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Invited review

JNK pathway: diseases and therapeutic potential[1]

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Abstract

c-Jun N-terminal protein kinases (JNK), also known as stress-activated protein kinases, were originally identified by their ability to phosphorylate the N-terminal of the transcription factor c-Jun and by their activation in response to a variety of stresses. JNK are multifunctional kinases involved in many physiological processes. The JNK pathway has been shown to play a major role in apoptosis in many cell death paradigms and its association with a variety of pathological processes is gradually been recognized. This review will concentrate on describing the involvement of the JNK pathway in the context of different diseases and the potential to adopt the JNK pathway components as therapeutic targets.

Introduction

Cells have to respond to environment stimuli properly to survive. These stimuli include physical stimulation such as heat, pH variation, radiation, redox, osmolarity, and chemical stimulation such as growth factors, cytokines, hormones, alterations in nutrient conditions, as well as other environment stresses[1]. These stimuli control many aspects of complex cellular processes, including the regulation of gene expression, cell survival, growth, differentiation and death. In these processes, many signal transduction pathways cooperate to relay, amplify, and integrate a diverse range of extracellular stimuli. The mitogen-activated protein kinase (MAPK) signaling pathway is one of the major signaling systems that transduce extracellular signals into cells[1,2] (Figure 1).

MAPK pathways are conserved in fungi, plants, and mammals. MAPK cascades contain at least 3 protein kinases in series. They are MAPK kinase kinases (MAPKKK, also known as MEKK), MAPK kinases (MAPKK, also known as MEK and MAPK). MAPKKK phosphorylate and activate the downstream MAPKK, which in turn phosphorylate and activate MAPK (Figure 1). These kinases are activated by dual phosphorylation[1–3]. As there are many different

extracellular signals in the cellular contexts, MAPKKK show high divergence in structure and gene number compared with MAPKK and MAPK. Typically, MAPKKK are regulated through receptor activation, membrane recruitment of adaptor molecules, activation of small GTP-binding proteins, and phosphorylation. The activation of the cascades may also require additional kinases upstream of this 3-tier kinase, as well as scaffold proteins that are able to manage the complexity and specificity of the pathway. The scaffold proteins can bind, organize, and facilitate specific interactions among the components so that specific stimuli can produce specific MAPK signaling responses (Figure 2)[2,4]. Because of the implication of MAPK in developmental processes and the survival/death responses of cells to their environment, the MAPK pathway has become the focus of attention in recent years[5].

In mammals, there are 3 subfamilies of MAPK that have been identified up to now: c-Jun N-terminal protein kinases (JNK)/stress-activated protein kinases (SAPK), p38 MAPK, and ERK (Figure 1)[2,6]. Following the same signaling arrangement, each individual MAPK pathway responds to specific stimuli and then regulates their specific substrates. This review will focus on the JNK MAPK pathway and its involvement in a variety of diseases.
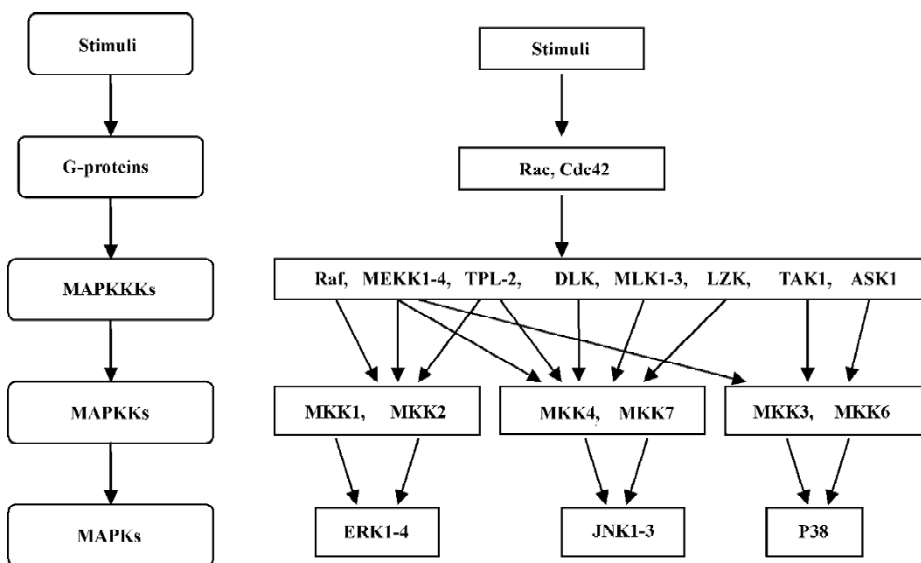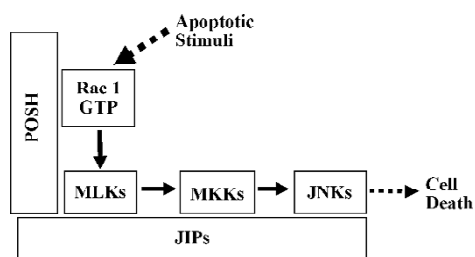
Figure 1. Components of the MAP kinase pathway.

Figure 2. Involvement of scaffold proteins in managing the complexity and specificity of the JNK pathway.

JNK MAPK pathway

The mammalian JNK were initially described as SAPK because they are activated in response to a variety of environmental stresses[7,8]. The JNK pathway is also recognized to respond to cytokines (eg, TNF and interleukin-1) and growth factors[9].

JNK is a multifunctional kinase involved in many physiological and pathological processes. The JNK pathway plays a major role in apoptosis in a variety of death paradigms. For example, the JNK pathway is required for neuronal cell death induced by many apoptotic stimuli, including nerve growth factor (NGF) deprivation, trophic support withdrawal, DNA damage, oxidative stress, α-amyloid exposure, low potassium, excitotoxic stress, 6-OHDA, UV irradiation, and tumor necrosis factor[10–19].

The JNK pathway cascade involved in neuronal cell death has been widely studied. It mainly contains activated Rac1/Cdc42, mixed-lineage kinases (MLK) and/or ASK1, MKK 4 and MKK7, and JNK in series[1,10,15–18,20]. In addition, other components are involved in the activation of the JNK pathway[16,18,19]. Furthermore, plenty of SH3 (POSH) and JNK interacting proteins (JIP) have been shown to act as a scaffold protein to form a multiprotein complex with other JNK apoptotic cascade elements[15,21,22].

In mammals, there are 3 JNK genes: *Jnk1, Jnk2*, and *Jnk3* on 3 different chromosomes, and each mammalian JNK gene has alternative splicing forms so there are at least 10 different JNK proteins of 46–55 kDa identified[23]. JNK1 and JNK2 are ubiquitously expressed, but the expression of JNK3 is mainly restricted to central nervous system (CNS) neurons (high level), cardiac smooth muscle, and testis (low levels)[10]. There is a different substrate affinity among these 10 isoforms[23]; therefore, different JNK isoforms could play specific roles in multicellular organisms, and there is also complementation between the JNK genes.

As to the substrates of JNK, initially studies found that JNK can interact with and phosphorylate the N-terminal transactivation domain of c-Jun and thereby enhance its ability to transactivate related gene expression, so it is called JNK[24]. However, ongoing studies show JNK can phosphorylate a variety of substrates, including additional transcription factors and even some non-nuclear proteins. In addition to c-Jun, JNK can phosphorylate transcription factors such as JunB, JunD, c-Fos, and ATF. These transcription factors, together with c-Jun, constitute activator protein-1 transcription factors, which can regulate the expression of several stress-responsive genes[25]. There are other tran-

scription factors such as ElK1, NFAT, and p53 that can also be phosphorylated by JNK[28]. JNK mediates apoptosis not only through its effects on gene transcription, but also through a transcriptional-independent mechanism[29]. Several studies have demonstrated that JNK is able to phosphorylate both pro- and anti-apoptotic proteins and regulate their activity. For example, JNK can phosphorylate Bcl-2 and Bcl-x_L and diminish their anti-apoptotic activity[30–32]. JNK can also phosphorylate the pro-apoptotic protein Bim and Bmf to promote their pro-apoptotic effects[33,34].

Involvement of the JNK pathway in diseases

The JNK pathway is involved in many physiological processes such as embryonic morphogenesis and naturally-occurring programmed cell death. The specific roles that JNK plays depend on the cellular context[10]. Under pathological conditions, the unusual activated JNK pathway can cause pathological cell death and different diseases.

JNK pathway and neurological disorders Mounting evidence suggests that the JNK pathway plays critical roles in the pathogenesis of many neurological disorders, including ischemic stroke, Parkinson's disease (PD), polyglutamine diseases, amyotrophic lateral sclerosis, auditory hair cell degeneration in addition to different tauopathies, which include Alzheimer's disease (AD), Pick's disease, progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, familial frontotemporal dementia, and parkinsonism.

Hyperphosphorylation and the accumulation of tau in neurons (and glial cells) is one of the main pathological hallmarks in AD. Increased levels of activated JNK have been found in brain homogenates in all the tauopathies. Strong active JNK immunoreactivity has been observed to be restricted to neurons and glial cells containing hyperphosphorylated tau, as well as in dystrophic neurites of senile plaques in AD[35]. The accumulation of amyloid beta peptide (Abeta) is believed to be an early and critical event leading to synapse and neuronal cell loss in AD. Abeta itself is toxic to neurons *in vitro*, and the expression of Abeta *in vivo* causes the loss of synapses and neurons in the brain in animal models[36]. Interestingly, the increased expression level of activated forms of JNK and p38 and hyperphosphorylated tau containing neurites has been observed in mice transgenic of human amyloid precursor protein-695 with the Swedish familial AD mutations (Tg2576) and a P264L familial AD mutation introduced by targeting the presenilin-1 gene[36]. In the Tg2576 model, JNK activity is associated with the 2 hallmarks of AD: the amyloid deposit and the hyper-phosphorylated Tau protein[36].

Reactive oxygen species (ROS) has been considered to be the major cause of Abeta toxicity and it is believed that the Abeta-triggered generation of ROS leads to the activation of the JNK pathway, which in turn phosphorylates tau in neurites surrounding amyloid deposits[35,37]. Corresponding evidence has been reported from the autopsies of AD patients who participated in the amyloid-beta immunization trial and died from the immunization-induced encephalitis. The activation of JNK was reduced together with decreased tau hyperphosphorylation of aberrant neurites in association with decreased amyloid plaques in both Tg2576 mice and human brains[35]. Thus, pharmacological inhibitors of JNK may also offer neuroprotection for AD.

JNK signaling has been implicated in the animal model of MPTP-induced degeneration of neurons in the substantia nigra. Following MPTP injection, the obvious activation of JNK, the downstream substrate of JNK, c-Jun, and the upstream kinase MKK4 have been detected in the substantia nigra, correlating with the death of dopaminergic neurons[12,38]. A peptide JNK inhibitor interferes with the interaction between JNK and JIP, and inhibits MPTP-induced JNK activation and cell death in the substantia nigra[39]. Gene targeting studies have shown that JNK2 and JNK3 play a vital role in MPTP-induced neuronal death in the substantia nigra. These results, along with the neuroprotective effects by the JNK-pathway inhibitor CEP-1347 in animal MPTP models, have propelled JNK signaling to be a promising target for the pharmacological treatment of the PD[40].

For ischemic stroke, increased activity of JNK was found to co-localize with TUNEL-labeling in the peripheral area of focal ischemia animal models[41]. JNK3 knockout mice are remarkably resistant to kainic acid-induced excitotoxicity[10]. Subsequent studies further showed that JNK3-deficient mice had increased resistance to a global ischemia-hypoxia model of stroke[42]. JNK3 deficiency renders reduced Bim and Fas expression after stroke, and JNK3-null hippocampal neurons have less cytochrome c release following oxygen-glucose deprivation[42]. Furthermore, mice lacking the JNK signaling scaffold protein JIP1 have increased resistance to the glutamate excitotoxicity[43] and reduced infarct size in a focal ischemia model of stroke[44]. Knockdown of another scaffold protein POSH is neuroprotective following cerebral ischemia in the rat hippocampus[45]. Taken together, these studies indicate that the JNK pathway may play an important role in ischemic cell death. It offers an opportunity to develop or apply inhibitors of JNK to treat neuronal cell death incurred by ischemia.

JNK pathway and other diseases It has been recognized

that the JNK pathway serves as a major "on" switch of programmed cell death in response to a variety of stimuli[46]. In addition to the well-established roles of JNK in neurological disorders, the JNK signaling pathway plays unexpected pathological roles in other diseases, including type 2 diabetes, cancer, stroke, heart disease, and inflammatory diseases.

Type 2 diabetes is the most prevalent and serious metabolic disease hallmarked by pancreatic beta-cell dysfunction and insulin resistance. Under diabetic conditions, oxidative stress and endoplasmic reticulum stress are induced in various tissues, leading to the activation of the JNK pathway[47].

Recent studies have demonstrated that JNK plays a central role in modulating insulin action and the pathogenesis of obesity, fatty liver disease, and type 2 diabetes[48]. JNK can directly phosphorylate IRS-1 at several sites, including Ser_{307}[49]. There is increased JNK-dependent IRS-1 Ser phosphorylation in obesity that leads to reduced insulin-stimulated IRS-1 Tyr phosphorylation. In obese animals, knockout of the JNK-1 gene, or the expression of a dominant-negative JNK isoform, results in the reversal of obesity-induced IRS-1 Ser phosphorylation and substantial protection against insulin resistance and defective insulin receptor signaling[4,48], while the exogenous expression of JNK in the adult liver results in severe insulin resistance in mice[4]. JIP1 in knockout mice creates a phenotype very similar to JNK-1 deficiency with reduced JNK activity and increased insulin sensitivity[50]. Meanwhile, a point mutation in JIP1 has been identified in type 2 diabetes patients, providing crucial genetic evidence for the role of JNK and JIP1 in the pathogenesis of type 2 diabetes in humans[51].

It is likely that JNK activity modulates islet function and/ or survival in different ways. First, JNK is involved in islet cell inflammation and death mediated by cytokines[52–54]. Second, JNK activation might cause β-cell dysfunction and defective insulin production, thereby contributing to the development of frank diabetes[55]. Third, administration of SP600125, an inhibitor of JNK, improves glucose-stimulated insulin production in isolated islets in the db/db model of obesity and diabetes[56]. Therefore, JNK might integrate defects in insulin secretion with peripheral insulin resistance in type 2 diabetes through its actions in pancreatic β-cells as well as peripheral sites of insulin action.

Taken together, there is very strong evidence that abnormal JNK activation is a critical event in the deterioration of glucose homeostasis and suppression of JNK in diabetic mice. Thus, the JNK pathway plays a central role in the pathogenesis of type 2 diabetes and could be a potential target for diabetes therapy.

Development of JNK pathway inhibitors

In light of the mounting evidence indicating the involvement of JNK signaling in different diseases, it is not surprising that JNK signaling has been of particular interest for drug development.

The complete elimination of JNK genes either alone or in combination has been the initial approach in understanding JNK function. It is very helpful for finding out the specific JNK target for diseases. For example, JNK3 is primarily localized in CNS neurons. Studies from JNK3 knockout mice indicate that JNK3 is an attractive target for neurodegeneration therapy in which neuronal cell death should be prevented[10]. In addition, JNK1 knockout can protect mice from obesity-induced insulin resistance, so JNK1 provides a target for the treatment of type 2 diabetes[48]. However, the "knockout" strategy can not be used in disease therapy because the long-term and complete loss of JNK pathway would cause unwanted side-effects. So many alternative approaches that can partly and temporally inhibit JNK function have been developed in recent years. These approaches include antisense techniques, RNAi techniques, and chemical and peptide inhibitors. Some commonly used inhibitors proved to be efficient for use both *in vitro* and *in vivo*, indicating that the JNK pathway is suitable for therapeutic use.

There are mainly 3 types of inhibitors thoroughly studied up to now. The first is the ATP-competitive inhibitor of the JNK pathway such as CEP-1347 and SP600125. Most of them are small organic compounds. Most drug discovery programs have focused on designing this kind of inhibitors. They occupy the ATP-binding site of the protein kinase, which is structurally similar in all kinases, so the phosphorylation of substrates is blocked. The ATP-competitive inhibition can be specific, with each inhibitor recognizing the specific ATP-binding site of each kinase. Up to now, at least 40 structurally-different small molecules have been described[40]. However, there is also the disadvantage that the efficacy of the ATP-competitive inhibitor can be reduced by the high endogenous levels of ATP. This has been observed in SP600125[57]. In addition to the ATP-binding site, other sites on the kinase can also provide the target for inhibition, so the second kind of inhibitor targets the substrate-binding site. Based on this mechanism, many peptide inhibitors and dominant-negative mutants against kinases have been designed. The non-phosphorylatable substrate analogue provides a good example of this kind. The third kind of inhibitor targets the allosteric regulatory sites. Either peptides or chemical non-peptides can be used if they can bind to the regulatory sites and block the phosphorylation.

CEP-1347 CEP-1347, originally named KT7515 or 3,9 *bis*-[(ethylthio)methyl]-K252a, was initially identified as a derivative of the natural compound K252a. The indolocarbazole K252a has both survival-promoting and neurotrophic effects. Compared to K252a, CEP-1347 is more suitable for therapeutic use because it retains the desirable properties of K252a and minimizes the undesirable effects[58]. For example, the natural compound K252a can inhibit the high affinity nerve growth factor receptor Trk, protein kinase C, myosin light chain kinase and cAMP-dependent protein kinase[58], and calmodulin-activated enzymes[59]. These inhibitory activities greatly limit its use as a neuronal therapeutic agent. CEP-1347 has much lower inhibitory activity towards these kinases.

Studies have shown that CEP-1347 can serve as a neurotrophic molecule to maintain the trophic status of neurons[60]. In addition, CEP-1347 can inhibit the activation of the JNK pathway and thereby prevent neuronal death in both cell culture and animal models. Pretreatment with CEP-1347 prevents β-amyloid-, NGF-withdrawal-, oxidative-stress-, and UV irradiation-induced cell death in PC12 cells and rat sympathetic neurons[11,13]. In the MPTP-model of PD, CEP-1347 can suppress JNK and MKK4 phosphorylation, as well as MPTP-induced cyclooxygenase-2 expression, and protects dopaminergic neurons[61,62]. In another model of PD-intrastriatal injection of 6-hydroxydopamine in rats, the CEP-1347 analogue CEP-11004 also diminishes the apoptotic death of dopaminergic neurons[40]. Taken together, these studies strongly implicate the protective functions of CEP-1347 in animal models of PD.

In addition to PD models, the subcutaneously delivery of CEP-1347 attenuates noise-induced hearing loss and prevents neomycin and aminoglycoside-induced hair cell death in guinea pigs[63,64]. CEP-1347 has also been applied to other disease models that result from JNK activation such as pancreatitis[65] and pulmonary fibrosis[66].

As to the mechanism of inhibition, Maroney *et al* found that the direct biochemical target of CEP-1347 was the MLK family, the upstream kinase activator of JNK in the JNK pathway[67]. By reducing the kinase activity of MLK, CEP-1347 selectively inactivates the JNK signaling pathway, but spares the ERK and p38 MAPK pathways[60].

CEP-1347 has undergone phase II/III clinical trials for neuroprotection in PD. It has been discontinued for lack of clinical efficacy. Although disappointing, we have to keep in mind that the dose of CEP-1347 used in the trials was not high enough in order to avoid potential adverse effects. In addition, although CEP-1347 can be effective in preventing neuronal cell death, it may not improve the symptoms since dopaminergic neurons were lost before treatment in PD patients.

SP600125 The structure of SP600125 (anthrax[1,9-cd] [yrazol-6{2H}-one]) is consistent with other known kinase inhibitors. SP600125 is a reversible ATP-competitive inhibitor of protein kinases. It targets all 3 JNK gene products (JNK1, JNK2, and JNK3) by competing with the ATP-binding site[57]. Since it inhibits JNK MAPK rather than upstream components of the pathway, SP600125 is likely to produce much more specificity of therapy effects than CEP-1347. SP600125 has been shown to be effective in suppressing cell death in the presence of different apoptotic stimuli[18]. In the MPTP model of PD, SP600125 can partially restore dopamine levels[68]. SP600125 has also been demonstrated in animal models of asthma[69]. However, there are also some disadvantages regarding this JNK inhibitor. First, it inhibits all JNK isoforms. The JNK gene knockout studies mentioned earlier have revealed isoform specific roles of JNK, so compounds showing isoform selectivity would be preferred for specific disease therapy. Second, at higher concentrations, SP600125 has more effects than expected since MKK3 and MKK6, and MKK4 and MKK7 are also inhibited[57]. As MKK3 and MKK6 are upstream activators of the p38 MAP kinase signaling pathway, the effects of SP600125 may not be entirely due to the inhibition of JNK signaling especially at higher concentrations. So we have to test the specificity of SP600125 towards other kinases to evaluate its therapeutic use. Third, because JNK is a terminal kinase in the cascade, the inhibition of JNK may produce more general effects compared to upstream kinase inhibition. Finally, SP600125 has very poor water solubility. That is why its application in neurological disorders and other disease models is limited so far.

AS601245 AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl)ethyl] amino]-4 pyrimidinyl) acetonitrile) has been shown to inhibit the JNK signaling pathway similar to SP600125. It inhibits JNK3 with IC_{50} of 70 nmol/L and IC_{50} for JNK1 and JNK2 at 150 and 220 nmol/L, respectively[70]. Thus, AS601245 appears to be a more selective inhibitor of JNK3 at a low dose. AS601245 provides significant protection against the delayed loss of hippocampal CA1 neurons in a gerbil model of transient global ischemia. A significant neuroprotective effect of AS601245 was also observed in rats after focal cerebral ischemia[70,71]. AS601245 has also been shown to decrease cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats[72]. The *in vitro* and *in vivo* anti-inflammatory potential of AS601245 was investigated recently and found to demonstrate efficacy in an experimental model of rheumatoid arthri-

tis[73]. It would be intriguing to investigate whether AS601245 has cytoprotective effects in animal models of neurodegenerative diseases.

Peptide inhibitors of the JNK pathway Peptide inhibitors target the protein/peptide substrate binding sites or regulatory sites of the protein kinases. This provides a more specific inhibition mechanism than ATP-competitive inhibitor. Many non-phosphorylatable substrate analogues have been designed on the basis of substrate-competition.

As to the regulatory site inhibition, a non-substrate JIP has been found in a 2-hybrid screen[21]. As mentioned earlier, JIP act as scaffold proteins to form a multiprotein complex with other JNK apoptotic cascade elements. However, JIP1 overexpression can block JNK activity in mammalian cells[21] and cell death in several physiological models[74]. This is probably because excess scaffold results from overexpression compared to the limited amounts of other JNK pathway elements that disrupt the integrity of each complex.

The D-JNKI-1 peptide, a cell penetrating JNK inhibitor, was constructed by linking the 20 amino acid segment of a JNK-binding domain (JBD) from JIP1 to a 10 amino acid HIV-TAT transporter sequence and by synthesizing a highly protease resistant form of the JBD that doubles its intracellular half-life[53]. It has been demonstrated to prevent loss of hearing following either electrode insertion trauma or loss of both hearing and hair cells following exposure to ototoxic levels of neomycin[75]. D-JNKI also showed a remarkable neuroprotective effect in both the transient and permanent middle cerebral artery occlusion (MCAO) model of stroke[76]. The protection correlated with the prevention of an increase in c-Jun activation and c-Fos transcription. Importantly, DJNKI reduces the infarct volume, even when administered at 6–12 h after transient MCAO. In view of its potency and long therapeutic window, this protease-resistant peptide is a promising neuroprotective agent for stroke.

Conclusions and perspectives

It has been recognized that the JNK pathway plays important roles in many physiological and pathological conditions. Therefore, JNK pathway components represent potential therapeutic targets for the treatment of related diseases, including neurodegenerative diseases, cancer, diabetes, hearing loss, heart diseases, inflammatory diseases, and autoimmune diseases. Several kinds of inhibitors have been developed, targeting different levels of the JNK signaling cascade and some of them, such as CEP-1347 and SP600125, have been used in clinical trials. Although there are still some problems limiting the use of these inhibitors such as the substrate specificity, the crosstalk between the

JNK and other signaling pathway, and potential side effects, including the possibility to induce tumor formation, we are hopeful that effective drugs targeting the JNK pathway will be developed for relevant diseases in the near future. To reach this goal, further efforts are required to learn more about the physiological roles of the JNK pathway components while attention should be paid to develop more specific inhibitors. In addition, long-term clinical studies are needed to observe whether JNK pathway inhibitors would lead to tumor formation and tumor metastasis in some circumstances.

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