalyzes the synthesis of the neurotransmitter, acetylcholine, and serves as a marker for motoneurons in spinal cord cultures (Phelps et al., 1988, 1990). This neurotrophic activity was optimized by examining novel K-252a analogs in embryonic rat spinal cord cultures and measuring ChAT activity. As reported elsewhere, an ethylthiomethyl analog of K-252a, CEP-1347 (for structure, see Fig. 1), exhibited greater efficacy (250% of control) and potency (EC_{50} = 50 nM) than K-252a in spinal cord ChAT assays (Kaneko et al., 1997).

The neurotrophic properties of CEP-1347 were not attributable to the inhibition of several known target kinases of K-252a. In contrast to the inhibition of several serine/threonine kinases by K-252a in the nanomolar range, the IC_{50} values of CEP-1347 for PKC, PKA, MLCK, and PI3K were all >10 μM (Table 1). K-252a inhibited *trk* tyrosine kinase activity with an IC_{50} of 2.5 nM, whereas the IC_{50} of CEP-1347 was >1 μM (Table 1). These results demonstrated a broad separation of neurotrophic activity from inhibition of several known target kinases of K-252a.

To examine whether the increased ChAT activity in spinal cord cultures was attributable, at least in part, to motoneuron survival, we assessed the activity of CEP-1347 in cultures of enriched motoneurons. Cell viability in untreated cultures decreased by 35% after 48 hr and by 65% after 72 hr. An inhibitor of p38, SB203580, did not rescue motoneurons from cell death (Fig. 2) (Cuenda et al., 1995). In contrast, viability in the presence of CEP-1347 did not differ substantially from cultures assayed at initial plating (Fig. 2).

Table 1. Comparison of *in vitro* kinase inhibitory activity between K-252a and CEP-1347

	IC_{50} (μM)	
Kinase	K-252a	CEP-1347
PKC	0.028	16.3
PKA	0.072	>10
MLCK	0.19	>10
PI3K	2.0	>10
TRK	0.002	>1

The morphology of motoneuron cultures was assessed in the presence of CEP-1347. In contrast to control cultures that rapidly underwent neurite retraction and cellular fragmentation, those cells treated with CEP-1347 displayed a flattened cell body morphology with extensive neuritic processes for at least 5 d (Fig. 3*a,b*). To determine whether cells in control cultures were dying by apoptosis, we examined chromatin condensation by staining the DNA with fluorescent Hoechst dye. By 48 hr a significant proportion of untreated motoneurons exhibited clear hallmarks of chromatin condensation, consistent with a previous report that cultures of enriched motoneurons in the absence of neurotrophic factors die in an apoptotic manner (Fig. 3*c*) (Comella et al., 1994; Milligan et al., 1994). In contrast, CEP-1347-treated cultures exhibited diffuse nuclear staining, consistent with the survival activity detected by the calcein assay (Fig. 3*d*).

Changes in the activities of members in the MAPK family have been implicated in neuronal survival and apoptosis (Xia et al., 1995). We examined whether neuronal survival induced by CEP-1347 was accompanied by changes in the activities of ERK1 and JNK1. The basal level of ERK1 activity did not change over time in untreated cultures, and 500 nM CEP-1347 had no significant effect on ERK1 activity (Fig. 4*A*). These data demonstrated that CEP-1347 promoted survival in the absence of a change in ERK1 activity. In contrast, JNK1 activity in untreated cultures increased approximately fourfold within 24 hr after plating. As early as 15 min after the addition of 500 nM CEP-1347, JNK1 activity sharply decreased to ~50% of control levels and continued to decrease for the next 24 hr (Fig. 4*B*).

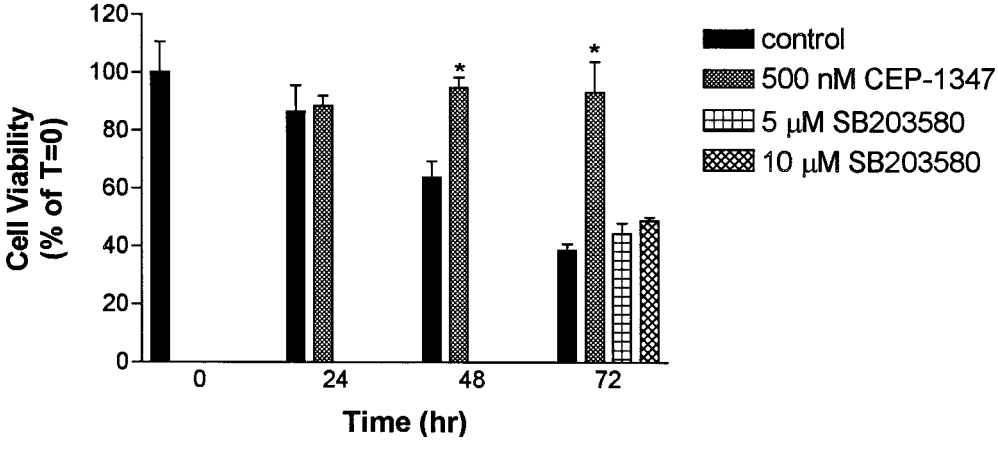

Figure 2. Time course of motoneuron death in the absence or presence of CEP-1347. Cells were plated at a density of 6×10^4 cells/cm^2 in chemically defined N2 medium. After 2 hr to allow for attachment, cells were incubated with 0.006% DMSO (*control*), 250 nM CEP-1347, or 5–10 μM SB203580 and monitored for cell viability over 3 d. Cell viability was measured by using the calcein AM assay as described in Materials and Methods. Experimental data represent the mean ± SD, n = 4; DMSO control data represent the mean ± SD, n = 12. Three independent experiments were performed; data presented are from one representative experiment. *$p < 0.05$, by Dunnett's t statistics, significantly different from control cultures.

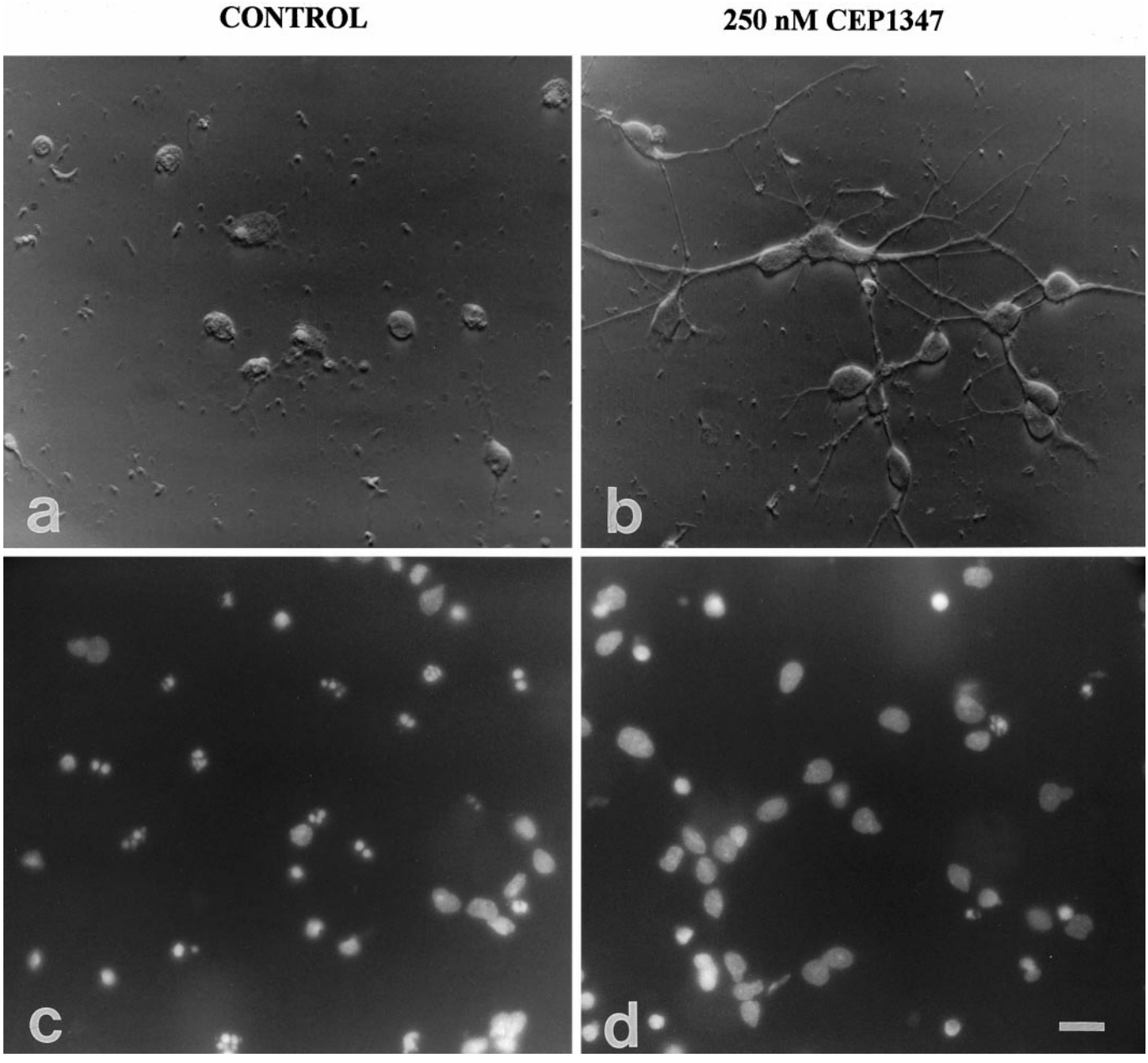

Figure 3. Apoptosis of enriched E14.5 motoneurons in the absence or presence of CEP-1347. Cells were plated at a density of 6×10^4 cells/cm^2 in chemically defined N2 medium. After 2 hr to allow for attachment, control cells were incubated with 0.006% DMSO control (*a, c*) or 250 nM CEP-1347 (*b, d*) for 5 d, followed by fixation and photography with Hoffman modulating contrast optics (*a, b*), or for 2 d, followed by staining with Hoechst dye (*c, d*) to detect condensed chromatin.

To resolve whether inhibition of JNK1 activity by CEP-1347 correlated with motoneuron survival, we compared the IC_{50} for JNK1 activity with the EC_{50} for survival by CEP-1347. Cultures enriched for motoneurons were grown in the presence of increasing concentrations of CEP-1347, and JNK1 activity and cell survival were determined. The IC_{50} for JNK1 activity measured at 22 hr was 21 ± 2 nM, whereas the EC_{50} for cell survival measured at 5d was 20 ± 2 nM (Fig. 5). These results suggested that cell survival and inhibition of JNK1 activity by CEP-1347 were integrally linked processes.

To determine whether the observed decrease in JNK1 activation was secondary to effects on neuronal survival or was an intrinsic property of CEP-1347, we examined the effect of CEP-1347 on JNK1 activation in Cos7 cells exposed to various external stresses. JNK1 activity increased after treatment with irradiation, sorbitol, and tunicamycin, consistent with previous studies (Derijard et al., 1994; Kyriakis et al., 1994; Rosette and Karin, 1996; Zanke et al., 1996) (Table 2a). CEP-1347 prevented the increase in JNK1 activity to a significant degree under all three stress conditions. Therefore, inhibition of JNK1 activation by CEP-1347 was not neuronal or stimuli-specific. In addition, inhibition of JNK1 activation was not a consequence of neuronal survival but appears to be an intrinsic property of CEP-1347.

Because p38 also has been implicated in neuronal apoptosis (Xia et al., 1995), it was of interest to determine whether CEP-1347 inhibited the p38 signaling pathway. MAPKAP2 is a sub-

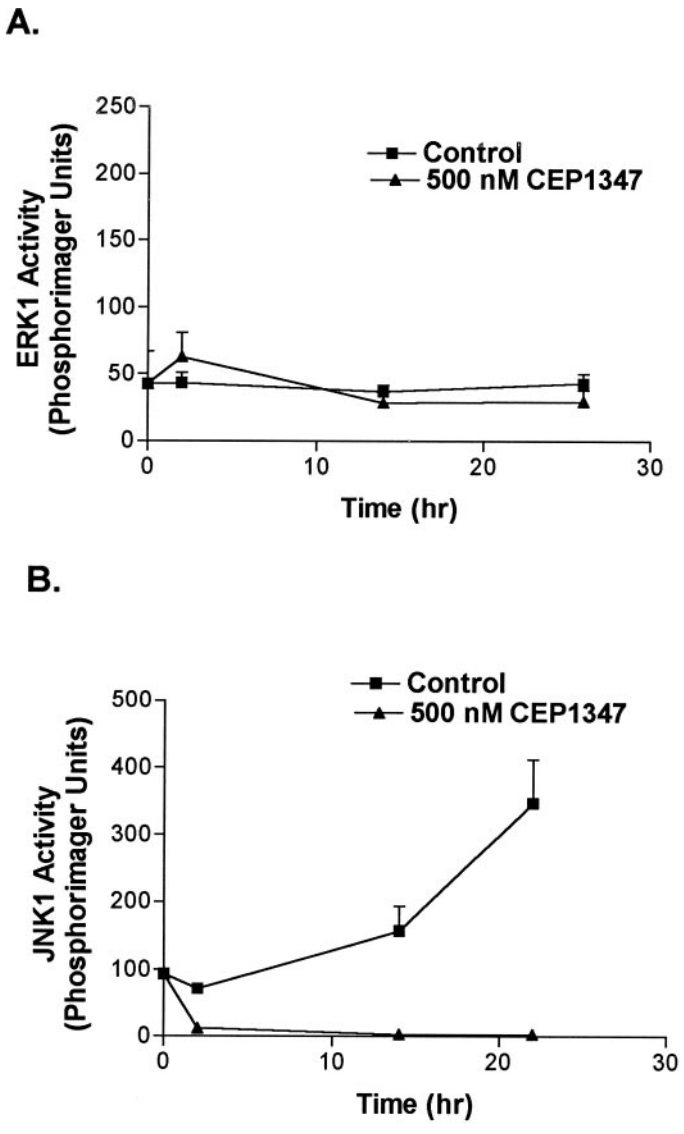

Figure 4. ERK1 and JNK1 activity in the absence or presence of CEP-1347. Cultures of enriched E14.5 motoneurons were treated with 0.01% DMSO control or 500 nM CEP-1347 for various times, as indicated. Cells were lysed in 1% Triton buffer, and the lysate was immunoprecipitated with the ERK1 (*A*) or JNK1 (*B*) antibody. The immunoprecipitates were assayed for kinase activity by using myelin basic protein or c-Jun, respectively, as substrates. Experiments were performed at least two times, and results from representative experiments are shown. *Points* represent the average of duplicate samples; error bars indicate the SEM.

strate of p38 and reflects p38 activation (Rouse et al., 1994). Attempts to measure p38 activity in motoneurons by assaying the activity of MAPKAP2 were unsuccessful, probably because of a lack of detection sensitivity in the low-density motoneuron cultures. We therefore tested the effect of CEP-1347 on MAPKAP2 activity in osmotically stressed Cos7 cells, a treatment that has been shown previously to activate p38 (Raingeaud et al., 1995). CEP-1347 had no effect on MAPKAP2 activity, whereas a p38 inhibitor, SB203580, completely blocked the stress-induced p38 activity (Table 2*b*) (Cuenda et al., 1995). These data suggested that CEP-1347 did not inhibit p38 directly or inhibit the upstream regulators of the osmotic shock-induced MAPKAP2 activity.

To determine whether JNK1 was a direct molecular target of CEP-1347 in Cos7 cells, we overexpressed cDNA constructs of HA–JNK1 alone or with an upstream activator of JNK1, MEKK1 (Lange-Carter et al., 1993; Minden et al., 1994), and examined JNK1 activity in the absence or presence of CEP-1347. After a 48 hr transfection, the JNK1 activity in Cos7 cells cotransfected with varying amounts of MEKK and HA–JNK1 constructs was ~threefold to 50-fold above the JNK1 activity in cells transfected with HA–JNK1 alone (Fig. 6). CEP-1347 did not prevent JNK1 activity under any of the conditions tested. These results indicated that JNK1 was not the direct molecular target of CEP-1347 and that the molecular target of CEP-1347 was either upstream of MEKK1 or independent of an MEKK1-activated JNK1 pathway.

DISCUSSION

We have shown that CEP-1347 rescues motoneurons from apoptotic death *in vitro* (see Fig. 2) and that survival correlates with the inhibition of JNK1 activation (see Figs. 4, 5). An endogenous substrate of JNK1 is the nuclear transcription factor *c-jun* (Hibi et al., 1993; Derijard et al., 1994; Kyriakis et al., 1994), and CEP-1347 suppressed *c-jun* mRNA in motoneurons at 24 hr after treatment (M. Glicksman, unpublished data). This is consistent with reports demonstrating that a dominant negative mutant of *c-jun* or a *c-jun* neutralizing antibody blocks apoptosis induced by neurotrophin withdrawal in SCG neurons and in PC12 cells (Estus et al., 1994; Ham et al., 1995; Xia et al., 1995). Furthermore, a dominant negative mutant of MEKK1, which is one upstream regulator of JNK activation, blocked apoptosis in NGF-withdrawn neuronally differentiated PC12 cells (Xia et al., 1995). These data provide convincing evidence to implicate the JNK signaling cascade in neuronal models of cell death. However, the sufficiency of JNK and *c-jun* activation to promote cell death is questionable. In SCG neurons undergoing prolonged NGF deprivation, suppression of elevated JNK activity by the readdition of NGF is insufficient to rescue all of the cells (Virdee et al., 1997). Furthermore, *c-jun* is elevated in cholinergic neurons after fornix–fimbria transection, and these cells do not die (Butterworth and Dragunow, 1996). These reports suggest that, in addition to JNK/*c-jun* activation, other signaling events may be involved in committing neurons to a death pathway.

A role for JNK in the induction of apoptosis also has been examined in non-neuronal systems. Inhibiting JNK or kinases upstream of JNK protects different cell types from death induced by a variety of stimuli such as camptothecin, thermal shock, *cis*-platinum, and ceramide (Verheij et al., 1996; Zanke et al., 1996; Ichijo et al., 1997; Seimiya et al., 1997). However, inactivation of the JNK signaling cascade does not protect against all types of stress-induced death. For example, high doses of arabinofuranosylcytosine lead to apoptosis in monocytic leukemia U937 cells, and this death is not blocked with a dominant negative mutant of c-Jun (Grant et al., 1996). Also, developmental PCD occurs in *c-jun* null embryos, suggesting that *c-jun* is not essential for apoptosis to occur in a variety of tissues (Roffler-Tarlov et al., 1996); in one study CD95- and CD3-mediated apoptosis was exacerbated in MEK4 null immature thymocytes (Nishina et al., 1997). Furthermore, the addition of tumor necrosis factor-α leads to apoptosis in many cell types and activates the JNK pathway; however, the role of JNK activation in these apoptotic models is controversial (Gardner and Johnson, 1996; Liu et al., 1996; Verheij et al., 1996; Natoli et al., 1997). Certainly, multiple pathways leading to cell death exist and may have different dependencies on the MAPKs for functional outcome, subject to the death stimulus and cellular environment.

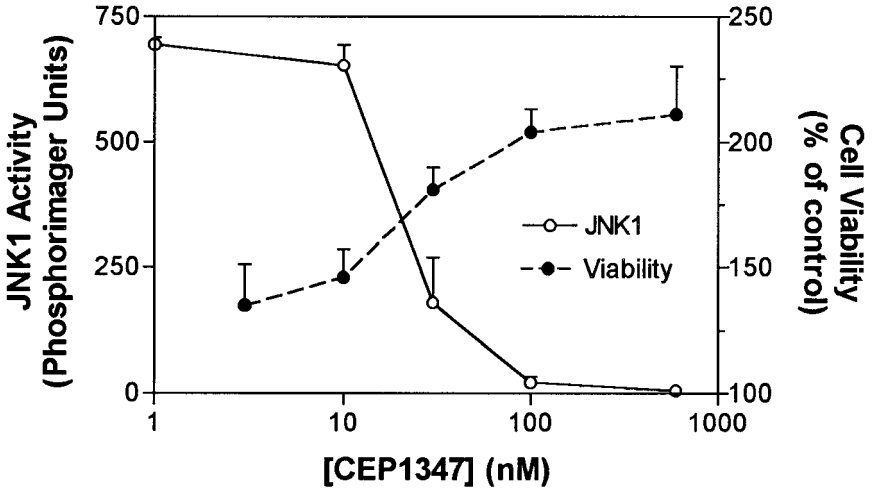

Figure 5. Dose–response of inhibition of JNK1 activity and cell survival by CEP-1347. Cultures of enriched E14.5 motoneurons were plated and allowed to adhere 2.5 hr before the addition of the indicated concentrations of CEP-1347. For JNK1 activity, cells were collected 22 hr after the addition of compound and assayed for kinase activity as described in Figure 4; cell viability was determined by calcein AM assay after 5 d in culture. The percentage of cell viability is relative to untreated controls, which is equivalent to 100%. *Points* represent the average of duplicate samples; the error bars indicate the SEM.

Dominant negative forms of various components of the JNK/p38 signaling pathway interfere with death, whereas constitutively active forms of components in the ERK signaling pathway promote survival after NGF withdrawal from neuronally differentiated PC12 cells (Xia et al., 1995). These results suggest that an opposing balance between the ERK and stress-activated kinases may be crucial for determining whether a cell survives or dies. However, NGF promotes survival in SCG in the presence of the MEK1 inhibitor, PD98059, which blocks ERK activation (Creedon et al., 1996; Virdee and Tolkovsky, 1996). Furthermore, sustained activation of ERK is insufficient to promote survival in hippocampal pyramidal neurons (Marsh and Palfrey, 1996). Because CEP-1347 promotes survival in the absence of any effect on ERK1 activity, results reported here support evidence that ERK activation is not necessary or essential for neuronal survival. Although we could not measure MAPKAP2 activity in the motoneuron model, CEP-1347 does not inhibit MAPKAP2 activity in sorbitol-treated Cos7 cells (Table 2). Furthermore, the p38 inhibitor, SB203580, inhibits MAPKAP2 activity in Cos7 cells (Table 2; Cuenda et al., 1995) but does not promote motoneuron survival (see Fig. 2). These data suggest that inhibition of p38 is not sufficient for motoneuron survival. Taken together, we conclude that the activity of the MAPK members is not tightly coupled in an opposing relationship during motoneuronal survival/death processes.

Table 2. Activity in Cos7 cells

JNK1 activity in stressed-induced Cos7 cells

Treatment	Control	CEP-1347
Untreated	1.0	1.0
Irradiation	5.3	2.4
Sorbitol	7.8	2.4
Tunicamycin	1.6	0.9

MAPKAP2 activity in osmotic shocked Cos7 cells

Treatment	Control	CEP-1347	SB203580
Untreated	1.0	1.4	0.6
Sorbitol	8.1	7.6	1.0

Cells were grown to confluency and pretreated with DMSO, 500 nM CEP-1347, or 10 μM SB203580 for 1 hr before treatment with UV irradiation (5 min in Stratolinker, followed by 1 hr incubation at 37°C), sorbitol (500 mM sorbitol for 1 hr), or tunicamycin (50 μg/ml for 5 hr). Lysates were collected, normalized for protein, and immunoprecipitated with the JNK1 or MAPKAP2 antibody, as described in Materials and Methods. Experiments were performed at least two times, and results from a representative experiment are shown. Results are expressed as the fold increase relative to untreated control.

The enhancement of motoneuronal survival by CEP-1347 *in vitro* is comparable to that elicited by optimal concentrations of protein growth factors (Arakawa et al., 1990; Henderson et al., 1993, 1994; Hughes et al., 1993). CEP-1347 does not exhibit selectivity for motoneurons but is also neurotrophic for neurons dissociated from other regions of the vertebrate embryo, for example, spinal cord and basal forebrain (Kaneko et al., 1997), dorsal root ganglia, striatum, basal forebrain, and entorhinal cortex (M. Glicksman, unpublished data).

Growth factors, such as BDNF and IGF-1, rescue motoneurons from trophic deprivation-induced cell death. In our hands the effect of BDNF on motoneuron survival was highly dependent on cell density. Under extremely stringent conditions (200 cells/cm^2) BDNF, as well as CEP-1347, rescued motoneurons. This low-density plating prohibited biochemical analysis of signaling pathways. However, at higher plating density, as presented in this manuscript, BDNF activated ERK1 but did not promote survival nor inhibit the rise in JNK1 activity (data not shown). Thus, BDNF-induced ERK1 activation was not sufficient for motoneuron survival in these cultures. The mechanism by which growth factors promote survival of neurons is unclear and perhaps may depend on the activation of other proteins such as phosphatidylinositol-3 kinase and Akt, which ultimately may lead to a decrease in JNK activity (Yao and Cooper, 1995; Dudek et al., 1997).

Inhibition of JNK1 activation appears to be an intrinsic property of CEP-1347, because the activation of JNK1 by multiple stress stimuli also was blocked by CEP-1347 in Cos7 cells (Table 2). Because different stimuli can activate JNK via distinct pathways, these data suggest that CEP-1347 is acting at a site at or proximal to JNK itself. Directly upstream of JNK is MEK4, which can be phosphorylated by a number of kinases, one of which is MEKK1 (Lange-Carter et al., 1993; Minden et al., 1994; Derijard et al., 1995; Lin et al., 1995). Transfection data reported here suggest that JNK1 is not the direct target of CEP-1347 and that the molecular target is either upstream or independent of MEKK1. Kinases of the germinal center and multiple lineage kinase families activate JNK independently of MEKK1 and are also potential targets for CEP-1347 action (for review, see Fanger et al., 1997). As has been shown in Table 1, CEP-1347 does not

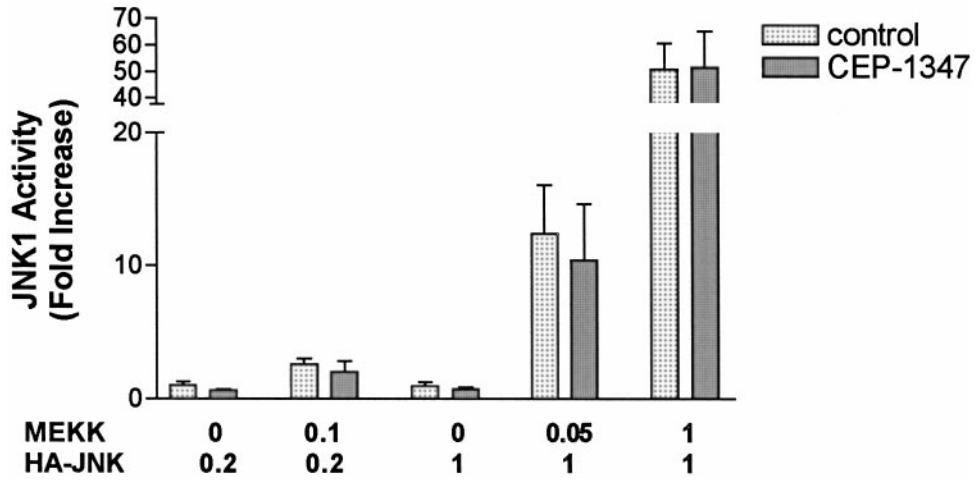

Figure 6. JNK1 activity in Cos7 cells overexpressing HA–JNK1 alone or with MEKK1. Cos7 cells were grown to 80% confluency and transfected with HA-tagged JNK1 alone or with MEKK1 at various amounts of cDNA, as indicated. After a 48 hr period the cells were incubated with 0.01% DMSO or 500 nM CEP-1347 for 2 hr, followed by lysis in 1% Triton buffer. Lysate was normalized to protein and immunoprecipitated with HA antibody. The immunoprecipitates were assayed for kinase activity with the *c-jun* substrate. Experiments were performed at least two times, and results from representative experiments are shown. Activity is expressed by fold increase relative to untreated HA–JNK-transfected cells. *Columns* represent the average of duplicate samples; the error bars indicate the SEM.

display the broad kinase inhibitory activities of K-252a. Although CEP-1347 appears to be more selective than K-252a, it also may have multiple cellular targets.

The recent discovery of stress-activated signaling pathways in dying neurons broadens the concept of neurotrophism, which classically has been defined by activation of the ras signaling cascade by NGF, resulting in the attenuation of death (Borasio et al., 1989; Nobes and Tolkovsky, 1995; Weng et al., 1996). We have demonstrated here that a nonpolypeptide organic molecule can attenuate neuronal death without the activation of a MAPK pathway. Importantly, CEP-1347-mediated motoneuron survival correlates with the inhibition of the JNK signaling cascade. These observations suggest that intervention in the JNK signaling cascade may offer opportunities for the development of therapeutic agents for neurodegenerative disease.

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