Chapter 13

JNK PATHWAY AS THERAPEUTIC TARGET TO PREVENT DEGENERATION IN THE CENTRAL NERVOUS SYSTEM

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Abstract: JNKs (c-Jun N- terminal kinases) are important transducing enzymes involved in many faces of cellular regulation such as gene expression, cell proliferation and programmed cell death. The activation of JNK pathway is critical for naturally occurring neuronal death during development as well as for pathological death of adult brain following different insults. In particular, JNKs play an important role in excitotoxicity and all related phenomena. Initial research concentrated on defining the components and organization of JNK signalling cascades, but more recent studies have begun to see JNK as the appropriate target for prevent cell loss. We used a specific JNK inhibitor, the cell permeable peptide D-JNKI1, to block JNK action in neuronal death following excitotoxicity in vitro and cerebral ischemia in vivo. Here we review our recent findings and we discuss the possibility of using D-JNKI1 as a therapeutic agent to prevent cell loss in the central nervous system.

Key Words: JNK inhibitors, excitotoxicity, neurodegeneration, ischemic damage

INTRODUCTION

Excitotoxicity is defined as the excessive activation of glutamate receptors, in which *N*-methyl-D-aspartate (NMDA) receptors play a key role, owing to their high Ca^{2+} permeability. The overactivation of the NMDA receptors induces downstream cascade of events resulting in neuronal death. Excitotoxicity is responsible for many brain injuries such as ischemia (43), epilepsy (30) and it plays an important role also in several neurodegenerative diseases (42), (22).

Excitotoxicity has been studied extensively, but the downstream mechanisms coupling increased intracellular Ca^{2+} to cell death are still poorly understood. Several studies have

tried to obtain neuroprotection by blocking NMDA receptors, the major agent representing this category is the NMDA-r antagonist MK-801: notwithstanding the promising results in animal models (reduction of the excitotoxic damage), they had to be abandoned for the emergence of unacceptable psychotomimetic side effects. These side effects are strongly correlated with the physiological NMDA receptor activity, which is essential for normal neuronal cell functions. A second possible approach in the prevention of excitotoxicity is to target specific intracellular pathways, which normally act downstream from the glutamate receptor, without blocking synaptic activity. A peptide that disrupts the interaction of NMDArs with the postsynaptic density protein PSD-95 has been used against excitotoxicity in cultured neurons and ischemic damage in vivo (1).

The first evidence of the JNK involvement in excitotoxicity derived from the analysis of JNK3 deficient mice: after a systemic injection of kainate these mice showed a reduction in seizures activity and a prevention of apoptosis (39). Moreover, mice with an inactive form of c-jun (Jun AA, in which serines 63 and 73 are mutated to alanines) also showed resistance to excitotoxic neuronal death, thus suggesting that preventing JNK from accessing c-jun might confer neuroprotection (3). It has been shown that in cortical neurons NMDA mediated neurotoxicity activates JNK in dependence on entry of extracellular calcium (23). Moreover in an immortalized rat hippocampal neuronal cell line, the post-synaptic density protein 95 (PSD95) links GluR6-mediated excitotoxicity with JNK via MLK2/3 (29). A recent paper by Brecht (11), has proved distinct roles for the different JNK isoforms in four models of neurodegeneration in vivo, including permanent ischemia and excitotoxicity by application of kainic acid.

JNK INHIBITORS

Several research laboratories have proposed JNK inhibitors in order to find effective drugs for the treatment of a variety of pathologies. These inhibitors could be divided in two classes: chemical compounds and cell permeable peptide inhibitors.

Chemical compounds

This approach consists in producing small organic compounds that are competitive inhibitors of the ATP-binding site of the kinase. This technique has given a couple of efficient JNK inhibitors. Two of them, CEP-1347 and SP600125 have been the subjects of peer-reviewed studies as described in Bozyczko-Coyne and Bogoyevitch reviews (10), (5) but the published information on their efficacy in clinical trials remains limited.

CEP-1347 is a semi-synthetic inhibitor of the MLK group of MAPKKK (see Figure 1). It blocks the upstream component in the JNK pathway, and derives from the naturally occurring small molecule K252a. It competes with ATP to bind MLKs, with IC_{50} values of 38nM (MLK1), 51nM (MLK2), and 23nM (MLK3) (26). CEP-1347 is able to prevent neuronal cell death in vivo models of Alzheimer's disease (AD), Parkinson's disease (PD) and cochlear hair cell death (37, 38).

SP600125, developed by Celgene, is an ATP competitive inhibitor of JNK (see Figure 1). It inhibits all three JNK isoforms, with IC_{50} values of 40, 40, and 90nM for JNK1, JNK2 and JNK3 respectively. It also has an inhibitory activity against other MAPKs, such as ERK and p38 (4). In vivo this compound inhibited leukocyte recruitment in a rat inflamed

lung model (16) and was active in the rat rheumatoid arthritis model (18).

It also protected dopaminergic neurons in the MPTP model of Parkinson's disease (38) and it was used to attenuate apoptosis in a model of Alzheimer's disease neurotoxicity (25).

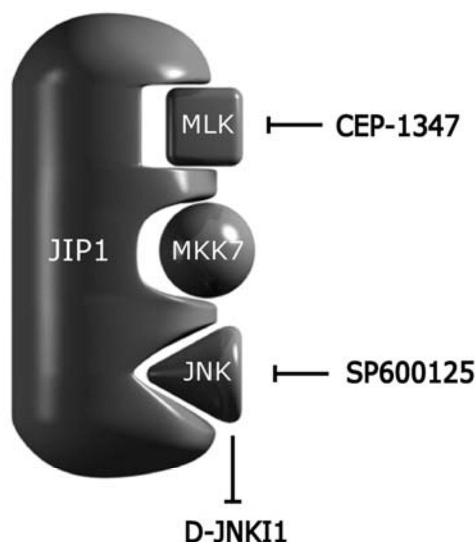

Figure 1. Modular organization of the JNK signaling pathway. JIP-1 scaffold protein binds to JNK, MKK7, one of the two JNK activators, and to members of the mixed-lineage protein kinases (MLK) group. The assembly of the JNK module by a scaffold may lead to a more efficient activation of JNK. The two small chemical inhibitors of the JNK pathway are acting at different levels of the cascade: whilst CEP-1347 inhibits MLK, SP600125 inhibits JNK activation. The cell permeable peptide D-JNKI-1 prevents JNK action without interfere with JNK activation/ phosphorylation.

Cell permeable peptide inhibitor

D-JNKI-1 inhibitor produces an allosteric modulation of JNK by blocking the access of the kinase to some of its targets, preventing protein-protein interactions, without interfering with its activation (Figure 1).

In 2001 Bonny showed that a new efficient and specific inhibitor of JNK action was able to protect pancreatic ß-cells from IL1 ß induced apoptosis. D-JNKI-1 peptide (which is the D-retro-inverso form, made of aminoacids in reversed sequence order) has been engineered by linking the 20 amino acid JNK-inhibitory sequence of IB1/JIP-1 scaffold protein (JBD_{20}) to the 10 amino acid HIV- transporter sequence (6). The $TAT_{48\text{-}57}$ peptide penetrates in a variety of cells and could be useful for delivering macromolecules, also to animal tissues (32). Between the TAT sequence and the JBD_{20} sequence two proline-residues were inserted as spacer to allow for maximal flexibility. JIP-1 scaffold protein and c-Jun, JNK main target, share a similar binding motif for interact with JNK. However JNK binding affinity to JIP-1 is about 100-fold higher than to c-Jun, for this reason the inhibitor peptide is able to block the access of JNK to its substrates. Barr and collaborators (2) found that a shorter peptide sequence (RPKRPTTLNLF=TI-JIP) based on amino acids 143-153 on the $JBD_{20}$of JIP-1, partly overlapped with the sequence described by Bonny (6) and was also able to prevent c-Jun phosphorylation. Thus the inhibitory action of D-JNKI-1 peptide is fundamentally different from that of classical small chemical inhibitors (7), (8). D-JNKI-1 does not inhibit JNK's enzymatic activity, but selectively blocks access to many of its substrates by a competitive mechanism

(6), (7), (8). To determine the specificity of the peptide in blocking JNK action, we characterized the effects of the peptide on the activity of 40 different kinases (10 µM peptide, 10 µM ATP) towards their respective substrates in cell free system. It did not interfere with the activities of the other kinases (7), (8), proving its exceptional superiority.

In a recent study we showed that the D-JNKI-1 peptide completely inhibited excitotoxicity in primary culture and strongly prevented neuronal loss against two different models, transient and permanent, of middle cerebral artery occlusion (MCAo) (8). However other authors have demonstrated powerful protection in different models of CNS injury: D-JNKI-1 protects against toxic drug and acoustic trauma induced auditory hair cell death (36) and against retinal ganglion cells death following optic nerve crush (34). This cell permeable peptide inhibitor offers interesting possibilities for therapeutic application in preventing neuronal loss.

OUR OWN RESULTS BY USING D-JNKI-1

We investigated the protective D-JNKI-1 action at three different levels: i) in vitro in cortical neurons cultures, ii) in organotypic hippocampal cultures, and iii) in vivo in focal cerebral ischemia.

Cortical neurons

D-JNKI-1 completely inhibited cell death induced by excitotoxicity (100µM NMDA) in primary cortical neurons (Figure 2)(8). JNK activation in NMDA-treated neurons appeared maximal after 30 min of NMDA, and resulted into an elevated c-Jun phosphorylation. Addition of D-JNKI-1 completely prevented the increase in phosphorylated c-Jun after 5h exposure to 100 µM NMDA (despite a normal level of JNK activation), bringing the level of P-c-Jun below even that in the control. NMDA-induced activation of the c-fos gene, transcription of which is under the positive control of the JNK target Elk-1, was also completely prevented by the peptide. In fact, c-fos expression in D-JNKI-1/NMDA treated neurons, evaluated by real-time RT-PCR, was comparable to control levels whereas in neurons treated with only NMDA it increased and strongly correlated with the NMDA time course.

Organotypic cultures

NMDA treatment (100 µM) in organotypic hippocampal cultures (9) resulted in death of pyramidal neurons in the CA1 and CA3 already detectable within two hours of NMDA administration. In the same regions, following two hours of NMDA, c-Jun was selectively phosphorylated and c-fos was up-regulated. Pretreatment of 2 µM D-JNKI-1 prevented pyramidal neurons cell death and completely inhibited c-Jun phosphorylation and c-fos expression (Figure 3).

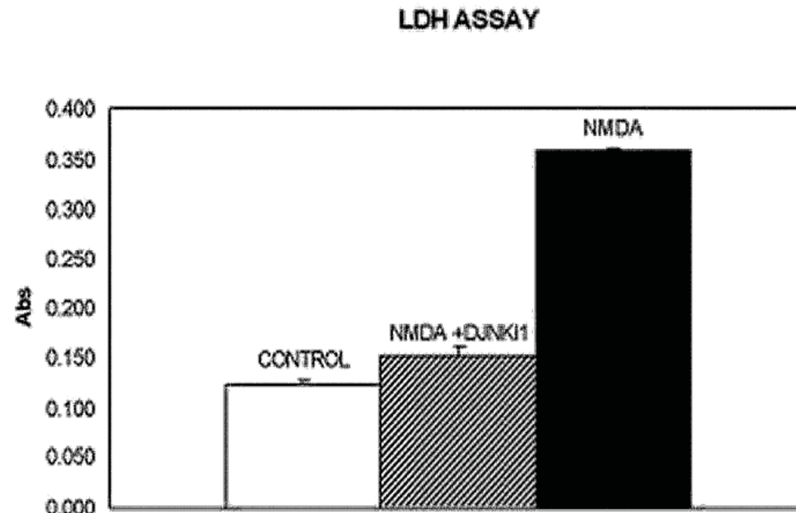

Figure 2. Neuronal death at 5 h after exposure to 100 μM NMDA evaluated by LDH activity in the culture medium in presence (black and white) or absence (black) of D-JNKI1 2 μM.

In vivo

D-JNKI-1 effect has been tested on two different models of cerebral ischemia (8), transient ischemia in adult mice and permanent focal ischemia in P14 rats. The peptide was able to confer strong protection against ischemic damage in both models. The infarcted volumes were traced on serial sections by using the Neurolucida software program after 24h from insult (17), allowing the quantification of brain damage (Figure 4). In the transient ischemia model we obtained a reduction of the infarct of 88% following an intracerebroventricular injection 1 hour before the lesion, and the strongest reduction (93%) following D-JNKI-1 administration 6 hours after ischemia. In the permanent middle cerebral artery occlusion (MCAo) model we injected the peptide intraperitoneally 30 min before, 6 hours after or 12 hours after ischemia and we obtained an infarcted volume decrease of respectively 68%, 78% and 49%. Actually, the only approved therapy for ischemic stroke is the administration of tissue plasminogen activator (tPA) within 3 hours of the onset of the ischemia, since after 3 hours the risk of a hemorrhage caused by tPA becomes too high. The most prominent feature of the protection observed with D-JNKI-1 is that the peptide is still effective when administered 6-12 hours after the onset of stroke. This is particularly relevant, as most patients will access medical centres only 6-10 hours following the onset of stroke.

To assess whether the peptide really prevented neuronal death or only delayed it, we analyzed lesion volumes one week (permanent ischemia) or two weeks (transient ischemia) after lesion in animals treated with D-JNKI-1 6 hours following ischemia, compared to control animals. In permanent ischemia D-JNKI-1-treated rats and control rats presented a shrunken infarct, but there was still a significant protection by the peptide (54%). In transient ischemia of adult mice we obtained 93% reduction of lesion volume, exactly the same

protection shown at the shorter survival time (24h after ischemia).

Furthermore, the powerful neuroprotection obtained is accompanied by behavioral benefits; we have not so far detected negative side effects at the dose-levels required for protection.

Therefore, JNK inhibition using D-JNKI-1 might prove useful in the treatment of stroke damage.

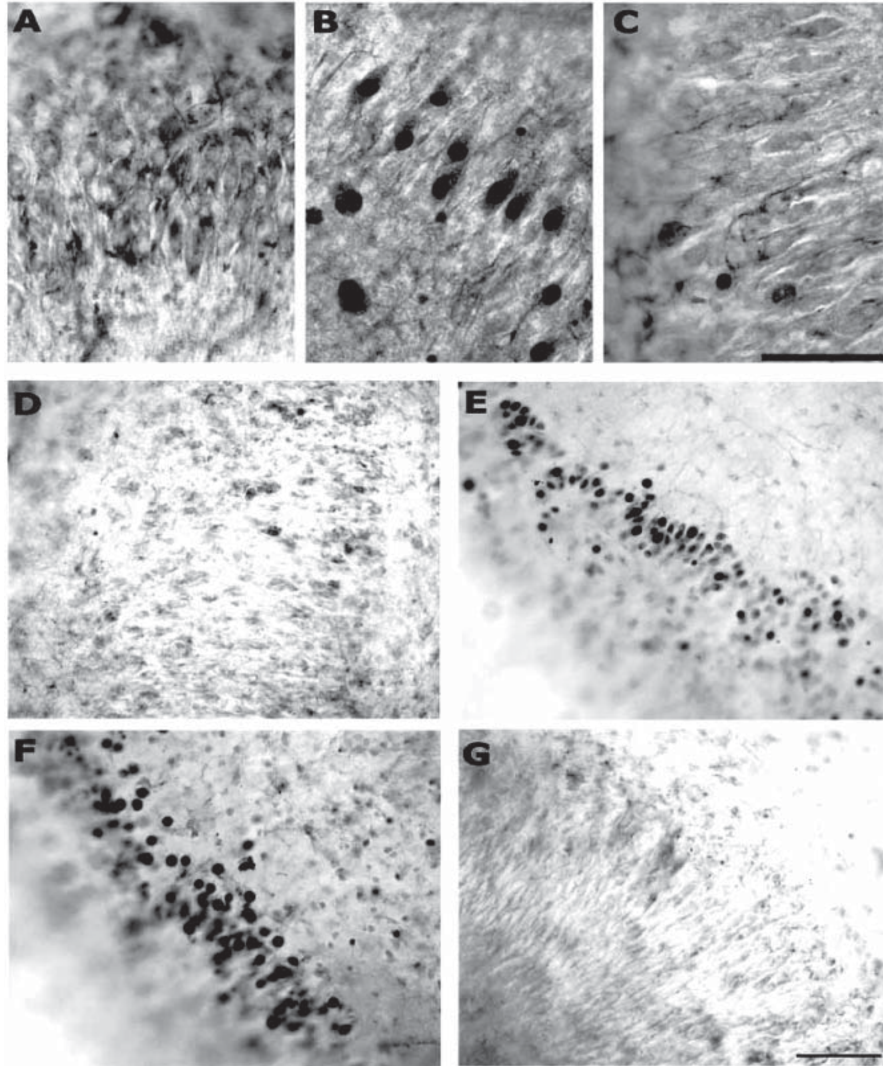

Figure 3. Immunohistochemistry in organotypic hippocampal cultures. (A-C):phosphorylated c-jun immunolabelling. (A): control condition, (B): 2h of 100μM NMDA, (C): 2h of 100μM NMDA in presence of 2μM D-JNKI1. (D-G): c-fos immunolabelling. (D): control condition, (E):1h of 100μM NMDA, (F): 2h of 100μM NMDA, (G): 2h of 100μM NMDA in presence of 2μM D-JNKI1. Reprinted, with permission from the Eur. J. Neurosci. 2003, 18(3): 473-85.

JNK TARGETS RELATED TO NEURODEGENERATION

JNK substrates related to cell death can be nuclear or cytoplasmic, and this means that JNK could act at transcriptional or at post-transductional level.

Concerning nuclear substrates, JNKs activate several members of the AP-1 group of transcription factors, the most important of which is c-Jun, activated by phosphorylation on Ser 63 and 73 (27). ATF-2 and Elk-1 are also activated by JNKs (21), (12). Transcriptional activity enhanced by these proteins is responsible for cells biologic response. JNK2 and JNK3 seem to be responsible for the activation of transcription factors whereas the basal constitutive presence of activated JNK1 is not effective in phosphorylation of transcription factors including c-Jun (13), (14). c-Jun phosphorylation was also linked to the protection or regeneration (19) and it has not been clarified if JNK can also contribute to pro-survival action.

Regarding cytoplasmic substrates, it has been shown that there is interdependency between JNK signaling and mitochondrial apoptosis (31). Oxygen-glucose deprivation in hippocampal neurons from Jnk3-null mice compared to WT mice suggests a critical JNK3 role in both the apoptotic process and the release of cytochrome c from mitochondria (24). The release of cytochrome c from mitochondria is controlled by specific members of the Bcl-2 family protein, whose phosphorylation could be regulated by JNKs (28), (35).

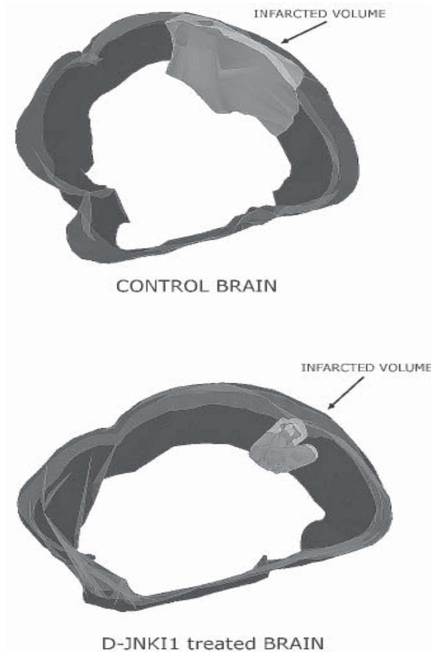

Figure 4. Three-dimensional reconstruction showing examples of lesion in control rat and D-JNKI-1 treated rat 6 hours after permanent ischemia (middle cerebral artery occlusion). This ischemic lesion results in a unilateral degeneration of the parietal cortex 24 hours following ischemia. The areas of the ischemic lesion and of the whole brain were traced using the Neurolucida software program for computer-aided microscopy.

DENN/MADD is also a JNK cytoplasmic substrate implicated in cell death. It has a death domain and was identified as a substrate for JNK3, which is the isoform mostly present in the CNS (41). The meaning of this phosphorylation is still unclear, but increasing evidences support a correlation between low MADD expression and neuronal loss: it has recently been shown that MADD down-regulation correlates with neuronal cell death in

Alzheimer's disease brain and hippocampal neurons (15).

Finally, JNK is involved in the phosphorylation of Tau and APP, two proteins strongly related to neurodegenerative diseases. In fact, hyperphosphorylation and accumulation of Tau in neurons (and glial cells) is one of the main pathologic hallmarks in Alzheimer's disease and other tauopathies. Many of the hyperphosphorylated sites are serine/threonine-proline sequences. Recently it has been shown that all three JNK isoforms phosphorylate Tau at many serine/threonine-prolines, as assessed by the generation of the epitopes of phosphorylation-dependent anti-Tau antibodies (40). Phosphorylation by JNK isoforms resulted in a greatly reduced ability of Tau to promote microtubule assembly, which would compromise neuronal transport and would result in neuronal dysfunction.

APP is efficiently phosphorylated in its cytoplasmic region by JNK3 in vitro (33) and APP phosphorylation by JNK is enhanced by the association of APP with scaffold protein JIP-1b (20). Moreover, an enhanced activation of JNK pathway and an attenuation of apoptosis by SP600125, the ATP competitive JNK inhibitor, through protection of mitochondrial dysfunction and reduction of caspase 9 activity have been demonstrated (25). These results put in evidence that JNK could play an important role in the pathogenesis of Alzheimer's disease.

CONCLUSIONS

JNK could provide a suitable target for the protection against neuronal loss and considerable effort is being directed to the development of JNK inhibitors. This resulted in a couple of chemical inhibitors: CEP-1347, an inhibitor of the MLK family of JNK pathway activators, and SP600125, a direct inhibitor of JNK activity. These commonly used inhibitors have demonstrated efficacy for use in vivo, with the successful intervention in animal models. An alternative approach is represented by the cell permeable peptide D-JNKI-1, which is a new powerful neuroprotective agent. D-JNKI-1 is an efficient and specific inhibitor of JNK action. It does not inhibit JNK's enzymatic activity, like the classical small chemical inhibitors, but it selectively blocks access to many of its substrates by a competitive mechanism and results as the most selective inhibitor proving its exceptional superiority.

D-JNKI-1 protects against several forms of excitotoxicity in vitro (8), (9), and it confers the strongest protection even reported before against ischemic cell loss in vivo with extended therapeutic window, since it still protects when administered 6-12 hours after the ischemic insult (8). Moreover it has been shown that it is really able to completely prevent the death program and not just to delay it, as a significant protection was still seen after one or two weeks following ischemic lesion in transient ischemia: this confirms that it could be a promising agent to obtain neuroprotection in stroke. D-JNKI-1 protective action has also been tested with positive results in two other models of CNS injury, hair cell death and hearing loss following ototoxic drug or acoustic trauma exposure (36) and retinal ganglion cells death following optic nerve crush (34): these results provide strong evidence that JNK inhibition with D-JNKI-1 could be a very interesting therapeuthic approach to prevent cell loss in the CNS.

Despite these promising results further studies will be necessary in order to detect D-JNKI-1 side effects, and caution is required in view of JNK's involvement in metabolic

regulation and neuronal plasticity and regeneration. In fact, it has to be admitted that the underlying cell biology of this powerful neuroprotection is still largely unknown.

A deep understanding of the organization and function of the JNK signaling cascade will be crucial to improve the specificity of the JNK inhibitors. In fact, due to the complex cross-talk within different signaling cascades, as well as peculiar neuronal response, it is difficult to predict potential adverse events that might arise from JNK inhibition. However, it appears clear that further studies are needed to offer greater specificity in order to prevent only the pathological responses of this signaling, that extent its physiological functions in such a large range.

Detailed studies about D-JNKI-1 toxicity and its potential side effects are now essential. Until these aspects are clarified the question will remain if D-JNKI-1 could represent an important molecule in human therapy and whether it can be extended to clinical trails.

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