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Review Article

Inhibitors of the JNK signaling pathway

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Introduction

Exposure of eukaryotic cells to various stresses causes activation of the jun-*N*-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway and p38 mitogen-activated protein kinase (MAPK). These two pathways are often activated in parallel, although the upstream activators of each pathway are different. In this review, we will focus on inhibitors that affect the JNK pathway, although many compounds also inhibit the p38 pathway.

Ultraviolet (UV) irradiation, osmotic stress, certain cytokines and mitogens activate a pathway involving small GTP-binding proteins and a cascade of protein kinases (see Figure 1 for overview of JNK/SAPK pathways). The upstream pathway prior to JNK activation is complex and offers many targets at which to intervene and inhibit the pathway. Evidence is accumulating to suggest that inhibiting this pathway may be beneficial in a number of disease states such as neurodegeneration and cardiac hypertrophy. Here, we will attempt to review endogenous inhibitors of this pathway, such as growth factors, JNK-interacting proteins and heat shock protein 72 (hsp72). In addition, we will review the current published and patent literature on chemical inhibitors of the pathway, such as CEP-1347, an MLK (mixed-lineage kinase) inhibitor, SB-203580 and SB-202190, combined p38 and JNK inhibitors, and SPC0009766, a recently reported JNK inhibitor from Signal Pharmaceuticals.

There are many different routes to the JNK pathway. One of the first and most well characterized is the pathway that is initiated by the TNF-α receptor (1, 2). From there, signal transduction proceeds through germinal center kinase (GCK), the MLKs, the MKKs (MAP kinase kinases; *e.g.,* MKK4 and MKK7), and finally to JNK. An alternative signal transduction cascade, upstream from JNK, is represented by a pathway that includes Rac1/2, Cdc42, MEKK1 and MKK4/MKK7 (Fig. 1). Thus, any one of these molecules can be thought of as a potential molecular target for the inhibition of JNK signaling. Downstream of JNK, the transcription factors c-jun, ATF2 and other members of the Jun family that are components of the AP-1 transcription factor complex are activated, leading to changes in gene expression (3-6).

The role of c-jun in neuronal apoptosis

Inhibiting the JNK pathway at the level of c-jun has proven to be advantageous in preventing neuronal apoptosis. For example, Ham *et al.* (7) showed that a dominant negative mutant of c-jun protects rat sympathetic neurons against programmed cell death induced by nerve growth factor (NGF) withdrawal. Likewise, rat cerebellar granule neurons deprived of serum and 25 mM KCl were also protected by a c-jun dominant negative mutant (8). A different experimental approach was utilized by Estus *et al.* (9), where they demonstrated that polyclonal neutralizing antibodies against c-jun could be used to show that c-jun

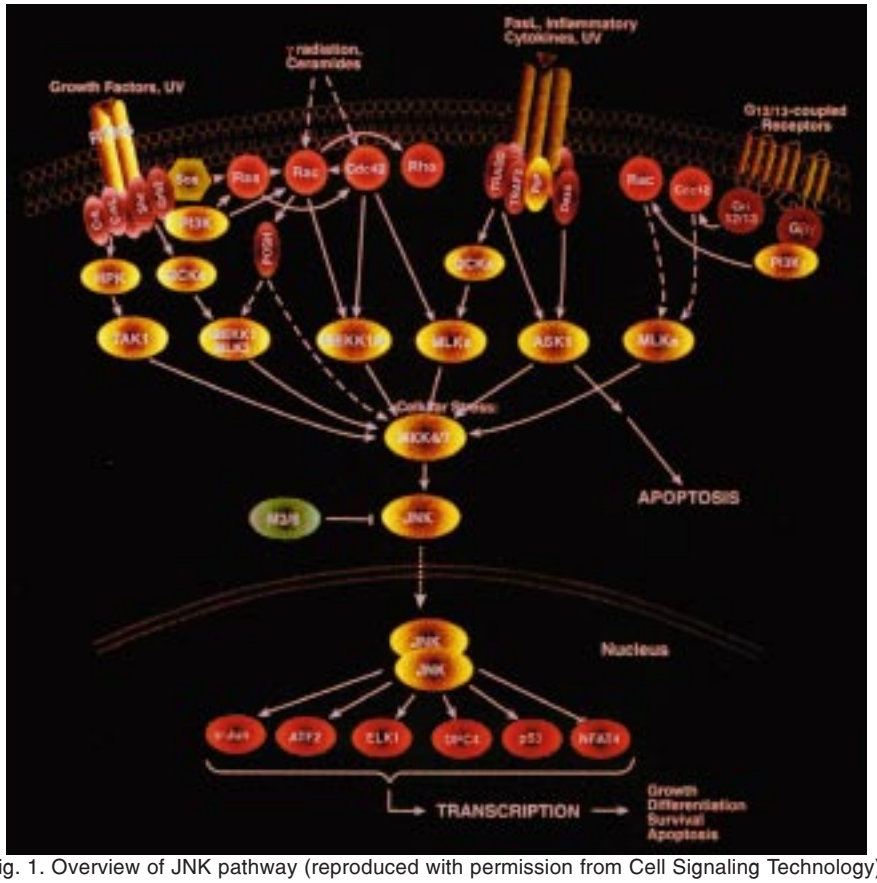

Fig. 1. Overview of JNK pathway (reproduced with permission from Cell Signaling Technology).

was necessary for neuronal apoptosis of rat sympathetic neurons. A third approach illustrating the importance of c-jun in neuronal survival was the use of c-jun phosphorylation site mutants. In those studies, Ser63 and Ser73 were mutated to alanine and transfected into rat cerebellar granule neurons. The authors reasoned that the Ala mutations prevented c-jun phosphorylation and apoptosis induced by KCl and serum withdrawal. In contrast, a constitutively active c-jun mutant, where Ser63 and Ser73 were both mutated to Asp, was transfected into cerebellar granule neurons and was found to be susceptible to apoptosis even in the presence of serum and KCl (8). Further proof of the validation of c-jun phosphorylation and stress-induced apoptosis comes from mice that have Ser63 and Ser73 mutated to Ala (10). The authors show that c-jun AA mutant mice are resistant to seizures and neuronal apoptosis induced by kainic acid. Taken together, all these data present a strong case for a critical role for c-jun and activation of the JNK pathway in neuronal apoptosis.

A role for JNK in cardiac hypertrophy and myocardial infarction

In addition to the extensive evidence supporting a role for JNK in neuronal cell death, there is now growing evidence that JNKs are involved in cardiac hypertrophy, both *in vitro* and *in vivo*. In rat ventricular myocytes, stimulation of the G-protein-linked α_1-adrenoceptor is associated with a hypertrophic response characterized by transcriptional activation of a number of genes, leading to expression of atrial natriuretic factor (ANF). Ras has been shown to be active in this signal cascade. Ramirez *et al.* (11) have shown that stimulation of α_1-adrenoceptors and Ras activates JNK in cardiomyocytes. Using ANF as a marker, they were able to show that dominant negative MEKK1 inhibited ANF expression induced by phenylephrine, while a constitutively active MEKK1 activated JNK and ANF reporter gene expression. Other workers have shown that dominant negative SEK1 was able to inhibit the hypertrophy and expression of ANF induced by endothelin-1 (12) by inhibiting the JNK pathway. These data suggest that the JNK pathway is necessary for agonist-induced cardiomyocyte hypertrophy. Interestingly, *in vitro*, activation of both JNK and p38 pathways, by coexpressing constitutively active MKK6 and MKK7, caused cell death (13). Furthermore, gene transfer of the dominant negative SEK1 *in vivo* to adult rat heart also prevented JNK activation and pressure overload-induced cardiac hypertrophy (14). Induction of myocardial infarction by coronary artery ligation caused JNK activation in both infarcted areas and in noninfarcted areas (15). These authors propose that JNK may be involved in

ventricular remodeling after myocardial infarction. Activation of JNK *in vivo* could be inhibited by treatment with an ACE (angiotensin-converting enzyme) inhibitor (temocapril) or an angiotensin receptor blocker (candesartan cilexitil). Angiotensin-converting enzyme inhibitors prevent ventricular dilation, dysfunction and cardiac hypertrophy by suppressing increases in the expression of ANF and other cytoskeletal proteins and, evidently, indirectly inhibit the JNK pathway. All of these data suggest that small-molecule inhibitors of the JNK pathway could have clinical use in the treatment of cardiac disorders.

The effect of JNK knockouts on the JNK signaling pathway

Of the three JNK isoforms, JNK1 and JNK2 are ubiquitously expressed, while JNK3 is restricted to neuronal tissues (16). Like the c-jun mutant mice, knockout studies for *jnk* have yielded important information on the function of JNK isoforms and the consequent JNK pathway inhibition that is manifested from these knockouts. Disruption of the gene encoding JNK3 in mice resulted in reduced seizure activity and severity upon treatment with kainic acid, as well as prevention of neuronal apoptosis as measured by Nissl and TUNEL staining (17). This result is a strong indication that the JNK3-mediated signaling pathway is an important component of neuronal apoptosis and survival, and implicates JNK3 as a potential target for small-molecule inhibition. It should be noted that the *jnk3* knockout mice were of normal size, fertile and showed no reduction in a number of neuronal markers as compared to wild-type animals, further emphasizing JNK3 as a potential drug target.

Like *jnk3*, *jnk1* and *jnk2* knockout mice were viable and showed no gross differences from wild-type animals. *jnk1*-deficient mice were of normal size and fertile, showing normal lymphocyte development with typical ratios of T-cells to B-cells, CD4 to CD8 cells, and naïve to memory cells in the periphery (18). Likewise, *jnk2*-deficient mice showed normal T- and B-cell development and no abnormalities were detected histologically in brain, liver or bone (19). Moreover, *jnk1/jnk3* and *jnk2/jnk3* double mutants all survived normally, whereas *jnk1/jnk2* compound mutants died at embryonic days E11-12, showing severe abnormalities in the brain (20, 21). Interestingly, the hindbrains of the *jnk1/jnk2* compound mutants showed a reduction in cell death, whereas the forebrain of these animals showed a 10-fold increase in the number of TUNEL-positive cells compared to wild-type animals. These results suggest that JNK1 and JNK2 regulate regiospecific apoptosis during early brain development (20). Tournier *et al.* (22) have generated mouse embryonic fibroblasts (MEF) from the JNK1/JNK2-null mice. These cells completely lack a functional JNK signaling pathway. The cells were resistant to UV-induced cell death, methylmethane sulfonate (MMS)-induced cell death and anisomycin-induced cell death. However, Fas-induced cell death was unaffected. Studies in these cells have also shown that JNK activation was required for cytochrome *c* release. However, microinjection of cytochrome *c* into MEF cells that lack JNK1 and JNK2 did induce death in the cells. This study shows the importance of JNK in the cell death pathway and links JNK with cytochrome *c* release, a pivotal point in the cell death cascade. The collective knockout data for the three JNK isoforms suggest that JNK3 may mediate neuronal responses to stress, whereas JNK1 and JNK2 play developmental roles in the nascent CNS, as well as impact on T-cell differentiation and apoptosis, but not normal lymphocyte development (19, 20, 23). Thus, it is interesting to speculate that any small-molecule inhibitors for the treatment of neuronal apoptosis may have to be JNK3-selective in order to protect against potential deleterious T-cell effects that JNK1 and/or JNK2 inhibition may cause.

Growth factors that inhibit JNK signaling

There is substantial evidence that growth or trophic factors, which maintain cells, do so at least in part by inhibiting “death pathways” such as JNK and p38. Evidence has shown that NGF inhibits activation of both JNK and p38 in PC12 cells (24) and JNK in sympathetic neurons (25, 26). Cheng *et al.* (27) report that JNK1 and JNK2 are activated in Schwann cells that have been deprived of serum, and that inhibition of this activation using dominant negative strategies promotes survival. Treatment of the Schwann cells with either a caspase 3 inhibitor (AcDEVD-Cho) or insulin-like growth factor-I (IGF-I) inhibits the death associated with serum deprivation. IGF-I has also been shown to inhibit JNK activation in neuroblastoma cells (28) exposed to high glucose, and cerebellar granule neurons subjected to potassium and serum deprivation (29). Vascular endothelial growth factor (VEGF), an endothelial cell mitogen which promotes endothelial cell survival and angiogenesis, inhibits the sustained activation of JNK caused by serum deprivation in human dermal microvascular endothelial cells (30). Thus, it is possible that activation of antiapoptotic pathways such as the ERK pathway in response to growth factors may inhibit the activation of the stress-activated JNK and p38 pathways. When the growth factor is removed, inhibition of the activation of JNK is attenuated and apoptosis occurs. In keeping with this hypothesis, Levresse *et al.* (31) have shown that in PC12 cells, phosphatidylinositol 3-kinase (PI3K) inhibitors cause activation of JNK and that IGF-I inhibits this activation (via PI3K and Akt), suggesting that growth factor signaling via PI3K and Akt chronically inhibits JNK activation. Further experiments were carried out utilizing a gain-of-function myristoylated form of Akt that was expressed using a retrovirus. JNK activation in cells expressing this myristoylated form of Akt was inhibited in response to UV or serum deprivation, suggesting that activation of Akt is sufficient to inhibit JNK activation. Recent data from Desbois (32) and Byon (33) suggested that MAPK phosphatase

(MKP1) may be involved in the inhibition of JNK signaling by insulin in CHO cells overexpressing insulin receptors.

Two naturally occurring neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), have been shown to be inhibitors of the JNK pathway. Both VIP and PACAP have been shown to inhibit lipopolysaccharide (LPS)-stimulated JNK signaling by inhibiting MEKK1 activity through a cAMP-dependent mechanism (34). In contrast to VIP and PACAP, a 20-amino-acid inhibitory domain of islet brain 1 (IB-1) and islet brain 2 (IB-2) proteins fused to the 10-amino-acid HIV TAT sequence to make them cell-permeable was shown to be a direct inhibitor of JNK. At a concentration of 25 μM, these peptides were shown to inhibit c-jun phosphorylation by JNK1 *in vitro,* as well as reducing IL-1β-induced apoptosis of βTC-3 cells (35). Thus, it appears that a variety of growth factors are able to inhibit the JNK pathway.

The role of the Bcl family in modulating JNK activity

The Bcl-2 family forms a crucial part of the cell death pathway. Recent evidence has shown that Bcl family members may be in the same pathway as JNK. The Bcl family of proteins contains both proapoptotic (Bad and Bax) and antiapoptotic (Bcl-2 and Bcl-x_L) (36, 37) proteins. Bcl-2 and Bcl-x_L are reported to prevent the release of cytochrome *c* from the mitochondria. The Bcl-x_L structure is similar to pore-forming domains of bacterial toxins. The Bcl-x_L channel is selective for monovalent cations, and thus, the proapoptotic Bcl family members could form a cytochrome *c*-conducting channel.

Several groups have now shown an association between JNK and the antiapoptotic members of the Bcl family Bcl-2 and Bcl-x_L. In addition, overexpression of these proteins in some systems resulted in inhibition of the activation of JNK. Pandey *et al.* (38) showed that JNK activation and cell death in U-937 cells treated with MMS, a monofunctional alkylating agent, could be inhibited by overexpression of Bcl-x_L. However, UV-induced cell death and JNK activation were not affected by Bcl-x_L expression. Treatment with MMS was associated with an increase in phosphorylation of related adhesion focal tyrosine kinase (RAFTK)/proline-rich tyrosine kinase-2 (PYK2), an upstream effector of JNK, and this was inhibited by overexpression of Bcl-x_L, suggesting that, in this case, Bcl-x_L was acting upstream of both JNK and PYK2. Vinblastine, another potent anticancer agent that inhibits microtubule formation, caused activation of JNK and phosphorylation, and therefore activation of Bcl-2 and Bcl-x_L, in human KB3 carcinoma cells. Inhibition of the expression of JNK1 and JNK2 with antisense oligonucleotides resulted in inhibition of vinblastine-induced phosphorylation of Bcl-x_L and Bcl-2 (39). Cheng *et al.* reported (27) that overexpression of Bcl-x_L in Schwann cell cultures inhibited activation of JNK following serum deprivation, in turn leading to increased survival. Similarly, in 293 cells, treatment with anticarcinogenic isothiocyanates caused activation of JNK, which could be inhibited by overexpression of either Bcl-2 or Bcl-x_L, again suggesting that Bcl family members are upstream of JNK (40). In U-937 cells, ionizing radiation caused a translocation of JNK to the mitochondria, where it interacts with Bcl-x_L (41). JNK phosphorylates Bcl-x_L at Thr47 and Thr115. Mutation of these residues, such that phosphorylation cannot take place, results in a more potent inhibition of radiation-induced apoptosis, implying that phosphorylation of Bcl-x_L is a critical step in signaling through the JNK pathway. Manna *et al.* (42) reported that in human promyelocytic lymphoma HL-60 cells, overexpression of Bcl-x_L inhibits TNF signaling at an early stage, downstream of the receptor. NF-κB, AP-1, MAPK and JNK were all inhibited by overexpression of Bcl-x_L, consistent with a general antiapoptotic effect of Bcl-x_L.

Bcl-2 overexpression in N18TG neuroglioma cells suppresses both cell death and JNK activation caused by etoposide, staurosporine, UV irradiation and anisomycin (43). Bcl-2 expression also inhibited activation of MEKK1, suggesting that Bcl-2 acts upstream of JNK and MEKK1. In this study, overexpression of JNK1 in Bcl-2-expressing cells was able to counteract the antiapoptotic effects of expression of Bcl-2. In PC12 cells, JNK activation caused by NGF withdrawal can be blocked by expression of Bcl-2 (44). Expression of Bcl-2 supports PC12 cells in the absence of trophic support. NGF deprivation in PC12 cells leads to activation of JNK. This activation can be inhibited by Bcl-2 expression, but not by zVADfmk, in spite of the fact that zVADfmk does promote survival in these cells. These data suggest that caspase activation in the course of cell death is either downstream of or parallel with JNK activation. In sympathetic neurons, Bcl family proteins act downstream of c-jun, with overexpression of Bcl-2 having no effect on c-jun levels, but inhibiting cell death due to NGF withdrawal (7). In cortical neurons treated with sodium arsenite to induce JNK activation and cell death, expression of either Bcl-2 or Bcl-x_L inhibits activation of JNK and cell death (45). The mechanism by which Bcl-2 promotes survival is still unclear, but it is now known to have a critical role in apoptosome formation and is involved in preventing the release of apoptogenic molecules from the mitochondria (37). All of these studies show that the Bcl family members are important participants in the cell death pathway and survival-promoting effects of individual members may be via inhibition of JNK.

Caspases as modulators of JNK activity

Overexpression of DED (death effector domain) caspases activates JNK via an interaction with TRAF (TNFR-associated factor) and subsequent activation of MEKK1, and suggests that caspase inhibitors may prevent JNK activation in certain circumstances (46). The latter work provided evidence that DED-containing proteins activated the JNK pathway by interacting with TRAF. However, two inhibitors of caspases and apoptosis, crmA and p35, did

not inhibit the activation of JNK by the prodomain of caspase 8. Overexpression of cFLIP in 293T cells caused apoptosis but failed to produce caspase activity (47). However, Chaudhary (46) found that crmA blocked cFLIP-induced JNK activation, suggesting JNK as an alternative pathway for death. Varfolomeev looked at JNK activation in fibroblast cells derived from caspase 8-null mice (48) and found that treatment of wild-type cells with TNF-α resulted in a peak activation of JNK, followed by sustained activation. In cells derived from the caspase 8-null mice, this sustained activation of JNK was not present, suggesting that caspase 8 plays a role in sustaining JNK activation. A further study of a Jurkat cell line deficient in caspase 8 showed the cells to be resistant to Fas-induced cell death due to an inability to activate either JNK or p38 pathways (49). Thus, it would appear that modulating caspase activity in certain circumstances impacts on JNK activity.

Heat shock proteins as regulators of JNK signaling

The heat shock proteins (hsps) function as chaperones and regulate protein translocation into organelles (50-53). The best-characterized hsps are those with a molecular weight of about 60, 70, 90 and 110 kDa. These major hsps are expressed at low levels in the absence of heat shock. The major function of hsp70 is as a chaperone in unstressed cells. Hsp70s carry newly formed unfolded proteins to members of the hsp60 family of chaperones for folding. Hsp70 also carries newly folded proteins for translocation to different organelles.

The two isoforms of hsp70 found in both cytoplasm and nucleus are known as hsp73 and hsp72. Hsp73 is synthesized constitutively in all mammalian cells, and is also known as hsp70 cognate, or hsc70. The hsp70 isoform that is only expressed in cells subject to stress is known as hsp72 (inducible). The two proteins share > 95% homology at the nucleotide level (54, 55). In response to cellular stresses, hsp72 prevents protein aggregation and induces refolding of damaged proteins.

Mild heat shock (stress tolerance) protects cells from severe heat shock and other noxious stimuli, such as hypoxia, ischemia and sepsis, *in vivo* and a wide range of stresses *in vitro* (50, 56), and these protective effects are mediated by hsp72. These reports have been substantiated by data showing that inhibition of hsp72 diminished cell survival (57). Mild heat shock activated JNK transiently at a low level, insufficient to elicit apoptosis but sufficient to induce the accumulation of hsp72 (58). After a recovery period, the accumulated hsp72 protected cells from further stress. Prolonged activation of JNK is required for apoptosis (59, 60), and therefore, the protective effects of expression of hsp72 may be due solely to effects on JNK activation.

Pretreatment of NIH3T3 cells with a short period of heat shock suppressed UV-stimulated JNK1 activity (61). Constitutive expression of hsp72 also inhibited activation of JNK1 and JNK-dependent apoptosis. The authors propose that hsp72 may, through binding to JNK, prevent the interaction between JNK and SEK1 and therefore the phosphorylation of JNK by SEK1. Volloch *et al.* (62) proposed that the magnitude of JNK activation was not as important as the duration. Constitutive expression of hsp72 in Rat-1 fibroblasts had no effect on the initial activation of JNK, but did reduce the duration of activity. This is consistent with the fact that brief activation of JNK does not cause cell death, but sustained phosphorylation of JNK results in activation of cell death pathways.

It has been proposed that the ability to express hsp72 diminishes with age and this could result in a higher rate of apoptosis in response to stress in older cells (63). Aged primary human fibroblasts, which have passed 62-67 population doublings, were unable to induce hsp72 following mild heat shock. Subjecting these cells to severe heat shock then induced strong activation of JNK and massive apoptosis (64). Younger cells accumulated hsp72 following mild heat shock. This led to suppression of JNK following severe heat shock and reduced the extent of apoptosis. Forced expression of hsp72 in aged cells suppressed JNK expression following severe heat shock and protected against apoptosis. This implicates hsp72 in susceptibility to apoptosis and suggests a possible role in aging. Gabai *et al.* (65) reported that in primary human fibroblasts, exposure to mild heat shock caused accumulation of hsp72 and a transient JNK activation. More severe heat shock caused long-lasting JNK activation and cell death. In addition, JNK activation in response to a variety of stresses, including IL-1, TNF or UV radiation, could be suppressed by heat shock and hsp72 was responsible for this. Similarly, myocardial cells subjected to transient energy deprivation, a model of myocardial infarction, exhibited cell death that had features of both necrosis and apoptosis and was independent of caspase activation (66). In these cells, inhibition of JNK using a dominant negative construct increased cell survival, whereas inhibition of p38 had no effect. These authors report that hsp72 inhibited activation of JNK via dephosphorylation of JNK itself rather than by inhibition of the upstream kinase pathway (67). To determine which parts of the hsp molecule are required for this effect on JNK activation, Yaglom *et al.* (68) deleted the ATP-binding domain critical for protein folding. This mutated hsp72 was still able to prevent activation of JNK by UV and IL-1 in fibroblasts and COS-7 cells, indicating that this effect of hsp72 was independent of its role in protein folding. All these data suggest that hsp72 plays a crucial role in regulating JNK activity in response to stress and that expression of hsp72 may determine whether a cell survives the stressful insult.

Ras family members as modulators of JNK activity

The Ras superfamily of small GTP-binding proteins have important regulatory roles in mitogenesis, cytoskeletal rearrangement, transcytosis and motility. The family is divided according to sequence homology into five

classes: Ras, Rab, Arf, Ran and Rho. The Ras family members (H-Ras, K-Ras, N-Ras, R-Ras, TC21, Rap1A/Rap1B and Rap2A/Rap2B) all play important roles in cell growth and development. The Rho family members (cdc42/G25K, Rac1, Rac2, RhoA, RhoB and RhoC) play a role in the regulation of the actin cytoskeleton (for review see 69-71). When bound to GTP, these proteins transduce signals to effector proteins, and when bound to GDP, they are inactive. These GTP-binding proteins are also subject to regulation. Guanine nucleotide dissociation stimulators (GDSs) catalyze the dissociation of GDP and thus favor the GTP, or active, form. Conversely, GTPase-activating proteins (GAPs) stimulate GTP hydrolytic activity and favor the formation of the GDP-bound inactive state.

Minden *et al.* (72) reported that expression of constitutively active forms of cdc42 or Rac caused activation of JNK in HeLa or NIH3T3 cells. Conversely, transfection of cells with RhoGAP, which inactivated all Rho family members, or dominant negative Rac1 inhibited activation of JNK by cytokines (73). The human T-cell lymphotrophic virus type I (HTLV-I) oncoprotein Tax can activate JNK. Jin *et al.* reported (74) a novel G-protein pathway suppressor (GSP2) that bound to Tax and inhibited the activation of JNK. This protein also inhibited TNF-α-induced activation of JNK. GSP2 had little effect on MEKK1, suggesting that it acts in the pathway between the TNF-α receptor and MEKK1. These data suggest that it is possible to inhibit the JNK pathway at the level of GTP-binding proteins.

Ste20 family as regulators of the JNK pathway

The Ste20 family of kinases are all related to the budding yeast Ste20p kinase (sterile20 protein). There are approximately 30 mammalian homologs, including the GCKs and p21-activated kinases (PAKs). These kinases activate the MAPK cascade and have effects on the regulation of apoptosis and cytoskeletal rearrangement, leading to changes in cell shape and motility (75). PAK2 activates Raf1 in the MAPK pathway and PAK1 phosphorylates MEK1. HPK1 (hematopoietic progenitor kinase 1), another member of this family, phosphorylates both MEKK1 and MLK3 (76, 77), thereby activating the JNK pathway. Overexpression of HPK1 activates the pathway. Expression of dominant negative forms of MEKK1, MKK4 and MLK3 inhibits the activation caused by overexpression of HPK1, placing HPK1 upstream of the other kinases. Another member of the GCK family, prostate-derived Ste20-like kinase (PSK), also phosphorylates MKK4 and MKK7 in the JNK pathway (78). There are a few reports of inhibitors of these kinases that affect the JNK pathway. Tassi *et al.* (79) reported the discovery of human JIK (JNK-inhibitory kinase), a member of the GCK-like subfamily, a 110-kDa protein which is ubiquitously expressed in human tissue. When expressed in COS-7 cells, it possessed serine threonine kinase activity. The authors were unable to find any stressful stimuli that increased the activity of JIK, although epidermal growth factor (EGF) receptor activation caused a decrease in JIK activity. Expression of JIK in COS-7 cells reduced basal JNK activity fourfold, and the stimulatory effect of EGF was partially inhibited by JIK. The authors were not able to determine where JIK was acting in the pathway and were unable to find evidence for a direct interaction between JNK and JIK. Shim *et al.* (80) reported that $p^{21WAF1/CIP1/Sdi1}$, a DNA damage-inducible cell cycle inhibitor, was able to inhibit the JNK pathway. The HSV gene product US3, a serine threonine kinase that shows homology to PAK, was activated by cdc42 or Rac. When this protein was expressed in HEP2 cells, it caused suppression of JNK activation (81), suggesting that the viral US3 gene product could act as an upstream inhibitor of JNK signal transduction. These data taken together suggest that certain members of the Ste20 family activate the JNK pathway and that endogenous or viral inhibitors are able to attenuate this activation.

Antioxidants as modulators of JNK activity

Treatment of cells with IL-1 or TNF-α results in the production of reactive oxygen species (ROS), which include superoxide, nitric oxide, hydrogen peroxide and hydroxyl radicals and are highly reactive, diffusible molecules known to be signaling intermediaries. They have been implicated in a number of diseases including Parkinson's disease and amyotrophic lateral sclerosis. As such, there has been much interest in antioxidants as neuroprotective agents. It has long been known that antioxidants protect against cell death, but it has only recently become apparent that they act at least in part via inhibition of the JNK pathway.

Treatment of bovine chondrocytes with cytokines leads to the production of ROS and activation of JNK and c-jun. Antioxidants such as *N*-acetylcysteine and ascorbic acid did not attenuate these effects (82). Conversely, treatment of the cells with H_2O_2 caused activation of JNK (83) and implied that ROS are important signaling intermediaries in the JNK pathway. DPI (diphenyleneiodonium), a potent inhibitor of flavenoid-containing enzymes, also inhibited JNK signaling, suggesting that enzymes such as NADPH oxidase and nitric oxide synthase (NOS) are important. Another report documented that *N*-acetylcysteine and 2-mercaptoethanol inhibited isothiocyanate-induced JNK activation (40). Thus, activation of JNK in several different cells and in response to different stressors can be attenuated with antioxidants.

The glutathione *S*-transferase (GST) family comprises a multigene family of which GST Pi (GSTp) is the most prevalent nonhepatic isozyme. Reduced glutathione binds to the "G" site of GSTp and plays an important role in detoxification of ROS and maintenance of the cellular redox state (84). GSTp has recently been identified as a JNK inhibitor by Adler *et al.* (85). The authors propose that GSTp may play an important role in maintaining low basal JNK and c-jun activity. The model proposed

suggested that JNK and c-jun were complexed to GSTp in nonstressed cells, preventing activation of the JNK pathway. In stressed cells, GSTp becomes oligomerized and dissociates from JNK and c-jun, allowing activation of these kinases. The model was strengthened by data from GSTp-null mice (86). MEF cells cultured from these animals have higher basal JNK activity than those from wild-type animals. This increased activity can be reduced by transfection of the cells with GSTp. Overexpression of GSTp was associated with tumorigenicity (84). Thus, cancer cells with high levels of expression of GSTp may avoid apoptosis by inhibiting the JNK pathway. Similarly, Wilhelm *et al.* (87) have reported that JNK activation following treatment of cells with monofunctional alkylating agents depended on levels of intracellular glutathione. Pretreatment of cells with GSH or *N*-acetylcysteine inhibited JNK activation, while depletion of intracellular GSH caused hyperinduction of JNK/SAPK activity.

Overexpression of apoptosis signal-regulating kinase 1 (ASK1) induces cell death with features of apoptosis (88, 89). Thioredoxin is a 12-kDa protein that is ubiquitously expressed and has a variety of roles related to cell proliferation and apoptosis (90). Saitoh *et al.* (91) reported that this protein is a physiological inhibitor of ASK1, binding in the *N*-terminal portion of the protein. Thioredoxin inhibited the kinase activity of ASK1 and also inhibited ASK1-dependent apoptosis. The authors proposed a model in which thioredoxin is bound to ASK1 in unstressed cells, inhibiting its activation. When cells are stressed, thioredoxin becomes oxidized and dissociates from ASK1, causing activation of the kinase. This model suggests a way in which intracellular redox status can lead to apoptosis through the JNK pathway, and how antioxidants may be protective.

Modulating JNK activity via a phosphatase

Recent reports suggest that JNK activation can be modified either via activating the upstream kinase cascade or by inhibiting a JNK phosphatase. Osmotic stress, anisomycin and UV radiation all cause activation of the kinase cascade. Heat shock, oxidative stress and ethanol may activate JNK through an alternative pathway. Arsenite, a tumor promoter, activates JNK through inhibition of a constitutively active JNK phosphatase (92). Recent reports on the effect of sodium arsenite on cortical neurons indicated that both JNK3 and p38 were activated upon treatment, whereas JNK1 and JNK2 appeared to play no role in this model (45). CEP-1347 (see below) was able to prevent the activation of c-jun downstream of JNK, suggesting that, at least in these neurons, arsenite does activate the kinase pathway, although it is possible that both inhibition of a phosphatase and activation of a kinase are taking place simultaneously. To date, however, the phosphatase that is responsible for dephosphorylating JNK has not been identified. When it is elucidated, however, it may represent another target for inhibiting the pathway.

Proteosome inhibitors as modulators of JNK activity

Inhibition of the major cytosolic proteolytic complex, the proteosome, is reported in some cells to induce apoptosis (93-96), while in other cells it is reported to protect against apoptosis (97, 98). Meriin *et al.* have studied the effect of a potent proteosome inhibitor, MG-132 (benzoyl-carbonyl-leucinyl-leucinyl-leucinal), on the human leukocyte cell line U-937 (99). Prolonged exposure of cells to MG-132 caused apoptosis, which was correlated with JNK activity, suggesting that MG-132 causes apoptosis via activation of JNK. Indeed, inhibiting JNK activation using dominant negative SEK1 suppressed the apoptosis caused by MG-132, confirming the importance of JNK activation in mediating the effects of MG-132. In the same U-937 cells, however, transient exposure to MG-132 followed by a period of withdrawal activated JNK only transiently, and was insufficient to induce apoptosis. During the recovery period, however, there was an accumulation of hsp72. Under these conditions, MG-132 was able to protect cells from severe heat shock via suppression of JNK activity. Thus, it seems likely that, in this instance, MG-132 modulation of JNK activity is via heat shock proteins.

Other pathway inhibitors that impact on JNK signaling

Park *et al.* have examined the effects of a variety of survival-promoting chemicals for effects on JNK activation (44). In naïve PC12 cells deprived of serum or differentiated PC12 cells deprived of NGF, JNK is activated. Diethylenetriamine nitric oxide (DETA.NO) a nitric oxide (NO) donor, aurin tricarboxylic acid (ATA), *N*-acetylcysteine (NAC), 8-bromo-cGMP and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) all attenuate JNK activity in PC12 cells deprived of serum or NGF. Endogenously produced NO inhibits JNK activation in murine microglial cells (100). The broad-spectrum caspase inhibitor zVADfmk does not inhibit JNK activation in this model, suggesting that caspase activation is either downstream of or parallel to JNK activation. All the above compounds that inhibit JNK activation are likely to be affecting different parts of the JNK signaling pathway. For example, ATA is known to stimulate PI3K and phospholipase C (PLC) activity and to activate the ERK pathway in PC12 cells (101). *N*-Acetylcysteine is known to increase phosphorylation of ERK (102) and this is likely to be its mechanism of action in these experiments. CPT-cAMP exerts its neuroprotective effects via activation of protein kinase A (PKA) (103). Nitric oxide donors and 8-bromo-cGMP appear to promote survival via a mechanism that involves elevation of intracellular cGMP. It appears that all of the above compounds modulate JNK activity upstream and via PKA and cGMP. These data again suggest that there is a balance between positive and negative signaling that determines whether a cell activates a stress-induced pathway and goes on to die, or whether signaling through

a "positive" growth factor-stimulated pathway overrides this and allows the cell to survive.

JNK-interacting proteins

Another level of control in the JNK signaling pathway is provided by the JNK-interacting proteins (JIPs). Murine JIP-1 was identified by Dickens and colleagues in 1997 (104) as a cytoplasmic protein that bound to JNK and regulated its activity. JIP-2 was identified shortly after as a member of the same family (105), and JSAP1 (JNK/SAPK-associated protein-1), or JIP-3, soon thereafter (106-108). These are scaffold proteins which mediate signal transduction through MLK, MKK and JNK pathways, perhaps by sequestering all of the necessary kinases (109). JIP-1 contains a phosphotyrosine-binding domain (PTB), as well as a JNK-binding domain and an src homology 3 domain (SH3) (110). Coexpression of JIP-1 with components of the JNK pathway enhances the activation of JNK (109). However, overexpression of JIP-1 inhibits JNK signal transduction, possibly by sequestering the components of the pathway into separate complexes.

JIP-1 mRNA is widely expressed in murine tissues, including testes, kidney and lung, and particularly brain (104). JIP-2 and JIP-1 mRNAs are both highly expressed throughout the nervous system and low levels of JIP-2 are seen in a limited number of other tissues (105). Recently, rat and human homologs of JIP-1 have been identified and termed islet brain 1 (IB-1) (111). Rat and human IB-1 are 97% identical to mouse JIP-1, with an insertion of 47 amino acids forming a putative helix loop helix and a phosphotyrosine-interaction domain. IB-1 expression is localized to the synaptic region of the olfactory bulb, hippocampus, cortex, cerebellar cortex and retina in the adult mouse (111). Expression levels peak at around two weeks after birth. The neuronal expression and synaptic localization suggest that IB-1 may be involved in cell signaling both during development and in the adult.

JSAP-1/JIP-3 is expressed in all cell bodies and axons of neurons in both the peripheral and central nervous system during embryonic development of the mouse, but its expression is limited to post-mitotic neurons (112). In the adult CNS, JSAP-1 is expressed in all neurons but restricted to cell bodies.

Kim *et al.* (113) have also reported novel splice variants of JIP-1 which have a putative phosphotyrosine-binding domain, a helix loop helix domain and an SH3 homology region in the *C*-terminus. These isoforms have been termed mJIP-1a (JIP-1), mJIP-1b, mJIP-2a, mJIP-2b and mJIP-3 in the mouse and rJIP-1b, rJIP-1c and rJIP-2a in the rat. These authors also report high levels of mRNA expression of all isoforms in the brain (hippocampus, cortex and cerebellum), and expression of JIP-1 in the kidney as well. JIP-1 expression has been reported in a number of neuronal cell lines. In the human neuroblastoma cell line SK-N-F1, a JIP-1 isoform consistent with the IB-1 form, migrating at 115 kDa, can be detected (110). Naïve PC12 cells do not express JIP-1, but upon differentiation with NGF, two proteins can be detected: one of 115 kDa and an additional protein of 180 kDa. In the mouse neuroblastoma cell line N1E-115, JIP-1 proteins are expressed in both naïve and differentiated cells, with the differentiated cells also expressing a phosphorylated form (110).

Accumulating experimental evidence indicates that overexpression of JIPs prevents activation of both JNKs and cell death. In neuroglioma cells (N18TG), overexpression of JIP-2a suppresses both JNK activation and cell death caused by etoposide (113). *In vivo* in rats subjected to transient middle cerebral artery occlusion, both phosphorylated JNK and JIP-1 are expressed at eight hours after reperfusion, suggesting a possible attempt by the cells to avoid cell death (114). Gillardon *et al.* (115) also report that JNK is upregulated following transient global ischemia, being detectable in the nuclei of hippocampal CA1 neurons within 24 hours. However, in this study, JIP-1 was unchanged. In the hippocampus, *jip-1* has been identified using microarrays as a gene that is upregulated following fimbria-fornix lesions (116). Levresse *et al.* (31) report that expression of a gain-of-function myristoylated form of Akt inhibits activation of JNK and promotes cell survival via induction of JIP-1. These data suggest that JIPs play an important role in the regulation of JNK signaling, and that modifying levels of expression of JIPs may prevent cell death.

Several recent reports have documented the use of the JNK-binding domain of JIPs to inhibit the JNK signaling pathway. Bonny *et al.* (35) have utilized a 20-amino-acid sequence from the JNK-binding domain of IB-1 or IB-2 linked to a 10-amino-acid carrier peptide from the HIV TAT sequence to carry the peptide into cells. These inhibitors block transcription of c-jun by JNK, and addition of the peptides to the insulin-secreting cell line βTC-3 results in inhibition of IL-1β-induced c-jun and c-fos expression. The peptides were also able to protect βTC-3 cells from apoptosis induced by IL-1β. Harding *et al.* (25) have reported that expression of the JNK-binding domain of JIP-1 in sympathetic neurons deprived of NGF prevented both phosphorylation of c-jun and neuronal cell death. All these studies suggest that JIPs play a role in regulating the JNK pathway, and that modulating signaling through JNK via the JIPs may be neuroprotective.

Inhibitors of the JNK signaling pathway

Given all the evidence implicating activation of the JNK pathway in cell death, inhibitors of this pathway may be beneficial in a wide range of diseases, such as Parkinson's disease, Alzheimer's disease and stroke. In recent years, several inhibitors of the JNK pathway have been reported. In the remainder of this review, we will discuss specific (and nonspecific) JNK pathway inhibitors. Unlike p38, for which a number of small-molecule

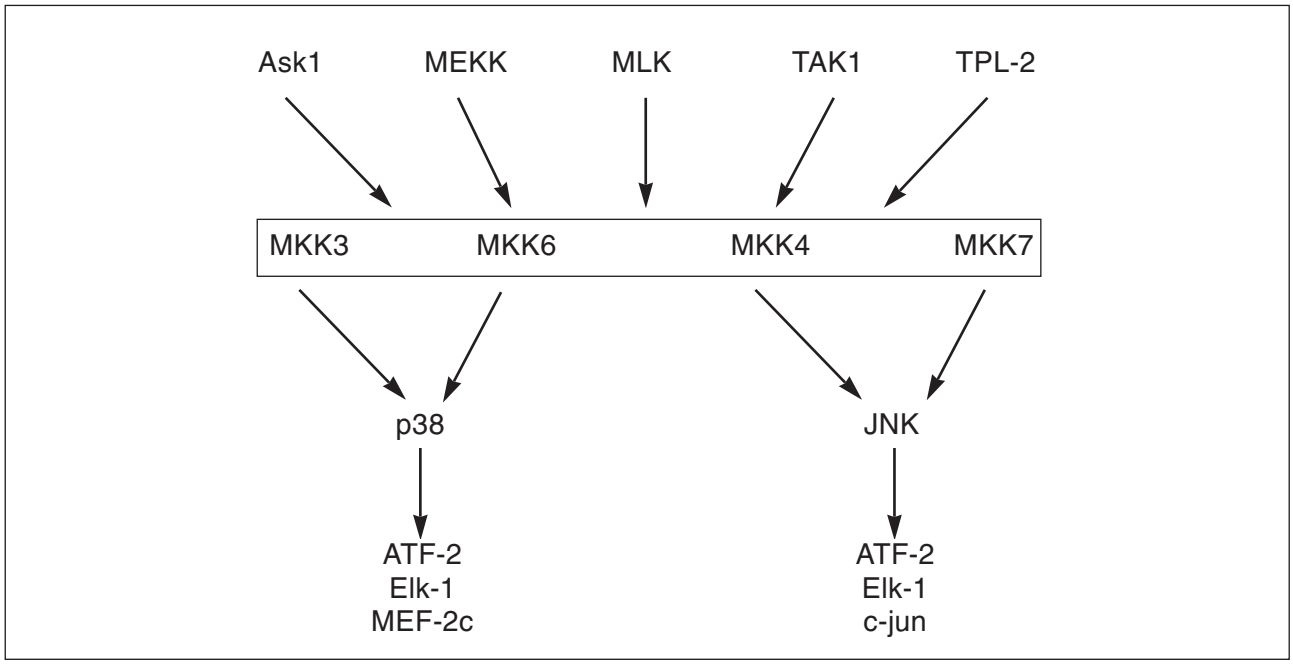

Fig. 2. Comparison of the upstream and downstream components of the JNK and p38 pathways.

inhibitors have been reported, many of them selective (117-121), very few potent JNK inhibitors have been published, and fewer still that show selectivity for JNK over other MAP kinase family members (120, 122, 123). Indeed, most of the JNK inhibitors reported thus far come from synthetic efforts to design p38 inhibitors. There are, however, some naturally occurring peptide inhibitors of the JNK pathway that have been reported (34, 35), the seleno-organic compound ebselen (124) and selenite, a direct JNK inhibitor (125). Park *et al.* showed that selenite was a direct inhibitor of JNK1 activity both *in vitro* and *in vivo,* having an *in vitro* IC_{50} between 10 nM and 50 nM. In a series of biochemical studies, they showed that selenite inhibited JNK1 through a thiol redox mechanism, as the inhibition could be reversed by reducing agents such as dithiothreitol and mercaptoethanol. They further confirmed the importance of sulfhydryl residues for JNK1 activity by demonstrating that *N*-ethylmaleimide, diamide and *o*-iodobenzoate abolished JNK1 activity *in vitro*. A final confirmation of the importance of a redox mechanism for JNK1 activity came from mutating Cys116 to Ser, which abolished the inhibitory effect of selenite (125).

The seleno-organic compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) was shown to suppress the lipopolysaccharide (LPS)-induced phosphorylation of JNK, but not p38, suggesting that ebselen exerts its JNK pathway-inhibitory properties not directly on JNK, but rather upstream from JNK. The authors reasoned that, since ebselen suppresses both NF-κB activation and JNK activation, but not p38 activation, a possible target for ebselen may be TAK-1 (TGF-β-activated kinase 1) (124) (Fig. 2).

The macrocyclic nonaketide antibiotic LL-Z1640-2 was shown to inhibit anisomycin-induced, but not TNF-induced, JNK activation. In these studies, concentrations of 25 ng/ml LL-Z1640-2 partially reduced JNK phosphorylation induced by anisomycin and 100 ng/ml fully inhibited JNK phosphorylation and subsequent c-jun phosphorylation. Since JNK phosphorylation was reduced in anisomycin-stimulated cells, the authors concluded that LL-Z1640-2 interferes with JNK signaling at the level of, or upstream from, the MKKs (126).

Small-molecule inhibitors of JNK signaling

As previously stated, the majority of published small-molecule inhibitors of JNK have stemmed from synthetic efforts for p38 MAP kinase. The compounds best characterized with respect to their inhibition of JNK isoforms are SB-203580 and its close analog SB-202190, the first pyridinylimidazole p38 inhibitors reported (117, 118, 127). The IC_{50} for SB-203580 for JNK3α1 is 790 ± 150 nM (122) and compares favorably to that determined for JNK2α1 (290 ± 120 nM) measured under similar conditions (120). There are reports, however, that SB-203580 is not an inhibitor of JNK1 (118, 128, 129). One explanation offered was a potential difference in the ATP K_m for the different JNK isoforms. The K_m (ATP) for JNK3α1 is 1.9 μM but the K_m (ATP) for JNK1 has not been reported, so confirmation of this hypothesis awaits experimental determination. An alternative explanation offered for the IC_{50} differences for SB-203580 was the significant difference in experimental conditions for determining the IC_{50}.

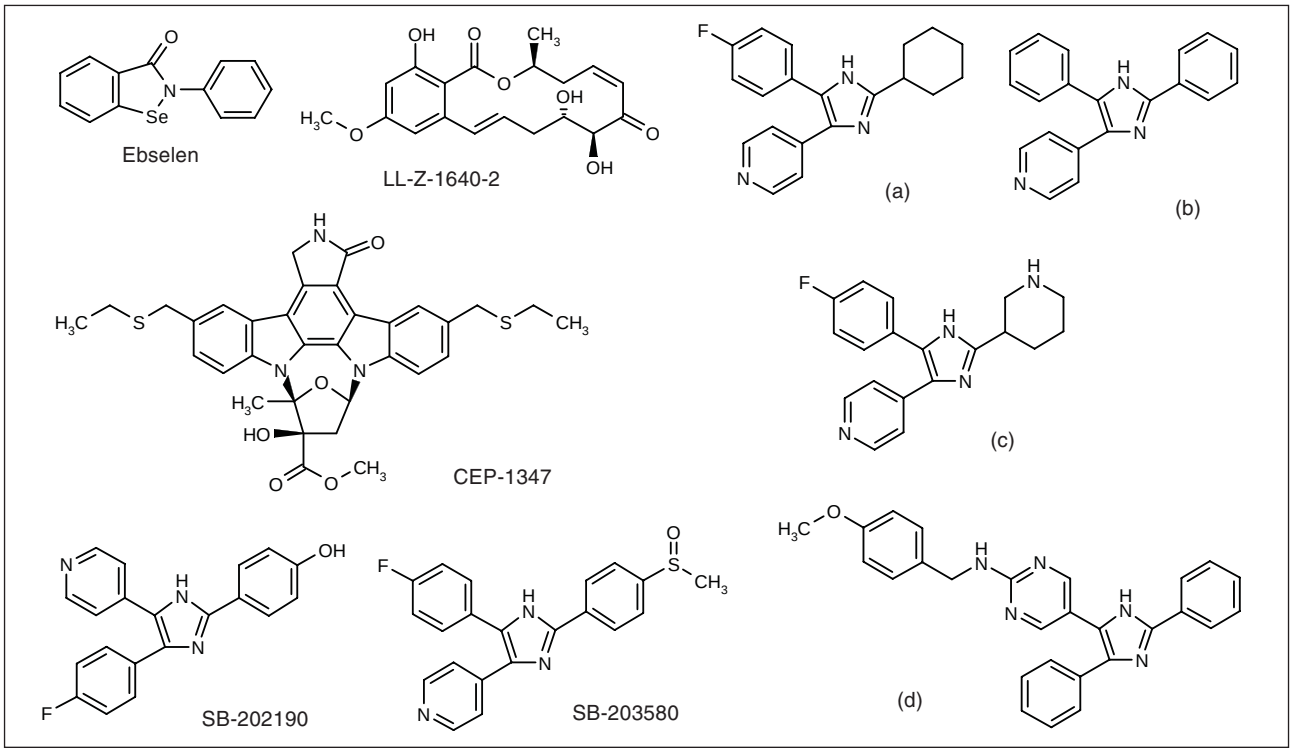

Fig. 3. A selection of compounds that inhibit the JNK pathway. a) 4-[5-(4-fluorophenyl)-2-cyclohexyl-1*H*-imidazol-4-yl]pyridine; b) 4-(2,5-diphenyl-1*H*-imidazol-4-yl)pyridine; c) 3-[5-(4-fluorophenyl)-4-(pyridin-4-yl)-1*H*-imidazol-2-yl]piperidine; d) [5-(2,4-diphenyl-1*H*-imidazol-5-yl)pyrimidin-2-yl](4-methoxybenzyl)amine. Also shown are the two GlaxoSmithKline compounds SB-203580 and SB-202190, the seleno-organic compound ebselen, the macrocyclic nonaketide LL-Z1640-2 and Cephalon's MLK inhibitor CEP-1347.

Gum *et al.* (129) measured the IC_{50} of SB-203580 at 50 μM ATP, whereas Lisnock *et al.* (122) and Liverton *et al.* (120) utilized an ATP concentration of 1 μM. Since it is likely that SB-203580 is ATP-competitive for JNK, these differences could explain the lack of inhibition seen by Gum *et al.* (129). Cuenda *et al.* (118) did not report the ATP concentration used in their assays, although it was likely to be > 20 μM, so a lack of JNK inhibition by SB-203580 can be expected. Recently, Eilers *et al.* (26) reported that SB-203580 was an effective inhibitor of recombinant rat JNK3, with an IC_{50} of 2 μM. Moreover, they showed that inhibition of JNK in sympathetic neurons prevents c-jun promoter activation and NGF withdrawal-induced death, further supporting the notion that SB-203580 is an inhibitor of JNK.

In addition to SB-203580, there are a number of compounds (Fig. 3) from a similar structural class that have been reported to be modestly potent inhibitors of JNK2α1, having IC_{50} values below 150 nM (120). These inhibitors are more potent than SB-203580, one example being 4-[5-(4-fluorophenyl)-2-cyclohexyl-1*H*-imidazol-4-yl]pyridine. This compound has an IC_{50} of 29 nM for JNK2α1 and is a more potent inhibitor of JNK2α1 than of p38 (120). Two other compounds from this class, 4-(2,5-diphenyl-1*H*-imidazol-4-yl)pyridine, and 3-[5-(4-fluorophenyl)-4-(pyridin-4-yl)-1*H*-imidazol-2-yl]piperidine, have IC_{50}s for JNK2α1 of 130 nM and 140 nM, respectively. A pyrimidinylimidazole, [5-(2,4-diphenyl-1*H*-imidazol-4-yl)pyrimidin-2-yl](4-methoxybenzyl)amine, has an IC_{50} for JNK2α1 of 98 nM (120). These data suggest that, while thus far no highly potent, selective JNK inhibitors have been reported, the likelihood of finding JNK-selective inhibitors is a reasonable possibility.

Structural basis for pyridinylimidazole selectivity

A fair amount of data from both X-ray crystallography studies, enzyme kinetics and site-directed mutagenesis experiments are available describing the structural basis for the specificity of pyridinylimidazole inhibitors for p38 MAP kinase (119, 130). Steady-state kinetic studies showed that SB-203580 and SB-202190 are ATP-competitive inhibitors of p38 (128, 131). These kinetic data are consistent with X-ray crystallography studies showing that SB-203580 binds in the ATP pocket of p38 (132). No kinetic or structural studies for this compound have been published for any of the JNK isoforms, but it is almost certain that this class of compound binds in the ATP pocket of JNK and exerts competitive inhibition with respect to ATP.

Site-directed mutagenesis studies, where Thr106 of p38α was mutated to either Met (the residue present in JNK) or Gln (the residue present in ERK), rendered p38α

insensitive to inhibitors with IC_{50} values < 10 nM, even at concentrations up to 3-10 μM (119, 130). This analysis suggested that the molecular basis for p38 inhibitor specificity could largely be attributed to Thr106 in the ATP-binding pocket.

The next question to be addressed was: Is the same residue in JNK, and other MAP kinase family members, the primary source of the insensitivity or low-potency inhibition of the pyridinylimidazoles? The answer clearly is no, since many of the pyridinylimidazoles described by Liverton *et al.* (120) were modestly potent (IC_{50} < 150 nM) versus JNK2α1. These data would suggest that there are many attributes of the ATP-binding pocket in JNK that may be responsible for selective inhibitor binding. The issue gets more complicated when structural features of the inhibitors are considered. For example, 4-[5-(4-fluorophenyl)-2-cyclohexyl-1*H*-imidazol-4-yl]pyridine is in the pyridinylimidazole class of inhibitors, like SB-203580, yet it is 10-fold more potent than SB-203580 for JNK2α1, suggesting that structural features of both the compound and enzyme active site will affect potency and selectivity with respect to JNK inhibition. Indeed, Gum *et al.* (129) have shown that JNK1, when assayed at 50 μM ATP, is insensitive to SB-203580 up to 100 μM. However, when Met106 was mutated to Thr (the corresponding residue from p38), 10 μM SB-203580 was able to inhibit the mutant JNK1. Furthermore, when the double mutant M106T,E107H was used, the concentrations of SB-203580 inhibiting JNK1 were reduced to the range 0.1-1 μM, suggesting that multiple features of the JNK1 active site contribute to SB-203580 binding. Like p38, data for ERK showed that a single amino acid in the ATP domain was largely responsible for the insensitivity of this compound towards ERK (133). The wild-type enzyme is largely insensitive to SB-203580, but a single point mutation changing the Gln residue at 105 to Thr (the residue in p38) rendered ERK sensitive to SB-203580, with an IC_{50} of 13 nM.

Taken together, the structural, kinetic, structure-activity-relationship and mutagenesis data suggest that, of the MAP kinase family members, the JNK isoforms have the most variability with regard to which features of the enzyme and pyridinylimidazoles affect inhibitor binding and potency. Therefore, since no one single determinant predominantly contributes to inhibitor binding and selectivity for JNK, the challenge of finding JNK-selective inhibitors may be greater than for other MAP kinase family members. Moreover, given the potential T-cell complications from inhibition of JNK1 and/or JNK2, it is likely that only a small-molecule inhibitor selective for JNK3 would have therapeutic benefit in the treatment of diseases associated with chronic neuronal apoptosis.

Inhibitors from the patent literature

Several compounds are reported in the patent literature to inhibit JNKs. Hoffmann-La Roche claimed 4-heteroaryl, 4-arylindolinones and annulated indolinones (Fig. 4, compounds **1-3**) (134-137). Vertex Pharma-ceuticals disclosed the oxime **4** (Fig. 4) as a JNK3 inhibitor (134, 138). Applied Research Systems has claimed benzazole derivatives (139) as JNK2 and JNK3 inhibitors, compound **6** (Fig. 4) having an IC_{50} of 70 nM for JNK3 and of 210 nM for JNK2. Thus, several companies are actively pursuing JNK as a possible therapeutic target.

CEP-1347

Until recently, there have been no published data on specific inhibitors of the JNK pathway. One of the first compounds to be discovered that inhibits the JNK pathway but is without effect on p38 is Cephalon's CEP-1347 (140). CEP-1347, a K-252a derivative, inhibits the JNK pathway at the level of MLK. The compound inhibits all five members of the MLK family (MLK1, MLK2, MLK3, dual leucine zipper kinase and leucine zipper-bearing kinase) and members of the GCK family (GCK, KHS and NIK) when expressed in COS-7 cells (141, 142). Therefore, any survival-promoting effects reported on treatment with this compound cannot be due to inhibition of p38.

CEP-1347 has "neurotrophic" activity on a number of populations of neuronal cells, including PC12 cells deprived of NGF (143), rat spinal motoneurons (144), chick sympathetic neurons, dorsal root sensory neurons, chick motoneurons (145) and rat sympathetic neurons (143). In addition, CEP-1347 causes an increase in choline acetyltransferase (ChAT) activity in cultured basal forebrain neurons and spinal motoneurons (140, 146). CEP-1347 prevents neomycin-induced hair cell loss in organotypic cochlear cultures and promotes survival of dissociated cochlear neurons (147).

CEP-1347 prevents both c-jun phosphorylation and cell death caused by treatment of cortical neurons with sodium arsenite (45) or the toxic β-amyloid (Aβ) peptide (148). Interestingly, CEP-1347 is not able to rescue cells from all types of cell death. For example, it promotes survival in differentiated PC12 cells deprived of NGF, but it does not block cell death caused by serum withdrawal in undifferentiated PC12 cells, although JNK is activated in this model (143). CEP-1347 does not rescue Jurkat T-cells following activation of Fas, suggesting that other pathways may also be involved in these models of cell death and that inhibition of JNK alone is not sufficient to attenuate the cell death (143). These data suggest that JNK pathway activation is important in neurotrophin deprivation in a number of neurons and that neurotrophins may promote survival effects by inhibiting the activation of this pathway.

CEP-1347 was found to be neuroprotective in a number of *in vivo* models of neurodegenerative disease. Following ibotenic acid infusion into the nucleus basalis magnocellularis, CEP-1347 attenuated the loss of cortical ChAT activity and the loss of ChAT-immunoreactive and retrogradely labeled FluoroGold-labeled neurons in the nucleus basalis (149). Animals treated with CEP-1347

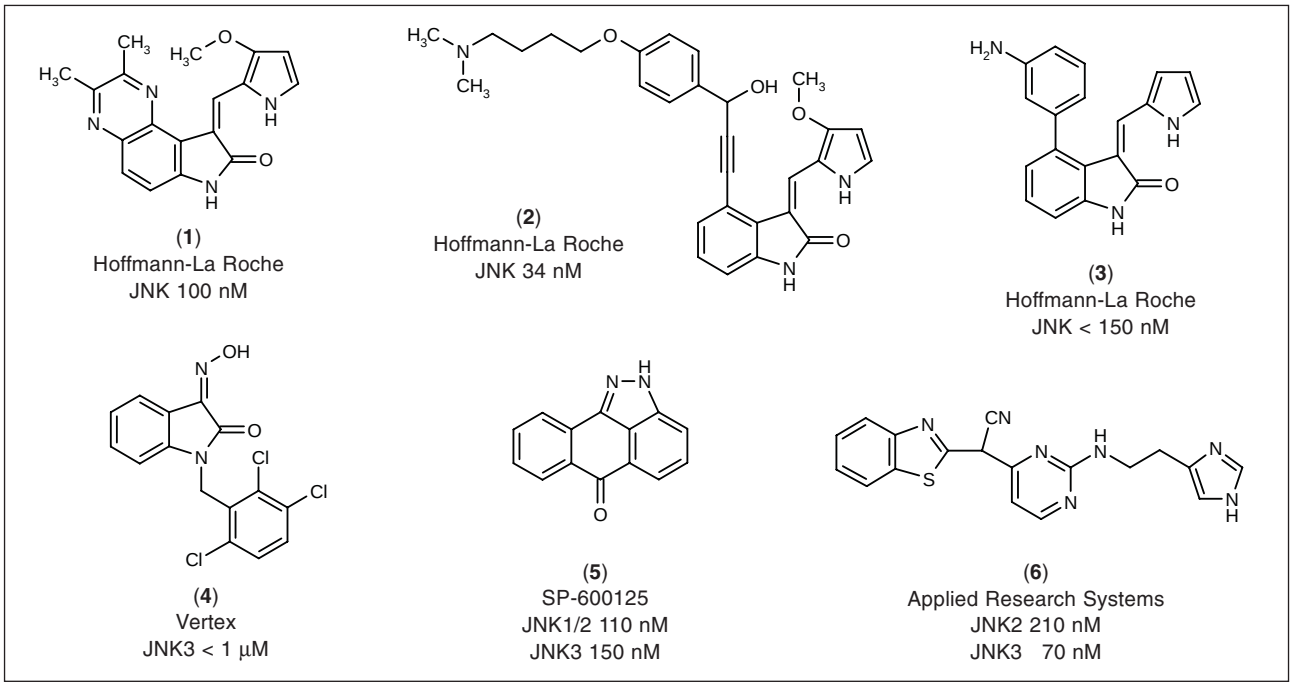

Fig. 4. A selection of compounds.

committed fewer errors in the T-test compared to animals treated with vehicle (150), indicating that the preserved neurons were functional. In animals in which the fimbria-fornix was transected, CEP-1347 was partially neuroprotective (146) and the compound was as efficacious as BDNF (brain-derived neurotrophic factor) in this model. In guinea pigs, CEP-1347 attenuated noise-induced hearing loss (147), suggesting a possible indication for JNK inhibitors in inner ear injuries. In the MPTP model of dopaminergic neuron loss, CEP-1347 attenuated the loss of striatal dopaminergic terminals and reduced the loss of dopaminergic cell bodies in the substantia nigra (151), suggesting a potential use in Parkinson's disease. In addition, CEP-1347 was recently shown to attenuate the MPTP-induced phosphorylation of both JNK and its upstream activator MKK4 following MPTP treatment (142). These data show the importance of JNK activation in neuronal cell death and that inhibition of JNK activation prevents cell death in a number of animal models of neurodegeneration. CEP-1347 is now in phase II trials for Parkinson's disease and it remains to be seen whether this JNK pathway inhibitor is successful in preventing further death of nigral neurons in man.

Compounds from Signal Pharmaceuticals

One of the major players in this area has been Signal Pharmaceuticals (now part of Celgene). Signal has described pyrazoloanthrone derivatives, such as SP-600125, as inhibitors of JNK (Fig. 4, compound **5**) (152). SP-600125 inhibits JNK1/2 with an IC_{50} of 110 nM and JNK3 with an IC_{50} of 150 nM, but is much less active at p38 (IC_{50} > 30 μM) (153). SP-600125 was shown to block IL-1-induced accumulation of phosphorylated JNK and expression of c-jun in cultured synoviocytes. AP-1 binding and collagenase mRNA expression were also reduced. *In vivo*, administration of SP-600125 was shown to inhibit JNK activation and collagenase expression in the joints of rats with adjuvant arthritis. Animals also showed a reduction in paw swelling and bone and cartilage damage. Thus, inhibition of JNK could be a potential therapy for diseases such as rheumatoid arthritis. Other reports, mainly from scientific meetings, have documented effects of SP-600125 in models of asthma, as well as models of arthritis and in culture models. As reported to the American Thoracic Society, SP-600125 was shown to reduce bronchoalveolar eosinophils and lymphocytes in animals undergoing repeated allergen exposure, and to reduce serum IgE, suggesting its possible use in the treatment of asthma (154, 155). At an Inflammation Research meeting, Bennett *et al.* (156) reported that SP-600125 inhibited T-cell activation by anti-CD3/CD28 and concentration-dependently inhibited the production of TNF-α, interferon γ, IL-10, IL-1β and IL-6 from Th1 cells, and IL-10 and TNF-α from Th2 cells. The compound also reduced TNF-α release in a mouse model of sepsis and kainic acid-induced seizures in rats (152).

There is a small amount of information on another JNK inhibitor, SP-105. This compound is reported to be selective, orally bioavailable and to show a good pharmacokinetic profile (155).

A third compound, SPC0009766, was presented at the Society for Neuroscience meeting in 2000 (157). Experiments demonstrated that exposure of primary ventral mesencephalic dopaminergic neurons to 30 μM 6-OHDA resulted in a 60% decrease in [^{3}H]-dopamine uptake and induced phosphorylation of c-jun, as determined by Western blot analysis. Pretreatment (40 minutes) of the cultures with SPC0009766 concentration-dependently increased dopamine uptake, which returned to control values at 30 μM. The IC_{50} for this effect was 15 μM. c-Jun phosphorylation was blocked by 30 μM SPC0009766. Other transcription factors (ATF2 and Elk-1) were unaffected. It remains to be seen whether this compound has activity *in vivo*. All these data suggest that the JNK inhibitors generated by Signal may have potential for the treatment of a number of diseases.

Conclusions

The JNK signal transduction pathway is activated in many different cell types and in response to many different stressful stimuli. Recently, inhibitors of this pathway, such as CEP-1347, which acts at the level of MLK, and the p38 inhibitor SB-203580, which also inhibits JNKs, have been reported. The evidence is now quite strong that inhibiting JNK promotes cell survival, particularly in neurons. However, there could be issues with JNK inhibitors as drugs. For example, it is not known what impact a JNK inhibitor would have on the immune system, especially if given as a long-term treatment. It is also not known what effects preventing cell death in both neurons and in the periphery would have, and whether this would promote cancer formation. The best prospects for JNK inhibitors in the clinic would appear to be for a neuronal target such as stroke or Parkinson's disease, where a specific JNK3 inhibitor could be given without effects on non-neuronal cells.

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