Targeting the JNK Signaling Pathway for Stroke and Parkinson's Diseases Therapy

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Abstract: The c-Jun NH2-terminal Kinase (JNK) signaling pathway is frequently induced by cellular stress and correlated with neuronal death. This unique property makes JNK signaling a promising target for developing pharmacological intervention. Among several neurological disorders, JNK signaling is particularly implicated in ischemic stroke and Parkinson's disease. The inhibitors of the JNK signaling pathway include upstream kinase inhibitors (for example, CEP-1347), small chemical inhibitors of JNK (SP600125 and AS601245), and peptide inhibitors of the interaction between JNK and its substrates (D-JNKI and I-JIP). The mechanisms by which JNK signaling induces apoptosis and evidence of cytoprotective effects of these JNK inhibitors are summarized in the present review.

INTRODUCTION

In very general terms, there are three lines of therapeutic targets for any neurological disorders. The first line of target is the specific source of cellular stress for individual neurological disease. For example, in the case of ischemic stroke, this means thrombolysis and recanalization to restore the cerebral perfusion. The second line of therapeutic strategy is to inhibit the stress-induced pro-death signaling and/or boost the stress-adaptive cytoprotective pathways. The third line of therapeutic strategy is to inhibit the final cell death mechanism such as applying caspase inhibitors to block apoptosis. Ideally, it is desirable to attack the first line of therapeutic targets but in many cases the source of cellular stress is ill defined and the damage is irreversible. There is also serious limitation for attacking the third line of therapeutic target as there are many paths leading to death and it is uncertain whether blocking caspases is sufficient to prevent the cell demise. Moreover, since cell death is usually the final manifestation of a chronic neurological disorder, the value of prolonging the life of terminally-ill neurons is open to question. In contrast, since a common set of pro-death and anti-death signaling pathways are frequently induced early in the course of disease progression, these are attractive therapeutic targets for a broad spectrum of neurological disorders.

Perhaps it is following the above mentioned rationale that there has been considerable interest in targeting the c-Jun NH_2-terminal Kinase (JNK) pathway for treating neurological disorders. In particular, several JNK inhibitors have been shown to be highly effective in pre-clinical animal studies of stroke, and a multi-center phase II/III clinical trial of Parkinson's disease using a JNK signaling pathway inhibitor (CEP-1347) is currently underway. The present review will summarize the current understanding of the JNK signaling pathway and its underlying mechanisms leading to cell death, the evidence that have implicated JNK signaling in stroke and Parkinson's disease, and the therapeutic effects of three types of JNK signaling pathway inhibitors in animal models. There are several recent excellent reviews to which the readers are advised to consult for full details of these subjects [1-3].

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JNK Signaling Pathway and Mechanism of Cell Death

JNK was initially identified as a stress-activated serine/threonine protein kinase that phosphorylates c-Jun on two sites (ser-63 and ser-73) in the NH2-terminal activation domain [4, 5]. Subsequent studies led to the cloning of *Jnk1*, *Jnk2* and *Jnk3*, and identification that JNKS is a branch of the mitogen-activated protein kinases (MAPK) group of signaling proteins [6]. Jnk1 and Jnk2 are ubiquitously expressed in all tissues whereas Jnk3 is predominantly expressed in the nervous tissue and heart [7. 8]. In addition, ten JNK isoforms are created by alternative splicing of the messenger RNAs derived from *Jnk1*, *Jnk2* and *Jnk3* [9]. The complexity of JNK isoforms and tissue distribution raises an important question of whether there is functional diversification of JNKs and what the underlying mechanisms are. Indeed, gene-targeting studies showed the neural- and heart-specific JNK3 is more closely involved in stress-induced neuronal apoptosis, whereas JNK1 and JNK2 have a critical role in neural development [7, 10]. Since there is no evidence for any JNK isoform having its unique substrates, the basis of functional diversification appears to lie in distinct tissue distribution patterns and upstream signaling activation of JNK isoforms.

Like all MAPK groups of kinases, the activation of JNK is mediated through a sequential kinase cascade that includes MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK) (Fig. **1**). JNK is activated by dual phosphorylation of the Thr-Pro-Tyr motif located in the activation loop by MKK4 and MKK7 [6]. Gene targeting studies demonstrate that both MKK4 and MKK7 are required for stress-induced JNK activation, and MKK7 is

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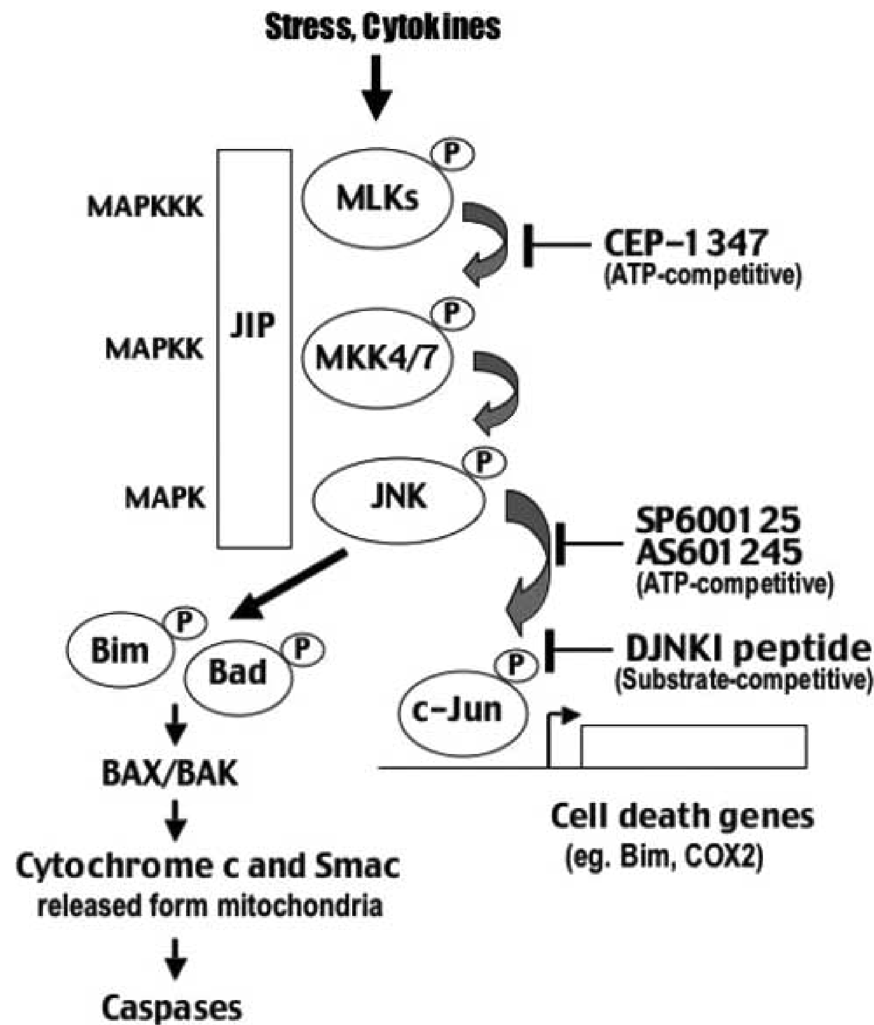

Fig. (1). Summary of the cascade of JNK signaling pathway, the downstream apoptotic mechanisms, and the actions of three classes of JNK inhibitors. Please note that certain details of the JNK signaling pathways are omitted for the simplicity of the diagram.

essential for JNK activation by TNF [11]. The JNK-activating kinases at the MAPKKK level are more diverse, including MEKKs (MAPK/ERK kinase kinases), MLKs (mixed-lineage kinases), DLKs (dual leucine-zipper kinases), ASK (activator of S-phase kinase), and TAK (transforming growth factor -activated kinase). JNKs and upstream kinases also interact with scaffold proteins, such as JIP1 that can assemble functional signaling modules [12]. In addition, JIPs are transported by the microtubule motor protein kinesins, through which JIPs may regulate localized activation of JNK within cells [13].

It is important to note that the function of JNK signaling in the nervous system is not solely for promoting apoptosis. Intriguingly, there is a high level of basal JNK signaling activity in the nervous system compared to other tissues, suggesting a role for normal physiological functions [14]. In addition, on top of the high basal activity, JNK signaling can be activated by stress or cytokines, which often leads to cell death. Increasing evidence suggests that the downstream events of JNK activation leading to apoptosis involve both transcription and mitochondrial mechanisms (Fig. **1**).

For the transcription mechanism, several lines of evidences suggest that c-Jun is the critical mediator of the pro-apoptotic mechanism. Once phosphorylated by JNK, c-Jun has increased transcription activity leading to up-regulation of many genes with AP-1 enhancer sequences. Transgenic mice carrying a mutated form of c-Jun that can no longer be phosphorylated by JNK (c-JunAA mutant in which serine 63 and 73 are changed to alanine) have been generated to test the role of JNK-induced c-Jun/AP-1-mediated transcription in apoptosis. Indeed, cJunAA mutant mice have increased resistance to glutamate excitotoxicity similar to *Jnk3*-null mice [15]. This result strongly suggests the induction of c-Jun/AP-1-mediated transcription is a major component of the apoptosis mechanism downstream of JNK signaling. The key cell death-promoting genes downstream of JNK signaling include Bim [16, 17] and Cox-2 [18]. However, c-Jun/AP-1 induction does not always lead to cell death. For example, there is good evidence that the c-Jun/AP-1 induction following axotomy is critical for axonal regeneration [16].

The mitochondrial mechanism of JNK signaling has only been elucidated in the past few years. The landmark study came from the demonstration that the absence of both Jnk1 and Jnk2 in murine embryonic fibroblasts (MEF) causes defects in the mitochondrial death signaling pathway, including the failure to release cytochrome c [20]. Subsequent studies showed that, in addition to

transcriptional up-regulation, JNK directly phosphorylates Bim, but not Bax, within the conserved dynein light chain (DLC) binding motif [21]. The phosphorylation of Bim leads to its dissociation from the dynein motor complex and potential engagement of the mitochondrial apoptotic pathway. In addition, transfection of a constitutive-active form of JNK (MKK7-JNK1 fusion) induces cell death in wildtype or Bid-null MEFs, but not in Bax/Bak double-null MEFs [22]. Similarly, exposure to UV in Jnk1/Jnk2-null MEFs fails to activate Bax, cause cytochrome c release, and induce cell death [22,23]. Together, these studies suggest a scenario that JNK directly phosphorylates Bim leading to activation of Bax/Bak-mediated cytochrome c-release and apoptosis (Fig. **1**). Besides Bim, there is also evidence that JNK phosphorylates Bad to trigger the mitochondrial cell death machinery [24]. Interestingly, JNK phosphorylates Bad at serine 128 residue whereas the pro-surviving Akt pathway phosphorylates Bad at serine 112 and 136 residues. Thus, Bad may be a converge point to integrate pro-survival and pro-apoptotic signals [24]. There is also a recent report that JNK can induce the release of another pro-apoptosis factor Smac from the mitochondria [25].

Evidences of JNK Signaling in Stroke, Parkinson's, and Alzheimer's Diseases

JNK signaling is induced by external stress and frequently associated with cell death. Thus, it has long been suspected that JNK signaling contributes to neuronal demise in many neurological disorders. In particular, there is strong evidence that JNK signaling is involved in ischemic stroke, Parkinson's diseases, and Alzheimer's disease.

For ischemic stroke, an elevated level of c-Jun phosphorylation co-localized with TUNEL-labeling was found in the peripheral area in an experimental model of focal ischemia [26]. Gene-targeting studies further showed that mice lacking the neural and heart-specific form of Jnk3 have remarkable resistance to the kainic acid-induced excitotoxicity [10]. Subsequent studies further showed that Jnk3-deficient mice have increased resistance to a global ischemia-hypoxia model of stroke [14]. It was shown that JNK3 deficiency causes reduced Bim and Fas expression after stroke, and Jnk3-null hippocampal neurons have less cytochrome c release following oxygen-glucose deprivation [14]. Furthermore, mice lacking the JNK signaling scaffold protein JIP1 have increased resistance to the glutamate excitotoxicity [27] and reduced infarct volume in a focal ischemia model of stroke [28]. Together, these studies suggest that JNK signaling may play an important role in determining cell death or survival for neurons-at-risk in the ischemic penumbra area.

For Parkinson's disease, JNK signaling has been implicated in the animal model of MPTP-induced degeneration of the substantia nigra neurons. Following MPTP injection, there is phosphorylation of JNK and the upstream kinase MKK4 in the substantia nigra, correlated with the death of dopaminergic neurons [29]. Adenoviral gene transfer of the JNK-binding domain (JBD) of JIP1 into the striatum, which acts as JNK inhibitor by preventing JNK to bind to the signaling scaffold modules, inhibits the MPTP-induced JNK activation and cell loss in the substantia nigra [30]. Gene targeting studies showed that Jnk2- and Jnk3-deficient mice, but not Jnk1-null mice, have increased resistance to MPTP-induced neuronal death in the substantia nigra, and the protective effect is even stronger in Jnk2 and Jnk3 double-null mice [18]. These results along with the neuroprotective effects inferred by the JNK-pathway inhibitors CEP-1347 have propelled JNK signaling to be a promising target for pharmacologic treatment of the Parkinson's disease.

It is also worth noting that JNK signaling may be involved in the pathogenesis of Alzheimer's disease in two ways. First, -amyloid peptide activates JNK signaling in neurons and the -amyloid-induced death is attenuated in cortical neurons from Jnk3-deficient mice [31]. Secondly, JNK may directly phosphorylate Tau and the amyloid precursor protein (APP) leading to the formation of neurofibrillary tangles and abnormal processing of APP, respectively [32]. Thus, pharmacological inhibitors of JNK may also offer neuroprotection in the Alzheimer's disease.

In light of the above-mentioned evidence implicating JNK signaling in several major neurological disorders, it is not surprising that JNK signaling has attracted the attention of drug development. The following is a summary of the effects of various JNK signaling pathway inhibitors (Fig. **1**).

Inhibitor of JNK Upstream Kinases: CEP-1347

The JNK pathway inhibitor CEP-1347, originally called KT7515 or 3, 9 bis-[(ethylthio)methyl]-K252a, was initially identified as a derivative of K252a [33]. K252a has distinct concentration-dependent effects in neurons. At higher concentrations, K252a inhibits the pro-survival effects of neurotrophins, whereas at low concentrations, it promotes neuronal survival and differentiation similar to the effects of neurotrophins [3]. CEP-1347 is a semi-synthetic derivative of K252a that promotes survival in chick dorsal root ganglion cultures, and induces choline acetyl transferase (ChAT) activity in cultures prepared from embryonic spinal cord and basal forebrain cultures [33]. It was also found that CEP-1347 promotes the survival of motorneurons in cultures along with inhibition of the JNK activity. Interestingly, CEP-1347 does not inhibit activated JNK, suggesting that it may target an upstream kinase of the JNK signaling pathway [35]. Subsequent studies showed that CEP-1347 is indeed an ATP-competitive inhibitor of the mixed-lineage kinases (MLK1, MLK2, and MLK3) with IC_{50} in the range of 20-50 nM *in vitro* [35]. By reducing the kinase activity of MLKs, CEP-1347 selectively inactivates the JNK signaling pathway, but spares the ERK and p38 MAP kinase pathways [34].

In the MPTP-model of Parkinson's disease, CEP-1347 attenuates JNK and MKK4 phosphorylation and protects dopaminergic neurons [36]. Moreover, CEP-1347 inhibits MPTP-induced cyclooxygenase (COX)-2 expression [37]. Furthermore, in a model characterized by apoptosis induced by intrastriatal injection of 6-hydroxydopamine in rats, the CEP-1347 analogue CEP-11004 also diminishes the number of dopaminergic apoptotic profiles [38]. Taken together, these studies strongly implicated the protective functions of CEP-1347 in animal models of Parkinson's disease. Currently, a large-scale multi-center phase II/III clinical trial of CEP-1347 for Parkinson's disease is underway, which is

expected to be completed in 2006 (the PRECEPT trial, for more information, see http://www.cephalon.com). Whether CEP-1347 has neuroprotective effects in ischemic strokes remains to be determined.

Inhibitor of JNK: SP600125 and AS601245

Signal Pharmaceuticals (now Celgene) has generated a series of pyrazoloanthrone derivatives including the compound SP600126 that inhibit the JNK signaling activity [1]. SP600125 (anthrax[1,9-cd][yrazol-6(2H)-one) inhibits JNK by competing with the ATP-binding site with IC_{50} of 40 nM for JNK1 and JNK2, 90 nM for JNK3 *in vitro* [39]. Although less potently, SP600125 also inhibits upstream MAP kinases including MKK4 (IC_{50}= 0.4 μM), MKK7 (IC_{50}= 5.1 μM), MKK3 (IC_{50}= 1.5 μM), and MKK6 (IC_{50}= 1.0 μM) [39]. As MKK3 and MKK6 are upstream activators of the p38 MAP kianse signaling pathway, the effects of SP600125 may not be entirely due to the inhibition of JNK signaling especially at higher concentrations.

Since its introduction in 2001, SP600125 has become a popular inhibitor of the JNK signaling pathway in studies of animal models of diseases. It was shown that SP600125 prevents joint destruction and reduces the AP-1 binding activity and metalloproteinase expression in rodent models of inflammatory arthritis [40]. Unfortunately, because SP600125 has very poor water solubility, its application in neurological disorder models is more limited. SP600125 has not been tested in animal models of Parkinson's disease. The study using intracerebroventricular (ICV) infusion of SP600125 decreases JNK and Bim interaction and translocation of Bax from the cytosol to the mitochondria in transient middle cerebral artery occlusion (MCAO) in rats. However, there is no reduction of the infarct volume after ICV-infusion of SP600125 [41].

Serono Pharmaceuticals also designed a series of benzazole-acetonnitrile compounds of JNK inhibitors [1]. Among these, AS601245 (1,3-Benzothiazol-2-yl(2-{[2-(3-pyridinyl)ethyl]amino}-4 pyrimidinyl) Acetonitrile) inhibits JNK3 with IC_{50} of 70 nM and IC_{50} for JNK1 and Jnk2 at 150 and 220 nM, respectively [42]. Thus, As601245 appears to be a more selective inhibitor of JNK3 at low dose. AS601245 has been shown to possess very impressive cytoprotective effects in a gerbil model of global ischemia [43], and rat model of myocardial infarction [43]. The cytoprotective effect of AS601245 has not been tested in animal models of Parkinson's disease.

JNK Inhibitor Peptides: DJNKI

In contrast to small chemical inhibitors like CEP-1347, SP600125, or AS601245, a completely different approach of design of JNK signaling inhibitors is based upon the discovery that over-expression of the JNK-binding domain (JBD) of the scaffold protein JIP1 will prevent the binding of JNK to c-Jun and some other substrates [12]. The JBD-domain has been expressed in adenoviral vectors to serve as an experimental tool to block JNK signaling [30]. A peptide inhibitor called D-JNKI that links the 10-amino acid HIV Tat (48-57) transporter sequence to the 20-amino acid JBD has been created as JNK inhibitors [1]. D-JNKI showed a remarkable neuroprotective effect in both transient and permanent MCAO model of strokes [44, 45]. Importantly, DJNK1 reduces the infarct volume even when administered at 6 hours after transient MCAO, suggesting it may be used as adjunct neuroprotective therapy after the completion of thrombolytic therapy or when the time window for thrombolytic therapy has passed.

A similar approach has also been undertaken to design small peptide inhibitor of JNK called I-JIP (Inhibitor of JNK-based on JIP-1), or TI-JIP (truncated I-JIP) [46]. Although the JNK inhibitor peptides have been demonstrated to possess impressive neuroprotective actions, it is important to bear in mind that whether D-JNKI and I-JIP are equally effective in blocking JNK binding to pro-apoptotic Bcl-2 family proteins has not been established. Thus, these JNK inhibitor peptides originally designed based on the JNK-docking domain of c-Jun may not completely inhibit the full spectrum of JNK substrates.

CONCLUSIONS

Given the intriguing property of stress-induced activity, the JNK signaling pathway is a promising target for developing pharmacologic intervention in a variety of disorders. As the phase II/III clinical trial of CEP-1347 for the Parkinson's disease will soon be completed, these results will attest whether inhibiting the JNK signaling pathway has therapeutic effects. In contrast, although several lines of evidence implicates JNK signaling in neuronal death in ischemic stroke, more JNK inhibitors with higher water solubility and better blood-brain-barrier permeability is needed to demonstrate the cytoprotective effects of JNK inhibition in stroke. Finally, as gene-targeting studies clearly show that different JNK isoforms have distinct functions in the nervous system and because the high level of basal JNK activity in the brain suggests some sort of physiological functions, it is important to ensure whether specific JNK isoforms have preferential roles in a given neurological disorder, and whether more selective JNK isoform inhibitors are needed for therapy.

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